

Modelling and systems approaches for elucidating information processing in multi-site phosphorylation systems

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Declaration

This thesis is a description of the work carried out by the author in the Department of Chemical Engineering, Imperial College London, under the supervision of Dr J Krishnan. I hereby certify that the work reported in this thesis was composed by and originated entirely from me. All information, which is not my own work, has been appropriately acknowledged in the text and references are given in the list of sources.

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Phosphorylation Systems

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Abstract

Multisite phosphorylation is a basic ingredient of cellular signalling and information processing and involves the modification of substrates by kinases and phosphatases at multiple locations. Multisite phosphorylation (and modification in general) has emerged as a key underlying mechanism by which cells exhibit control different aspects of their functioning and decision making. In the past decade, various studies have focussed on the behaviour of multisite phosphorylation, theoretically, analysing both distributive and processive mechanisms of multisite phosphorylation. There have also been a number of experimental studies in multisite phosphorylation in a range of cellular pathways and contexts. While these studies have provided important insights, there is a significant gap in understanding the role of multisite phosphorylation and its role in signalling pathways. In this thesis, a structured modelling and systems engineering approach is employed to elucidate different aspects of multisite phosphorylation and its role in cellular pathways. This is developed through a series of investigations.

The first investigation demonstrates that distributive multisite phosphorylation systems can exhibit a biphasic dose response thus demonstrating an in-built competing effect in the modification mechanism, a feature not shared by processive mechanisms. A series of analyses is able to pin down when this biphasic response will and will not occur.

In the second investigation, the main focus is on the effect of the modification mechanism on information processing characteristics. A framework is developed to analyse the interplay between distributive and processive mechanisms leading to mixed mechanisms. Analysis of mixed mechanisms shows that bistability and biphasic dose response curves can be eliminated as a consequence of the interplay of distributive and processive mechanisms.

On the other hand, the interplay between these two mechanisms can result in sustained oscillations.

In the third investigation, a structured systems framework is developed to bridge the gap between the multisite phosphorylation module in isolation and as part of networks and pathways. To this end, a comprehensive *in silico* analysis is performed to examine the effect of enzyme activation, the effect of retroactivity associated with an output of the multisite module participating in downstream pathways, the effect of substrate (or enzyme) production and degradation, and also the effect of embedding the multisite module in different network modules. These studies provide a whole range of insights into qualitative transitions in behaviour associated with the effect of ambient network on the multisite module.

The final investigation focuses on examining the effect of multiple kinases and/or phosphatases in multisite phosphorylation. Through an examination of ordered and random mechanisms this study demonstrates how the presence of a common substrate can couple different pathways. This study also demonstrates what kinds of information processing capabilities and constraints arise with multiple kinases.

Taken together, a series of investigations provide a consolidated systems view of different aspects of multisite phosphorylation and as part of networks. This provides a consolidated platform and framework from which to investigate the effect of multisite phosphorylation in a range of specific natural contexts and to rationally engineer different aspects of biochemical pathways containing multisite phosphorylation.

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List of Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
Cdc	Cell division cycle protein
c-FFW	Coherent feed-forward regulation
Ca ²⁺	Calcium ion
CMC	Covalent modification cycle
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinases
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
Hopf	a critical point where a system's stability switches and a periodic solution arises
i-FFW	Incoherent feed-forward regulation
IP ₃	Inositol trisphosphate
JNK	c-Jun N-terminal kinases
K ⁺	Potassium ion
LP	Limit points
MAPK	Mitogen-activated protein kinases
mRNA	Messenger Ribonucleic acid
NFB	Negative feedback regulation
PFB	Positive feedback regulation

PKC	Protein kinase C
PTMs	Post translational modifications
RTKs	Receptor tyrosine kinases
SAPK	Stress-activated protein kinases
Sic1	Stoichiometric inhibitor protein of Cdk1-Clb in <i>S. cerevisiae</i>
Wee1	Protein kinase in <i>S. pomb</i>

List of Publications

Some of the research presented in this thesis can be found in the following list of publications:

- [1] **Suwanmajo, T.** and Krishnan, J. (2013) Biphasic responses in multi-site phosphorylation Systems. *Journal of the Royal Society Interface*. **10**: 20130742.
- [2] **Suwanmajo, T.** and Krishnan, J. (2015) Mixed mechanisms in multi-site phosphorylation. *Journal of the Royal Society Interface*. **12**: 20141405.
- [3] Krishnan, J., Mois, K. and **Suwanmajo, T.** (2014) The behaviour of basic autocatalytic signalling modules in isolation and embedded in networks. *The Journal of Chemical Physics*, **141**: 175102.
- [4] **Suwanmajo, T.** and Krishnan, J. (2018a) A bottom-up approach to bridging the gap between multi-site modification systems in isolation and as part of signalling pathways. *Journal of the Royal Society Interface*.
- [5] **Suwanmajo, T.** and Krishnan, J. (2018b) Open model of multi-site substrate modification. (*in preparation*).
- [6] **Suwanmajo, T.** and Krishnan, J. (2018c) Multi-site substrate modification through multiple kinases and phosphatases (*in preparation*).

Chapter 1

Introduction

Most aspects of cellular life and response to the environment are dynamically regulated by cellular signalling networks. Cell signalling underpins a broad spectrum of cellular processes *via* complex reaction networks of “nano machines” (proteins): growth, division, differentiation are all controlled by complex cell signalling (and genetic) networks. In recent years, the major open question of how cells process a multitude of signals (both extra and intracellular) to yield specific and desired responses has always been on the forefront of researchers’ attention owing to its basic importance as well as broad application. One class of applications is in the biomedical arena. Scientific evidence suggests that abnormalities of cellular signal processing are causatively implicated in a diverse spectrum of pathological processes; for example, cancers (Irish et al., 2006), diabetes (Hart, 2000), Alzheimer’s diseases (LaFerla, 2002) and even AIDS (Lavrik et al., 2009). As such, not only does systematic understanding of cell signaling networks provide a basic insight into cellular processes, but it also opens up a novel avenue for medical therapy, leading to medical and pharmaceutical breakthroughs. However, a deep and effective understanding of the functioning of such networks cannot be achieved purely intuitively because of their organisational complexities.

Cell signalling is a complex process and several steps organized in different pathways are involved. Although in the previous decades, a great deal of progress has been made in this field, there are many aspects of cell signalling which are still poorly understood. A brief examination of the landscape of cell signalling pathways and processes reveals the reason why this is the case. Firstly cell signalling processes and pathways involve a fairly large

number of components. Secondly, instead of being isolated, different signalling pathways are interconnected into hierarchical networks which not simply linear pathways, but may involve complex organization including different kinds of feedback. Despite their nano-sized structure of the constituent elements, such networks are exceedingly complex because of a large number of interacting components, feedback and intricate organization (shaped by evolution) which results in the emergence of complex dynamic behaviour. Under opportune conditions, they can exhibit highly nonlinear behaviours, for example, ultrasensitive monostable switching, bistable memory switching and oscillations. All these behaviours have been reported in multiple biological contexts and are actively exploited in cell biology. Consequently the qualitative analysis of cell signalling networks is extremely complex, sophisticated and subtle, so it requires an integration of mathematical modelling and system engineering approaches.

In the last decade and a half, modelling and systems engineering approaches have been widely used in the context of interdisciplinary biological studies in systems biology. Particularly they seem to be a gold standard technique to gain insight into the bewildering complexities of cell signalling networks. The reason that one needs modelling and systems engineering approaches is that the knowledge of signalling system components obtained by experiment by a variety of currently existing technologies does not provide sufficient understanding of how the signalling processes work. To gain insight into this, one need to understand the relationships among components of signalling networks, which can be obtained by modelling and systems engineering approaches.

While some sources of complexity in cell signalling networks have been discussed above, there is one aspect which has not been mentioned: the complexity of enzymatic mechanisms. This thesis focuses on one aspect of this complexity: multisite modification wherein enzymes modify/demodify the substrate at more than one location. Far from being an

isolated occurrence, this multisite modification (multisite phosphorylation to be specific) is a recurrent occurrence in cell signalling pathways. This mechanism plays an important role in many aspects of cell signalling. For instance, it modulates protein's activity, protein's subcellular location, and increases the possibilities for integrating signals from different pathways (Cohen, 2000). In fact the modification status of a protein is sometimes referred to as a "molecular barcode" since the knowledge of the modification status is an important indicator of different aspects of the functioning of the substrate. In multisite phosphorylation the modification of substrates (phosphorylation) is performed by enzymes called kinases and the de-modification of substrates is performed by enzymes called phosphatases. There are different mechanisms by which a single enzyme (kinase/phosphatase) may effect multiple modifications. One example is a distributive mechanism, wherein every modification results in the release of the modified substrate along with relevant enzyme. A subsequent rebinding of this substrate and enzyme is necessary for further modification. Another example is the processive mechanism, where multiple modifications occur while the enzyme remains bound to the substrate. Both mechanisms have been studied extensively in the literature. The reason why each mechanism is employed is not clear.

A variety of studies on multisite phosphorylation have been performed in the literature. Multisite phosphorylation has been studied in multiple biological contexts experimentally. This includes multisite phosphorylation of different cell cycle phosphorylation such as Sic 1, Cdc25 and Wee1 (Nash et al., 2001; Perry and Kornbluth, 2007). Furthermore a very broad study of multi-site modification and its consequence for cellular function have also been studied experimentally (Walsh 1996). These various studies provide a number of insights into how the modification mechanisms occur, how they affect protein function and how that in turn affects the functioning of cellular pathways.

From the theoretical and systems side, a number of basic studies have been undertaken to study the dynamics and information processing behaviour of multisite phosphorylation. Previous studies have shown how two site modification of a substrate by a single kinase phosphatase pair can give rise to bistability in a distributive mechanism, while also showing that such bistability is ruled out in a processive mechanism. Additionally, the fact that multiple site modification gives rise to a threshold behaviour has also been demonstrated. The fact that unlimited multistability may be observed in distributive multisite modification has also been studied. Different studies have been performed on the so-called ordered and random mechanisms.

The previous studies performed before the start of this thesis provide an important platform for understanding different aspects of multisite phosphorylation. However when one examines the importance of multisite phosphorylation on one hand and their presence in cell signalling pathways on the other hand, a number of questions arise. (i) What are the ranges of qualitative information processing behaviours which can be exhibited by different multisite modification mechanisms? Are there other behaviours besides those already discussed? (ii) What is the effect of more complex enzymatic modification mechanisms, beyond simple distributive and simple processive? (iii) How do these multisite modification mechanisms function in signalling pathways, and what is the interplay between the enzymatic modification module and the pathway/network? (iv) How does the presence of multiple modification enzymes affect the modification dynamics of the substrate?

This thesis addresses these various questions and is organized as follows. In the next chapter the background for the work is presented. The results are organized in four subsequent chapters. In the first of these, the focus is on simple distributive and processive mechanisms and examining different aspects of the dose response curve in multisite modification in detail. In the subsequent chapter the effect of the interplay between

distributive and processive mechanisms leading to mixed mechanisms is dissected. This study is an investigation of more complex combinations of mechanisms and their effect of information processing. The subsequent chapter focuses on many aspects of bridging the gap between the enzymatic modification module in isolation and as part of a signalling pathway. This leads us to examine in detail how different aspects of the behaviour of the enzymatic module are affected by its presence in a signalling pathway, and conversely, how it affects the behaviour of the signalling pathway. This is done through a sequence of focussed investigations adding up a comprehensive systems picture. The last results chapter focuses on certain aspects of multiple enzyme modification of a substrate. Our focus right through this thesis is on basic qualitative behaviour. The analysis undertaken involved a range of tools, including numerical simulations, bifurcation analysis and analytical work

Multisite phosphorylation is a common constituent sub-unit of a number of biological networks. Therefore, the systematic understanding of different mechanisms of the multi-phosphorylation could provide basic insights into a broad spectrum of cellular pathways and processes involving multi-site mechanisms such as MAPK pathways (Ferrell & Bhatt, 1997; Zhao & Zhang, 2001; Cargnello and Roux, 2011), p53 pathway (Meek, 1998; Harris and Levine, 2005; Khoo et al., 2014), dark adaptation in humans (Kennedy et al., 2001; Ohguro et al., 2005; Travis et al., 2007; Huang et al., 2011). Other examples include check point control in the cell cycle which is completed by proteins with multiple sites (Kõivomägi et al, 2011; Verdugo et al, 2013). In this study, a series of investigations of multisite phosphorylation constitutes a dedicated systems approach to unravelling different facets of the complexity and behavior of this enzymatic module in isolation and in pathways. This serves as a platform for further detailed investigations and expansions in a wide range of specific contexts.

Chapter 2

Background and literature

2.1 Fundamentals of cellular signal processing

2.1.1 Cell signalling mechanisms

Cellular signalling – a dynamic process used by cells to receive a variety of extracellular information and convert it into appropriate biological responses – is an important component of both unicellular and multicellular organisms. It underlies a number of cellular processes (e.g. cell growth, cell division and apoptosis) *via* complex reaction networks of proteins and genes. Timely and efficient cellular signal processing enable cells to develop and work regularly. Dysfunctional cell signalling events; however, might lead to clinical abnormalities and illness. Therefore, one can reasonably argue that all living entities including animals, plants and even microorganisms require this “efficient processing” for their survival.

The features of signalling principles are common to all organisms (Hancock, 2010). Basically there are three main stages in signal processing (as depicted in figure 2.1). The first stage (reception) involves receptor activation *via* binding of an extracellular input (signal molecule); for instance, hormones, growth factors and neurotransmitters, to a receptor molecule embedded in either the membrane or cytoplasm of the target cell. Since the cell has the ability to recognize signals, each type of receptor binds to specific signaling molecules. The second stage is signal transduction. In this stage, a conformational change of receptor

arising from the reception process enables signal to be transmitted to the interior of the cells through intracellular signaling pathway. This complex pathway is associated with a protein cascade which is a series of biochemical reactions in which the product of the upstream reaction plays an enzymatic role for the next downstream reaction. In the final stage (response), the output of the pathway instigates an appropriate intracellular response.

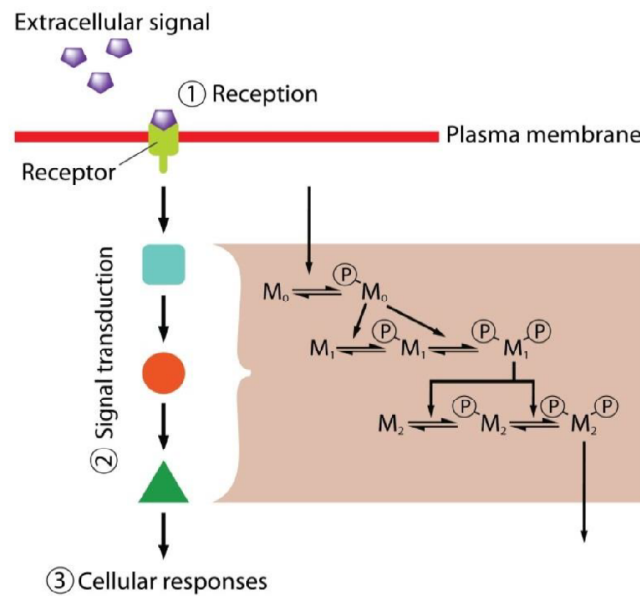


Figure 2.1 Schematic representation of the cellular signal processing. *The soft pink background photo shows a MAPK pathway, a ubiquitous signalling module in eukaryotes.*

However, there are essential two points to note. Firstly, very often, rather than being isolated; many cascades interact with one another (Bhalla and Iyengar, 1999; Eungdamrong and Iyengar, 2004). This is termed as crosstalk (figure 2.2). The linkages between pathways enable the cell to process a large variety of signals by sharing the functions of interacting components inside the cell, leading to different effects (Goitre et al., 2012; Vinod and Venkatesh, 2008).

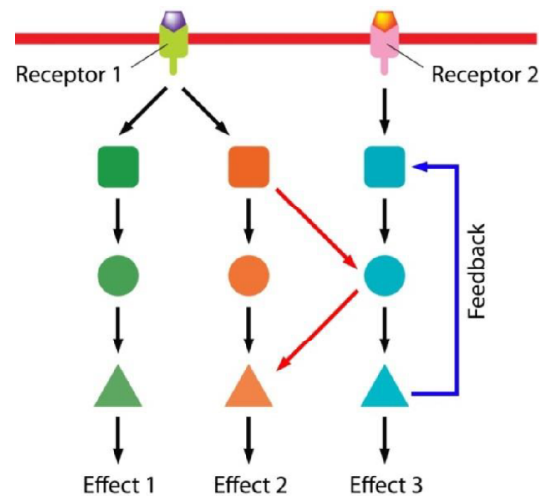


Figure 2.2 The cross-talk between signalling pathways (red arrow)

Secondly, biological control systems consist of a set of regulatory interactions such as feedback regulation— the process in which the output signals are sent back to their input. A key function of feedback loops is to shape the dynamic responses in time and space (Brandman and Meyer, 2008), which plays diverse roles in different regulatory systems. Feedback can cause different kinds of qualitative behaviour (which is important for cellular processes) such as ultrasensitivity (Reth and Brummer 2004), adaptation (Levchenko 2002), biological switching and oscillation.

Depending upon the net effect around the signalling circuits, feedbacks can be dichotomized into positive and negative feedback (Tyson et al. 2003). Based on two component systems, feedback control loops can be represented diagrammatically as in figure 2.3.

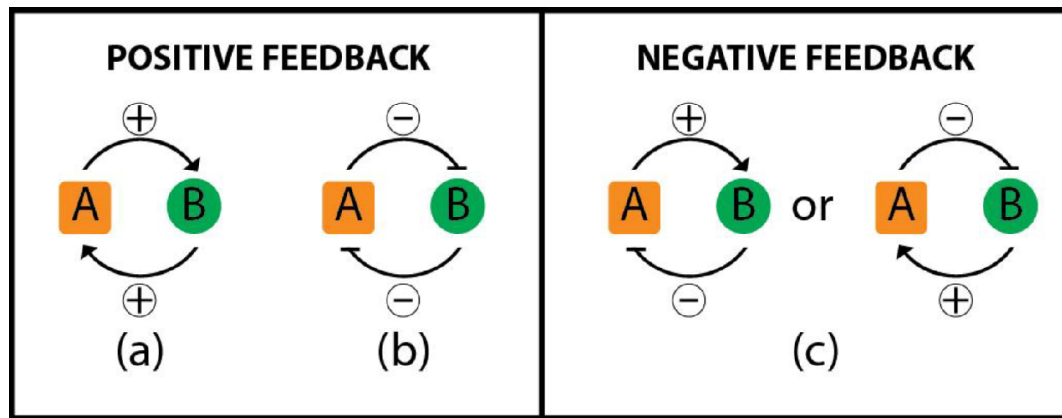


Figure 2.3 Possible feedback loops between two interacting components. (a) mutual activation, (b) mutual inhibition and (c) activation/inhibition.

Considering two component systems in figure 2.3, there are three possible core topologies: (a) mutual activation, (b) mutual inhibition and (c) activation/inhibition. It can be seen that protein A can be a positive modulator or negative modulator of protein B. Feedback loops of the mutual activation and mutual inhibition are positive whilst the net effect around the activation/inhibition loop is negative. In signaling circuits, there are a large number of shared components and intertwined feedback and feed-forward loops. Consequently the dynamic behaviour of the system is extremely complicated.

2.1.2 Types of signal processing

There are two types of cellular signal processing:

- **Intracellular signalling** in which signals are processed within one cell.
- **Intercellular signalling** which refers to cell-to-cell communication.

In intercellular signalling, a sender cell is referred to as the “signalling cell” whilst a receiver cell is the “target cell”. In general, signals released from the signalling cell can be

received both locally and distantly. Examples of cell-to-cell communication include endocrine, paracrine, autocrine and juxtacrine.

Endocrine is a long-distance signalling system where the message is carried *via* the circulatory system. As shown in figure 2.4, signalling molecules from the signalling cell are carried a long distance (again *via circulation*) in the extracellular medium to the target cell.

Paracrine is a local signalling system where the message is carried *via* diffusion. In this system, the signalling molecules released from inducing cells act on nearby target cells.

Autocrine is the local signalling system where a signal sender and a signal receiver are of the same cell type.

Juxtacrine is a direct cell-cell signalling by physical contact between adjacent cells. Such cells communicate with each other through a special channel for communication.

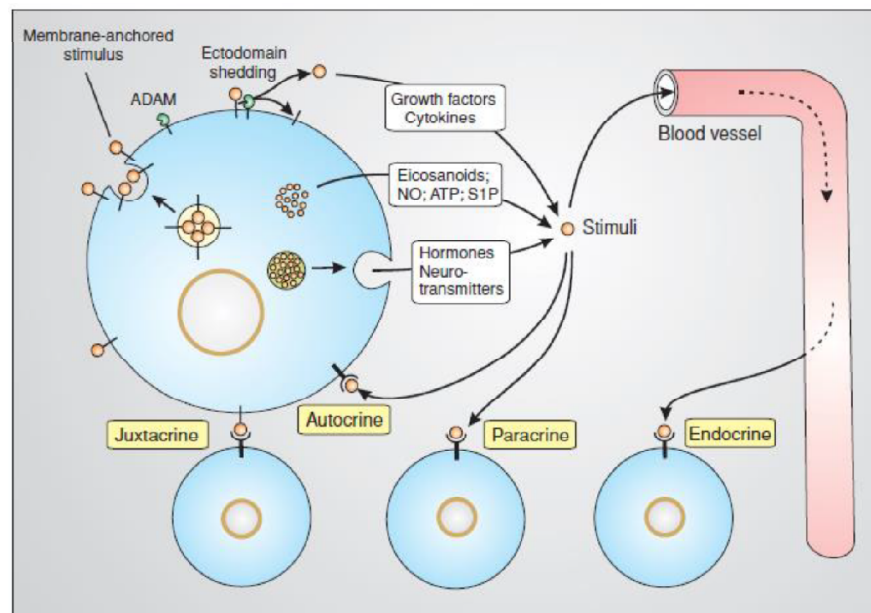


Figure 2.4 *Forms of an intercellular signalling (Berridge, 2009).*

2.1.3 Types of signalling molecules

In the cellular system, signalling proteins exist either in active forms (on state) or inactive forms (off state). Signalling proteins cannot transmit signals down the signalling pathway unless they are in the active state. While activating signals activate proteins, deactivating signals are responsible for modulating the lifetime of the active state of their protein substrates (Krauss, 2003). In general signalling molecules can be categorised according to their chemical modification.

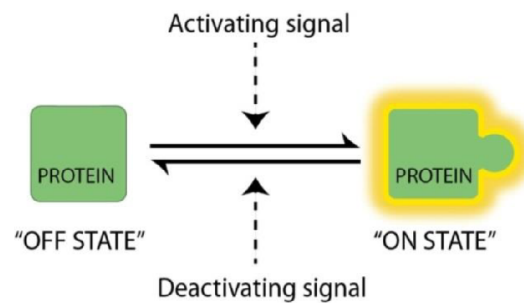


Figure 2.5 Activation and deactivation of signalling protein.

Protein kinases

Protein kinases can be regarded as the “workhorses” of cell signalling (Marks et al., 2009). Without these signal molecules, cellular information cannot be processed (Tan et al., 2009). Kinases alter other proteins’ activities by instigating the transfer of the phosphoryl group from ATP to the amino acid side chains of their protein substrates, a process termed phosphorylation (Berg et al., 2002). This turns on the activity of those proteins. In eukaryotes, kinases phosphorylate their protein substrates at the serine, threonine and tyrosine residues (Manrai & Gunawardena, 2008). The phosphorylation mechanism will be discussed in section 2.3.

Protein phosphatases

Phosphatases undo phosphorylation, referred to as dephosphorylation. They catalyse the hydrolytic cleavage of phosphoryl group from residues in their protein substrate. In the context of cell signalling phosphatases can shut down signalling events through dephosphorylation (Imboden & Koretsky, 1995). Both phosphatase and kinase, in general, work together to modulate cellular response (Tonks, 2006).

G-proteins

Like protein kinases, G-proteins can be regarded as “the molecular switches” (Berridge, 2009; Krauss, 2003). Bound to GDP, G-proteins deactivate. By contrast, their activity is turned on *via* the exchange of GTP for GDP, which is facilitated by GEF. G-proteins are thus often called “GTP-binding proteins”. The active form of such proteins is of significance in terms of transmitting cellular information from activated cell surface receptors (G protein-coupled receptors) to the downstream signalling elements, thereby instigating a diverse range of signal transduction mechanisms.

Second messenger

Second messengers or intracellular signal are low molecular weight and diffusible substances, which facilitates signal relay from cell surface receptors to target molecule within the cell interior (Kramer et al., 2009). The activity of a number of proteins is dependent of them. For instance, DAG and Ca^{2+} regulate protein kinase C, and cyclic AMP regulates protein kinase A. In general, second messengers can be dichotomized into hydrophobic messenger and hydrophilic messenger. While hydrophobic messengers are localized in the membrane, hydrophilic messengers exist in the cytosol (Krauss, 2003). In the context of cell

signalling, this type of signalling proteins is critical in terms of amplification of their upstream signals.

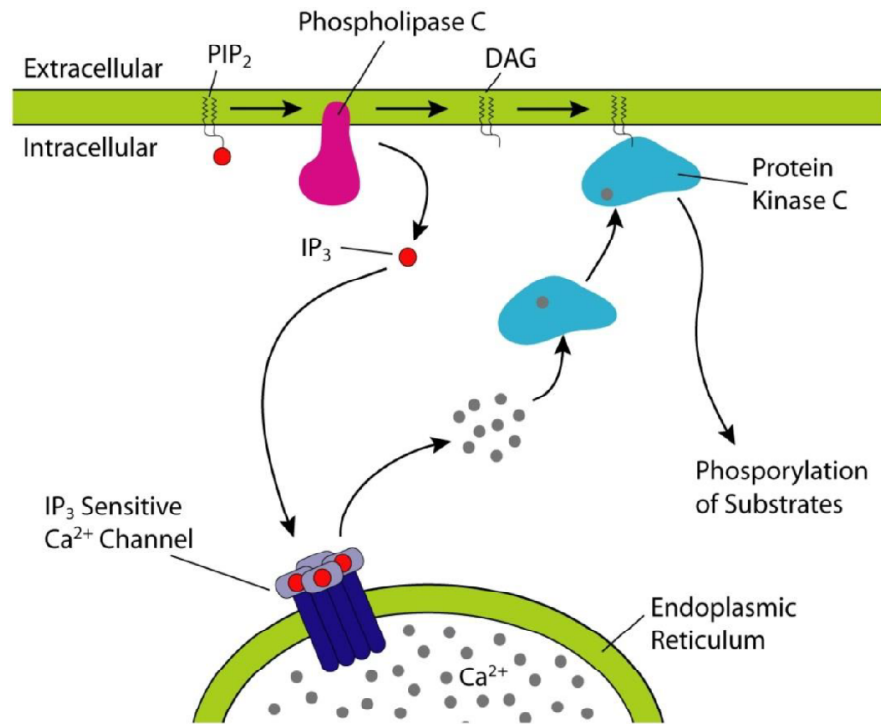


Figure 2.6 Activation of cell surface receptors leads to the synthesis of a large number of second messenger molecules such as IP₃, Ca²⁺ and DAG. These messengers then activate their target proteins (Korte, 2007).

2.1.4 Types of Receptors

Cell signalling receptors can be broadly classified into two types: (i) cell surface receptors or (ii) intracellular receptors.

2.1.4.1 Cell surface receptors

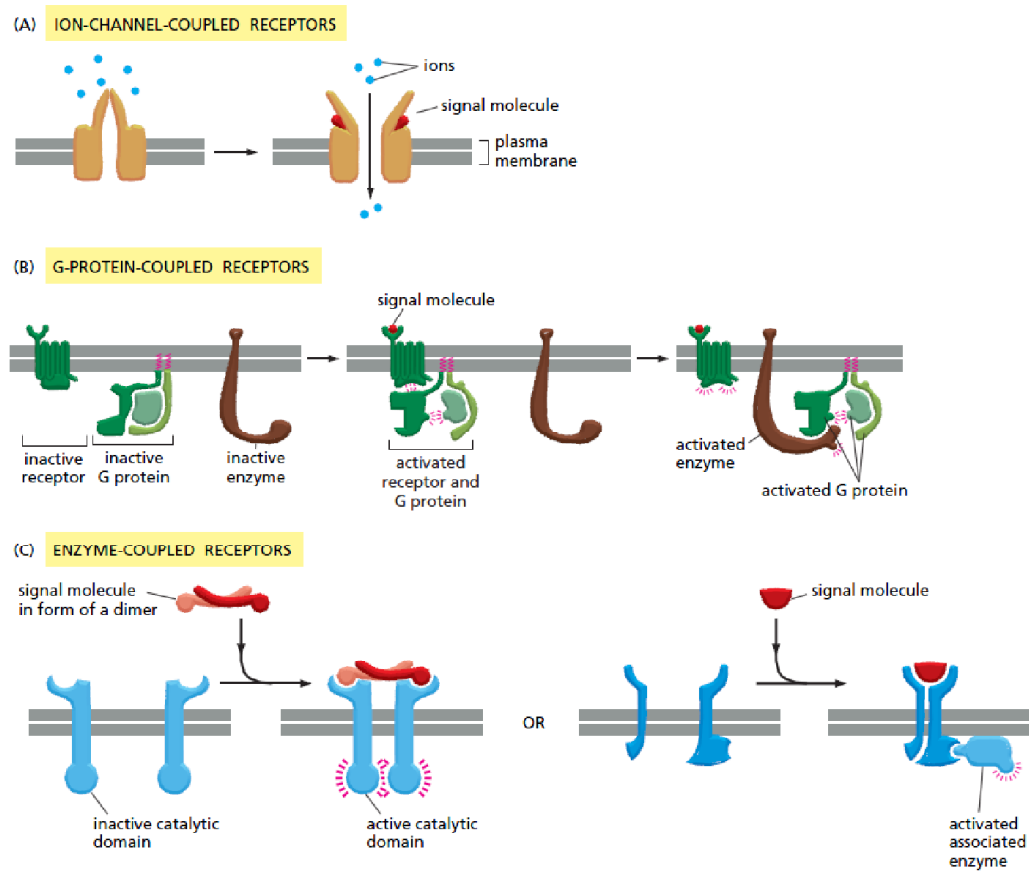


Figure 2.7 Three classes of cell surface receptors (Alberts et al. 2008)

G-protein linked receptor

G-protein linked receptors are the largest group of proteins embedded in a plasma membrane. Being in active, both G-protein and receptor are isolated from each other. When the receptor is activated by external cues, there is a change in its conformation and, as a

result, it binds to G-protein. This gives rise to an activation of trimeric G-protein, thereby instigating the next stage of signalling processes. This activation leads to multiple consequences:

- Stimulus of enzymes embedded in the plasma membrane to release second messengers, such as cAMP, IP₃ and Ca²⁺.
- Stimulus of kinase activity.
- Turning on or turning off certain ion channels (K⁺, Ca²⁺) in order to control/allow the release of certain neurotransmitters (Raingo et al., 2007).

Ion channel linked receptor

This type of receptor often involves synaptic signalling. In the un-stimulated state, the ion-permeable pores remains closed. By contrast, receptor activation by ligand binding results in the channel opening, allowing an influx of charged ions. This event, however, is short-lived. The ions that flow through the channel are positive, for instance, K⁺, Ca²⁺ and Na⁺.

Enzyme-coupled receptor

This type of receptor is unique. That is it possesses intrinsic enzymatic activity (Hancock, 2010). This includes receptor tyrosine kinases (RTKs) which have attracted a great deal of attention from researchers owing to their medical significance. Not only can RTKs phosphorylate other proteins, they can phosphorylate themselves. Binding of their ligands (e.g. hormones, cytokines and growth factors) results in receptor dimerization and consequently multisite autophosphorylation (at tyrosine residues). These ensuing phosphorylated tyrosines provide docking sites for a number of signalling proteins, which

leads to the activation of signal transduction cascades, for example, the MAPK/ERK pathway and SAPK/JNK pathway.

2.1.4.2 Intracellular receptors

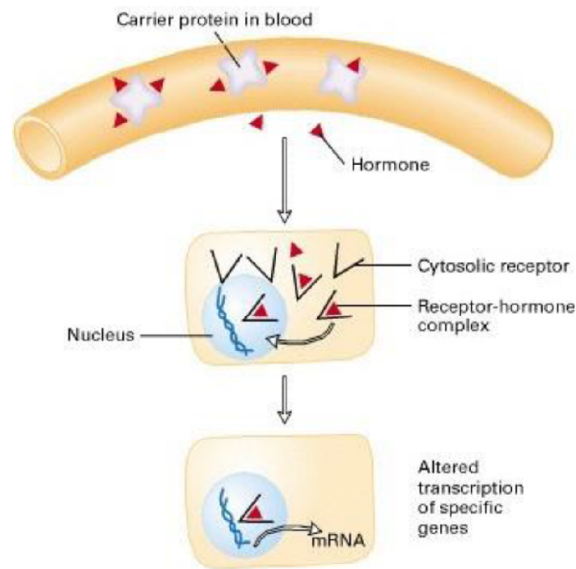


Figure 2.8 The intracellular receptor altering gene expression (Lodish et al, 2000).

Intracellular receptors refer to receptors that function in cytoplasm or nucleus. They are activated by lipid soluble signal molecules whose size is small enough to pass through the transmembrane. This type of receptor can induce a broad spectrum of cellular response; for example, some are involved in gene regulation in eukaryotes. As can be seen in figure 2.8, transport of steroid hormones are facilitated by carrier proteins in the blood. After dissociation from these carriers, steroid hormones pass through the target cell's plasma membrane *via* diffusion and subsequently bind to the intracellular receptor, either in cytoplasm or in the nucleus. The receptor-hormone complex then binds to the sequence-specific DNA fragments, which instigate gene transcription (Lodish et al, 2000).

2.2 Qualitative behaviour of signalling networks

Cellular processes depend on information processing networks. These are intricate protein and genetic networks, processing the vast array of information about the cell's environment, allowing cells to make appropriate responses/decisions. They range from simple networks to those with high levels of complexity (e.g. regulatory feedback/feed-forward processes). Such complexities enable the networks to exhibit diverse types of complex dynamic behaviour which cells use for specific purposes. This section briefly summarizes certain qualitative behaviours ubiquitously seen in cellular signalling networks. These include switch-like behaviour, adaptive behaviour and oscillatory dynamic behaviour.

2.2.1 Switch-like behaviour

2.2.1.1 Ultrasensitivity (monostable switching)

In certain situations, cells require a highly sensitive and therefore rapid response to upstream stimuli, rather than a gradual response, to regulate their functions/activities. The term “ultrasensitivity” refers to a nonlinear signalling behaviour where a relatively small change in input gives rise to a dramatic change in output (as shown in figure 2.9a). The curve of ultrasensitive response has a threshold and steep response: it can be conceptualised as an electronic switch in the sense that the signalling systems can transition from the “off”-state to the “on”-state.

This form of signal processing is reversible and is observed in a number of signal transduction pathways containing ultrasensitive motifs. One example of these motifs, a ubiquitous means by which common protein activities are regulated, is the zero-order

covalent modification cycle, the unmodified and modified substrate is interconverted by a pair of opposing enzymes (such as kinase/phosphatase). If both enzymes operate near saturation, minimal changes in the ratio of the opposing enzymes leads to nontrivial changes in the number of modified substrates. Other ultrasensitive motifs include positive cooperative binding, homo-multimerization, molecular titration and multi-step signalling (Buchler and Cross, 2009; Zhang et al., 2013). All these examples are of interest as they show how the mechanism (by itself) can generate qualitative behaviour – the behaviour arises from internal protein-protein interactions without complex regulation such as feedback or feed-forward regulation. Ultrasensitivity is significant for signal amplification in many pathways (i.e. Janus kinase (JNK) signalling cascade (Bagowski et al., 2003) and the mitogen activated protein kinase (MAPK) cascade (Huang and Ferrell, 1996)) and is considered to be a necessary prerequisite for other kinds of qualitative behaviour (Ferrell and Ha, 2014).

2.2.1.2 Bistability

Bistability is associated with complexities which results in more sophisticated switch-like behaviour. It is a common property of cell signalling and is an example of irreversible switching in biochemical networks, playing an important role in many biological contexts.

Unlike a monostable switch, a bistable switch is either completely or partially reversible. There is region of input where the system has two stable steady states (figure 2.9b). It is believed that the basic ingredient to achieve bistability is positive feedback. Recently, however, it has been reported that systems without feedback are capable of exhibiting bistability. These include simple covalent modification systems where protein substrates share the same catalysing enzymes (Feliu and Wiuf, 2012) and where converter enzymes are regulated in a reciprocal manner (Straube and Conradi, 2013). Bistable switches are thought to be essential for irreversible cellular decision making. For instance it provides

‘check point control’ in cell cycle transition in eukaryotic cells (Verdugo et al., 2013). It also plays important roles in modulating memory and plasticity in cellular differentiation (Wang et al., 2009) and in decision-making processes in the cell apoptosis (Trotta et al., 2012; Ho and Harrington, 2010).

2.2.2 Adaptation

Cells use adaptive signalling to adapt to persistent perturbations from their surrounding environments. Many biological systems can generate adaptive signalling —the ability to reset themselves following a step change in input. In perfect adaptation, a steady-state output is constant irrespective of steady-state input: although the output makes change transiently, it returns to the same steady state. Basically, the adaptive feature involves converting a step input into a pulse output. There are multiple modes of adaptation, ranging from perfect adaptation to partial adaptation (under adaptation or over adaptation).

Adaptation is seen in a number of cellular contexts and can be achieved through networks with feedback processes. For instance, adaptation to osmotic stress conditions in yeast is achieved *via* the MAPK pathway coupled with negative feedback regulation (Hao et al., 2007). In this case, a downstream component phosphorylates an upstream receptor, leading to desensitization of the pathway. In addition, adaptation is also seen in networks with feed-forward regulation such as chemotactic signalling systems (Levchenko and Iglesias, 2002) and sensory systems.

2.2.3 Oscillation

Oscillations are important signal processing characteristics in which information is processed and encoded in terms of periodic change in the system with time. This is one of the most obvious properties of life. For example it is observed in circadian rhythms (in, for example, respiration, heart beat and brain rhythmic) (Zhang and Kay, 2010). Cell cycle oscillators guarantee that the appropriate order of specific stages of development in cell division cycle is sustained: thus DNA replication happens and occurs only once before chromosome segregation per cell cycle (Pomerening et al., 2003).

Although it is not clearly understood, one of indispensable core ingredients for generating oscillatory control is through the negative feedback effect (with time delay). For example, it has been shown that a single negative feedback loop in a three layer MAPK cascade can induce oscillatory dynamic behaviour in enzyme activities (Kholodenko, 2000). In real systems, networks are often coupled with complexities which affect the behaviour of the negative feedback oscillator, for example, ERK pathway with both positive and negative feedback loop (Matallanas et al., 2011). In general there are many factors affecting the oscillatory behaviour such as ultrasensitivity, coupling of feedback, a number of intermediate reactions and spatial effects (Lenz and Sogaard-Andersen, 2011).

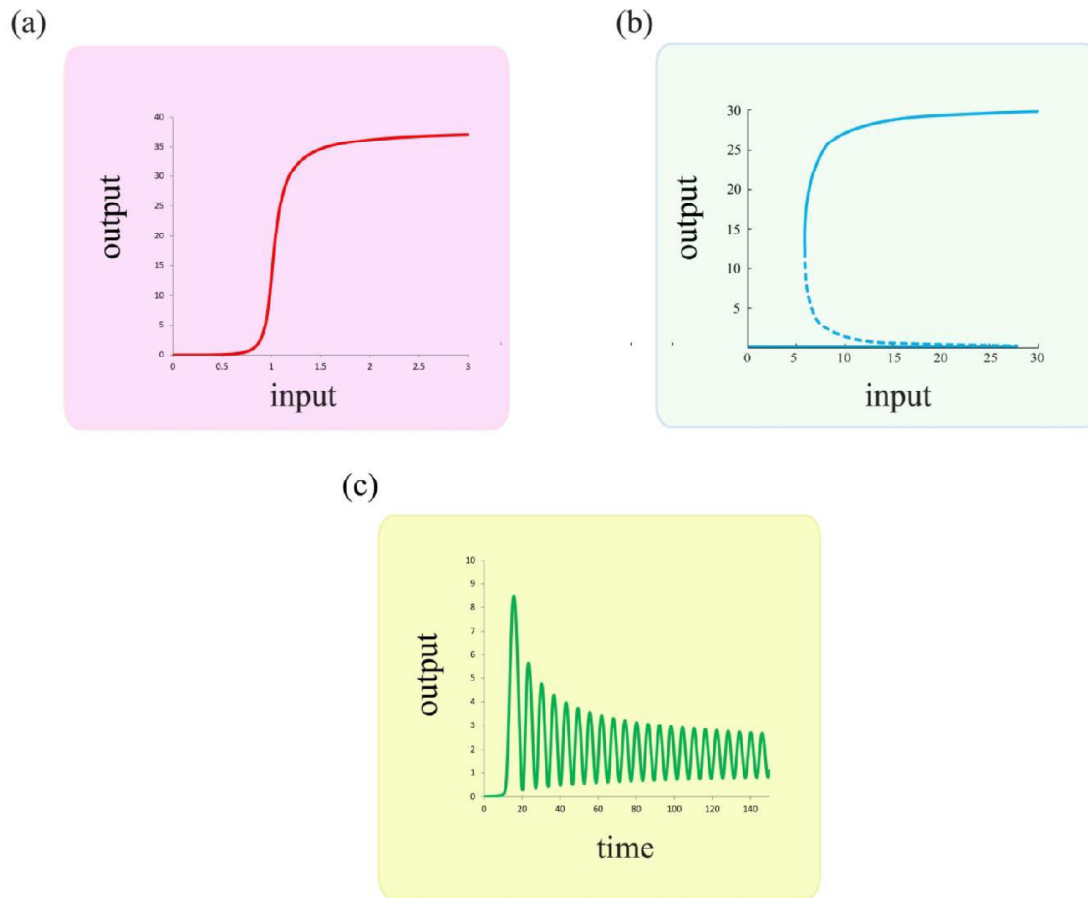


Figure 2.9 Different kinds of qualitative behaviour playing important roles in cellular processes. (a) ultrasensitivity, (b) bistability and (c) oscillation

2.3 Multi-site phosphorylation

2.3.1 Background

Cells are dynamic entities – they sense and respond to external stimuli continuously for their functioning and survival. The cellular response to environmental change is often triggered by various types of posttranslational modifications (PTMs); this describes a reversible process whereby two opposing enzymes modify their substrates by adding/removing functional groups to make them suitable for regulatory purpose. These include acetylation, methylation, ubiquitilation, hydroxylation and phosphorylation. Among these PTMs, phosphorylation is the most extensively characterised by both experimental and modelling studies (Humphrey et al., 2015).

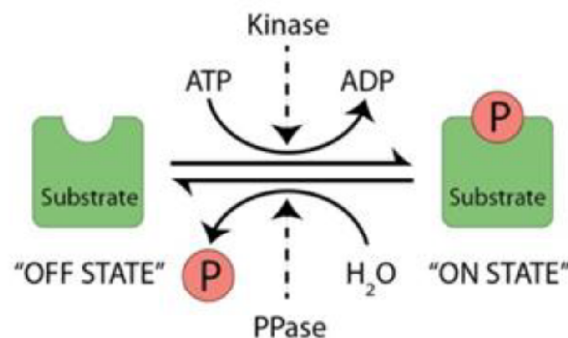


Figure 2.10 Schematic representation of reversible phosphorylation/dephosphorylation

Reversible phosphorylation is one type of covalent modification regulating signalling in both prokaryotic and eukaryotic organisms. It works as a “modulator” of protein function and is a basic element in a number of cellular signalling networks. Phosphorylation refers to the transfer of phosphoryl groups from a donor molecule (ATP) to residues of a receptor

molecule (target protein), rendering proteins more active. While kinases catalyse the attachment of a phosphate to substrates, enzymes called phosphatase (PPase) activate the removal of a phosphate group (dephosphorylation) *via* hydrolysis. More than half of all proteins in a body exists either in phosphorylated molecule (on state) or dephosphorylated molecules (off state). For this reason, this control mechanism is called the “molecular switch” (Ishiai et al. 2008). Experimentally the importance of phosphorylation for regulating proteins was firstly reported in an *in vitro* study of rat liver mitochondria by Kennedy in 1954 (Burnett & Kennedy, 1954). His work has sparked a great deal of research interest in the role of this covalently modified mechanism in controlling protein function.

Nearly all aspects of cellular processes are associated with phosphorylation. It is believed that almost a third of human proteins are covalently modified by phosphorylation on at least one active site, either transiently or permanently (Cohen, 2002). Most phosphorylated proteins possess several docking sites for kinase to act on. The process whereby proteins are phosphorylated at more than one location is referred to “*Multisite phosphorylation*”.

2.3.1.1 Types of multi-site phosphorylation

Although multisite phosphorylations in multiple systems vary, the basic principle of operation and the core elements are the same. Based on enzyme processivity, multi-phosphorylation is broadly categorised as processive and distributive (see figure 2.11). Processive systems require just one enzyme-substrate binding event to complete full multisite modification while distributive employs one enzyme-substrate binding event for single site modification. Mixed mechanisms which combine features of these two scenarios are also possible.

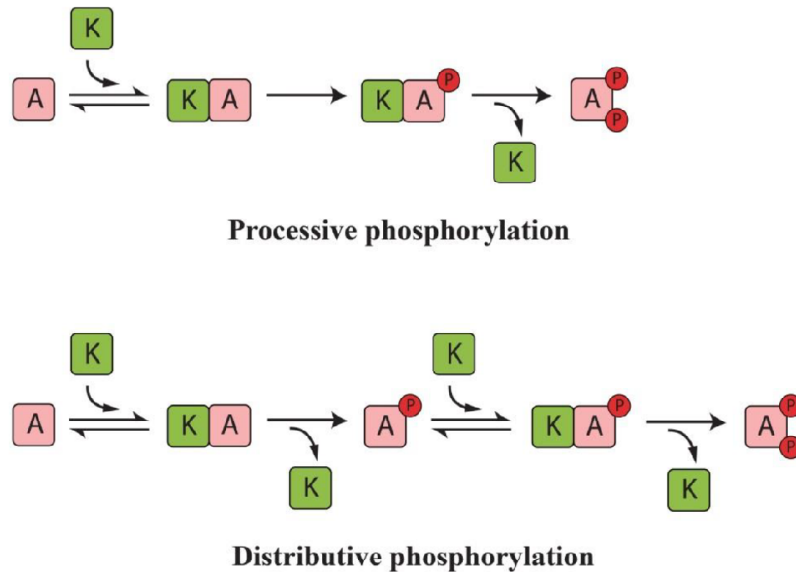


Figure 2.11 Multisite phosphorylation mechanisms based on enzyme processivity.

In addition, multiple variants of these mechanisms exist: sequential, random and cyclic mechanisms. The difference among these three mechanisms lies in the order of phosphate group addition/removal to/from the substrate. In the sequential mechanism, phosphorylation follows a fixed order in that the last site phosphorylated by kinase is the first dephosphorylated by phosphatase. The cyclic mechanism has the same order of phosphorylation as the sequential mechanism whereas the order of dephosphorylation is the reverse. The random mechanism is the most complicated in that given proteins can be modified in an apparently random basis regardless of the phosphorylation state of the other sites. Difference in the phosphate processing leads to different qualitative signal processing characteristics. Combinations of different orders of phosphate transfer have also been observed experimentally.

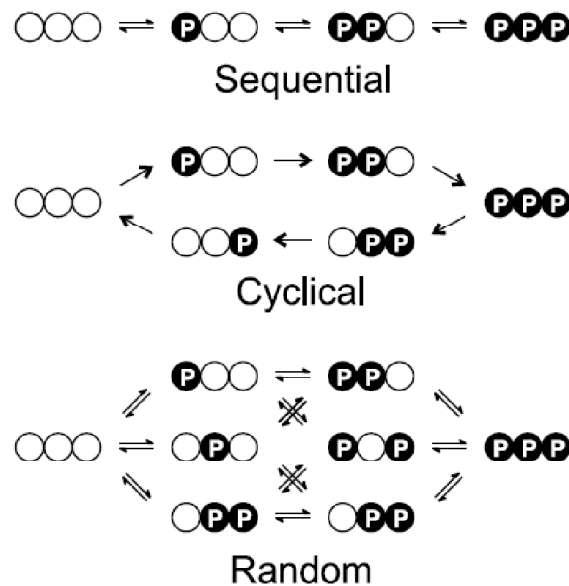


Figure 2.12 Multiple variants of multisite phosphorylation mechanisms based on order of phosphate processing (Salazar & Hofer, 2007).

Multisite phosphorylation is a ubiquitous building block of signalling networks, found in almost all organ systems and in most cell types, ranging from those found in bacteria, plants, and animals to humans. In eukaryotes, proteins are reversibly phosphorylated on the serine (Ser), threonine (Thr) and tyrosine (Tyr) residues. Experimental evidence shows that eukaryotic proteins can be multi-phosphorylated at as many as 100 sites, allowing for multiple regulations.

Multisite phosphorylation is of great significance since it is the way a number of cellular processes are controlled. It plays important roles in many contexts such as protein translocation, protein degradation and signal integration. Depending on the location and number of modifications, proteins behave differently. For instance, protein Sic1 must be multi-phosphorylated at at least six sites during the G1/S Transition of the cell cycle so that it can be degraded and the S phase happen in an appropriate time, which is referred to as a

multi-site switch (figure 2.3). Overall, multi-site phosphorylation is not just activating protein activity but also making protein suited for multi-specific purposes. Interesting examples of phospho-proteins are summarised in Table 2.1

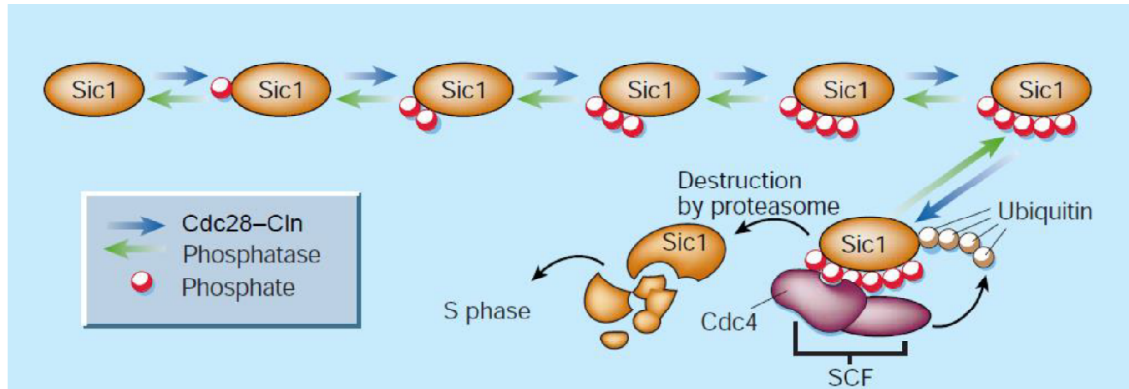


Figure 2.13 Protein degradation in Sic1, triggered by multisite phosphorylation. Sic1 must be modified at least six times so that it can be destroyed and the S phase occur at an appropriate time (Ferrell Jr, 2001)

Table 2.1 Examples of multisite phosphorylation events.

Phospho-protein	Biological Role	The number of phosphorylated sites
Tumor protein 53	The “cellular gatekeeper” of genome integrity that helps prevent the genome mutation which is the main cause of cancer formation (Strachan and Read, 1999; Levine, 2000; Bazigou et al., 2007)	16 (Holmberg et al., 2002)
Epidermal growth factor receptor	The anti-cancer drug target	10 (Schulze et al., 2005)
Hyper-phosphorylated microtubule-associated protein tau	The cause of Alzheimer’s disease, so tau is considered as a therapeutic target	39 – 45 (Hanger et al., 2009)

2.3.2 Signal transduction in single-site modification cycle

In this section, some basic results of existing *in silico* studies in single-site substrate modification are summarised. This includes the single-site modification module in isolation, which is then expanded to include additional regulation.

Single-site covalent modification cycles (CMCs), such as phosphorylation, acetylation, methylation and ubiquitination, are the building blocks of many regulatory networks in biological systems. An important qualitative aspect of the intrinsic information processing capabilities of such CMCs was theoretically demonstrated in (Goldbeter and Koshland, 1981), showing that CMCs is capable of generating zero-order ultrasensitivity (highly sigmoidal response behavior). This qualitative behaviour is occurred under enzyme saturating conditions. Ultrasensitivity is important as it is a pre-requisite for signalling networks to generate bistability and sustained oscillations (Ferrell and Ha, 2014). It was experimentally observed in several enzyme systems (*in vitro* study) (LaPorte and Koshland, 1983; Meinke et al., 1986; Huang and Ferrell, 1996).

In biological systems, bistability is thought to be essential for irreversible cellular decision making (Ozbudak et al., 2004). It has been known that CMCs by itself cannot generate such behaviour. Generally, a pre-requisite for single-site mechanism to generate bistability is positive feedback regulation (Kholodenko, 2006). However, numerous *in silico* studies have recently been reported that CMCs without positive feedback regulation are capable of exhibiting bistability. These include simple covalent modification systems where protein substrates share the same catalysing enzymes (Feliu and Wiuf, 2012) and where converter enzymes are regulated in a reciprocal manner (Straube and Conradi, 2013). In (Feng et al., 2016), it has been shown that bistability can exist in CMCs *via* multi-state

enzymes (figure 2.14c). They demonstrated theoretically that bistability arises due to a consequence of competition between the two states of the enzyme for their substrates.

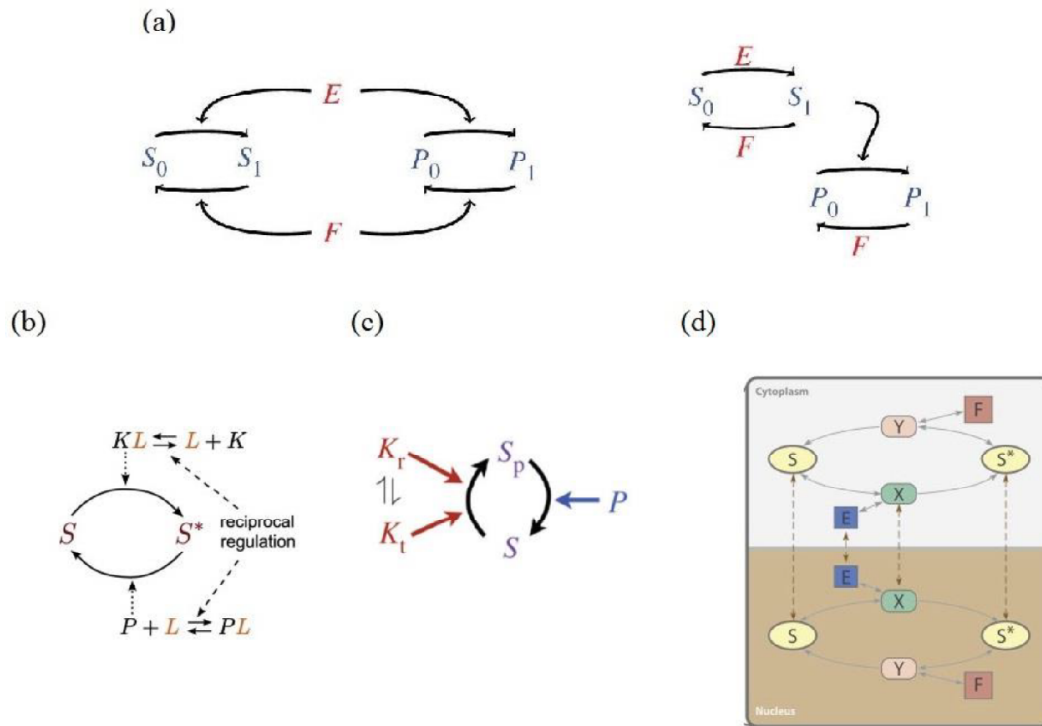


Figure 2.14 Additional regulation which enables single-site phosphorylation to generate bistability. (a) enzyme sharing. (b) reciprocal enzyme regulation, (c) multi-state enzymes and (d) cellular compartments. (image adapted from (Feliu and Wiuf, 2012; Straube and Conradi, 2013; Harrington et al., 2014; Feng et al., 2016))

Recently, the work of Harrington et al (2014) has theoretically investigated how cellular compartments influence information-processing capacities in covalent modification cycle. In their study, the single-site enzymatic reactions are assumed to occur in both the nucleus and the cytoplasm, with both substrate and enzyme shuttling reactions between these two compartments in the proposed model (see figure 5.14(c)). Their results have shown the

way in which the compartmentalization increases the number of stable steady states which can become simultaneously accessible, conferring greater flexibility and plasticity to such systems (Harrington et al., 2014).

A number of *in silico* studies focus on cascades of single-site modification (Ortega et al., 2002; Thattai and Oudenaarden, 2002; Blüthgen et al., 2006). Such studies demonstrated that the cascades have nontrivial effects in signal transduction in terms of signal amplification, noise reduction, accelerating the speed of the response and introducing time delay. In addition, the effect of retroactivity in single-site modification cascades has also been investigated in (Ossareh et al., 2011), showing that phosphorylation cascades are robust to retroactivity and the length of the cascade plays important role in attenuating the retroactive effect. The retroactive effect on phosphorylation cascades was also theoretically investigated in (Ventura et al., 2008). They showed that the cascades can exhibit damped temporal oscillations and this effect was treated as a “hidden feedback” to the upstream.

2.3.3 Intrinsic behaviour of multisite phosphorylation

Although phosphorylation was discovered for almost 60 year, *in silico* studies on multisite phosphorylation has been very active in the mid-2000s. This part will briefly discuss on the modelling work involving intrinsic capability of multisite phosphorylation in generating qualitative dynamic behaviour

Ultrasensitivity

The first report on ultrasensitivity in multisite phosphorylation has been published by Gunawardena in 2005. He first demonstrated theoretically that the multi-phosphorylation can generate good threshold behaviour but cannot function as a sharp switch (Gunawardena, 2005). His research focuses on an ordered, distributive mechanism. When a threshold is crossed, the response increased in a hyperbolic manner.

Salazar & Hofer (2007) theoretically analysed the information processing in multisite phosphorylation systems. Their work covers different mechanisms (ordered, random and cyclic mechanisms). They found that the response curve in sequential mechanism is steeper than that of random mechanism; however, the response kinetics of sequential phosphorylation is slower than those of random mechanism. This lies in the fact that there are more phosphorylation routes in random mechanism.

Recently, the modelling work of Liu et al (2010) looked at how the substrate sequestration influences the steady-state property of multisite phosphorylation. To this, they proposed the model incorporating distributive multisite phosphorylation and substrate sequestration. In this study, in order to prove that multisite phosphorylation can generate ultrasensitivity, the developed model was based on the assumption that opposing enzymes operate under unsaturated condition. The authors concluded that the key property of multisite

phosphorylation which gives rise to good thresholding and switching is that both unphosphorylated and fully phosphorylated proteins must be sequestered (as shown in figure 2.15).

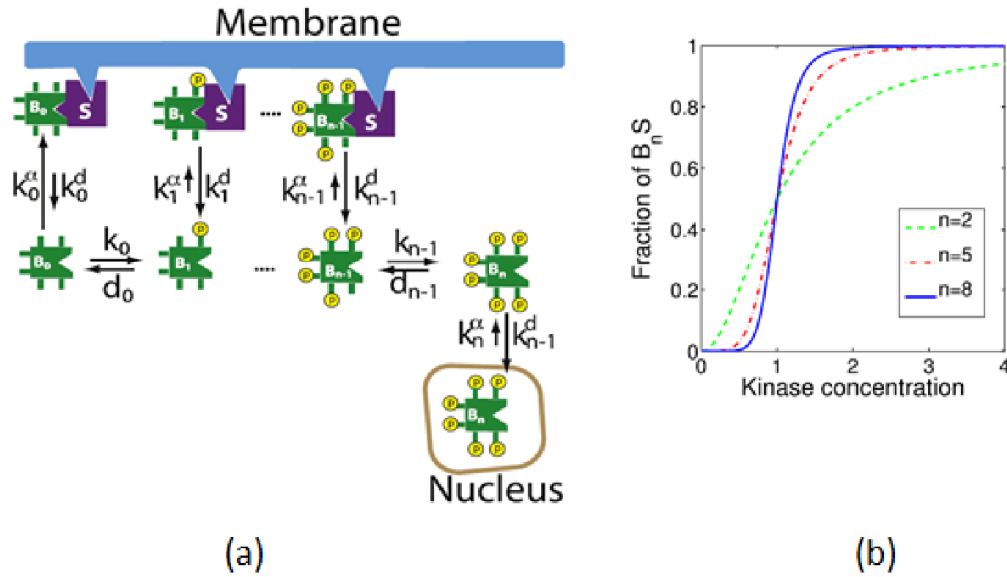


Figure 2.15 Multisite phosphorylation model proposed by Liu et al (2010). (a) Model. (b)

Dose response curve showing ultrasensitive behaviour arises from sequestration

Bistability

It was Markevich et al (2004) who first performed an *in silico* analysis of the intrinsic information processing capability of multisite phosphorylation, showing that simple ordered, double distributive phosphorylation per se can induce bistability (Markevich et al., 2004). In addition, their work also focused on the double phosphorylation of ERK (the sub-unit module of MAPK pathway), in which kinase (MEK) modifies substrate randomly while phosphatase (MKP3) dephosphorylates the phospho-forms as shown in figure 2.15. Their computational work demonstrated that dual phosphorylation of ERK is capable of generating bistability for realistic parameter values retrieved from experimental data.

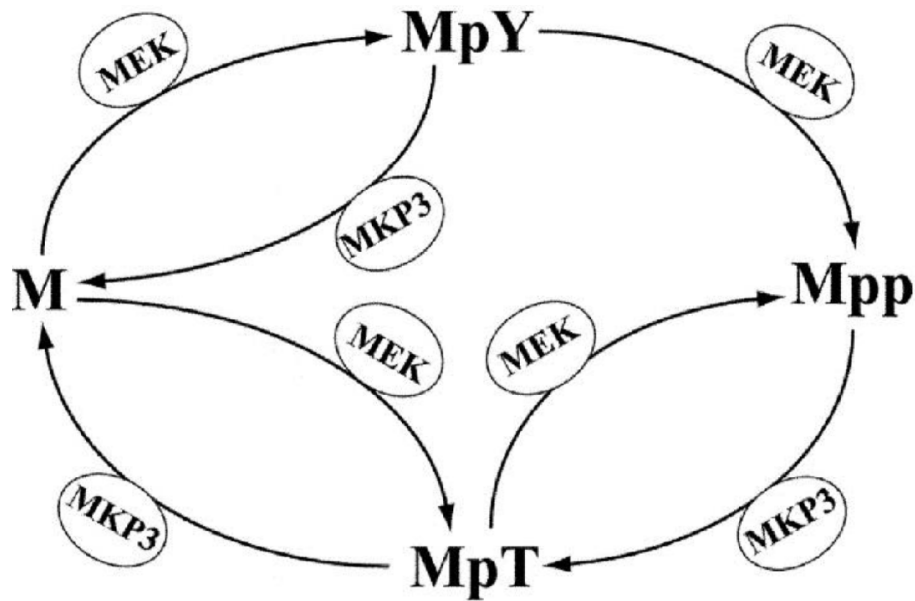


Figure 2.16 Double distributive phosphorylation cycle of ERK (Markevich et al., 2004).

A related theoretical modelling work was published by Ortega et al in 2006 showing how the multi-site phosphorylation can generate bistability per se. In this study they investigated the condition where double distributive phosphorylation can exhibit bistability. They concluded that in the absence of feedback loops double phosphorylation can exhibit bistable switching if:

- Substrate is doubly phosphorylated or dephosphorylated by the same enzyme.
- At least one modification step operate within the zero-order regime.
- The ratio of the catalytic constant of the phosphorylation and dephosphorylation step in the second modification cycle is higher than this ratio in first cycle.

The prerequisite condition for generating bistability in ordered, double distributive phosphorylation has theoretically been analysed in (Conradi and Mincheva, 2014), showing that differences in catalytic constants enable the emergence of bistability in double phosphorylation cycle. Furthermore, Thomson & Gunawardena (2009) demonstrated theoretically that multi-phosphorylation systems are capable of showing unlimited multi-stability, and that as the number of sites increases, the number of realizable stable steady states also increases.

Oscillations

So far, the ability of sequential (ordered), double distributive phosphorylation to exhibit sustained-oscillations has not been reported in the literature. It has been demonstrated theoretically that the Michaelis-Menten approximation of the sequential, double distributive phosphorylation does not admit oscillations (Bozeman and Morales, 2017). As information can be lost in exchange for ease of algebra in Michaelis-Menten approximation, to rule out the oscillatory behaviour in sequential, double distributive phosphorylation requires future theoretical work.

While oscillatory behaviour in ordered distributive phosphorylation has not been reported in the literatures, there is *in silico* work showing that double phosphorylation can generate sustained oscillation where enzymes modify their substrates in a random manner. Jolly et al (2012) demonstrated that a random two-site distributive mechanism is capable of exhibit oscillations, for specific combinations of parameters. A strong kinetic asymmetry (in the cycle) in the random mechanism was identified as a key ingredient for oscillations

2.4 Open questions

Surveying the landscape of work in the literature, one important point to note is that all these published papers deal with the complexity of multisite phosphorylation in isolation. All of these studies were mainly centred on the intrinsic information processing capability of multisite phosphorylation wherein the modification mechanisms are purely distributive or processive and substrates are modified by the same pair of enzyme.

While these studies have provided an important platform for understanding different aspects of multi-site phosphorylation, there is a significant gap in understanding the role of multisite phosphorylation and its role in signalling pathways. This leads to a number of questions: (i) Are there other qualitative behaviours which can be exhibited by purely distributive or processive mechanisms, in addition to those already demonstrated in the literature? (ii) What is the effect arising from interplay between distributive and processive mechanisms when substrates are modified by “mixed mechanisms”? (iii) How do these multisite modification mechanisms function in signalling pathways, and what is the interplay between the multi-site phosphorylation module and the pathway/network? (iv) How does the presence of multiple kinase/phosphatases affect the internal dynamics of multi-site phosphorylation systems?

2.5 Aim and objectives

As multi-site phosphorylation is a basic building block of a number of biological networks, a systematic understanding of information processing of various aspects of the multi-phosphorylation will provide basic insights into a number of cellular processes. The aim of this thesis was to bridge the gap between the current understanding of multisite phosphorylation and its role in signalling pathways. The primary focus of this thesis was on investigating qualitative information processing capabilities of multi-site phosphorylation module in isolation, on one hand and their presence in cell signalling pathways on the other hand. The work was based on numerical simulation, numerical bifurcation analysis and analytical work. The aim of this thesis will be achieved by addressing the following four objectives:

- (1) Investigating new information processing capabilities in purely distributive and processive phosphorylation.
- (2) Investigating information processing capabilities in mixed mechanisms of multi-site phosphorylation.
- (3) Investigating the effect of the pathway/networks on internal dynamics of multi-site phosphorylation in order to bridge the gap between the module in isolation and as part of a signalling pathway/network
- (4) Investigating information processing capabilities of multi-site substrate modification through multiple enzyme systems

2.6 Outline of the thesis

The results in this thesis are organized as follows:

Chapter 3 focuses on examining simple distributive and processive mechanisms and examining different aspects of the dose response curve in multisite modification in detail. This chapter mainly focuses on the capability of multi-phosphorylation to exhibit biphasic dose response.

Chapter 4 focuses on examining in detail the effect of the interplay between distributive and processive mechanisms leading to mixed mechanisms. This study is an investigation of more complex combinations of mechanisms and their effect of information processing.

Chapter 5 focuses on a number of aspects of bridging the gap between the enzymatic modification module in isolation and as part of a signalling pathway. These aspects are explicit activation, retroactivity, open systems and embedding enzymatic module in networks. Studying this leads us to examine in detail how different aspects of the behaviour of the enzymatic module are affected by its presence in a signalling pathway, and conversely, how it affects the behaviour of the signalling pathway. This is done through a sequence of focused investigations adding up a comprehensive systems picture.

Chapter 6 focuses on information processing in multi-site modification through different enzymes. The effect of multiple kinases on multi-site phosphorylation through a sequence of scenario proceeding from the simple to the complex will be investigated.

Finally, the conclusions and recommendations for future work will be presented in chapter 7.

Chapter 3

Biphasic responses in multi-site phosphorylation systems

3.1 Introduction

Multisite phosphorylation systems represent a particular extension of the basic covalent modification cycle wherein the same pair of enzymes (kinases and phosphatases) may provide multiple modifications for the basic substrate. Thus, this may be seen as a concatenation of covalent modification cycles with the same pair of enzymes. The multi-site modification status of a protein has been referred to as a dynamic ‘molecular barcode’ (Patwardhan and Miller, 2007), controlling signal transduction and downstream effects.

This chapter focuses only on studying the case where modifications are effected by only one enzyme pair, whereas the ‘molecular barcode’ usually refers to modification by multiple enzyme pairs. While the same enzyme pairs may be involved in multiple modifications, there are qualitatively different molecular mechanisms by which such modifications may be effected. Thus, multi-site phosphorylation systems may be classified as distributive, wherein each modification requires a new enzyme binding event, and processive where a single binding can lead to a sequence of modifications (Salazar and Hofer, 2007). Mixed mechanisms that combine features of these two scenarios are also observed (Komeili and O’Shea, 1999). Even within distributive mechanisms, multiple variants such as ordered (Salazar and Hofer, 2009; Zhao and Zhang, 2001) and random (Deshaies and Ferrell, 2001; Burack and Sturgill, 1997; Wagner et al., 1985) mechanisms exist. These differences arise from what kinds and sequences of modifications are permitted: an ordered mechanism allows

only a specific order in the modifications, whereas a completely random mechanism allows any sequence of modifications.

Given their importance, multi-site phosphorylation systems have been the focus of a number of experimental and modelling studies. The experimental studies usually focus on the effect of multi-site modifications in specific contexts such as in cell cycle proteins and inflammation processes (Hanger et al., 2007; Luciano et al., 2003; Nash et al., 2001). Because proteins that are modified at specific locations can have different functions, there is naturally a special interest in the details, mechanisms and consequences of these multi-site mechanisms. There is also a considerable body of work on pathways such as the mitogen-activated protein kinase (MAPK) pathway in its various contexts (Pitzschke et al., 2009; Zhang and Liu, 2002; Kim and Choi, 2010). Recent experimental efforts aim to go beyond qualitative descriptions and quantify different modifications of substrates using tools such as mass spectrometry (Derouiche et al., 2012; Choudhary and Mann, 2010).

The wide-ranging applications and complexity of multisite phosphorylation have attracted a range of modelling and theoretical studies. Modelling ranges from studying different aspects of multi-site phosphorylation in individual contexts such as the MAPK and p53 pathways, to examining multi-site phosphorylation in cell cycle control and multi-site modification as a controller for protein degradation (Barik et al., 2010; Orton et al., 2005; Varedi et al., 2010). Other theoretical studies examine the consequences of different mechanisms, and the signal transduction capabilities of such systems (Manrai and Gunawardena, 2008). The ability of such systems to produce threshold and ‘ultrasensitive’ behaviour was the focus of a number of studies (Gunawardena, 2005). The ability of distributive systems to generate bistability was demonstrated in (Markevich et al., 2004). Subsequently, it was shown that multi-phosphorylation systems were capable of showing unlimited multi-stability, and that as the number of sites increases, the number of realizable

stable steady states also increases (Thomson and Markevich, 2009). The effects of other aspects such as sequestration were also examined (Liu et al., 2010; Zhu et al., 2010).

This chapter focuses on examining the intrinsic capability of multisite phosphorylation to exhibit biphasic (bell-shaped) and partial biphasic (henceforth referred to as biphasic) dose-response characteristics. To this end, multiple variants of multi-site phosphorylation mechanisms, to examine the mechanisms which give rise to this behaviour are examined. Numerical simulations, bifurcation analysis and analytical work are used to understand the origins of this behaviour and the factors which accentuate it. The models employed in this chapter are presented in section 3.2 and the results are discussed in section 3.3. Analytical results are briefly presented in section 3.4. The chapter is concluded with a synthesis and discussion of the relevance of the findings. An appendix discusses analysis of different variants of mechanisms discussed above in more detail.

3.2 Modelling and methods

Multi-site phosphorylation mechanisms are present in different contexts in signal transduction, sometimes combined with other factors. In this chapter, an *in silico* analysis is performed to examine the intrinsic behaviour of multi-site phosphorylation mechanisms, and use generic models for this purpose. There are a few variants of multi-site phosphorylation mechanisms that are relevant here. Herein, models of distributive multi-site phosphorylation are primary used, and processive mechanisms are discussed to serve as a contrast. In examining distributive mechanisms, one point to note is that multiple variants of distributive mechanisms also exist: completely ordered, completely random and other intermediate cases. Examination is performed on both ordered as well as random mechanisms.

By performing *in silico* analysis on fairly generic models, it is possible to understand intrinsic complexities of multi-site phosphorylation in a controlled manner, disentangling this from other complicating factors. Simulation results and analysis for ordered as well as random mechanisms are presented, considering double-site modifications for the most part. What happens if more than two different modifications may occur is briefly considered. This study is based on ordinary differential equation (ODE)-based models of multi-site modification. These models are based on a deterministic and lumped description of the multi-site modification process. This is done because the ODE models can be analysed effectively and transparently, and serve as a platform for examining additional issues such as stochastic effects. As the focus of this chapter is on a basic steady-state characteristic of multi-site mechanisms, ODE description in this study suffices.

Herein, the ODE models include standard kinetic descriptions of enzymes reversibly binding to substrates to form complexes, and the catalytic conversion of the complex to the modified substrate. A basic model is an ordered mechanism of double-site phosphorylation by a kinase–phosphatase pair. Thus, the same kinase is involved in both the first-site and the second-site modification, and the phosphatase acts to undo each of the conversions. All the elementary steps are described by mass-action kinetics. The model is thus a mathematical depiction of the scenario depicted in figure 3.1 with the assumption above. In the case of random mechanisms, the kinase may modify the unmodified substrate to two different singly modified substrates (with potentially different rates), and can convert each of these to the doubly modified substrate. The phosphatase can likewise act to reverse each of these modifications. These scenarios generalize in a straightforward way to an arbitrary number of modification sites. Both models represent generic descriptions of multi-site modifications and have been used repeatedly in the literature.

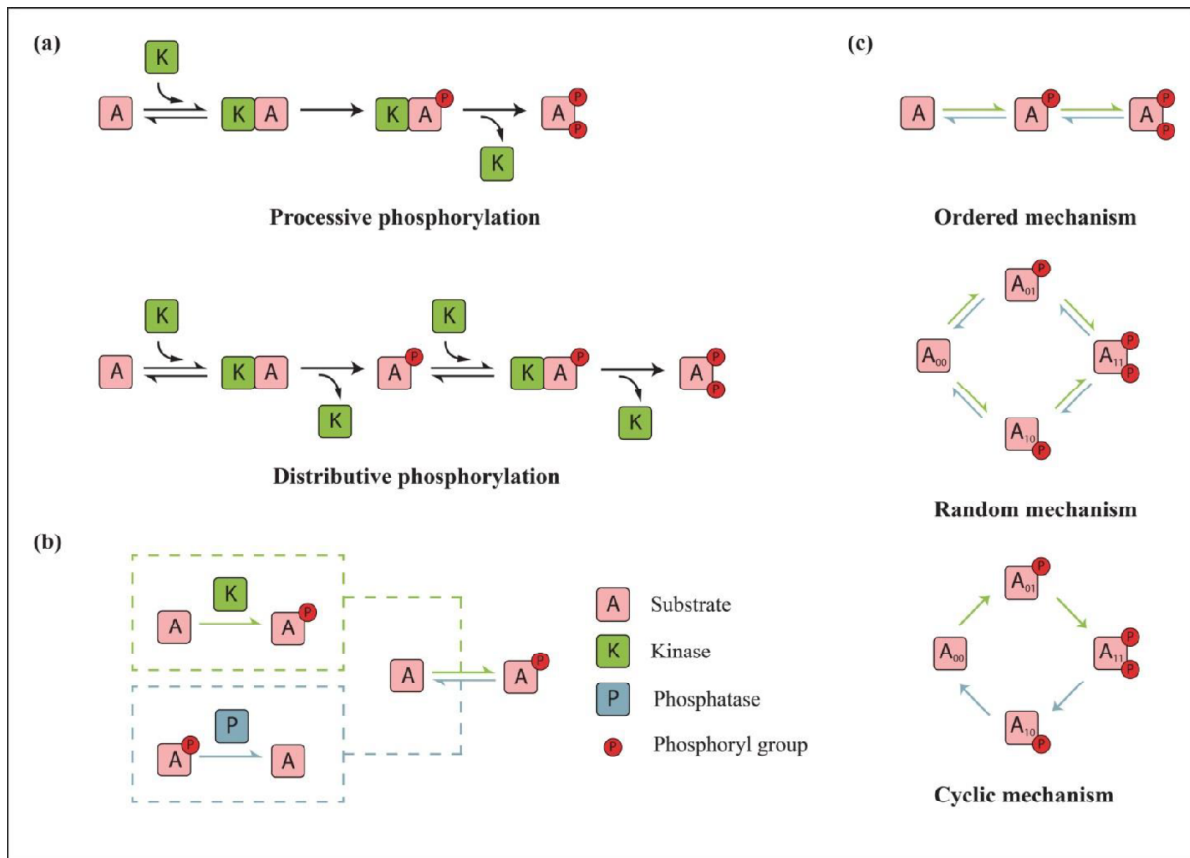


Figure 3.1 Schematic of models. Schematic of double phosphorylation model without additional regulation. (a) shows that based on enzyme processivity double phosphorylation can be dichotomized into a processive and distributive mechanism. In processive phosphorylation, only one enzyme–substrate binding event leads to full phosphorylation, conversely, each modification requires a new enzyme binding event in distributive phosphorylation. (b) shows the simplest model of the single phosphorylation cycle which is a basic building block of all mechanistic aspects of multi-site phosphorylation. A kinase (K) catalyses the phosphorylation reaction while a phosphatase (P) undoes phosphorylation. (c) shows the variety of distributive double phosphorylation structures based on order of phospho-site processing.

The model is discussed in a little more detail. The equations for a two-site-ordered distributive mechanism (figure 3.2d) are

$$\frac{d[A]}{dt} = k_2[AK] - k_1[A][K] + k_{12}[A_pP]$$

$$\frac{d[K]}{dt} = (k_2 + k_3)[AK] - k_1[A][K] + (k_5 + k_6)[A_pK] - k_4[A_p][K]$$

$$\frac{d[AK]}{dt} = k_1[A][K] - (k_2 + k_3)[AK]$$

$$\frac{d[A_p]}{dt} = k_3[AK] - k_4[A_p][K] + k_5[A_pK] + k_9[A_{pp}P] + k_{11}[A_pP] - k_{10}[A_p][P]$$

$$\frac{d[A_pK]}{dt} = k_4[A_p][K] - (k_5 + k_6)[A_pK]$$

$$\frac{d[A_{pp}]}{dt} = k_6[A_pK] + k_8[A_{pp}P] - k_7[A_{pp}][P]$$

$$\frac{d[P]}{dt} = (k_8 + k_9)[A_{pp}P] - k_7[A_{pp}][P] + (k_{11} + k_{12})[A_pP] - k_{10}[A_p][P]$$

$$\frac{d[A_{pp}P]}{dt} = k_7[A_{pp}][P] - (k_8 + k_9)[A_{pp}P]$$

$$\frac{d[A_pP]}{dt} = k_{10}[A_p][P] - (k_{11} + k_{12})[A_pP]$$

In this equation $[A]$, $[A_p]$, and $[A_{pp}]$ are respectively the concentrations of unphosphorylated, the singly phosphorylated and the doubly phosphorylated substrate, $[AK]$ and $[A_pK]$ describe the concentrations of relevant substrate–kinase complexes and $[A_pP]$ and $[A_{pp}P]$ describe the concentrations of the relevant substrate–phosphatase complexes. Such a model may be regarded as one describing a mechanism where the order of dephosphorylation is opposite to the order of phosphorylation. The equations for the free enzymes (kinase/phosphatase) are also shown. In the above model, $[K]$ and $[P]$ refer to the active kinase and phosphatase concentrations, respectively. In general, both kinases and phosphatases are subject to different control mechanisms and either of them may be activated

Figure 3.2 Depiction of multiple distributive and processive models. Schematic models of double phosphorylation with variation of order of phosphate processing. (a–c) present ordered-, cyclic and random processive phosphorylation models, respectively, whereas (d,e) present ordered- and cyclic distributive phosphorylation models, respectively.

(or made available) to trigger and control signal transduction. In this model, for the sake of simplicity, an explicit description of this activation is avoided and thus the enzymes represent active modification-capable enzymes. It is worth emphasizing that the model is a fairly generic model for describing ordered distributive multi-site phosphorylation. It is worth mentioning that the behaviour focused on here is readily observed in more complex models that describe explicit activation of the kinase by the upstream signal.

In this model, the total (active) kinase and phosphatase concentrations are parameters (they are conserved in the dynamical evolution of the system) and likewise the total substrate concentration is also a constant. In an n -site-ordered modification system (a generalization of the above model to include n modifications), there are thus n independent substrate concentrations, along with n kinase–substrate complexes and n phosphatase–substrate complexes. The random mechanism allows for all possible ordering of modification, and thus accounts for all possible partial phosphoforms and their dynamics explicitly, allowing for different modifications to take place at different rates. This naturally leads to a substantially more complicated kinetic model. In the random mechanism, there are $2^n - 1$ independent substrate concentrations (for all possible phosphoforms), and an equal number of kinase–substrate and phosphatase complexes. The equations for random two-site mechanisms and ordered n -site mechanisms are shown in the appendix A. One may also identify mechanisms which fall between the descriptions outlined above. One such mechanism is a ‘cyclic’ mechanism shown for two-site modification in figure 3.1. This is essentially an ordered mechanism where the order of phosphorylation and dephosphorylation is not the exact opposite of one another: thus, it is important to explicitly account for the different partially phosphorylated species. The kinetic model of this cyclic mechanism and its analysis is presented in the appendix A.

The model of multi-site modification is specified by the choice of mechanism and the parameters. The parameters include total concentrations of substrate and (active) kinase and phosphatase. Because both kinases and phosphatases may be subject to different control mechanisms and be seen in modifying different substrates, it is possible to encounter different relative amounts of these quantities. For purposes of analysis, the investigation starts with a scenario where the phosphatase concentration is less than the total substrate (the effects of varying total phosphatase concentration will be examined subsequently). Situations involving relatively limited amounts of phosphatase are seen, for instance, in the case of phosphatase Cdc14 in metaphase yeast cells and (depending on the level of stimulus) in the agonist-induced dephosphorylation of protein kinase isotypes (Hansra et al., 1999; Bouchoux and Uhlmann, 2011 al., 1999). The sharing of the same phosphatase between many signalling pathways, as well as other control mechanisms regulating phosphatase availability/activity (Virshup and Shenolikar, 2009) could also result in a scenario akin to this. The total (active) kinase concentration is a parameter which will be varied directly in the analysis. With regard to the kinetic parameters, analysis is performed, based on kinetic parameters where the enzyme–substrate binding and unbinding rates are equal, and normally choose the catalytic rate to be less than the binding and unbinding rates. This is similar to parameters used in the literature (Markevich et al., 2004; Salazar and Hofer, 2006; Conradi and Mincheva, 2014). Results focus on how biphasic dose–response characteristics may arise by varying certain parameters, and for the most part, the other parameters are kept fixed and in the background. Results do not depend strongly on the particular choice of auxiliary kinetic parameters.

Having chosen a representative choice of parameters (similar to others chosen in the literature), the way how the variation of certain fixed parameters may give rise to biphasic behaviour is examined. Bifurcation analysis, simulations and analytical approaches are used to understand this effect. Simulations were performed using both ODE15s in MATLAB and

COPASI for comparison. The equations are directly programmed in the former, while generated by the software automatically in the latter. This is complemented by bifurcation analysis in MATCONT. Finally, analytical work is used to examine the effects observed, and how it changes when other parameters are varied and when different enzymatic mechanisms are used. This multi-pronged approach allows us to see how the insights from different approaches add up to a consistent picture.

3.3 Results

Here, the computational analysis of the model is discussed. Complementary analytical work is presented in section 3.4. For the remainder of this chapter, enzyme concentrations refer to active enzyme concentrations. The focus is on studying the steady-state response of the distributive multi-site phosphorylation system as a function of the total kinase concentration, for the basal set of parameters chosen. An investigation begins with analysing ordered multi-site phosphorylation systems, and examining random mechanisms subsequently. For purposes of illustration, double-site modifications are discussed, and other aspects associated with the number of sites are briefly examined subsequently.

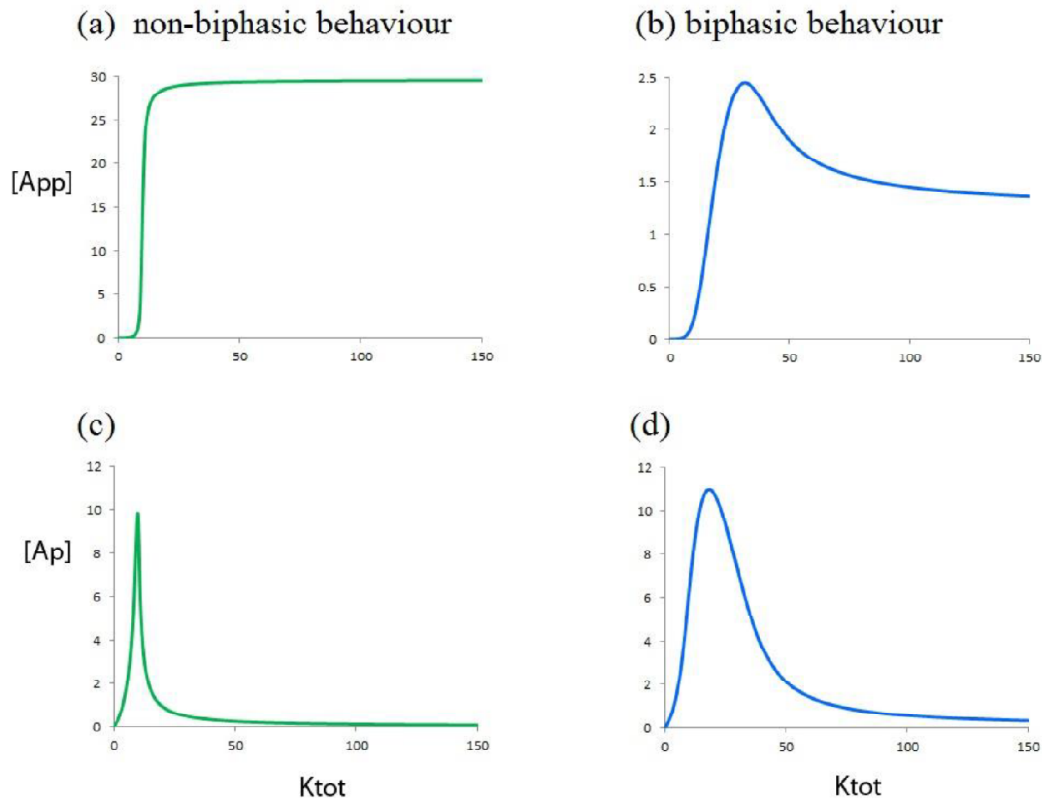


Figure 3.3 Steady-state dose–response curves in ordered distributive mechanisms.

Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , and partially phosphorylated substrate, A_p , to the total concentration of kinase, K_{tot} , when the catalytic rate constant of the first and second phosphorylation step is the same (a,c) and when the catalytic rate constant of the second phosphorylation step is smaller than the first step (b,d).

Figure 3.3 presents simulations showing how the maximally phosphorylated substrate concentration at steady state varies with total kinase concentration. Figure 3a shows that as the total kinase concentration increases, the concentration of the double phosphorylated substrate increases monotonically and asymptotes to its maximum level. The single phosphorylated substrate concentration exhibits a biphasic response: the initial increase at low kinase levels reaching a maximum, before a decrease at higher kinase concentrations. This is simply understood from the fact that the dominant effect at higher kinase

concentrations is the conversion of the single modified substrate to the double modified substrate. Examination is then performed on the same system for a case where the catalytic rate of the second phosphorylation step is reduced. In this case, it is found (figure 3.3b) that the concentration of the double phosphorylated form increases and appears to plateau off, but actually reduces for higher kinase concentrations. In other words, the maximally phosphorylated substrate exhibits a (partial) biphasic response to the input. This result is interesting as it demonstrates that a simple distributive multi-site phosphorylation mechanism is intrinsically capable of generating such a biphasic mechanism.

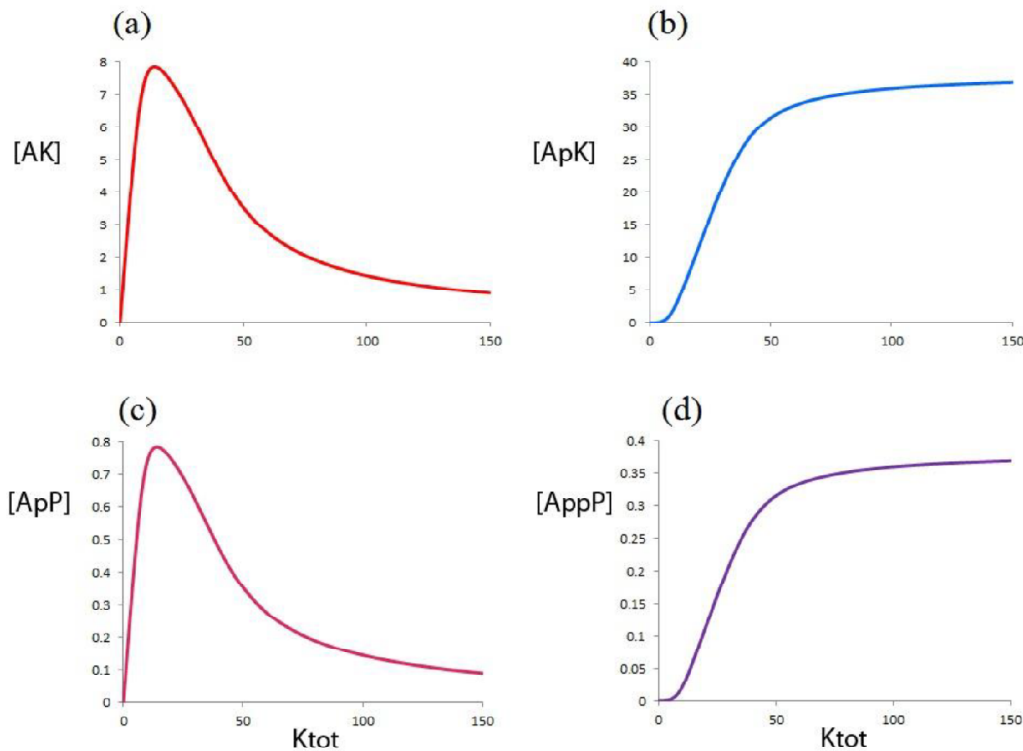


Figure 3.4 Dose-response curves: variation of complexes. Steady-state dose-response curve showing the concentration of four enzyme – substrate complexes as a function of the total concentration of kinase, K_{tot} , in the double-site-ordered phosphorylation network corresponding to figure 3.3b. (a,b) show the kinase – substrate complexes (AK and ApK , respectively), whereas (c,d) show the two phosphatase – substrate complexes (ApP and $AppP$, respectively)

Further insights into the origins of this behaviour can be obtained by looking at the variation of the four enzyme–substrate complexes as a function of total kinase concentration. This is shown in figure 3.4 which demonstrates that while both enzyme–substrate complexes of the first cycle are biphasic as expected, the complexes of the second cycle exhibit a monotonic variation, which saturates at high kinase concentration.

When the trends exhibited by the enzyme–substrate complexes are examined, it can be seen that those of the second are in qualitative contrast to the double phosphorylated substrate. In fact, it immediately raises the question: what causes the concentration of the double phosphorylated substrate to decrease? A closer examination of the results reveals that there are, in fact, two competing effects. An increase in the kinase concentration means more conversion of substrate into the kinase–substrate complex of the second cycle. However, this occurs at the expense of the single phosphorylated substrate. This conversion or ‘sequestration’ of the single modified substrate into the kinase complex now means that there is a reduction in the amount of the single modified substrate for the phosphatase to bind to. In other words, an increase of kinase leads not only to an increase of the A_pK complex which saturates, but also frees up phosphatase which might otherwise also bind to A_p . This increased availability of phosphatase now means that the extra phosphatase can bind to A_{pp} and degrade it more efficiently. This explains the origins of the biphasic behaviour. A close inspection of the graph reveals that the complex A_pP actually decreases quite substantially (in the region where the A_{pp} curve turns around), consistent with this picture. It is also worth pointing out that while a (relatively) low catalytic constant in the second cycle causes this biphasic effect, a low binding constant (of kinase to A_p) does not. This again is consistent with the explanation for the reason of the biphasic behaviour, as a low binding constant does not provide an effective ‘sequestration’ of substrate in the A_pK complex.

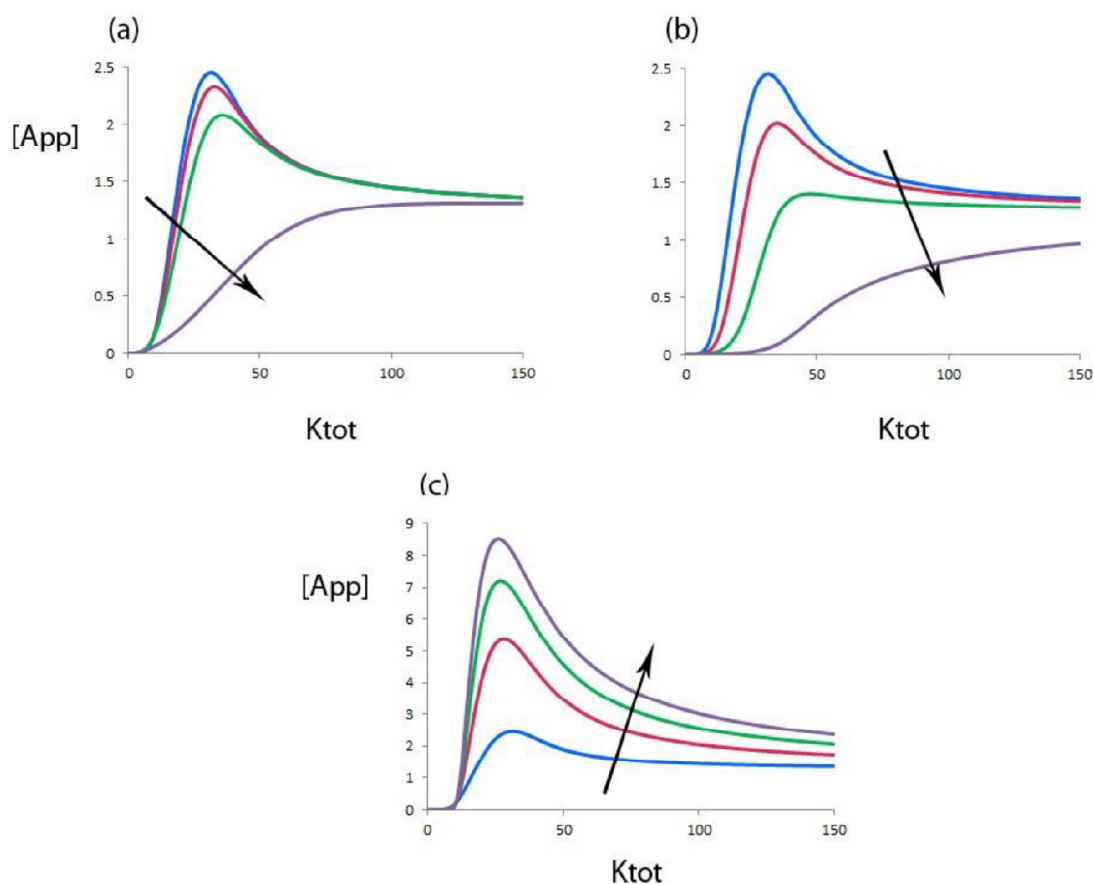


Figure 3.5 Effect of kinetic parameters. Effect of kinetic parameters on biphasic response behaviour for the fully modified substrate in ordered, double-site phosphorylation. (a) Decreasing the binding and (b) catalytic constant of the phosphorylation in the first cycle can diminish and lead to the abolishment of the biphasic response, while increasing the binding constant of the dephosphorylation of the first cycle can accentuate the biphasic behaviour. In (a,b), the black arrow denotes the direction of decreasing kinetic parameters, while the black arrow in (c) denotes increasing value of kinetic parameters.

After the origins of this biphasic behaviour have been investigated, the effects of different parameters on this response are then examined. Figure 3.5a,b shows the effects of varying the binding and catalytic constants of the first cycle. It is found that decreasing each

of these can lead to the abolishment of this biphasic response. The reason for this is that when these constants are low (and comparable with the catalytic constant of the second cycle), there is no longer a clear cut sequestration effect of substrate in the A_pK complex as before. On the other hand, increasing the binding constant of the dephosphorylation of the first cycle can strongly accentuate the biphasic behaviour (figure 3.5c) as it only more strongly accentuates the reduced availability of phosphatase. It is also found that reducing the dephosphorylation catalytic constant for the second cycle tends to diminish and abolish the biphasic behaviour (reducing the impact of the greater available phosphatase, by having significant phosphatase sequestered in the $A_{pp}P$ complex). Again, a reduction of the binding constant of the kinase to A_p tends to reduce or abolish the biphasic behaviour, as it reduces the relative effect of sequestration of substrate (and in particular A_p) in the complex A_pK .

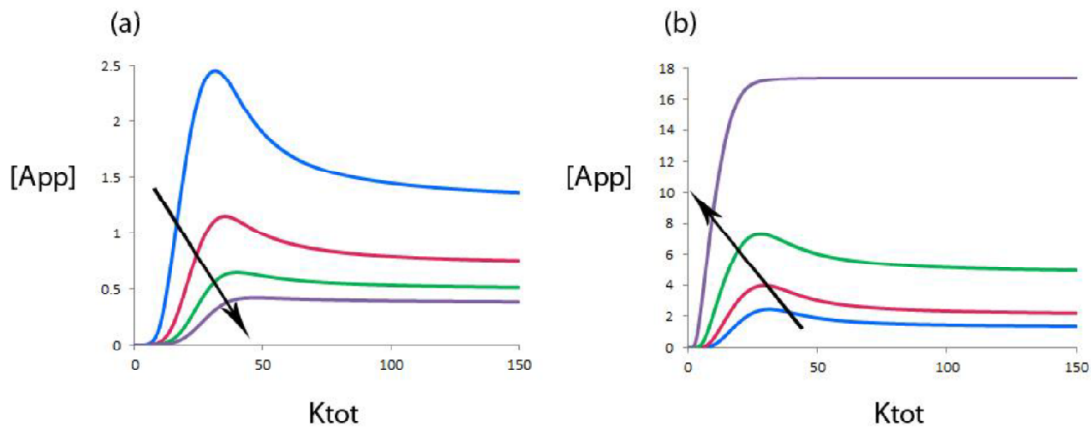


Figure 3.6. Effect of total phosphatase. Effect of the total concentration of phosphatase, P_{tot} , on biphasic response behaviour for the fully modified substrate in ordered, double phosphorylation. (a) Shows that increasing concentration of phosphatase can lead to abolishment of the biphasic response. (b) Shows that the biphasic response is abolished if the concentration of phosphatase is too low. The black arrow in (a) denotes the direction of increasing concentration of phosphatase, whereas the black arrow in (b) denotes decreasing size of concentration of phosphatase.

It is worth pointing out that while biphasic behaviour can be achieved by reducing the catalytic constant of the second phosphorylation, it can, in fact, be obtained when catalytic constants of both phosphorylation reactions are equal, but with a relatively high dephosphorylation rate constant of A_{pp} (results not shown).

The effect of the total phosphatase concentrations is now investigated. As seen in figure 3.6a an increased availability of phosphatase actually reduces or abolishes this biphasic effect, which is again consistent with the ‘release’ of phosphatase being a role in generating the behaviour mentioned above. It is worth pointing out, however, that if the available phosphatase is too low, then a biphasic behaviour is not seen (figure 3.6b). In fact, the explanation of the biphasic behaviour shows two competing effects, one being the strong enough effect of the release of phosphatase. If this is too weak, then the biphasic behaviour is not seen.

After the reasons for the biphasic behaviour in the ordered double phosphorylation mechanism have been clearly understood, it is interesting to examine whether such behaviour may occur in slightly more complex multi-site modification mechanisms. Figure 3.7a shows the results of analysis of a random double-modification mechanism. Here, the catalytic constant of only one of the end cycles is reduced. It is found that the biphasic behaviour is not only present, but in fact enhanced. Again, the effects of varying other kinetic parameters (in the relevant leg of the cycle) in this more complex system are broadly the same as the simpler ordered system. Because this random mechanism (with lowered catalytic constant of one of the second steps) can be thought of as a more complex version of the ordered mechanism, it is worth asking what the role of the extra pathway is. It is found that in the regime of kinase concentration where the biphasic behaviour occurs, very little of the intermediate modified substrate in the second cycle exists. Overall, numerical analysis shows that the biphasic characteristic is more pronounced, because of the presence of an extra pathway for the fully

phosphorylated substrate to be unmodified. It is worth pointing out that if the catalytic constants of conversion of both singly modified forms (A_{01} , A_{10}) are reduced, then a behaviour is very similar to an ordered mechanism.

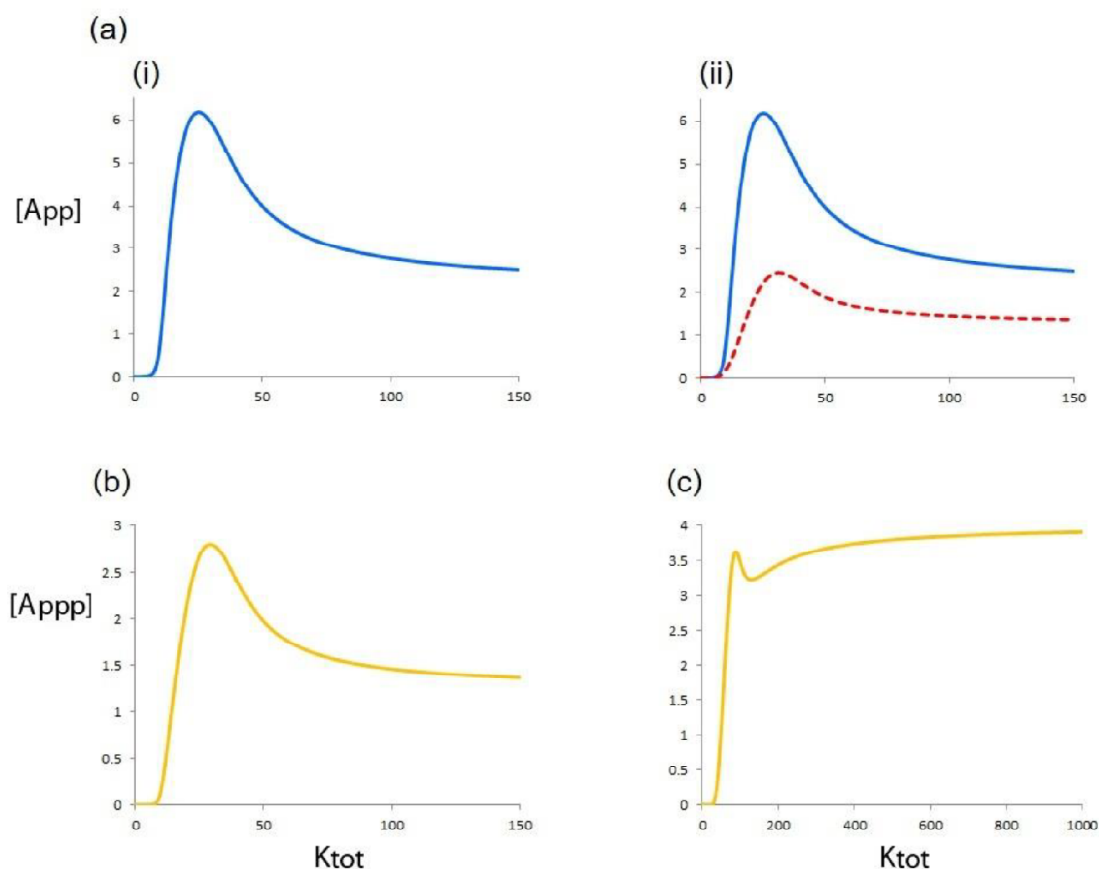


Figure 3.7 Random double-site and ordered triple-site phosphorylation. Steady-state input– output response curve of the concentration of maximally phosphorylated substrate in various multi-site phosphorylation networks. (a) Shows the biphasic response from random double phosphorylation in (i), whereas (ii) contrasts the biphasic response from random double phosphorylation with that from ordered double phosphorylation (dashed line). (b) shows biphasic response from triple ordered phosphorylation. (c) Shows that under certain conditions triple ordered phosphorylation leads to a limited biphasic response.

To study the effect of the number of sites, the investigation is then performed on another more complex version of the basic model that has been studied: an ordered triple phosphorylation system. As seen in figure 3.7b, this triple phosphorylated system can, indeed, also show the biphasic behaviour. The reason for this is very similar to that of the double phosphorylated system studied above. Furthermore, it is possible under some conditions (for example if the catalytic constants of the forward reaction of the last two cycles were reduced) that the maximally phosphorylated form exhibits a limited biphasic response, which reduces in a certain range before increasing again (figure 3.7c). Clearly, the presence of more sites can lead to more complex behaviour building on the basic mechanism which was analysed in the double phosphorylation system.

After studying the origins of this partial biphasic behaviour, an interesting question arises whether this could be combined with other qualitatively different features of signal transduction in multi-site phosphorylation. Figure 3.8a shows how the biphasic behaviour can be combined with a threshold behaviour characteristic of such systems, even in double-site phosphorylation systems. This demonstrates how the multi-site phosphorylation system can exhibit a complex dose–response curve comprising both thresholds and biphasic behaviour. Figure 3.8(b-c) also shows how both ordered triple and random double phosphorylation can exhibit bistable behaviour that can be combined with such biphasic behaviour. This clearly demonstrates how tuning parameters in the final cycle giving rise to/accentuating biphasic behaviour can be combined with other intrinsic complex features of signal transduction.

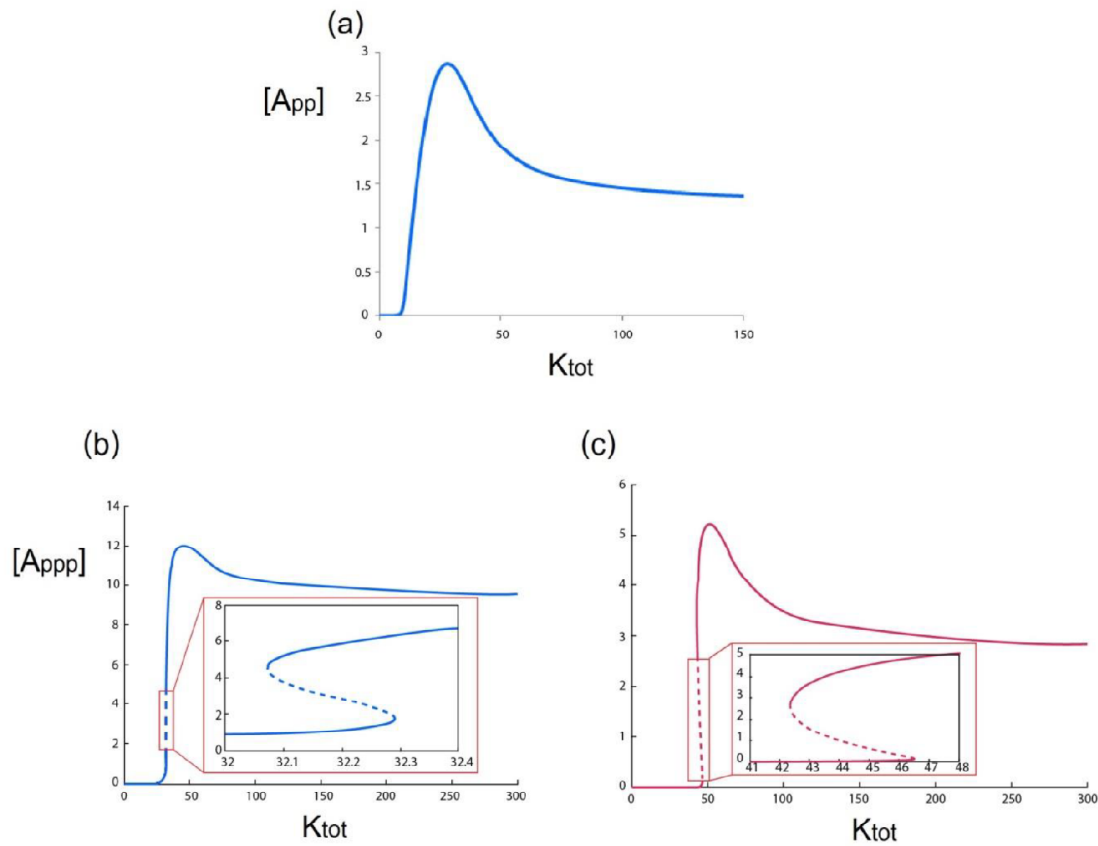


Figure 3.8 Combination of signal processing characteristics. The biphasic dose-response behaviour may be combined with other signal processing characteristics. (a) shows how threshold behaviour is combined with biphasic behaviour in ordered double phosphorylation. (b,c) shows how bistable behaviour is combined with biphasic behaviour in random double phosphorylation and ordered triple phosphorylation, respectively.

3.4 Model analysis

Section 3.3 presented an *in silico* analysis demonstrating the presence of hidden competing effects which give rise to biphasic dose-response characteristics. Here, numerical results are complemented with some analytical results (which is presented in (Suwanmajo and Krishnan, 2013) and reproduced in this section) that build on and consolidate the insights obtained. The main focus is on the ordered distributive model for two-site modifications.

It is worth examining a simpler case first. This simpler case is that of the same model where all the catalytic constants are high. In this case, there is negligible hold-up of enzyme in complexes, and each of the modification reactions can be described *via* mass-action kinetics. The analysis of this situation can be easily performed explicitly, noting that essentially all the enzyme is free (i.e. the free enzyme concentration is essentially the total enzyme concentration) and also that in the ordered model, the steady state involves equilibrium for each modification/demodification cycle. It is easy to see that in this limit

$$[A_{pp}] = \left(\frac{k_4}{k_7}\right) \left(\frac{[K]}{[P]}\right) [A_p]$$

and

$$[A_p] = \left(\frac{k_1}{k_{10}}\right) \left(\frac{[K]}{[P]}\right) [A]$$

leading to a hyperbolic response for the maximally phosphorylated form

$$[A_{pp}] = \frac{(k_1 k_4 / k_7 k_{10}) ([K]/[P])^2 A_{\text{tot}}}{1 + (k_1 / k_{10}) ([K]/[P]) + (k_1 k_4 / k_7 k_{10}) ([K]/[P])^2}$$

It can thus be seen that no biphasic response (as total kinase concentration is varied) occurs in this case.

The factors responsible for the biphasic response will be demonstrated analytically. In order to do this, it is worth noting that even the ordered double phosphorylation model is quite complicated to analyse explicitly. Therefore, the focus is on some limiting cases which retain the main effects, and simplify others. Thus, the analysis is based on the assumption that the first phosphorylation reaction occurs *via* mass-action kinetics (high catalytic constant and hence negligible kinase hold-up in the complex) and the same assumption is used for the

dephosphorylation reaction for the second step. Thus the focus is on a slightly simpler situation, which can be analysed more transparently.

Analysing the steady state of the system results in the conservation conditions

$$[P] + [A_pP] = P_{\text{tot}} \quad , \quad [K] + [A_pK] = K_{\text{tot}}$$

and

$$[A] + [A_p] + [A_{pp}] + [A_pK] + [A_pP] = P_{\text{tot}}$$

along with the steady-state equations

$$\left. \begin{aligned} [A_pK] &= \frac{k_4}{k_5+k_6} [A_p][K] \\ [A_pP] &= \frac{k_{10}}{k_{11}+k_{12}} [A_p][P] \\ k_6[A_pK] &= k_7[A_{pp}][P] \\ k_{12}[A_pP] &= k_1[A][K] \end{aligned} \right\} \quad (3.1)$$

After some algebra (Suwanmajo & Krishnan, 2013), this results in the steady state

$$\left. \begin{aligned} [P] &= P_{\text{tot}} / (1 + (k_{10}/(k_{11} + k_{12}))[A_p]) \\ [K] &= K_{\text{tot}} / (1 + (k_4/(k_5 + k_6))[A_p]) \\ [A_{pp}] &= A_{\text{tot}} / (1 + \alpha_1[P] + \alpha_2[P]/[K] \\ &\quad + \alpha_3[P]^2/[K] + \alpha_4[P]^2/[K]^2) \end{aligned} \right\} \quad (3.2)$$

and

where the various coefficients in the equation for $[A_{pp}]$ can be obtained explicitly in terms of kinetic constants (see appendix A). Note that if the second and fourth terms in the denominator were absent, then this would reduce to a form similar to the case considered above.

Equation (3.2) reveals the presence of the competing effects. First, it can be seen that the free enzyme concentrations are not constant (as the parameter K_{tot} is changed) and depend on concentration of the partial phosphorylated species. Further by looking at the denominator of the expression for $[A_{pp}]$ the competition between the second and third terms can be seen.

As the total kinase concentration increases, the concentration of the partial phosphorylated species decreases eventually increasing the free phosphatase concentration. The free kinase concentration increases both because the total kinase concentration increases and because the partial phosphorylated species concentration decreases. It can thus be seen that the second and third terms in the denominator are in competition, and that it is possible in certain parameter regimes that the increase of the free phosphatase is the dominant contribution, thus decreasing the overall concentration of A_{pp} (the last term in the denominator is small relative to the others in this regime). This is in agreement with the intuitive explanation provided earlier, providing a more explicit demonstration. Further, it can be seen from the analytical expression that this arises only when there is significant sequestration of substrate in the A_pK complex. Finally, a relatively small catalytic constant for the second phosphorylation (k_6) has the effect of increasing the relative effect of the free phosphatase as the total kinase concentration becomes high. This is because α_1 is inversely proportional to k_6 (see appendix A). While α_2 also increases, the effect is offset by a high free kinase concentration.

Taken together, the analytical analysis shows how such a biphasic effect arises when one accounts for significant substrate sequestration in the complex. It is worth emphasizing that while such an effect gives rise to biphasic behaviour in multi-site modification, such behaviour will not be seen in single modification cycles. This is shown analytically in the appendix A. Thus, it can be seen that the biphasic behaviour relies on a combination of multiple modifications and certain reactions where the kinetics is far from mass action. Further insight is obtained by examining a slightly different model: the cyclic model, which describes ordered phosphorylation, but where the order of dephosphorylation is the same as the order of phosphorylation (resulting in different partially phosphorylated forms). Here, the ‘sequestration’ of the singly phosphorylated form in the kinase complex does not result in any biphasic response. This is demonstrated analytically in the appendix A, where it is shown that

biphasic responses of the maximally phosphorylated species as a function of total kinase concentration cannot exist. In this case, the phosphatase does not bind to the singly phosphorylated form (A_{01}) in the forward modification, and thus the competing effects described earlier do not come into play. This provides further consolidated understanding of the role of the mechanism which has been described earlier. Finally, it is also demonstrated analytically in the appendix A, that different processive models of multi-site phosphorylation will not exhibit such biphasic behaviour.

Taken together, the analysis undertaken pins down both the competing effects and shows how making changes in the mechanism completely removes the competing effects and prevents the possibility of biphasic dose–response characteristics.

3.5 Conclusion

Systematically, understanding the effects of multi-site modification in different contexts is challenging because of their intrinsic complexity, context-specific factors as well as the coupling and interaction with other pathways and cellular entities. It is clear that disentangling such complexity requires a systematic elucidation of these different elements and their interplay, and that understanding the intrinsic complexity and coupling to other cellular factors is relevant in multiple specific contexts.

This chapter focused on elucidating a specific aspect of signal transduction which is intrinsic to multi-site phosphorylation. This elucidation is done *in silico* by investigating a generic model of (distributive) multi-site phosphorylation, and its variants at steady state. The assumptions in the model are minimal, and on par with those typically made for modelling signal transduction (steady state and a closed system for enzymes and substrates). It could happen that in some specific contexts, even these assumptions are not strictly met.

Nevertheless, the description above can be regarded as fairly generic descriptions of distributive multisite phosphorylation, without the consideration of other factors.

Here, the analysis undertaken focused on one aspect of the intrinsic complexity of distributive multi-site phosphorylation. Results showed how distributive multi-site phosphorylation is intrinsically capable of generating (partial) biphasic dose–response characteristics in the maximally phosphorylated substrate, even under monostable conditions. Using a combination of numerical simulations, bifurcation analysis and analytical work, it was able to uncover a hidden competing effect which is responsible for this. Such behaviour was obtained by considering parameter regimes where the catalytic rate of phosphorylation in the last cycle was small, compared with the others, though this could be obtained also in other parameter regimes. This allowed for the substrate to become converted and ‘sequestered’ in the enzyme–substrate complex of the last cycle, as the total amount of active kinase was increased. This led to the generation of increased phosphatase by removing the intermediate phosphoforms to which it could bind, which could act on the maximally dephosphorylated substrate, providing the competing effect. It is noteworthy that while the model used in this study does not explicitly describe the activation of enzymes, the same behaviour is readily observed in an expanded model that explicitly describes activation of kinase enzyme by upstream signals (results discussed in Chapter 5).

Such biphasic behaviour was readily obtained in both ordered and random mechanisms, and in fact, random mechanisms showed a greater accentuated biphasic behaviour. Increasing the number of modifications (for instance considering an ordered triple phosphorylation system) could still readily lead to such biphasic behaviour. It was also able to demonstrate that this biphasic behaviour could be readily combined with other studied behaviours of multi-site phosphorylation mechanisms such as thresholds and bistability. This

demonstrates that a multi-site modification system, in addition to possessing a larger ‘state space’ is also intrinsically capable of more complex signal processing.

As the behaviour observed depends on ‘sequestration’ of the substrate in the enzyme–substrate complex of the last cycle, it can be seen (and analysis confirms) that such behaviour would not be obtained by modelling each modification step using mass-action kinetics (this neglects the complex). Some simple analysis (see appendix A) immediately reveals that a single covalent modification cycle by itself is not capable of demonstrating biphasic behaviour either. Thus the behaviour observed relies on both multiple modifications as well as sufficient hold-up in complexes.

To further consolidate such insights, a number of variants of enzymatic mechanisms are examined. In the appendix A, it was demonstrated analytically how the biphasic behaviour is destroyed in ordered mechanisms, when the order of phosphorylation and dephosphorylation is altered. This shows how a structural change in the network, which bypasses the mechanism described above, is incapable of intrinsically exhibiting a biphasic dose–response characteristic. By systematically analysing a series of processive multi-site phosphorylation mechanisms, it was able to demonstrate that such mechanisms were not capable of exhibiting such behaviour. Taken together, this analysis indicates that if a biphasic (even partial) response is observed in a specific multi-site phosphorylation system, then either certain mechanisms of multi-site phosphorylation can be immediately ruled out, or the effect has to be extrinsic to the multi-site phosphorylation. Thus, this analysis may prove useful in certain contexts to exclude certain mechanisms based on the observation of this simple to observe behaviour. Such analysis could prove to be of broader relevance in systems (bio)chemistry, as it focuses on one aspect of the intrinsic complexity of multi-site modification of substrates by an enzyme pair.

3.6 Discussion

In this study, the analysis undertaken was performed on a model of multi-site phosphorylation, which represents multi-site phosphorylation in a well-controlled environment. Multi-site modification is repeatedly seen in cellular contexts, and both kinase and phosphatase activity/concentration may be regulated by multiple factors. It is being increasingly realized that phosphatase activity is regulated in a number of ways in cells (Virshup and Shenolikar, 2009). This includes regulatory control mechanisms and localization. Furthermore, there are different contexts where the active phosphatase concentration may be relatively low. One example is that of Cdc14 in metaphase budding yeast (Bouchoux and Uhlmann, 2011) (in fact, a gradual increase in phosphatase concentration occurs at different stages of the cell cycle). A similar example can be seen in PKC signal transduction (Hansra et al., 1999). The alkaline phosphatase (involved in multi-site phosphorylation) concentration is considerably reduced in certain disorders (Lum., 1995). In general, a combination of factors could reduce the available phosphatase. The effect of coupling of pathways through shared components is a recurring theme in signalling, and analysis suggests that phosphatase sharing may in fact be responsible for coupling and crosstalk in different cellular contexts (Rowland et al., 2012). In this study, the hidden competing effect which was examined arises from the fact that the same phosphatase is shared between different phosphoforms. A simple extension of this analysis shows that it is possible to obtain a biphasic response in concentration of the unphosphorylated substrate as (active) phosphatase concentration is increased, keeping the (active) kinase concentration fixed, for exactly analogous reasons. In fact, for fixed kinetic parameters, it is possible to obtain biphasic responses of maximally phosphorylated substrate (as active kinase concentration is increased) and unmodified substrate (as active phosphatase concentration is increased). This reveals that such competing effects can be at play in both directions. In some

contexts, the entity of interest may be the dephosphorylated substrate, and this analysis directly applies here too.

The above-mentioned analysis demonstrates how the sharing of phosphatase (and kinase) enzyme between different phosphoforms can give rise to the behaviour observed. It is worth extending the analysis above to reveal another consequence of such coupling in a basic model used in this study. If the total enzyme concentrations are kept fixed, and the total substrate concentration is increased, then in some cases, the concentration of the maximally phosphorylated substrate can also exhibit a biphasic response. In this case, increasing the substrate concentration has two competing effects: while it tends not only to increase the maximally phosphorylated substrate concentration, it also has the effect of sequestering the kinase in complexes. This reduces the free kinase concentration and has a competing effect. This is discussed in the appendix A. Thus, kinase sequestration rather than phosphatase release plays a role in the biphasic response.

From the above-mentioned discussion, it is very plausible that the mechanism described may be at work in specific contexts. In order to clearly and unambiguously demonstrate this experimentally careful experiments are required, which are able to disentangle any complexities from extraneous factors. Phosphatase sharing is believed to be an important source of crosstalk and coupling (Cohen, 1992; Brady et al., 1999; Rowland et al., 2012), which is also true for kinases. The effect of coupling of processes through shared components is a pervasive theme in current systems biology (see (Seaton and Krishnan, 2011) and references therein). Experiments have demonstrated that such coupling through shared components can have significant effects *in vivo*, and the addition of other substrates could have significant effects in MAPK signalling (Kim et al., 2010; Kim et al., 2011). Further, recent experiments in the MAPK system (Perrett et al., 2013) varying the total amount of ERK (substrate) demonstrate a biphasic response of the maximally phosphorylated

form of ERK, and the experiments are shown to be consistent with a mechanism involving kinase sequestration by the substrate. This is an experimental example in multi-site phosphorylation suggesting sequestration of kinase as a mechanism for a biphasic response (Perrett et al., 2013) and is a concrete example of how sharing of a common enzyme between different phosphoforms can result in a biphasic response. In this study, analysis provides a framework for understanding how and under what conditions this may happen. In addition, experiments exploring tau (multi-site) phosphorylation reveal a biphasic response of tau phosphorylation on forskolin (Tian et al., 2009). The experiments indicate that at high levels of forskolin, the phosphatase PP2-A expression and activity is increased, giving rise to this effect. While additional regulatory mechanisms which give rise to this may indeed exist, the obtained results in this study indicate how phosphatase release may itself be able to give rise to biphasic effects.

While biphasic behaviour has been observed in many contexts in cell signalling, they have in fact been observed elsewhere in signalling pathways also involving multi-site phosphorylation systems (Zhu et al., 2010). The experimental study in (Zhu et al., 2010) clearly shows a biphasic-type response in the doubly modified phosphoform. While there could be multiple reasons for this behaviour, results obtained from this study indicate possible ways in which the multi-site phosphorylation mechanism itself could contribute to this.

A biphasic response represents an auto-shutdown/reduction in response at high input levels, and is observed in many biological contexts. Such a response is referred to as hormesis in some contexts. It arises in many contexts in signal transduction itself and can sometimes be related to or be partially responsible for dose-dependent downstream decision-making. Indeed, the biphasic response results in an ‘optimal’ range of stimulus for downstream effects and may be desirable for different reasons. Biphasic responses can arise

due to scaffolds, or incoherent feed-forward pathways (Levchenko et al., 2000; Kim et al., 2008), and are often associated with these factors. It is interesting to find this capability built into the basic multi-site modification mechanism, and not requiring additional mechanisms such as scaffolds or other regulation. The biphasic response found in this chapter is due to a hidden competing effect in multi-site phosphorylation. It is worth pointing out, incidentally, that in some cases different modifications can have opposing effects on downstream pathways, which could be a different source of biphasic signal transduction characteristic in signalling, involving multi-site phosphorylation. The results herein indicate that the biphasic behaviour may be accentuated by other factors such as additional negative feedback or scaffolds. It is also possible that such biphasic behaviour is built-in at many levels in signal transduction and that such behaviour built into the multi-site phosphorylation mechanism can increase the robustness of biphasic responses in pathways. Finally, it is worth emphasizing that multi-site modification mechanisms are part of many signal transduction pathways, which are complex and process signals in a highly nonlinear way. Uncovering of the hidden competing effect allows for a better understanding of the signal transduction characteristics, capabilities and constraints in pathways (in multiple contexts) involving multi-site modification or interacting with them.

In this study, the results suggest how multi-site phosphorylation, in addition to acting as sophisticated biological control mechanisms, could act as complex signal processors. By combining effects involving one part of the cycle (giving rise to thresholds) and those at the other end of the cycle, it is possible for such mechanisms to exhibit complex signal processing characteristics. This has implications for both the signal processing capability of multi-site phosphorylation and their engineering using synthetic means.

Chapter 4

Mixed mechanisms of multi-site phosphorylation

4.1 Introduction

In the previous chapter, the study undertaken has focused on how purely distributive multisite phosphorylation can exhibit a biphasic dose response curve while all variations of purely processive mechanisms cannot do so. As enzymatic mechanisms can affect information processing and hence pathway/network behaviour, multiple studies in the literature, including the work undertaken in the chapter 3, focus on intrinsic information-processing capabilities in multi-site phosphorylation systems (Gunawardena, 2005; Markevich et al., 2004; Thomson and Gunawardena, 2009; Salazar and Hofer, 2009; Jolley et al., 2012). All these works deal with the intrinsic capabilities of multi-site phosphorylation in information processing where the modification mechanisms are purely distributive or processive.

While existing studies have resulted in an extensive dissection of distributive and processive mechanisms, there are other so-called mixed mechanisms which exist (Komeili and O'Shea, 1999). Mixed mechanisms can arise for multiple reasons. One possibility is that phosphorylation and dephosphorylation occur through different mechanisms. Another possibility is that phosphorylation and/or dephosphorylation on multiple sites could act through a combination of distributive and processive mechanisms, and this has been implicated in particular contexts (Komeili and O'Shea, 1999; Jeffery et al., 2001). It is also

possible in certain contexts that both distributive and processive routes to modification are available (Koivomagi et al., 2011). In such a case, purely distributive and purely processive mechanisms may be seen as extreme limiting cases, where one mechanism dominates the other. While different mixed mechanisms have been postulated or employed in models in particular contexts, there has been no systematic study of the interplay between distributive and processive mechanisms.

In this chapter, a framework is developed to examine the behaviour of mixed mechanisms. The aim is to study, in detail, the effects of combining processive and distributive mechanisms from the viewpoint of chemical information processing. A hierarchy of models is developed to study such effects. Firstly, the investigation focuses on mechanisms where phosphorylation is distributive and dephosphorylation is processive, and the other way round. Then the focus is on examining the effects of phosphorylation occurring through a combination of processive and distributive steps, while dephosphorylation is either processive or distributive. Finally, the relationship between the results of such models and a more comprehensive model that allows for both processive and distributive mechanisms at each step are also discussed. In this manner, a bridge between purely processive and purely distributive models is created, isolating clear patterns associated with their interplay, as discussed below. The particular focus in this chapter is on key qualitative signatures of information processing in mixed systems, such as bistability, biphasic dose-responses and oscillatory behaviour.

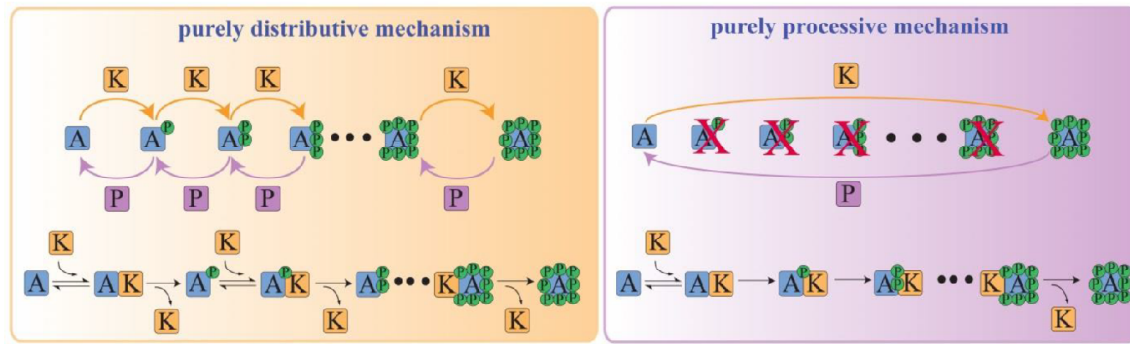
Such a framework is useful for multiple reasons. Firstly, mechanisms that are mixed exist in nature (Komeili and O'Shea, 1999; Jeffery et al., 2001; Koivomagi et al., 2011). Secondly, there are multiple factors that can effectively result in a switching between distributive and processive mechanisms, including crowding agents, presence of scaffolds and applied stress (Aoki et al., 2013; Kocieniewskia et al., 2012; Levchenko et al., 2000).

Depending on conditions, this might result in an effectively mixed mechanism. Thirdly, in different cases, both distributive and processive routes to modification may be available, and the mechanism may be regarded as being fully mixed (Koivomagi et al., 2011). Finally, tools from protein engineering, nanoengineering and synthetic biology/chemistry can allow for manipulating chemical mechanisms in novel ways, and this study provides a platform for understanding the kinds of kinetics and chemical information-processing behaviour that can emerge from enzyme/substrate engineering. Models and methods are presented in section 4.2, followed by the results (section 4.3) and a summary of analytical results, which is presented in (Suwanmajo and Krishnan, 2015) and reproduced in the section 4.4. The chapter concludes with a discussion, with analytical work in appendix B.

4.2 Models

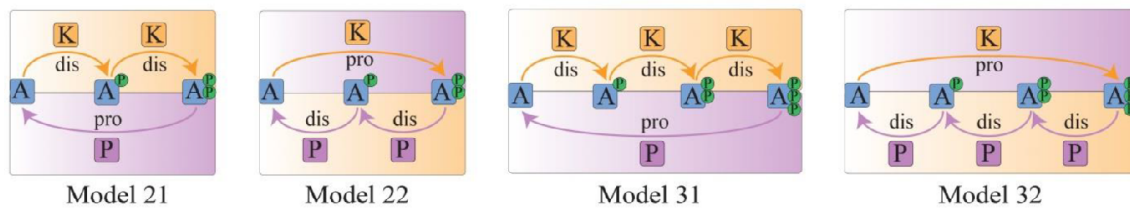
In this study, the aim is to understand the interplay between distributive and processive mechanisms in multi-site phosphorylation and approach this through a broad hierarchy of three classes of models. All models are described through widely used descriptions of individual modifications involving enzyme binding to substrate to form the complex which irreversibly converts the substrate to yield the product: an abundance of ATP (adenosine triphosphate) is implicitly assumed. In the case of distributive mechanisms, such descriptions of individual modifications are used for all modifications with enzyme binding/unbinding to substrate being involved in each modification. In the case of processive mechanisms, the enzyme binds to a substrate, forms a complex, and this leads to the sequence modifications of the substrate while the enzyme is still bound to it (figure 4.1a). It is possible to have models of processive modification where the intermediate product is released along the way, but this is not studied in detail here. This extra feature does not affect any of the qualitative results presented in this study. This is discussed subsequently in the context of a comprehensive mixed model (also see appendix B). In all the models, an explicit activation of kinase and/or phosphatase is not employed, for simplicity. The essential results are not dependent on this aspect. All the models are formulated in terms of ordinary differential equations, and can be the basis for subsequent investigations of stochastic effects that build on these results. The conservation of total substrate, kinase and phosphatase implies that these quantities are all parameters. For simplicity, this study focuses on ordered mechanisms, which impose a specific order on phosphorylation and dephosphorylation (also called sequential mechanisms: see diagram in the previous chapter).

(a) Purely multi-site phosphorylation mechanisms (based on enzyme processivity)

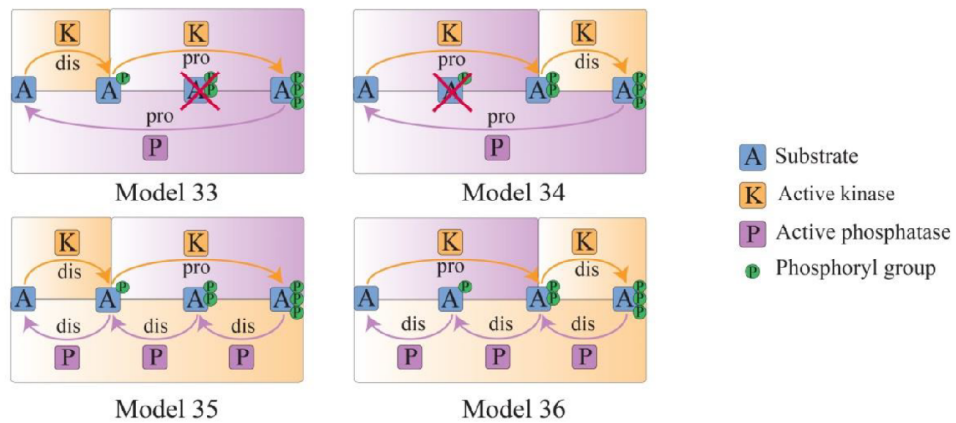


Mixed mechanism frameworks

(b) Distributive kinase/processive phosphatase and processive kinase/distributive phosphatase



(c) Mixed mechanism in phosphorylation



(d) Mixed composite mechanism

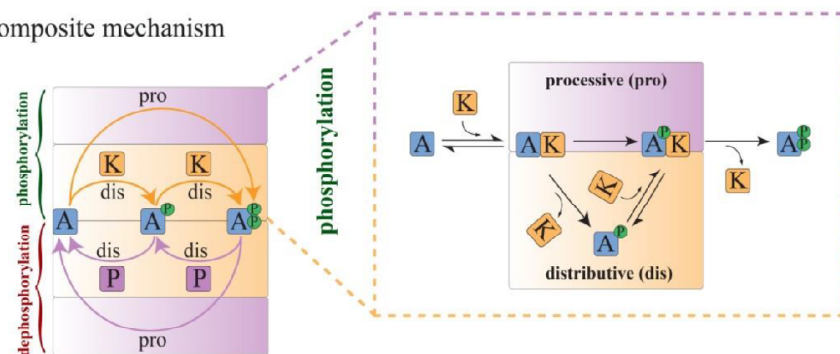


Figure 4.1. Schematic of ordered phosphorylation models. (a) Two types of pure ordered multi-site phosphorylation mechanisms: (i) a distributive and (ii) a processive mechanism. (b)

Mixed double- and triple-site models where one direction is distributive and another is processive. (c) Mixed triple-site models where there is a combination of distributive and processive steps in phosphorylation. (d) A mixed composite phosphorylation model where there is a combination of both processive and distributive mechanisms in all steps.

At the outset, the approach in analysing mixed systems is summarised. Mixed systems can, in principle, arise through a combination of sequences of modifications (phosphorylation/dephosphorylation) that are essentially purely distributive/processive, or involve combinations of both components in each modification. A model that incorporates both distributive and processive routes in each step covers all cases. However, studying such a model in detail parametrically is challenging, becoming rapidly more so as the number of sites increases. While exhaustive parametric analysis is useful, it is not necessarily the easiest way to isolate key patterns associated with information processing. Therefore, a hierarchy of models is employed to study a sequence of special cases (e.g. distributive phosphorylation/processive dephosphorylation), alongside the comprehensive model. These special cases can often be analytically studied, revealing features that generalize to different numbers of modification sites. This reveals certain features associated with particular parametric choices/regimes of the comprehensive model.

With the above discussion as a backdrop, a brief description of the various models is discussed. In this study, models focus on mixed two- and three-site modification systems. The detailed equations for some representative models are included in appendix B. In the description of models, a sequence of mixed models where a given step is either purely distributive or purely processive (the first and second class of models below) is presented, followed by comprehensive mixed models.

The first class of models (21 and 22) involves double-site modification where either the kinase acts distributively and the phosphatase processively, or the other way around (figure 4.1b). The essential results of the first case can be mapped onto the second case. The models are obtained as mentioned above.

The second class of models involves situations where the kinase (or phosphatase) can act through a combination of (purely) distributive and processive mechanisms. For this to occur, the minimum number of modification sites is three. Multiple variants of models of this class are considered, as shown in figure 4.1c. In principle, if one allows for such mixed mechanisms involving one enzyme, one can study (apart from three site analogues of the distributive/processive models of the first class above, which is referred to as models 31 and 32) four other models (models 33, 34, 35, 36). Here, the models involve a combination of distributive and processive modification, the latter involving two sites, in phosphorylation. Depending on the location of the processive mechanism in this multi-site modification sequence, there are two possibilities for the mixed phosphorylation. For each of these cases, dephosphorylation can occur in either a processive or distributive manner. This leads to a set of four models, which is illustrated in figure 4.1c.

The third class of models contains a combination of processive and distributive mechanisms at each step. This model, depicted graphically in figure 4.1d, combines elements of each of the mechanisms at each step. While for the most part these mixed models are not studied in detail thoroughly (though some analysis of such models of two-site mechanisms is discussed), these models are examined to see whether their limiting behaviour (when either processive/distributive steps is made weak) approaches that of the previous classes of models. Since the models of the previous two classes are naturally contained within this more comprehensive model, when the earlier classes of models are studied, they are used as a bridge to this more comprehensive model. All mixed models are depicted in appendix B.

Taken together, this study allows for a detailed examination of mixed mechanisms. Each of these models has parameters which naturally affects its information processing capabilities. Representative parameters are chosen for each model (presented in the Appendix B), which encapsulates different behaviour of the mechanisms, and focus on the interaction between processive and distributive steps. In this study, the focus is largely on qualitative signatures. Mixed models are analysed in different parametric regimes, where the catalytic constants are varied primarily: these regimes are chosen based on the distinct behaviour seen in pure mechanisms (e.g. bistability), to serve as a contrast. In cases where certain behaviour (e.g. bistability) is ruled out in the mixed model, an analytical proof of this assertion is presented. In other cases where new behaviour is found (such as oscillations), it is able to explicitly demonstrate such a qualitative feature through simulations and bifurcation analysis (and have checked that such behaviour is possible in plausibly broad parameter ranges). Then the combination of parameters that can give rise to this is discussed. A broad range of parametric analysis has also been performed underpinning this study. This is not, however, discussed in detail except to illuminate specific points. Some parametric analysis is performed on a comprehensive two-site mixed model, where the ‘crossover’ between distributive and processive models is studied. Thus, through numerical simulation, bifurcation analysis and analytical work, one can consolidate his understanding of the interaction of processive and distributive mechanisms. Simulations were performed using both ode15s (a stiff ordinary differential equation solver) in MATLAB and COPASI (Hoops et al., 2006) for comparison. The equations are directly programmed in the former, while generated by the software automatically in the latter. This is complemented by bifurcation analysis in MATCONT (Dhooge et al., 2003) (which uses the same code for the model as the one used in MATLAB).

At various points, the behaviour of mixed (ordered) mechanisms is compared with that of ‘related’ (ordered) distributive and processive mechanisms. In certain cases, the effect of the order of distributive and processive steps is investigated, when they occur together in phosphorylation. In certain situations, to compare a distributive modification step (which has extra binding and unbinding steps) with a processive modification step, it is reasonable chose the catalytic constants to be the same. The binding and unbinding rates are kept fixed for any sequence of forward or reverse modifications (unless otherwise mentioned). This ensures that sensible comparisons can be made between different sequences of forward and/or reverse modifications, involving distributive and processive steps. Of course, it is possible that binding/unbinding rates can be different for different phosphoforms, but in the absence of specific experimental information regarding this, one can adopt the above approach to start with. The effects of these other factors can be studied subsequently. In what follows, the investigation focuses only on ordered mechanisms.

4.3 Results

At the outset, the focal point of results is discussed. In this study, the primary goal is identifying and elucidating the presence of key qualitative signatures and capabilities (and constraints) in information processing in mixed systems, such as bistability, oscillations, biphasic dose-responses (as the kinase concentration is varied) or certain combinations thereof. This section presents a sequence of results for the models above showing ‘representative’ and possible qualitative behaviour in this regard. If certain behaviour is not seen, analytical proof is performed to rule out such behaviour, which is presented in (Suwanmajo and Krishnan, 2015) and reproduced in section 4.4 and the Appendix B. Examining these models in sequence also brings to light differences between partial phosphoform behaviour, effects of the order of mechanisms and contrasts with pure models

with ‘corresponding’ parameters. The focus is not on a detailed parametric analysis of these models here.

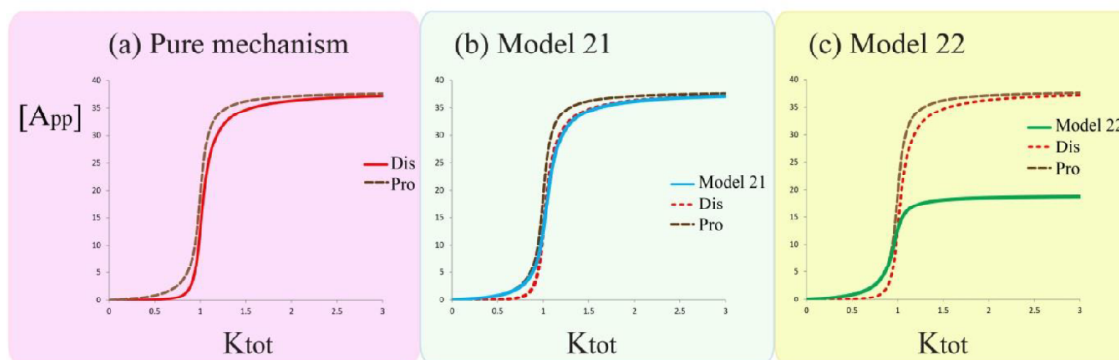
The discussion starts with two-site models with different phosphorylation and dephosphorylation mechanisms followed by three-site models with mixed mechanisms of phosphorylation. The relationship between these models and mixed composite models, where each modification can have processive and distributive pathways, is also discussed. Throughout this chapter, the following notation is used (explained further in appendix B): A refers to the substrate, and the subscript ‘p’ denotes a phosphorylated substrate. A_p and A_{pp} are thus singly and doubly modified substrates, respectively. K refers to the free kinase, P to the free phosphatase and K_{tot} denotes total kinase concentration. Relevant kinase and phosphatase complexes are denoted by concatenations of relevant substrate symbols with K or P : e.g. A_pK denotes the kinase complex where kinase is bound to A_p .

4.3.1. Two-site modification

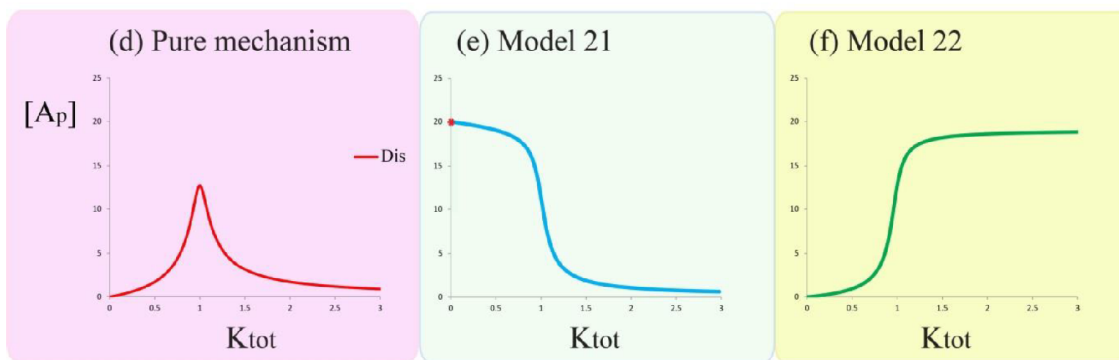
This section starts by discussing the two double-site modification models: model 21 refers to the case where the phosphorylation is (purely) distributive and dephosphorylation is (purely) processive, and model 22 describes the opposite scenario. Figure 4.2 shows the variation of the partial and fully modified substrate as a function of total kinase concentration. This mixed mechanism is first examined in a parameter regime, where the ‘analogous’ purely distributive mechanism is monostable. It is found that while the fully modified substrate exhibits a sigmoidal response similar to both distributive and processive models (due to zero-order ultrasensitivity), certain differences are observed. In model 21, it is found that the partial phosphoform exhibits a decreasing profile asymptoting to zero for large values of K_{tot} . This is what is generically expected in a distributive mechanism, with all available substrate (including partial phosphoform) getting converted to the fully

phosphorylated form. On the other hand as K_{tot} approaches zero the partial phosphoform A_p does not approach zero (figure 4.2). In fact, examining the steady state of $A_p + A_pK$ reveals

Completely phosphorylated substrate



Singly phosphorylated substrate



that $[A][K]$ is proportional to $[A_p][K]$ (also see appendix B). In other words A and A_p are proportional.

Figure 4.2. Steady-state dose–response curves in ordered double phosphorylation models for purely distributive (dis), purely processive (pro) and mixed mechanisms (models 21 and 22). (a–c) show the steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of the total concentration of kinase, K_{tot} , while (d–f) shows the corresponding curve for the partially phosphorylated substrate, A_p . (Completely phosphorylated substrate: (a) pure mechanism, (b) model 21 and (c) model 22. Singly phosphorylated substrate: (d) pure mechanism, (e) model 21 and (f) model 22.)

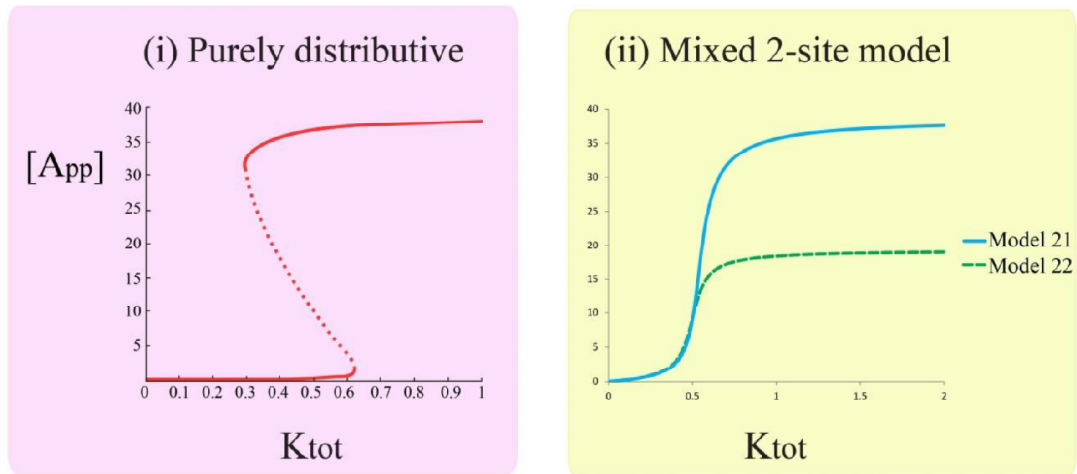
It is worth noting that introducing a weak distributive component in the dephosphorylation steps (for instance, by considering a fully mixed model as mentioned above) actually shows that, for small values of K_{tot} , the concentration of A_p increases very rapidly, reaching a peak for a small value of K_{tot} and then following a decreasing trend as suggested by the above analysis. This can be summarised by saying that the behaviour of A_p as a function of total kinase concentration is essentially monotonically decreasing, except for small values of K_{tot} where small background levels of other mechanisms can result in a sharp increase. Thus, there is a boundary layer-like behaviour (in parametric space) at $K_{\text{tot}} = 0$ in the fully mixed model. This can be seen from an analysis of the fully mixed model, which is briefly discussed. In this model, the steady state of $A_p + A_pK$ results in $c_1[A][K] - c_2[A_p][K] + c_3[A_{pp}P] - c_4[A_p][P] + c_5[A_pP] = 0$, where all the c 's are constants. By neglecting the distributive component to dephosphorylation, the above result is achieved (obtained by setting $c_3=c_4=c_5=0$). For simplicity, if a high catalytic conversion of A_pP is assumed, the concentration of A_pP is negligible. Then $[A_p] = (c_1[A][K] + c_3[A_{pp}P])/(c_2[K] + c_4[P])$. This indicates that, when K_{tot} approaches 0, $[K]$ approaches 0 and $[A_p]$ does indeed approach zero, owing to the presence of the extra term $c_4[P]$ in the denominator. Even if c_4 is small in magnitude, the presence of this term results in $[A_p]$ approaching zero as K_{tot} becomes zero. Physically, this can be understood as follows. In the absence of a distributive component in dephosphorylation, a small amount of kinase can move the substrate towards modifications and the cycle is completed through dephosphorylation, though not directly of A_p . With weak distributive dephosphorylation, this is more or less the case except when the kinase levels are low enough for direct dephosphorylation (of A_p) to compete.

In the case where the phosphorylation is processive, an analysis of the dephosphorylation (the distributive leg of the pathway) in appendix B shows that the concentration of A_p is proportional to that of A_{pp} (by considering the steady state of $A_p +$

A_pP). This explains the sigmoidal increasing variation of A_p , which is different from a pure distributive mechanism (figure 4.2). It also explains the reduced asymptotic amplitude of A_{pp} as K_{tot} becomes high: a fixed fraction of the substrate is in the partially phosphorylated form. Again it is worth noting that if one examines a comprehensive mixed mechanism, introducing a weak distributive step in phosphorylation, then the profile of A_p saturates as above for a broad range of K_{tot} before decreasing to 0 as K_{tot} becomes very high (results not shown). This can be summarised by saying that the comprehensive mixed model follows essentially the same behaviour as model 22, with the possibility of a difference at very high values of K_{tot} (weakening the distributive component above increases the value of K_{tot} at which this happens). This can be understood from analytical work.

The focus is now on examining these two mixed mechanisms in parameter regimes, where the purely distributive analogues are bistable. Note that all potential binding/unbinding constants are fixed at the same nominal value. Analogous distributive mechanism refers to the distributive mechanism with these binding/unbinding constants and the same sets of catalytic constants. A choice of sufficiently different catalytic constants in phosphorylation can result in bistability in a purely distributive model, as shown in figure 4.3a (also see (Conradi and Mincheva, 2014)). By contrast, the two mixed models exhibit monostable behaviour. In appendix B, it is demonstrate analytically how bistability is ruled out in such mixed models, when catalytic conversion steps are irreversible. This analysis also extends, in a straightforward way, to such mixed models with more than two site modifications as discussed there, and thus this analysis of a two-site mixed model reveals a common pattern valid for n -site modifications.

(a)



(b)

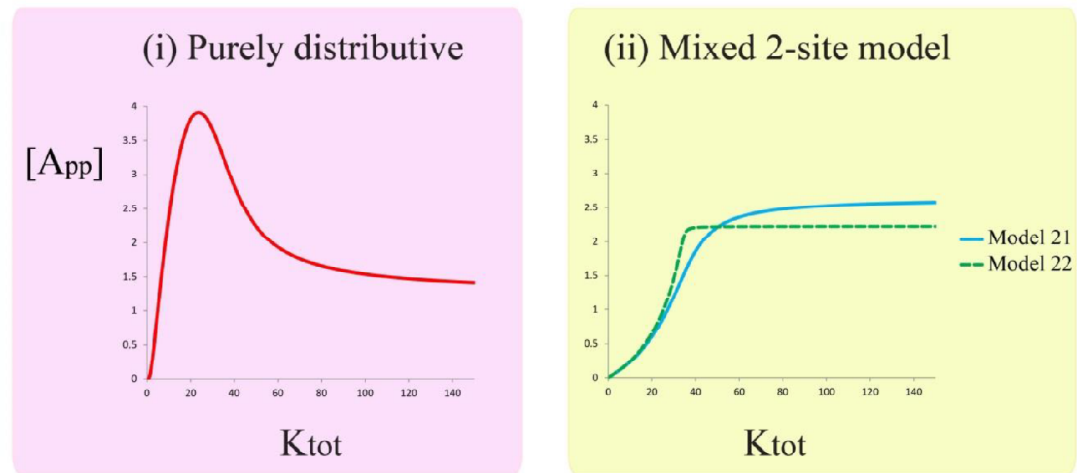


Figure 4.3 Steady-state dose – response curves in various ordered double phosphorylation models with different catalytic constants of phosphorylation reactions.

(a) Bistability may be obtained in a purely distributive model (i), while this behaviour is destroyed in both double-site mixed models (ii). (b) The biphasic behaviour which can arise from a purely distributive model (i) is not found in both mixed double-site models (ii).

Figure 4.3b shows a dose–response curve of a distributive model in a different regime, indicating a biphasic response for the fully modified phosphoform. The origins of

such biphasic responses are discussed in the previous chapter, where it is shown that distributive mechanisms are intrinsically capable of generating such biphasic responses in the absence of product inhibition, even when the catalytic conversion step is irreversible. It is found that the ‘corresponding’ mixed mechanisms do not exhibit such biphasic dose–response curves (for the fully modified substrate). In appendix B, it is shown analytically that biphasic responses under the conditions of irreversible catalytic steps can be ruled out.

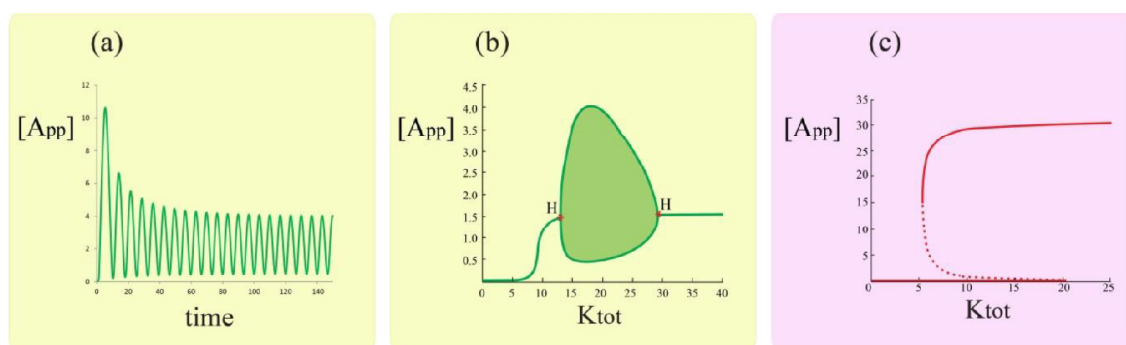


Figure 4.4 Oscillatory behaviour in mixed double-site phosphorylation models. (a) The mixed model 22 is capable of sustained oscillations. (b,c) Bifurcation analysis for the mixed mechanism (model 22) and a distributive mechanism with corresponding parameters. Bifurcation analysis shows that as the total kinase concentration, K_{tot} , is changed, (b) the steady state loses stability *via* a Hopf bifurcation in the mixed system, while (c) the purely distributive system exhibits bistability (simulations result in a steady state).

Figure 4.4 presents another aspect of mixed mechanisms. In contrast to the ‘corresponding’ pure distributive mechanism, which reaches a steady state, it is found that in a mixed model (for specificity model 22) sustained oscillations can occur (figure 4.4a). A bifurcation diagram shows that, as the parameter K_{tot} is changed, the steady state does indeed lose stability to a Hopf bifurcation. A Hopf bifurcation denotes a transition in dynamical

systems resulting in the ‘birth’ of oscillations (Stogatz, 2000). Sustained-oscillations in a two-site ordered distributive mechanism was not reported in the literature and a detailed parametric search has shown no example of such behaviour. However, a random two-site distributive mechanism has been shown to exhibit oscillations, for specific combinations of parameters (Jolley et al., 2012). Here it is found that both ordered as well as random two-site mixed models are able to exhibit oscillations and that the random mechanism is capable of generating oscillations of greater amplitude (results not shown). In a sense, the mixed ordered two-site modification mechanism could be the simplest enzymatic modification scheme that can intrinsically exhibit oscillation, in isolation, without additional explicit feedback regulation. A completely mixed model is also able to exhibit oscillatory behaviour. Naturally, model 21 also exhibits oscillatory behaviour.

4.3.1.1. Oscillations

It is worth examining the source of these oscillations. These oscillations are found in model 22 in parametric regimes where the binding constant of A_{pp} to the phosphatase is quite high, and when the catalytic constant for the dephosphorylation of A_p is reasonably high, with other parameters all of similar magnitude (order 1). In fact, oscillations are also obtained when the dephosphorylation step of A_p acts *via* mass action kinetics (i.e. the limit of a very high catalytic constant). It is found that, in such a scenario, a significant amount of the phosphatase is sequestered in the complex $A_{pp}P$. However, such a scenario appears not to result in oscillations in two-site ordered distributive models. If the effect of increasing K_{tot} is examined, it is found that this produces more A_{pp} which largely gets converted to the phosphatase complex $A_{pp}P$ (high binding constant of A_{pp} to P), and eventually to A_p . However, the paucity of free phosphatase means that this A_p is hardly converted back to A . Thus, increasing the total kinase concentration has an effect of depleting/moving away

substrate, which contributes a negative feedback effect. In an ordered distributive model, on the other hand, A_p can be directly converted back to A_{pp} by the kinase, something that is not possible/negligible here. This suggests how a combination of enzyme sequestration and modification characteristics combines to readily generate oscillations here.

Further analysis of a mixed composite two-site modification model is dissected in appendix B, showing how this bridges the distributive and processive scenarios.

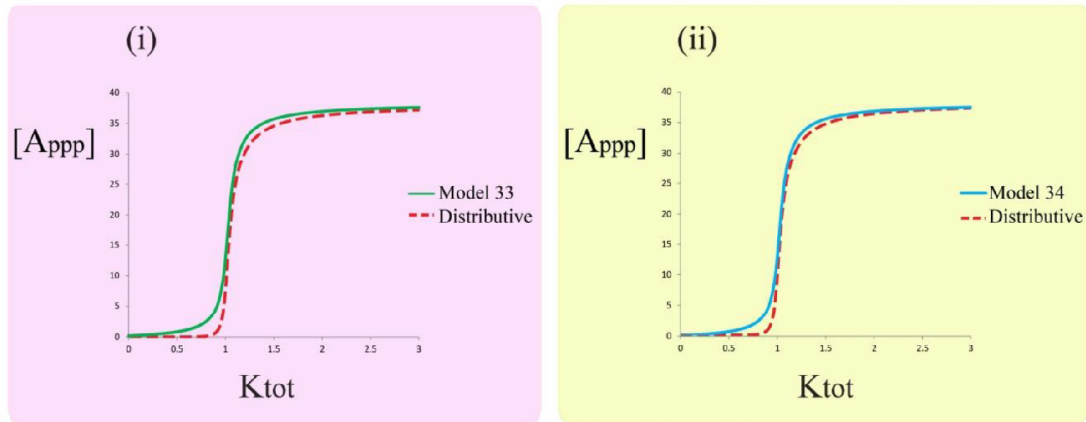
4.3.2. Three-site modification

Three-site modification schemes are now considered. In this case, it is possible for either phosphorylation and/or dephosphorylation to combine (purely) processive and distributive mechanisms. A selection of mixed mechanisms is examined as follows. This study examines only those cases where the dephosphorylation is either purely processive or purely distributive. For simplicity, this study does not consider cases where both phosphorylation and dephosphorylation occur through a combination of mechanisms. Therefore, there are six mixed mechanisms left, which can be described as: model 31, dis—pro; model 32, pro—dis; model 33, dis pro pro—pro; model 34, pro pro dis—pro; model 35, dis pro pro—dis; model 36, pro pro dis—dis. In these models, the horizontal line separates the phosphorylation mechanism from the dephosphorylation mechanism. Models 31 and 32 have a uniform mechanism for phosphorylation and this is hence referred to by one label. In all other models, the order of modification in phosphorylation is explicitly listed through the sequence of three labels (see figure 4.1 for a schematic depiction of kinetic steps). Analysing these models allows us to examine the effects of the interplay and ordering of processive and distributive steps in one direction.

Models 31 and 32 are three-site analogues of the two-site models 21 and 22. While the additional modification site enlarges the parameter space, the qualitative behaviour of

these models is essentially the same as the two-site models, owing to a similar structure. It can be shown analytically (appendix B) that, if the catalytic step is irreversible, bistability and biphasic dose–response curves are ruled out.

(a) Completely phosphorylated substrate



(b) Partially phosphorylated substrate

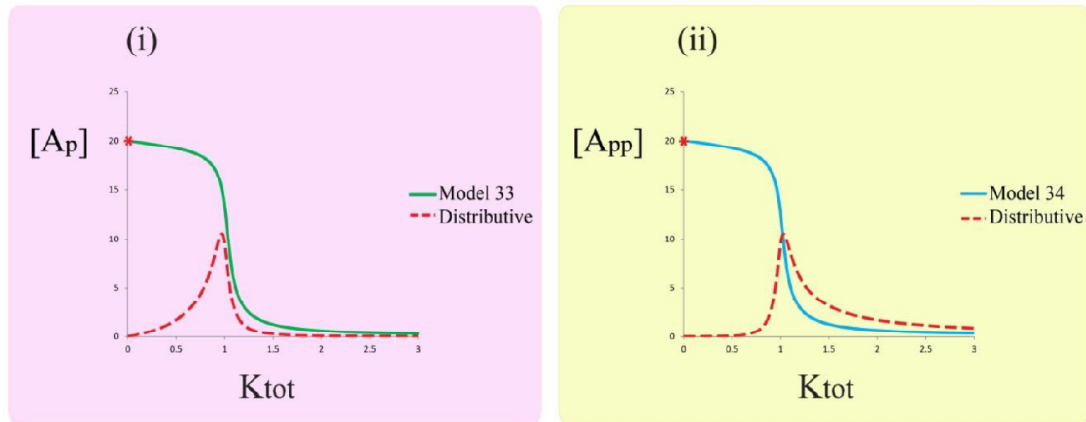


Figure 4.5 Steady-state dose – response curves in triple-site phosphorylation models with mixed phosphorylation and processive dephosphorylation. Panel (a) contrasts the steady-state dose –response curve of the maximally phosphorylated substrate, A_{pp} , in mixed triple-site models (model 33 (i) and model 34 (ii)) and that of pure distributive triple-site phosphorylation. (b) The corresponding information for the partial phosphoform.

The focus of the study now turns to models 33 and 34, involving mixed distributive and processive steps in phosphorylation differing only in order (figure 4.5), with processive dephosphorylation. Figure 4.5a shows that the fully modified phosphoform exhibits a sigmoidal dependence on total kinase concentration. Again, it is found that the relevant partial phosphoforms, A_p , for model 33 exhibits monotonic decreasing variation. This is explained by noting that it is proportional to A (see analysis in appendix B). In model 33, by considering the steady state of $A_{pp} + A_pK$, it is found that $[A][K]$ is proportional to $[A_p][K]$ (A_p is only produced from AK in this case, and the conversion of A_pK is proportional to its concentration) and hence the concentration of A is proportional to that of A_p . Again, it is essential to note that, as K_{tot} becomes very small, this suggests a non-zero asymptote for A_p . However by including some weak distributive component in the dephosphorylation steps, it can be seen that A_p actually increases quite sharply for small K_{tot} and thereafter the response is essentially the same as that depicted. The reason for this behaviour is the same as described previously. By similar reasoning, it is found that, in model 34, A_{pp} exhibits an essentially monotonic decreasing profile and this is because A_{pp} is proportional to A . This is seen by considering the steady state of $A_pK + A_{pp}K$.

As both these models have processive dephosphorylation, they are modified variants of the distributive phosphorylation/processive dephosphorylation models discussed above (models 31 and 32). In those models, bistability and biphasic responses for the fully modified phosphoform are ruled out. The current models involve part of the phosphorylation mechanisms being processive, and this further simplifies the model and reduces possible behaviour which may be obtained. An analysis of these scenarios (appendix B) shows that both bistability and biphasic behaviour can indeed be ruled out.

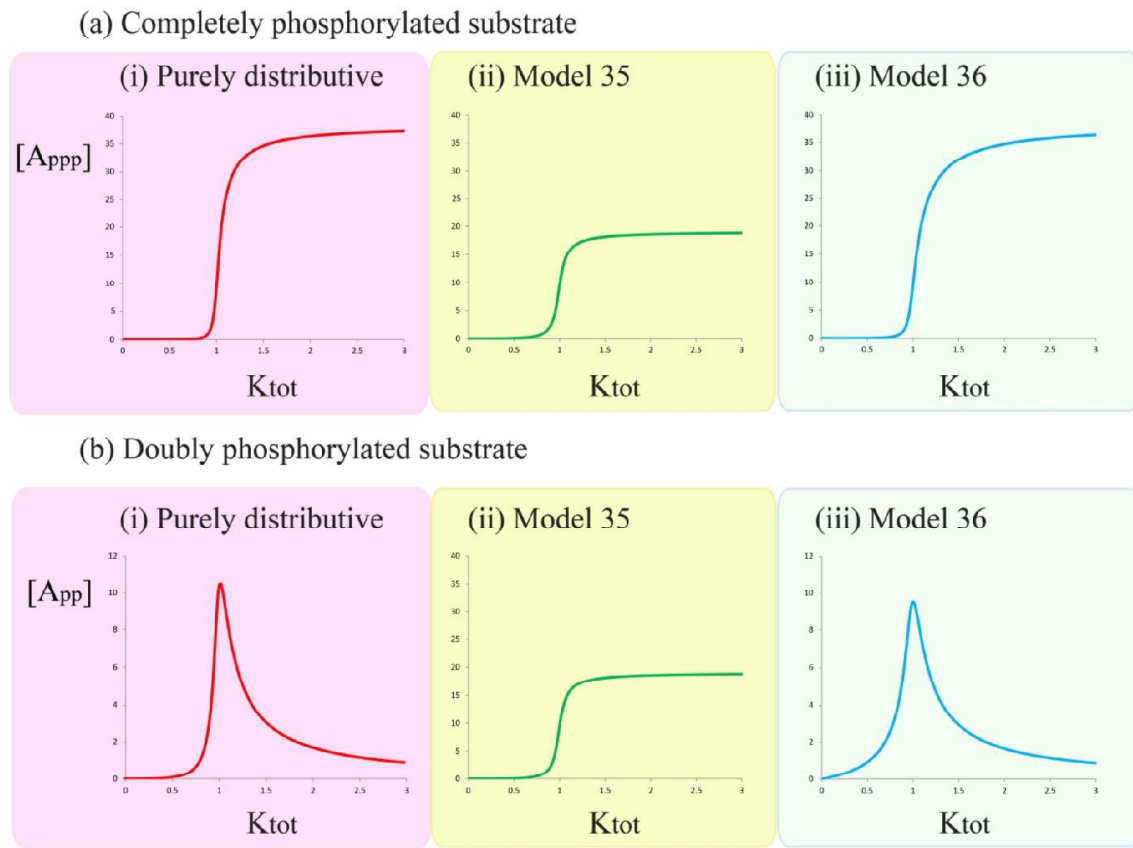


Figure 4.6 Steady-state dose–response curves in pure and mixed triple-site phosphorylation models with distributive dephosphorylation (models 35 and 36) under monostable conditions. The steady-state dose – response curves for the maximally phosphorylated substrate, A_{ppp} , in these three models are shown in (a) while those for the doubly phosphorylated substrate in these three models are presented in (b). ((a) Completely phosphorylated substrate: (i) purely distributive, (ii) model 35 and (iii) model 36. (b) Doubly phosphorylated substrate: (i) purely distributive, (ii) model 35 and (iii) model 36.)

Models 35 and 36 involve distributive dephosphorylation. These two mixed mechanisms are first examined in the monostable parameter regime (figure 4.6). Figure 4.6a shows that the fully modified phosphoform exhibits a sigmoidal dependence of the total

kinase concentration, with the clear presence of a threshold. This is true for both models 35 and 36, and is in fact seen in the fully distributive analogue as well. However, a difference between these models is seen at this stage itself. In model 35 (the processive steps are the last two steps in the phosphorylation sequence), A_{pp} exhibits a sigmoidal curve which mirrors A_{ppp} (figure 4.6b). This can be understood in a similar manner to the cases above, since A_{pp} is proportional to A_{ppp} . It is worth mentioning that, by varying the catalytic constant of the last phosphorylation, it is possible to get a biphasic dose–response curve for both A_{ppp} and A_{pp} . In contrast to model 36, A_{pp} (but not A_{ppp}) exhibits a biphasic response. This shows how the order of processive and distributive steps affects phosphoform characteristics.

These two models are then examined in a bistable parameter range. Since the dephosphorylation is distributive and since there are at least two different binding/unbinding pairs of events in the phosphorylation sequence, this system is readily capable of exhibiting bistability (figure 4.7a). This study shows how the location of the distributive and processive steps can affect the bistable range. In this case, it is found that having the first two steps in the phosphorylation as processive (model 36) leads to a much greater range of bistability, compared with model 35. This conclusion could of course depend on the choice of enzyme–substrate binding/unbinding constants. Finally, figure 4.7b also shows how varying the catalytic constant in the last phosphorylation step can result in a biphasic dose–response curve for the maximally phosphorylated substrate. This is much more pronounced when the last step is distributive

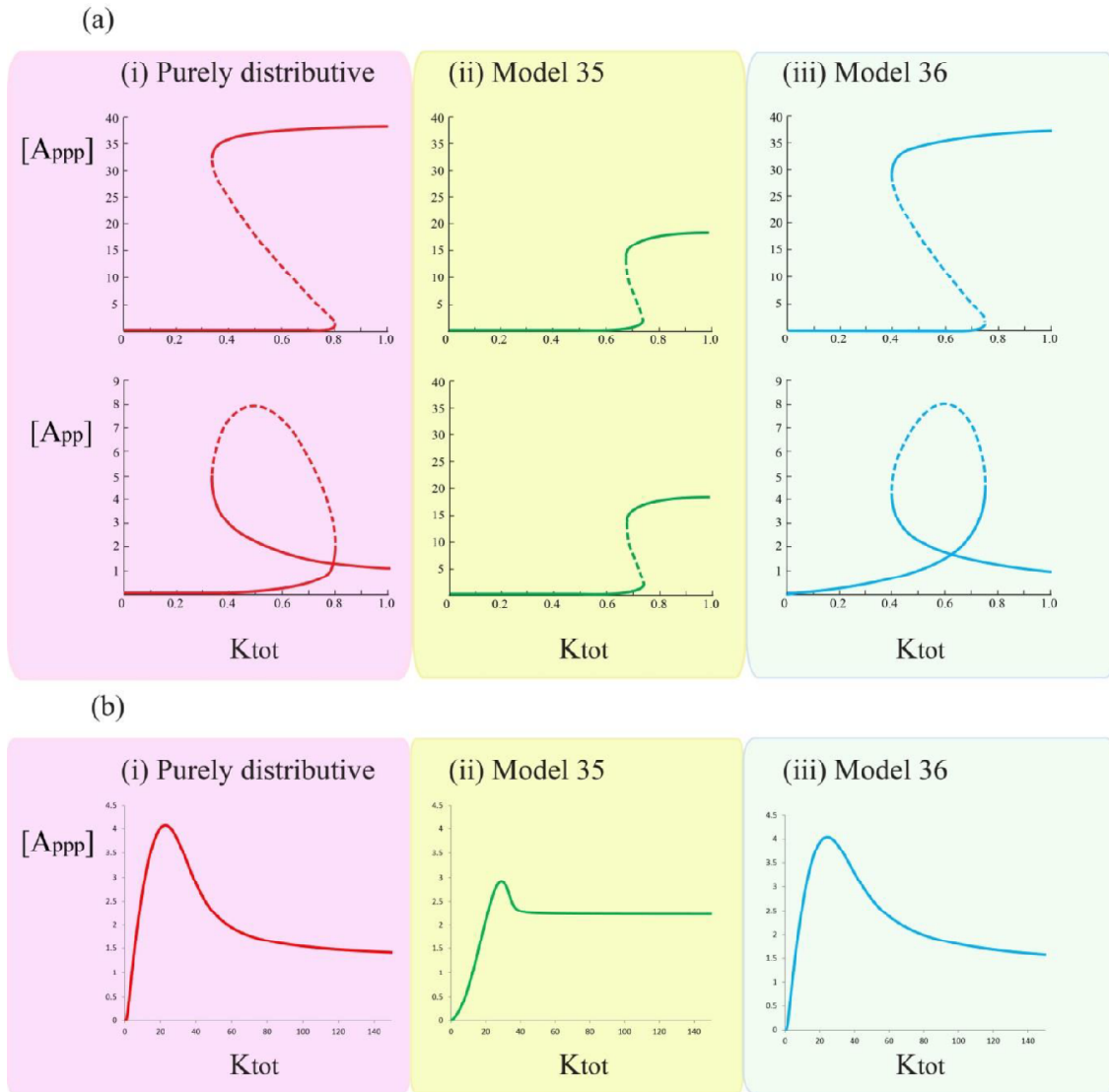


Figure 4.7 Steady-state dose–response curves in triple-site phosphorylation models with distributive dephosphorylation: (purely distributive model, models 35 and 36) exhibiting bistability and biphasic responses. (a) The steady-state dose – response curves of fully and doubly phosphorylated substrates, A_{ppp} and A_{pp} , in these models under bistable conditions. (b) The dose – response curve of the fully phosphorylated substrate, A_{ppp} , in these three models demonstrating biphasic behaviour. ((a):(i) Purely distributive, (ii) model 35 and (iii) model 36. (b):(i) Purely distributive, (ii) model 35 and (iii) model 36).

Figure 4.8 contrasts three mixed models (with same catalytic constants) with distributive dephosphorylation revealing new facets. When the phosphorylation is purely processive (model 32), oscillations are observed over a very broad range of kinase concentrations (figure 4.8a(i) and 4.8b(i)). It can be seen in figure 4.8b(ii) that model 35 exhibits both bistability and oscillations arising through a Hopf bifurcation of the ‘upper’ branch of steady states. The parameters combine sufficiently different catalytic constants in phosphorylation (giving bistability) and those conducive to oscillations. With the same parameter regime, figure 4.8b(iii) shows that model 36 exhibits bistable behaviour with no oscillations, showing how ordering of processive and distributive steps can result in qualitatively different behaviour.

One important thing to note is that all three-site mixed mechanisms can exhibit oscillations and that oscillations are readily obtained in the relevant parameter regimes of comprehensive mixed three-site models as well. However, the combination of oscillations and bistability occurs only in models 35 and 36, and does not occur in models 21 and 22, since these other models are not capable of exhibiting bistability as discussed above. This shows how the number of sites, as well as the number of essentially distributive steps in both phosphorylation and dephosphorylation, can introduce new modes of information processing.

The discussion now turns to the comprehensive mixed models (figure 4.1d) and models which have been analysed as above. If a sequence of processive modifications is examined (for instance in dephosphorylation in two site modification), it is found that incorporating a distributive pathway introduces extra elements: (i) a pathway converting $A_{pp}P$ to A_p and (ii) a binding of A_p to P to form the complex A_pP and a corresponding unbinding step. It is worth mentioning that, even when the rate constant involved in the reaction converting $A_{pp}P$ to A_p is not small, the qualitative behaviour of the model is essentially similar. Thus, having a modified processive model allowing for the release of intermediate

phosphoforms results in essentially similar conclusions. If, however, the binding/unbinding of A_p to P is not small, then the pathway behaves like a distributive pathway.

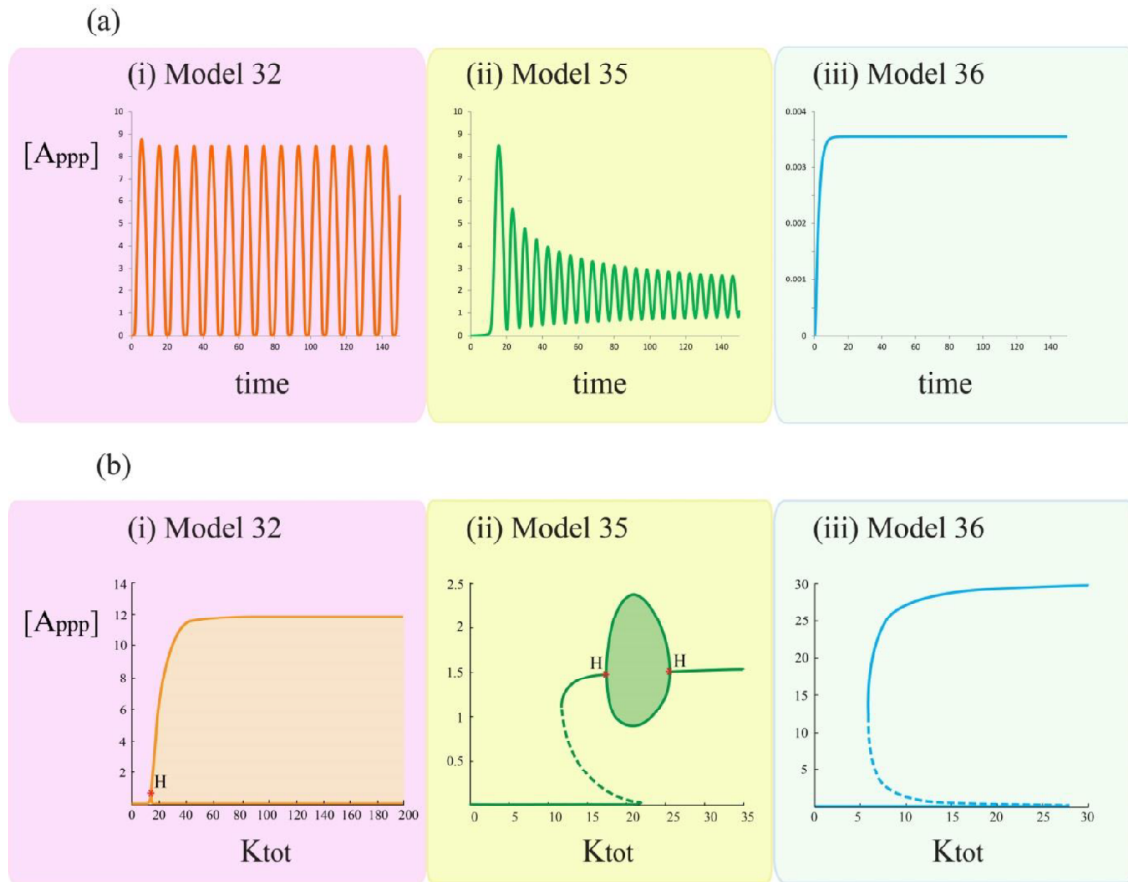


Figure 4.8 Effect of distributive and processive phosphorylation steps on oscillatory dynamic behaviour in mixed ordered triple-site phosphorylation models with distributive dephosphorylation. (a) With the same catalytic constant in all steps, both (i) model 32 and (ii) model 35 can exhibit sustained oscillation with different frequency and amplitude while (iii) model 36 does not, for these parameters. (b) The steady state loses stability to a Hopf bifurcation in model 32 (i) and model 35 (ii), while bistability with no oscillatory behaviour is seen in (iii) model 36.

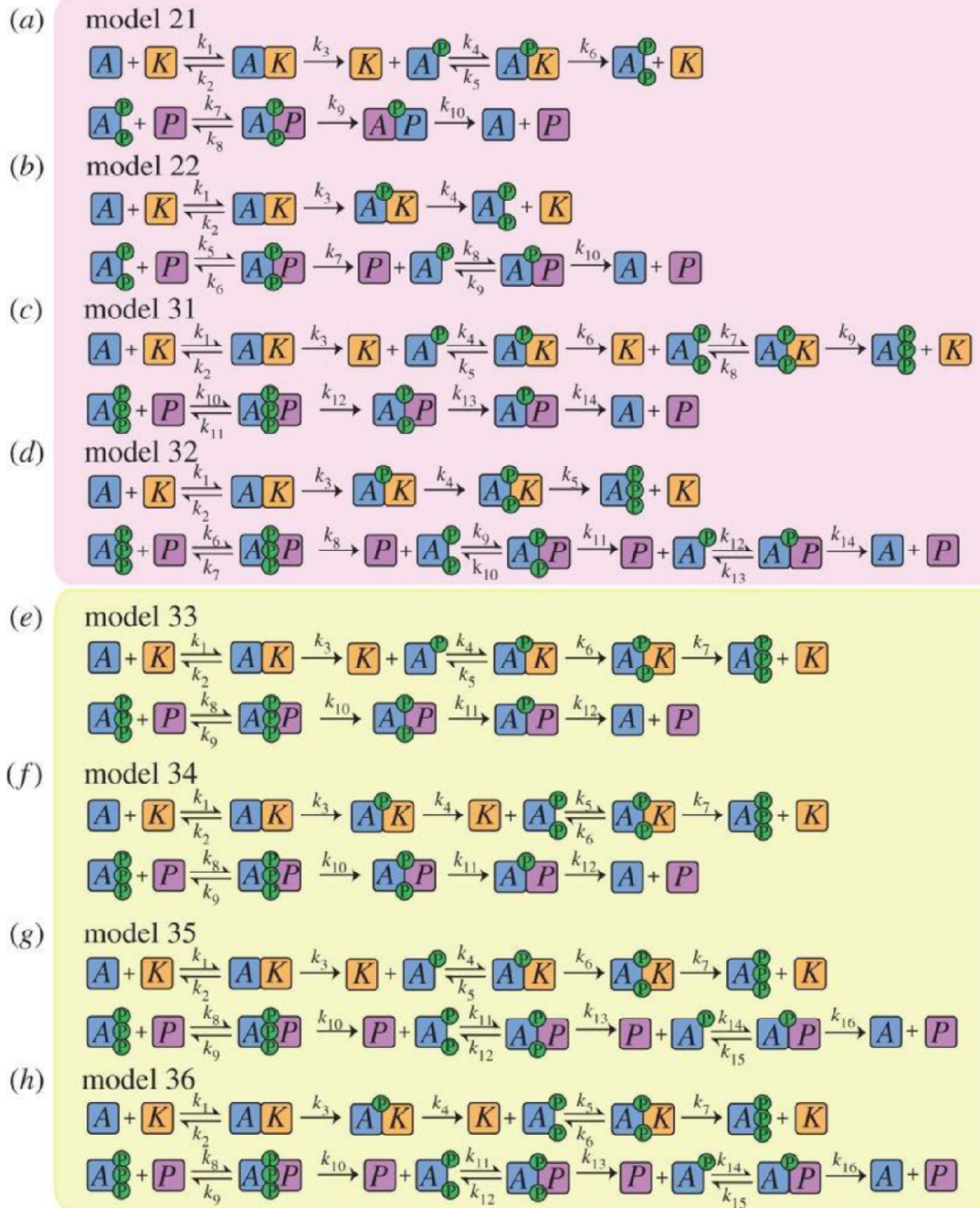


Figure 4.9 Pictorial depiction of mixed ordered phosphorylation models. (a–d) Mixed models where one direction is distributive and another is processive, whereas (e–h) present mixed models where there is a combination of distributive and processive steps in phosphorylation. The various k 's denote rate constants (binding, unbinding, catalytic) as appropriate.

4.4. Analytical studies

Herein a summary of some analytical studies performed on these systems is presented in (Suwanmajo and Krishnan, 2015) and reproduced in this section and appendix B. The analytical approach relies on obtaining steady-state concentrations of species through the solution of three coupled equations, involving free kinase concentration $[K]$, free phosphatase concentration $[P]$ and concentration of maximally modified phosphoform A_{pn} . This is done, firstly, by eliminating concentrations of all complexes in terms of products of substrate concentrations and those of K or P . Then all substrate concentrations are written in terms of that of A_{pn} , P and K .

In the two-site models 21 and 22 (see figure 4.9), it is found that the nature of equations which results is of the form

$$\begin{aligned} [K] &= K_{\text{tot}} - \alpha[A_{pp}][P] \\ [P] &= \frac{P_{\text{tot}}}{1 + \beta[A_{pp}]} \\ [A_{pp}] &= \frac{A_{\text{tot}}}{\gamma_1 + \gamma_2[P] + \gamma_3[P]/[K]} \end{aligned} \quad (4.1)$$

where α , β , γ_1 , γ_2 and γ_3 are constants. Analysis of this equation (see appendix B) shows that bistability and biphasic responses for A_{pp} are ruled out. An n -site analogue of these models results in exactly the same form of equation, resulting in the same conclusions.

Three-site modification systems where phosphorylation occurred through a combination of processive and distributive steps are also considered. If the dephosphorylation is processive (models 33 and 34), again, the resulting equation is of the form above, and similar conclusions hold good.

An extension of such a result is also discussed in the case of an n -site (ordered) modification system consisting of purely processive and purely distributive steps. If

distributive steps in phosphorylation and dephosphorylation are disjoint (i.e. no intermediate phosphoform is released in both phosphorylation and dephosphorylation), then the steady-state equations again simplify to a form similar to the above, and bistability is again ruled out. This indicates that it is necessary to have common distributive steps in phosphorylation and dephosphorylation to produce bistability.

Noting the relationship between these models and comprehensive mixed models, this analysis reveals various aspects of qualitative behaviour of the latter in different limiting situations.

4.5. Conclusion

Multi-site phosphorylation is a basic building block of signalling and cellular information processing. It is well known that the nature of enzymatic modification can significantly affect information processing and behaviour of signalling pathways, as seen from studies of distributive and processive systems. Multiple scenarios can result in mixed mechanisms. The natural question that arises is: how does information processing occur through mixed systems and how does this depend on the number of modification sites? In this chapter, a framework was developed to elucidate different aspects of the interplay between distributive and processive mechanisms, resulting in mixed models

4.5.1. A hierarchy of models

Since increasing the number of modifications increases the number of parameters involved, mixed mechanisms were analysed by considering a hierarchy of models. Investigation was first performed on the scenario where phosphorylation and dephosphorylation occur by different mechanisms (in two-site modification systems). This was then followed by examining mixed scenarios for phosphorylation, with

dephosphorylation being either processive or distributive (in three-site modification systems). Finally, a more comprehensive model involving both processive and distributive elements at every stage was also briefly studied. This includes the other models (which serve as a bridge to this) as special cases. It was able to check that the behaviour of this model essentially reduces to those of the earlier models when the rates of individual pathways are made small, and also elucidate the source of any differences. Thus, this study simultaneously involves more specific models (that also have the advantage of being more tractable) alongside comprehensive models. It is essential to move back and forth between these models, extracting relevant insights therefrom in a consolidated manner. The analysis of the specific cases allows us to identify patterns of information processing applicable to the n -site case

4.5.2. Two-site mixed systems

When opposing steps follow different mechanisms, it was found that behaviour such as bistability and biphasic behaviour in the maximally phosphorylated substrate (as total kinase concentration is increased) is ruled out, when all catalytic conversions are irreversible. This is established analytically and carries through to similar mechanisms involving multiple modification sites. The behaviour of intermediate phosphoforms is quite different from that in pure mechanisms due to the fact that they are bound to the enzyme in the processive leg of the reaction. It was found that mixed mechanisms were intrinsically capable of exhibiting oscillatory behaviour. Distributive twosite modifications have been reported to exhibit oscillations in a region of parameter space, when the mechanism is random (Jolley et al., 2012). However, oscillations in ordered distributive (or processive) double-site phosphorylation have not been reported. Random mechanisms involve more species with additional scope for sequestration effects, and can in general produce behaviour not seen in ordered mechanisms. A strong kinetic asymmetry (in the cycle) in the random mechanism

was identified as a key ingredient for oscillations (Jolley et al., 2012). In contrast it was found that, in a mixed model, even ordered mechanisms exhibit oscillations and do so when one of the enzymatic steps acts close to mass action kinetics (high catalytic constant). The presence of a processive leg can combine with the distributive step, in certain parameter regimes (involving enzyme sequestration), and introduce a substrate depletion effect generating oscillations. Thus, two-site ordered mixed mechanisms may be among the simplest multi-site enzymatic systems to intrinsically generate oscillations. Oscillations were also seen with both distributive/processive routes to modification at each step.

4.5.3. Three-site mixed systems

This section focuses on studying mixed mechanisms in phosphorylation when dephosphorylation occurred through a uniform mechanism. In certain scenarios, it was found that bistable behaviour can be ruled out. Bistability can be observed in these mixed mechanisms, but this requires distributive elements of both phosphorylation and dephosphorylation. The ordering and relative locations of distributive/processive steps can strongly affect system behaviour. Oscillatory behaviour was observed in all variants of three-site mixed mechanisms in this study, and, in some cases, was combined with bistability.

4.6. Discussion

4.6.1. The interplay of distributive and processive steps

This study reveals different facets of the interplay between processive and distributive mechanisms (see table 1 and related comments in appendix B). Even when processive and distributive mechanisms exhibit a simple quantitatively similar sigmoidal behaviour, a mixed

mechanism can give a quantitatively very different response. While the presence of processive steps can prevent or reduce the capacity of multi-stability, it can also introduce oscillations. Increasing the number of modification sites leads to an increasing number of new information-processing modes. It was able to determine analytically that, if no partial phosphoform was simultaneously associated with distributive steps in phosphorylation and dephosphorylation, then bistability would not be obtained in the model, irrespective of the number of sites. This analysis is relevant for multiple reasons. The first reason is that mixed mechanisms are seen in nature. For instance, it is known that the phosphorylation of protein Pho85 occurs through a mixed mechanism (in phosphorylation itself), and this is part of cell cycle regulation. While the modelling of this specific system in its cellular context is not undertaken here, developed framework and results in this study provide a basis for understanding what kind of dynamic patterns can result from the interweaving of processive and distributive steps in phosphorylation. Given the widespread occurrence of distributive and processive mechanisms, it is also to be expected that scenarios where the phosphorylation and dephosphorylation occur through different mechanisms and that there will be naturally occurring instances of this.

It is also known that due to various cellular factors (e.g. molecular crowding, scaffold, stress) various distributive mechanisms can behave effectively like processive mechanisms. When one examines this alongside the multitude of scenarios involving multi-site phosphorylation, it can be seen that there are multiple ways for a given multi-site mechanism to behave like a mixed mechanism. Even if some scenarios resulting in such behaviour necessarily result from stochastic effects, having a kinetic framework is useful, as it provides a basis for understanding what arises from the kinetics alone. This can be extended to examine stochastic effects. It is likely that, in some contexts, both routes are available, in which case distributive and processive steps are just limiting cases. Such models have also

been invoked in different biological studies (Kõivomägi et al., 2011). The analysis of comprehensive mixed models in this study is directly relevant here as well. In fact, studying a number of specific cases allows us to isolate key patterns in specific parametric regimes, which bookends a detailed parametric study.

Table 4.1 A summary of qualitative features of mixed mechanisms in two- and three-site modifications. The results, based on the models studied, for various scenarios of mechanisms (distributive, processive and mixed) in phosphorylation and dephosphorylation are discussed. The interplay between mixed phosphorylation and mixed dephosphorylation is not discussed in detail in the text (see comments in appendix B regarding this entry).

Ordered multi-site modification				
		Phosphorylation →		
Dephosphorylation ↓	Mechanism	Distributive	Mixed	Processive
	Distributive	Bistability Biphasic response	Bistability Biphasic response Oscillation	No Bistability No Biphasic response Oscillation
	Mixed	Bistability Biphasic response Oscillation	Bistability* Biphasic response* Oscillation**	No Bistability No Biphasic response Oscillation
	Processive	No Bistability No Biphasic response Oscillation	No Bistability No Biphasic response Oscillation	Simple Steady state

The analysis undertaken provides a framework for analysing the information-processing capabilities of mixed modification systems. Since these modification systems are embedded in signalling networks at different stages, this is directly relevant to information

processing in signalling networks as well, indicating what behaviour can arise purely from the modification mechanism. For instance, oscillatory behaviour has been observed in multiple signalling contexts. The combination of factors that generates oscillations has varied from context to context. In cyanobacterium, *in vitro* studies report oscillation at a covalent modification level *via* a three-protein network: proteins KaiA, KaiB and KaiC (Nakajima et al., 2005). A model incorporating feedback regulation in this context has been developed in conjunction with experiments (Rust et al., 2007). The multi-site phosphorylation of KaiC has been shown to be an important ingredient in this oscillatory circuit. This study shows how oscillation can arise from the enzymatic mechanism itself (and hence this can be ‘transmitted’ to other signalling entities). Likewise, this suggests under which conditions the mechanism of multisite phosphorylation may or may not generate bistability or biphasic behaviour, which has a bearing on pathway behaviour in which this is embedded. More generally, certain behaviour in signalling pathways could be made more robust or significantly altered by the behaviour of the enzymatic mechanisms themselves. It is worth mentioning that this study focuses on the intrinsic behaviour of the mechanism in isolation, in well-controlled conditions. It is possible that, in individual cellular contexts, significant deviations from these conditions can occur. In such situations, having a firm basis for understanding the intrinsic behaviour of the mechanisms helps, as it allows us to systematically infer what the effects of the deviations are, rather than misattribute observations to hypothesized mechanisms.

Finally, the analysis undertaken provides a framework for suggesting new modes of chemical information processing that arise through the interplay of distributive and processive mechanisms, nonlinearity as well as the number of sites. Using tools of synthetic chemistry and nanoengineering, it is likely that different aspects of enzymatic mechanisms will be engineered in the future. Indeed, engineering chemistry for the purposes of new modes of information processing is not new and is pursued in multiple contexts (Katz, 2012),

both biomolecular as well as non-biomolecular. A number of studies aim to harness or control biomolecular information processing, towards the generation of robust information-processing capabilities and applications. These may, in turn, be seen as elementary building blocks for biomolecular computing systems. This study points to capabilities and constraints in harnessing mixed mechanisms and multi-site modification mechanisms in general for chemical information processing, through the manipulation of enzymes, substrates, modification sites and protein structures, as well as reaction environments.

Taken together, this study shows that mixed modification mechanism can exhibit substantially different modes of chemical information processing when compared with pure mechanisms. This is of relevance in a number of scenarios ranging from the elucidation of signal transduction in concrete cellular contexts to the design of new modes of molecular information processing in biomolecular computing.

Chapter 5

Bridging the gap between the multi-site phosphorylation module in isolation and as parts of signalling networks

5.1 Introduction

So far in the thesis the study has focused on how multisite phosphorylation in isolation can exhibit a biphasic dose response curve where the modification mechanism is distributive. Then the effect of the change in mechanism was also studied. In particular, the previous chapter focussed on studying mixed mechanisms which involve the interplay of distributive and processive modification, demonstrating that this can give rise to different kinds of behaviour (abolishment of bistability and biphasic response and introduction of oscillation). Looking at various works in the previous chapter and various works in the literature (Markevich et al, 2004; Yang et al., 2004; Ortega et al., 2006; Salazar and Höfer, 2006; Manrai and Gunawardena, 2008; Kapuyet al., 2009; Thomson and Gunawardena, 2009; Jolley et al., 2012; Conradi and Mincheva, 2014), the important thing to note is all these papers deal with the complexity of multisite phosphorylation in isolation. All of these studies are focusing on the intrinsic information processing capability of multisite phosphorylation. In other words, all these papers conceptualize and deal with multisite phosphorylation as the closed compartment of substrate and enzyme where reaction happens (similar to a reaction in a test tube).

One of the important reasons for studying multisite phosphorylation is that it is a chemical key controller of many signalling pathways (Cohen, 2000). It plays very important roles in information propagation in these pathways. It has both basic and applied interest. However to understand the information processing of multisite phosphorylation as parts of signalling pathways, it is necessary to go beyond studying multisite phosphorylation just by itself.

There are multiple challenges in bridging the gap between the intrinsic behaviour of multisite phosphorylation and their functioning in pathway. For example, in all pathways the enzymes are not fixed pools but they are regulated by signal. In some cases, there is a production and degradation of the substrates and enzymes also and some of this may be relevant in the time scale of interest. Interestingly, the output of multisite phosphorylation is important in cellular outcomes. When the output participates in downstream pathways, it can also get sequestered and this can affect the upstream pathway behaviour, which is called retroactivity (Del Vecchio et al., 2008). Furthermore multi-site phosphorylation can be parts of different kinds of simple and complex networks and finally there are other complexities like spatial regulation and other factors.

The aim in this chapter is to bridge the gap between multisite modification modules in isolation and as parts of signalling pathways. Noting all the challenges and ingredients listed above, it is important to approach the goal in a very structured and systematic way. In this study, these various challenges are addressed one by one. In the first step, the effect of enzyme activation on the behaviour of multisite phosphorylation systems is considered. The second step focuses on examining the effect of the retroactivity which results from one of the phosphoform participating in downstream pathways. In the third step, the effect of the inflow and outflow of the enzyme and substrates (so that the modification module is no longer a closed system) is considered. In the last step to see the behaviour of multisite phosphorylation

in the networks in which they are embedded, simple kinds of modules with the multi-site phosphorylation embedded in them are examined. In particular, two kinds of feed-forward modules, coherent and incoherent feed-forward, and two kinds of feedback module, positive and negative feedback, are considered. All these modules have been studied in the simple biochemical cases (only simple single-site modification). In this study, multisite-phosphorylation is introduced as a basic element involve this pathway to study what the effect of multisite phosphorylation is on the network and what the effect of the network is on multisite phosphorylation

5.2 Models

The models which will be employed in this chapter are presented in four sections below. The first section presents the explicit activation model while the retroactive model is discussed in the second section. In the subsequent section, the focus is on describing the open systems model where there is an inflow and outflow of the enzymes or substrates. The final section presents feedback and feed-forward network models. With regard to the multisite modification model, the standard model of ordered double distributive phosphorylation (which is previously described in chapter 3) is mainly used.

5.2.1 Explicit enzymatic activation

In this section a basic model is developed to study the effect of explicit activation on the dynamic behaviour of multisite phosphorylation (see figure 5.1). To achieve this, an explicit enzymatic activation is introduced to the basic model of double distributive phosphorylation (see the standard distributive model in chapter3). The equations for kinase activation and phosphatase activation are:

$$\frac{d[K_o]}{dt} = k_{i,K}[K] - k_{a,K}S_1[K_o]$$

$$\frac{d[P_o]}{dt} = k_{i,P}[P] - k_{a,P}S_2[P_o]$$

In this equation $[K_o]$ and $[P_o]$ are respectively the concentrations of inactive kinase and phosphatase concentration whereas $[K]$ and $[P]$ refer to active kinase and phosphatase concentration, respectively. The activation/deactivation rate constant ($k_{i,K}$, $k_{a,K}$, $k_{i,P}$ and $k_{a,P}$)

are denoted as appropriate. The total amount of kinase is conserved, and so is the total amount of phosphatase:

$$K_{\text{tot}} = [K_0] + [K] + [AK] + [A_pK] \quad ,$$

$$P_{\text{tot}} = [P_0] + [P] + [A_{pp}P] + [A_pP]$$

where K_{tot} and P_{tot} are the total concentration of kinase and phosphatase, respectively. $[AK]$, $[A_pK]$, $[A_{pp}P]$ and $[A_pP]$ denote enzyme-substrate complex concentration in double phosphorylation (see in chapter 3). The substrate conservation (A) is the same as that in a standard double distributive phosphorylation model.

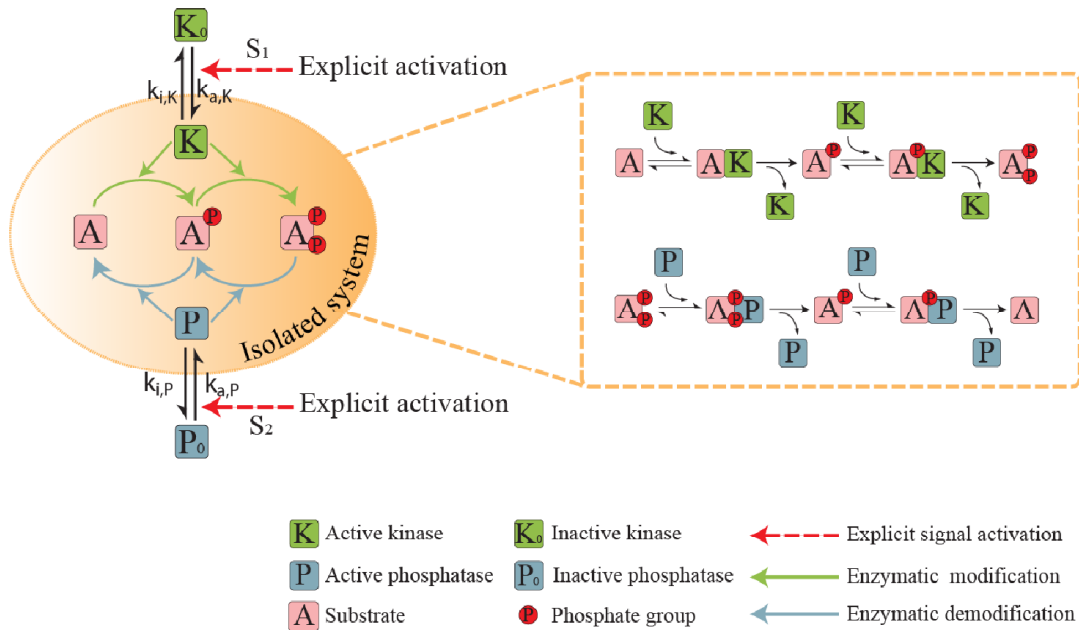


Figure 5.1 Schematic of model with explicit activation. Schematic of double distributive phosphorylation model with explicit activation. Explicit activation model involves kinase (K) and phosphatase (P) activation by external signals, S_1 and S_2 , respectively, whereas both enzymes are in active form in a closed system model considered previously

5.2.2 Retroactivity

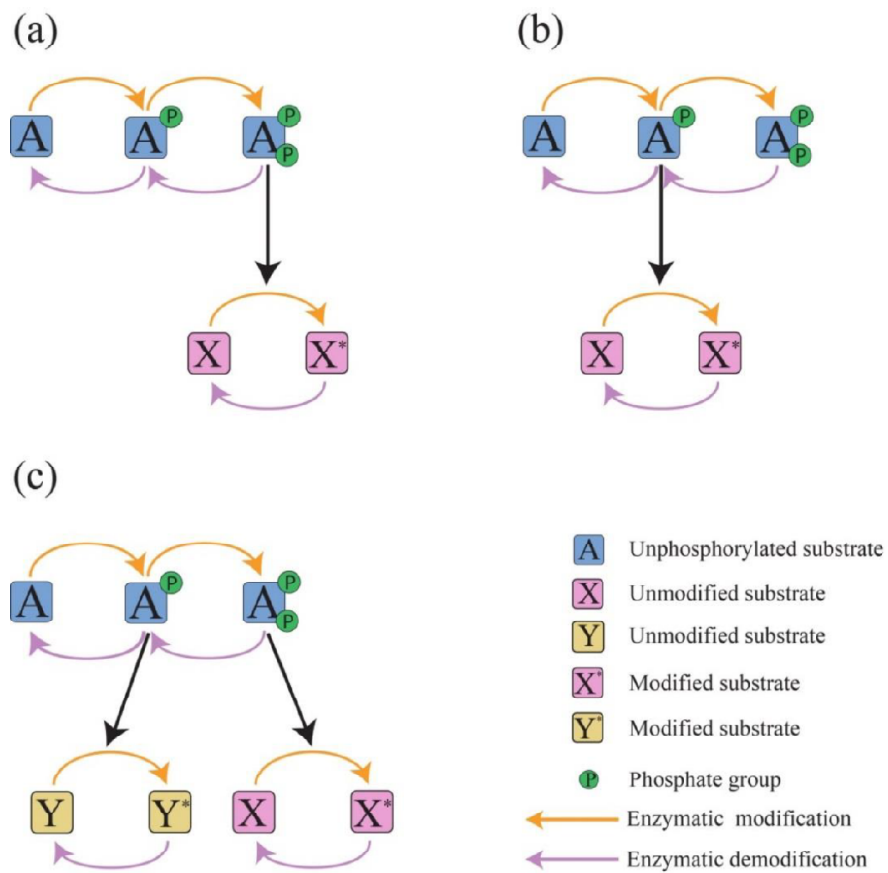


Figure 5.2 Schematic of retroactive models. Schematic of retroactive models of ordered double distributive phosphorylation. (a) and (b), respectively, represents the retroactive model where maximally and (b) partially phosphorylated substrates are sequestered to the downstream single covalent modification cycle (CMC). (c) represents retroactive model where all phosphoforms are sequestered to the different downstream CMC.

Retroactivity is essentially the perturbation of an upstream component as a consequence of a connection to a downstream component (Del Vecchio et al 2008). In the context of signalling, the effect of retroactivity on single-site covalent modification cycle was theoretically analysed in multiple studies, most of which focus on covalent modification cycles and as an extension, on kinase cascades (Ventura et al., 2008; Ossareh et al., 2011; Catozzi et al., 2016). To author's knowledge, no attempt has been made to systematically investigate the retroactive effect on the internal dynamics of multi-site phosphorylation, accounting for the potential intrinsic behaviour of the multisite substrate modification on one hand, and the multiple substrate species contributing to this, on the other.

In this thesis, a basic model is employed to study the effect of retroactivity which results from one of the phosphoforms (in multi-site phosphorylation) participating in the downstream covalent modification cycle (CMC). This model depicts, in a minimal way, the effect of sequestration of phosphoform in a downstream reaction. A basic model of double distributive phosphorylation is mainly used here..

Model R1

The maximally phosphorylated substrate (A_{pp}) catalyses a downstream substrate, X , producing X^* (the modified form of X), as shown in figure 5.2a. The downstream CMC is modelled using a standard mechanism, describing enzyme substrate binding and modification. The equations for the downstream CMC are:

$$\frac{d[X]}{dt} = k_{14}[A_{pp}X] - k_{13}[A_{pp}][X] + k_{18}[X^*F]$$

$$\frac{d[XA_{pp}]}{dt} = k_{13}[A_{pp}][X] - (k_{14} + k_{15})[XA_{pp}]$$

$$\frac{d[X^*]}{dt} = k_{15}[XA_{pp}] + k_{17}[X^*F] - k_{16}[X^*][F]$$

$$\frac{d[X^*F]}{dt} = k_{16}[X^*][F] - (k_{17} + k_{18})[X^*F]$$

The total amount of de-modifying enzyme (F) is conserved, and so is the total amount of downstream (X_{tot}) and upstream substrates (A_{tot}).

$$X_{\text{tot}} = [X] + [X^*] + [XA_{\text{pp}}] + [X^*F] \quad ,$$

$$F_{\text{tot}} = [F] + [X^*F]$$

$$A_{\text{tot}} = [A] + [A_p] + [A_{\text{pp}}] + [AK] + [A_pK] + [A_{\text{pp}}P] + [A_pP] + [XA_{\text{pp}}]$$

where X_{tot} and F_{tot} are the total concentration of downstream substrate and de-modifying enzyme, respectively. $[XA_{\text{pp}}]$ and $[X^*F]$ denote enzyme-substrate complex in downstream CMC as appropriate. The kinase and phosphatase conservation is the same as that in a basic double distributive phosphorylation. As a result of downstream sequestration, for the maximally phosphorylated substrate, A_{pp} , the kinetic equation governing its time evolution becomes:

$$\frac{d[A_{\text{pp}}]}{dt} = k_6[A_pK] + k_8[A_{\text{pp}}P] - k_7[A_{\text{pp}}][P] - k_{13}[A_{\text{pp}}][X] + (k_{14} + k_{15})[XA_{\text{pp}}]$$

Model R2

Model R2 describes a system in which the partial phosphorylated substrate, A_p , is sequestered in the downstream module (figure 5.2b). The equations for the downstream CMC are:

$$\frac{d[X]}{dt} = k_{14}[A_pX] - k_{13}[A_p][X] + k_{18}[X^*F]$$

$$\frac{d[XA_p]}{dt} = k_{13}[A_p][X] - (k_{14} + k_{15})[XA_p]$$

$$\frac{d[X^*]}{dt} = k_{15}[XA_p] + k_{17}[X^*F] - k_{16}[X^*][F]$$

$$\frac{d[X^*F]}{dt} = k_{16}[X^*][F] - (k_{17} + k_{18})[X^*F]$$

The total amount of de-modifying enzyme (F) is conserved, and so is the total amount of the substrate in downstream (X_{tot}) and upstream module (A_{tot}):

$$X_{tot} = [X] + [X^*] + [XA_p] + [X^*F] ,$$

$$F_{tot} = [F] + [X^*F]$$

$$A_{tot} = [A] + [A_p] + [A_{pp}] + [AK] + [A_pK] + [A_{pp}P] + [A_pP] + [XA_p]$$

where X_{tot} , A_{tot} and F_{tot} are the total concentration of downstream, upstream substrate and de-modifying enzyme, respectively. $[XA_p]$ and $[X^*F]$ denote enzyme-substrate complex in covalent modification cycle as appropriate. The kinase and phosphatase conservation is the same as that in a standard double distributive phosphorylation model. As a result of downstream sequestration, for the partial phosphorylated substrate, A_p , the kinetic equation governing the time evolution of this phosphoform becomes:

$$\begin{aligned} \frac{d[A_p]}{dt} = & k_3[AK] - k_4[A_p][K] + k_5[A_pK] + k_9[A_{pp}P] + k_{11}[A_pP] - k_{10}[A_p][P] - k_{13}[A_p][X] \\ & + (k_{14} + k_{15})[XA_p] \end{aligned}$$

Model R3

Model R3 presents a system in which maximally phosphorylated substrate (A_{pp}) catalyses a downstream substrate, X , producing X^* (the modified form of X) while the partially phosphorylated substrate (A_p) catalyses another downstream substrate, Y , producing Y^* (see figure 5.2c). The downstream covalent modifications are modelled using a standard mechanism, describing enzyme substrate binding and modification: The equations for the downstream covalent modification are:

$$\frac{d[X]}{dt} = k_{14}[A_{pp}X] - k_{13}[A_{pp}][X] + k_{18}[X^*F_1]$$

$$\frac{d[XA_{pp}]}{dt} = k_{13}[A_{pp}][X] - (k_{14} + k_{15})[XA_{pp}]$$

$$\frac{d[X^*]}{dt} = k_{15}[XA_{pp}] + k_{17}[X^*F_1] - k_{16}[X^*][F_1]$$

$$\frac{d[X^*F]}{dt} = k_{16}[X^*][F_1] - (k_{17} + k_{18})[X^*F_1]$$

$$\frac{d[Y]}{dt} = k_{20}[YA_p] - k_{19}[A_p][Y] + k_{24}[Y^*F_2]$$

$$\frac{d[YA_p]}{dt} = k_{19}[A_p][Y] - (k_{20} + k_{21})[YA_p]$$

$$\frac{d[Y^*]}{dt} = k_{21}[YA_p] + k_{23}[Y^*F_2] - k_{22}[Y^*][F_2]$$

$$\frac{d[Y^*F_2]}{dt} = k_{22}[Y^*][F_2] - (k_{22} + k_{23})[Y^*F_2]$$

The total amount of de-modifying enzyme (F_1 and F_2) is conserved, and so is the total amount of the downstream (X_{tot} and Y_{tot}) and upstream substrate (A_{tot}).

$$F_{1,tot} = [F_1] + [X^*F_1] ,$$

$$F_{2,tot} = [F_2] + [Y^*F_2] ,$$

$$X_{\text{tot}} = [X] + [X^*] + [XA_{\text{pp}}] + [X^*F_1] \quad ,$$

$$Y_{\text{tot}} = [Y] + [Y^*] + [YA_p] + [Y^*F_2] \quad ,$$

$$A_{\text{tot}} = [A] + [A_p] + [A_{\text{pp}}] + [AK] + [A_pK] + [A_{\text{pp}}P] + [A_pP] + [XA_{\text{pp}}] + [YA_p]$$

where X_{tot} , Y_{tot} and $F_{1,\text{tot}}$ and $F_{2,\text{tot}}$ are the total concentration of downstream substrate and demodifying enzyme, respectively. $[XA_{\text{pp}}]$, $[X^*F_1]$, $[YA_p]$ and $[Y^*F_2]$ denote enzyme-substrate complexes in covalent modification cycle as appropriate. The kinase and phosphatase conservation is the same as that in a standard double distributive phosphorylation. As a result of downstream sequestration, for the maximally and partially phosphorylated substrate, A_{pp} and A_p , the kinetic equations governing the time evolution becomes:

$$\frac{d[A_{\text{pp}}]}{dt} = k_6[A_pK] + k_8[A_{\text{pp}}P] - k_7[A_{\text{pp}}][P] - k_{13}[A_{\text{pp}}][X] + (k_{14} + k_{15})[XA_{\text{pp}}]$$

$$\begin{aligned} \frac{d[A_p]}{dt} = & k_3[AK] - k_4[A_p][K] + k_5[A_pK] + k_9[A_{\text{pp}}P] + k_{11}[A_pP] - k_{10}[A_p][P] - k_{19}[A_p][Y] \\ & + (k_{20} + k_{21})[YA_p] \end{aligned}$$

5.2.3 Open systems

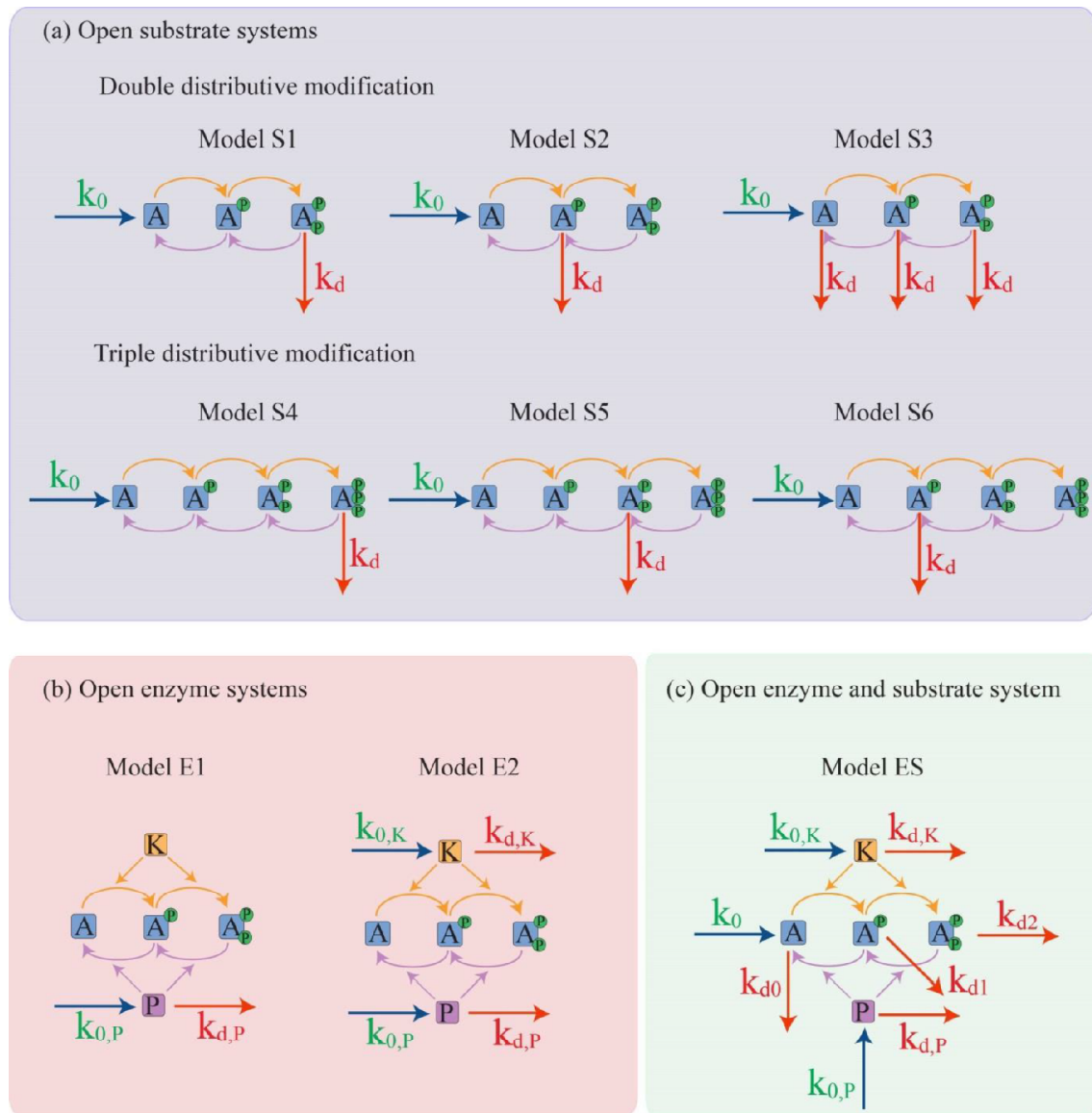


Figure 5.3 Schematic of open system models. (a) represents a set of open substrate system models. (b) represents multiple open enzyme system models. (c) represents an open system model where there is an inflow and outflow of both substrates and enzymes.

In the previous chapters, studies consider the case where substrate and enzyme is a fixed pool. In this section, a sequence of models is developed to study the effect of the inflow and outflow of the enzyme or substrates on dynamic behaviour of multisite phosphorylation. Basically, analysis is performed on a basic model of double distributive phosphorylation which is used in chapter 3, extending it to triple site modification, when necessary.

5.2.3.1 Open substrate systems

This section considers the systems in which there is production of unmodified substrate (A) and degradation of different modified substrates in a standard model of 2-site distributive phosphorylation. Note that the total amount of substrate is not conserved in open substrate systems (model S1-S6) while the conservation conditions for both kinase and phosphatase are the same. In all the model variants below, the focus is on the equations which are modified relative to the closed system models presented in chapter 3, so as to avoid repeating the entire set of equations.

Model S1

Model S1 represents a system in which there is production of substrate (A) and degradation of the fully modified substrates (A_{pp}) in ordered double distributive phosphorylation. For $[A]$ and $[A_{pp}]$, the kinetic equations governing the time evolution becomes:

$$\begin{aligned}\frac{d[A]}{dt} &= k_0 + k_2[AK] - k_1[A][K] + k_{12}[A_pP] \\ \frac{d[A_{pp}]}{dt} &= k_6[A_pK] + k_8[A_{pp}P] - k_7[A_{pp}][P] - k_d[A_{pp}]\end{aligned}$$

where k_0 and k_d denote the production rate constant and degradation rate constant, respectively.

Model S2

Model S2 represents a system in which there is production of substrate (A) and degradation of the singly modified substrates (A_p) in double distributive phosphorylation. For $[A]$ and $[A_p]$, the kinetic equations governing the time evolution becomes:

$$\begin{aligned}\frac{d[A]}{dt} &= k_0 + k_2[AK] - k_1[A][K] + k_{12}[A_pP] \\ \frac{d[A_p]}{dt} &= k_3[AK] - k_4[A_p][K] + k_5[A_pK] + k_9[A_{pp}P] + k_{11}[A_pP] - k_{10}[A_p][P] - k_d[A_p]\end{aligned}$$

where k_0 and k_d denote the production rate constant and degradation rate constant, respectively.

Model S3

Model S3 represents a system in which there is production of substrate (A) and degradation of both substrate and all modified phospho-form (A , A_p and A_{pp}) in double distributive phosphorylation. For $[A]$, $[A_p]$ and $[A_{pp}]$, the kinetic equations governing the time evolution becomes:

$$\begin{aligned}\frac{d[A]}{dt} &= k_0 + k_2[AK] - k_1[A][K] + k_{12}[A_pP] - k_{d0}[A] \\ \frac{d[A_p]}{dt} &= k_3[AK] - k_4[A_p][K] + k_5[A_pK] + k_9[A_{pp}P] + k_{11}[A_pP] - k_{10}[A_p][P] - k_{d1}[A_p] \\ \frac{d[A_{pp}]}{dt} &= k_6[A_pK] + k_8[A_{pp}P] - k_7[A_{pp}][P] - k_{d2}[A_{pp}]\end{aligned}$$

where k_0 denotes the production rate constant while k_{d0} , k_{d1} and k_{d2} refer to degradation rate constant of A , A_p and A_{pp} , respectively.

Model S4

Model S4 represents a system in which there is production of substrate (A) and degradation of the maximally phosphorylated substrates (A_{ppp}) in (ordered) triple distributive phosphorylation. The equation of standard model is presented in the chapter3. For [A] and $[A_{ppp}]$, the kinetic equations governing the time evolution become modified and result in:

$$\begin{aligned}\frac{d[A]}{dt} &= k_0 + k_2[AK] + k_{18}[A_P P] - k_1[A][K] \\ \frac{d[A_{ppp}]}{dt} &= k_9[A_{pp}K] + k_{11}[A_{ppp}P] - k_{10}[A_{ppp}][P] - k_d[A_{ppp}]\end{aligned}$$

where k_0 and k_d denote the production rate constant and degradation rate constant, respectively.

Model S5

Model S5 represents a system in which there is production of substrate (A) and degradation of the doubly phosphorylated substrates (A_{pp}) in (ordered) triple distributive phosphorylation. The equation of standard model is presented in the chapter3. For [A] and $[A_{pp}]$, the kinetic equations governing the time evolution becomes:

$$\begin{aligned}\frac{d[A]}{dt} &= k_0 + k_2[AK] + k_{18}[A_P P] - k_1[A][K] \\ \frac{d[A_{pp}]}{dt} &= k_6[A_P K] + k_8[A_{pp}K] - k_7[A_{pp}][K] + k_{12}[A_{ppp}P] + k_{14}[A_{pp}P] - k_{13}[A_{pp}][P] \\ &\quad - k_d[A_{pp}]\end{aligned}$$

where k_0 and k_d denote the production rate constant and degradation rate constant, respectively.

Model S6

Model S6 represents a system in which there is production of substrate (A) and degradation of the singly phosphorylated substrates (A_p) in (ordered) triple distributive phosphorylation. The equation of standard model is presented in the chapter3. For [A] and $[A_p]$, the kinetic equations governing the time evolution becomes:

$$\begin{aligned}\frac{d[A]}{dt} &= k_0 + k_2[AK] + k_{18}[A_pP] - k_1[A][K] \\ \frac{d[A_p]}{dt} &= k_3[AK] - k_4[A_p][K] + k_5[A_pK] + k_{15}[A_{pp}P] + k_{17}[A_pP] - k_{16}[A_p][P] \\ &\quad - k_d[A_p]\end{aligned}$$

where k_0 and k_d denote the production rate constant and degradation rate constant, respectively.

5.2.3.2 Open enzyme systems

This section focuses on studying the systems where enzyme is not a fixed pool. There is inflow and outflow of the enzyme in a basic model of 2-site distributive phosphorylation. Note that the total amount of enzyme is not conserved here while the conservation condition for substrate is the same as that in closed multisite phosphorylation systems.

Model E1

Model E1 represents a system in which there is production and degradation of phosphatase, P. For [P], the kinetic equations governing the time evolution becomes:

$$\frac{d[P]}{dt} = k_{0,K} + (k_8 + k_9)[A_{pp}P] - k_7[A_{pp}][P] + (k_{11} + k_{12})[A_pP] - k_{10}[A_p][P] - k_{d,P}[P]$$

where k_0 and k_d denote the production and degradation rate constant, respectively.

Here the total amount of kinase is conserved similar to the closed 2-site distributive model while the total amount of phosphatase is not.

Model E2

Model E2 represents a system in which there is production and degradation of both kinase, K, and phosphatase, P. For [K] and [P], the kinetic equations governing the time evolution becomes:

$$\frac{d[K]}{dt} = k_{0,K} + (k_2 + k_3)[AK] - k_1[A][K] + (k_5 + k_6)[A_pK] - k_4[A_p][K] - k_{d,K}[K]$$

$$\frac{d[P]}{dt} = k_{0,P} + (k_8 + k_9)[A_{pp}P] - k_7[A_{pp}][P] + (k_{11} + k_{12})[A_pP] - k_{10}[A_p][P] - k_{d,P}[P]$$

where k_0 and k_d denote the production and degradation rate constant, respectively.

The total amount of kinase and the total amount of phosphatase are not conserved in this scenario.

5.2.3.3 Open enzyme and substrate system (model ES)

Model ES represents the scenario where neither enzymes nor substrates are part of a fixed pool. There is inflow and outflow of all enzymes and all substrates in a basic model of 2-site distributive phosphorylation. For [A], $[A_p]$, $[A_{pp}]$, [K] and [P], the kinetic equations governing the time evolution becomes:

$$\frac{d[A]}{dt} = k_0 + k_2[AK] - k_1[A][K] + k_{12}[A_pP] - k_{d0}[A]$$

$$\frac{d[A_p]}{dt} = k_3[AK] - k_4[A_p][K] + k_5[A_pK] + k_9[A_{pp}P] + k_{11}[A_pP] - k_{10}[A_p][P] - k_{d1}[A_p]$$

$$\frac{d[A_{pp}]}{dt} = k_6[A_pK] + k_8[A_{pp}P] - k_7[A_{pp}][P] - k_{d2}[A_{pp}]$$

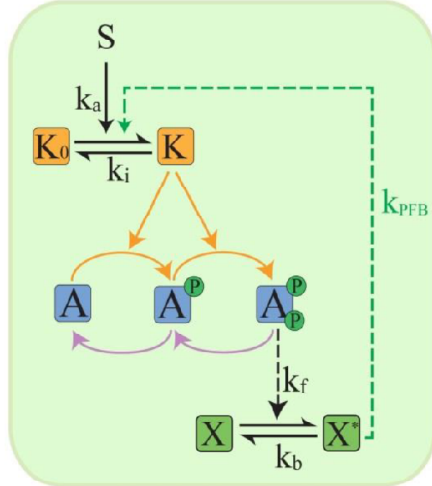
$$\frac{d[K]}{dt} = k_{0,K} + (k_2 + k_3)[AK] - k_1[A][K] + (k_5 + k_6)[A_pK] - k_4[A_p][K] - k_{d,K}[K]$$

$$\frac{d[P]}{dt} = k_{0,P} + (k_8 + k_9)[A_{pp}P] - k_7[A_{pp}][P] + (k_{11} + k_{12})[A_pP] - k_{10}[A_p][P] - k_{d,P}[P]$$

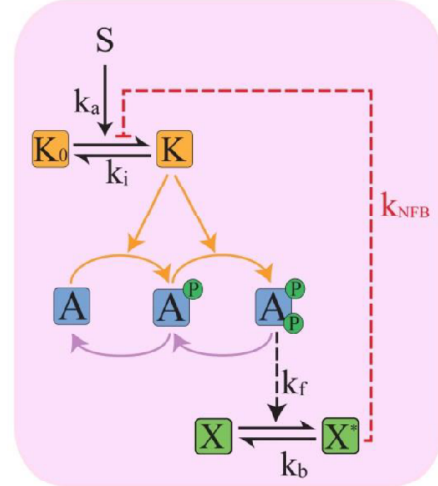
where k_0 , $k_{0,K}$ and $k_{0,P}$ respectively denote the production rate constant of A, K and P while k_{d0} , k_{d1} and k_{d2} , $k_{d0,K}$ and $k_{d0,P}$ refer to degradation rate constant of A, A_p , A_{pp} , K and P respectively. Note that the total amount of enzyme is not conserved here and neither is the total amount of substrate.

5.2.4 Effect of the modules embedded in the networks

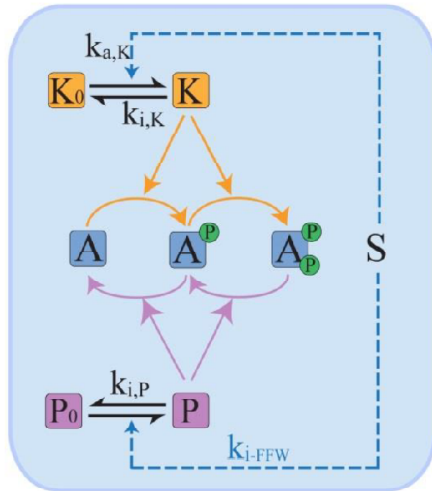
(a) Positive feedback (PFB)



(b) Negative feedback (NFB)



(c) Incoherent feed-forward (i-FFW)



(d) Coherent feed-forward (c-FFW)

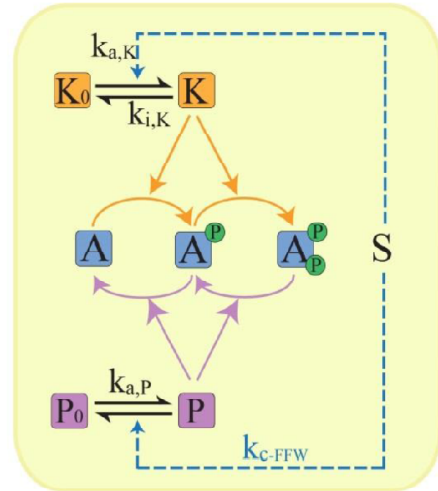


Figure 5.4 Schematic of phosphorylation modules embedded in different kinds of network modules. Schematic of modules of ordered double distributive phosphorylation embedded in different kinds of network modules (a) positive feedback module, PFB (b) negative feedback module, NFB, (c) incoherent feed-forward module, i-FFW, and (d) coherent feed-forward module, c-FFW.

In this section models are constructed to investigate the behaviour of multisite phosphorylation embedded in networks. In particular, the focus is on two kinds of feedback modules, positive and negative feedback and two kinds of feed-forward modules, coherent and incoherent feed-forward. An ordered double distributive phosphorylation is introduced as a basic element into such modules. The substrate modification follows the same equation as in a basic double phosphorylation model, which is previously described in chapter3.

5.2.4.1 Positive feedback module (PFB)

This section focuses studying the effect of positive feedback module which basically involves explicit activation of kinase (by external signal(S)) as well as positive feedback regulation. In PFB model, the maximally phosphorylated substrate, A_{pp} , regulates the activation of a downstream substrate, X , producing X^* (the modified form of X) and X^* in turn regulates the activation of kinase of the multisite phosphorylation module. The equations for kinase activation and downstream substrate activation are:

$$\frac{d[K_0]}{dt} = k_i[K] - k_a[S][K_0] - k_{PFB}[K_0][X^*]$$

$$\frac{d[X]}{dt} = k_b[X^*] - k_f[X][A_{pp}]$$

$$\frac{d[X^*]}{dt} = k_f[X][A_{pp}] - k_b[X^*]$$

The equation for the active kinase $[K]$ also reflects the effect of the positive feedback (not shown here). In this equation, $[K_0]$, $[K]$ is respectively the concentrations of inactive and active kinase. The conservation of the total amount of kinase follows in exactly the same way as in the explicit activation model in section 5.2.1 while the conservation condition of phosphatase follows in the same way as in a basic 2-site model. The activation/deactivation rate constants (k_i , k_a , k_b , k_f and k_{PFB}) denote rate constants for the relevant steps (deactivation,

signal based activation, forward reaction of downstream modification, reverse reaction of downstream modification, positive feedback reaction

The total amount of downstream substrate (X_{tot}) is conserved:

$$X_{\text{tot}} = X + X^*$$

5.2.4.2 Negative feedback module (NFB)

The Negative feedback model (figure 5.4b) follows in the same way as in the positive feedback model with the only difference that the downstream product, X^* , inactivates the kinase. As a result the equation for the (inactive) kinase is

$$\frac{d[K_0]}{dt} = k_i[K] - k_a[S][K_0] + k_{\text{NFB}}[X^*][K]$$

The activation/deactivation rate constants (k_i , k_a , k_{NFB}) denote appropriate reaction rate constants associated with the activation/deactivation of the kinase (see figure 5.4b). Note that k_{NFB} is the constant which presents the strength of the negative feedback regulation.

5.2.4.3 Incoherent feed-forward module (i-FFW)

In the section 5.2.1, an explicit activation module was presented. This section focuses on studying an incoherent feed-forward module (figure 5.4c) which basically involves explicit activation of kinase and phosphatase simultaneously by the same signal. Therefore, the equations for kinase activation and phosphatase activation become:

$$\frac{d[K_0]}{dt} = k_{i,K}[K] - k_{a,K}S[K_0]$$

$$\frac{d[P_0]}{dt} = k_{i,P}[P] - (k_{i\text{-FFW}}S + \beta)[P_0]$$

In this equation, K_o and P_o , are respectively the concentrations of inactive kinase and phosphatase concentration whereas $[K]$ and $[P]$ refer to active kinase and phosphatase concentration, respectively. The conservation of the total amount of kinase and phosphatase follow exactly the same way as in the explicit activation model in section 5.2.1. The activation/deactivation rate constants ($k_{i,K}$, $k_{a,K}$, $k_{i,P}$, β and k_{i-FFW}) are denote rate constants of appropriate steps (see figure 5.4d). Note that k_{i-FFW} is the constant which presents the strength of the incoherent feed-forward regulation.

5.2.4.4 Coherent feed-forward module (c-FFW)

The coherent feed-forward module (figure 5.4d) basically involves explicit activation of kinase and deactivation of phosphatase simultaneously by the same signal. Therefore, the equations for kinase activation and phosphatase activation become:

$$\frac{d[K_o]}{dt} = k_{i,K}[K] - k_{a,K}S[K_o]$$

$$\frac{d[P_o]}{dt} = (k_{c-FFW}S + \beta)[P] - k_{a,P}[P_o]$$

The activation/deactivation rate constants ($k_{i,K}$, $k_{a,K}$, $k_{a,P}$ and k_{c-FFW}) denote rate constants of appropriate steps (see figure 5.4d). The conservation of the total amount of kinase and phosphatase follows in exactly the same way as in the explicit activation model in section 5.2.1. Note that k_{c-FFW} is the constant which presents the strength of the coherent feed-forward regulation.

5.3 Results

This chapter aims to study the behaviour of multisite phosphorylation as a part of signalling networks. The focal point of this study is mainly on qualitative features; therefore, a detailed parametric analysis of these models is not the focus here. The results are divided into four sections, which are organized as follows. In the first part, the effect of explicit activation will be discussed. Then the effect of retroactivity will be dissected in the second section. In the subsequent section, the focus is on discussing open systems. Last, but definitely not least, the effect of the modules embedded in the networks will be discussed in the fourth section.

Method of analysis: The approach is emphasized at the outset. This is relevant for all the different sets of investigations below. Considering the effect of coupling the multisite module to the environment in each of its aspects (as discussed above), this study aims to address the question: what are the qualitative effects and changes (either capabilities or constraints in information processing) introduced by the coupling to the environments? Thus all the analysis performed in this chapter are oriented towards this question.

Consequently, the approach is to isolate qualitative effects and changes introduced by the coupling. The goal is not to perform an exhaustive numerical analysis of each model coupled to the environment. The analysis has indeed been underpinned by detailed parametric searches and analysis, but results reported here focuses on key qualitative behaviour and changes introduced by the coupling.

As mentioned earlier, the multisite phosphorylation module in isolation has a number of parameters which affects the quantitative aspects of the response. While this is the case, there are still a small number of basic qualitative responses, and so the focus is on the

multisite module when it exhibits these responses, and studying the coupling to the environment. In all these cases, similar or equivalent trends can be observed when different parameter sets representing the same qualitative behaviour are chosen. Given particular qualitative behaviour of the multisite module, the author then focus on trends (rather than numbers) associated with the coupling. This basically allows us to isolate qualitative signatures associated with the coupling.

It is worth commenting on the focus of this chapter, in relation to other related studies in signalling. The focus is on multisite modification as see how the behaviour is perturbed by different ways in which the surrounding network environment perturbs the behaviour. It has already been pointed out that the effect of downstream pathways on upstream modules has been studied in a range of settings (Ventura et al., 2008; Ossareh et al., 2011; Catozzi et al., 2016). The effect of feedback and feed-forward regulation on simple covalent modification cycles, and (in the case of feedback) on enzymatic cascades has also been undertaken (Kholodenko, 2000; Levchenko and Iglesias, 2002; Seaton and Krishnan, 2011). The interaction of a module with the network environment has been also studied in (Menon and Krishnan, 2016). In this thesis, the focus, by contrast, is to start with the multisite module and take a unified approach towards examining how different interactions can perturb this.

In this work numerical simulations and numerical bifurcation analysis are performed (and this is already underpinned by performing analysis in different parameter regimes). It is also complemented by analytical work which is presented in relevant publications in preparation.

Taken together this study provides a broad view of the qualitative behaviour and typical transitions (as well as unexpected transitions) associated with the coupling of the

module to the environment. This provides a framework and reference for subsequent detailed computational parametric investigations

5.3.1 Effect of explicit enzyme activation

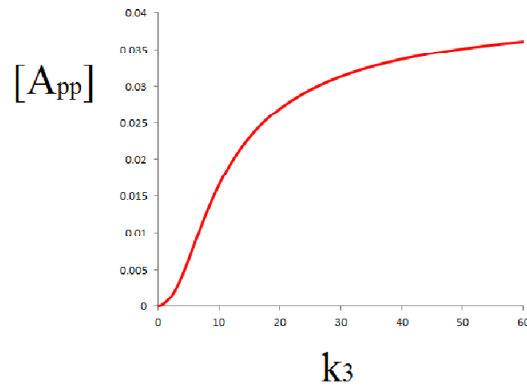
In this section, the focus is on studying the effect of explicit enzymatic activation on dynamic behaviour of multi-site phosphorylation. The behaviour of the phosphorylation model with explicit activation is contrasted with that without enzyme activation (with ‘corresponding’ parameters).

The main goal is to identify and elucidate the presence of key qualitative signatures and capabilities in information processing arising from explicit enzymatic activation in multi-site phosphorylation. This study does not focus on a detailed parametric analysis of these models here. While enzymes, both kinase and phosphatase, are active in the isolated model, the explicit activation model is additionally composed of explicit activation of both enzymes (as shown in figure 5.1).

An ordered 2-site distributive phosphorylation is used to study the effect of explicit activation. The study starts with examining explicit activation model in parameter regimes, where the ‘corresponding’ isolated systems present a monotonic dose response. Explicit activation systems are different from isolated systems in that both enzymes (kinase and phosphatase) are active in isolated systems whereas both enzymes can be active or inactive in explicit activation model. Figure 5.5a and 5.5b(i)) present simulations showing how the maximally phosphorylated substrate concentration at steady state varies with the catalytic constant of the first phosphorylation step, k_3 . In contrast to the ‘corresponding’ isolated system, which reaches a steady state, it is found that in the explicit activation model sustained oscillations can occur (figure 5.5b(ii)). A bifurcation diagram shows that, as the parameter k_3

is changed, the steady state does indeed lose stability to a Hopf bifurcation. This shows that explicit activation can completely introduce oscillation whereas isolated system may even present monotonic dose response.

(a) without explicit activation



(b) with explicit activation

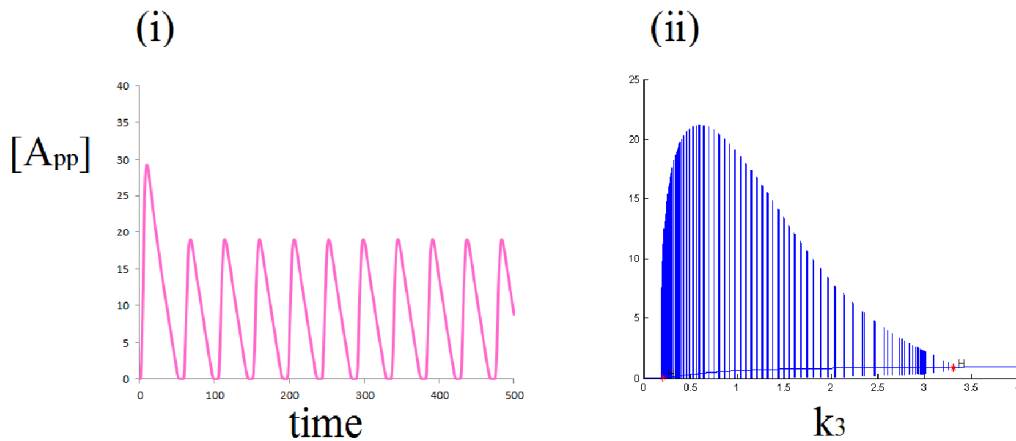
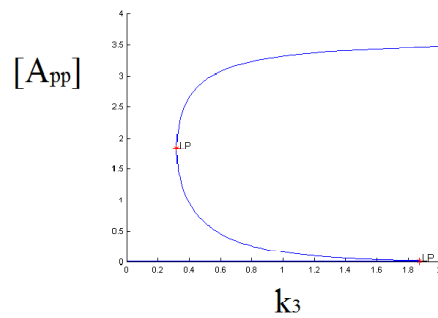


Figure 5.5 Oscillatory dynamic behaviour in explicit activation model (ordered double distributive modification). With the corresponding parameters, the ordered double distributive phosphorylation model (in isolation) exhibits monotonic response (a) whereas the explicit activation model exhibits sustained oscillation (b(i)). Bifurcation analysis in b(ii) shows that as k_3 is changed, the steady state loses stability *via* a Hopf bifurcation in explicit activation model.

The focus now is on examining explicit activation system in parameter regimes, where the isolated system is bistable. A choice of sufficiently different catalytic constants in (de)phosphorylation can result in bistability in an ordered distributive model, as shown in figure 5.6a. By contrast, the explicit activation model introduces sustained oscillation whereas bistability is completely destroyed in this parameter range (as shown in figure 5.6b).

(a) without explicit activation



(b) with explicit activation

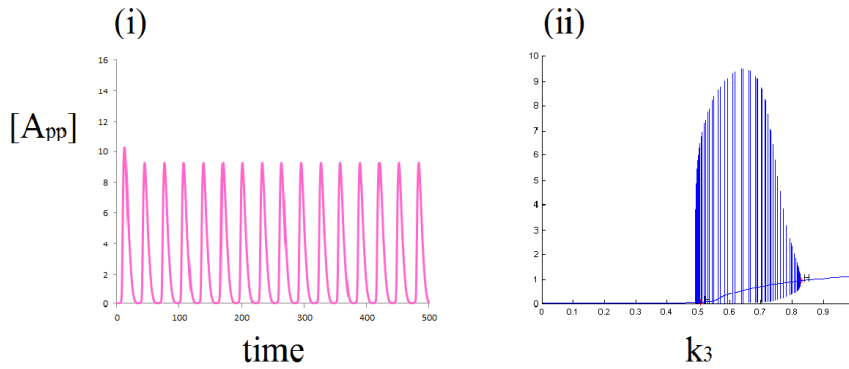


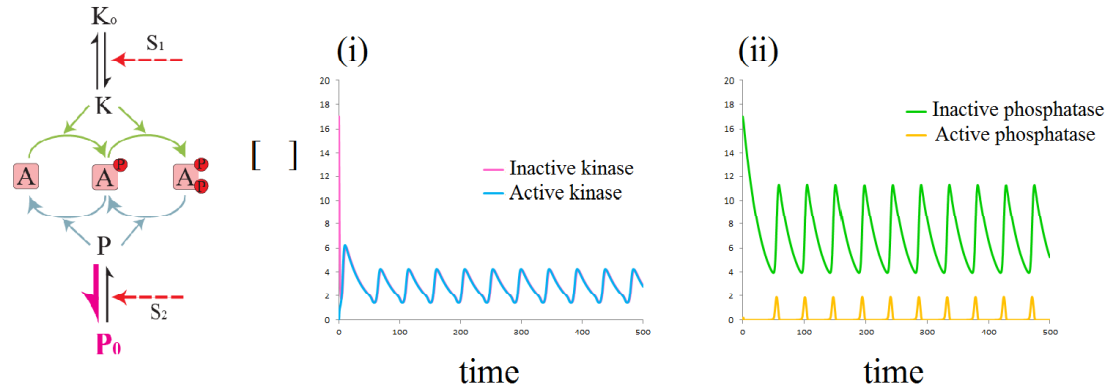
Figure 5.6 Oscillatory dynamic behaviour in ordered double distributive phosphorylation with explicit activation. With the corresponding parameters, ordered double distributive phosphorylation model in isolation exhibits bistability (a) whereas the explicit activation model exhibits sustained oscillation (b(i)). Bifurcation analysis in b(ii) shows that as k_3 is changed, the steady state loses stability *via* a Hopf bifurcation in explicit activation model.

Explicit activation is a very simple additional ingredient and is ubiquitous in signalling pathways. In spite of this, this study shows that it can create an important qualitative difference. It can introduce sustained oscillation whereas isolated system may even presents monotonic response or bistability. In the previous chapter, sustained oscillations have not been observed in the isolated system even with detailed parametric search (two-site ordered distributive phosphorylation) and such kinds of behaviour have not been reported in the literature. This means that explicit activation by itself can completely introduce new behaviour which is different from the isolated modification system.

Further insights into the origins of oscillation can be obtained by looking at the variation of both enzymes as a function of time. Figure 5.7 presents simulations showing how the enzyme concentration varies with time. The explicit activation model is first examined in parameter regimes, which are used in figure 5.5b. As seen in figure 5.7a, simulations show that by introducing explicit activation the system can present oscillation when phosphatase is far from active form while kinase is not (and hence, by symmetry, similar behaviour can be seen in the other way around). Furthermore, the explicit activation model is investigated in other parameter regimes. It was found that oscillations can occur when both kinase and phosphatase are far from active fully (figure 5.7b).

Overall by introducing explicit activation, it is found that it is necessary for at least one enzyme to be not fully activated to introduce oscillation in ordered 2-site phosphorylation. When all enzymes are in active form, the explicit activation model will become like the isolated system model and sustained oscillation is not observed. In a sense, a basic enzyme sequestration per se can introduce oscillation in multisite phosphorylation both in monostable and bistable range of parameters. Since sequestration of only one enzyme is sufficient for inducing oscillation, it can be inferred that models with explicit activation of only kinase or phosphatase is sufficient for inducing oscillation as well (result not shown).

(a) $[P_0] \gg [P]$ or $[K_0] \gg [K]$



(b) $[P_0] \gg [P]$ and $[K_0] \gg [K]$

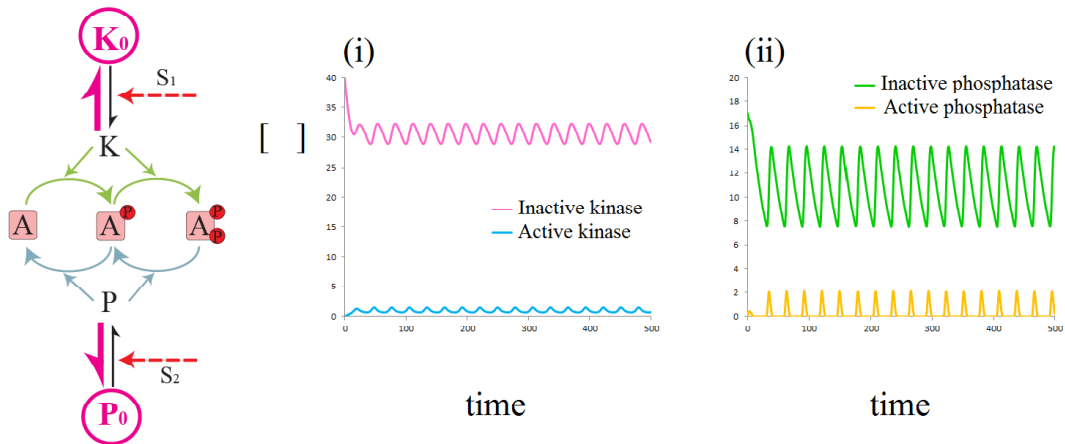


Figure 5.7 The time-dependent response profiles of the concentration of various enzymes in explicit activation model. Simulations show that by introducing explicit activation to ordered double distributive phosphorylation, the system can present sustained oscillation when one (a) or both enzymes (b) is far from the fully active form.

After investigating simple oscillations with only large or only small amplitudes arising from multi-phosphorylation with explicit enzymatic activation, it is interesting to examine whether slightly more complex temporal patterns may arise in a sustained manner. The focus is now on examining explicit activation model in kinetic parameter regimes, which is used in figure 5.5b (with different signal intensity). Figure 5.8 presents simulations

showing how the fully phosphorylated substrate concentration varies with time. As seen in figure 5.8a, it is found that the system can generate sustained oscillation with two separated amplitudes, a so-called double-period oscillation. This shows how explicit activation can completely introduce more complex temporal behaviour and how it has profound effect on the dynamic behaviour of multisite phosphorylation.

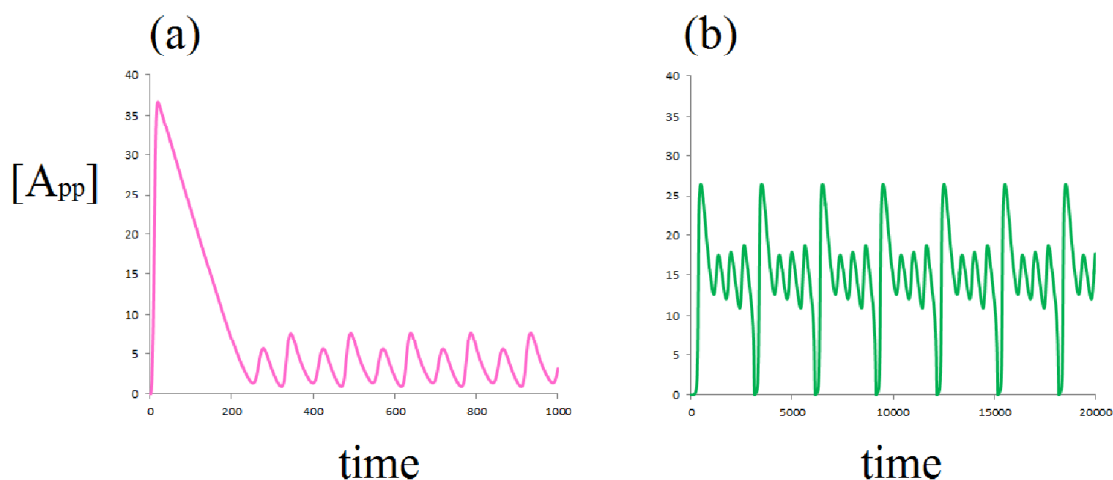


Figure 5.8 Complex oscillatory behaviour in explicit activation model. The completely phosphorylated substrate, A_{pp} , concentration as a function of time. (a) Double period oscillation and (b) Mixed mode oscillation

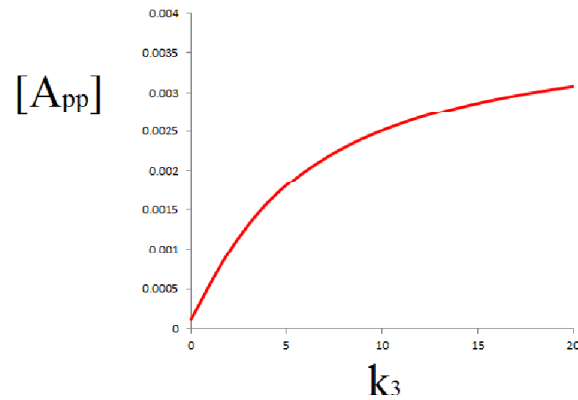
It is also interesting to ask whether the systems with explicit activation can exhibit more complex oscillatory dynamic behaviour. As seen in figure 5.8b, it is found that at proper condition the system can exhibit ‘mixed mode oscillation’ wherein there is an alternation between oscillations of distinct large and small amplitude. Mixed-mode oscillations can be observed in both experiments and models appropriate to chemical or biological systems (Petrov et al., 1992). Examples include calcium signalling which involves negative feedback regulation (Berridge et al., 2000; Wilson & Callaway, 2000). Here this study shows that

mixed mode oscillation can arise due to the double-site modification without feedback regulation (and thus this can be ‘transmitted’ to other signalling entities). To the best of the author’s knowledge, this would be the first study to show that mixed mode oscillation can arise due to simple enzymatic mechanism without feedback regulation.

After investigating oscillatory behaviour arising due to explicit activation in the ordered mechanism, the focus now is on examining the other scenario where the order of phospho-site processing is random. Based on the parameter regime where the kinetic rate constants are symmetric (in the cycle), monostable behaviour is observed in the isolated model, as shown in figure 5.9a. By contrast, oscillations are observed in random model with explicit activation in this parameter regime. Bifurcation analysis in figure 5.9b(ii) shows that as the catalytic constant of the first phosphorylation step (upper branch), k_3 , is changed, the steady state loses stability *via* a Hopf bifurcation in the explicit activation model.

In biological contexts, kinases/phosphatases can modify their substrate in either ordered or random manner. Random mechanisms involve more species with additional scope for sequestration effects, and can in general produce behaviour not seen in ordered mechanisms. Recently have there been studies of oscillatory behaviour in random 2- site phosphorylation (Jolley et al., 2012). A strong kinetic asymmetry (in the cycle) in the random mechanism was identified as a key ingredient for oscillations. Here this study shows that explicit activation can introduce oscillation in random mechanisms although the kinetic parameters (in the cycle) are symmetric. This result emphasizes the capability of explicit activation to introduce oscillation in various multisite phosphorylation mechanisms although the order of phosphate processing is different.

(a) without explicit activation



(b) with explicit activation

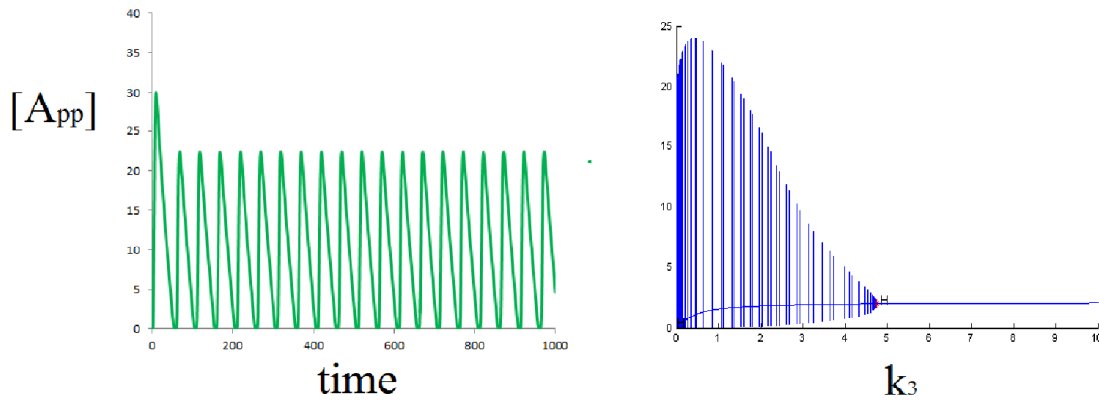


Figure 5.9 Oscillatory dynamic behaviour in random double distributive phosphorylation with explicit activation. In contrast to the isolated system which reaches a steady state (a), introducing explicit activation can induce sustained oscillation (b) in random double phosphorylation. (c) Bifurcation analysis shows that as the catalytic constant of the first phosphorylation step (upper branch), k_3 , is changed, the steady state loses stability *via* a Hopf bifurcation in the explicit activation model.

It is worth mentioning that explicit activation can also affect other qualitative behaviour in multisite phosphorylation. For example, it can destroy bistability in isolated systems in certain range of parameters in earlier results (see figure 5.6). In addition, it is found that it can also induce bistability under certain conditions, as shown in figure 5.10. Bistability is believed to play an important role in cellular decisions and is observed in isolated module of double phosphorylation. Although explicit activation can induce oscillation, it is not sufficient for introducing tri-stability, which is analytically proved in (Suwanmajo and Krishnan, 2018a)

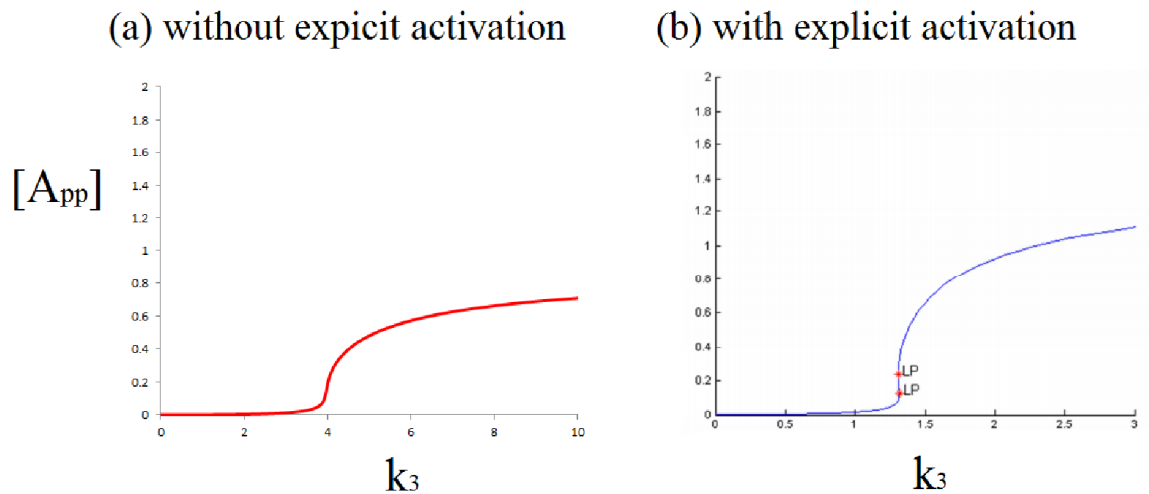


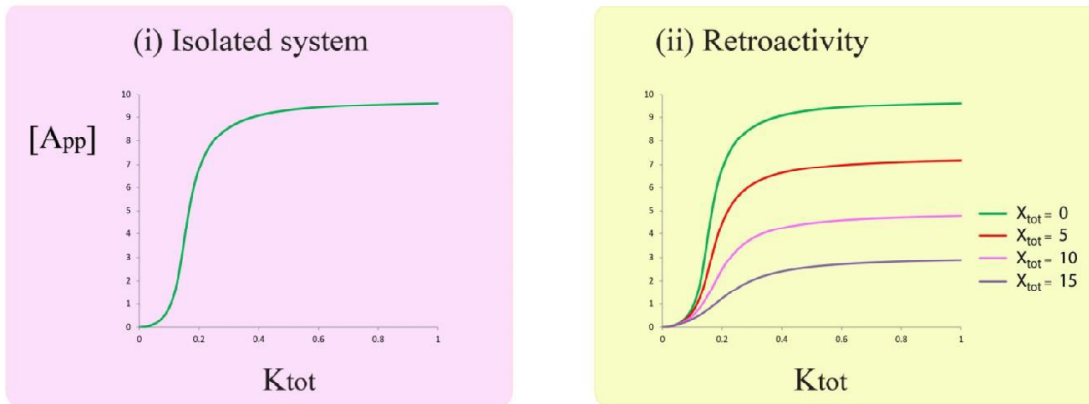
Figure 5.10 Explicit activation and bistability in ordered double distributive phosphorylation. Steady-state dose-response of the fully modified substrate, A_{pp} , as a function of the catalytic constant of the first phosphorylation step (upper branch), k_3 . In contrast to the isolated system which exhibits a steady state (a), introducing explicit activation can induce bistability (b) in ordered double distributive phosphorylation.

5.3.2 Effect of retroactivity

This section focuses on investigating the effect of retroactivity which arises from the fact that the output of multisite phosphorylation can get sequestered in different downstream pathways. This is studied in the case of 2-site phosphorylation where products, modified substrates, are sequestered in the downstream cycle (as shown in figure 5.2). The behaviour of retroactivity model will be contrasted with that of isolated mechanism with ‘corresponding’ parameters. The results will be presented in this section, which focuses on some examples of how downstream sequestration can play a role in completely distorting the behaviour of multisite modification. One of the big challenges in understanding this kind of issue is that multisite phosphorylation can be in different parameter regimes having different kinds of qualitative behaviour. Here the focus is only on how downstream sequestration can affects ‘qualitative’ behaviour in multisite phosphorylation. A detailed parametric analysis of these models is not focused here. The results are representative in the sense that similar trends are seen for different underlying parameter sets exhibiting the same qualitative behaviour.

The way in which the upstream module is coupled to the downstream module has a significant role both in the dynamics and the steady state of species in the preceding module. If the coupling reaction follows mass action kinetics, the downstream module will not have any effect on the upstream module. In this study, (ordered) 2-site phosphorylation is coupled to the downstream single covalent modification cycle (CMC), and this study considers the case where catalytic reaction is not in the mass action regimes. Consequently, the sequestration of the upstream product by the downstream target might induce a change in the preceding module.

(a) Completely phosphorylated substrate



(b) Partially phosphorylated substrate

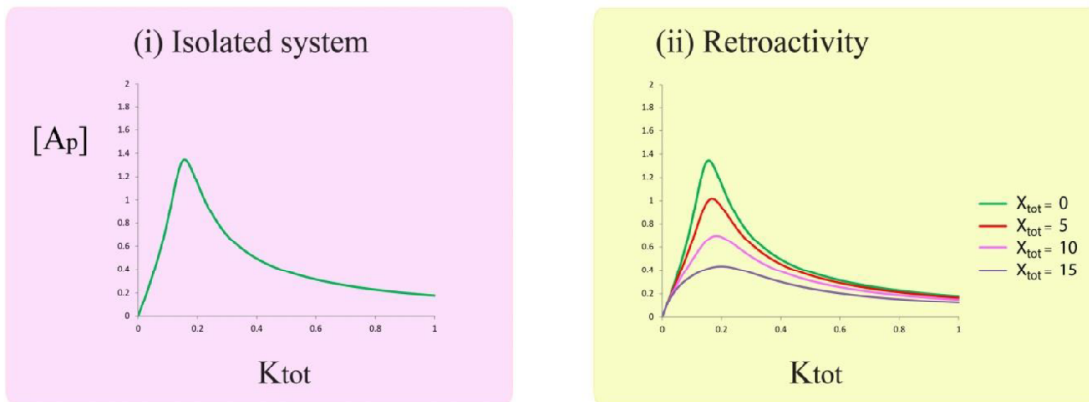


Figure 5.11 Steady-state dose-response curves in ordered double phosphorylation: distributive module in isolation and retroactive model (model R1). (a) shows dose-response curves of the fully phosphorylated substrate, A_{pp} , as a function of the total kinase concentration, K_{tot} , for different values of the total amount of downstream substrate, X_{tot} , while (b) shows the corresponding curve for the partially phosphorylated substrate, A_p . (Fully phosphorylated substrate: (i) isolated module and (ii) model R1. Singly phosphorylated substrate: (i) isolated module and (ii) model R1)

This section starts with a study of retroactive model where the fully modified substrate (A_{pp}) catalyses a downstream substrate, X , producing X^* (the modified form of X) *via* CMC (Model R1). The focus is first on examining the retroactive model in parameter regimes, where the ‘corresponding’ isolated system presents a monotonic dose response (for the fully modified phosphoform). As seen in figure 5.11a(i), as the total kinase concentration, K_{tot} , increases, the concentration of the double phosphorylated substrate increases monotonically and asymptotes to its maximum level. However when the isolated system is coupled to downstream CMC, the system presents monotonic response with different saturation levels (figure 5.11a(ii)). Gradual difference is seen when the total amount of downstream substrate, X_{tot} , is varied. If X_{tot} is zero, there is no retroactivity. As X_{tot} increases, the system presents a monotonic dose response with lower saturation level. This is completely due to the effect of the downstream module (sequestering of upstream species in the downstream module) on the multisite phosphorylation module.

Figure 5.11b(i) shows that the single phosphorylated substrate concentration in isolated module exhibits a biphasic response: the initial increase at low kinase levels reaching a maximum, before a decrease at higher kinase concentrations. This is simply understood from the fact that the dominant effect at higher kinase concentrations is the conversion of the single modified substrate to the double modified substrate. Figure 5.11b(ii) shows how steady state response of partially phosphorylated substrate varies with the total kinase concentration, K_{tot} , for different values of the total amount of downstream substrate, X_{tot} . It is found that increased availability of X_{tot} tends to diminish such biphasic response due to sequestering in the downstream module.

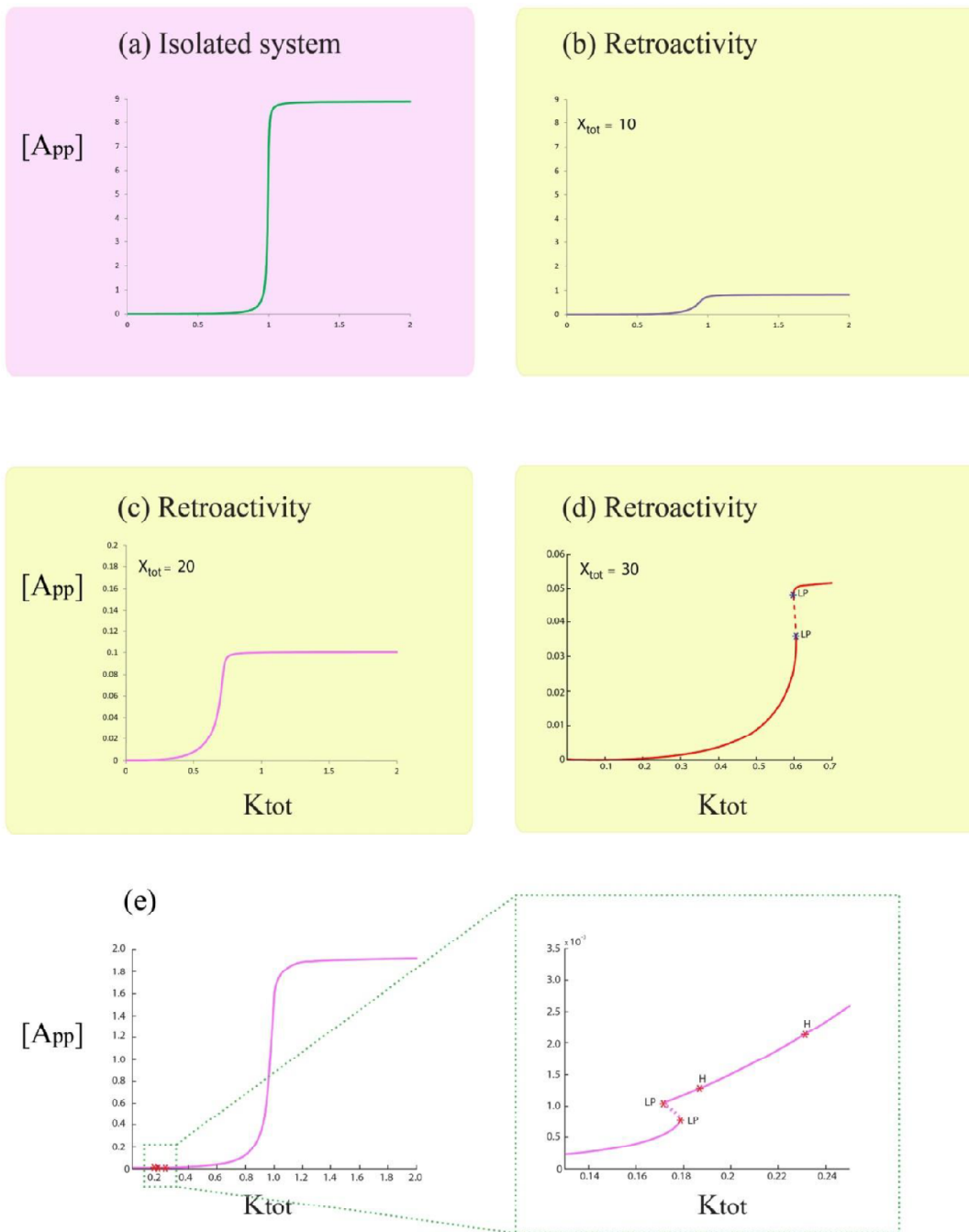


Figure 5.12 Effect of retroactivity on ultrasensitive behaviour in an ordered, double distributive phosphorylation (model R1). (a) shows dose-response curves of the fully phosphorylated substrate, A_{pp} , as a function of the total kinase concentration, K_{tot} in distributive phosphorylation in isolation. (c-d) shows how change in the total amount of downstream substrate, X_{tot} , can affect the behaviour in model R1. (e) shows the retroactive effect due to sequestration in the downstream CMC whose kinetic parameters are different.

The investigation is now performed on the retroactive model in parameter regimes, where the isolated system exhibits monostable switching behaviour. Here an even more striking retroactive effect is observed. While the isolated module presents monostable switching (figure 5.12a), qualitative differences are observed when the isolated module is coupled to the downstream CMC. Qualitative differences are observed when the total amount of downstream substrate, X_{tot} , is increased. Figure 5.12b shows that when the value of X_{tot} is equal to 10, the system exhibits a monotonic response with the clear presence of a threshold but a poor switch (the response becomes less steep). By contrast, the system presents monotonic response without threshold when X_{tot} is increased to 20 (figure 5.12c). Interestingly, the systems can present bistability when X_{tot} is increased to 30 as shown in figure 5.12d (even the value of X_{tot} is higher than this the systems can present bistability (result not shown)). Overall downstream sequestration can completely alter behaviour even qualitatively from monostable to bistable switching. This shows that downstream sequestration in pathway can cause very significant changes in multisite phosphorylation.

In (Ossareh et al., 2011), it was theoretically shown that for certain parameter conditions, phosphorylation cascades consisting of only single site modification are generally robust to retroactivity and the length of the cascade has been shown to have significance in attenuating the retroactive effect. Here this study focuses on retroactive effect in multi-site phosphorylation and results showed that at certain conditions multi-site mechanism coupled to a downstream CMC cycle (single-site modification), model R1, significant changes can be induced. The reason for this qualitative change is due to the fact that with a greater number of sites, there is more nonlinearity (along with a broader range of information processing capabilities), and this can contribute to the possibility of retroactivity both abolishing certain qualitative behaviour as well as inducing other kinds of behaviour.

The increased complexity of multisite substrate modification implies that the retroactive effect can be quite subtle. This is further exemplified by examining the effect of a downstream CMC, whose kinetics is altered. Figure 5.12 (e) shows that the system can present bistability combined with sustained oscillations as a result of retroactive effect. This shows that a sufficiently large substrate concentration along with appropriate kinetic parameters in the downstream cycle can have nontrivial effects on the multi-site phosphorylation cycle's internal dynamics. Therefore, to reshape signalling dynamics or to minimise the retroactive effect in multi-site phosphorylation, the total substrate (and enzyme) concentrations as well as kinetic parameter in the downstream cycle should be considered carefully in conjunction.

The retroactivity model is also examined in parameter regimes, where the 'corresponding' isolated systems exhibit bistability, which is shown in figure 5.13a. Here a striking effect is also seen. Figure 5.13b shows how steady state response of fully phosphorylated substrate varies with the total kinase concentration, K_{tot} , for different values of the total amount of downstream substrate, X_{tot} . As shown in this figure, the system presents bistability with different saturation levels and different bistability ranges as a result of downstream sequestration. Furthermore, such an effect can induce tristability when the value of X_{tot} is higher, which is shown in figure 5.13c. Taken together, this result shows how downstream sequestration can completely alter behaviour qualitatively from bistability to multi-stability although the downstream network (CMC) is not itself bistable. Ordered double distributive phosphorylation in isolation is not capable of generating tristability according to (Thomson & Gunawardena, 2009). Therefore, it can be stated that downstream sequestration by itself can completely introduce new behaviour which is different from isolated systems.

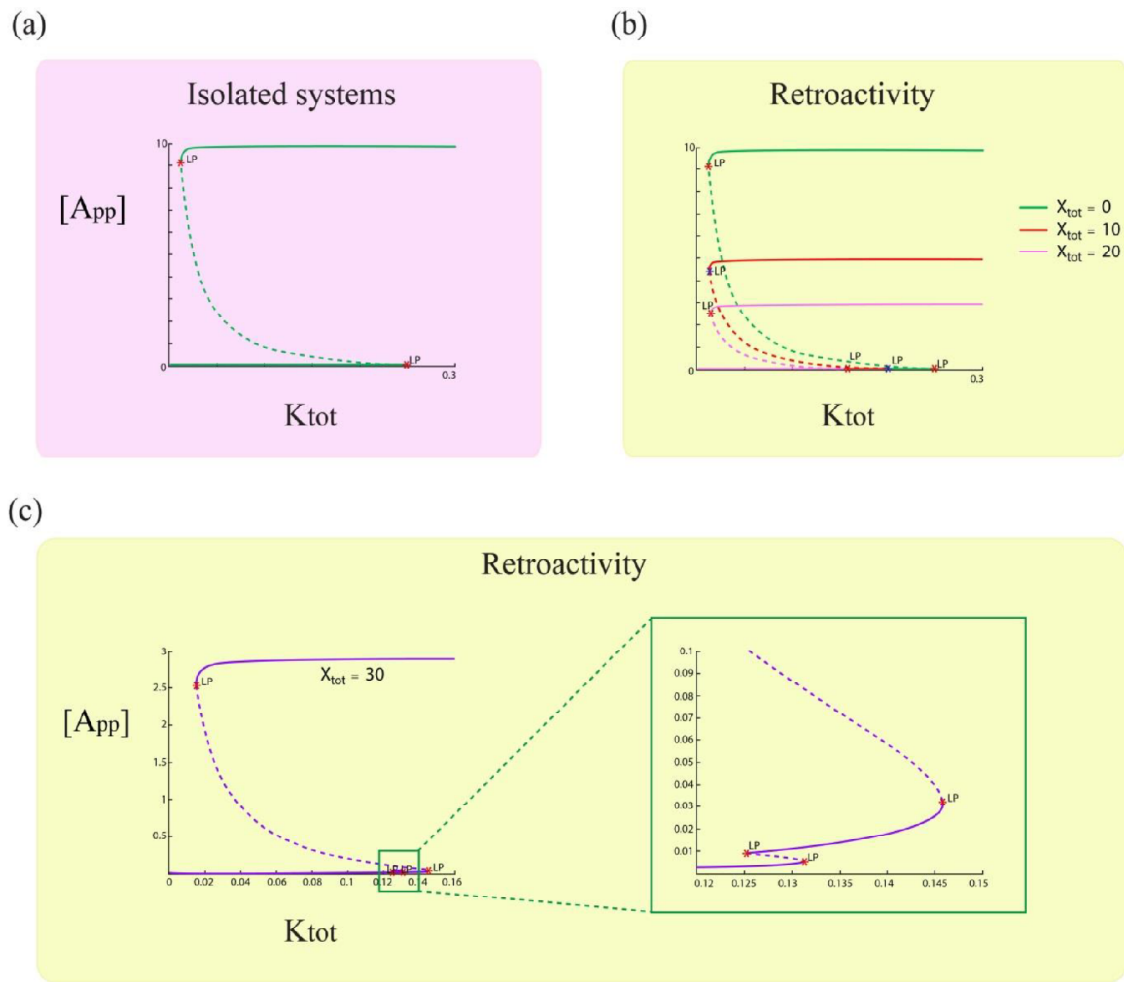
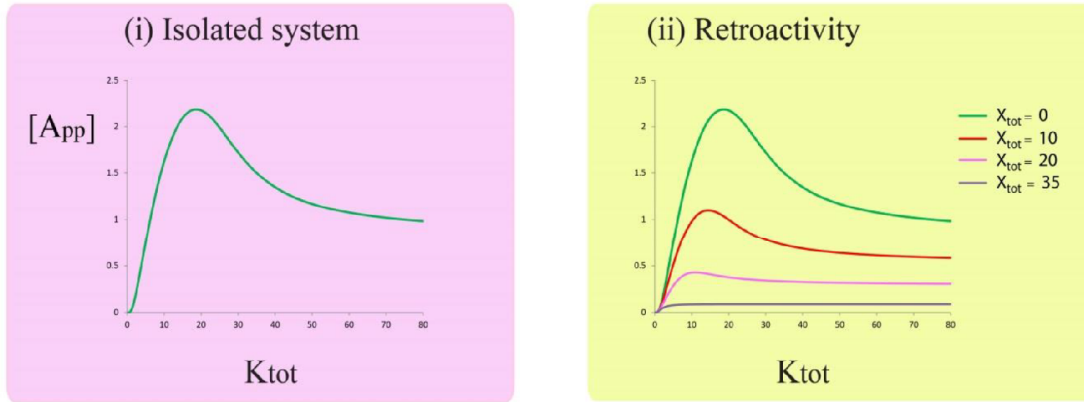


Figure 5.13 Effect of retroactivity on bistable behaviour in ordered 2-site distributive phosphorylation. (a) shows dose-response curves of the fully phosphorylated substrate, A_{pp} , as a function of the total kinase concentration, K_{tot} , in double distributive phosphorylation in isolation. (c-d) shows how change in the total amount of downstream substrate, X_{tot} , can affect the behaviour in model R1.

It is worth mentioning that, when different parameter regimes where the ‘corresponding’ isolated systems are bistable are chosen, downstream sequestration tends to diminish and eventually leads to an abolishment of bistability (result not shown).

(a)



(b)

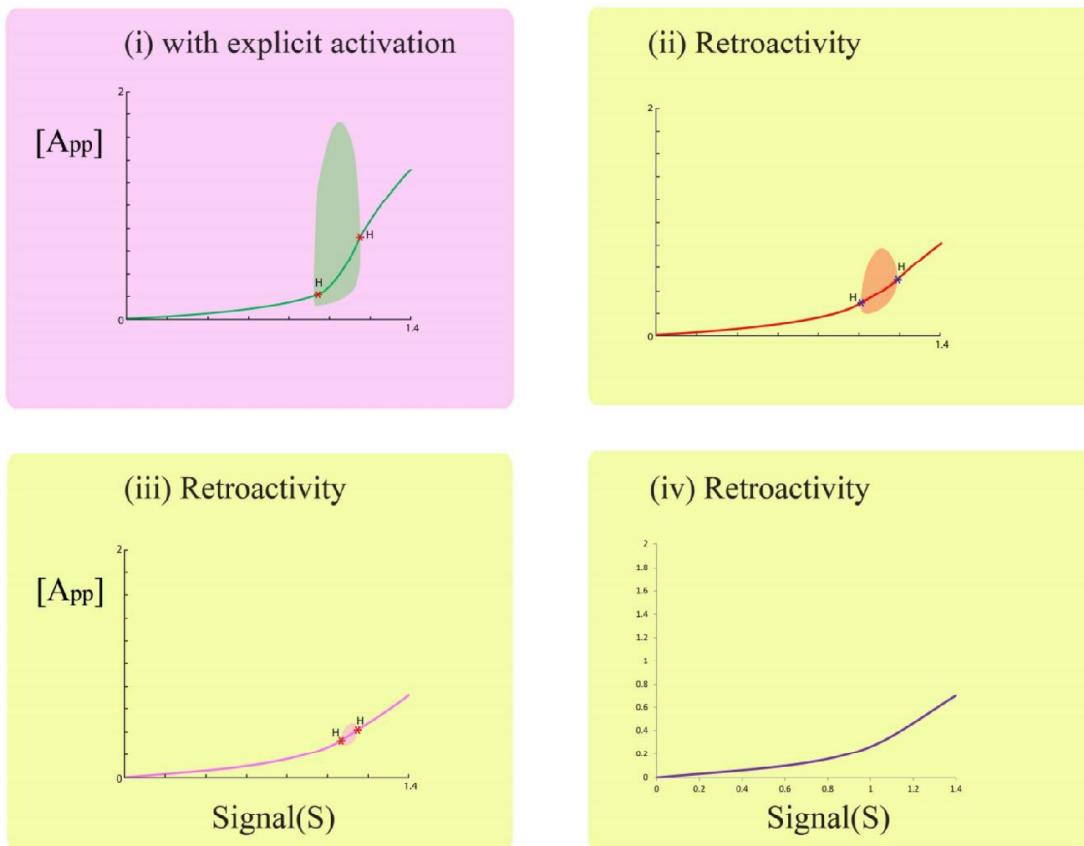


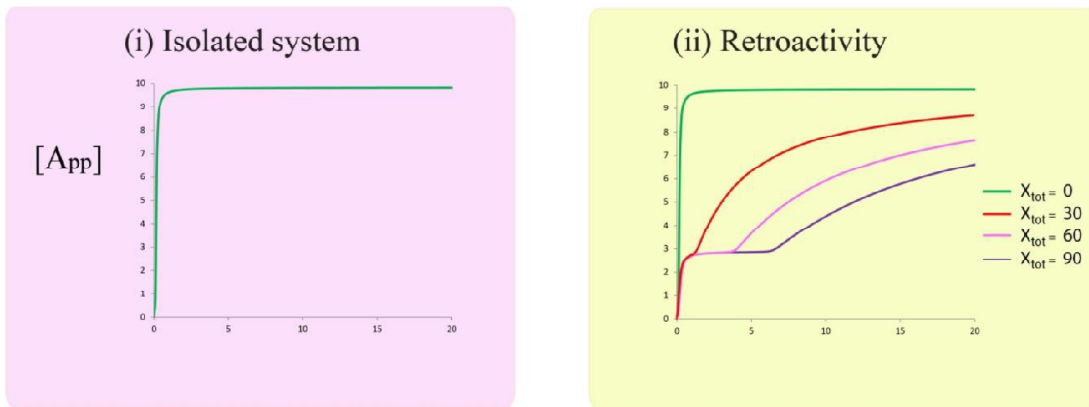
Figure 5.14 Retroactive effects on biphasic behaviour in 2-site distributive phosphorylation and oscillatory behaviour in model with explicit activation. (a) shows dose-response curves of the fully phosphorylated substrate, A_{pp} , as a function of the total kinase concentration, K_{tot} , for different values of the total amount of downstream substrate,

X_{tot} . (b): (i) shows oscillatory behaviour which arises from introducing explicit activation in ordered 2-site distributive phosphorylation. (ii-iv) bifurcation analysis show that as X_{tot} increases, the input range for oscillations becomes narrower, and eventually, oscillatory behaviour is completely abolished.

The investigation now focuses on how participation in the downstream pathway distorts the non-monotonic response in isolated module. Figure 5.14a(i) shows a dose-response curve of isolated system in a different regime, indicating a biphasic response for the fully modified substrate. The origins of such responses are previously discussed in the previous chapter. As seen in figure 5.14a(ii), increased availability of the total amount of the downstream substrate, X_{tot} , tends to diminish and can abolish this biphasic behaviour in multisite phosphorylation.

It is interesting to examine the effect of retroactivity on double distributive phosphorylation with explicit activation. To this explicit kinase activation is introduced in model R1 (henceforth referred to as model R1-E). Here the behaviour of model R1-E is contrasted with the ‘related’ explicit activation model with ‘corresponding’ parameters. For explicit activation model, as the input signal is changed, the steady state loses stability *via* a Hopf bifurcation (figure 5.14b(i)). The bifurcation diagram in figure 5.14b(ii)-(iv) show that increased downstream sequestration tends to reduce the input range for oscillatory behaviour and eventually can abolish the oscillatory dynamic behaviour. The ability of explicit activation to introduce oscillatory behaviour in ordered 2- site distributive phosphorylation was previously discussed in the section 5.2.1. Here this study shows how downstream sequestration can completely alter behaviour even from oscillation to monotonic dose response in multi-site phosphorylation with explicit activation.

(a)



(b)

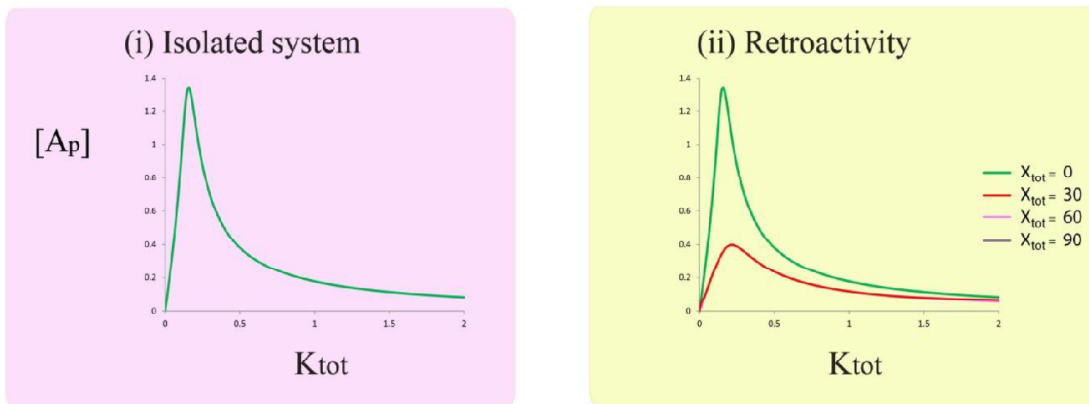


Figure 5.15 Steady-state dose-response curves in ordered double phosphorylation: distributive module in isolation and retroactive model (model R2). (a) shows dose-response curves of the fully phosphorylated substrate, A_{pp} , as a function of the total kinase concentration, K_{tot} , for different values of the total amount of downstream substrate, X_{tot} , while (b) shows the corresponding curve for the partially phosphorylated substrate, A_p . (Fully phosphorylated substrate: (i) isolated module and (ii) model R2. Singly phosphorylated substrate: (i) isolated module and (ii) model R2)

After investigating the effect of downstream sequestration in the case where the fully modified substrate catalyses the downstream substrate, the next step is to examine the case where partially phosphorylated substrate, A_p , catalyses the downstream substrate, X , producing X^* (the modified form of X) *via* CMC (henceforth referred to as model R2). The model R2 is firstly examined in parameter regimes, where the ‘corresponding’ isolated system presents a monotonic dose response (corresponding to figure 5.11a(i)). As seen in figure 5.15a(i), as the total kinase concentration, K_{tot} , increases, the concentration of the double phosphorylated substrate, A_{pp} , increases monotonically and asymptotes to its maximum level. However with the presence of retroactivity, A_{pp} increases more gradually and starts acquiring a plateau before it increases (figure 5.15a(ii)). This plateau change is more pronounced as the total amount of downstream substrate, X_{tot} , increases.

Figure 5.15b presents how the single phosphorylated substrate varies with the total kinase concentration. The isolated module presents a biphasic response, as seen in figure 5.15b(i). Due to coupling to the downstream module, it is found that increased availability of X_{tot} tends to diminish such a biphasic response and the plateau-like structure is not observed in the dose-response curve of A_p (as seen in figure 5.15b(ii)).

The investigation now focuses on how participation in the downstream pathway distorts the monostable switching (kinetic parameter corresponding to figure 5.12a) in isolated module. While the isolated module presents monostable switching (figure 5.16a(i)), certain differences are observed when the isolated module is coupled to the downstream covalent modification cycle. As shown in figure 5.16a(ii), it can be seen that the threshold aspect is maintained and once the response passes the threshold, it presents the plateau-like behaviour before further increases. This behaviour is much more pronounced as the total amount of downstream substrate increases.

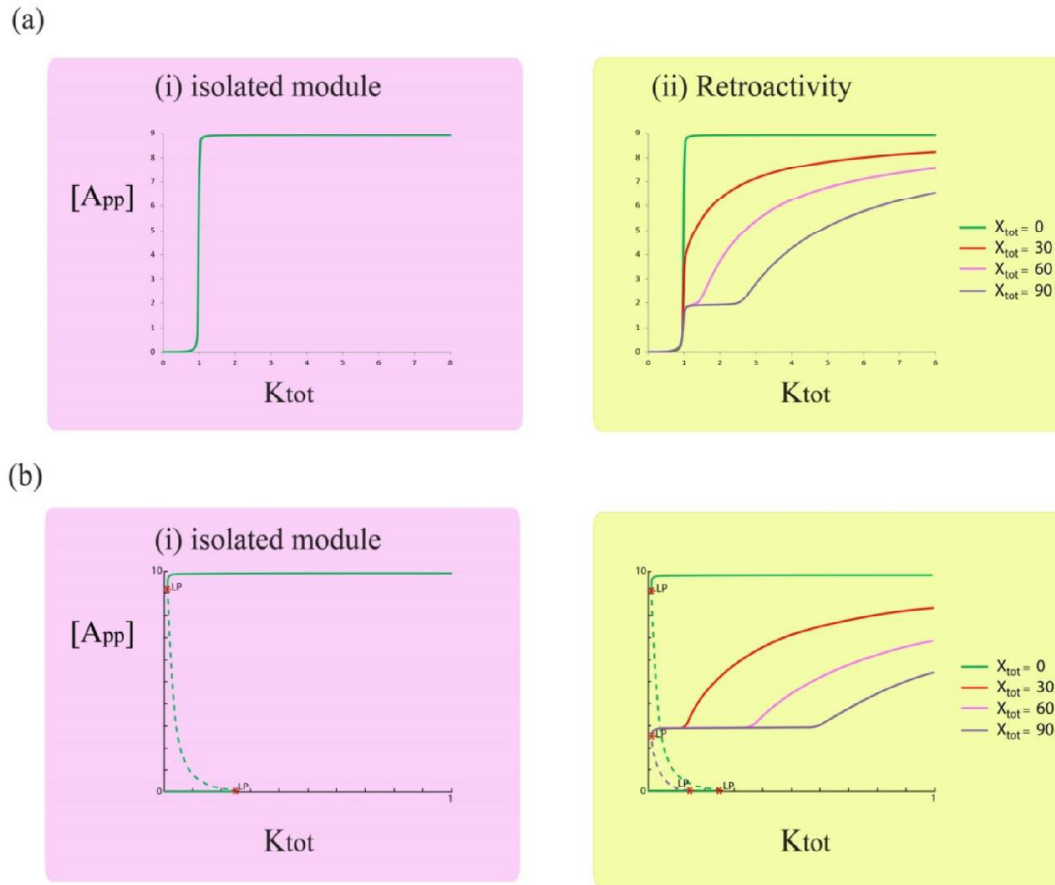


Figure 5.16 Effect of retroactivity on ultrasensitive and bistable behaviour in ordered, double distributive phosphorylation (Model R2). Steady state dose-response curves of the fully modified substrate, A_{pp} , as a function of the total kinase concentration, K_{tot} , for different values of X_{tot} . (a) Monostable switching: (i) isolated system and (ii) Model R2. (b) Bistable switching: (i) isolated system and (ii) Model R2.

The effect of the downstream sequestration on distorting the behaviour of multi-site modification is also examined in model R2 in parameter regimes, where the isolated system is bistable, as shown in figure 5.16b(i). As shown in figure 5.16b(ii), the system can exhibit bistability with decreased threshold. Once the system switches from the lower to upper state, it presents the plateau-like behaviour before it increases. This plateau-like behaviour is much more pronounced as the total amount of the downstream substrate, X_{tot} , increases.

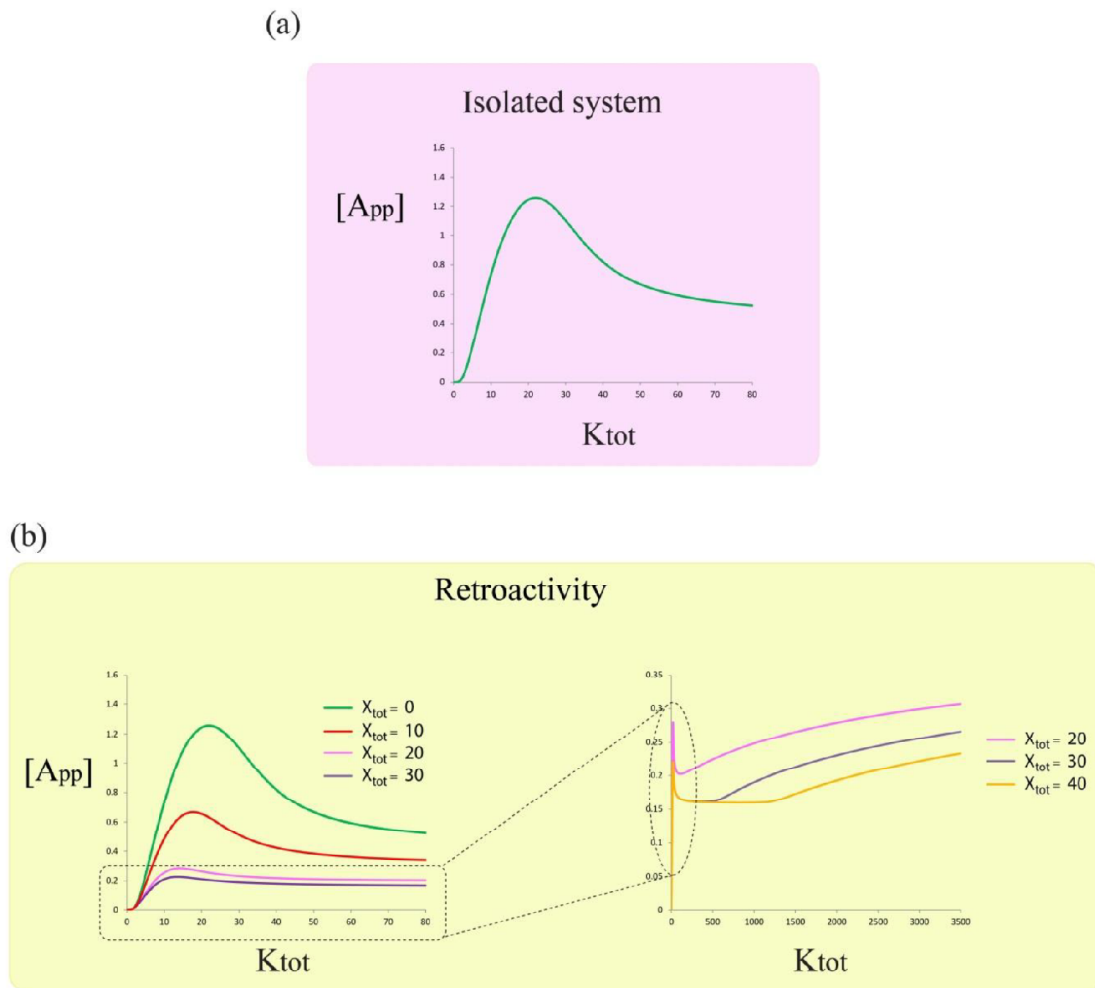


Figure 5.17 Effect of retroactivity on biphasic behaviour in ordered, double distributive phosphorylation (Model R2). (a) Biphasic dose response in isolated system. (b) Dose-response curves of A_{pp} as a function of K_{tot} , for different values of X_{tot} in Model R2.

Figure 5.17a shows a dose–response curve of the isolated model in a different parameter regime, indicating a biphasic response for the fully modified phosphoform. The downstream sequestration of the partial phosphoform can distort this behaviour, which can be seen in figure 5.17b. The systems can present a limited biphasic response for small value of K_{tot} and this behaviour tends to diminish as the total amount of downstream substrate increases. However once the biphasic curve reach its minimum, it presents the plateau-like behaviour before increasing again (for large value of K_{tot}), as seen in figure 5.17b (right). The more the amount of downstream substrate, the more prominent is the plateau characteristic.

Following a study of the case where only one species is sequestered to the downstream module, for completeness, the final step is to investigate the case when both A_{pp} and A_{p} are sequestered to downstream covalent modification cycles (A_{pp} catalyses the downstream substrate, X , producing X^* (the modified form of X) while A_{p} catalyses the downstream substrate, Y , producing Y^* (the modified form of Y). Here the model R3 is examined in parameter regimes, where the isolated system is in a monostable switch regime, which is shown in figure 5.18a. The effect of downstream sequestration is shown in figure 5.18(b)-5.18(j). It can be seen that sequestration of both A_{p} and A_{pp} can distort the behaviour of multi-site phosphorylation. It can alter behaviour from monostable switching to a hyperbolic response. Although sequestration of A_{pp} can result in bistability (figure 5.18h), this bistable behaviour is abolished as a result of A_{p} sequestration (see figure 5.18(h-j)).

Taken together, results in this section show how downstream sequestration of modified phosphoforms can play a nontrivial role in distorting the behaviour of multisite modification. The way in which the downstream sequestration affects qualitative behaviour in multisite phosphorylation is discussed and results indicate that downstream sequestration in pathways of one or more phosphoform can cause very significant changes in multisite phosphorylation.

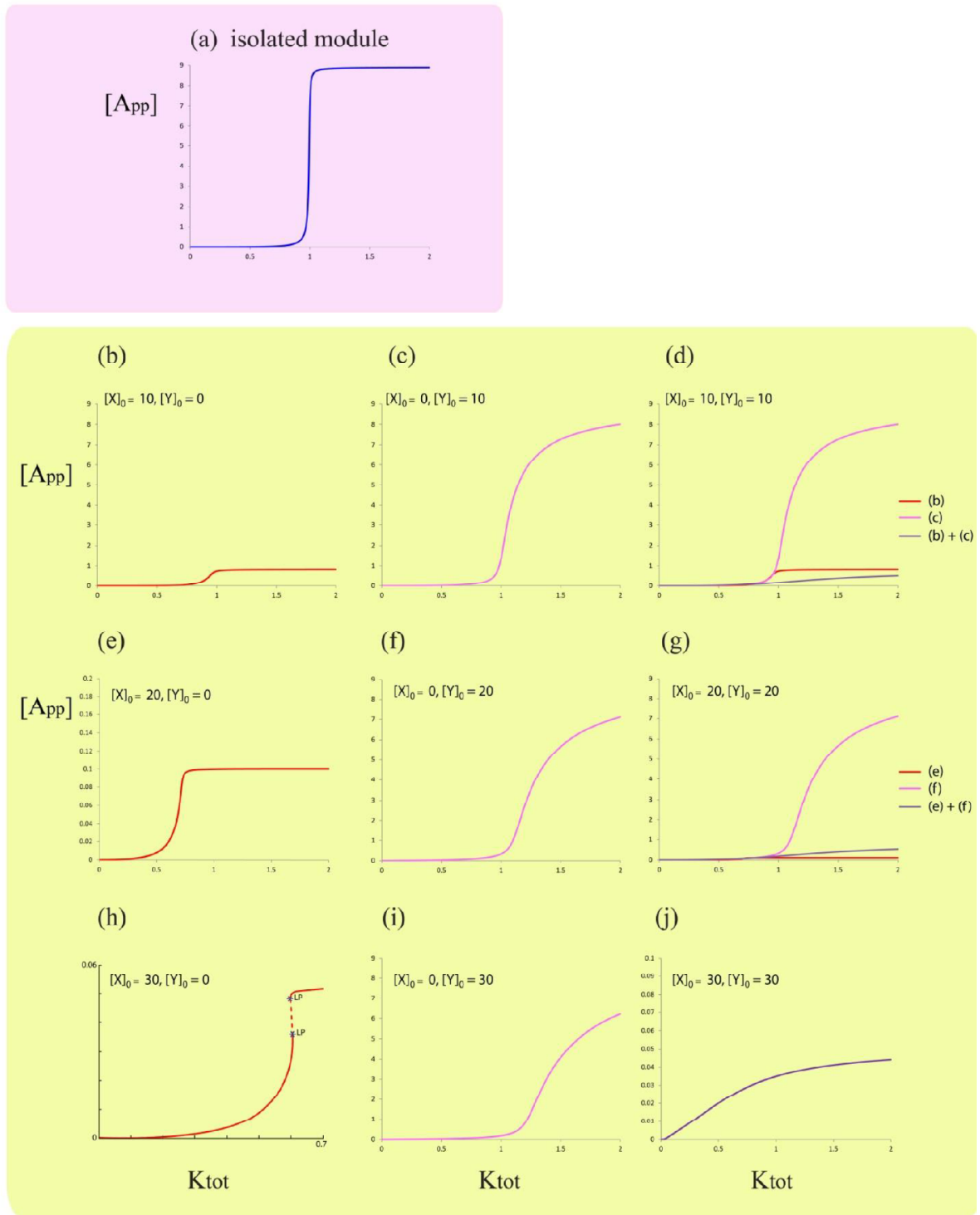


Figure 5.18 Effect of retroactivity on internal dynamics of double distributive phosphorylation: both partially and fully modified substrates are sequestered to different downstream cycle. Dose-response curves of A_{pp} as a function of K_{tot} : (a) isolated system. (b-j) systems with retroactivity (model R3)

In this chapter, section 5.3.1 and 5.3.2, respectively, focus on the way in which explicit enzymatic activation and retroactivity affect the internal dynamics of multisite phosphorylation. Such studies bridge the gap between the intrinsic behaviour of multi-site phosphorylation and their functioning in realistic signalling pathways. Table 5.1 summarises the qualitative differences between double site phosphorylation systems in isolation and coupled with upstream pathway (*via* explicit activation) or downstream covalent modification cycles (retroactivity). This result sheds light on the way how internal dynamics in multi-site phosphorylation are reshaped. For example, simple explicit activation can change the system from monostable behaviour to bistability and even oscillations. Likewise, another way to tune the systems to exhibit oscillation can be done *via* retroactivity, though this may not be as preferable as the former case. On the other hand, enzymatic activation is not sufficient for introducing tristability, which is analytically discussed in (Suwanmajo and Krishnan, 2018a). Overall, table 5.1 shows the qualitative differences between multisite phosphorylation module in isolation and as parts of signalling pathways. This allows us to gain insight into not only the dynamic behaviour of multisite phosphorylation in signalling pathway but also ways to engineer the internal behaviour of the system or to design the synthetic biological circuits (further discussed in Suwanmajo and Krishnan, 2018a)

Table 5.1 A summary of the qualitative differences between double site phosphorylation systems in isolation and coupled with upstream pathway (*via* explicit activation) or downstream covalent modification cycles (retroactivity).

Qualitative behaviour of multi-phosphorylation in isolation	Qualitative behaviour observed in multisite phosphorylation in pathways			
	Monostable behaviour	Bistability	Tristability	Oscillation
Monostable behaviour	Explicit activation Retroactivity	Explicit activation Retroactivity		Explicit activation Retroactivity
Bistability	Explicit activation Retroactivity	Explicit activation Retroactivity	Retroactivity	Explicit activation

5.3.3 Effect of open systems

This study focuses on investigating the effect of inflow and outflow of substrate and enzyme on the dynamic behaviour of multisite phosphorylation. The primary goal is to identify and elucidate the presence of key qualitative signatures and capabilities in information processing in open systems (distributive double phosphorylation). This section presents a sequence of results for the models (see figure 5.3) showing representative and possible qualitative behaviour in this regard.

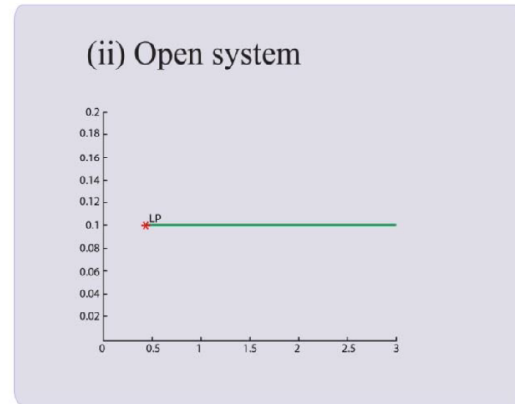
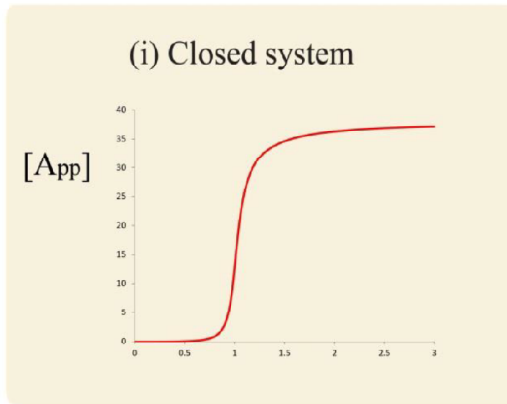
This section starts with the study of the influence of the substrate inflow/outflow on the behaviour of ordered two-site phosphorylation, followed by three-site phosphorylation models (Figure 5.3a). To this, the behaviour of the open systems is contrasted with that of the ‘related’ closed systems with ‘corresponding’ kinetic parameters (without inflow/outflow). The dynamic behaviour of the open enzyme systems (figure 5.3b) is then examined. The study then focuses on examining the combined effects of both substrate and enzyme open systems (Figure 5.3c). Note that this study does not focus on a detailed parametric analysis of these models.

5.3.3.1 Open Substrate systems

In the substrate open system, there is production of unmodified substrate and degradation of products. This section begins with the case when the fully phosphorylated substrate, A_{pp} , leaks out/is degraded in the ordered 2-site system (Model S1, see in figure 5.2). Figure 5.19 presents simulations showing how the maximally and partially phosphorylated substrate concentration at steady state varies with total kinase concentration. The open system is first examined in a parameter regime, where the ‘analogous’ closed

distributive mechanism is monostable. It is found that qualitative differences between the behaviour of open and closed systems. Figure 5.19b shows that the system never reaches a steady state for low values of K_{tot} , a behaviour different from closed system (figure 5.19a). However, the fully modified substrate can exhibit adaptation like-behaviour: the fully phosphoform exhibits a steady profile for sufficiently large values of K_{tot} (for which a steady state is obtained). This can be understood from analytical work in (Suwanmajo & Krishnan, 2018b). Adaptation behaviour is generically expected in the systems with negative feedback or feed-forward regulation (Tyson, 2003), though open systems are known to also exhibit this behaviour. On the other hand, figure 2d shows that A_p does not exhibit such an adaptive behaviour. Overall open systems by itself can completely introduce new behaviour which is different from isolated systems even in the simplest case.

(a)



(b)

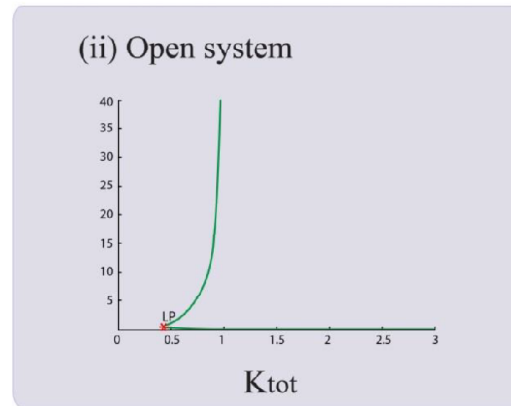
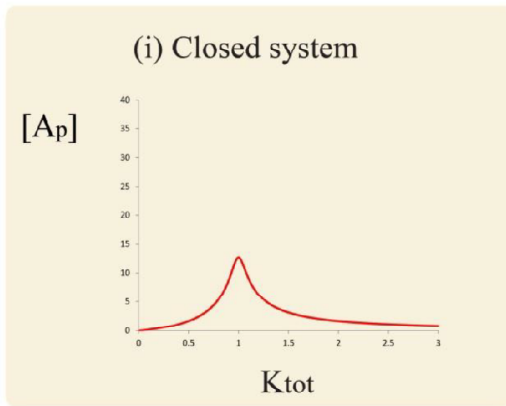


Figure 5.19 Dose–response curves in ordered, double distributive phosphorylation models: closed and open systems (model S1). (a) show the steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of the total concentration of kinase, K_{tot} , while (b) shows the corresponding curve for the partially phosphorylated substrate, A_p . ((a) Fully phosphorylated substrate: (i) closed system and (ii) open system (model S1). (b) Singly phosphorylated substrate: (i) closed system and (ii) open system (model S1)).

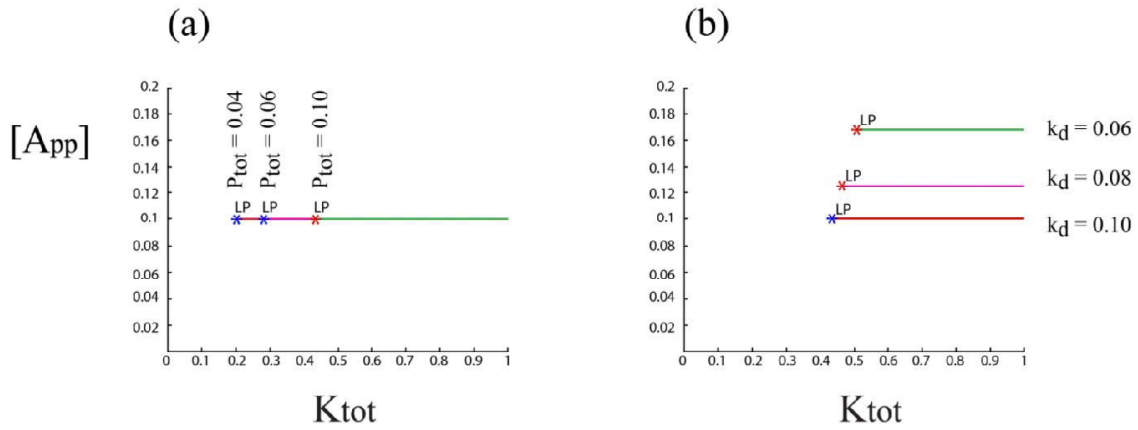
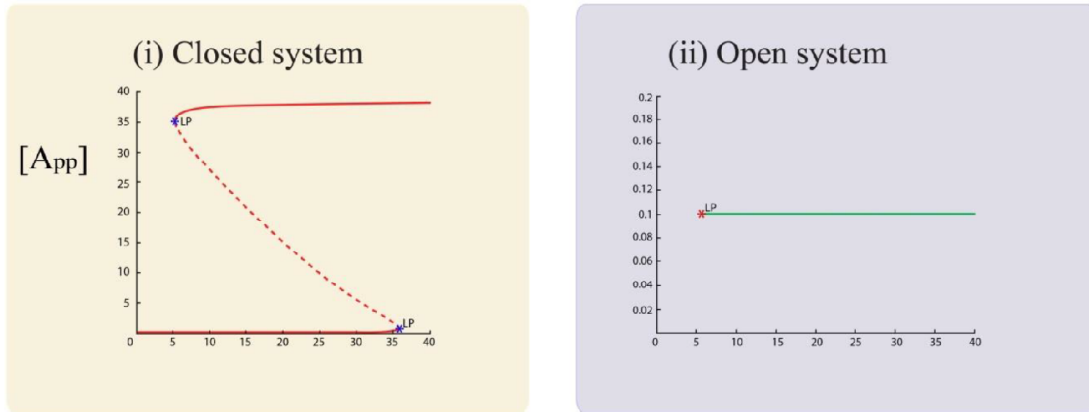


Figure 5.20 Effect of parameters. Effect of parameters on adaptation behaviour in open systems corresponding to figure 5.19a(ii) (ordered, double-site phosphorylation). (a) decreased availability of phosphatase can increase the range of K_{tot} that the systems can present adaptation behaviour (b) As the degradation rate of the fullyphosphoform (k_d) decreases, the systems tend to present adaptation at higher level for large values of K_{tot} .

After investigating the open system in figure 5.19, where the system can exhibit adaptation behaviour, the effects of different parameters on this response (kinetic parameters corresponding to figure 5.19a(i)) are then examined. Figure 5.20a presents the effects of varying the total phosphatase concentrations (P_{tot}). As seen in this figure, decreased availability of phosphatase can increase the range of K_{tot} over which the systems can present adaptation behaviour. However the fully phospho-form still exhibits a steady profile at the same level irrespective to the change of the total phosphatase concentrations. Figure 5.20b shows the effects of the degradation rate of the fullyphosphoform (k_d) on the adaptation behaviour. It is found that as k_d decreases, the systems tend to present adaptation at higher level for large values of K_{tot} . This can be understood from (Suwanmajo and Krishnan, 2018b) that the response of fully phosphorylated substrate at steady state is simply equal to k_0/k_d and does not rely on other parameters.

(a)



(b)

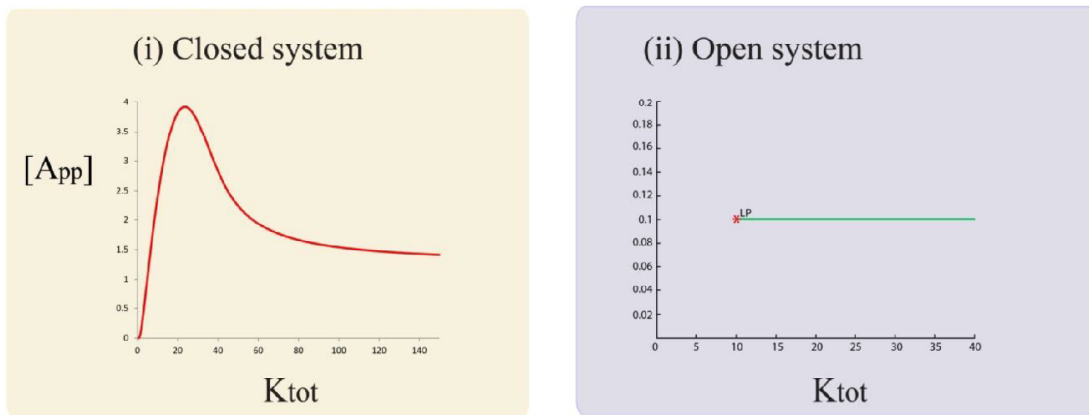
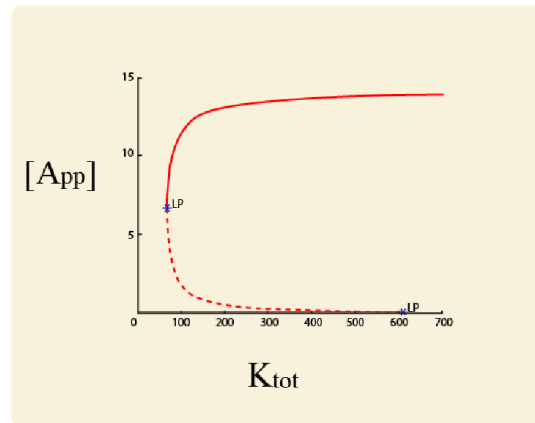


Figure 5.21 Dose–response curves in ordered, double distributive models with different catalytic constants of phosphorylation reactions: closed and open system (model S1). (a) Bistability may be obtained in a (closed) distributive model (i), while this behaviour is destroyed in open system, model S1 (ii). (b) The biphasic behaviour which can arise from a distributive model (i) is not found in model S1.

The model S1 is now examined in parameter regimes, where the (closed) distributive analogues are bistable. A choice of appropriate catalytic constants in (de)phosphorylation can result in bistability in a closed distributive system, as shown in figure 5.21a(i) (Conradi & Mincheva, 2014). In contrast, figure 5.21a(ii) shows that the open system model does not exhibit bistability. (Bistability can be ruled out in this case, which is analytically demonstrated in (Suwanmajo and Krishnan, 2018b)). Instead, the presence of inflow and outflow can abolish the possibility of steady states at low values of K_{tot} and results in adaptive behaviour (for A_{pp}) under monostable condition for sufficiently large values of K_{tot} (as seen in the figure). This shows how the open system can alter behaviour qualitatively from bistability to monostable condition and can completely introduce new behaviour which is different from isolated systems.

Figure 5.21b(i) shows a dose–response curve of the closed distributive systems in a different regime, indicating a biphasic response for the fully modified phosphoform. The origins of such biphasic responses are discussed in chapter 3, where it is shown that distributive mechanisms are intrinsically capable of generating such biphasic responses in the absence of product inhibition, even when the catalytic conversion step is irreversible. By contrast, it is found that the ‘corresponding’ distributive open systems (model S1) do not exhibit such biphasic dose–response curves for the fully phosphorylated substrate (figure 5.21b(ii)). Instead it presents adaptation behaviour for large value of K_{tot} . Biphasic dose response for fully modified phosphoform can be ruled out, which is analytically demonstrated in (Suwanmajo & Krishnan, 2018b).

(a) Closed system



(b) Open system

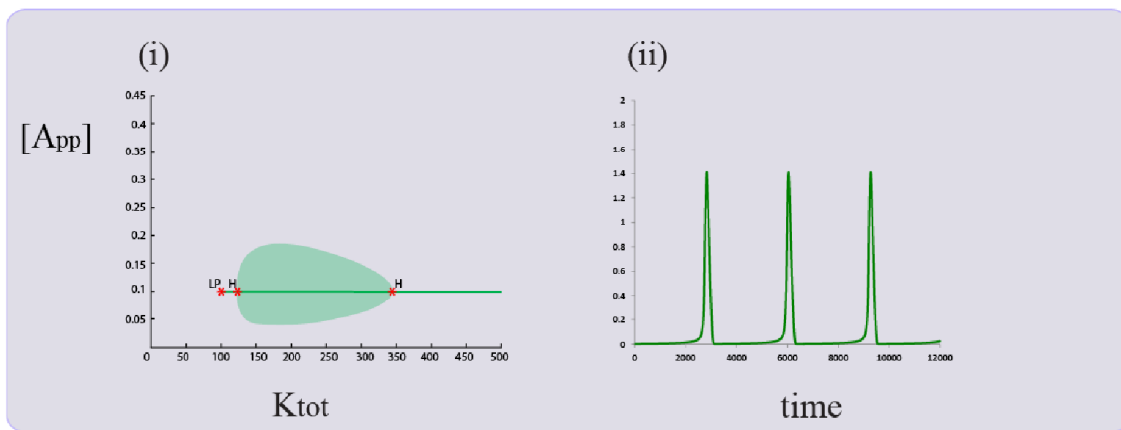


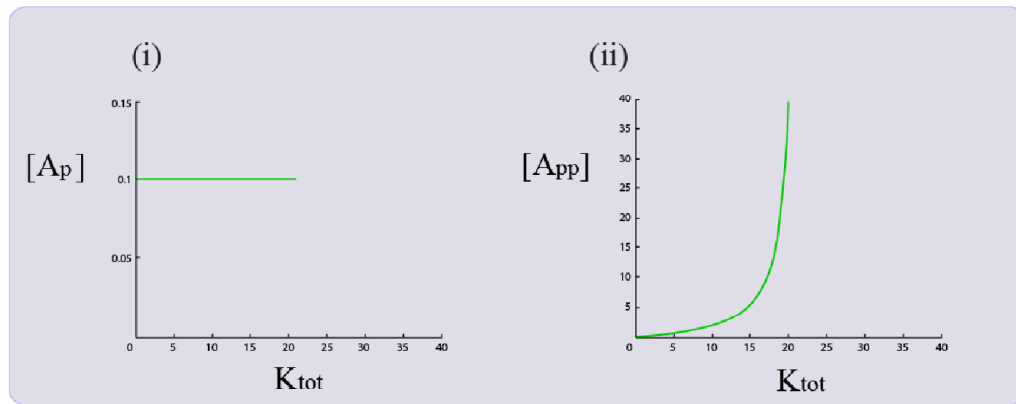
Figure 5.22 Oscillatory behaviour in open double phosphorylation system (model S1). In contrast to closed system which exhibits bistability (a), model S1 is capable of generating sustained oscillation (b(ii)). Bifurcation analysis in b(i) shows that as the total kinase concentration, K_{tot} , is changed, the steady state loses stability *via* a Hopf bifurcation in open substrate system.

It is interesting to ask whether open substrate systems could result in more striking effects. Figure 5.22 presents another aspect of open systems. In contrast to the ‘corresponding’ closed distributive system, which has multiple stable steady states (figure 5.22a), it is found that the open system (Model S1) exhibits both adaptation and sustained oscillations. A bifurcation diagram in figure 5.22b (i) shows that, as the parameter K_{tot} is changed, the steady state does indeed lose stability to a Hopf bifurcation. As mentioned in the previous chapter, sustained oscillations have not been observed in a closed system of ordered, double phosphorylation and this was not reported in the literature. This shows how open systems by itself can introduce new kinds of qualitative behaviour in multi-site phosphorylation systems. Taken together, the effect of production and degradation of species was shown to be capable of completely altering the behaviour of the multisite phosphorylation module in multiple aspects: for example abolishing bistability, even abolishing the possibility of steady states and introducing adaptive behaviour (and oscillation).

After investigating open systems where degradation occurs in the fully phosphorylated substrate, the focus now is on examining the case of the partial phosphoform involving degradation (Model S2). The study starts with the investigation of the open system in a parameter regime which is used in figure 5.19a (where the ‘analogous’ closed distributive system is monostable). Figure 5.23a presents simulations showing how the maximally and partially phosphorylated substrate concentration at steady state varies with total kinase concentration. As seen in this figure, the partial phospho-form (A_p) presents adaptation for values of K_{tot} below a threshold level, while the fully phospho-form exhibits an increasing trend in the same range. Related analytical work is discussed in (Suwanmajo and Krishnan, 2018b).

To investigate whether this open system can exhibit other qualitative dynamic behaviour, model S2 is examined in a parameter regime where the ‘corresponding’ closed distributive system presents bistability. Figure 5.23b shows that the partially phosphorylated substrate (A_p) presents both adaptation and sustained oscillation for an intermediate range of K_{tot} . It is worth mentioning that bistability and biphasic response (for fully phosphoforms) is not observed in this model after a detailed parametric search. In fact, analytical work in (Suwanmajo and Krishnan, 2018b) shows that bistability and biphasic responses can be ruled out in this model.

(a)



(b)

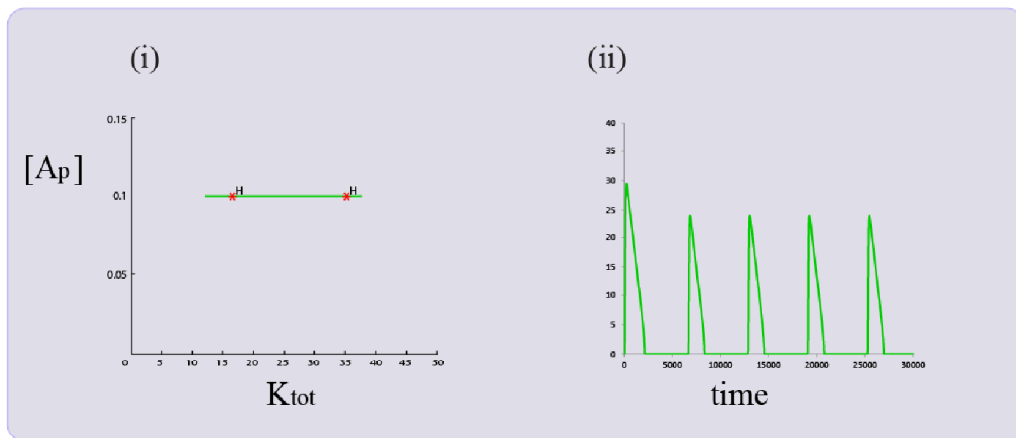
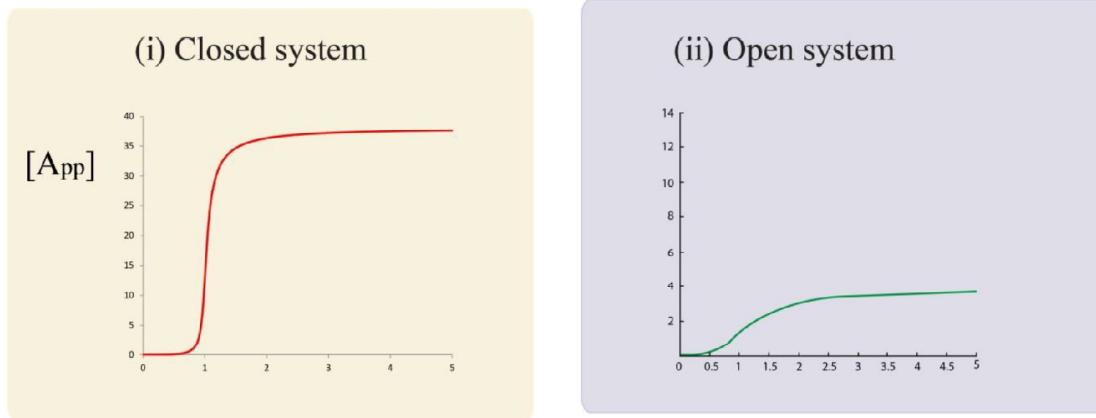


Figure 5.23 Dynamic behaviour in open double phosphorylation model where the partial phosphoform degrades (model S2). (a) steady-state input–output response in model S2 with the same catalytic constants of phosphorylation reactions: (i) A_{pp} and (ii) A_p dose

response curve as a function of K_{tot} . (b) shows that, at certain parameter regimes, model S2 is capable of generating sustained oscillation (ii). Bifurcation analysis shows that as K_{tot} is changed, the steady state loses stability *via* a Hopf bifurcation in the open system (i).

(a)



(b)

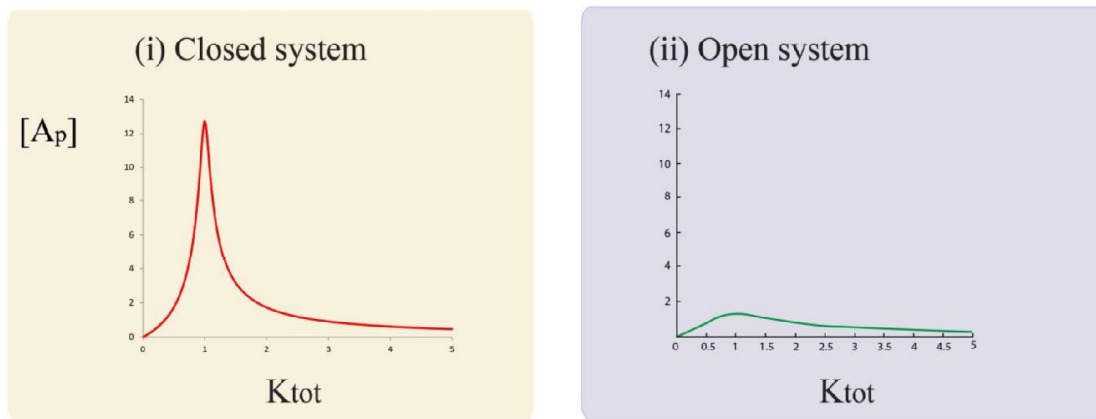


Figure 5.24 Steady-state dose–response curves in open double phosphorylation models

(model S3). (a) shows the steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of the total concentration of kinase, K_{tot} , while (b) shows the corresponding curve for the partially phosphorylated substrate, A_p . ((a) Fully phosphorylated substrate: (i) closed system and (ii) open system (model S3). (b) Singly phosphorylated substrate: (i) closed system and (ii) open system (model S3)).

After investigating the open systems where the degradation of only one substrate occurs, the focus now is on investigating the case where all substrates degrade (Model S3). Figure 5.24 presents simulations showing how the maximally and partially phosphorylated substrate concentration at steady state varies with total kinase concentration. The first investigation focuses on the behaviour of the open system in a parameter regime, where the ‘analogous’ closed system exhibits monostable switching. As seen in figure 5.24a, it is found that the fully modified substrate exhibits a monotonic response (figure 5.24a(ii)). A clear difference is seen in open system compared to the ‘related’ closed systems which present monostable switching (figure 5.24a(i)). By contrast, the partial phosphoform (A_p) exhibits biphasic response similar to the closed system (figure 5.24b). Generally, in the closed (distributive) systems, the partial phosphoform presents biphasic behaviour as discussed earlier.

Figure 5.25(a) shows a dose–response curve of a closed model in a different regime, indicating a biphasic response for the fully modified phosphoform. It is found that the ‘corresponding’ open systems (model S3) do not exhibit such biphasic dose–response curves (for the fully phosphorylated substrate) and a detailed parametric search has shown no example of biphasic response.

The open system (Model S3) is now examined in different parameter regimes, where the closed analogues are capable of producing bistable behaviour. As seen in figure 5.25(b), the open system can exhibit bistability similar to the closed system. However, figure 5.25(c) show that bistability is abolished in the open system but sustained oscillation occurs, using different parameter regimes, where the ‘related’ closed system presents bistability. This shows how open substrate systems can either maintain or destroy bistability.

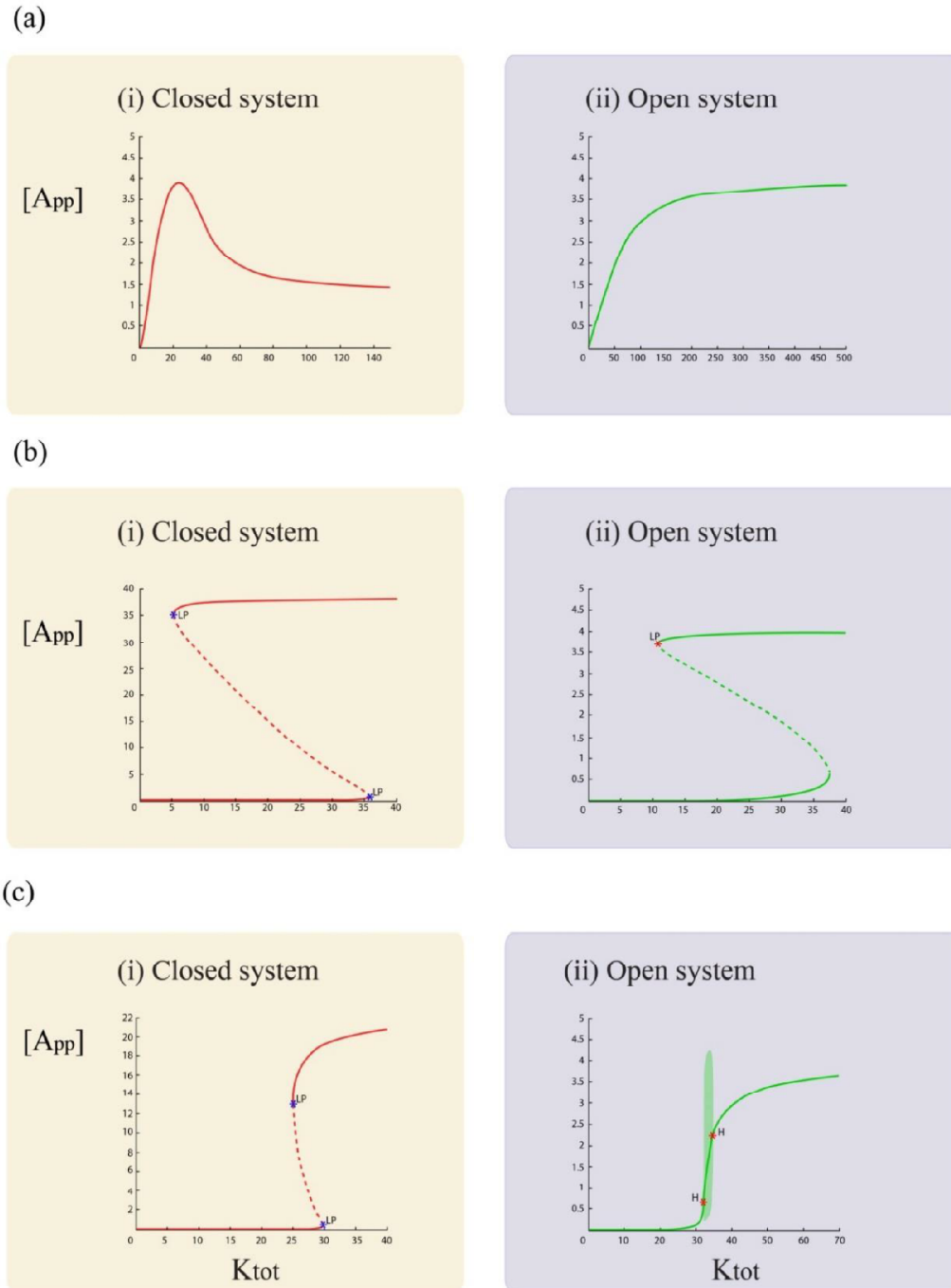
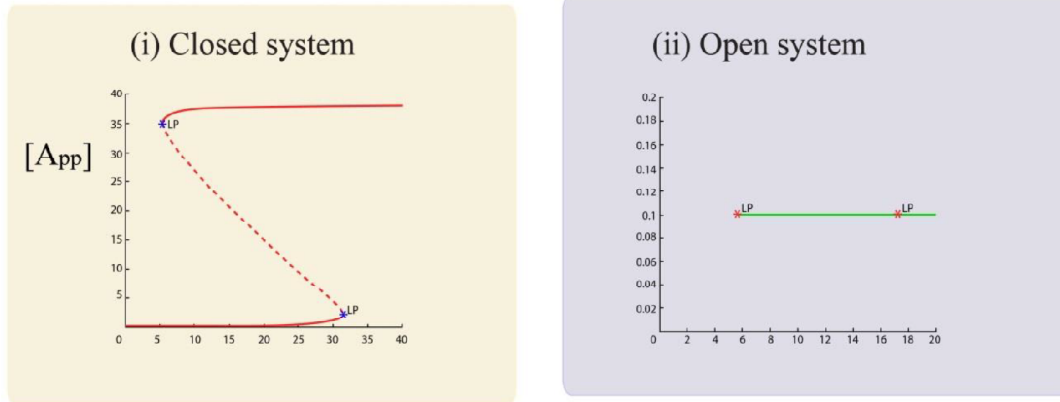


Figure 5.25 Steady-state dose–response curves in model S3 with different catalytic constants of phosphorylation reactions. Closed double distributive systems: a(i) biphasic behaviour, b(i) and c(i) bistable behaviour. Open systems: a(ii) the system does not exhibit biphasic response, b(ii) bistability is observed. c(ii) oscillation is observed.

(a)



(b)

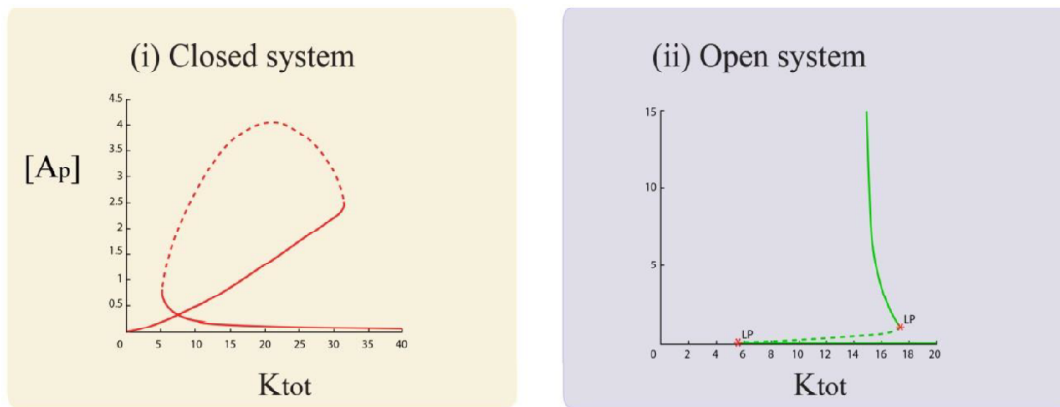


Figure 5.26 Steady-state dose–response curves in ordered triple phosphorylation models: closed and open system (model S4). (a) show the steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of the total concentration of kinase, K_{tot} , while (b) shows the corresponding curve for the doubly phosphorylated substrate, A_{pp} . ((a) Fully phosphorylated substrate: (i) closed system and (ii) open system (model S4). (b) Doubly phosphorylated substrate: (i) closed system and (ii) open system (model S4)).

After understanding the behaviour of 2-site open phosphorylation systems, it is interesting to study slightly more complex multi-site modification mechanisms (ordered 3-site modification). The focus is now on investigating the case where the fully phosphorylated

substrate degradation occurs (Model S4). This model is examined in parameter regimes, where the closed system is bistable. A choice of different catalytic constants in phosphorylation can result in bistability in a (closed) distributive model, as shown in figure 5.26a(i). It is also found that the open system (model S4) can also exhibit bistability combined with adaptation. This shows how the presence of open systems as well as an increase number of sites can introduce new modes and new combinations of information processing in (distributive) multisite phosphorylation systems.

It is worth mentioning that, if the degradation occurs at the singly (model S5) or doubly (model S6) phosphorylated substrate, bistability is not observed after a detailed parameter search. This can be analytically demonstrated in (Suwanmajo & Krishnan, 2018b) that bistability can be ruled out in such models. This shows how the location (in the module) of “inflow” and “outflow” had a significant effect in determining what kind of behaviour resulted, in conjunction with the kinetic parameters of the multisite modification module

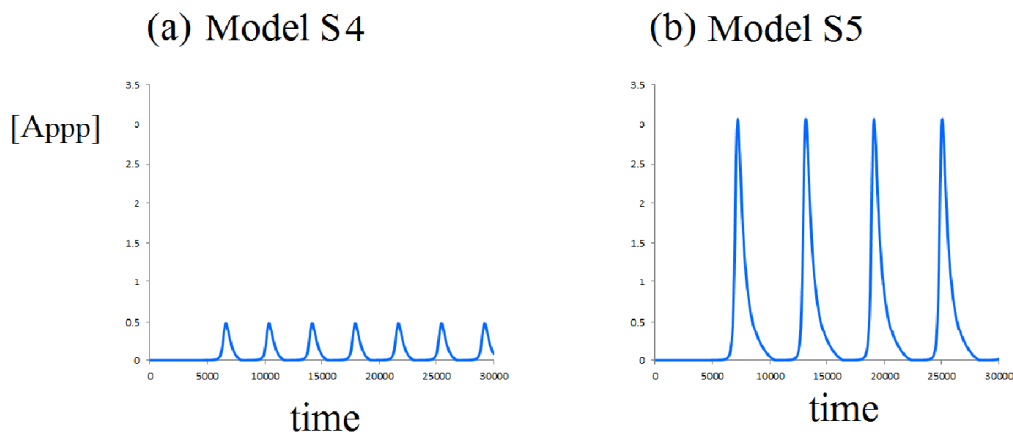


Figure 5.27 Oscillatory behaviour in open triple-site phosphorylation models. With the same catalytic constant in all steps, both (a) model 32 and (b) model 35 can exhibit sustained oscillation with different frequency and amplitude.

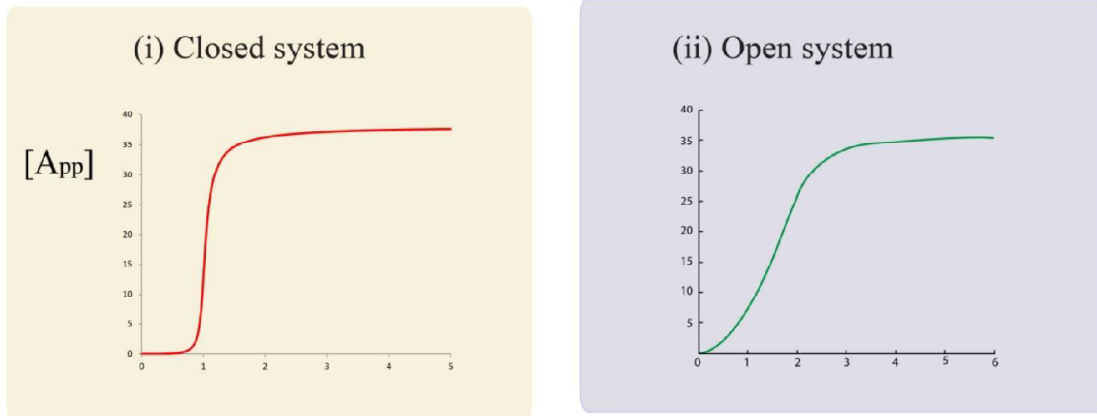
After investigating bistability in 3-site distributive open systems, it is interesting to ask whether these models can generate oscillatory dynamic behaviour. Figure 5.27 presents simulations showing how the maximally phosphorylated substrate concentration varies with time. These (open systems) models are contrasted by using the same kinetic parameters. As seen in this figure, model S4, model S5 can exhibit sustained oscillation with different frequency and amplitude while model S6 does not (not shown), for this parameter range. This shows how the location (in the module) of “inflow” and “outflow” had a significant effect in determining the oscillatory behaviour in multi-site modification. Sustained oscillation is actually observed in model S6, in other parameter regimes. Oscillatory behaviour has been observed in multiple signalling contexts. The combination of factors that generates oscillations has varied from context to context. In the previous chapter, 3-site phosphorylation mechanisms have been reported to exhibit oscillations in a region of parameter space, when the mechanism is ‘mixed’ while oscillation in 3-site (purely) distributive phosphorylation is not observed. As such, results in this section show how the outflow location significantly affects the qualitative behaviour of the open system.

5.3.3.2 Open Enzyme systems

After investigating the effects of open substrate systems on behaviour of multisite-phosphorylation, the focus now is on examining the effect of open enzyme systems on the behaviour of such mechanisms. Enzyme degradation has been observed in multiple signalling contexts (Lin et al., 2003). A striking example includes the phosphatase degradation in MAPK pathway which is a key control in a number of cellular functions, including cell proliferation, differentiation and stress responses (Choi et al., 2006; Kondoh and Nishida, 2007). The study starts with the investigation on how open phosphatase systems affect the behaviour of 2-site modification (see Model E1).

Figure 5.28 presents simulations showing how the completely and partially phosphorylated substrate concentration at steady state varies with total kinase concentration. The behaviour the open enzyme system (Model E1) is examined in a parameter regime, where the ‘analogous’ closed system is monostable. Here certain differences are observed. As seen in figure 5.28a, it is found that the fully modified substrate exhibits a monotonic response different from the ‘related’ closed systems which present monostable switching. This means that open enzyme systems can diminish the monostable switching characteristics. By contrast, the partial phospho-form (A_p) exhibits biphasic response similar to the closed system (figure 5.28b).

(a)



(b)

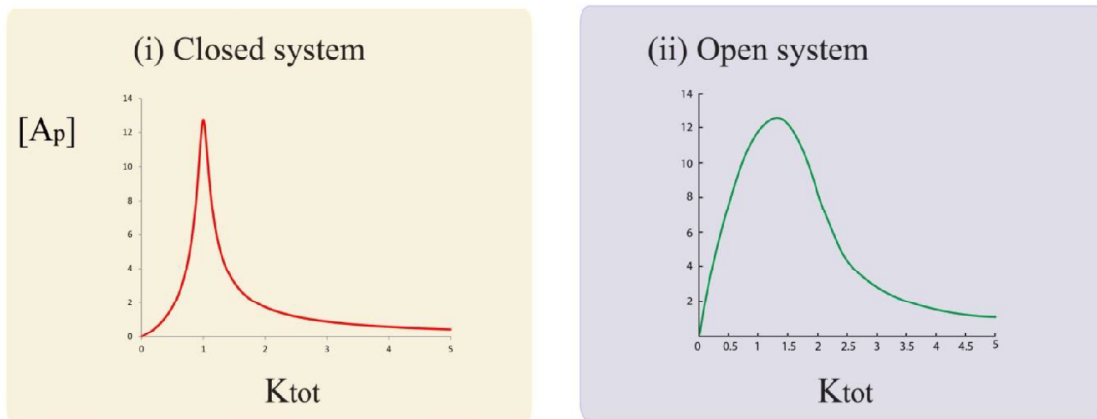
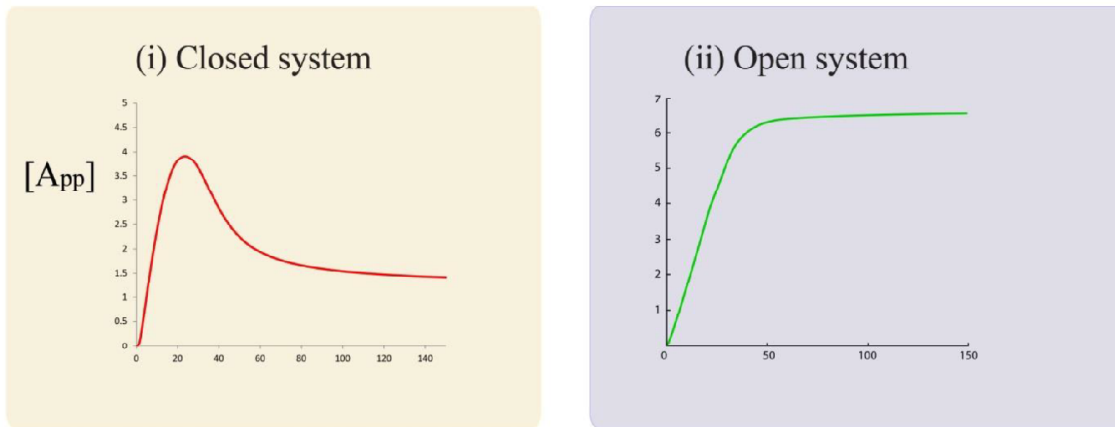


Figure 5.28 Steady-state dose–response curves in double phosphorylation models: closed and open enzyme system (model E1). (a) show the steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of the total concentration of kinase, K_{tot} , while (b) shows the corresponding curve for the partially phosphorylated substrate, A_p . ((a) Fully phosphorylated substrate: (i) closed system and (ii) open system (model E1). (b) Singly phosphorylated substrate: (i) closed system and (ii) open system (model E1)).

(a)



(b)

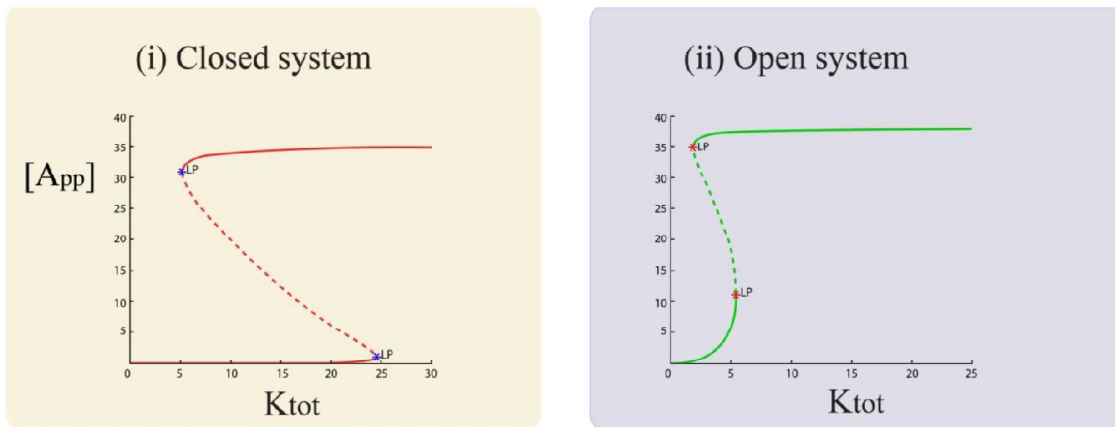


Figure 5.29 Steady-state dose–response curves in ordered double distributive phosphorylation models with different catalytic constants of phosphorylation reactions: closed and open enzyme systems (model E1). (a) The biphasic behaviour which can arise from a distributive model (i) is not found in model E1 (ii). (b) The (closed) distributive model exhibits bistability (i), while the input range for bistability becomes narrower in model E1 (ii).

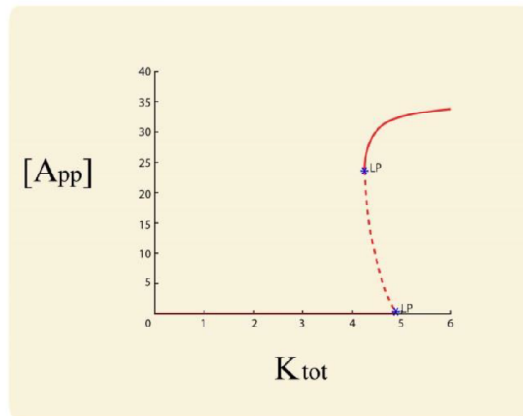
This open system (Model E1) is also examined in parameter regimes, where the closed systems are capable of producing other key qualitative signatures of information

processing. Figure 5.29a shows a dose–response curve of a closed model in a different regime, indicating a biphasic response for the fully modified phospho-form. It is found that the ‘corresponding’ open systems do not exhibit such biphasic dose–response curves (for the fully phosphorylated substrate), and analytical work in (Suwanmajo and Krihsnan, 2018) shows that when 2-site distributive phosphorylation associated with open phosphatase systems (model E1), biphasic responses can be ruled out. Note that the origins of the biphasic response discussed in chapter 3, are bypassed in this case.

The model E1 is also investigated in a parameter regime, where the ‘analogous’ closed systems is bistable (figure 5.29b(i)). It is found that the fully modified phosphoform can exhibit bistability with narrow range, compared to the case of corresponding closed systems, as shown in figure 5.29b(ii).

Figure 5.30 presents another aspect of open phosphatase systems (Model E1). In contrast to the ‘corresponding’ closed system, which exhibits bistability (figure 5.30a), it is found that model E1 is capable of generating sustained oscillation under monostable conditions. A bifurcation diagram in figure 5.30b(ii) shows that, as the parameter K_{tot} is changed, the steady state does indeed lose stability to a Hopf bifurcation. At the Hopf bifurcation point, a system's stability switches and a periodic solution arises (Strogatz, 2000). Overall the phosphatase production/degradation by itself can alter behaviour even from bistable to monostable response and can completely introduce new behaviour which is different from the closed multi-phosphorylation systems.

(a) Closed system



(b) Open system

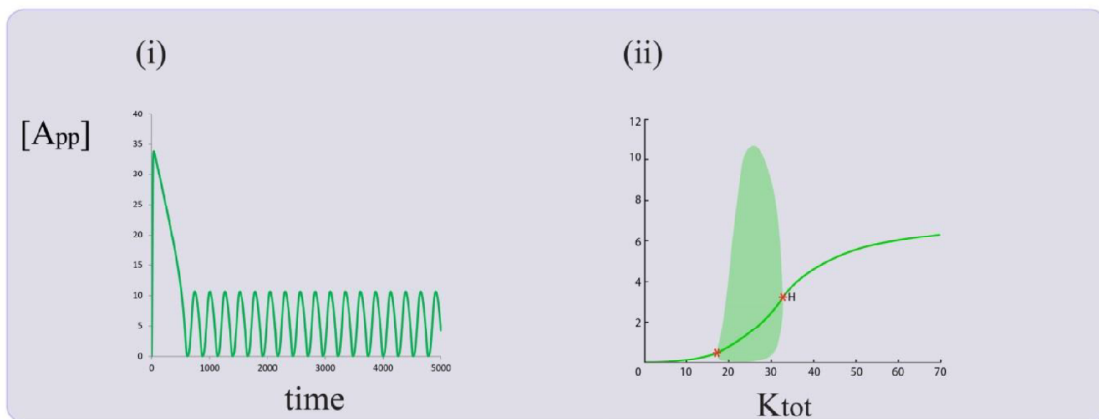


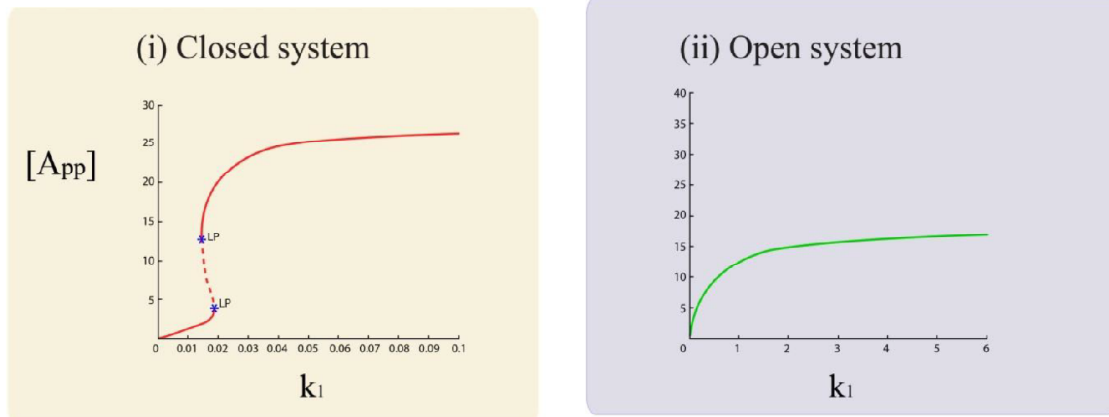
Figure 5.30 Oscillatory behaviour in open enzyme model (Model E1). While (a) the closed double distributive system exhibits bistability (simulations result in a steady state), b(i) the open enzyme model is capable of generating sustained oscillations. b(ii) Bifurcation analysis shows that as the total kinase concentration, K_{tot} , is changed, the steady state loses stability *via* a Hopf bifurcation in the model E1.

After investigating the effect of open phosphatase systems on multi-site phosphorylation, the focus is now on examining the case where both phosphatase and kinase are open systems (Model E2). It is interesting to ask whether the systems can present key qualitative signatures, such as bistability and oscillations.

Model E2 is first examined in parameter regimes, where the closed analogues are bistable. Figure 5.31a presents simulations showing how the maximally phosphorylated substrate concentration at steady state varies with binding constant of the first phosphorylation step (k_1). A choice of sufficiently different catalytic constants in phosphorylation can result in bistability in double distributive phosphorylation, as shown in figure 5.31a(i). By contrast, the ‘corresponding’ open system (model E2) exhibits monostable in this parameter regime as seen in figure 5.31a(ii). It is worth mentioning that model E2 cannot exhibit bistability, which is analytically demonstrated in (Suwanmajo and Krishnan, 2018b).

Another aspect of model E2 is now investigated. Figure 5.31b presents simulations showing how the maximally phosphorylated substrate concentration at steady state varies with binding constant of the first dephosphorylation step (k_7). In contrast to the ‘corresponding’ closed system, which exhibits bistability (figure 5.31b(i)), a bifurcation diagram in figure 5.31b(ii) shows that, as the parameter k_7 is changed, the steady state does indeed lose stability to a Hopf bifurcation. In the literature, sustained-oscillations in closed (ordered) double distributive phosphorylation systems have not been reported. This result shows that the open enzyme systems can cause oscillatory behaviour in multisite modification and this can be ‘transmitted’ to other signalling entities. The result is significant since it allows us to systematically infer the effects of the deviations which induce oscillation in multisite phosphorylation, rather than misattribute observations to hypothesized mechanisms.

(a)



(b)

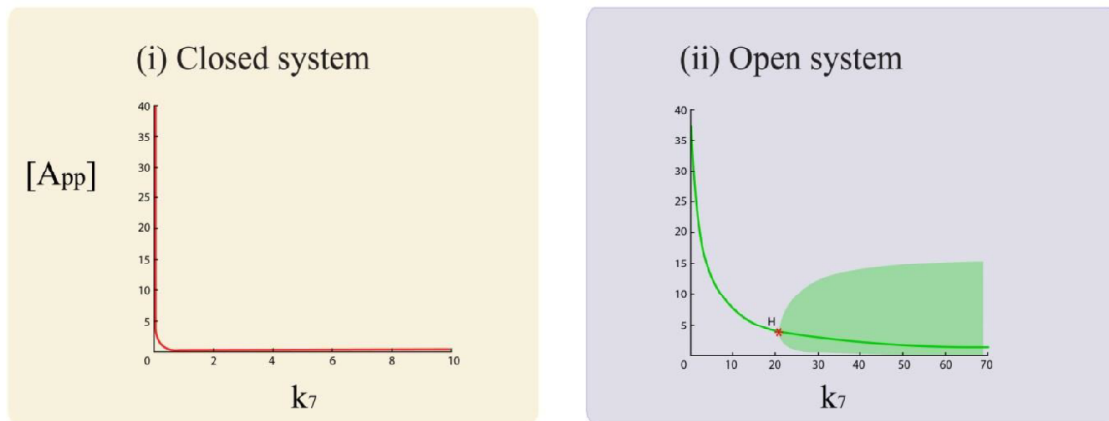


Figure 5.31 Simulations showing how the maximally phosphorylated substrate concentration at steady state varies with kinetic parameters in phosphorylation reaction: closed and open system (model E2). (a) The bistable behaviour which can arise from a distributive model (i) is not found in model E2 (ii). (b) In contrast to the (closed) distributive model which is monostable (i), model E2 can exhibit oscillatory behaviour (ii).

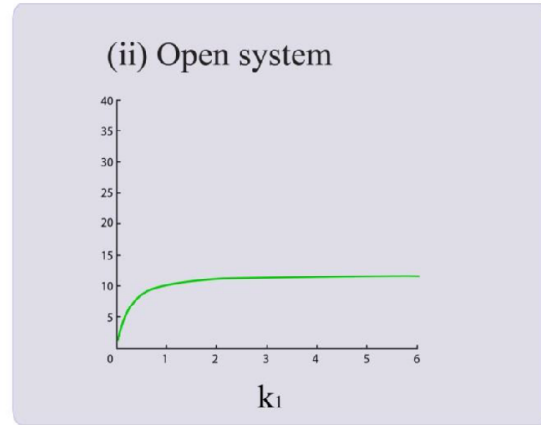
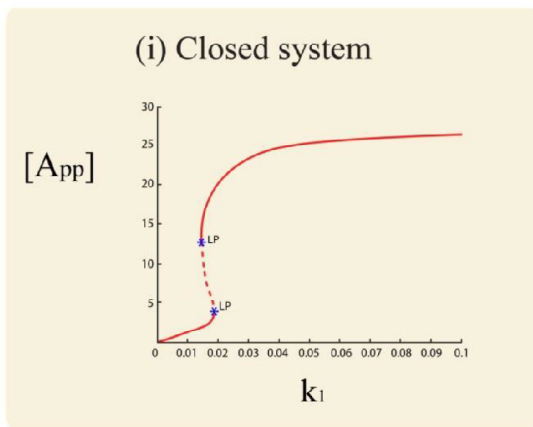
5.3.3.3 Open substrate and enzyme system

This section focuses on investigating the case where both enzyme and substrate systems are not closed (Model ES). The goal is to investigate whether such systems can present key qualitative signatures, such as bistability and oscillations.

Model ES is first examined in parameter regimes, where the closed distributive analogues are bistable. Figure 5.32a presents simulations showing how the maximally phosphorylated substrate concentration at steady state varies with binding constant of the first phosphorylation step (k_1). It is found that the closed system exhibit bistability, as shown in figure 5.32a(i). By contrast the ‘corresponding’ open system exhibits hyperbolic response, as seen in figure 5.32a(ii). It is worth mentioning that model ES cannot exhibit bistability, which is analytically demonstrated in (Suwanmajo and Krishnan, 2018b).

Here, another aspect of model ES is now investigated. Figure 5.32b presents simulations showing how the maximally phosphorylated substrate concentration at steady state varies with binding constant of the first dephosphorylation step (k_7). In contrast to the ‘corresponding’ closed system, which exhibits bistability (figure 5.32b(i)), a bifurcation diagram in figure 5.32b(ii) shows that, as the parameter k_7 is changed, the steady state does indeed lose stability to a Hopf bifurcation. This result shows that the enzyme and substrate open systems can introduce oscillation behaviour in multisite phosphorylation system.

(a)



(b)

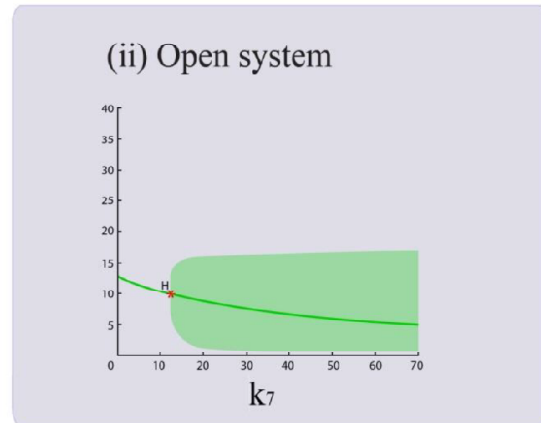
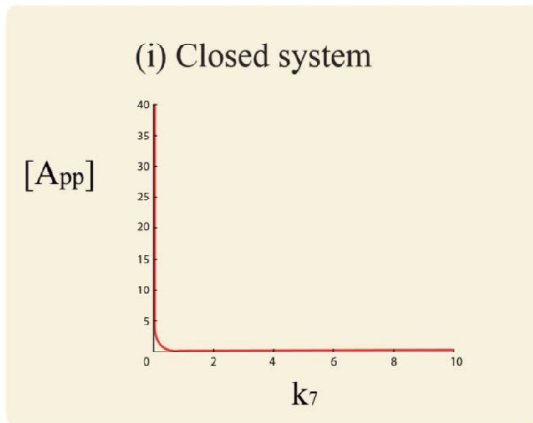


Figure 5.32 Simulations showing how the maximally phosphorylated substrate concentration at steady state varies with kinetic parameters in phosphorylation reaction: closed and open system (model ES). (a) The bistable behaviour which can arise from a closed distributive model (i) is not found in model ES (ii). (b) In contrast to the (closed) distributive model which is monostable (i), model ES can exhibit oscillatory behaviour (ii).

Overall, section 5.3.3 focuses on studying the effect of open systems (i.e. production or degradation of substrate and enzymes) on the internal dynamic behaviour in multi-site phosphorylation. Qualitative differences in the open systems (of ordered, double distributive phosphorylation) when compared with closed systems are summarised in table 5.2. For instance, new kinds of qualitative behaviour such as sustained oscillation and adaptation can be introduced by open systems. Certain kinds of qualitative behaviour which can be ruled out in the open system case are also discussed in (Suwanmajo and Krishnan, 2018b).

Table 5.2 Qualitative differences in the open system (of ordered, double distributive phosphorylation) when compared with closed systems.

Systems	Outflow species	Qualitatively Essential differences from closed system		Behaviour of partial phospho-form	Other qualitative characteristics observed
		SEEN	RULED OUT		
Substrate open system (Inflow species: unmodified substrate, A)	App	Oscillation No steady state for certain ranges of parameter regimes Adaptation (for App) Adaptation combined with oscillation (for App)	Bistability Biphasic behaviour (for App as Ktot is varied)	Oscillation No steady state for certain ranges of parameter regimes Two steady states for certain ranges of parameter regimes A decrease at higher kinase concentrations	
	Ap	Oscillation No steady state for certain ranges of parameters Adaptation (for Ap) Adaptation combined with oscillation (for Ap)	Bistability Biphasic behaviour (for App as Ktot is varied)	Oscillation No steady state for certain ranges of parameter regimes Adaptation (for Ap) Adaptation combined with oscillation (for Ap)	
	App and Ap	Oscillation	Biphasic behaviour (for App as Ktot is varied)	Biphasic behaviour Biphasic behaviour which presents biphasic dose-response which exhibits a range of oscillation	Bistability
	App, Ap and A	Oscillation	Biphasic behaviour (for App as Ktot is varied)	Biphasic behaviour Biphasic behaviour which presents biphasic dose-response which exhibits a range of oscillation	Bistability
Enzyme open system Phosphatase open system (Inflow species: P) Kinase & phosphatase open system (Inflow species: P and K)	P	Oscillation	Biphasic behaviour (for App as Ktot is varied)	Biphasic behaviour Biphasic behaviour which presents biphasic dose-response which exhibits a range of oscillation	Bistability
	P and K	Oscillation	Bistability	Biphasic behaviour Biphasic behaviour which presents biphasic dose-response which exhibits a range of oscillation Oscillation	Bistability
Enzyme & substrate open system (Inflow species: A, P and K)	App, Ap, A, K and P	Oscillation	Bistability	Oscillation	

5.3.4 Effect of the modules embedded in the signalling networks

Multi-site phosphorylation modules are ubiquitous in cellular processes and do not operate in isolation. It can be part of different kinds of simple and complex networks. This section aims to bridge the gap between intrinsic behaviour in multisite phosphorylation and its function in signalling pathways. To gain insight into the dynamic behaviour of multisite phosphorylation in the networks, simple kinds of network modules with the ordered, double distributive phosphorylation module embedded in them, are examined. In particular, this study considers two kinds of different feed-forward modules, coherent (c-FFW) and incoherent feed-forward (i-FFW), and two kinds of different feedback modules, positive (PFB) and negative feedback (NFB). All these modules have been studied where the constituent mechanisms involve simple modification (Alon, 2006; Kholodenko, 2006; Levchenko and Iglesias, 2002). The aim of this section is to study what the effect of multisite phosphorylation is on the network and what the effect of the ambient network is on multisite phosphorylation. One of the big challenges in understanding this kind of issue is the multisite phosphorylation can be in different parameter regimes having different kinds of qualitative behaviour. Here the focus is mainly on qualitative behaviour and trends; therefore, a detailed parametric analysis of these models is not focused here.

Results are organized as follows. In the first section, the effect of the positive feedback module (PFB) will be discussed while the second section focuses on discussing the effect of the negative feedback module (NFB). In the subsequent section, the effect of incoherent feed-forward module will be dissected. Finally, the effect of coherent feed-forward module will be discussed in the fourth section.

5.3.4.1 Positive feedback module (PFB)

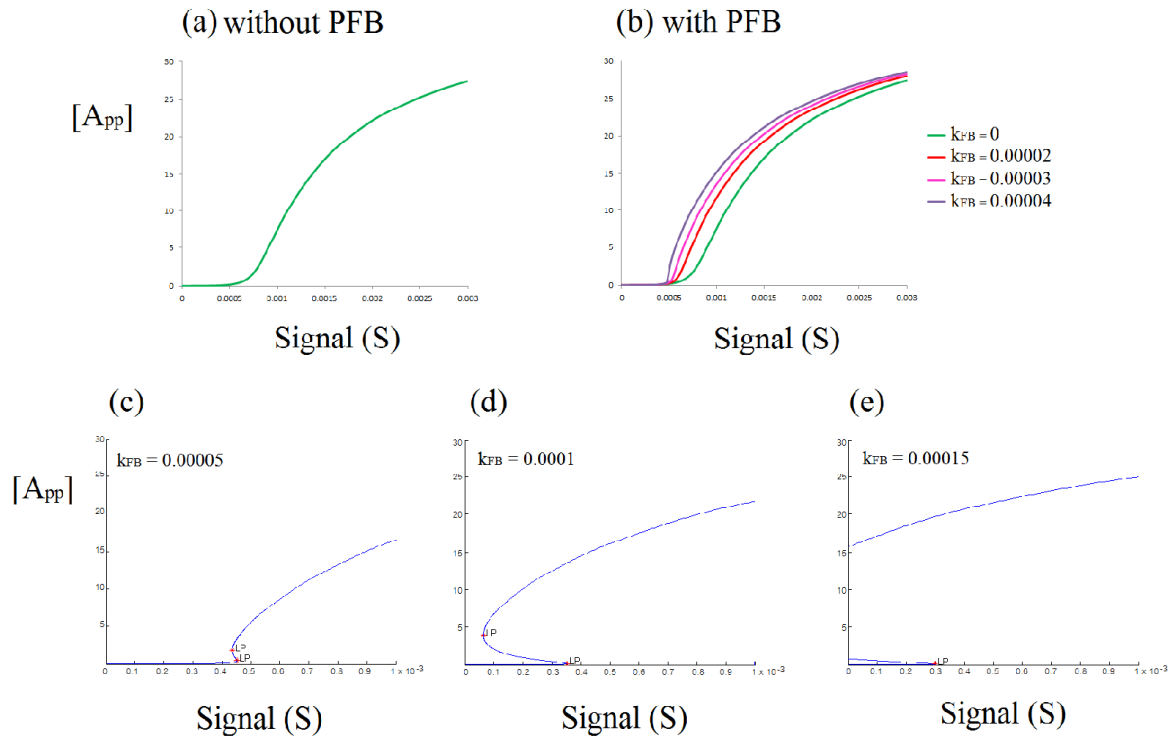


Figure 5.33 Effect of PFB on monotonic response in ordered double distributive phosphorylation. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S) . (a) double phosphorylation model without PFB. (b-e) shows how dose response curves of A_{pp} varies with the input signal for different values of the activation constant, k_{PFB} (k_{PFB} is activation constant which represents the strength of the positive feedback).

In many biological contexts, multi-site phosphorylation is embedded in feedback loops. For example, in many cell types, the MAPK cascades (which involves double phosphorylation) are imbedded in feedback loops, negative or positive or negative, depending on whether the terminal kinase stimulates or inhibits the activation of the initial level

(Kholodenko, 2000). In this section, the goal is to bridge the gap between intrinsic behaviour in multisite phosphorylation and its functioning in positive feedback module (PFB). This study starts by examining the effect of PFB on the intrinsic behaviour of multisite phosphorylation in parameter regimes, where the system without PFB is monostable (as seen in figure 5.33a). Figure 5.33b shows how steady state response of maximally phosphorylated substrate varies with the input signal for different values of the activation constant, k_{PFB} (k_{PFB} is activation constant which represents the strength of the positive feedback while S denotes input signal which activates inactive kinase). As seen in this figure, as k_{PFB} increases, the systems are monostable and the threshold feature is maintained whereas the response becomes steeper. By contrast, the systems exhibit partial reversible bistable switching when k_{PFB} is increased (figure 5.33c) and the input range for bistability becomes wider when k_{PFB} increases even further. Taken together, with the presence of PFB the behaviour in multisite phosphorylation can be altered even qualitatively from monostable to partially reversible and irreversible bistable switching.

The investigation now focuses on how PFB distorts the biphasic response (for the fully modified phosphoform) in multi-site phosphorylation without PFB (figure 5.34a). It is found that as k_{PFB} increases, the systems present biphasic behaviour with the similar amplitude. However, the systems can exhibit both biphasic response as well as irreversible switching when k_{PFB} increases even further (figure 5.34(c) and (d)). This shows how PFB can induce bistability while biphasic behaviour is retained.

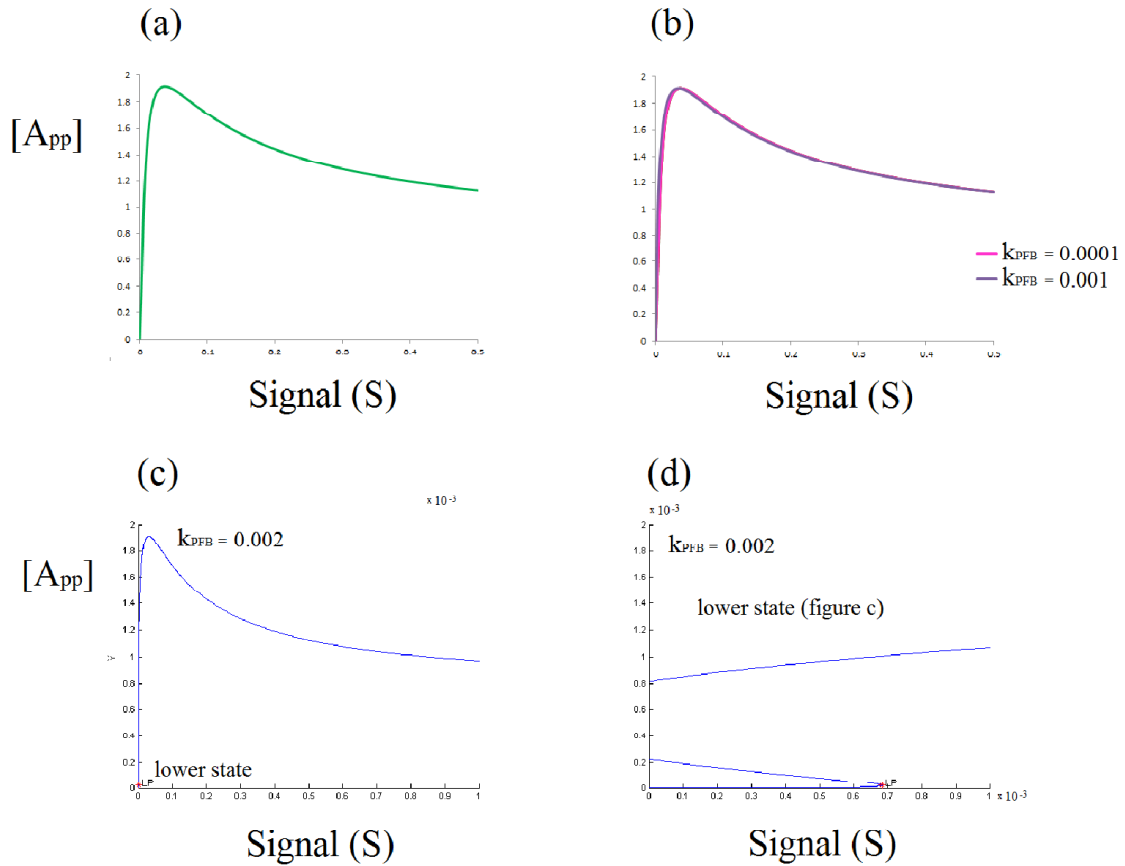


Figure 5.34 Effect of PFB on biphasic behaviour in ordered double distributive phosphorylation. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S) . (a) double phosphorylation model without PFB. (b-d) shows how dose response curves of A_{pp} varies with the input signal for different values of k_{PFB} .

Following investigation of the effect of PFB on distorting the monotonic response in multi-site phosphorylation, the next step is to consider the case where the isolated system exhibits bistability (as shown in figure 5.35). It is found that introducing PFB to multisite phosphorylation can alter reversible bistable switching (figure 5.35a(i)) to irreversible bistable switching (figure 5.35a(ii)). Furthermore, combination of reversible and irreversible bistable switching is observed (figure 5.35b(ii)).

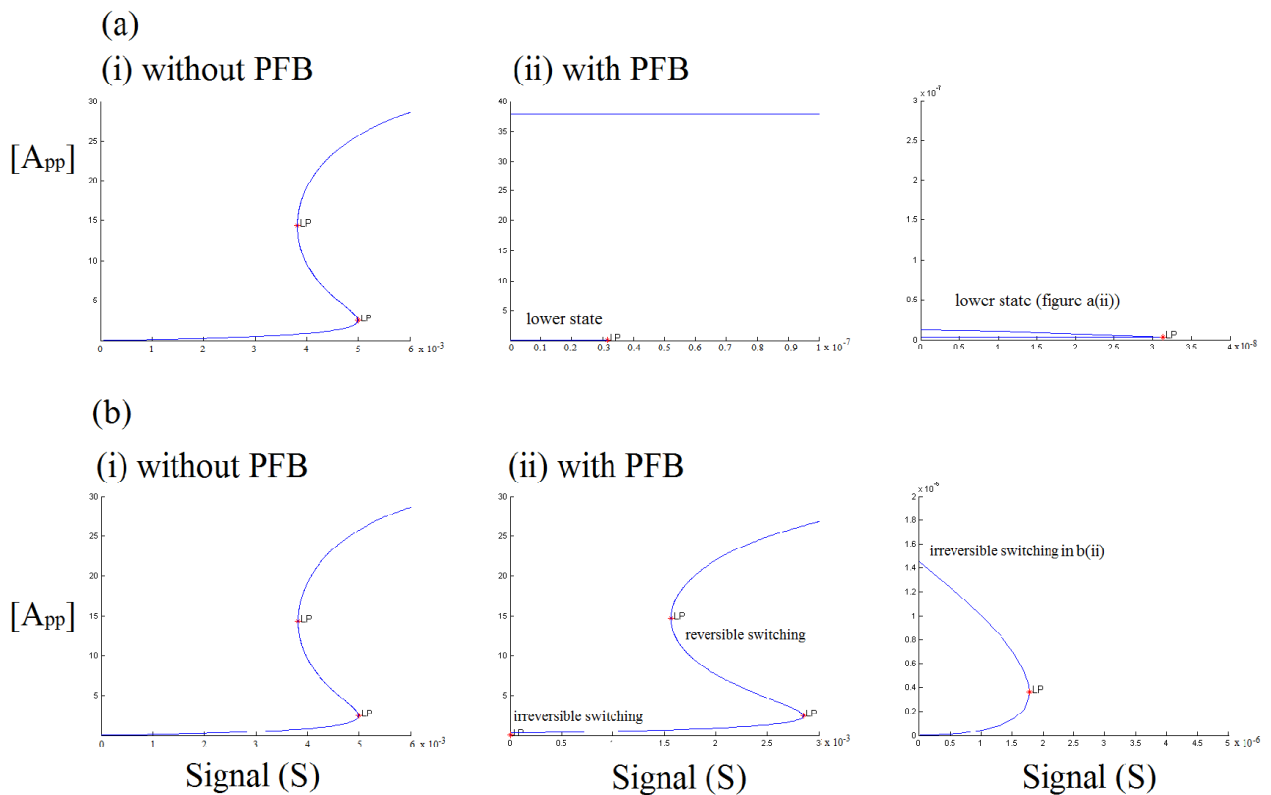


Figure 5.35 Effect of PFB on bistable behaviour in ordered double distributive phosphorylation. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a): double phosphorylation model (i) without PFB. (ii) with PFB. (b): double phosphorylation model (i) without PFB. (ii) with PFB.

The focus is still on examining the the effect of PFB on bistable behaviour in multisite phosphorylation. Here a more striking effect is seen. Figure 5.36 shows that introducing PFB to double distributive phosphorylation can qualitatively alter bistability to tristability in multisite phosphorylation. Ordered double phosphorylation by itself cannot present tristability (Thomson & Gunawardena, 2009). Therefore, introducing PFB to multisite phosphorylation can introduce new kind of behaviour which is not observed in systems without PFB.

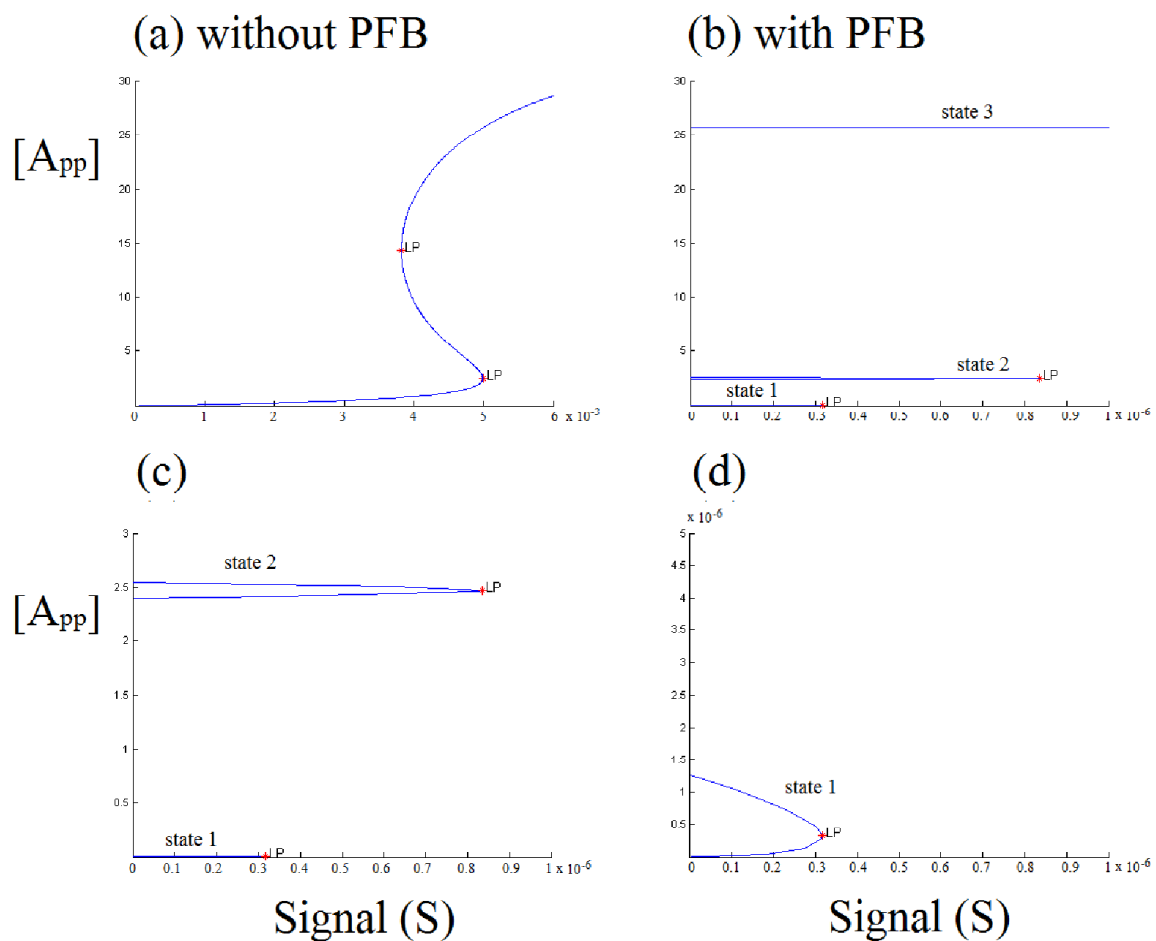


Figure 5.36 Tristability in ordered double distributive phosphorylation with PFB. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a) double distributive phosphorylation model

without PFB. (b) double phosphorylation with PFB. (c) shows the middle state while (d) shows the lower state of tristability in (b).

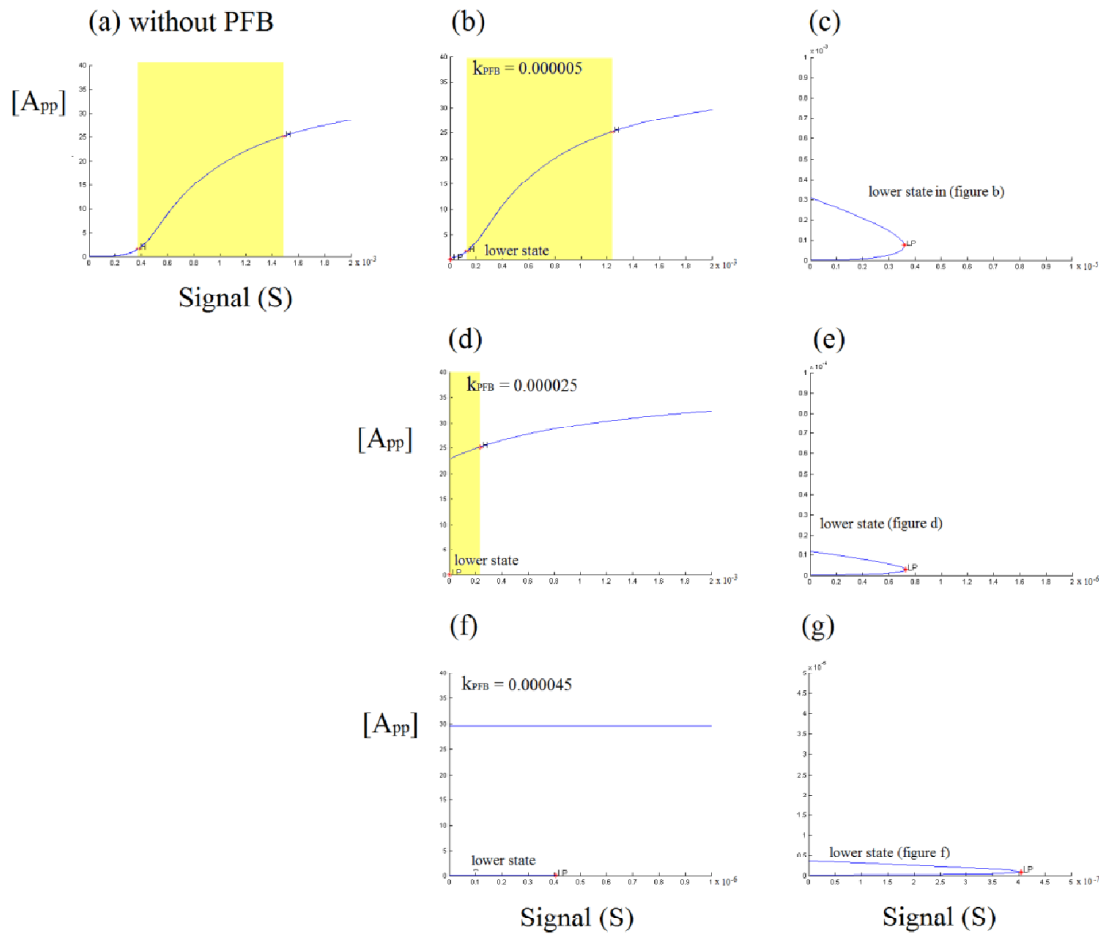


Figure 5.37 Effect of PFB on oscillatory behaviour in ordered double distributive phosphorylation. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a) double phosphorylation model without PFB. (b-g) shows how dose response curves of A_{pp} varies with the input signal for different values of k_{PFB} . (yellow background denotes input range for oscillation)

In the previous section, it was shown that explicit activation can induce oscillation in ordered double distributive phosphorylation when kinase or phosphatase are far from active form. Now the investigation focuses on how PFB affects the oscillatory behaviour due to explicit activation in double distributive phosphorylation. While systems without PFB exhibit sustained oscillation under monostable condition, striking differences are observed when PFB is introduced to the systems. For lower value of k_{PFB} , the systems present both sustained oscillation and irreversible switching (figure 5.37b and figure 5.37c). Both Hopf bifurcation points move to lower values of input signal. When k_{FB} increases further, the systems present both sustained oscillation and irreversible switching and the input range for oscillations is reduced (figure 5.37d and figure 5.37e). When k_{FB} increases furthermore, the systems retain irreversible switching but oscillatory behaviour is eliminated (figure 5.37f and figure 5.37g). Over all introducing PFB can qualitatively alter the behaviour of multisite phosphorylation system. Increasing the strength of PFB can induce bistability but reduce the input range for oscillations and eventually destroys oscillatory behaviour.

5.3.4.2 Negative feedback module (NFB)

This section aims to bridge the gap between intrinsic behaviour in multisite phosphorylation and their functioning with a negative feedback module (NFB). The focus is first on examining the effect of NFB on the behaviour of multisite phosphorylation in a parameter regime, where the system without NFB is monostable.

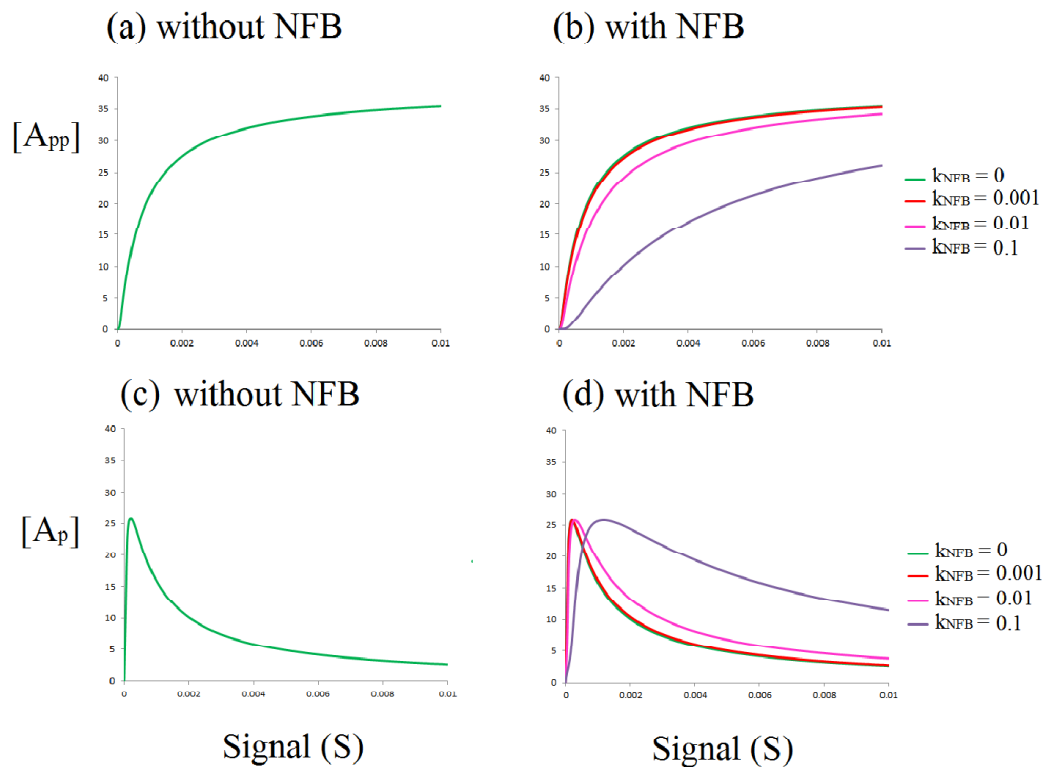


Figure 5.38 Effect of NFB on monotonic response in ordered double distributive phosphorylation. (a) steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S) in phosphorylation model without NFB. (b) shows how dose response curves of A_{pp} varies with the input signal for different values of k_{NFB} (k_{NFB} is a constant which represents the strength of the negative feedback). (c) and (d) show the corresponding curve for the partially phosphorylated substrate, A_p .

Figure 5.38 shows how the steady state responses of maximally and partially phosphorylated substrate varies with the input signal, S , for different values of k_{NFB} . As seen in figure 5.38a, as the input signal, S , increases, the concentration of the double phosphorylated substrate increases monotonically and asymptotes to its maximum level. However introducing NFB to the system, the system presents a monotonic response with different saturation levels (figure 5.38b). A gradual difference is seen when k_{FB} is varied. As k_{FB} increases, the system presents a monotonic dose response at lower saturation level. This is completely due to the effect of NFB on the multisite phosphorylation module. Figure 5.38 (c) shows that the single phosphorylated substrate, A_p , concentration exhibits a biphasic response: the initial increase at low signal levels reaching a maximum, before a decrease at higher input signal in the systems without NFB. Introducing NFB results in A_p exhibiting a biphasic response over a wider range of input signal (figure 5.38d).

The investigation now focuses on NFB model in different parameter regimes, where the system without NFB presents a biphasic response for the fully modified phosphoform (figure 5.39a). Figure 5.39b and 5.39c show how steady state response of maximally phosphorylated substrate varies with the input signal for different values of k_{NFB} . As the NFB strength is increased, the biphasic behaviour is maintained. Compared to the system without NFB, the A_{pp} dose response increases more gradually initially and reaches a maximum at higher level of input signal, due to the effect of NFB on the multisite phosphorylation module.

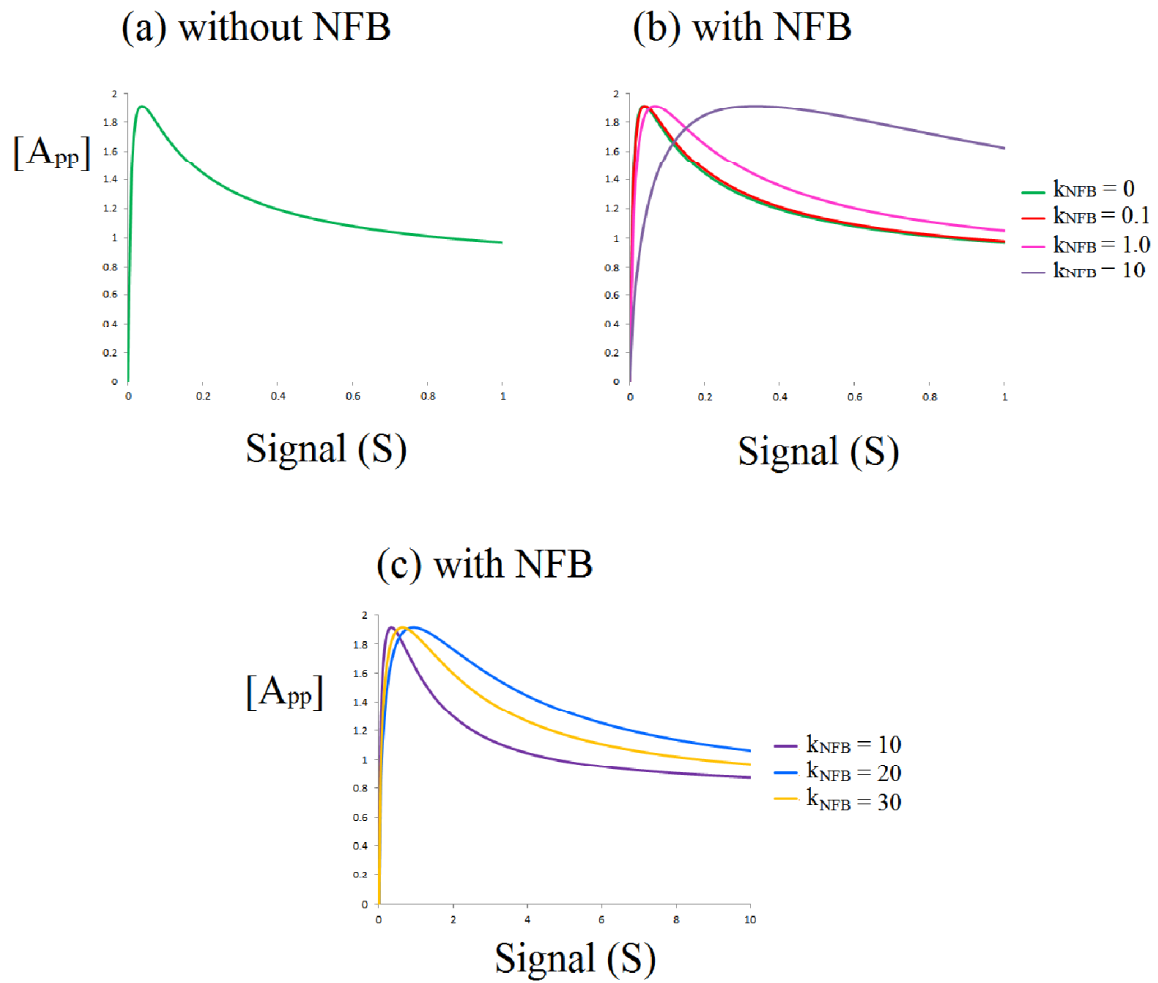


Figure 5.39 Effect of NFB on biphasic behaviour in ordered double distributive phosphorylation. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a) double phosphorylation model without NFB. (b-c) shows how dose response curves of A_{pp} varies with the input signal for different values of k_{NFB} .

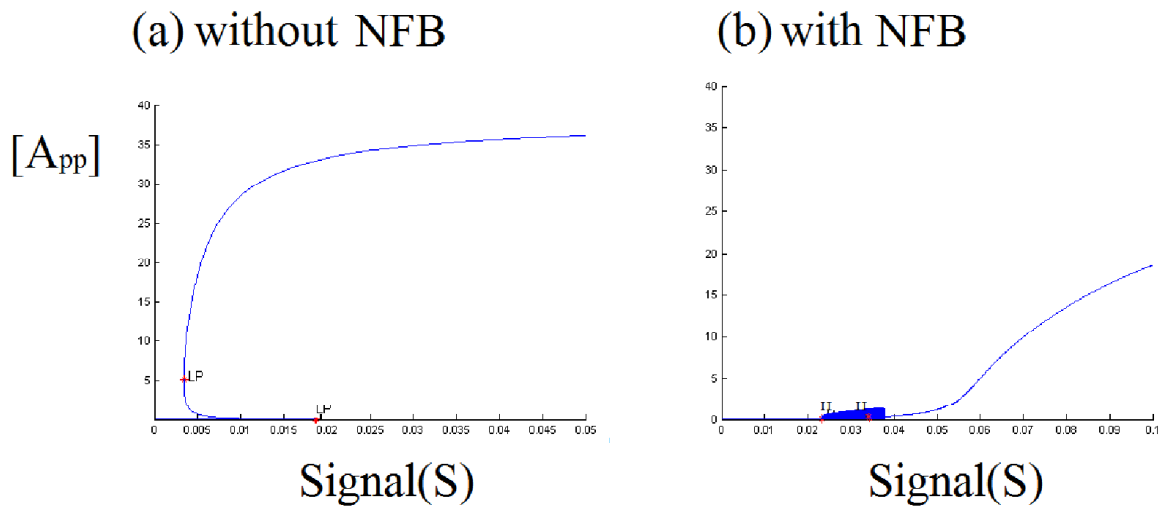


Figure 5.40 Effect of NFB on bistable behaviour in ordered double distributive phosphorylation. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a) double phosphorylation model without NFB. (b) shows how NFB induces oscillatory behaviour in ordered double distributive phosphorylation.

The focus now is on investigating the case where the system without NFB exhibits bistability. Here a more striking effect is seen. Figure 5.40 shows that introducing NFB to ordered double distributive phosphorylation can qualitatively alter bistability to oscillation (under monostable condition) in multisite phosphorylation. In the previous chapter, sustained oscillations were not observed in isolated (ordered) double phosphorylation. Therefore, introducing NFB to multisite phosphorylation can introduce new kind of behaviour which is not observed in systems without NFB. Note that in this example in the absence of feedback there is no oscillation, while increasing the feedback strength can trigger oscillations.

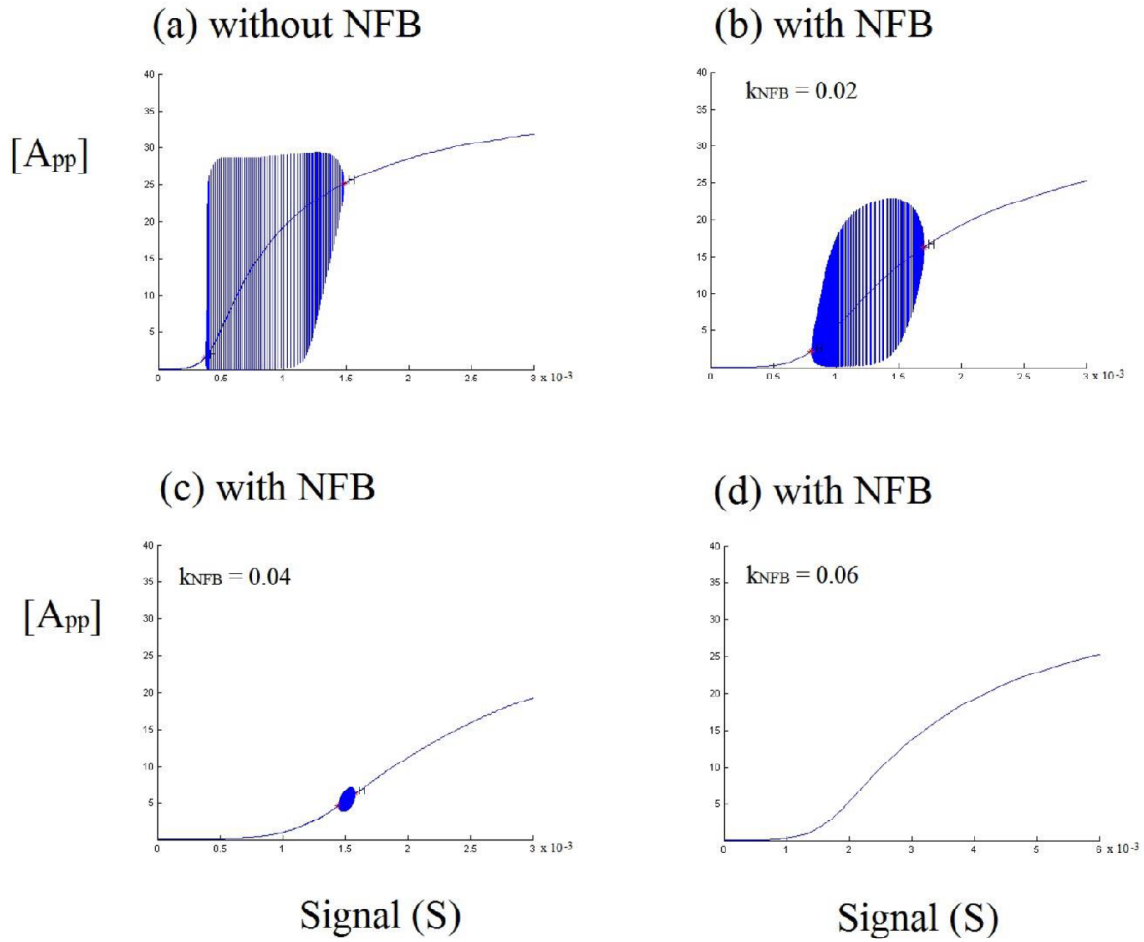


Figure 5.41 Effect of NFB on oscillatory behaviour in ordered double distributive phosphorylation model. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a) double phosphorylation model without NFB. (b-d) shows how dose response curves of A_{pp} varies with the input signal for different values of k_{NFB} .

In the previous section, it was shown that explicit activation can induce oscillation in ordered double distributive phosphorylation. The investigation now focuses on how NFB affects the oscillatory behaviour due to explicit activation in double phosphorylation. While

the systems without NFB exhibit sustained oscillation under monostable condition, striking differences are seen when NFB is introduced to the systems. The input range for oscillatory behaviour varies significantly as the strength of NFB, k_{NFB} , increases. For $k_{\text{NFB}} = 0.02$, the systems exhibit sustained oscillation and the input signal range for oscillations is reduced (Figure 5.41b). When k_{NFB} is increased to 0.04, the input (signal) range for oscillations is further reduced (Figure 5.41c). The complete disappearance of oscillations is observed when k_{NFB} is increased to 0.06 (Figure 5.41c). Over all introducing NFB can qualitatively alter the behaviour of multisite phosphorylation system in a somewhat unexpected way, given that the previous example showed that negative feedback could generate oscillations. Increasing the strength of NFB can reduce both the input range for oscillations and the amplitude of the oscillations, and eventually destroys oscillatory behaviour.

5.3.4.3 Incoherent feed-forward module (i-FFW)

This section aims to bridge the gap between intrinsic behaviour in multisite phosphorylation and their functioning in an incoherent feed-forward network module (i-FFW). This starts by examining the effect of i-FFW on the behaviour of multisite phosphorylation in a parameter regime, where the system without i-FFW is monostable (as seen in figure 5.42a(i)). Figure 5.42a(ii) shows how steady state response of maximally phosphorylated substrate varies with the input signal for different values of the activation constant, $k_{\text{i-FFW}}$ ($k_{\text{i-FFW}}$ is a constant which represents the strength of the incoherent leg of the feed-forward regulation). As seen in this figure, introducing i-FFW to multisite phosphorylation enables the systems to present partial biphasic dose–response characteristics. Such behaviour is much more pronounced as $k_{\text{i-FFW}}$ increases.

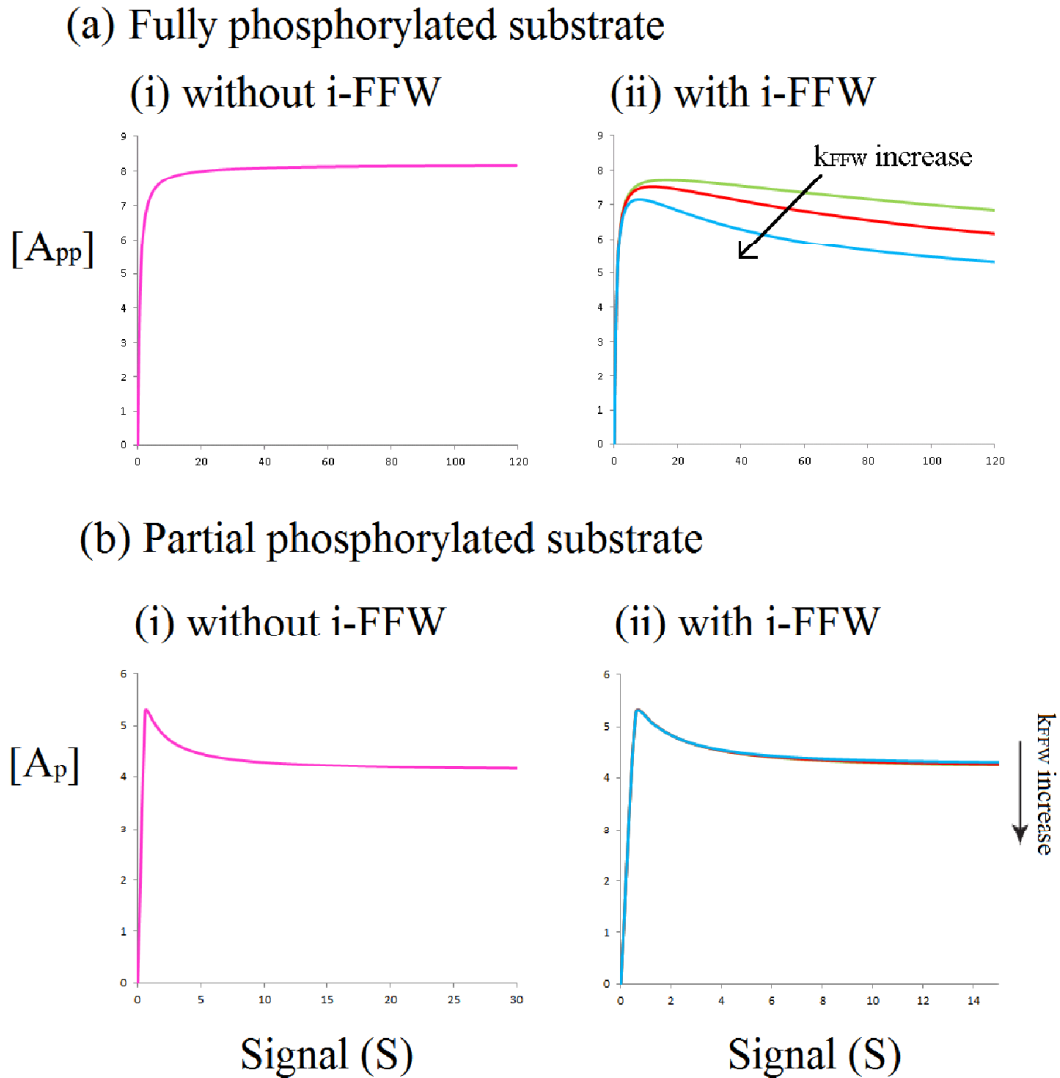


Figure 5.42 Effect of i-FFW on monotonic response in ordered double distributive phosphorylation. (a): (i) steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S) in phosphorylation model without i-FFW. (ii) shows how A_{pp} dose response varies with the input signal for different values of k_{i-FFW} . (b) shows the corresponding curve for the partially phosphorylated substrate, A_p .

Figure 5.42b(i) shows that the single phosphorylated substrate, A_p , concentration exhibits a biphasic response: the initial increase at low signal levels reaching a maximum, before a decrease at higher input signal in the systems without i-FFW. Introducing i-FFW to double distributive phosphorylation, results in the partial phosphoform exhibiting biphasic response with the same amplitude, as shown in figure 5.42b(ii)). In this case, the i-FFW has negligible effect on the partial phosphoform.

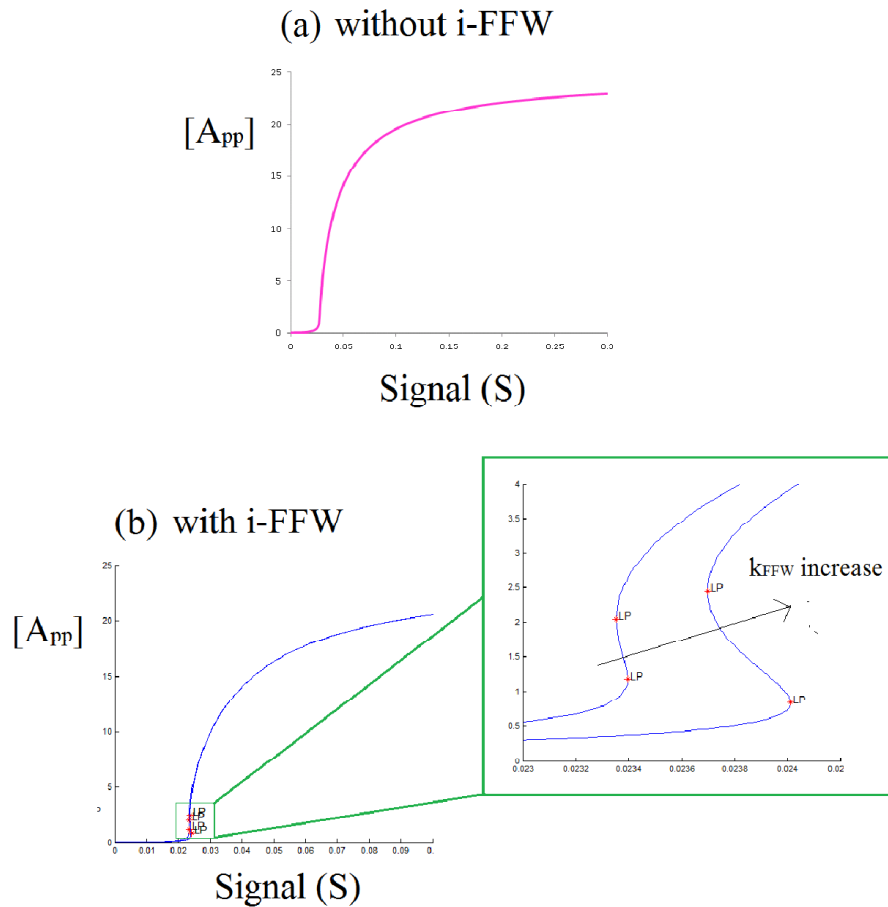


Figure 5.43 Effect of i-FFW on threshold behaviour in ordered double distributive phosphorylation. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal, S . (a) Model without i-FFW. (b) Model with with i-FFW.

The i-FFW model is now examined in different parameter regimes, where the systems without i-FFW can exhibit threshold behaviour (figure 5.43a). Here a more striking effect is seen. Figure 5.43b shows how steady state response of maximally phosphorylated substrate varies with the input signal for different values of $k_{i\text{-FFW}}$. As the $k_{i\text{-FFW}}$ is increased, the systems can exhibit bistability with a wider bistable range (of input). This shows that i-FFW can completely alter behaviour even qualitatively from monostable to bistable switching, even though the iFFW brings an in-built competition by itself.

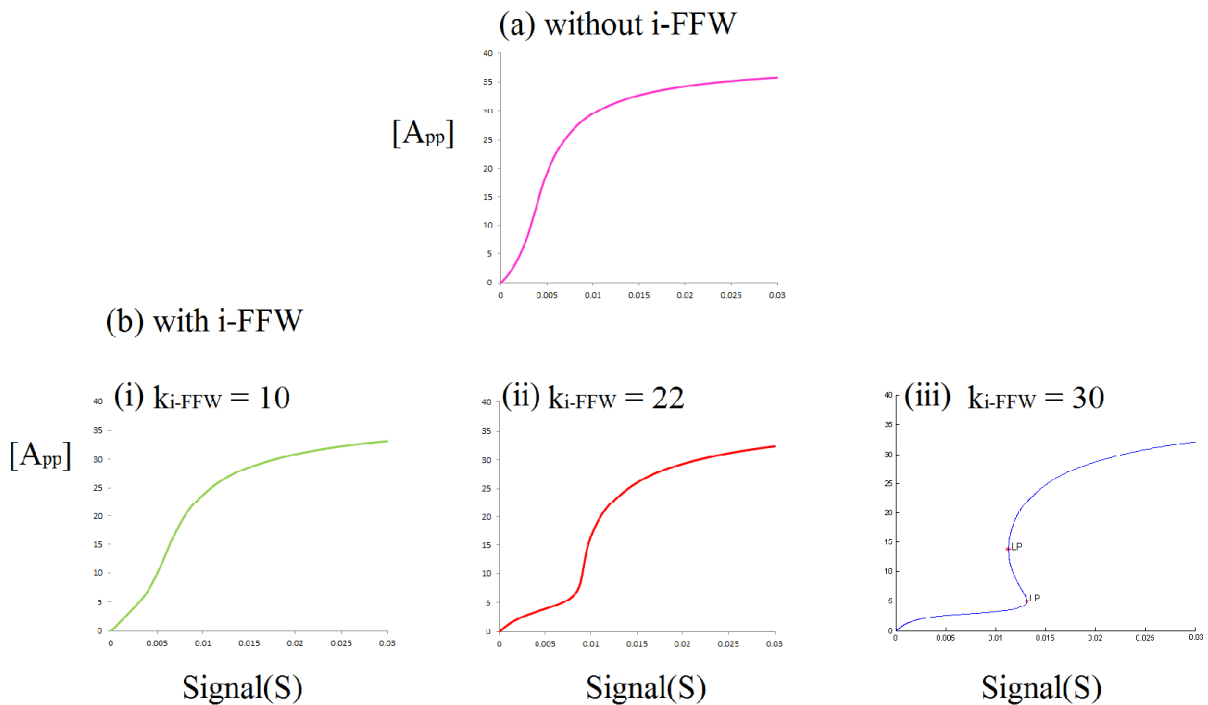


Figure 5.44 Effect of i-FFW on inducing bistability in ordered double distributive phosphorylation. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal, S . (a) Model without i-FFW. (b) Model with with i-FFW: (i) $k_{i\text{-FFW}} = 10$, (i) $k_{i\text{-FFW}} = 22$ and (i) $k_{i\text{-FFW}} = 30$.

Figure 5.44 shows how i-FFW can qualitatively alter the behaviour of multisite phosphorylation in the parameter regimes where the systems without i-FFW exhibit hyperbolic response (figure 5.44a). Figure 5.44b shows that introducing i-FFW to the systems can induce 2 phases of monotonic response, and eventually induce bistability.

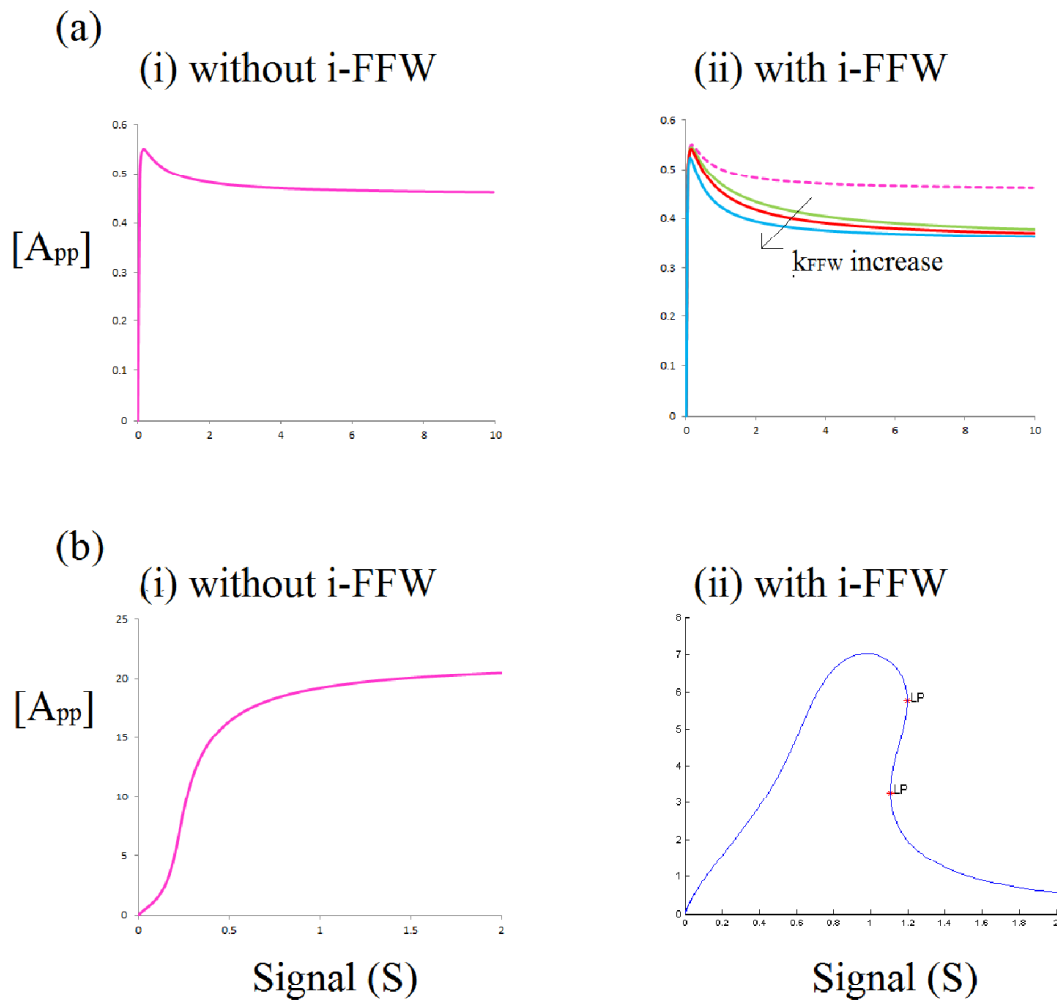


Figure 5.45 Biphasic behaviour and i-FFW in ordered double distributive phosphorylation. Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal, S . (a): (i) Model without i-FFW. (ii) Model with with i-FFW. (b): (i) Model without i-FFW. (ii) Model with with i-FFW.

Figure 5.45a(i) shows a dose–response curve of model without i-FFW in a different regime, indicating a biphasic response for the fully modified phosphoform. . Figure 5.45a(ii) shows the steady state response of maximally phosphorylated substrate as a function of input signal, S , for different values of $k_{i\text{-FFW}}$. It is found that increasing the value of $k_{i\text{-FFW}}$ can accentuate such biphasic behaviour in multisite phosphorylation systems. This is due to the effect of feed-forward regulation.

After investigating that i-FFW can induce biphasic response and bistability in ordered 2-site phosphorylation systems; it is interesting to ask whether these two kinds of qualitative behaviour could be combined together. Figure 5.45b shows that i-FFW can alter monotonic response in systems without i-FFW to bistability combined with biphasic responses. This means that i-FFW can induce a new complex mode of information processing.

The investigation now focuses on the case where the systems without i-FFW exhibit bistability. Figure 5.46 shows the steady state response of maximally phosphorylated substrate, A_{pp} , as a function of input signal, S , for different values of $k_{i\text{-FFW}}$. Compared to the systems without i-FFW which exhibit bistability (figure 5.46a), it is found that increasing the value of $k_{i\text{-FFW}}$ can accentuate such bistable behaviour in multisite phosphorylation systems. As $k_{i\text{-FFW}}$ increases, input range for bistability becomes wider (figure 5.46b). This is even though the feed-forward network itself brings an inbuilt competition.

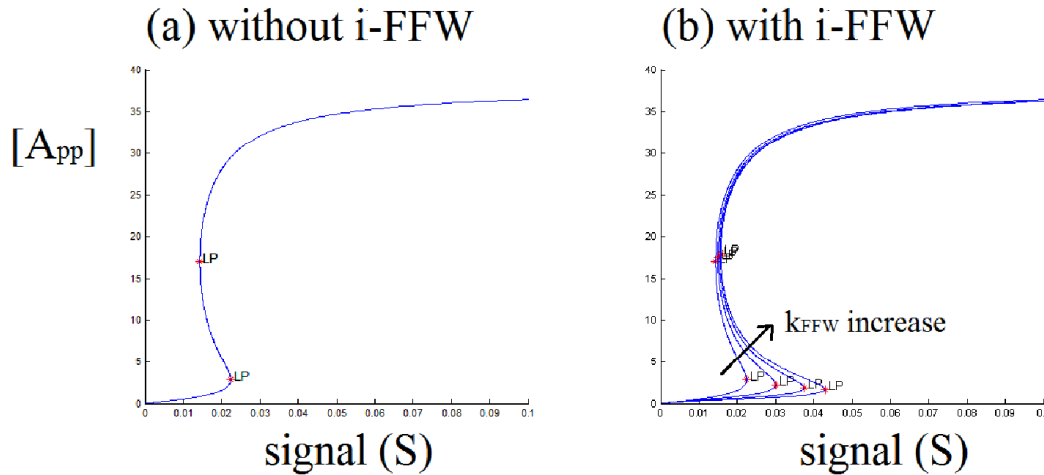


Figure 5.46 Effect of i-FFW on bistable behaviour in ordered double distributive phosphorylation. Steady-state dose-response curves of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a) double phosphorylation model without i-FFW. (b) double phosphorylation model with i-FFW.

In section 5.3.1, it was shown that explicit activation can induce oscillation in ordered double distributive phosphorylation when kinase or phosphatase are far from active form. Now the focus is on investigating how i-FFW affects the oscillatory behaviour due to explicit activation in ordered double phosphorylation. While systems without i-FFW exhibit sustained oscillation (5.47a), striking differences are seen when i-FFW is introduced to the double site systems. Figure 5.47(b)-(c) show that the systems exhibit both biphasic response and sustained-oscillation for $k_{i-FFW} = 0.02$ and 0.04 . The input signal range for oscillations becomes wider and two distinct oscillatory regions of stable limit cycles that coexist over a range of input signal are observed. The oscillatory regions merge as k_{i-FFW} increases (figure 5.47(d)-(g)), followed by the complete disappearance of oscillations (figure 5.47h) but biphasic behaviour is retained.

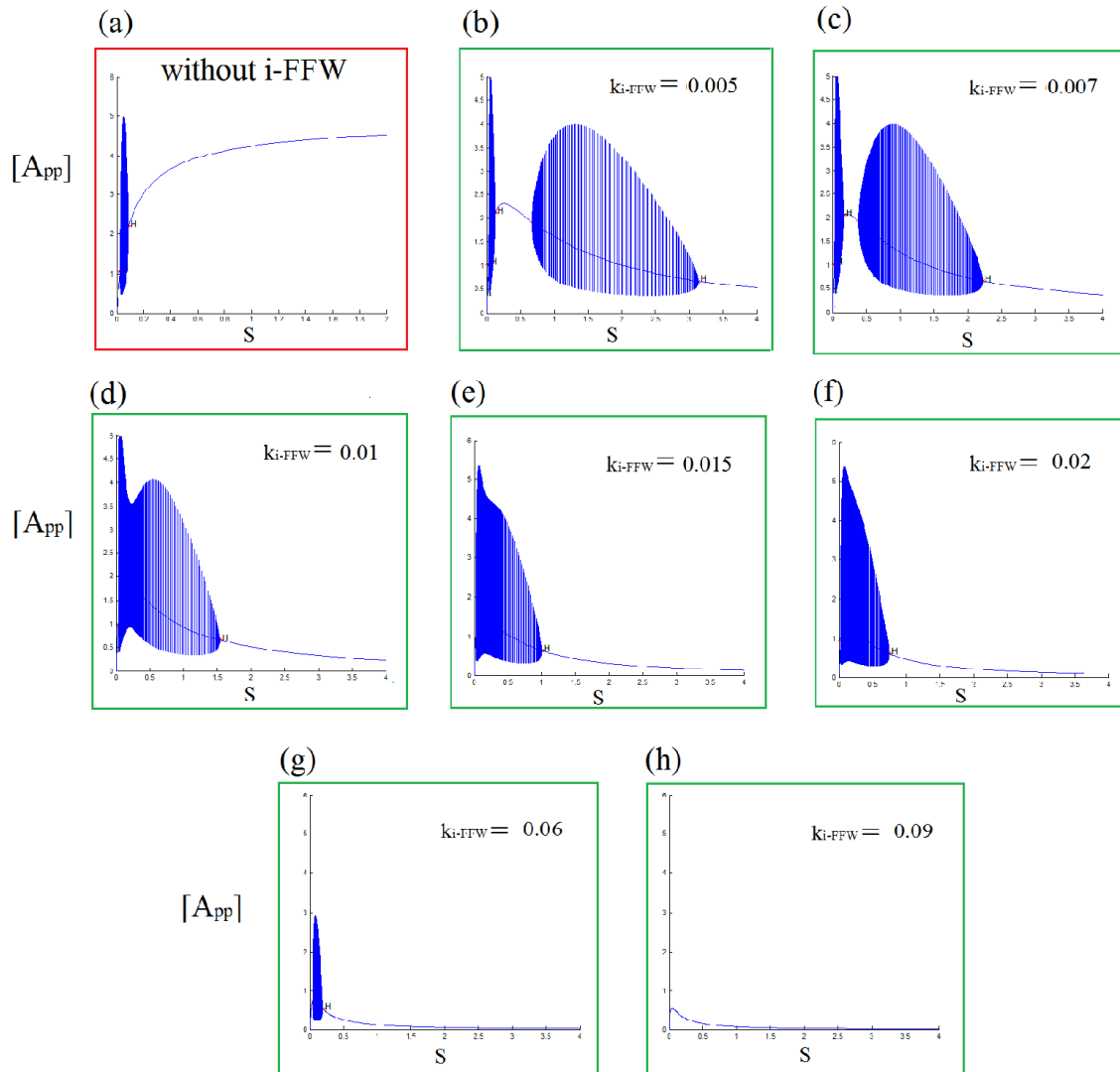


Figure 5.47 Effect of i-FFW on oscillatory behaviour in ordered double distributive phosphorylation model. Steady-state dose-response curves of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a) double phosphorylation model without i-FFW. (b-h) show how dose-response curves of A_{pp} varies with the input signal for different values of k_{i-FFW} .

5.3.4.4 Coherent feed-forward module (c-FFW)

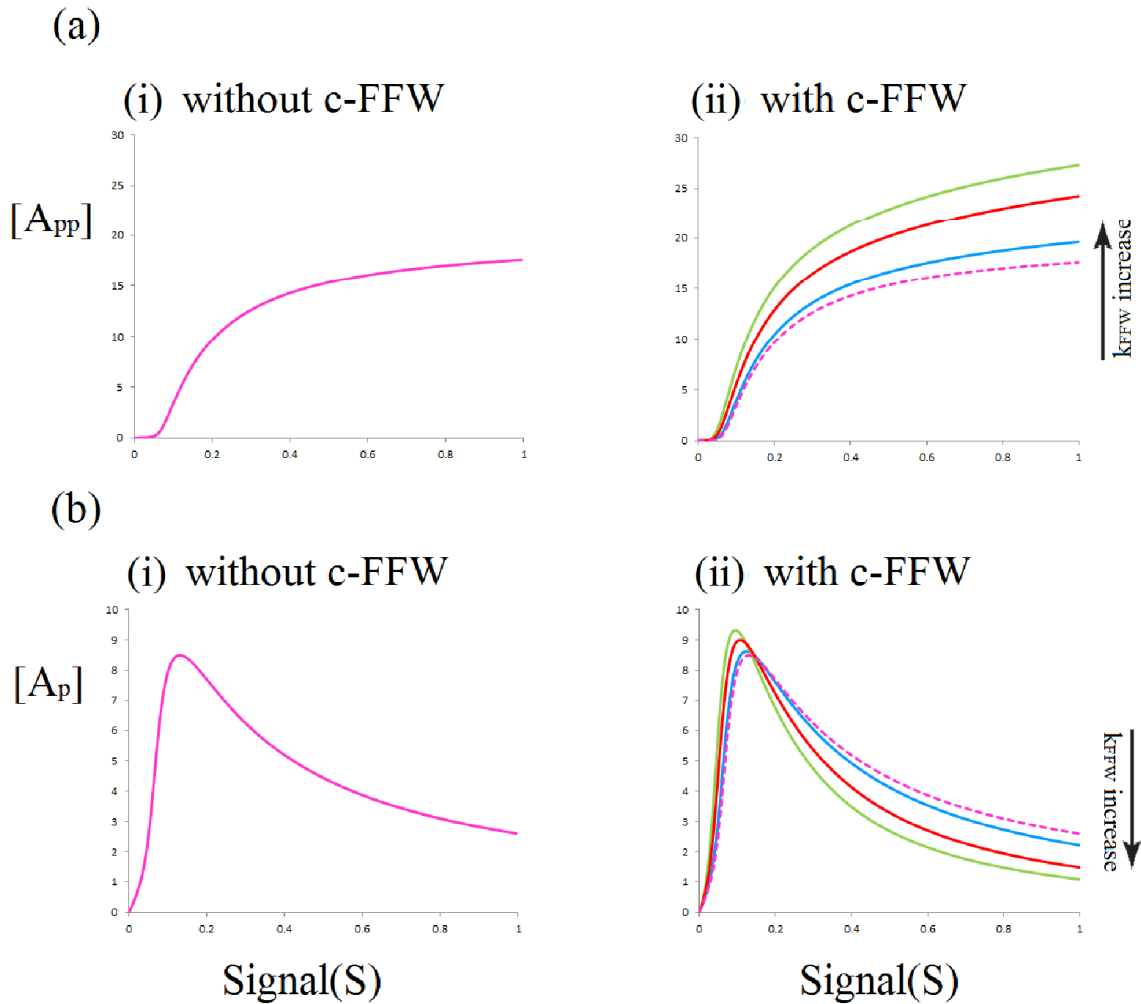


Figure 5.48 Effect of c-FFW on monotonic response in ordered double distributive phosphorylation. a(i) Steady-state input–output response curve of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S) in phosphorylation model without c-FFW. a(ii) shows how dose response curves of A_{pp} varies with the input signal for different values of k_{c-FFW} (k_{c-FFW} is a constant which represents the strength of the coherent feed-forward regulation). b(i-ii) show the corresponding curve for the partially phosphorylated substrate, A_p .

This section aims to bridge the gap between intrinsic behaviour in multisite phosphorylation and their functioning in coherent feed-forward module (c-FFW). This starts by examining the effect of c-FFW on the behaviour of multisite phosphorylation in a parameter regime, where the system without the c-FFW is monostable (as seen in figure 5.48a(i)). Figure 5.48a(ii) shows how steady state response of maximally phosphorylated substrate varies with the input signal for different values of the activation constant, $k_{c\text{-FFW}}$ ($k_{c\text{-FFW}}$ is the constant which represents the strength of the coherent feed-forward regulation). As seen in this figure, when 2- site phosphorylation is embedded in a c-FFW module, the system presents a monotonic response with different saturation levels (figure 5.48a(ii)). A gradual difference is observed when $k_{c\text{-FFW}}$ is varied. As $k_{c\text{-FFW}}$ increases, the systems present monotonic dose response with higher saturation level. This is completely due to the effect of c-FFW on the behaviour of multisite phosphorylation.

Figure 5.48b(i) shows that the single phosphorylated substrate, A_p , concentration exhibits a biphasic response: the initial increase at low signal levels reaching a maximum, before a decrease at higher input signal in the systems without c-FFW. Introducing c-FFW to multisite phosphorylation, the partial phosphoform exhibits biphasic response with the higher amplitude (5.48b(ii)).

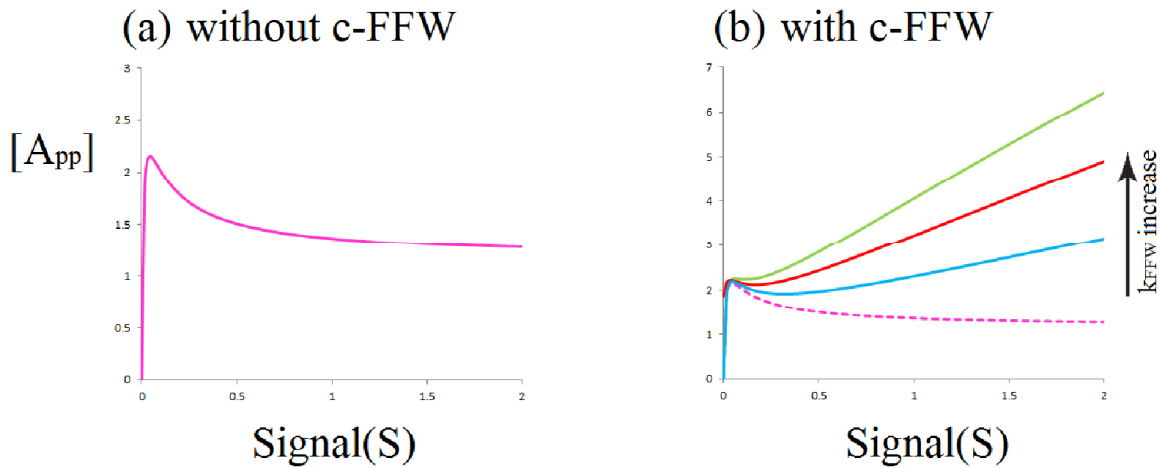


Figure 5.49 Effect of c-FFW on biphasic behaviour in ordered double distributive phosphorylation. Steady-state dose-response curves of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a) Double phosphorylation model without c-FFW. (b) Double phosphorylation model with c-FFW.

Figure 5.49a shows a dose-response curve of model without c-FFW in a different regime, indicating a biphasic response for the maximally phosphorylated substrate. Figure 5.49b shows the steady state response of maximally phosphorylated substrate as a function of input signal, S , for different values of k_{c-FFW} . It is found that increasing the value of k_{c-FFW} tends to diminish and abolish biphasic behaviour in multisite phosphorylation systems. In fact, this result presents the qualitative contribution of the coherent feed-forward loop, which leads to an increased maximal phosphoform for higher signal strengths.

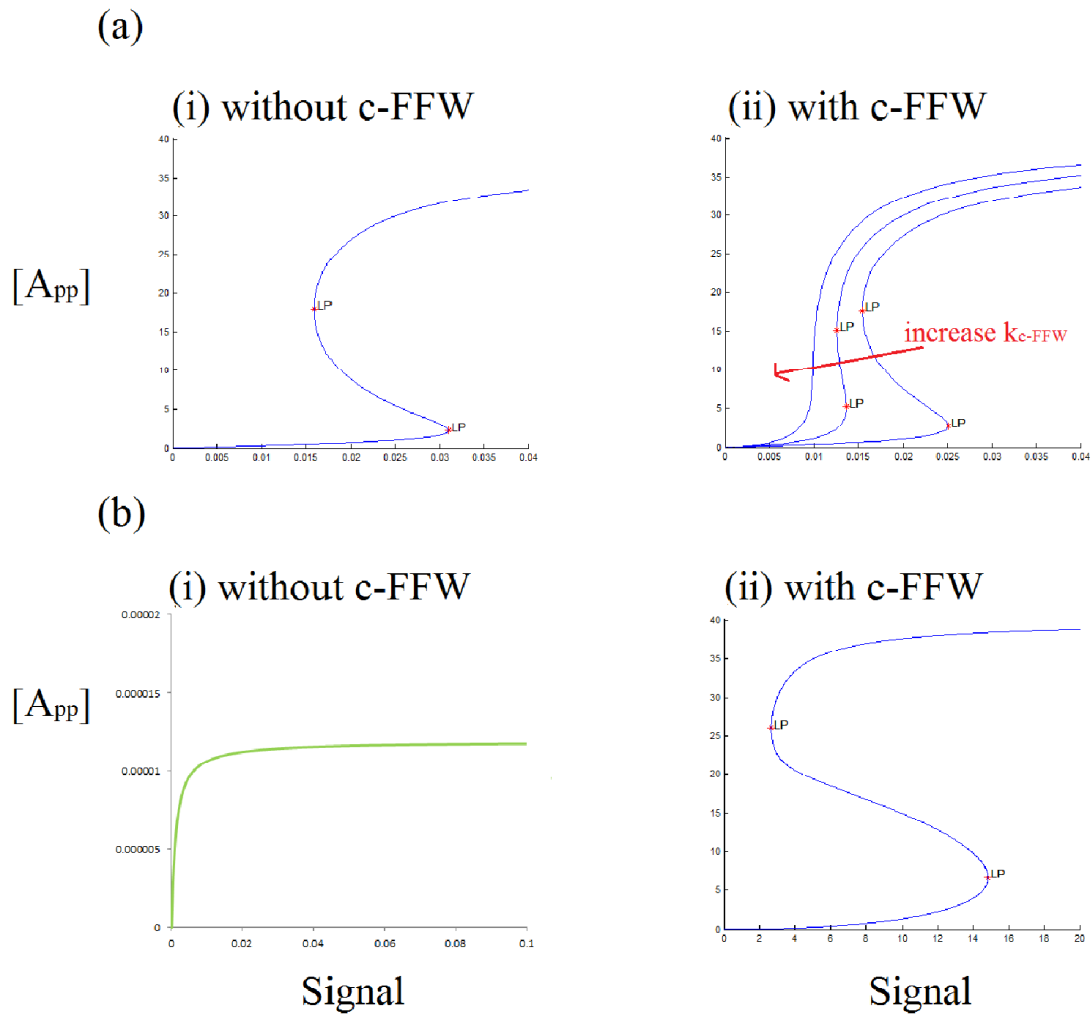


Figure 5.50 Effect of c-FFW on bistable behaviour in ordered double distributive phosphorylation. Steady-state dose-response curves of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a): (i) Model without c-FFW. (ii) Model with c-FFW. (b): (i) Model without c-FFW. (ii) Model with c-FFW.

The study now focuses on the effect of c-FFW in different parameter regimes, where the system without c-FFW is bistable, which is shown in figure 5.50a(i). Figure 5.50a(ii) shows the steady state response of fully phosphorylated substrate as a function of the input signal, S , for different values of $k_{c\text{-FFW}}$. As shown in this figure, it is found that increasing the value of $k_{c\text{-FFW}}$ tends to diminish and abolish bistability in multisite phosphorylation systems.

The above case shows that c-FFW can destroy bistability in multisite phosphorylation. By contrast, it is also found that, in certain conditions, c-FFW can give rises to the opposite effects. In some parameter regimes, in which the systems without c-FFW exhibit monotonic response, as shown in figure 5.50b(i), c-FFW can qualitatively alter such response to bistability to the systems (figure 5.50(ii)).

It is interesting to investigate how c-FFW affects the oscillatory behaviour due to explicit activation in ordered double phosphorylation. While oscillations are observed over a very broad range of input signal in the systems without c-FFW (figure 5.51a), striking differences are seen when c-FFW is introduced to the double distributive phosphorylation. Figure 5.51(b) shows that the input range for oscillatory behaviour changes significantly as $k_{c\text{-FFW}}$ is increased. Increasing $k_{c\text{-FFW}}$ tends to diminish both the input range for oscillations and the amplitude of the oscillations, and eventually abolish oscillatory behaviour.

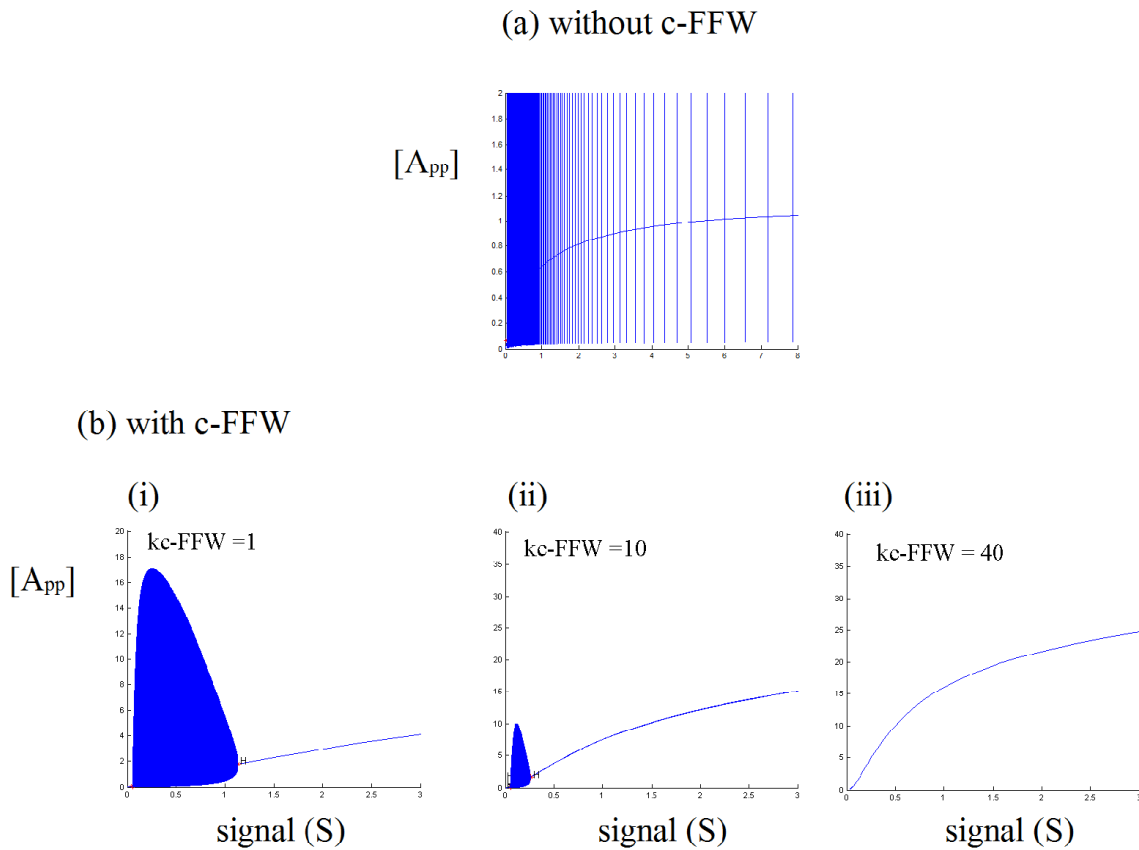


Figure 5.51 Effect of c-FFW on oscillatory behaviour in ordered double distributive phosphorylation. Steady-state dose-response curves of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S). (a) Double distributive phosphorylation model without c-FFW. (b) Double distributive phosphorylation model with c-FFW with different.

The c-FFW model is now examined in different parameter regimes, where the systems without c-FFW presents a monotonic dose response (figure 5.52a(i)). In contrast to the system without c-FFW, which reaches a steady state, it is found that in the model with c-FFW sustained oscillations can occur. A bifurcation diagram in figure 5.52a(ii) shows that, as the input is changed, the steady state does indeed lose stability to a Hopf bifurcation. This shows that c-FFW can completely introduce oscillation whereas isolated system may even

present monotonic dose response. Figure 5.52b(ii) shows that oscillatory behaviour in the system with c-FFW arises from the fact that phosphatase is far from active form while active phosphatase is much more than inactive form in systems without c-FFW (5.52b(i)).

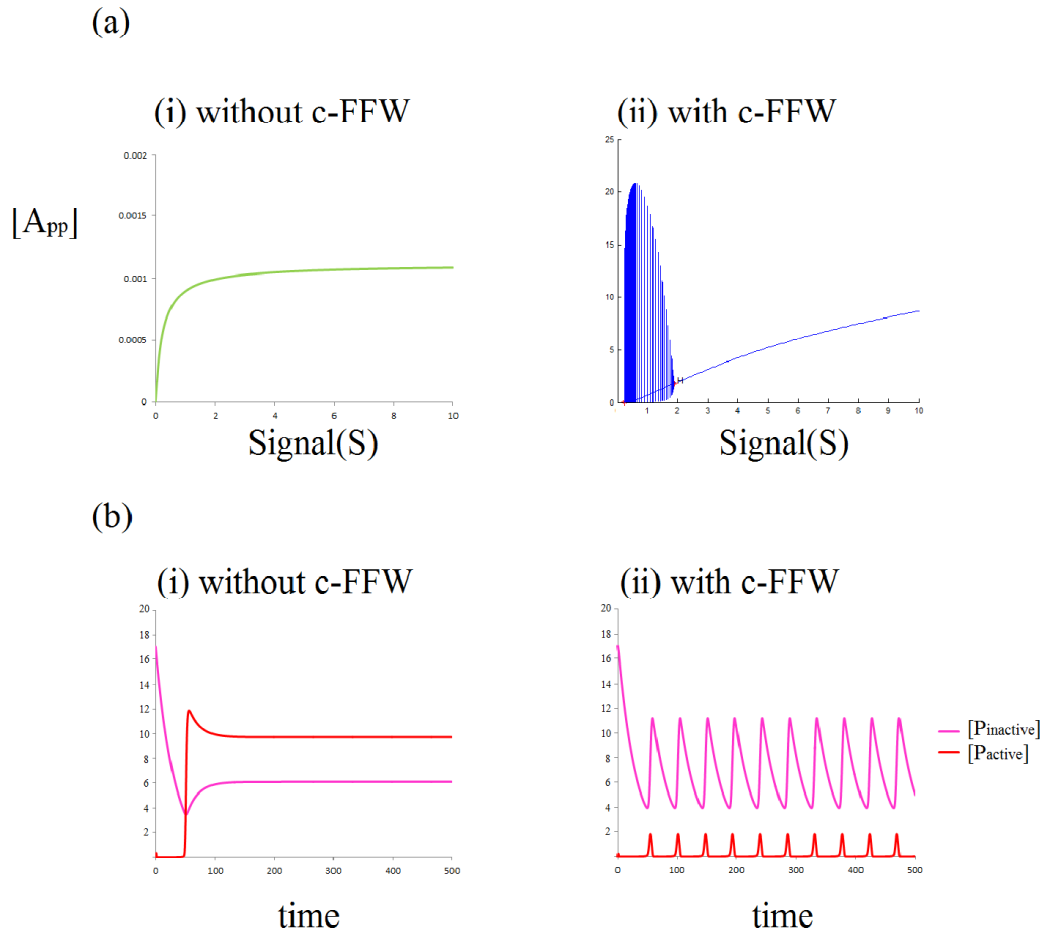


Figure 5.52 Oscillatory behaviour arising from c-FFW in ordered double distributive phosphorylation. (a) Steady-state dose-response curves of the concentration of maximally phosphorylated substrate, A_{pp} , as a function of input signal (S): (i) model without c-FFW and (ii) model with c-FFW. (b) shows that oscillatory behaviour in systems with c-FFW arises from the fact that phosphatase is far from active form (ii) while active phosphatase is much more than inactive form in common mechanisms without c-FFW (i).

Overall, the analysis in section 5.3.4 focuses on the effect of embedding a multi-site phosphorylation module in various network modules such as coherent (c-FFW) and incoherent feed-forward (i-FFW) module, negative (NFB) and positive feedback (PFB) module. Table 5.3 summarises the results showing how the internal dynamics in an ordered, double distributive phosphorylation can be qualitatively changed/reshaped by such various kinds of network modules in different parameter regimes. The qualitative changes in dynamic behaviour of single-site modification, as a result of embedding in such network modules, are also presented in this table. This shows not only what the effect of multisite phosphorylation is on each network, but also what the effect of each network is on multisite phosphorylation. These results are of relevance to both systems biology and synthetic biology.

Table 5.3 A summary of qualitative features observed when double-site distributive modification is embedded in various networks. The results, based on the models studied, for various scenarios of network modules with the multi-phosphorylation embedded in them, are discussed in the main text.

systems	Qualitative behaviour in isolated systems without explicit activation	Qualitative behaviour in isolated systems with explicit activation	Qualitative behaviour observed in networks			
			PFB	NFB	i-FFW	c-FFW
Double phosphorylation	monostable behaviour	monostable behaviour	monostable behaviour bistability	monostable behaviour	monostable behaviour bistability biphasic behaviour biphasic behaviour combined with bistability	monostable behaviour bistability oscillation
	biphasic behaviour	biphasic behaviour	biphasic behaviour biphasic response combined with bistability	biphasic behaviour	biphasic behaviour	monostable behaviour biphasic behaviour
	bistability	bistability	bistability Combination of bistable switching tristability	bistability oscillation	bistability	bistability monostable behaviour
		oscillation	oscillation oscillation + bistability bistability	oscillation monostable behaviour	oscillation Oscillation (two range) Oscillation + biphasic response Biphasic behaviour monostable behaviour	monostable behaviour oscillation
single phosphorylation	monostable behaviour	monostable behaviour	monostable behaviour bistability	monostable behaviour	monostable behaviour biphasic behaviour	monostable behaviour

5.4 Conclusion

Multi-site phosphorylation has been the focus of numerous studies: they are typically focused on the module in isolation. These various studies have isolated many important aspects of multi-site phosphorylation. However the key point is all of them study is module in isolation and there is a significant gap between studying the module in isolation and as parts of cellular pathway and in cellular contexts. There is a real need to bridge the gap between module in isolation and as parts of pathways. In this chapter, different aspects are studied to bridge the gap of the modification pathway in isolation and as parts of the networks.

The first part focuses on studying the effect of enzyme activation on the pathways. It was found that enzyme activation can introduce many quantitative effects on the behaviour of the pathway. Most importantly, it was found that enzyme activation by itself with (ordered) distributive double phosphorylation can introduce sustained-oscillation. This is something which has not been observed in many studies in isolated system. It was found that actually activation of just the kinase or phosphatase is sufficient for this. Furthermore it was found that it is possible to get complex oscillation from this very simple set up. Furthermore, the presence of enzyme activation could results in destroying bistability or inducing bistability of the intrinsic multisite phosphorylation systems.

In the second part, the effect of retroactivity which arises from the fact that the output of multisite phosphorylation can get sequestered in a downstream pathway was investigated. The focus is on the case of double site phosphorylation where the maximal phosphoform is sequestered, the intermediate phosphoform is sequestered or both of phosphoforms are sequestered in downstream covalent modification cycle. It was found that retroactivity can introduce many qualitative effects on the behaviour of the pathway, which includes a change in qualitative behaviour in the multisite phosphorylation module, including a loss of

bistability and introduction of tristability. Furthermore it was found that behaviour of the system depends on the phosphoform being sequestered. This is of clear relevance in cell signalling pathways as one or more phosphoforms may indeed be sequestered in downstream pathways as they are involved in signal transduction through those pathways

The third section considers the effect of the open system, i.e. production or degradation of substrate and all enzymes. In the case of production of substrate, this study considers the case of inflow of unmodified substrate and the outflow of the products (maximal phosphoform/intermediate phosphoform/both of them). It was found that the production and degradation of species was shown to be capable of completely altering the behaviour of the multisite phosphorylation module in multiple aspects: for example abolishing bistability, even abolishing the possibility of steady states and introducing adaptive behaviour and oscillation. Furthermore it was found that the location (in the module) of “inflow” and “outflow” had a significant effect in determining what kind of behaviour resulted, in conjunction with the kinetic parameters of the multisite modification module. In particular it allows for new combinations of information processing (e.g. bistability and exact adaptation).

In the last section, the effect of embedding a multisite module in a network module (coherent and incoherent feed-forward modules, negative and positive feedback modules) is investigated. It is able to assess both the effect of the ambient network on the multisite module, as well as the effect of the multisite module on the network. It was found that in the case of feedback, it is possible to introduce or destroy steady states, significantly alter the range of bistability. Furthermore, it was possible to introduce multistability in ordered double site phosphorylation. In the case of feed-forward modules, the combination of feed-forward regulation with the multisite module gave rise to a range of behaviour which involved a significant interplay between both modules. For example, incoherent feed-forward module

could induce biphasic behaviour while coherent feed-forward tended to diminish such kinds of behaviour. Furthermore, it was possible to alter the range of bistability and oscillatory behaviour in both modules.

Taken together, various studies reveal multiple facets of bridging the gap between the module in isolation and parts of the network. Upstream activation itself can introduce important effect in multisite phosphorylation. Retroactivity is important and the combination of retroactivity and multisite phosphorylation lead to very important qualitative effect. While many systems are considered to be closed, the production and degradation of substrates or enzymes can completely alter the behaviour of the systems. Thus this is relevant over the timescale of interest and is important to account for this. Finally numerous studies have focused on the intrinsic behaviour of multisite phosphorylation in isolation and feedback and feed-forward module have also been studied in the simple modification. In this chapter, various studies pinpoint how a network structure impact on multisite phosphorylation and how multisite phosphorylation affect the network. All of these provide the foundation for considering complex effect of all of these in specific contexts.

Chapter 6

Multi-site substrate modification through multiple kinases and phosphatases

6.1 Introduction

So far in this thesis, the investigations have looked at the range of scenarios of increasing complexity starting with ordered double-site modification proceeding to mixed mechanisms in ordered double-site modification, leading to understanding the behaviour of double-site modification systems embedded in networks and within cellular contexts. These various studies start to give a consolidated view of different aspects of multi-site phosphorylation involving a common kinase and phosphatase. It was how behaviour such as biphasic responses are seen in simple ordered mechanisms and how the nature of interaction of processive and distributive mechanism can introduce new behaviour while ruling out other kinds of behaviour and how different aspects of the modification mechanisms and the surrounding networks of cellular contexts can profoundly affect the behaviour.

In these various studies, many of the main results arise precisely because the kinase and phosphatase for two modification reactions are the same. When one steps back and looks at cellular signalling, it can be seen that there are many instances where different kinases perform modifications on the same substrate. In these cases, the phosphatase involved could be the same or different (typically the number of phosphatases is less than the number of kinases in the cells (Donella-Deana et al., 2003)). Consequently, if one wants to understand different aspects of the complexity of cell signalling networks and multi-site modification, it is necessary to study the effect of different enzymes performing different modifications on

the same substrate. Ultimately, the effect of different enzymes is a sequence of modification on the substrate. The sequence of modifications on the substrate is sometimes referred to as a molecular barcode (Patwardhan and Miller, 2007), by analogy with the electronic barcode. There is a real need to understand the effect of different enzymes on multisite modification mechanisms and how it impacts the sequence of substrate modifications in a systematic way.

As the outset, a number of important questions arise. Is it possible to get the kinds of qualitative behaviour which have been seen earlier (bistability, oscillation and biphasic dose response curves) when the enzymes are different? Does this result depend on whether the modification mechanism is ordered or random? How is this altered if kinase is different but phosphatase is the same? How is the dose response curve of each kinase pathway dependent on the other? Are there new kinds of behaviour different from what have been seen earlier possible?

This chapter focuses on selected aspects of multisite phosphorylation behaviour associated with different kinases, focusing on the qualitative behaviour of the system motivated by the question above. Here, a detailed parametric analysis of all aspects of the two pathways and their coupling (the coupling occurring through common substrates) is not performed. Rather this study tries to address these basic questions about the qualitative behaviour in a step by step manner proceeding from the simple to the complex. Thus this study starts with ordered 2-site mechanisms involving different kinases and considers both the case of different phosphatases and common phosphatase. This study then proceeds to case of random mechanism to assess what the role of mechanism is in this regard. Finally noting that multiple modifications can happen on the substrate, the study is then expanded to consider 3-site modification involving two different kinase pathways. The approaches will be to isolate the whole range of qualitative behaviour which is either possible or rule out in this system. Taken together this leads to the consolidated view of some of the information

processing capability and constraint associated with multiple kinase pathways acting on the same substrate.

6.2 Models

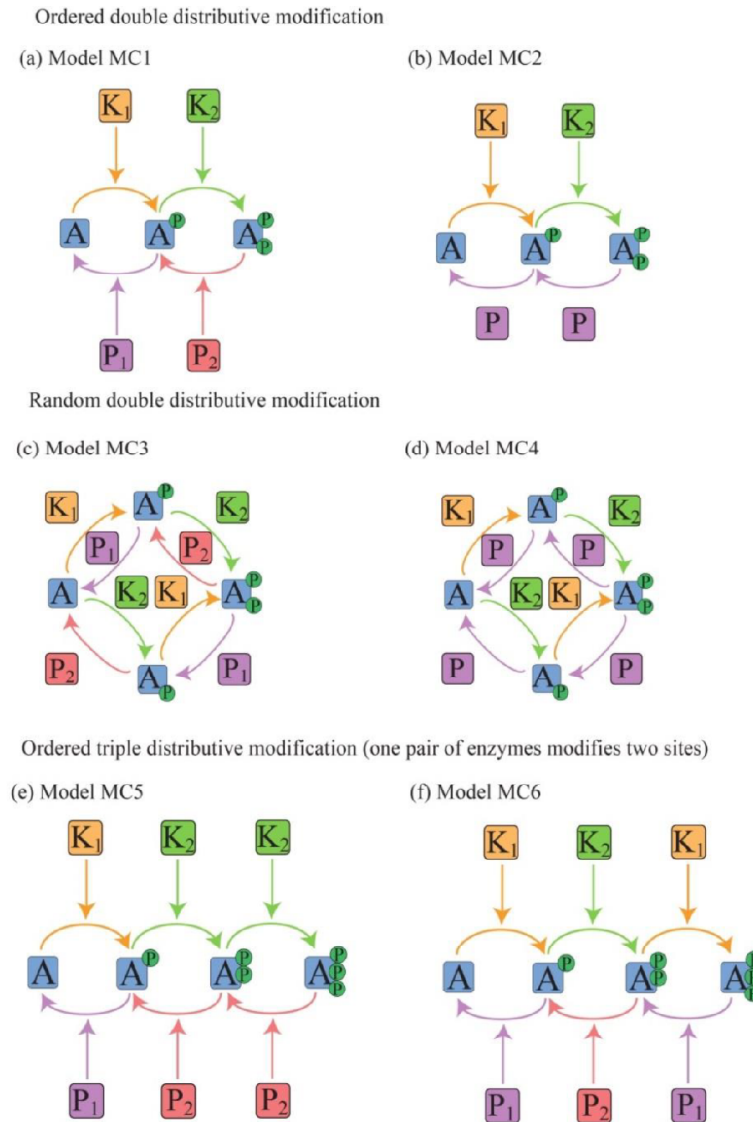


Figure 6.1 Schematic of multi-site phosphorylation models (associated with multiple enzymes). (a) and (b) represent 2-site modification models where different kinases modify substrates in an orderly distributive manner. (c) and (d) represent 2-site models where

different enzymes modify substrates in a random manner. (e) and (f) show 3-site models which involve multiple enzyme systems.

Here, the models employed in this study are presented and discussed. The goal of this work is to investigate the behaviour of another aspect of multisite modification mechanisms involving multiple enzymatic systems. The models which will be studied are divided into three sections. The first section presents ordered 2-site modification models while the second section focuses on random 2-site modification models. In the third section, 3-site modification model involving multiple enzymatic systems is presented. This study is based on ordinary differential equation (ODE)-based models of multi-site modification with multiple enzymatic systems. These models are based on a deterministic and lumped description of the multi-site modification process.

6.2.1 Ordered modification models

This section discusses the case of ordered double phosphorylation with multiple enzymatic systems: (a) all enzymes are different (model MC1) and (b) two kinases but one phosphatase. Note that, by symmetry, model MC2 can also represent a scenario where substrates are modified by one kinase but two phosphatases

Model MC1

Here, a model of a double-site distributive phosphorylation system with different kinases and different phosphatases for each phosphoform modification is presented (figure 6.1a). This model includes standard kinetic descriptions of enzymes reversibly binding to substrates to form complexes, and the catalytic conversion of the complex to the modified

substrate. In this model, there is an ordered distributive mechanism where enzyme K_1 and P_1 are involved in the first cycle while K_2 and P_2 are involved in the second cycle. All the elementary steps are described by mass-action kinetics. The model is thus a mathematical depiction of the scenario depicted in figure 6.1a with the assumption above. The equations for model MC1 (figure 6.2a) are:

$$\begin{aligned}
 \frac{d[A]}{dt} &= k_2[AK_1] - k_1[A][K_1] + k_{12}[A_pP_1] \\
 \frac{d[K_1]}{dt} &= (k_2 + k_3)[AK_1] - k_1[A][K_1] \\
 \frac{d[AK_1]}{dt} &= k_1[A][K_1] - (k_2 + k_3)[AK_1] \\
 \frac{d[A_p]}{dt} &= k_3[AK_1] - k_4[A_p][K_2] + k_5[A_pK_2] + k_9[A_{pp}P_2] - k_{10}[A_p][P_1] + k_{11}[A_pP_1] \\
 \frac{d[K_2]}{dt} &= (k_5 + k_6)[A_pK_2] - k_4[A_p][K_2] \\
 \frac{d[A_pK_2]}{dt} &= k_4[A_p][K_2] - (k_5 + k_6)[A_pK_2] \\
 \frac{d[A_{pp}]}{dt} &= k_6[A_pK_2] + k_8[A_{pp}P_2] - k_7[A_{pp}][P_2] \\
 \frac{d[P_2]}{dt} &= (k_8 + k_9)[A_{pp}P_2] - k_7[A_{pp}][P_2] \\
 \frac{d[A_{pp}P_2]}{dt} &= k_7[A_{pp}][P_2] - (k_8 + k_9)[A_{pp}P_2] \\
 \frac{d[P_1]}{dt} &= (k_{11} + k_{12})[A_pP_1] - k_{10}[A_p][P_1] \\
 \frac{d[A_pP_1]}{dt} &= k_{10}[A_p][P_1] - (k_{11} + k_{12})[A_pP_1]
 \end{aligned} \tag{6.1}$$

In this equation $[A]$, $[A_p]$, and $[A_{pp}]$ are respectively the concentrations of unphosphorylated, the singly phosphorylated and the doubly phosphorylated substrate, $[AK_1]$ and $[A_pK_2]$ describe the concentrations of relevant substrate–kinase complexes and $[A_pP_1]$ and $[A_{pp}P_2]$ describe the concentrations of the relevant substrate–phosphatase complexes.

Such a model may be regarded as one describing a mechanism where the order of dephosphorylation is opposite to the order of phosphorylation. The equations for the free enzymes (kinase/phosphatase) are also shown. In the above model, $[K_1]$, $[K_2]$, $[P_1]$ and $[P_2]$ refers to the active kinase and phosphatase concentrations as appropriate. In general, both kinases and phosphatases are subject to different control mechanisms and either of them may be activated (or made available) to trigger and control signal transduction. In this model, for the sake of simplicity, an explicit description of this activation is avoided and thus the enzymes in the model represent active modification-capable enzymes. It is worth emphasizing that the model is a fairly generic model for describing ordered distributive multi-site phosphorylation with multiple enzymatic systems.

In this model, the total concentration of enzymes ($K_{1,tot}$, $K_{2,tot}$, $P_{1,tot}$ and $P_{2,tot}$) is conserved, and so is the total concentration of the substrate (A_{tot}):

$$K_{1,tot} = [K_1] + [AK_1] \quad ,$$

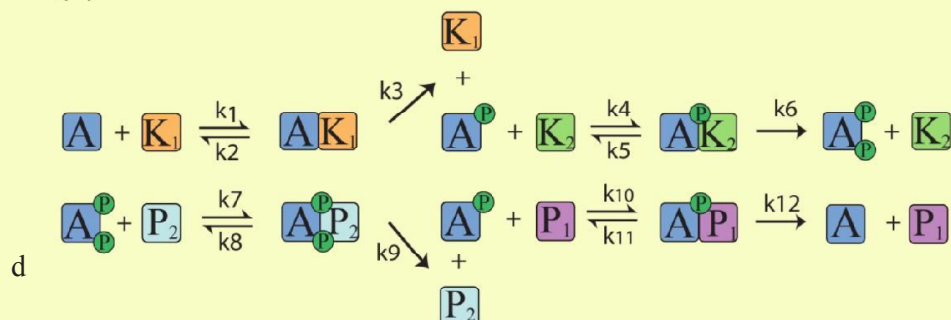
$$P_{1,tot} = [P_1] + [A_pP_1] \quad ,$$

$$K_{2,tot} = [K_2] + [A_pK_2] \quad ,$$

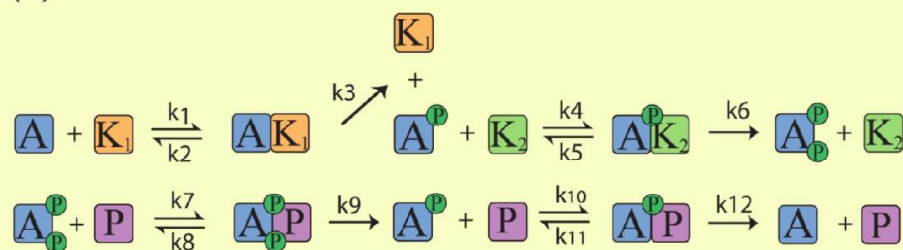
$$P_{2,tot} = [P_2] + [A_{pp}P_2] \quad ,$$

$$A_{tot} = [A] + [A_p] + [A_{pp}] + [AK_1] + [A_pK_2] + [A_{pp}P_2] + [A_pP_1] \quad (6.2)$$

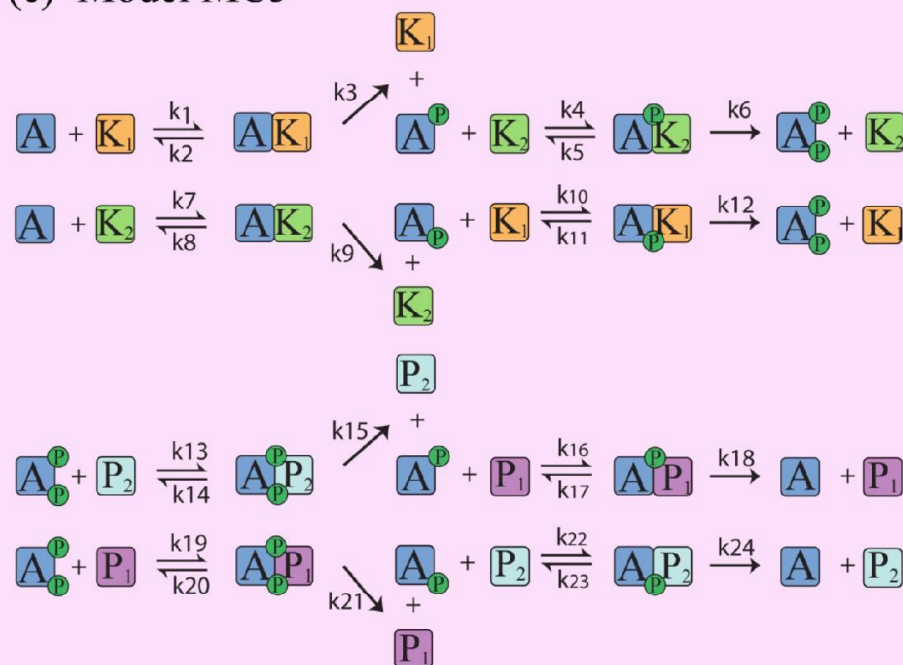
(a) Model MC1



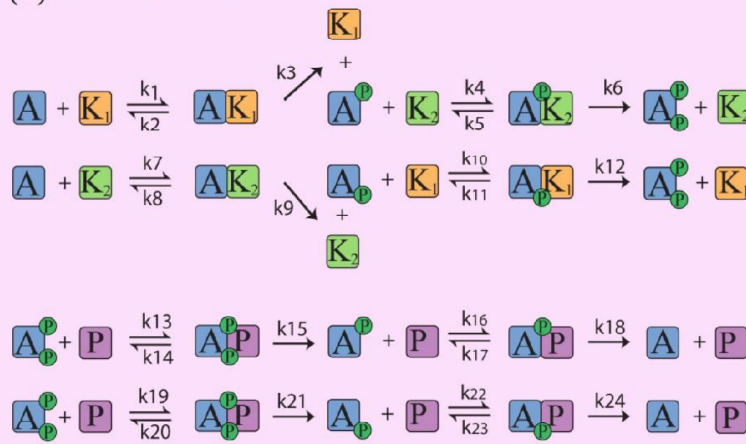
(b) Model MC2



(c) Model MC3



(d) Model MC4

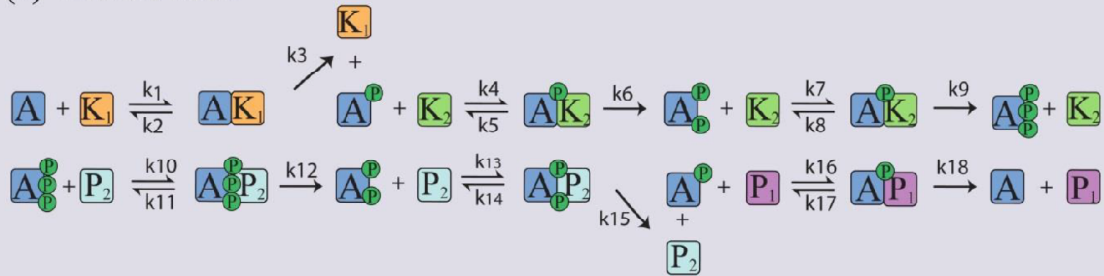


Substrate
 Kinase
 Phosphatase
 Phosphate group

Model MC3 and MC4

$A_{11} = A_{11}$
 $A_{01} = A_{01}$
 $A_{10} = A_{10}$

(e) Model MC5



(f) Model MC6

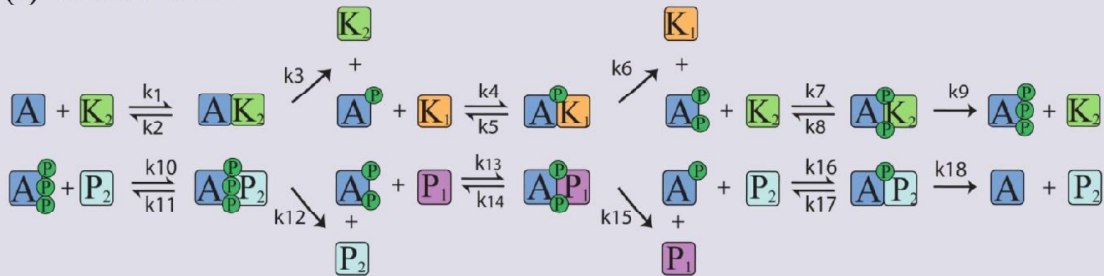


Figure 6.2 Pictorial depictions of phosphorylation models (with multiple enzymes). (a–b)

Double-site models where enzymes modify substrates in a specific order. (c–d) Double-site models where enzymes modify substrates in a random manner. (e–f) Triple-site models with different orders of single site modification and double site modification. The various k 's denote rate constants (binding, unbinding, catalytic) as appropriate.

Model MC2

Here, a model of an ordered two-site phosphorylation system with different kinases but same phosphatase for each phosphoform demodification is presented (figure 6.1b). In this model, there is an ordered distributive mechanism where different kinases, K_1 and K_2 , respectively, modify the first and second site while the same phosphatase acts to undo each of the conversions. The equations for model MC2 (figure 6.2b) are:

$$\begin{aligned}
 \frac{d[A]}{dt} &= k_2[AK_1] - k_1[A][K_1] + k_{12}[A_pP] \\
 \frac{d[K_1]}{dt} &= (k_2 + k_3)[AK_1] - k_1[A][K_1] \\
 \frac{d[AK_1]}{dt} &= k_1[A][K_1] - (k_2 + k_3)[AK_1] \\
 \frac{d[A_p]}{dt} &= k_3[AK_1] - k_4[A_p][K_2] + k_5[A_pK_2] + k_9[A_{pp}P_2] - k_{10}[A_p][P_1] + k_{11}[A_pP_1] \\
 \frac{d[K_2]}{dt} &= (k_5 + k_6)[A_pK_2] - k_4[A_p][K_2] \\
 \frac{d[A_{01}K_2]}{dt} &= k_4[A_p][K_2] - (k_5 + k_6)[A_pK_2] \\
 \frac{d[A_{pp}]}{dt} &= k_6[A_pK_2] + k_8[A_{pp}P] - k_7[A_{pp}][P] \\
 \frac{d[P]}{dt} &= (k_8 + k_9)[A_{pp}P] - k_7[A_{pp}][P] + (k_{11} + k_{12})[A_pP] - k_{10}[A_p][P] \\
 \frac{d[A_{pp}P]}{dt} &= k_7[A_{pp}][P] - (k_8 + k_9)[A_{pp}P] \\
 \frac{d[A_pP]}{dt} &= k_{10}[A_p][P] - (k_{11} + k_{12})[A_pP]
 \end{aligned} \tag{6.3}$$

In this model, the conservation condition for kinases (K_1 and K_2) follows the same equation as in model MC1. The conservation condition for phosphatase and substrate becomes:

$$P_{\text{tot}} = [P] + [A_{\text{pp}}P] + [A_pP] ,$$

$$A_{\text{tot}} = [A] + [A_p] + [A_{\text{pp}}] + [AK_1] + [A_pK_2] + [A_{\text{pp}}P] + [A_pP] \quad (6.4)$$

6.2.2 Random modification models

Model MC3

Here a model of a random two-site phosphorylation system with two kinases and two phosphatases, model MC3, is presented (figure 6.1c). In this model, there is a random double distributive mechanism where different kinases (K_1 and K_2) and different phosphatases (P_1 and P_2), modify substrates randomly. The equations for model MC3 (figure 6.2c) are:

$$\frac{d[A]}{dt} = k_2[AK_1] - k_1[A][K_1] + k_8[AK_2] - k_7[A][K_2] + k_{18}[A_{01}P_1] + k_{24}[A_{10}P_2]$$

$$\frac{d[K_1]}{dt} = (k_2 + k_3)[AK_1] - k_1[A][K_1] - k_{10}[A_{10}][K_1] + (k_{11} + k_{12})[A_{10}K_1]$$

$$\frac{d[K_2]}{dt} = (k_8 + k_9)[AK_2] - k_7[A][K_2] - k_4[A_{01}][K_2] + (k_5 + k_6)[A_{01}K_2]$$

$$\frac{d[AK_1]}{dt} = k_1[A][K_1] - (k_2 + k_3)[AK_1]$$

$$\frac{d[AK_2]}{dt} = k_7[A][K_2] - (k_8 + k_9)[AK_2]$$

$$\frac{d[A_{01}]}{dt} = k_3[AK_1] - k_4[A_{01}][K_2] + k_5[A_{01}K_2] + k_{15}[A_{11}P_2] - k_{16}[A_{01}][P_1] + k_{17}[A_{01}P_1]$$

$$\frac{d[A_{10}]}{dt} = k_9[AK_2] - k_{10}[A_{10}][K_1] + k_{11}[A_{10}K_1] + k_{21}[A_{11}P_1] - k_{22}[A_{10}][P_2] + k_{23}[A_{10}P_2]$$

$$\frac{d[A_{01}K_2]}{dt} = k_4[A_{01}][K_2] - (k_5 + k_6)[A_{01}K_2]$$

$$\frac{d[A_{10}K_1]}{dt} = k_{10}[A_{10}][K_1] - (k_{11} + k_{12})[A_{10}K_1]$$

$$\frac{d[A_{11}]}{dt} = k_6[A_{01}K_2] + k_{12}[A_{10}K_1] - k_{19}[A_{11}][P_1] + k_{20}[A_{11}P_1] - k_{13}[A_{11}][P_2] + k_{14}[A_{11}P_2]$$

$$\begin{aligned}
\frac{d[P_1]}{dt} &= (k_{20} + k_{21})[A_{11}P_1] - k_{19}[A_{11}][P_1] - k_{16}[A_{01}][P_1] + (k_{17} + k_{18})[A_{01}P_1] \\
\frac{d[P_2]}{dt} &= (k_{14} + k_{15})[A_{11}P_2] - k_{13}[A_{11}][P_2] - k_{22}[A_{10}][P_2] + (k_{23} + k_{24})[A_{10}P_2] \\
\frac{d[A_{11}P_1]}{dt} &= k_{19}[A_{11}][P_1] - (k_{20} + k_{21})[A_{11}P_1] \\
\frac{d[A_{11}P_2]}{dt} &= k_{13}[A_{11}][P_2] - (k_{14} + k_{15})[A_{11}P_2] \\
\frac{d[A_{01}P_1]}{dt} &= k_{16}[A_{01}][P_1] - (k_{17} + k_{18})[A_{01}P_1] \\
\frac{d[A_{10}P_2]}{dt} &= k_{22}[A_{10}][P_2] - (k_{23} + k_{24})[A_{10}P_2]
\end{aligned} \tag{6.5}$$

In this equation $[A]$, $[A_{01}]$ (or $[A_{10}]$) and $[A_{11}]$ are respectively the concentrations of unphosphorylated, the singly phosphorylated and the doubly phosphorylated substrate, $[AK_1]$, $[AK_2]$, $[A_{10}K_1]$ and $[A_{01}K_2]$ describe the concentrations of relevant substrate–kinase complexes and $[A_{01}P_1]$, $[A_{11}P_1]$, $[A_{11}P_2]$ and $[A_{10}P_2]$ describe the concentrations of the relevant substrate–phosphatase complexes.

In this model, the total concentration of enzymes ($K_{1,tot}$, $K_{2,tot}$, $P_{1,tot}$ and $P_{2,tot}$) is conserved, and so is the total concentration of the substrate (A_{tot}):

$$\begin{aligned}
K_{1,tot} &= [K_1] + [AK_1] + [A_{10}K_1] , \\
P_{1,tot} &= [P_1] + [A_{11}P_1] + [A_{01}P_1] , \\
K_{2,tot} &= [K_2] + [AK_2] + [A_{01}K_2] , \\
P_{2,tot} &= [P_2] + [A_{11}P_2] + [A_{10}P_2] , \\
A_{tot} &= [A] + [A_{01}] + [A_{10}] + [A_{11}] + [AK_1] + [AK_2] \\
&\quad + [A_{10}K_1] + [A_{01}K_2] + [A_{01}P_1] + [A_{11}P_1] + [A_{11}P_2] + [A_{10}P_2]
\end{aligned} \tag{6.6}$$

Model MC4

Here a model of a random two-site phosphorylation system with two separated kinases but one phosphatase, model MC4, is presented (figure 6.1d). In this model, there is a random double distributive mechanism where different kinases (K_1 and K_2) and common phosphatase (P), modify substrates *via* a random mechanism. The equations for model MC4 (figure 6.2d) are:

$$\frac{d[A]}{dt} = k_2[AK_1] - k_1[A][K_1] + k_8[AK_2] - k_7[A][K_2] + k_{18}[A_{01}P] + k_{24}[A_{10}P]$$

$$\frac{d[K_1]}{dt} = (k_2 + k_3)[AK_1] - k_1[A][K_1] - k_{10}[A_{10}][K_1] + (k_{11} + k_{12})[A_{10}K_1]$$

$$\frac{d[K_2]}{dt} = (k_8 + k_9)[AK_2] - k_7[A][K_2] - k_4[A_{01}][K_2] + (k_5 + k_6)[A_{01}K_2]$$

$$\frac{d[AK_1]}{dt} = k_1[A][K_1] - (k_2 + k_3)[AK_1]$$

$$\frac{d[AK_2]}{dt} = k_7[A][K_2] - (k_8 + k_9)[AK_2]$$

$$\frac{d[A_{01}]}{dt} = k_3[AK_1] - k_4[A_{01}][K_2] + k_5[A_{01}K_2] + k_{15}([A_{11}P])_1 - k_{16}[A_{01}][P] + k_{17}[A_{01}P]$$

$$\frac{d[A_{10}]}{dt} = k_9[AK_2] - k_{10}[A_{10}][K_1] + k_{11}[A_{10}K_1] + k_{21}([A_{11}P])_2 - k_{22}[A_{10}][P] + k_{23}[A_{10}P]$$

$$\frac{d[A_{01}K_2]}{dt} = k_4[A_{01}][K_2] - (k_5 + k_6)[A_{01}K_2]$$

$$\frac{d[A_{10}K_1]}{dt} = k_{10}[A_{10}][K_1] - (k_{11} + k_{12})[A_{10}K_1]$$

$$\frac{d[A_{11}]}{dt} = k_6[A_{01}K_2] + k_{12}[A_{10}K_1] - k_{19}[A_{11}][P] + k_{20}([A_{11}P])_2 - k_{13}[A_{11}][P] + k_{14}[A_{11}P]$$

$$\begin{aligned} \frac{d[P]}{dt} = & (k_{14} + k_{15})([A_{11}P])_1 - k_{13}[A_{11}][P] - k_{22}[A_{10}][P] + (k_{23} + k_{24})[A_{10}P] \\ & + (k_{20} + k_{21})([A_{11}P])_2 - k_{19}[A_{11}][P] - k_{16}[A_{01}][P] + (k_{17} + k_{18})[A_{01}P] \end{aligned}$$

$$\frac{d([A_{11}P])_2}{dt} = k_{19}[A_{11}][P] - (k_{20} + k_{21})([A_{11}P])_2$$

$$\begin{aligned}
\frac{d([A_{11}P])_1}{dt} &= k_{13}[A_{11}][P] - (k_{14} + k_{15})([A_{11}P])_1 \\
\frac{d[A_{01}P]}{dt} &= k_{16}[A_{01}][P] - (k_{17} + k_{18})[A_{01}P] \\
\frac{d[A_{10}P]}{dt} &= k_{22}[A_{10}][P] - (k_{23} + k_{24})[A_{10}P]
\end{aligned} \tag{6.7}$$

In this model, the conservation condition for each kinase (K_1 and K_2) follows the same equation as in model MC3. The conservation condition for phosphatase and substrate becomes:

$$\begin{aligned}
P_{\text{tot}} &= [P] + ([A_{11}P])_1 + ([A_{11}P])_2 + [A_{10}P] + [A_{01}P] \quad , \\
A_{\text{tot}} &= [A] + [A_{01}] + [A_{10}] + [A_{11}] + [AK_1] + [AK_2] \\
&\quad + [A_{10}K_1] + [A_{01}K_2] + [A_{01}P] + ([A_{11}P])_1 + ([A_{11}P])_2 + [A_{10}P]
\end{aligned} \tag{6.8}$$

6.2.3 Ordered 3-site phosphorylation involving multiple enzyme systems

This section presents models of ordered, 3-site phosphorylation systems in which one kinase/phosphatase pair modifies two sites while another modifies one site. This means that the 3-site models arise from coupling of single site modification and 2-site modification cycle. This study considers two cases: (i) single modification occurs at the first cycle (Model MC5) and (ii) single modification occurs at the middle cycle of 3-site phosphorylation (Model MC6).

Model MC5

This model (MC5) describes an ordered 3-site phosphorylation system in which the first cycle is modified by a single kinase/phosphatase pair (K_1/P_1) while the middle cycle as well as the last cycle is modified by another kinase/phosphatase pair (K_2/P_2), as seen in figure 6.1e. The equations for model MC5 (figure 6.2e) are:

$$\begin{aligned}
 \frac{d[A]}{dt} &= k_2[AK_1] - k_1[A][K_1] + k_{18}[A_pP_1] \\
 \frac{d[AK_1]}{dt} &= k_1[A][K_1] - (k_2 + k_3)[AK_1] \\
 \frac{d[A_p]}{dt} &= k_3[AK_1] - k_4[A_p][K_2] + k_5[A_pK_2] + k_{15}[A_{pp}P_2] + k_{17}[A_pP_1] - k_{16}[A_p][P_1] \\
 \frac{d[A_pK_2]}{dt} &= k_4[A_p][K_2] - (k_5 + k_6)[A_pK_2] \\
 \frac{d[A_{pp}]}{dt} &= k_6[A_pK_2] - k_7[A_{pp}][K_2] + k_8[A_{pp}K_2] + k_{12}[A_{pp}P_2] + k_{14}[A_{pp}P_2] \\
 &\quad - k_{13}[A_{pp}][P_2] \\
 \frac{d[A_{pp}K_2]}{dt} &= k_7[A_{pp}][K_2] - (k_8 + k_9)[A_{pp}K_2] \\
 \frac{d[A_{ppp}]}{dt} &= k_9[A_{pp}K_2] - k_{10}[A_{ppp}][P_2] + k_{11}[A_{ppp}P_2] \\
 \frac{d[A_{ppp}P_2]}{dt} &= k_{10}[A_{ppp}][P_2] - (k_{11} + k_{12})[A_{ppp}P_2] \\
 \frac{d[A_{pp}P_2]}{dt} &= k_{13}[A_{pp}][P_2] - (k_{14} + k_{15})[A_{pp}P_2] \\
 \frac{d[A_pP_1]}{dt} &= k_{16}[A_p][P_1] - (k_{17} + k_{18})[A_pP_1]
 \end{aligned} \tag{6.9}$$

This model is supplemented by conservation of the total concentration of kinase ($K_{1,tot}$, $K_{2,tot}$), phosphatase ($P_{1,tot}$, $P_{2,tot}$) and substrate (A_{tot}).

Model MC6

Model MC6 represents an ordered 3-site phosphorylation system in which the middle cycle is modified by a single kinase/phosphatase pair (K_1/P_1) while the first cycle as well as the last cycle is modified by another kinase/phosphatase pair (K_2/P_2) as seen in figure 6.1f.

The equations for model MC6 (figure 6.2f) are:

$$\begin{aligned}
 \frac{d[A]}{dt} &= k_2[AK_2] - k_1[A][K_2] + k_{18}[A_pP_2] \\
 \frac{d[AK_2]}{dt} &= k_1[A][K_2] - (k_2 + k_3)[AK_2] \\
 \frac{d[A_p]}{dt} &= k_3[AK_2] - k_4[A_p][K_1] + k_5[A_pK_1] + k_{15}[A_{pp}P_1] + k_{17}[A_pP_2] - k_{16}[A_p][P_2] \\
 \frac{d[A_pK_1]}{dt} &= k_4[A_p][K_1] - (k_5 + k_6)[A_pK_1] \\
 \frac{d[A_{pp}]}{dt} &= k_6[A_pK_1] - k_7[A_{pp}][K_2] + k_8[A_{pp}K_2] + k_{12}[A_{ppp}P_2] + k_{14}[A_{pp}P_1] \\
 &\quad - k_{13}[A_{pp}][P_1] \\
 \frac{d[A_{pp}K_2]}{dt} &= k_7[A_{pp}][K_2] - (k_8 + k_9)[A_{pp}K_2] \\
 \frac{d[A_{ppp}]}{dt} &= k_9[A_{pp}K_2] - k_{10}[A_{ppp}][P_2] + k_{11}[A_{ppp}P_2] \\
 \frac{d[A_{ppp}P_2]}{dt} &= k_{10}[A_{ppp}][P_2] - (k_{11} + k_{12})[A_{ppp}P_2] \\
 \frac{d[A_{pp}P_1]}{dt} &= k_{13}[A_{pp}][P_1] - (k_{14} + k_{15})[A_{pp}P_1] \\
 \frac{d[A_pP_2]}{dt} &= k_{16}[A_p][P_2] - (k_{17} + k_{18})[A_pP_2]
 \end{aligned} \tag{6.10}$$

This model is supplemented by conservation of the total concentration of kinase ($K_{1,tot}$, $K_{2,tot}$), phosphatase ($P_{1,tot}$, $P_{2,tot}$) and substrate (A_{tot}).

6.3 Results

In this section, the computational analysis of the model of multi-site modification involving multiple enzymatic systems is discussed. Results are organised as follows. First, the focus is on the ordered double-site distributive mechanisms involving different kinases, considering both the case of different phosphatases and that of common phosphatase (model MC1 and MC2). This study then proceeds to the case of double-site distributive model with, random mechanisms, to assess what the role of mechanism is in this regard (model MC3 and MC4). Finally noting that multiple modifications can happen on the substrate this study is then expanded to consider 3-site distributive modification (model MC5 and MC6) involving two different kinase pathways (2 sites are modified by a single kinase/phosphatase pair and one site is modified by another pair of enzymes). Here, the focus is on studying the steady-state response of these models as a function of the total kinase concentration (the dose response curve). Since, in the models below, there is more than one kinase, one of the kinase concentrations is kept fixed at a basal value and the other is varied. The study is then focus on the effect of the basal value of the kinase whose concentration is fixed. In certain cases, the behaviour of these models (by multiple enzymes) to ‘related’ distributive mechanisms (by same kinase and phosphatase) with ‘corresponding’ parameters, is contrasted as appropriate. This allows us to clearly demonstrate the difference between the case of single kinase/phosphatase pair (as studied in earlier chapters), and that of multiple kinases and/or phosphatases.

Method of analysis: The methodology is described at the outset. This is relevant for all the investigations below. The primary goal in this chapter is to address the question: what are the qualitative characteristics (either capabilities or constraints in information processing) in the

different variants of the multi-site phosphorylation models (by multiple enzymes)? The analysis undertaken in this chapter is oriented towards this question.

In this regard and building from the previous chapters, this study particularly focuses on qualitative characteristics such as biphasic dose response curves, bistability and oscillations. The reason for doing this is that these characteristics simultaneously represent both basic as well as qualitatively different modes of information processing. Consequently these responses are easy to discern experimentally. In addition these characteristics have potentially important roles to play. For instance the possibility of bistability gives rise to a switch-like behaviour with irreversibility/hysteresis. This is believed to underlie binary decision making, with irreversibility, such as occurs in cell fate determination, transitions in cell types, or even transitions in cell cycle models as seen in *Xenopus Oocytes* (Ferrell and Machleder, 1998; Laurent and Kellershohn, 1999). In the case of oscillations, this represents a distinct dynamic mode of information in response to static conditions. This has been observed in a range of cellular contexts such as circadian clocks driven by cell-autonomous transcriptional-translational oscillators (Dibner et al., 2010; Reppert and Weaver, 2002; Young and Kay, 2001). Oscillatory circuits based on phosphorylation reactions have also been realized experimentally (Nakajima et al., 2005). Finally a biphasic dose response curve implies a degree of response reduction beyond a certain level of input. This could be useful in tuning the range of input over which a substantial response may be observed. Biphasic dose response curves have been studied in a range of cellular contexts, including in the context of Hormesis (Calabrese and Baldwin, 2003; Calabrese, 2013; Cook and Calabrese, 2006).

The goal is to check if such behaviour can be obtained in the different models of multi-site phosphorylation by multiple enzymes. If a particular behaviour is seen in simulations, it is reported computationally, and bifurcation analysis is performed to consolidate the findings. The computational analysis undertaken has indeed been

underpinned by detailed parametric searches and analysis, but this study reports results which demonstrate the behaviour in sample parameter regimes (noting that such behaviour can be obtained even when other parameters are altered). If a certain kind of behaviour is not observed, this can be explained analytically, and the author references papers in preparation where this is demonstrated. In some cases, additional complex behaviour observed in these systems is reported through computational studies.

In this manner, it is able to map out what kinds of qualitative behaviour and characteristics are associated with each variant of a multi-phosphorylation (by multiple enzymes) model. Taken together, this also demonstrates the necessary (and in fact the minimal) ingredients for a particular behaviour to be observed. In a similar way, in some cases below, computational results demonstrate how different pathways sharing a common substrate (which is a characteristic of all models below) can result in new effects not seen in either of the pathways.

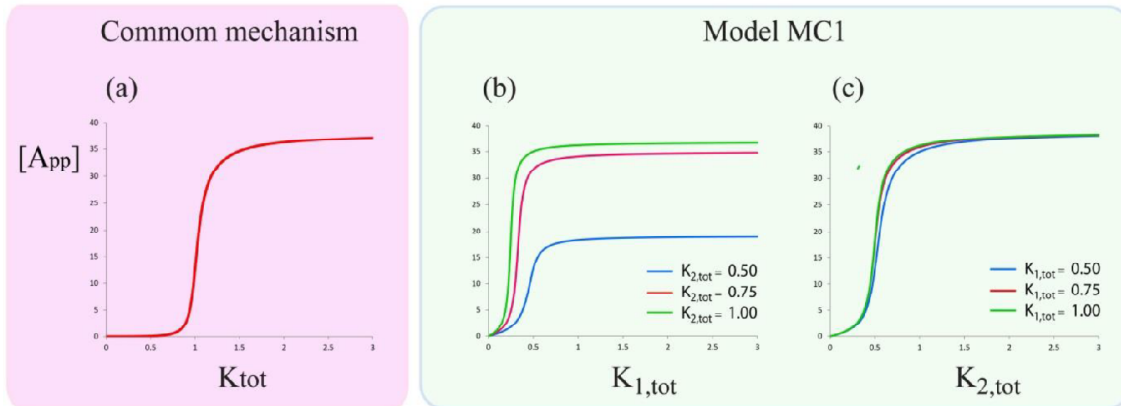
As mentioned earlier, the multi-site phosphorylation modules have a number of kinetic parameters which affect the quantitative aspects of the response. While this is the case, there are still a small number of basic qualitative responses, and this is the main focus here. In all these cases, similar or equivalent trends can be observed when different parameter sets representing the same qualitative behaviour are chosen.

Taken together this study provides a broad view of the qualitative behaviour and information processing capabilities and constraints associated with multiple kinases and/or phosphatases in multisite modification and the difference between ordered and random mechanisms. This provides a platform for subsequent detailed computational parametric investigations.

6.3.1 Ordered double-site modification schemes

Herein, ordered 2-site distributive phosphorylation is mainly used to study the behaviour of the multi-site phosphorylation through multiple enzymes. This section first considers model MC1, where ordered phosphorylation/dephosphorylation is modified by different kinases/phosphatases at each site. Figure 6.3 shows the variation of the partially and fully modified substrate as a function of total concentration of kinase (for model MC1, the dose response curve as a function of either kinase is presented). Model MC1 is first examined in a parameter regime, where the ‘analogous’ common distributive mechanism is monostable (Analogous distributive mechanism means that the distributive mechanism with the same sets of binding/unbinding and catalytic constants). It is found that while the fully modified substrate, A_{pp} , exhibits a sigmoidal dependence of the total kinase concentration, with the clear presence of a threshold in common double distributive mechanisms (figure 6.3a), the responses become monotonic with loss of threshold in model MC1. In model MC1, the first site is modified by K_1 while the second site is modified by K_2 . As seen in figure 6.3b, the A_{pp} dose response to $K_{tot,1}$ becomes less steep as $K_{2,tot}$ increases. The same trend was also observed in the A_{pp} dose response (as a function of $K_{tot,2}$), as shown in figure 6.3c.

Completely phosphorylated substrate



Singly phosphorylated substrate

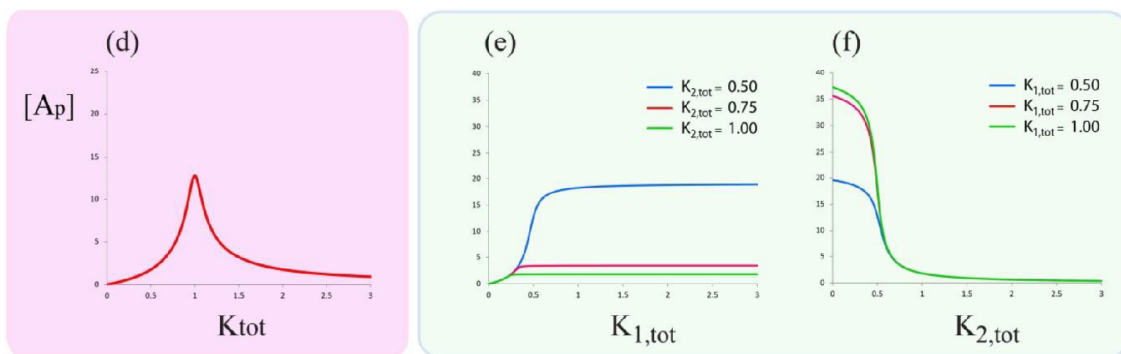


Figure 6.3 Steady-state dose-response curves in ordered double-site phosphorylation models: common distributive model and model MC1. (a–c) show the steady-state input–output response curves of the concentration of fully phosphorylated substrate, A_{pp} , as a function of the total concentration of kinase (K_{tot} or $K_{1,tot}$ or $K_{2,tot}$ as appropriate) while (d–f) show the corresponding curve for the partially phosphorylated substrate, A_p . (Completely phosphorylated substrate: (a) common mechanism, (b–c) model MC1. Singly phosphorylated substrate: (d) common mechanism, (e–f) model MC1).

Certain differences are also seen in the partial phospho-form, A_p , steady-state dose response. In model MC1 (Figure 6.3f), it is found that the partial phospho-form presents a decreasing profile asymptoting to zero for large values of $K_{2,tot}$. This is what is commonly expected in a distributive phosphorylation, with all available substrate (including partial phospho-form) getting converted to the maximally phosphorylated form. By contrast, A_p exhibits a sigmoidal response to $K_{1,tot}$, as shown in figure 6.3e, which is different from common distributive mechanism which exhibit biphasic dose response (figure 6.3d). Analytical work in (Suwanmajo & Krishnan, 2018c) shows that A_p cannot exhibit biphasic response to the $K_{tot,1}$.

The focus is now on examining the model MC1, where the common distributive analogues are bistable. A choice of sufficiently different catalytic constants in phosphorylation can result in bistability in a common distributive phosphorylation model, as shown in figure 6.4a. By contrast, the model MC1 exhibits only a monostable response. Figure 6.4b shows how the steady state response of fully phosphorylated substrate (A_{pp}) varies with the total concentration of the kinase modifying the first site, $K_{1,tot}$, for different values of that of the kinase modifying the second site, $K_{2,tot}$. As shown in this figure, the system exhibits a monotonic response with the same saturation level but the response becomes less steep as $K_{2,tot}$ decreases. Figure 6.4c shows how A_{pp} varies with $K_{2,tot}$ for different values of $K_{1,tot}$. The system exhibits a monotonic response to $K_{2,tot}$ similar to the case in figure 6.4b. Bistability is ruled out in model MC1, which is analytically demonstrated in (Suwanmajo & Krishnan, 2018c). A different approach, based on algebraic methods, to analysing this two-site case (to prove that bistability is ruled out) was presented in (Feliu & Wiuf, 2012).

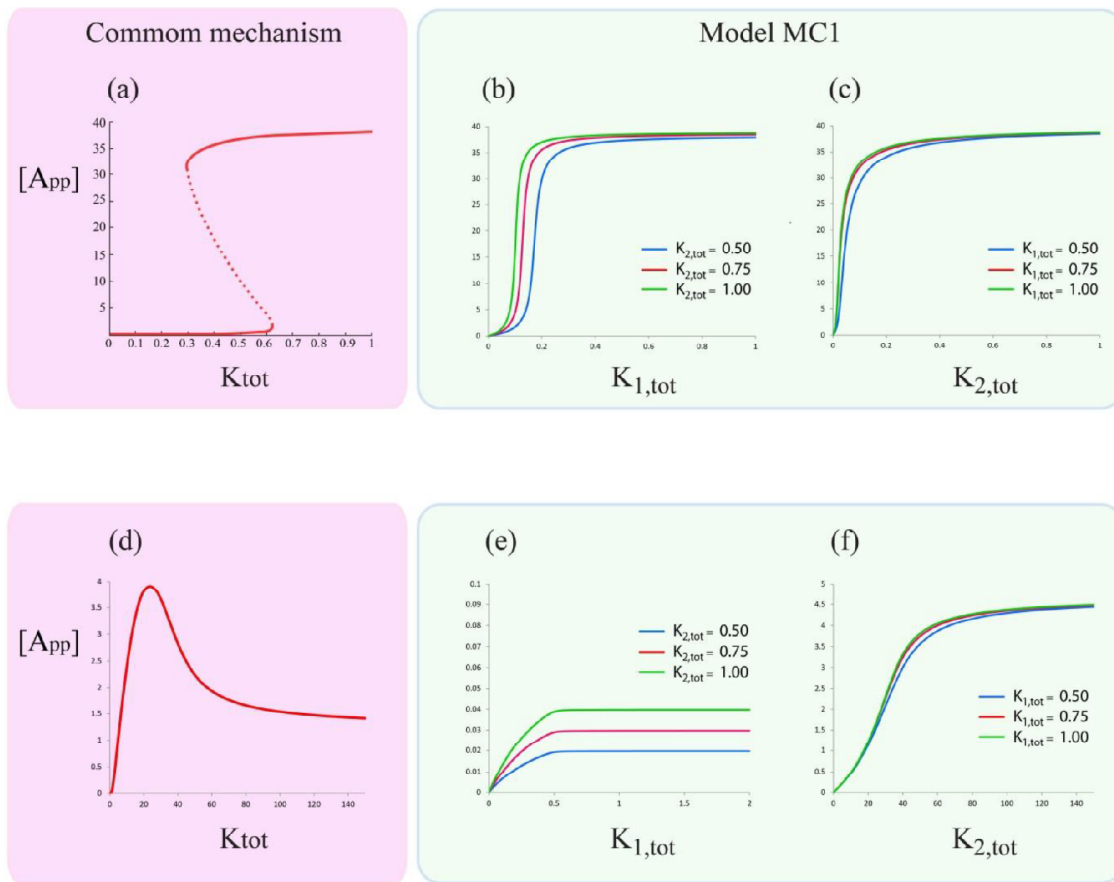


Figure 6.4 Steady-state dose-response curves in various ordered double phosphorylation models with different catalytic constants of phosphorylation reaction. (a–c) show the steady-state input–output response curves of the concentration of fully phosphorylated substrate, A_{pp} , as a function of the total concentration of kinase (K_{tot} or $K_{1,tot}$ or $K_{2,tot}$ as appropriate) while (d–f) show the corresponding curve for the partially phosphorylated substrate, A_p . (Completely phosphorylated substrate: (a) common mechanism, (b–c) model MC1. Singly phosphorylated substrate: (d) common mechanism, (e–f) model MC1)

Figure 6.4d shows a dose–response curve of a common distributive model in a different regime, indicating a biphasic response for the completely phosphorylated substrate. The origins of such biphasic responses were discussed in the chapter 3. It is found that the ‘corresponding’ mechanisms associated with different kinases and phosphatases (model MC1) do not exhibit such biphasic response (for the fully phosphorylated substrate) to $K_{1,tot}$ (figure 6.4e) and $K_{tot,2}$ (figure 6.4f). Analytical work in (Suwanmajo & Krishnan, 2018c) shows that biphasic responses for the maximally phosphorylated substrate (as a function of $K_{2,tot,1}$ or $K_{1,tot}$) in this model can be ruled out.

Following investigation of the case where de-phosphorylation is performed by different phosphatases, the next step is to investigate (ordered) 2-site mechanism which is modified by different kinases and same phosphatase (Model MC2). The case where the ‘analogous’ common distributive mechanism is monostable (see figure 6.3a) is first considered. Figure 6.5a and 6.5b, respectively shows how steady state response of fully and partially phosphorylated substrate typically varies with the total concentration of the kinase which modifies the first site, $K_{1,tot}$. As shown in figure 6.5a, when $K_{2,tot}$ is equal to zero, $[A_{pp}]$ becomes zero (the second site is not modified). When $K_{2,tot}$ is equal to 0.5, A_{pp} exhibits a sigmoidal response and such response becomes steeper compared to that of model MC1 (figure 6.3b). Similarly, A_p also exhibits a sigmoidal response when $K_{2,tot} = 0.5$ and it will reach the saturation at higher level when there is no K_2 because all available substrate is getting converted to the partially phosphorylated form. By contrast, in the common distributive mechanism the partial phospho-forms generally exhibit biphasic dose response (as seen earlier) and such behaviour can be ruled out in the partial phospho-form response to $K_{1,tot}$ in model MC2, which is demonstrated in (Suwanmajo and Krishnan, 2018c).

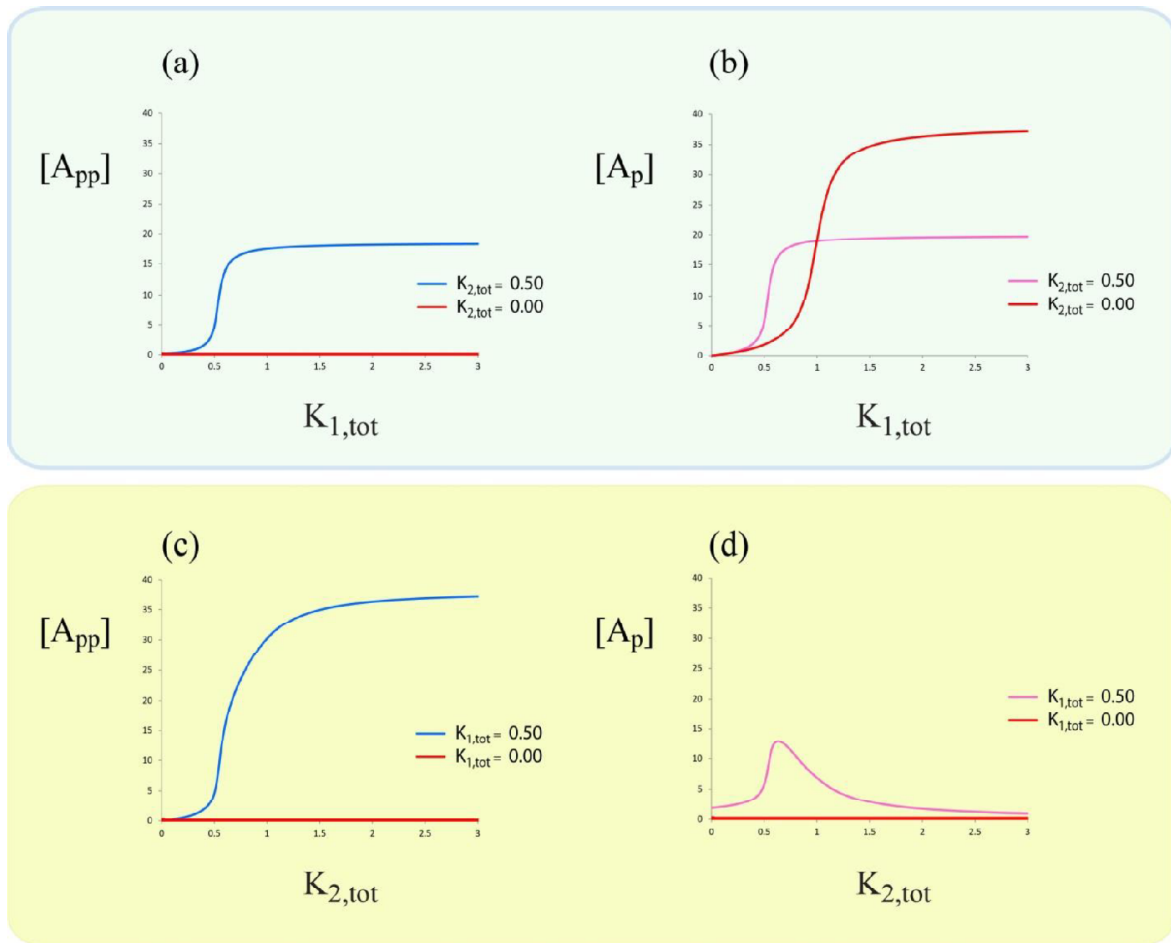


Figure 6.5 Steady-state dose-response curves in ordered 2-site model (model MC2) where dephosphorylation is performed by the same phosphatase. (a–b) respectively show the steady state input–output response curve of the concentration of maximally, A_{pp} , and partially phosphorylated substrate, A_p , as a function of the total concentration of kinase modifying the first site ($K_{1,tot}$) while (c–d) show the corresponding curve as a function of the total concentration of kinase modifying the second site ($K_{2,tot}$).

Figure 6.5c and 6.5d, respectively, shows how steady state response of fully and partially phosphorylated substrate varies with the total concentration of the kinase which modifies the second site, $K_{2,tot}$. For $K_{1,tot} = 0.5$, A_{pp} exhibits a sigmoidal response while A_p shows biphasic response as a function of $K_{2,tot}$. The red line in figure 6.5 (c-d) shows that phosphorylation cannot operate in the absence of first kinase which modifies the first site ($K_{1,tot} = 0$).

The effect of multiple kinase systems on distorting the internal dynamics of multi-site modification mechanisms is also examined in model MC2 in parameter regimes, where binding/unbinding and catalytic constant in each modification step is the same. Figure 6.6b shows how steady state response of fully phosphorylated substrate (A_{pp}) varies with the total concentration of the kinase modifying the first site, $K_{1,tot}$, for different values of that of the kinase modifying the second site, $K_{2,tot}$. As seen in this figure, as $K_{2,tot}$ increases, the behaviour can qualitatively be altered from a monotonic monostable response to a bistable response. Figure 6.6c shows steady state response of A_{pp} as a function of $K_{2,tot}$, for different values of $K_{1,tot}$. As seen in this figure, a decreased availability of $K_{1,tot}$ alters the behaviour from monotonic monostable response to a bistable response.

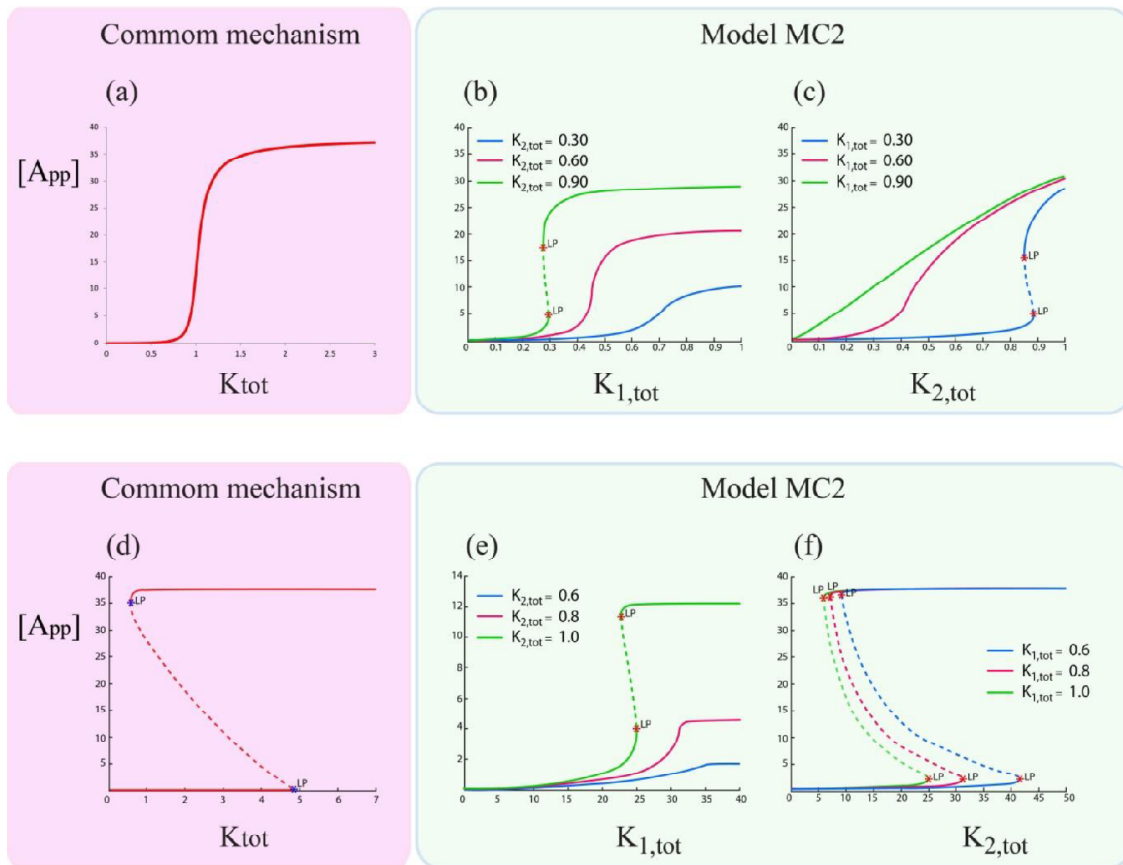


Figure 6.6 Steady-state dose-response curves in various ordered double phosphorylation models with different catalytic constants of phosphorylation reactions.

(a–c) show dose-response curves of the concentration of fully phosphorylated substrate, A_{pp} , as a function of the total concentration of kinase (K_{tot} or $K_{1,tot}$ or $K_{2,tot}$ as appropriate) while (d–f) show the corresponding curve for the partially phosphorylated substrate, A_p . (Completely phosphorylated substrate: (a) common mechanism, (b–c) model MC2. Singly phosphorylated substrate: (d) common mechanism, (e–f) model MC2)

Sufficiently different catalytic constant is a prerequisite for multistationarity and bistability in ordered double distributive phosphorylation by the same enzymes (Conradi & Mincheva, 2014). Here it is shown that ordered double phosphorylation by different kinase can cause bistability although the catalytic constants in all modification steps are similar in contrast to the earlier chapter (e.g. chapter 3). A bistable dose response curve can be seen by varying either enzyme but this also depends on the total concentration of the other enzyme. Depending on the total concentration of the other enzyme, the possibility of a bistable dose response curve can be completely eliminated.

It is worth mentioning that in addition to the amount of the other kinase affecting the bistable behaviour and dose response characteristic of the system, the specific identity of the enzymes also affects such behaviour (in other words, the sequence/location of which enzymes modify the substrate can affect the behaviour). As seen in figure 6.6b, when the value of $K_{2,tot} = 0.9$, the system can exhibit bistability over small range of $K_{1,tot}$. By contrast, a monotonic response to $K_{2,tot}$ is observed when the value of $K_{1,tot} = 0.9$ (figure 6.6c).

The investigation now focuses on the model MC2, where the common distributive analogues are bistable, as shown in figure 6.6d. Figure 6.6e shows how steady state response of A_{pp} varies with $K_{1,tot}$ for different values of $K_{2,tot}$. As seen in this figure, lowering $K_{2,tot}$ tends to diminish and eventually leads to an abolishment of bistability. By contrast, a reduced availability of $K_{1,tot}$ can widen bistable range with respect to $K_{2,tot}$, as seen in figure 6.5f. Taken together a bistable dose response curve can be seen by varying either enzyme but this also depends on the concentration of the other enzyme.

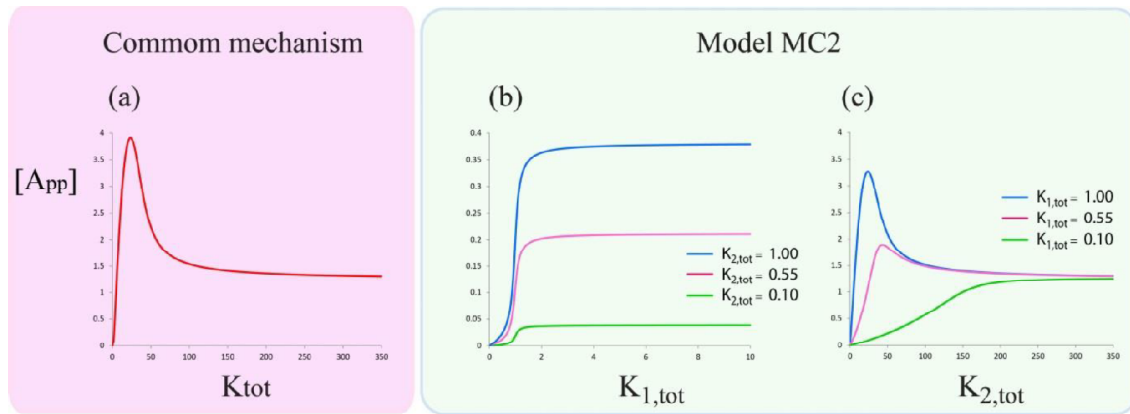


Figure 6.7 Biphaseic behaviour in various ordered double distributive phosphorylation models. The biphasic dose response curve of the fully phosphorylated form which can be observed in a common distributive model (a) is not found in the dose-response curve of fully phosphoform, A_{pp} , to $K_{1,tot}$ in model MC2 (b). However, A_{pp} can exhibit biphasic response as a function of $K_{2,tot}$ in model MC2 and lowering $K_{1,tot}$ tends to diminish and eventually abolish this behaviour (c).

Figure 6.7 shows a dose–response curve of a common distributive model in a different regime, indicating a biphasic response for the fully modified substrate. It can be seen in figure 6.7b that in ‘corresponding’ mechanisms (with different kinases and sharing phosphatase), a biphasic relationship between the maximally modified substrate concentration at steady state, A_{pp} , and the total concentration of kinase modifying the first site, $K_{1,tot}$, is not observed. The biphasic behaviour can be ruled out for this scenario, which is analytically demonstrated in (Suwanmajo & Krishnan, 2018c). However, A_{pp} can exhibit a biphasic response to the total concentration of kinase modifying the second site, $K_{2,tot}$, and lowering $K_{1,tot}$ tends to diminish and eventually abolish such behaviour (figure 6.7c). This observation further consolidates the insights of the chapter 3 which pinpoint the reason for a

biphasic response: this is equally relevant here, precisely because of the common phosphatase.

It is interesting to ask whether this model (model MC2) can exhibit other kinds of qualitative behaviour when explicit enzymatic activation is introduced (figure 6.8a). Figure 6.8b shows that the model with explicit activation is capable of generating sustained oscillations. Bifurcation analysis in figure 6.8c shows that as the input signal which activates the first kinase, S_1 , is changed, the steady state loses stability *via* a Hopf bifurcation.

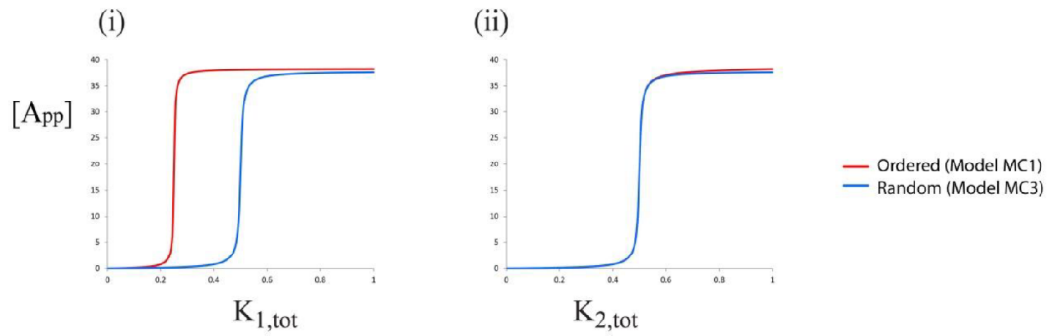
The effect of explicit activation on the dynamic behaviour of multi-site phosphorylation was previously discussed in the chapter 5, where it was shown that explicit activation can induce oscillatory behaviour in (ordered) double distributive phosphorylation by a single kinase/phosphatase pair. This study shows that with explicit activation, sustained oscillation is obtained even though the substrates are phosphorylated by different kinases (dephosphorylated by the same phosphatase). By symmetry, model MC2 also represents an ordered 2-site distributive mechanism performed by sharing kinase and different phosphatases. This means that, with explicit activation, one enzyme being shared in multisite modification is sufficient for generating sustained oscillation. In case of model MC1, by introducing explicit activation, sustained oscillation has not been observed after a detailed parametric search.

6.3.2 Random double-site modification schemes

This section focuses on studying the multi-site modification schemes where mechanisms occur in a random manner. The focus is first on considering the model MC3 where random distributive phosphorylation is modified by different kinases and different phosphatases at each site. Figure 6.9(a) contrasts the behaviour of random phosphorylation model with ‘related’ ordered phosphorylation mechanism with ‘corresponding’ parameters (kinetic symmetry in the cycle) where the system is monostable. As seen in figure 6.9a(i) which presents the steady state response of A_{pp} as a function of the total concentration of the first kinase, $K_{tot,1}$, it is found that random structure (model MC3) exhibits monostable switching with higher threshold compared to ordered structure (model MC1). By contrast, the threshold aspects in these two models are similar in the dose response curves of A_{pp} as a function of the total concentration of the second kinase, $K_{tot,2}$, as shown in figure 6.9a(ii).

The steady-state dose-response curves of partial phospho-form as a function of $K_{1,tot}$ in these two models are shown in figure 6.9b(i)-(iii). In model MC3, A_{01} refers to the partial phospho-form of the upper pathway while A_{10} refers to the partially phosphorylated substrate at the lower pathway. While the partial phospho-form presents monotonic response in model MC1, one of the partial phospho-form, A_{01} , in random mechanism exhibit a sigmoidal response with the clear presence of a threshold and the other phospho-form (A_{10}) presents a decreasing profile asymptoting to zero for large values of $K_{1,tot}$. Figure 6.9b(iv)-(vi) present the steady-state dose-response curves of partial phospho-form as a function of $K_{2,tot}$ in these two models. It is found that A_p in model MC1 and A_{10} in model MC3 present a decreasing profile asymptoting to zero for large values of $K_{2,tot}$ while A_{01} in model MC3 exhibits a sigmoidal response.

(a) Completely phosphorylated substrate



(b) Singly phosphorylated substrate

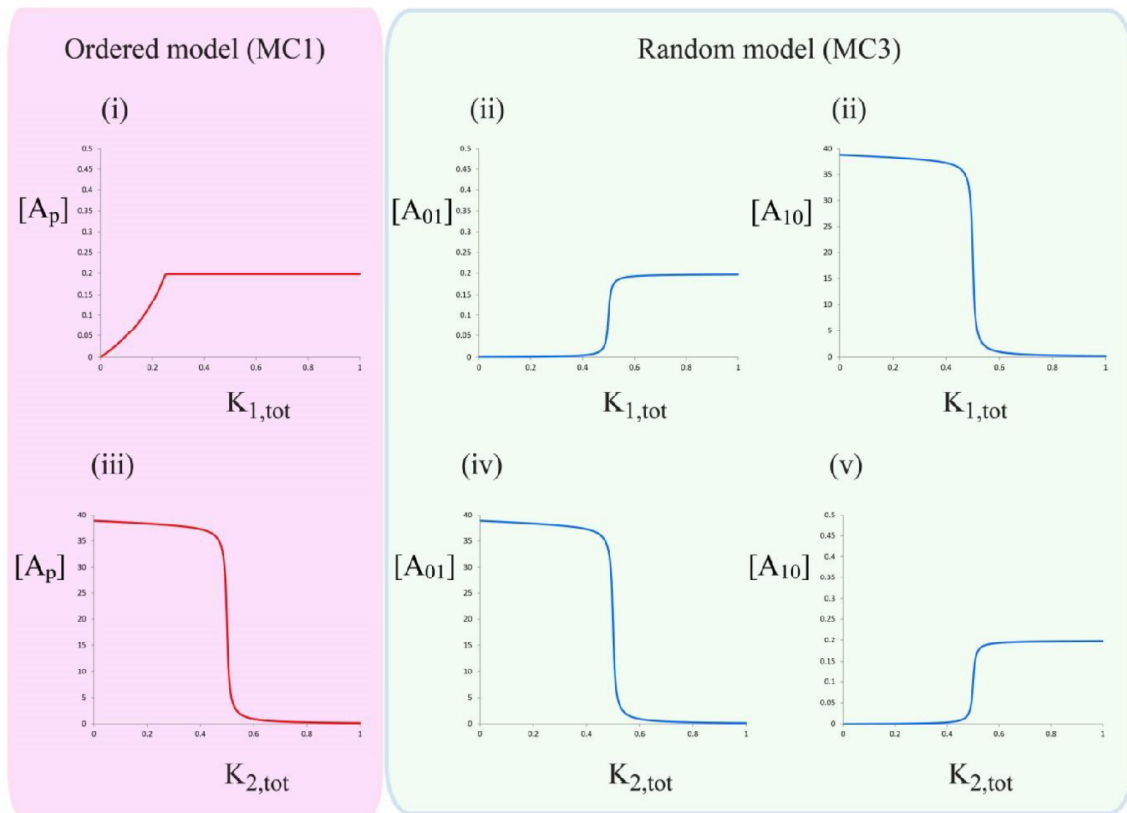


Figure 6.9 Steady-state dose-response curves in various double phosphorylation models with different kinases and phosphatases. (a) contrasts the dose response curve of A_{pp} in model MC3 with model MC1 with corresponding parameters. The dose response curves of A_p as a function of $K_{1,tot}$ in these two models are shown in (b(i)-(iii)) while the response curves of A_p as a function of $K_{2,tot}$ are shown in (b(iv)-(vi)).

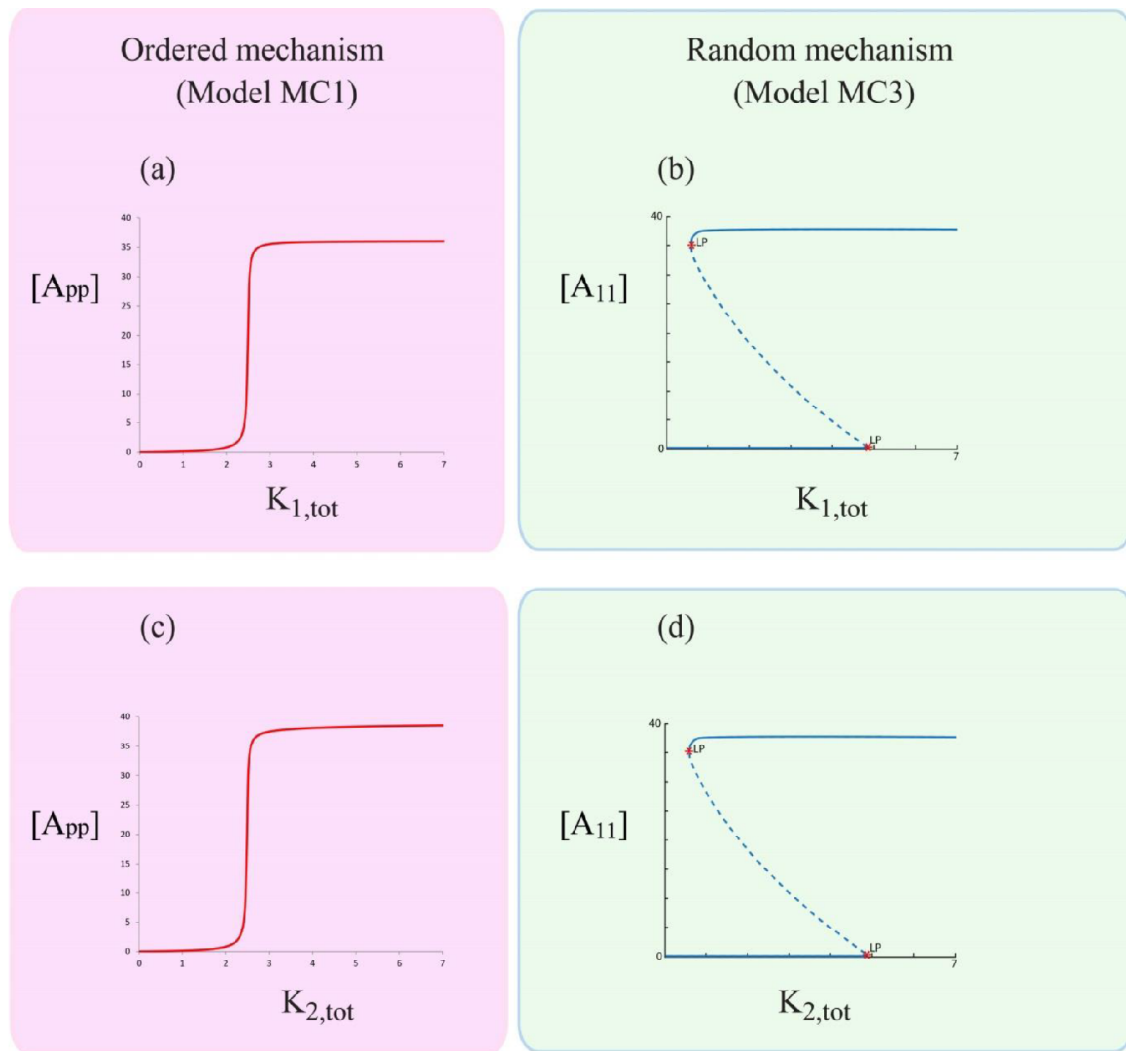


Figure 6.10 Steady-state dose-response curves in random phosphorylation model (MC3) and ordered phosphorylation model (MC1) with corresponding parameters. (a) and (c), respectively, present steady-state dose response curve of A_{pp} as a function of $K_{1,tot}$ or $K_{2,tot}$ in model MC1 while (b) and (d) present steady state response curve of the fully modified phosphoform (A_{11}) as a function of $K_{1,tot}$ or $K_{2,tot}$ in model MC3, respectively.

Model MC3 is now examined in parameter regimes, where the ‘relating’ ordered mechanism (model MC1) is monostable switching (figure 6.10a and 6.10c). While the ‘related’ ordered phosphorylation model presents monostable switching, it is found that, with

corresponding parameters (kinetic symmetry in the cycle), random model (MC3) can exhibit a bistable response curve as a function of $K_{1,tot}$ (figure 6.10b) and $K_{2,tot}$ (figure 6.10d). In the previous section, bistability is not observed in ordered mechanism where the substrates are modified by different kinases and separated phosphatases (model MC1) and such bistable behaviour can be ruled out in that model. Here this study shows that random mechanism modified by different kinases and phosphatases can exhibit bistability, completely in contrast to the ordered mechanism. This shows that the order of phospho-site processing has a qualitative effect on behaviour of multi-site modification mechanisms with different kinases and phosphatases.

In general, the random structure involves more species and sequestration, which could allow the systems to process more information and this leads to additional complexity. It is interesting to ask whether other kinds of qualitative behaviour can be seen in random model (MC3). Figure 6.11a and 6.11b shows a dose–response curve of a model MC3 in a different regime, indicating a biphasic response for the fully modified substrate as a function of total kinase concentration ($K_{1,tot}$ or $K_{2,tot}$). In the previous results it was found that biphasic response for the fully phosphoform is not observed in ordered mechanism (model MC1) where substrates are (de)phosphorylated by different kinases and separated phosphatases and such behaviour can be ruled out for that scenario (Suwanmajo and Krishnan, 2018c). Here simulation results show that random structure with different kinases and separated phosphatases can exhibit such biphasic behaviour in the maximally phosphoform as a function of either enzymes in contrast to the ordered case.

Furthermore, it is found that it is possible to get oscillation even with two kinases and two phosphatases even without any explicit activation. Figure 6.11(c-d) present another aspect of random phosphorylation with multiple enzymes. Figure 6.11c shows that model MC3 is capable of exhibiting sustained oscillation. A bifurcation diagram in figure 6.11d

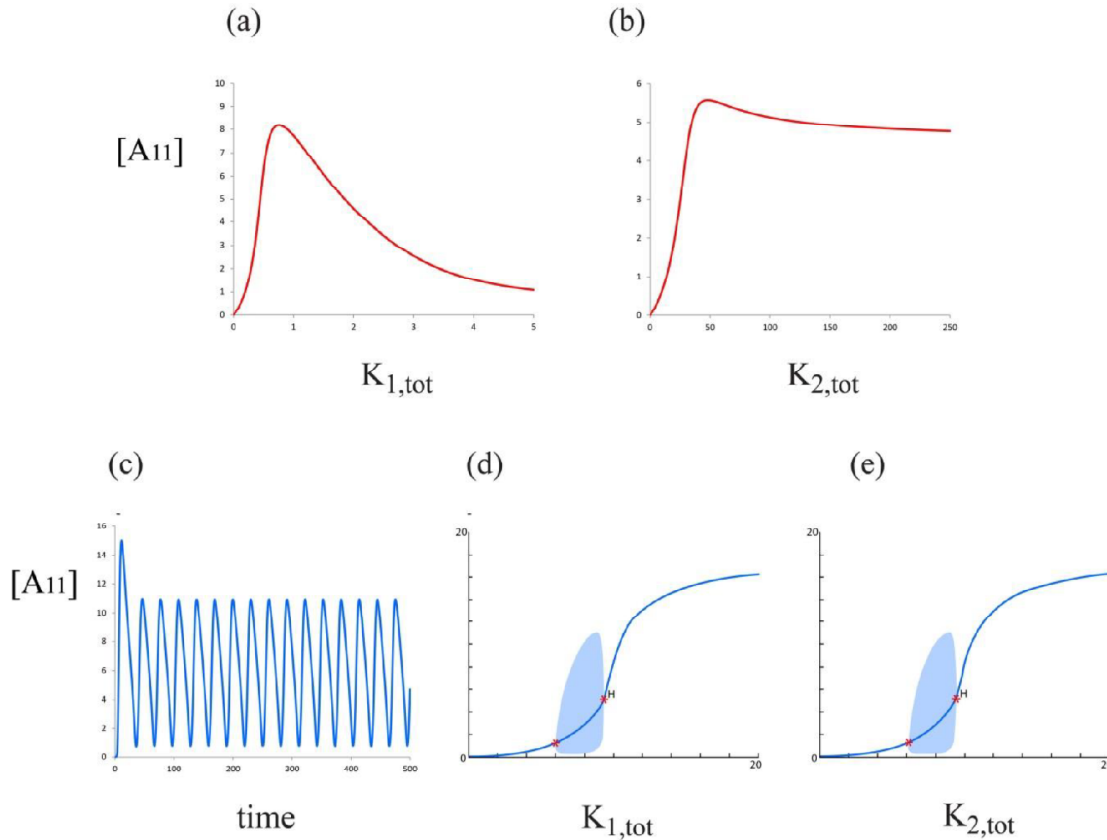


Figure 6.11 Biphasic and oscillatory behaviour in random modification model (model MC3). (a) Biphasic dose response for A_{11} as a function of $K_{1,tot}$ (b) Biphasic dose response for A_{11} as a function of $K_{2,tot}$. (c) Model MC3 is capable of generating sustained oscillations. (d-e) Bifurcation analysis for the random modification scheme (model MC3). Bifurcation analysis shows that the steady state loses stability *via* a Hopf bifurcation as the total kinase concentration ($K_{1,tot}$ or $K_{2,tot}$) is changed.

(and 6.11e) shows that, as the parameter $K_{1,tot}$ (and $K_{2,tot}$) is changed, the steady state does indeed lose stability to a Hopf bifurcation. Random double distributive modifications have been reported to exhibit sustained oscillations in a region of parameter space, when the mechanism is modified by a single kinase/phosphatase pair (Jolley et al, 2012). Here, results show that random double phosphorylation even catalysed by different kinases and separate

phosphatases is capable of generating sustained-oscillation. In the earlier result, sustained oscillations have not been observed in an ordered mechanism (model MC1) after a detailed parameter search. This means that the order of phospho-site processing has profound effect on dynamic behaviour of multisite phosphorylation. The effect of the order of phospho-site processing on information processing in common multi-site modification mechanisms was previously reported in (Salazar & Hofer, 2007).

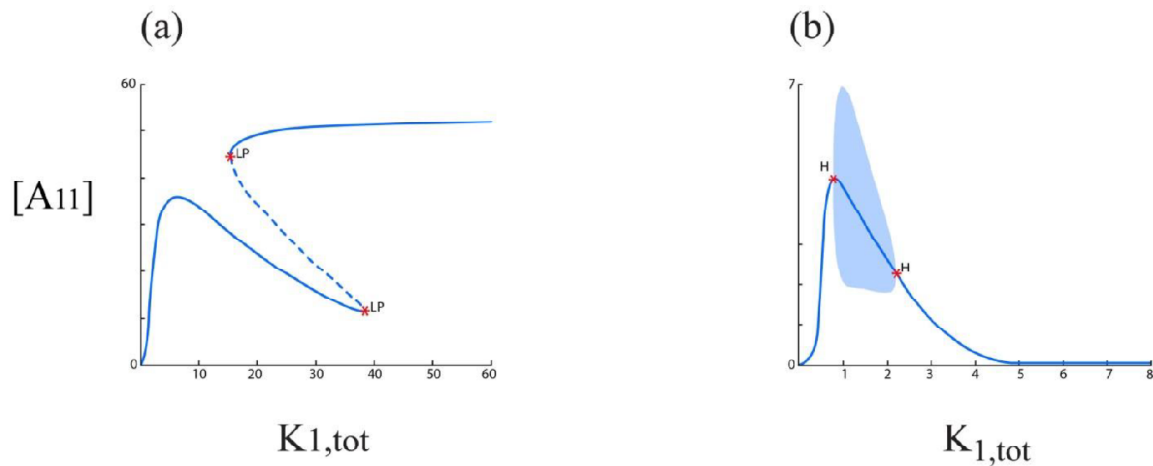


Figure 6.12 Complex dynamic behaviour in the random modification model (model MC3). (a) Biphasic dose response combined with bistability. (b) Biphasic dose response combined with sustained oscillation.

After investigating the qualitative behaviour (such as bistability, oscillation and biphasic response) in random mechanism (model MC3), it is interesting to ask whether the combination of such behaviour can be observed in this model. Figure 6.12 presents the combination of such behaviour can be observed in this model. Figure 6.12 presents the maximally phosphorylated substrate concentration at steady state, A_{11} , as a function of the total concentration of the kinase modifying the first site ($K_{1,tot}$). Figure 6.12a shows how biphasic behaviour can be combined with bistability, in model MC3. This demonstrates how the random phosphorylation system with different kinases and separated phosphatases can

exhibit a complex dose–response curve comprising of both biphasic behaviour and bistability. Furthermore, biphasic behaviour can also be combined with other qualitative behaviour. Figure 6.12b shows how biphasic behaviour can be combined with sustained oscillation. It can be seen that there are three phases of the steady state response. In the first phase, the response gradually increases as $K_{1,tot}$ increases. In the second phase, the systems exhibit oscillation over input range. In the third phase, the systems present a decreasing profile asymptoting to zero for large values of $K_{1,tot}$. Taken together this shows how the order of phosphor-site processing as well as multiple enzyme systems can introduce new modes of information processing.

While the above analysis involves investigating the random mechanisms where dephosphorylation is catalysed by different phosphatases, the focus now is on considering the random mechanism where dephosphorylation is catalysed by the same phosphatase (Model MC4). Figure 6.13(a-b), contrast the behaviour of random modification model (MC4) with ‘related’ ordered modification mechanism (model MC2) with corresponding parameters. Compared to ordered model which exhibits bistability in figure 6.13a, it is found that the input range ($K_{1,tot}$) for bistability becomes wider in random model with phosphatase sharing (figure 6.13b). The effect of enzyme-sharing on inducing bistability was theoretically discussed in (Feliu & Wiuf, 2011).

Figure 6.13c and 6.13d show that model MC4 can exhibit new mode of information processing in certain parameter regimes. As seen in figure 6.13c, the random model is capable of generating sustained oscillation. Bifurcation analysis in figure 6.13d shows that the system actually exhibits sustained oscillation associated with threshold behaviour. As the total kinase concentration, $K_{1,tot}$, is changed, the steady state loses stability *via* a Hopf bifurcation. Here it can be seen that an oscillatory region exists in the ‘off’ state of the monostable switching. This means that the system will begin oscillating when the input to the

system is under a certain threshold. If the input surpasses the threshold, oscillation will not occur but a significant change in output will be observed. A combination of oscillation and threshold behaviour is observed in asymmetric division of the *C. elegans* zygote (Pecreaux et al., 2006).

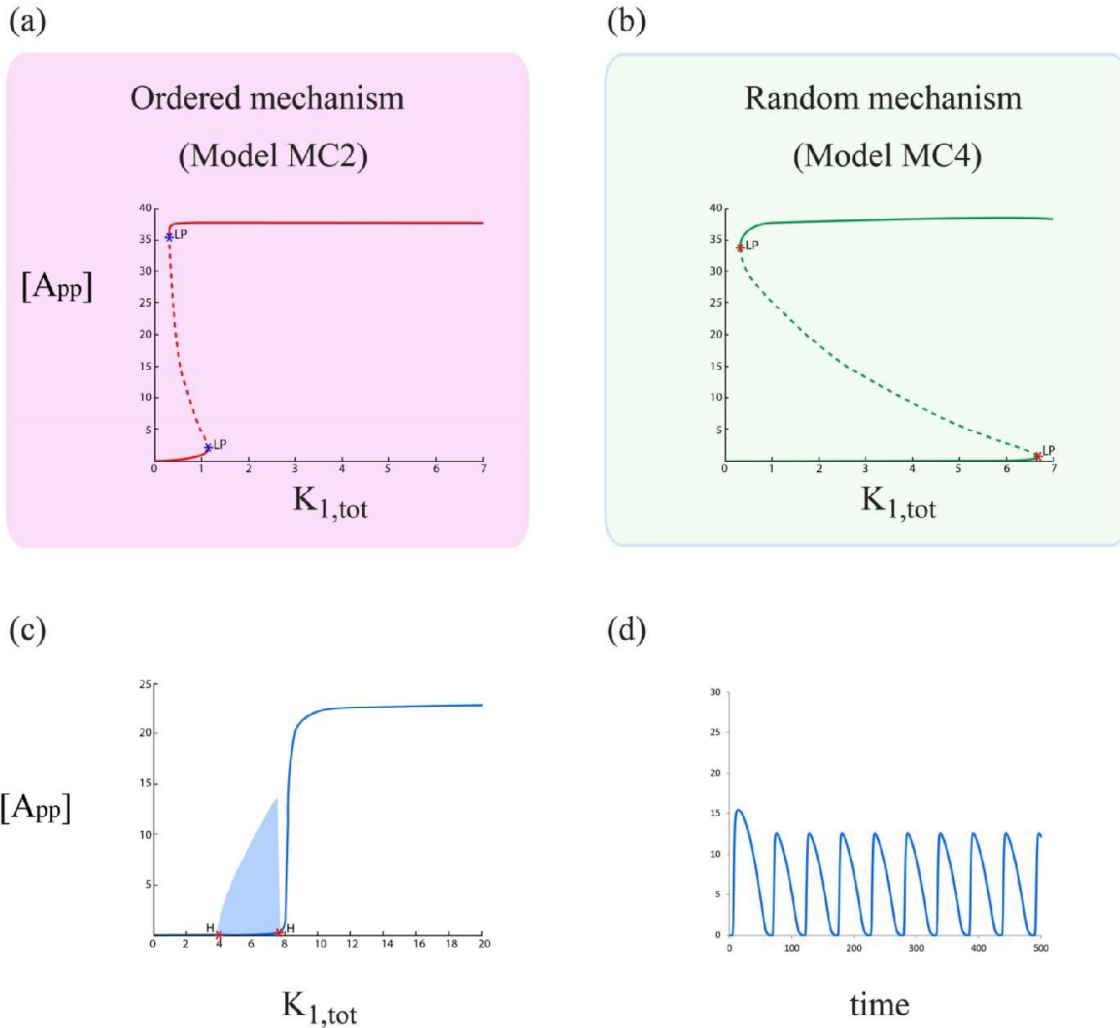


Figure 6.13 Bistable and oscillatory behaviour in random double phosphorylation with different kinases and sharing phosphatase (model MC4). (a) and (b), respectively, present bistable response curves in ordered (MC2) and random model (MC4) with corresponding parameters. (c) Random model (MC4) is capable of generating sustained oscillation. (d) Bifurcation analysis shows that as the total kinase concentration, $K_{1,tot}$, is changed, the steady state loses stability *via* a Hopf bifurcation in model MC4.

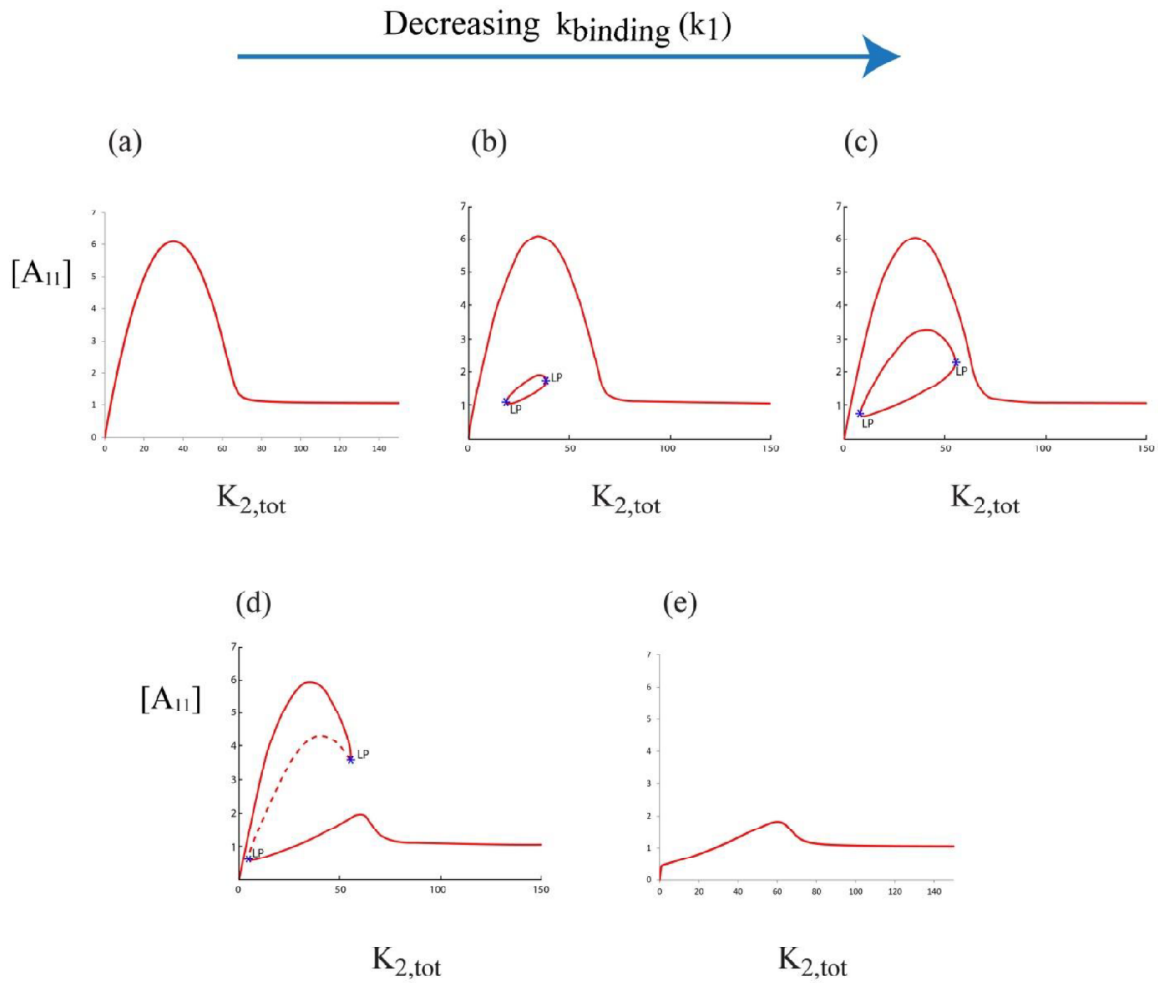


Figure 6.14 Biphasic behaviour with the presence of isola in random double phosphorylation with different kinases and sharing phosphatase (model MC4). (a) – (e) show how changes in the binding constant of the first phosphorylation step, k_1 , affect steady state behaviour: (a) Biphasic response. (b-e): There is an occurrence of isola loop separated from the biphasic curve, followed by merging isola and biphasic curve, and eventually, the loss of isola (bistability) (biphasic behaviour is retained).

Figure 6.14a shows a dose–response curve of the model MC4 in a different regime, indicating a biphasic response for the fully modified substrate, A_{11} , as a function of $K_{2,\text{tot}}$. Figure 6.14(b) - (e) show how changes in the binding constant of the first phosphorylation

step in the upper pathway, k_1 , affect such behaviour. By decreasing k_1 , there is an occurrence of a closed isolated loop separated from the biphasic response curve; such a loop is called 'isola' (as seen in figure 6.14b). Lowering k_1 can cause the input range for isola to become wider in (figure 6.14c), followed by merging isola and biphasic curve which leads to the complete disappearance of separated isola loop (two lower branches can join each other) and introduction of common bistability (figure 6.14d), and eventually, the loss of bistability while the biphasic behaviour is retained (figure 6.14e). This shows how the order of phospho-site processing as well as enzyme (phosphatase) sharing can introduce a new complex mode of information processing. In the literature, an isola was observed in certain biological contexts such as the induction of long-term memory (Song et al., 2006) and calcium signalling (two coupled calcium oscillators) (Bindschadler & Sneyd, 2001). Such systems involve complexities such as feedback and spatial regulation. To the best of author's knowledge, this thesis might be the first study showing that 2-site enzymatic mechanism by itself is capable of generating such kinds of behaviour (isola).

6.2.3 Ordered, triple phosphorylation involving multiple enzyme systems

After investigating the 2-site modification mechanisms, this section focuses on a slightly more complex mechanism, ordered 3-site modification involving one kinase/phosphatase pair performing a double-site modification and another kinase/phosphatase pair performing single-site modification (model MC5 and MC6). The focus is first on contrasting the singly modified substrate (A_p) dose response curve in single phosphorylation with that in model MC5, with corresponding parameters (All binding/unbinding and catalytic constants in each cycle are equivalent). Combination of single-site modification cycle (by K_1 and P_1) and double site phosphorylation cycle (by K_2

and P_2) results in model MC5. It can be seen that, when both cycles are isolated from one another, single phosphorylation cycle exhibits a sigmoidal response, as seen in figure 6.15a. By contrast, varying the input to the single site mechanism exhibits a bistable response to the single site modification when this single-site cycle is coupled to double phosphorylation cycle (which results in model MC5) as shown in figure 6.15b. In this case, the qualitative change is entirely due to the coupling effect. Sufficiently different catalytic constant is a prerequisite for bistability in double distributive phosphorylation by single kinase/phosphatase pair (Conradi & Mincheva, 2014). In our case, bistability can happen even though the catalytic constant of the double site modification are the same (in such a case the isolated double site mechanism does not exhibit bistability). Furthermore, single site modification by itself cannot exhibit bistability. Taken together, this suggests that bistable switching found in figure 6.15b arises from coupling of single phosphorylation and double phosphorylation rather than their intrinsic bistable switch-like behaviour. Therefore our result suggests that bistability (and other qualitative behaviour) in signalling pathways need not necessarily result from the intrinsic capability in that pathway, but can stem from coupling of pathway through shared components.

Figure 6.15c shows steady state dose response curve of A_{pp} as a function of $K_{1,tot}$ in model MC6, using corresponding parameters used in figure 6.15b. In the middle cycle, enzyme K_1 modifies its substrate (A_p) at one site, resulting in A_{pp} . It can be seen in this figure that A_{pp} dose response is monostable. In this case coupling of single site modification and double site modification does not have qualitative effects (using the same value of total concentration of other enzymes). Taken together this shows how changing the order of modification of the kinase can substantially alter the qualitative behaviour (in the case of 6.15b).

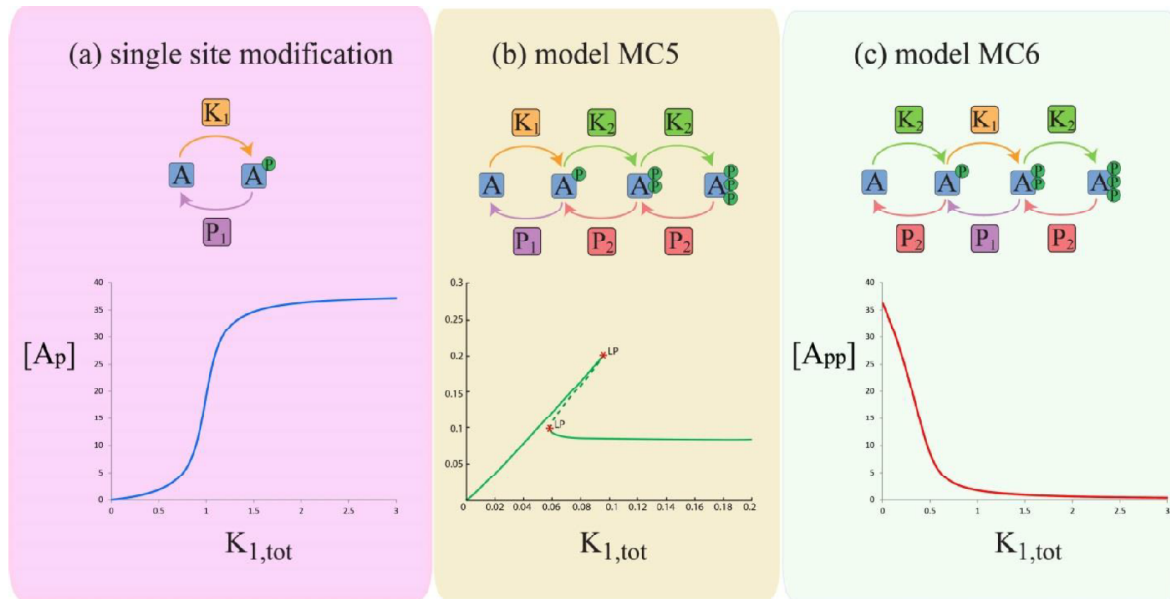


Figure 6.15 Steady state dose response curves in single phosphorylation and triple site modification with the same catalytic constants of phosphorylation reaction. (a) Dose response curve of A_p to $K_{1,tot}$ in single phosphorylation. (b) Dose response curve of A_p to $K_{1,tot}$ in model MC5. (c) Dose response curve of A_{pp} to $K_{1,tot}$ in model 36.

Figure 6.16 shows the effect of changing the order of modification of the enzyme pair on qualitative behaviour in 3-site modification models. The steady state dose response curve of the concentration of triply phosphorylated substrate, A_{pp} , as a function of $K_{tot,2}$ in model MC5 and MC6 is shown in this figure. As seen in figure 6.16a, both models can exhibit bistability and the order of modification of the kinase/phosphatase pair can affect the locations of the limit points (LPs) in the steady state input-output curve. Figure 6.16b shows a dose-response curve of a model MC5 and MC6 in a different regime, indicating a biphasic response for the fully modified substrate, A_{pp} , as a function of total kinase concentration

($K_{2,\text{tot}}$). It can be seen that the biphasic behaviour is more pronounced in model MC5 compared to model MC6. This shows how changing the order of modification of the enzyme can substantially alter the qualitative behaviour in multi-site mechanisms with multiple enzyme systems.

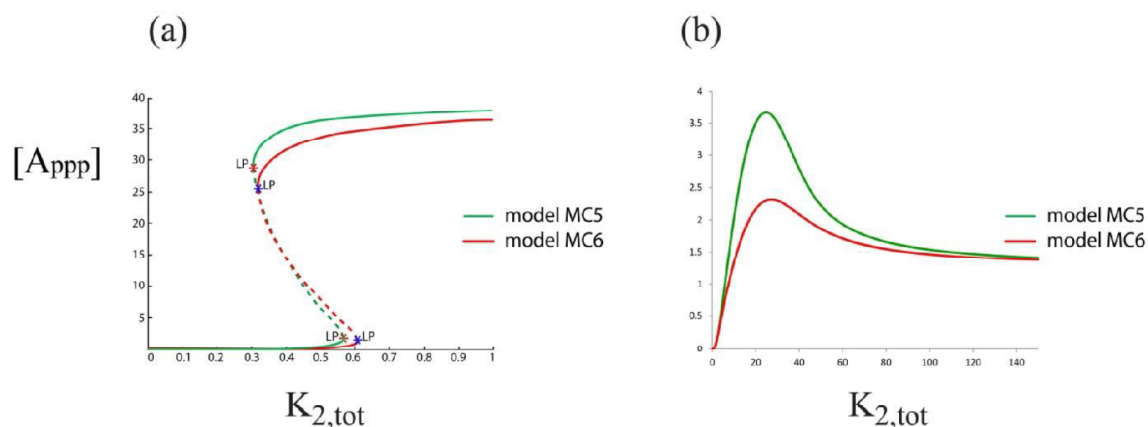


Figure 6.16 Steady-state dose-response curves in various ordered triple phosphorylation models with different catalytic constants of phosphorylation reaction. (a)-(b) contrast the qualitative behaviour (dose response curve of A_{ppp} as a function of $K_{2,\text{tot}}$) in model MC5 with that in model MC6 with corresponding parameters: (a) bistability and (b) biphasic dose response.

6.4 Conclusions

In this chapter, the focus is on studying the internal dynamic behaviour of multi-site phosphorylation through different enzymes. The motivation for this comes from the fact that many substrate modifications occur through different sets of enzymes. Since all the previous examples which have been studied earlier in the thesis involves only a single kinase and single phosphatase, this represents a new dimension to the study of multi-site phosphorylation. This chapter focuses on studying the effect of multiple kinases in phosphorylation through a sequence of scenarios proceeding from the simple to the complex. Table 6.1 summarises the qualitative features of double-site distributive phosphorylation through multiple enzymes, which emerge in this study. The results, based on the models studied, for various scenarios of mechanisms (ordered and random) in multisite phosphorylation are summarized below. The results start with the case of double site ordered phosphorylation. Here it was found that when the kinases and the phosphatases are distinct, the possibility of bistable and biphasic behaviour (and oscillations, though this is not easy to demonstrate) are all ruled out. Even in the simple case, if the investigation is performed on the case of two different kinases and a common phosphatase, a number of surprising results are found. Firstly, it was found that it is possible to get bistability just due to the common phosphatase. Secondly, it was found that it is possible to generate a biphasic dose response curve of the maximally phosphorylated form in response to the second kinase but not to the first. Thirdly it was found that introducing explicit activation in this model can generate oscillation similar to what was shown in the previous chapter. All these results demonstrate how the oscillation and bistability require only a common phosphatase and can occur even though kinase is different. Interestingly in this case bistability can occur even when catalytic constants are similar in contrast to the previous chapter. A bistable dose response curve can

be seen by varying either enzyme but this also depends on the concentration of the other enzyme. Depending on the concentration of the other enzyme, the possibility of a bistable dose response curve can be completely destroyed.

Table 6.1 A summary of qualitative features of double-site distributive phosphorylation through multiple enzymes studied in this chapter. The results, based on the models studied, for various scenarios of mechanisms (proceeding from ordered to random phosphate processing) in multisite phosphorylation are discussed in detail in the text.

systems	Qualitative behaviour in common mechanism	Qualitative behaviour observed in multi-site phosphorylation with different kinases			
		Different phosphatase		Common phosphatase	
		K1,tot as in put	K2,tot as input	K1,tot as in put	K2,tot as input
<u>Ordered mechanism</u>	monostable behaviour without biphasic response	monostable behaviour without biphasic response	monostable behaviour without biphasic response	monostable behaviour without biphasic response	monostable behaviour without biphasic response
	biphasic response	monostable behaviour without biphasic response	monostable behaviour without biphasic response	monostable behaviour without biphasic response	monostable behaviour without biphasic response
	bistability	monostable behaviour without biphasic response	monostable behaviour without biphasic response	monostable behaviour without biphasic response	monostable behaviour without biphasic response
<u>Random mechanism</u>		monostable behaviour bistability biphasic response oscillation	monostable behaviour bistability biphasic response oscillation	monostable behaviour bistability biphasic response oscillation isola	monostable behaviour bistability biphasic response oscillation isola

Following this, the next step is to study the random modification scenario. In this case, it was notice that even when the kinases and phosphatases are different, the system can exhibit bistability, completely in contrast to the ordered mechanism. Similarly it was possible to get biphasic responses in the maximally modified phosphoform as a function of either enzyme in contrast to the ordered case. Finally it was found that it is possible to get oscillation even with two kinases and two phosphatases even without any explicit activation. All of these conclusions already demonstrate the increased possibility of new qualitative behaviour in random mechanisms relative to ordered mechanisms. Building on this, the study then proceeds to the case where the phosphatase is common to all dephosphorylation steps. Here again, surprising results were found. As expected, the systems could generate oscillations as well as bistability in this case. Even more interestingly in certain parameter regimes, the system was capable of generating oscillations associated with threshold

behaviour. Furthermore, it was also able to see the presence of isola in the dose response curve. This further illustrates the fact that random mechanisms are able to demonstrate subtlety and complexity in behaviour even when the kinases are different.

Finally, for completeness, this study also considers ordered triple-site mechanisms involving one kinase/phosphatase pair performing a double site modification and another kinase/phosphatase pair performing a single site modification. In this case, it was found that triple site modification mechanism is capable of exhibiting bistability. Interestingly, this can happen even though the catalytic constants of the double site modification are the same (in such a case the isolated double site mechanism does not exhibit bistability). Furthermore, varying the input to the single site mechanism, results in a bistable response to the single site modification. This is entirely due to the coupling to the double site modification. The dose response curves with respect to each enzyme depend on the basal level of other enzymes. Finally changing the order of modification of the kinases can substantially alter the qualitative behaviour. These various results clearly illustrate new trends and insights when one encounters multiple kinases performing different kinds of modification even in ordered mechanisms.

Taken together, in this chapter, the effect of different kinases performing substrate modifications has been studied. Quite surprisingly, it was found that just having a common phosphatase is sufficient for a range of qualitative behaviour. Furthermore when the study proceeds from ordered to random mechanism, the whole range of qualitative behaviour is possible even with separated kinases and separated phosphatases. Finally coupling of the single site and double site modification can result in behaviour which is not present in either. All these aspects provide a basal reference framework for understanding different aspects of the qualitative information processing in multisite phosphorylation with multiple different enzymes.

Chapter 7

Conclusions and Recommendations

7.1 Conclusions

Cellular functioning, regulation and their response to the environment are all controlled by complex signalling (and gene regulatory networks). These networks perform a whole host of important tasks in a robust manner. The complexity and sophistication of these networks stems from multiple sources: the large number of components, their hierarchical organization, the presence of abundant nonlinearity and widespread feedback, and their organization shaped by evolution. Unravelling and elucidating the functioning of these networks is a major theme of systems biology, with important ramifications in basic cell biology, biomedical and biotechnological applications, and in synthetic biology as well. Accordingly a number of dedicated modelling and systems approaches have been deployed, in collaboration with experimental work in a wide array of contexts.

An important source of the complexity of signalling networks, which is also central to functioning and molecular regulation of cellular processes, is the complexity of enzymatic modification mechanisms. In cellular biology, there are a number of ways in which this may arise. One of the most common sources of complexity is the fact that proteins may be modified at multiple locations by enzymes. A very widespread and representative example is multisite phosphorylation, where enzymes (kinases and phosphatases) modify protein substrates at a number of locations. This multisite modification, far from being a mere curiosity, is in fact central to the control of substrate and related downstream processes where a modified form of the substrate acts as a key controller. The modification status of a protein

is often referred to as a molecular bar code and this encodes different aspects which determine the proteins function, localization etc. Given the central importance of multisite phosphorylation in cell signalling networks, and the importance of multisite phosphorylation generally, this topic really merits focussed dedicated systems studies.

Prior to this thesis, there had been a number of studies of multisite phosphorylation in multiple biological contexts, focussing on the phosphorylation and its consequences. There were also a number of modelling studies focussing on different aspects of multisite phosphorylation. These had focussed on different aspects of information processing in multisite modification. In spite of these studies, a number of questions presented themselves: (i) What are the range of qualitative information processing behaviours which can be exhibited by different multisite modification mechanisms? Are there other behaviours besides those already discussed? (ii) What is the effect of more complex enzymatic modification mechanisms, beyond simple distributive and simple processive? (iii) How do these multisite modification mechanisms function in signalling pathways, and what is the interplay between the enzymatic modification module and the pathway/network? (iv) How does the presence of multiple modification enzymes affect the modification dynamics of the substrate?

In this thesis, the main goal was to address some of these questions, with a view towards elucidating a number of “systems” aspects of multisite phosphorylation. The approach, right through this thesis, was to place a primary focus on qualitative aspects. In other words, the analysis was primarily concerned with the information processing capabilities and constraints associated with multisite phosphorylation. While these studies are of course qualitative, they are ultimately developed with the underlying viewpoint that elucidating the qualitative capabilities and constraints provides a proper foundation and reference from which other detailed quantitative (experimental and computational) studies could be undertaken. Keeping this in mind, the tools which were used in this study were

numerical simulation, numerical bifurcation analysis and analytical work. Numerical simulation is a basic tool to computationally explore behaviour (both steady state and dynamic). In this thesis, mechanistic models were implemented in MATLAB, and crosschecked with the same models generated in COPASI, which are generated automatically by the software, given the network. Numerical bifurcation analysis allowed for the assessment of the effect of parameters both sensitivity and stability. This was performed in MATCONT. Both simulation and bifurcation analysis are complementary tools in analysing dynamic systems. Finally analytical work, when possible, allows for a clear cut demonstration of effects, dependence of parameters, and for an explicit demonstration of capabilities and constraints in information processing.

The main results of the thesis are as follows. In the first investigation, it was found that simple distributive double site modification mechanisms were capable of exhibiting biphasic dose response curves. This study revealed an in-built competing effect which was the source of this behaviour. This could be seen in both ordered and random mechanisms. It was further showed that this behaviour could not be seen in processive mechanisms. Further subtle changes in the modification scenario, which changed the ordered distributive mechanism to a cyclic mechanism, abolished the possibility of a biphasic response. This further underscored the source of the behaviour. It was also shown that with an increase in phosphorylation sites would allow for this behaviour to be combined with other features such as bistability.

Most studies on multisite phosphorylation had focussed on either distributive or processive mechanisms. In the second investigation, the main focus was on examining the interplay between distributive and processive mechanisms leading to mixed mechanisms. A detailed investigation was performed on mixed mechanisms in both double site ordered mechanisms and also triple site ordered mechanisms. The consideration of triple-site

mechanisms allowed for the possibility of sequences of distributive and processive mechanisms in either phosphorylation or dephosphorylation or both. It was revealed that when phosphorylation was distributive and dephosphorylation was processive (or the other way around), biphasic and bistable behaviour could be ruled out. On the other hand this interplay could result in oscillatory behaviour even in an ordered double site mechanism. Ordered triple site mechanisms could result in the combination of bistability and oscillatory behaviour.

Building on the previous studies, the next investigation focussed on developing a systems approach to bridge the gap between the module in isolation and as part of a network. Noting the ways in which interaction with the ambient network arise, there were four aspects which are considered in this study: (i) The effect of enzyme activation (ii) The effect of retroactivity due to a phosphorylated substrate sequestered in some downstream pathway (iii) The effect of production and degradation of some substrate species and (iv) The effect of embedding a multisite module in some typical network modules.

A detailed study based on such framework led to an array of results elucidating each of these facets. It was found that explicit activation itself could induce oscillations even though the module (an ordered double site distributive model) did not intrinsically exhibit oscillatory behaviour. Analysis showed how the retroactive effect could introduce a change in qualitative behaviour in the multisite phosphorylation module, including a loss of bistability. Additional analysis showed how this depended on the phosphoform being sequestered. This is of clear relevance in cell signalling pathways as one or more phosphoforms may indeed be sequestered in downstream pathways as they are involved in signal transduction through those pathways. The effect of production and degradation of species was shown to be capable of completely altering the behaviour of the multisite phosphorylation module in multiple aspects: for example abolishing bistability, even abolishing the possibility of steady states

and introducing adaptive behaviour. The location (in the module) of “inflow” and “outflow” had a significant effect in determining what kind of behaviour resulted, in conjunction with the kinetic parameters of the multisite modification module. Finally the effect of embedding a multisite module in a network module (coherent and incoherent feed-forward modules, negative and positive feedback modules) was also examined. Based on the developed structured-systems framework, it was possible to assess both the effect of the ambient network on the multisite module, as well as the effect of the multisite module on the network. It was found that in the case of feedback, it was possible to introduce or destroy steady states, significantly alter the range of bistability and so on. In the case of feed-forward modules, the combination of feed-forward regulation with the multisite module gave rise to a range of behaviour which involved a significant interplay between both modules.

In the final investigation, the effect of multiple (different) kinase pathways (and possibly phosphatase pathways) performing the substrate modification was investigated. This study focused on both ordered and random mechanisms. It was found that in an ordered mechanism, even with different kinases and common phosphatases, it was possible to get bistability. It was able to demonstrate the effect of pathway interaction through regulation of a common substrate. In the scenario of a random mechanism, it was found that bistability in 2-site modification could occur even with separate kinases and phosphatases, and that oscillations could arise as well. The possibility of biphasic responses was studied as well, demonstrating both the presence (and in some cases absence) of this phenomenon. Other complex behaviour of these modules was also studied.

7.2 Recommendations for Future work

This thesis has presented a range of studies seeking to understand various aspects of multi-site phosphorylation-based signalling systems. The different studies undertaken provide a consolidated systems view of different aspects of multisite phosphorylation. Building on the work here, a number of avenues could be investigated.

7.2.1 Parametric analysis of multi-site phosphorylation systems

To start with, the thesis methodology has focussed on qualitative behaviour of multisite phosphorylation studied using simulation, bifurcation analysis and analytical work. In this regard, representative parameters sets demonstrating different kinds of behaviour have been chosen. One future direction of work is to perform exhaustive parameter sampling to investigate the global parameter dependence of the module behaviour. This will complement the approach taken in this thesis in the different investigations and provide insights into the global parameter dependence.

7.2.2 Investigating the effect of the dynamic signals on internal dynamic of multi-site phosphorylation systems

A second aspect which has been considered only briefly at select stages of the thesis is the effect of the transient behaviour of the module, and characterizing this. In many cellular contexts, cells encounter the temporal variation of external signals. Such signals are ubiquitous in stress responses (El-Samad et al. (2005); Muzzey et al. (2009); Ni et al. (2009)), and also arise in sensory systems (Friedlander and Brenner (2009)) and chemotactic systems (Levchenko and Iglesias (2002)).

The activation of switches in multi-site phosphorylation by transient signals differs from the activation of switches by static signals. Numerous studies on multisite phosphorylation in the literature have been mostly centred on static signals. There is, however, no study investigates the effect of the temporal signal on activating multisite phosphorylation systematically. Since important aspects of signal transduction rely on transient characteristics, in this context it would be a natural aspect to study. Understanding such effects might have relevance to the manipulation of particular pathways through transient signals.

7.2.3 Modelling multi-site phosphorylation in concrete systems

Other possible avenues of research involve using these results as a platform to investigate concrete systems. One example is the modelling of ERK signalling and MAPK pathways. The MAPK pathway is also ubiquitous in eukaryotic signal transduction. This module consists not only of single phosphorylation, but also multisite (double) phosphorylation. There is evidence that both MAPKK and MAPK are (de)phosphorylated in a distributive manner (Ferrell & Bhatt, 1997; Zhao & Zhang, 2001). In nature there are crosstalk regulations between different MAPK pathways, depending on the cellular context. It is clear that the present work (and extensions outlined above) can be used as a platform to investigate these pathways and crosstalk between these pathways, in conjunction with appropriate experiments.

There are various types of post-translational modifications (PTMs) that occur in the human eye lens. Dysfunctional regulations of such PTMs are implicated in human eye diseases (Travis et al., 2007). Among these PTMs, it seems that phosphorylation plays a

major role in phototransduction mechanisms (Kennedy et al., 2001; Travis et al., 2007; Huang et al., 2011).

Phosphorylation-dephosphorylation of rhodopsin is common in dark adaptation (Kennedy et al., 2001). In fact, dark adaptation involves many mechanisms; for example, phosphorylation, arrestin binding, reduction of photolysed chromophore (all-trans retinal), dephosphorylation and regeneration with 11-cis retinal. Among these mechanisms, reversible phosphorylation is a major step in modulating dark adaptation (Ohguro et al., 2005), so a number of *in vivo* researches regarding multi-phosphorylation of rhodopsin have been done in the last decade. There are however, a number of unanswered questions associated with dark adaptation, some of which can be addressed starting from the vantage point of this thesis.

Multisite phosphorylation is a common constituent sub-unit of a number of biological networks. Therefore, the systematic understanding of different mechanisms of the multi-phosphorylation could provide basic insights into a broad spectrum of cellular processes. In this thesis, a series of investigations of multi-site phosphorylation undertaken constitutes a dedicated systems approach to unravelling different facets of the complexity and behavior of this enzymatic module in isolation and in pathways. This serves as a platform for further detailed investigations and expansions in a wide range of specific contexts.

Appendix A

Biphasic responses in multi-site phosphorylation systems

In this section, a number of aspects are discussed in turn: (i) The models used in the analysis undertaken (ii) Analytical explanation of certain results and (iii) The parameters employed in the main text. This section starts by discussing the model equations. The computational results presented in chapter 3 are complemented by analytical work, which is presented in (Suwanmajo and Krishnan, 2013) and reproduced in the appendix A.

A.1. Model equations for random distributive mechanism

Here, the models used in the analysis undertaken are presented. This section presents and discusses the n -site-ordered distributive model and the two-site random mechanism.

The n -site distributive-ordered mechanism is a simple extension of the two-site mechanism to incorporate n modifications. The equations for the n -site modification can be obtained in an exactly analogous way, and can be succinctly written as follows:

$$\left. \begin{aligned}
 \frac{d[A_0]}{dt} &= k_{ub,p(1)}[A_0K] - k_{b,p(1)}[A_0][K] + k_{cat,dp(1)}[A_1P] \\
 \frac{d[A_i]}{dt} &= k_{cat,p(i)}[A_{i-1}K] + k_{ub,p(i+1)}[A_iK] + k_{b,p(i+1)}[A_i][K] \\
 &\quad + k_{cat,p(i+1)}[A_{i+1}P] + k_{ub,dp(i)}[A_iP] + k_{b,dp(i)}[A_i][P] \\
 &\quad ; (i = 1, 2, 3, \dots, n-1) \\
 \frac{d[A_iK]}{dt} &= (-k_{ub,p(i+1)} - k_{cat,p(i+1)})[A_iK] + k_{b,p(i+1)}[A_i][K] \\
 &\quad ; (i = 0, 1, \dots, n-1) \\
 \frac{d[A_iP]}{dt} &= (-k_{ub,dp(i)} - k_{cat,dp(i)})[A_iP] + k_{b,dp(i)}[A_i][P] \\
 &\quad ; (i = 1, 2, \dots, n) \\
 \frac{d[A_n]}{dt} &= k_{cat,p(n)}[A_{n-1}K] + k_{ub,dp(n)}[A_nP] - k_{b,dp(n)}[A_n][P]
 \end{aligned} \right\} \quad (A. 1)$$

These equations describe the concentration dynamics of the unmodified, partially modified and completely modified substrates, the various kinase and phosphatase complexes. The equations for the free kinase and phosphatase are written in a manner similar to the double-site modification and are not shown explicitly here

The random double-site modification mechanism model is obtained in a similar way to the ordered model, except for the fact that two different partially modified substrates are incorporated. The equations for the model are

$$\left. \begin{aligned}
\frac{d[A]}{dt} &= k_2[AK_1] - (k_1 + k_7)[A][K] + k_{18}[A_{01}P] + k_{24}[A_{01}P] + k_8[AK_2] \\
\frac{d[AK_1]}{dt} &= k_1[A][K] - (k_2 + k_3)[AK_1] \\
\frac{d[AK_2]}{dt} &= k_7[A][K] - (k_8 + k_9)[AK_2] \\
\frac{d[A_{01}]}{dt} &= k_2[AK_1] - k_4[A_{01}][K] + k_5[A_{01}K] + k_{15}[A_{11}P_1] + k_{17}[A_{01}P] + k_{16}[A_{01}][P] \\
\frac{d[A_{10}]}{dt} &= k_9[AK_2] - k_{10}[A_{10}][K] + k_{11}[A_{10}K] + k_{21}[A_{11}P_2] + k_{23}[A_{10}P] + k_{22}[A_{10}][P] \\
\frac{d[A_{01}K]}{dt} &= k_4[A_{01}][K] - (k_5 + k_6)[A_{01}K] \\
\frac{d[A_{10}K]}{dt} &= k_{10}[A_{10}][K] - (k_{11} + k_{12})[A_{10}K] \\
\frac{d[A_{11}]}{dt} &= k_6[A_{01}K] + k_{12}[A_{10}K] - (k_{13} + k_{19})[A_{11}][P] + k_{14}[A_{11}P_1] + k_{20}[A_{11}P_2] \\
\frac{d[A_{11}P_1]}{dt} &= k_{13}[A_{11}][P] - (k_{14} + k_{15})[A_{11}P_1] \\
\frac{d[A_{01}P]}{dt} &= k_{16}[A_{01}][P] - (k_{17} + k_{18})[A_{01}P] \\
\frac{d[A_{11}P_2]}{dt} &= k_{19}[A_{11}][P] - (k_{20} + k_{21})[A_{11}P_2] \\
\frac{d[A_{10}P]}{dt} &= k_{22}[A_{10}][P] - (k_{23} + k_{24})[A_{10}P]
\end{aligned} \right\} \text{(A. 2)}$$

A.2. Double-site-ordered distributive mechanisms and single-site mechanism

The analysis undertaken in the chapter3 (reproduced from (Suwanmajo and Krishnan, 2013)) shows how at steady state the concentration of the doubly modified substrate may be obtained as

$$[A_{pp}] = \frac{A_{tot}}{1 + \alpha_1[P] + \alpha_2[P]/[K] + \alpha_3[P]^2/[K] + \alpha_4[P]^2/[K]^2} \quad \text{(A 3)}$$

This was obtained by imposing steady-state conditions for concentrations of all relevant components and imposing conservation of total substrate, kinase and phosphatase.

In equation (A 3), the various constants may be written explicitly in terms of kinetic constants as

$$\left. \begin{aligned} \alpha_1 &= \frac{k_7}{k_6} \\ \alpha_2 &= \frac{(k_5 + k_6)k_7}{k_4k_6} \\ \alpha_3 &= \left[\frac{k_{10}}{k_{11} + k_{12}} \right] + \left[\frac{(k_5 + k_6)k_7}{k_4k_6} \right] \\ \text{and } \alpha_4 &= \left(\frac{k_{12}}{k_1} \right) \alpha_3 \end{aligned} \right\} \quad (\text{A } 4)$$

In the main text, it was asserted that biphasic behaviour would not be possible in a single modification cycle (the isolated system without product inhibition). Using the same notation for the single cycle, as it is done for the first cycle but without making any assumptions about rate constants, it is easy to see that at steady state

$$\left. \begin{aligned} [\text{AK}] &= \left(\frac{k_1}{k_2 + k_3} \right) [\text{A}][\text{K}] \\ \text{and } [\text{A}_\text{p}\text{P}] &= \left(\frac{k_{10}}{k_{11} + k_{12}} \right) [\text{A}_\text{p}][\text{P}] \end{aligned} \right\} \quad (\text{A } 5)$$

Thus, we have from a conservation of enzyme that

$$[\text{K}] = K_{\text{tot}} \frac{1}{1 + (k_1[\text{A}]/(k_2 + k_3))}$$

and

$$[\text{P}] = P_{\text{tot}} \frac{1}{1 + (k_{10}[\text{A}_\text{p}]/(k_{11} + k_{12}))}$$

The conservation of total substrate results in

$$[A] \left(1 + K_{\text{tot}} \frac{\alpha}{1 + \alpha[A]} \right) + [A_p] \left(1 + P_{\text{tot}} \frac{\beta}{1 + \beta[A_p]} \right) = A_{\text{tot}} \quad (\text{A } 6)$$

Where $\alpha = k_1/(k_2+k_3)$, $\beta = k_{10}/(k_{11}+k_{12})$ (only the functional form is required). Further, at steady state:

$$k_3 K_{\text{tot}} \left(\frac{\alpha[A]}{1 + \alpha[A]} \right) - k_{12} P_{\text{tot}} \left(\frac{\beta[A_p]}{1 + \beta[A_p]} \right) = 0 \quad (\text{A } 7)$$

By differentiating the conservation equation for substrate with respect to K_{tot} , it can be seen that if $dA_p/dK_{\text{tot}} = 0$, then it should follow that $dA/dK_{\text{tot}} = 0$. Suppose it is assumed the possibility of $dA_p/dK_{\text{tot}} = 0$. Then by differentiating the steady-state equation above, the author arrives at a contradictory conclusion that $\alpha = 0$. Thus, the single modification cycle is incapable of exhibiting a biphasic dose–response characteristic.

A.3. The cyclic distributive mechanism

This section focuses on analysing a variation of the ordered mechanism where the order of phosphorylation is not opposite to the order of dephosphorylation (figure 3.2), and again consider a two-site mechanism. This mechanism is almost identical to the ordered mechanism considered in the paper, except that the order of phosphorylation and dephosphorylation implies that there are different singly phosphorylated forms. This means, the model has to explicitly account for the two different singly phosphorylated forms A_{01} and A_{10} . The model for this mechanism can be written in a very similar manner to those above.

$$\left. \begin{aligned}
\frac{d[A]}{dt} &= k_2[AK] - k_1[A][K] + k_{12}[A_{10}P] \\
\frac{d[K]}{dt} &= (k_2 + k_3)[AK] - k_1[A][K] + (k_5 + k_6)[A_{01}K] + k_4[A_{01}][K] \\
\frac{d[AK]}{dt} &= k_1[A][K] - (k_2 + k_3)[AK] \\
\frac{d[A_{01}]}{dt} &= k_3[AK] - k_4[A_{01}][K] + k_5[A_{01}K] \\
\frac{d[A_{01}K]}{dt} &= k_4[A_{01}][K] - (k_5 + k_6)[A_{01}K] \\
\frac{d[A_{11}]}{dt} &= k_6[A_{01}K] - k_7[A_{11}][P] + k_8[A_{11}P] \\
\frac{d[P]}{dt} &= (k_8 + k_9)[A_{11}P] - k_7[A_{11}][P] + (k_{11} + k_{12})[A_{10}P] + k_{10}[A_{10}][P] \\
\frac{d[A_{10}]}{dt} &= k_9[A_{11}P] - k_{10}[A_{10}][P] + k_{11}[A_{10}P] \\
\frac{d[A_{11}P]}{dt} &= k_7[A_{11}][P] - (k_8 + k_9)[A_{11}P] \\
\frac{d[A_{10}P]}{dt} &= k_{10}[A_{10}][P] - (k_{11} + k_{12})[A_{10}P]
\end{aligned} \right\} \quad (A\ 8)$$

Again, this model is supplemented by conservation conditions for total kinase, phosphatase and substrate. The kinetic constants in this model refer to similar conversion and binding/unbinding reactions as in the ordered model considered in the main text, with the only difference that there are two separate singly modified phosphor-forms, with only A_{01} being further phosphorylated, and A_{10} being dephosphorylated.

A brief analysis of this model is presented. In what follows, the focus is on the functional relationships between different phospho-forms. First, note that at steady state:

$$[AK] = \left(\frac{k_1}{k_2 + k_3} \right) [A][K] \quad (A\ 9)$$

$$[A_{01}K] = \left(\frac{k_4}{k_5 + k_6} \right) [A_{01}][K] \quad (A\ 10)$$

$$[A_{11}P] = \left(\frac{k_7}{k_8 + k_9} \right) [A_{11}][P] \quad (A\ 11)$$

and
$$[A_{10}P] = \left(\frac{k_{10}}{k_{11} + k_{12}} \right) [A_{10}][P] \quad (\text{A } 12)$$

By noting that, steady state implies

and
$$\left. \begin{aligned} k_3[AK] &= k_6[A_{01}K] \\ k_6[A_{01}K] &= k_9[A_{11}P] \\ k_9[A_{11}P] &= k_{12}[A_{10}P] \end{aligned} \right\} \quad (\text{A } 13)$$

From above, it can be seen that all steady-state complex concentrations are directly proportional to that of $A_{11}P$ (proportionality constant only dependent on kinetic constants), which in turn is proportional to the product of the concentrations of A_{11} and P . Further, it can be seen that in a similar way, $[A_{11}]$ and $[A_{10}]$ are proportional, and similarly, so are $[A]$ and $[A_{01}]$. Finally, from the proportionality of complexes, we see that both $[A]$ and $[A_{01}]$ are proportional to $[A_{11}][P]/[K]$ from above.

Now, the conservation of kinase results in

$$[K] = K_{tot} - \alpha[A_{11}][P] \quad (\text{A } 14)$$

for a suitable constant α which is a function of kinetic parameters (obtained by rewriting all other kinase complexes in terms $[A_{11}P]$). Similarly, a conservation of phosphatase results in

$$[P] = \frac{P_{tot}}{(1 + \beta[A_{11}])} \quad (\text{A } 15)$$

for a suitable constant β obtained in terms of kinetic parameters. This is obtained by writing both phosphatase complexes in terms of $[A_{11}][P]$ directly from above.

For the conservation of the substrate, it should be noted here that the substrate may be present in different modified forms as well as complexes. Noting from the comments above, how all of them can be related to $[A_{11}]$ the author ends up with an equation of the form

$$[A_{11}] = \frac{A_{tot}}{\gamma_1 + \gamma_2[P] + \gamma_3[P]/[K]} \quad (A\ 16)$$

for suitable constants $\gamma_1, \gamma_2, \gamma_3$. Now suppose $d[A_{11}]/dK_{tot} = 0$ at some value of K_{tot} . This means that the derivative of the denominator in the expression for $[A_{11}]$ must necessarily be zero. It can be seen by differentiating the expression of $[P]$ that at this value of K_{tot} that $d[P]/dK_{tot} = 0$. Further, as $d([P]/[K])/dK_{tot} = -([P]/[K]^2)dK/K_{tot} + (1/[K])d[P]/dK_{tot}$, it can be seen that the only way the derivative of the denominator is zero is if $d[K]/dK_{tot} = 0$. However, by direct differentiation of the expression for $[K]$, at this location, it can be seen that $d[K]/dK_{tot}$ at this location cannot be zero. Thus, it can be seen that A_{11} cannot be a biphasic function of K_{tot} .

A.4. The Processive mechanism

A.4.1. Processive ordered mechanism

Now the focus is on examining a two-site-ordered processive mechanism. The processive mechanism is realized by kinase binding to substrate, forming a complex which is catalytically converted to A_pK , which then becomes converted to A_{pp} , releasing the kinase. An analogous chain of events happens in the dephosphorylation. The key point here is that the enzyme is held in complexes and is released only in the final step.

The equations for a two-site processive mechanism are (figure 3.2)

$$\left. \begin{aligned}
\frac{d[A]}{dt} &= k_2[AK] - k_1[A][K] + k_8[A_pP] \\
\frac{d[K]}{dt} &= k_2[AK] - k_1[A][K] + k_4[A_pK] \\
\frac{d[AK]}{dt} &= k_1[A][K] - (k_2 + k_3)[AK] \\
\frac{d[A_pK]}{dt} &= k_3[AK] - k_4[A_p][K] \\
\frac{d[A_{pp}]}{dt} &= k_4[A_pK] - k_5[A_{pp}][P] + k_6[A_{pp}P] \\
\frac{d[P]}{dt} &= k_6[A_{pp}P] - k_5[A_{pp}][P] + k_8[A_pP] \\
\frac{d[A_{pp}P]}{dt} &= k_5[A_{pp}][P] - (k_6 + k_7)[A_{pp}P] \\
\frac{d[A_pP]}{dt} &= k_7[A_{pp}P] - k_8[A_pP]
\end{aligned} \right\} \quad (A 17)$$

These reflect the mechanism described above.

Now, at steady state,

$$[AK] = \left(\frac{k_1}{k_2 + k_3} \right) [A][K]$$

$$[A_pK] = \left(\frac{k_3}{k_4} \right) [AK]$$

$$[A_{pp}P] = \left(\frac{k_5}{k_6 + k_7} \right) [A_{pp}][P]$$

and

$$[A_pP] = \left(\frac{k_7}{k_8} \right) [A_{pp}P]$$

Further, from the steady state, it is easy to see that $[A_{pp}P]$ is proportional to $[A_pK]$.

Now, as $[K] + [AK] + [A_pK] = K_{tot}$, kinase concentration satisfies

$$[K] = K_{tot} - \alpha [A_{pp}][P] \quad (A 18)$$

for a suitable constant α which is a function of kinetic parameters (concentrations of all kinase complexes, proportional to $[A_{pp}P]$). Phosphatase also satisfies

$$[P] = \frac{P_{tot}}{(1 + \beta[A_{pp}])} \quad (\text{A } 19)$$

for a suitable constant β . This is obtained from the conservation of phosphatase, writing concentrations of all phosphatase complexes in terms of $[A_{pp}][P]$. Finally, from the relations above and conservation of total substrate, $[A_{pp}]$ satisfies

$$[A_{pp}] = \frac{A_{tot}}{\gamma_1 + \gamma_2[P] + \gamma_3[P]/[K]} \quad (\text{A } 20)$$

for suitable constants $\gamma_1, \gamma_2, \gamma_3$. That such a functional form arises is seen by noting that concentrations of all complexes (kinase and phosphatase) are proportional to $[A_{pp}][P]$, whereas (from above) the concentration of free A is proportional to $[A_{pp}][P]/[K]$.

Now, suppose $d[A_{pp}]/dK_{tot} = 0$. It can be seen from above (very similar to the previous case) that $d[P]/dK_{tot} = 0$. However, differentiating the expression for $[A_{pp}]$, it can be seen that it is necessary that $d[K]/dK_{tot} = 0$, which is impossible from the expression for $[K]$.

This leads to the conclusion that the two-site-ordered processive mechanism cannot exhibit biphasic behaviour. This analysis can be extended in a very straightforward way to ordered n -site processive mechanisms. The only difference is the presence of more intermediate kinase and phosphatase complexes. However, at steady state, the concentrations of all these intermediate forms are proportional to the product of the concentrations of the maximal phosphorylated form and the free phosphatase. It can thus be seen that the argument carries through in exactly the same way also in that case.

A.4.2. Processive cyclic mechanism

This section briefly discusses a cyclic processive two-site mechanism, which is processive, but where the order of phosphorylation and dephosphorylation are different. This results in different intermediate complexes (figure 3.2).

The analysis can be performed in a very similar way to the previous cases. Note that at steady state, for reasons exactly as above (i) the concentration of all kinase complexes is proportional to $[A][K]$. (ii) The concentration of all phosphatase complexes is proportional to $[A_{II}][P]$. This follows in a very simple way by considering the steady state of all components. Further, it is found by balancing the net rates of the forward and backward reaction (or equivalently considering the steady state of $A_{II} + A_{II}P$) that $[A][K]$ is proportional to $[A_{II}][P]$. Now using this information and considering conservation of kinase, phosphatase and substrate results in exactly the same kind of functional relationship as before:

$$[K] = K_{tot} - \alpha[A_{11}][P] \quad (\text{A } 21)$$

$$[P] = \frac{P_{tot}}{(1 + \beta[A_{11}])} \quad (\text{A } 22)$$

$$[A_{11}] = \frac{A_{tot}}{\gamma_1 + \gamma_2[P] + \gamma_3[P]/[K]} \quad (\text{A } 23)$$

for suitable constants α , β , γ_1 , γ_2 , γ_3 . The conclusions follow exactly the same way as in previous sections.

A.4.2. Processive random mechanism

Random mechanisms are generally more complicated than ordered mechanisms. This section shows that a random double-site processive mechanism will not exhibit any partial biphasic behaviour either. A look at the random processive model reveals a more complicated

structure (figure 3.2). However, by considering the steady state for all complexes, the author arrives at the following conclusion: (i) the concentration of all kinase complexes is proportional to $[A][K]$ and for exactly the same reasons (ii) the concentration of all phosphatase complexes is proportional to $[A_{II}][P]$. Now, at steady state (for example by considering the steady state of $[A_{II}] + [(A_{II}P)_I] + [(A_{II}P)_2]$), there is a balance between the net forward and backward reactions:

$$k_4[A_{01}K] + k_8[A_{10}K] = k_{12}[A_{01}P] + k_{16}[A_{10}P] \quad (\text{A } 24)$$

Noting the facts mentioned above regarding the kinase and phosphatase complexes, we see that $[A][K]$ is proportional to $[A_{II}][P]$ just as before. Here again, from the conservation of kinase, phosphatase and substrate, accounting for all complexes, the variables $[K]$, $[P]$ and $[A_{II}]$ satisfy

$$[K] = K_{tot} - \alpha[A_{11}][P] \quad (\text{A } 25)$$

$$[P] = \frac{P_{tot}}{(1 + \beta[A_{11}])} \quad (\text{A } 26)$$

$$[A_{11}] = \frac{A_{tot}}{\gamma_1 + \gamma_2[P] + \gamma_3[P]/[K]} \quad (\text{A } 27)$$

for suitable constants α , β , γ_1 , γ_2 , γ_3 . The conclusions follow exactly the same way as in previous sections. It can thus be seen that the extra complexity of the random mechanism actually does not alter the conclusions.

A.5 Parameters

In this section, all parameter values in each module are outlined. All parameters listed here are dimensionless.

In all the numerical simulation and bifurcation analysis, unless otherwise mentioned, the initial conditions of substrate, $[A]_{t=0}$ ($=A_{\text{tot}}$), and phosphatase, $[P]_{t=0}$ ($=P_{\text{tot}}$), are 40 and 1, respectively, while those for other variables are zero. Furthermore, kinetic parameters (k) such as binding, unbinding and catalytic constant are equal to 1. All simulation results from phosphorylation models here correspond to the distributive mechanism. Simulation results are complemented by analytical results to explain key trends. In addition to the results shown, other sensitivity analysis was also performed.

Figure 3.3 – Parameters (catalytic constants) for the ordered double site phosphorylation model.

(a) *Non-biphasic behaviour*
 $k_3 = 0.1$ and $k_6 = 0.1$

(b) *Biphasic behaviour*
 $k_3 = 0.1$ and $k_6 = 0.01$

Figure 3.4 – Parameters (catalytic constants) for the ordered double site phosphorylation model.

$k_3 = 0.1$ and $k_6 = 0.01$

Figure 3.5 – Parameters for the ordered double site phosphorylation model.

(a) *Effect of the binding constant of phosphorylation in the first cycle (k_1)*
 $k_3 = 0.1$, $k_6 = 0.01$ and k_1 (the binding constant of the first phosphorylation step) was varied, taking values 0.01, 0.34, 0.67 and 1.

(b) *Effect of the catalytic constant of phosphorylation in the first cycle (k_3)*
 $k_6 = 0.01$ and k_3 (the catalytic constant of the first phosphorylation step) was varied, taking values 0.01, 0.04, 0.07 and 0.1.

(c) *Effect of the binding constant of dephosphorylation in the first cycle (k_{10})*

$k_3 = 0.1$, $k_6 = 0.01$ and k_{10} (the binding constant of dephosphorylation in the first cycle) was varied, taking values = 1, 4, 7 and 10.

Figure 3.6 – Parameters for the ordered double site phosphorylation model.

In (a) and (b), $k_3 = 0.1$ and $k_6 = 0.01$.

(a) *Total concentration of phosphatase, P_{tot} , (also the initial condition of phosphatase) was varied, taking values 1, 1.5, 2 and 2.5.*

(b) *Total concentration of phosphatase, P_{tot} , (also the initial condition of phosphatase) was varied, taking values 0.25, 0.50, 0.75 and 1.*

Figure 3.7 – Parameters for various models.

(a) *Random double site phosphorylation model (left)*

Catalytic constants of the two phosphorylation steps in upper pathway are 0.1 and 0.01, respectively, while those in the lower pathway are 0.1 and 0.1, respectively.

Ordered double phosphorylation model (right)

$k_3 = 0.1$, $k_6 = 0.01$

(b) *Ordered triple site phosphorylation model*

The catalytic constants of the three phosphorylation steps are 0.1, 0.1 and 0.01, respectively.

(c) *Ordered triple site phosphorylation model*

The total concentration of substrate (A_{tot}) is 100, while the catalytic constants of the three phosphorylation steps are 0.035, 0.007 and 0.007, respectively.

Figure 3.8 – Parameters for various models.

(a) Ordered double phosphorylation model (demonstrating threshold behaviour)

$k_1 = 15$, $k_3 = 0.1$, $k_6 = 0.01$

(b) Random double phosphorylation model (demonstrating bistable behaviour)

Both catalytic constants of the two phosphorylation steps in upper pathway are 0.01, while those in the lower pathway are all equal to 0.1. In addition, the total concentration of substrate (A_{tot}) is 60 and the binding constant of the first phosphorylation step in the upper pathway is 5.

(c) Ordered triple phosphorylation model (demonstrating bistable behaviour)

The total concentration of substrate (A_{tot}) is 60, while the catalytic constants of the three phosphorylation steps are 0.01, 0.1 and 0.01, respectively. The binding constant of the first phosphorylation step is 4.

Figure 3.9 – Parameters for the ordered double site phosphorylation model.

(a) *Total kinase concentration (K_{tot}) is less than that of phosphatase (P_{tot})*

$$K_{tot} = 5, P_{tot} = 20 \text{ and } k_3 = k_6 = k_9 = k_{12} = 0.1$$

(b) *Total kinase concentration (K_{tot}) is higher than that of phosphatase (P_{tot})*

$$K_{tot} = 20, P_{tot} = 5, k_3 = 0.01, k_6 = 0.1 \text{ and } k_9 = k_{12} = 0.15$$

Appendix B

Mixed mechanism of multisite phosphorylation

In this section, a number of aspects are discussed in turn: (i) analytical explanation of certain results, (ii) the models which are used in analysis and (iii) the parameters used in the main text. The computational results presented in chapter 4 are complemented by analytical work, which is presented in (Suwanmajo and Krishnan, 2015) and reproduced in this section.

B.1 Analysis of models

B.1.1 Mixed mechanisms where one direction is purely processive.

To complement some of the results presented earlier, analytical approaches are employed in a few cases. In this section, it is shown that (i) a mixed mechanism with phosphorylation distributive and dephosphorylation processive (or the other way around) will not result in a biphasic dose–response curve for the maximally modified substrate as a function of total kinase concentration. This is true irrespective of the number of modification sites. (ii) A two-site modification equivalent of the scenario considered in (i) is not capable of exhibiting bistability and this is extended to n -site analogues. (iii) A three site modification mechanism with mixed phosphorylation and processive dephosphorylation does not exhibit either bistable behaviour or a biphasic dose–response curve of the kind considered above. This is extended to n -site modification mechanisms with processive dephosphorylation and mixed mechanisms of phosphorylation.

This section begins by analysing potential biphasic dose–response characteristics in the maximally modified substrate as the total kinase concentration is varied, when the phosphorylation is distributive and dephosphorylation is processive. By examining the steady state of the two-site model (described explicitly in §B.2), it is able to note the following facts:

- (1) All kinase complex concentrations are proportional to the product of the relevant substrate and the free kinase concentrations. This simply follows by considering the steady state of the complexes.
- (2) All phosphatase complex concentrations are proportional to that of $[A_{pp}P]$. This follows by examining the steady state of the relevant phosphatase complexes (noting that dephosphorylation is processive) and relating them to $[A_{pp}P]$. It also follows that the concentration of $A_{pp}P$ is proportional to the concentration of A_{pp} and that of P . This follows by considering the steady state of $A_{pp}P$.
- (3) The concentration of A_pK is proportional to that of $A_{pp}P$. This follows by considering the steady state of $A_{pp} + A_{pp}P$. Along with the previous facts, it follows that A_p is proportional to $[P]/[K]$.
- (4) By considering the steady state of $[A_p] + [A_pK]$, it can be seen that $[AK] \propto [A_pK]$, from which it follows that $[A] \propto [A_p]$ (also see discussion in the text).
- (5) Finally, it can be seen that $[A_{pp}P] \propto [AK]$ (by considering the steady state of $[A] + [AK]$).

Now, the conservation equations are

$$\left. \begin{aligned} [A] + [AK] + [A_pK] + [A_p] + [A_{pp}] + [A_{pp}P] + [A_pP] &= A_{tot} \\ [K] + [AK] + [A_pK] &= K_{tot} \\ [P] + [A_pP] + [A_{pp}P] &= P_{tot} \end{aligned} \right\} \quad (B\ 1)$$

and

By taking account of the above facts and incorporating them in the above equations, it can be seen that $[K] = K_{tot} - \alpha[A_{pp}][P]$ for some constant α (noting that all kinase complexes

are proportional to $[A_{pp}P]$). Note that $[P] = P_{tot}/(1 + \beta[A_{pp}])$, for a suitable constant β . This follows by writing concentrations of all phosphatase complexes in terms of $[A_{pp}P]$. Simplifying the conservation equation for the substrate, the concentration of the maximally phosphorylated substrate can be written as

$$[A_{pp}] = \frac{A_{tot}}{1 + \gamma[P] + \zeta[P]/[K]}, \quad (B\ 2)$$

where γ, ζ are constants.

This is done by eliminating all variables in terms of $[A_{pp}]$, $[P]$ and $[K]$. Suppose that there is a biphasic response for the maximally phosphorylated substrate as a function of K_{tot} . This means that at some location $d[A_{pp}]/dK_{tot} = 0$. It can be seen from the equation for $[P]$ that if $d[A_{pp}]/dK_{tot} = 0$ (the requirement for a biphasic response), then it follows that $d[P]/dK_{tot} = 0$. By differentiating the equation for $[K]$ with respect to K_{tot} it can be seen that, if such a condition were to hold good, then it immediately follows that $dK/dK_{tot} = 1$. However, by differentiating the equation for $[K]$ with respect to K_{tot} , it is found that if $d[P]/dK_{tot} = 0$ and $d[K]/dK_{tot} = 1$, then $d[A_{pp}]/dK_{tot}$ can never be zero, contradicting the assumption. Hence biphasic responses for the maximally phosphorylated substrate are ruled out.

While the above analysis was discussed for two-site modification, it follows in an essentially identical way for more than two sites. Multiple modifications result in more kinase and phosphatase complexes. In fact, all the points mentioned above carry through if $[A_{pp}]$ is replaced by the concentration of the maximally phosphorylated substrate. All additional kinase complex concentrations are still proportional to that of $[AK]$, and hence all additional intermediate phosphoforms are proportional to that of $[A]$. All phosphatase complexes are proportional to that of the phosphatase complex of the maximally phosphorylated substrate.

Noting this, it can be seen that the argument above carries through in an essentially identical way when the number of sites is increased.

It is able to consider the case where the phosphorylation is processive and dephosphorylation distributive. Using the same approach and reducing the relevant steady-state equations to those involving A_{pp} , P and K , it can be seen that a similar functional form of equations from which it follows that a biphasic dose–response curve of the kind above is ruled out.

The focus now returns to the case of distributive phosphorylation and processive dephosphorylation. This considers the issue of potential multi-stability in this model. The starting point here will be the expression for $[A_{pp}]$ in terms of $[P]$, $[K]$ and other parameters derived above. This requires the functional form of this equation. Inverting this equation results in:

$$1 + \gamma[P] + \frac{\zeta[P]}{[K]} = \frac{A_{tot}}{[A_{pp}]} \quad (B\ 3)$$

In this equation, it is able to substitute for $[K]$ and $[P]$ in terms of $[A_{pp}]$ from above. By rearranging terms, an expression of the functional form is obtained:

$$1 + \frac{\gamma P_{tot}}{K_{tot} + (\beta K_{tot} - \alpha P_{tot})[A_{pp}]} + \frac{\zeta P_{tot}}{1 + \beta[A_{pp}]} = \frac{A_{tot}}{[A_{pp}]} \quad (B\ 4)$$

By clearing various constants, this can be reduced to an equation of the functional form

$$\frac{A_{tot}}{x} = \frac{a}{b+x} + \frac{c}{d+x} + 1 \quad (B\ 5)$$

where x refers to $[A_{pp}]$, where a , b , c , d are positive constants. This assumes the constant $\beta K_{tot} - \alpha P_{tot}$ is positive. If this constant is negative, the functional form of the equation is

$$\frac{A_{tot}}{x} = \frac{a}{b-x} + \frac{c}{d+x} + 1 \quad (\text{B } 6)$$

Each of these cases will be examined separately to assess the possibility of multi-stability.

Case 1: $\beta K_{tot} - \alpha P_{tot} > 0$

In this case make a change of variable $y = I/x$. This results in an equation of the functional form

$$A_{tot}y = \frac{c_1y}{c_2+y} + \frac{c_3y}{c_4+y} + 1 \quad (\text{B } 7)$$

Note that the RHS starts at 1 (when $y = 0$) and is an increasing saturating function of y (y takes on only positive values). It is straightforward to see that the derivative of the RHS is a decreasing function of y , since it involves linear combinations of $I/(c_2 + y)^2$ and $I/(c_4 + y)^2$. Since the LHS increases from zero to infinity as y increases, it can be seen that there is at least one intersecting point of these functions. Furthermore, it can be seen that the derivative of the RHS has to be less than that of the LHS at this location (since the RHS is larger at $y = 0$). But since the derivative of the RHS is a decreasing function of y and that of the LHS is a constant it can be seen that subsequently the LHS will always be greater than the RHS, and hence only one positive root results.

Case 2: $\beta K_{tot} - \alpha P_{tot} < 0$

Here the equation is written in terms of x directly. Note that any feasible root has to satisfy $b - x > 0$ to ensure positive free enzyme concentrations.

Writing out the equation results in an equation of the form

$$A_{tot}(b - x)(d + x) = x[a(d + x) + c(b - x) - (b - x)(d + x)] \quad (\text{B } 8)$$

This is a cubic equation which has potentially three roots. By moving the RHS to the LHS, it is found that the coefficient of x^3 and that of the constant term have the same sign (all constants are positive here). This means that at least one of the roots is negative since the product of roots is negative. Furthermore, the equation is rewritten in terms of the variable $u = b - x$. This results in an equation of the form

$$A_{tot}u(b + d - u) = (b - u)[a(b + d - u) + cu + u(b + d - u)] \quad (\text{B } 9)$$

Taking all terms to one side, it is found that by similar reasoning one of the roots for u is negative. This corresponds to a second unfeasible steady state. This is easily understood from a graphical inspection as well. Thus, it can be seen that there can be only one feasible steady state and multi-stationarity is ruled out.

While the above analysis has been performed for a two-site mixed model with phosphorylation distributive and dephosphorylation processive, it directly generalizes to the case for n -site modification with the same mechanism under the same assumptions. This is because the procedure used to eliminate all complexes and intermediate phosphoforms results in the same functional form of equation. Naturally switching the mechanism of phosphorylation and dephosphorylation results in the same conclusion.

B.1.2 Mixed mechanism in phosphorylation

This section focuses on discussing two mixed three-site mechanisms where the dephosphorylation is processive. The two mechanisms differ only in whether the first two or the last two phosphorylation steps are processive. This section starts by consider the latter case. Here it can be seen that

- (1) $[AK] \propto [A][K]$ while $[A_pK], [A_{pp}K] \propto [A_p][K]$.
- (2) All phosphatase complex concentrations are proportional to that of $[A_{ppp}P]$.
- (3) The concentration of $A_{pp}K$ is proportional to that of $A_{ppp}P$. This follows by considering the steady state of $A_{ppp} + A_{pp}P$. Then it follows that A_p is proportional to $[A_{ppp}][P]/[K]$.
- (4) By considering the steady state of $[A_p] + [A_pK]$, it can be seen that $[AK] \propto [A_pK]$, from which it follows that $[A] \propto [A_p]$.
- (5) Finally, it can be seen that $[A_{ppp}P] \propto [AK]$.

Therefore, it can be seen that the free phosphatase satisfies an equation of the form $[P] = P_{tot}/(1 + \beta[A_{ppp}])$ and the free kinase satisfies an equation $[K] = K_{tot} + \alpha[A_{ppp}][P]$. In the conservation condition for substrate, in addition to the complexes, only $[A]$, $[A_p]$, $[A_{ppp}]$ are included.

The procedure for analysing this is exactly as above and again results in an equation

$$[A_{ppp}] = \frac{A_{tot}}{1 + \gamma[P] + \zeta[P]/[K]} \quad (\text{B } 10)$$

The analysis performed in the above case carries through here as well, and it can be concluded that there is no bistability or biphasic response of A_{ppp} as a function of K_{tot} .

Following this, the next step is to examine the situation where the first two steps are processive and the last phosphorylation step is distributive. In the above, the only changes are as follows:

- (1) $[AK], [A_pK] \propto [A][K]$ while $[A_{pp}K] \propto [A_{pp}][K]$.
- (2) The concentration of $A_{pp}K$ is proportional to that of $A_{pp}P$. Then it follows that $[A_{pp}]$ is proportional to $[A_{pp}][P]/[K]$.

The equations for free kinase/phosphatase have similar functional forms as before. The only change in the substrate conservation is that, in addition to the complexes, only $[A]$, $[A_{pp}]$, $[A_{ppp}]$ are included.

Again this results in an equation for $[A_{ppp}]$ that is of the same form as before and is analysed in exactly the same way, with the same conclusions.

B.1.2.1. Mechanisms with mixed phosphorylation and processive dephosphorylation

The analysis above examines the combination of processive and distributive steps in three-site phosphorylation with processive dephosphorylation. This section focuses on considering an n -site analogue of this situation, with an arbitrary combination of distributive and processive steps in phosphorylation. It is noted that

- (1) All phosphatase complex concentrations are proportional to that of $[A_{p^n}P]$. This follows by examining the steady state of the relevant phosphatase complexes (noting that dephosphorylation is processive) and relating them to $[A_{p^n}P]$. It also follows that the concentration of $A_{p^n}P$ is proportional to the product of concentrations of $A_{p^n}P$ and that

of P. This follows by considering the steady state of $A_{p^n}P$ ($A_{p^n}P$ refers to the maximally phosphorylated phosphoform).

- (2) The concentration of $A_{p^{n-1}}K$ is proportional to that of $A_{p^n}P$, which in turn is proportional to $[A_{p^n}][P]$. This follows by considering the steady state of $A_{p^n} + A_{p^n}P$
- (3) The steady state of $[A] + [AK]$ implies that $[A_{p^n}P] \propto [AK]$. $[AK] \propto [A][K]$.
- (4) By considering the steady states for $A_{p^m} + A_{p^m}K$, for $m = 1, 2, \dots, n-1$ sequentially, it can be seen that all $[A_{p^m}K] \propto [AK]$, for $m = 1, 2, \dots, n-1$. If the formation of this kinase complex was via a distributive step, then the concentration of this kinase complex is proportional to $[A_{p^m}][K]$ and hence $[A_{p^m}] \propto [A]$. In this manner, all kinase complexes can be related to one another and all free phosphoforms can be related to $[A_{p^m}]$, $[P]$, $[K]$. The relations between these phosphoforms are similar to that observed above.
- (5) By incorporating the conservation of substrate, kinase and phosphatase, it can be seen that the free phosphatase satisfies an equation of the form $[P] = P_{\text{tot}}/(1 + \beta[A_{p^n}])$ and the free kinase satisfies an equation $[K] = K_{\text{tot}} - \alpha[A_{p^n}][P]$. In the conservation condition for substrate, in addition to the complexes, the relevant individual phosphoforms are incorporated

The procedure for analysing this is exactly as above and again results in an equation

$$[A_{p^n}] = \frac{A_{\text{tot}}}{1 + \gamma[P] + \zeta[P]/[K]} \quad (\text{B } 11)$$

The analysis performed in the above case carries through here as well, and it can be concluded that there is no bistability or biphasic response of A_{p^n} as a function of K_{tot} .

Therefore, if dephosphorylation is processive, the above conclusions continue to hold good, for any sequence of distributive and processive mechanisms in phosphorylation.

B.1.3 Mixed distributive and processive phosphorylation and dephosphorylation

This section demonstrates the following statement: Consider a model of ordered n -site modification mechanism, comprised of a combination of purely processive and purely distributive steps. If the mechanism is such that no single partial phosphoform (apart from the completely unmodified and completely modified species) is associated with both distributive phosphorylation and distributive dephosphorylation (i.e. it is not released in both phosphorylation and dephosphorylation), then the resulting model will not exhibit bistability or biphasic behaviour for the maximally phosphorylated substrate. Note that if there is one phosphoform associated with both distributive phosphorylation and dephosphorylation, the resulting model (in some parameter regime) has the qualitative capability of an ordered 2-site distributive model and the capacity to exhibit bistability and biphasic behaviour. This assertion is demonstrated through a sequence of steps. It is shown that, at steady state

(i) The concentrations of all partial phosphoforms released during phosphorylation are proportional.

(ii) The concentrations of all partial phosphoforms released during dephosphorylation are proportional to one another.

(iii) All kinase complex concentrations are proportional to that of AK .

(iv) All phosphatase concentrations are proportional to that of $A_{pn}P$

(v) $[AK]$ is proportional to $[A_{pn}P]$

Suppose $A_{pm1}, A_{pm2} \dots A_{pmk}$, represent the partial phosphoforms released during phosphorylation, and suppose $A_{pn1}, A_{pn2} \dots A_{pni}$ represent those released during dephosphorylation. These sets of partial phosphoforms are disjoint. To establish (i) and (iii) the author starts by noting that phosphorylation occurs through a sequence of distributive and

processive steps. In the distributive steps, phosphoforms may be released, and these can rebind to the kinase. Now the concentration of all kinase complexes connected through a sequence of processive modifications are proportional to one another at steady state, for the same reason as studied earlier. Now consider the sequence of species involved between two successive distributive steps: if one analyse the condition for the steady state of $A_{pm1} + A_{pm1}K + \dots + A_{pm2-1}K$, it is found that the production must match removal. This leads to the fact that $A_{pm1-1}K$ is proportional to $A_{pm2-1}K$: in other words the 4 concentrations of two kinase complexes separated by a distributive step are also proportional. By repeating this for the entire sequence of phosphorylation transitions, it is found that at steady state all kinase complex concentrations are proportional, and proportional to that of AK . This established (iii).

Furthermore, since all the kinases complexes associated with phosphoforms which are released, are proportional to the product of the corresponding phosphoform and the free kinase concentrations, and since all kinase complexes are proportional, it can be seen that concentrations of all released phosphoforms are proportional to one another. Since all kinase concentrations are proportional to that of AK , it can be seen that the concentration of these released phosphoforms is proportional to A . This establishes (i).

Now by a parallel argument, applied to dephosphorylation, it is immediately seen that all phosphatase complex concentrations are proportional to $A_{pn}P$ and, the concentrations of all partial phosphoforms released during dephosphorylation are proportional to one another at steady state. Finally examining the steady state of $[A] + [AK]$, reveals that $[AK]$ is proportional to $[A_{pn}P]$, as discussed previously.

Conservation of phosphatase and kinase concentration results in $[P] = P_{tot}/(1 + \beta[A_{pn}])$ and the free kinase satisfies an equation $[K] = K_{tot} - \alpha[A_{pn}][P]$, for exactly the reasons studied previously. Substrate conservation results in an equation of the form $\alpha_1[A] + \alpha_2[A_{pn}]$

$+ \alpha_3[AK] + \alpha_4[A_{pn}][P] = A_{tot}$. The contributions of the kinase and phosphatase complexes are contained in the last two terms, while those of the phosphoforms released during phosphorylation and dephosphorylation are represented in the first two terms. Now using the relationships above, $A_{pn} = A_{tot}/(\gamma_1 + \gamma_2[P] + \gamma_3[P]/[K])$, which is exactly the form of equations studied earlier. Thus it can be concluded that the systems cannot exhibit bistability or biphasic behaviour for the maximally phosphorylated substrate (A_{pn}).

B.2 Model

Equations of a representative model are represented in the Appendix. This is complemented by presenting the equations of other models. Note that these other models are all contained within the comprehensive mixed model. First, model 22 is presented. This model is the translation of the diagrammatic depiction of the model (Figure 4.9) into mathematical terms. Here as usual $[A]$, $[A_p]$ and $[A_{pp}]$ represent the concentrations of the unmodified, singly modified and doubly modified substrate, $[K]$ and $[P]$ refer to the free kinase and phosphatase concentrations, $[AK]$, $[A_pK]$ to the concentrations of the kinase complexes and $[A_{pp}P]$ and $[A_pP]$ to the phosphatase complexes. The various rate constants here correspond to those in Figure 4.9 in the chapter 4.

$$\frac{d[A]}{dt} = k_2[AK] - k_1[A][K] + k_{10}[A_pP]$$

$$\frac{d[K]}{dt} = k_2[AK] - k_1[A][K] + k_4[A_pK]$$

$$\frac{d[AK]}{dt} = k_1[A][K] - (k_2 + k_3)[AK]$$

$$\frac{d[A_pK]}{dt} = k_3[AK] - k_4[A_pK]$$

$$\frac{d[A_{pp}]}{dt} = -k_5[A_{pp}][P] + k_6[A_{pp}P] + k_4[A_pK]$$

$$\frac{d[P]}{dt} = (k_6 + k_7)[A_{pp}P] - k_5[A_{pp}][P] + (k_9 + k_{10})[A_pP] - k_8[A_p][P]$$

$$\frac{d[A_{pp}P]}{dt} = k_5[A_{pp}][P] - (k_6 + k_7)[A_{pp}P]$$

$$\frac{d[A_p]}{dt} = -k_8[A_p][P] + k_7[A_{pp}P] + k_9[A_pP]$$

$$\frac{d[A_pP]}{dt} = k_8[A_p][P] - (k_9 + k_{10})[A_pP]$$

Model 33 is then presented: this is described by the following equations.

$$\frac{d[A]}{dt} = k_2[AK] + k_{12}[A_pP] - k_1[A][K]$$

$$\frac{d[K]}{dt} = (k_2 + k_3)[AK] + k_7[A_{pp}K] - k_1[A][K] + k_5[A_pK] - k_4[A_p][K]$$

$$\frac{d[AK]}{dt} = k_1[A][K] - (k_2 + k_3)[AK]$$

$$\frac{d[A_p]}{dt} = k_3[AK] + k_5[A_pK] - k_4[A_p][K]$$

$$\frac{d[A_pK]}{dt} = k_4[A_p][K] - (k_5 + k_6)[A_pK]$$

$$\frac{d[A_{pp}K]}{dt} = k_6[A_pK] - k_7[A_{pp}K]$$

$$\frac{d[A_{ppp}]}{dt} = k_7[A_{pp}K] + k_9[A_{ppp}P] - k_8[A_{ppp}][P]$$

$$\frac{d[P]}{dt} = k_9[A_{ppp}P] + k_{12}[A_pP] - k_8[A_{ppp}][P]$$

$$\frac{d[A_{ppp}P]}{dt} = k_8[A_{ppp}][P] - (k_9 + k_{10})[A_{ppp}P]$$

$$\frac{d[A_{pp}P]}{dt} = k_{10}[A_{ppp}P] - k_{11}[A_{pp}P]$$

$$\frac{d[A_pP]}{dt} = k_{11}[A_{pp}P] - k_{12}[A_pP]$$

Again this is a diagrammatic depiction of the model presented in Figure 4.9. The notation is the same as above, with $[A_{ppp}]$ being the concentration of maximally phosphorylated substrate, $[A_{ppp}P]$ that of the phosphatase complex and $[A_{pp}K]$ that of the kinase complex which produces the maximally phosphorylated substrate. Again, the rate constants correspond to those depicted in figure 4.9. Model 34 which is described by:

$$\frac{d[A]}{dt} = k_2[AK] + k_{12}[A_pP] - k_1[A][K]$$

$$\frac{d[K]}{dt} = k_2[AK] + k_4[A_pK] + (k_6 + k_7)[A_{pp}K] - k_1[A][K] - k_5[A_{pp}][K]$$

$$\frac{d[AK]}{dt} = k_1[A][K] - (k_2 + k_3)[AK]$$

$$\frac{d[A_pK]}{dt} = k_3[AK] - k_4[A_pK]$$

$$\frac{d[A_{pp}]}{dt} = k_4[A_pK] + k_6[A_{pp}K] - k_5[A_{pp}][K]$$

$$\frac{d[A_{pp}K]}{dt} = k_5[A_{pp}][K] - (k_6 + k_7)[A_{pp}K]$$

$$\frac{d[A_{ppp}]}{dt} = k_7[A_{pp}K] + k_9[A_{ppp}P] - k_8[A_{ppp}][P]$$

$$\frac{d[P]}{dt} = k_9[A_{ppp}P] + k_{12}[A_pP] - k_8[A_{ppp}][P]$$

$$\frac{d[A_{ppp}P]}{dt} = k_8[A_{ppp}][P] - (k_9 + k_{10})[A_{ppp}P]$$

$$\frac{d[A_{pp}P]}{dt} = k_{10}[A_{ppp}P] - k_{11}[A_{pp}P]$$

$$\frac{d[A_pP]}{dt} = k_{11}[A_{pp}P] - k_{12}[A_pP]$$

The notation is the same as that above, with rate constants corresponding to that depicted in Figure 4.9. The comprehensive mixed model equations are not presented in detail here.

B.3 Parameter values in Figures

This section presents some details regarding the parameters employed. Non-dimensionless parameters are used in all cases. Chapter 4 analyses the information processing capabilities of mixed multi-site phosphorylation systems in which substrate is in excess over enzyme. In all the numerical simulation and bifurcation analysis, unless otherwise mentioned, the initial conditions of substrate, also equal to total substrate (A_{tot}), and phosphatase, (equal to total phosphatase, P_{tot}), are 40 and 1, respectively, while those for other variables are zero. Furthermore, kinetic parameters (k) such as binding, unbinding and catalytic constant are equal to 1, unless otherwise mentioned. All simulation results from phosphorylation models here correspond to both pure and mixed mechanisms, which is illustrated in Figure B1 (in the appendix B) and Figure 4.9 (in chapter 4). The parameters are summarised in the table which follows. In places where relatively high catalytic constants are chosen, a mass-action description of the same step gives similar results.

Figure 4.3 Parameters (catalytic constants) for ordered double-site phosphorylation models: purely distributive and mixed phosphorylation (model 21 and model 22) (a) Relatively high catalytic constant for the second phosphorylation step (i) Purely distributive model: $k_6 = 100$ (see supplementary figure) (ii) Model 21: $k_6 = 100$ Model 22: $k_4 = 100$ (b) Relatively low catalytic constant for the second phosphorylation step (i) Purely distributive model: $k_6 = 0.01$ (ii) Model 21: $k_6 = 0.01$ (blue line) Model 22: $k_4 = 0.01$ (green-dashed line)

Figure 4.4 Parameters for ordered double-site phosphorylation models: purely distributive and mixed phosphorylation (model 22) (a) Unsteady state property (Oscillation in 2-site mixed systems) Total kinase (K_{tot}) and total phosphatase (P_{tot}) is equivalent to 17.5 and 5, respectively; Model 22: $k_5 = 100$, $k_7 = 0.9$, $k_8 = 3$ and $k_{10} = 100$. In (b), K_{tot} is varied. (c) Purely distributive model: $k_7 = 100$, $k_9 = 0.9$, $k_{10} = 3$ and $k_{12} = 100$. The same P_{tot} as above.

Figure 4.7 Parameters (catalytic constants) for ordered triple-site phosphorylation models: purely distributive and mixed phosphorylation (model 35 and model 36) (a) Relatively high in the catalytic constant for the last phosphorylation step (i) Purely distributive model: $k_9 = 100$ (ii) Model 35: $k_7 = 100$ (iii) Model 36: $k_7 = 100$ (b) Relatively low in the catalytic constant for the last phosphorylation step (i) Purely distributive model: $k_9 = 0.01$ (ii) Model 35: $k_7 = 0.01$ (iii) Model 36: $k_7 = 0.01$

Figure 4.8 Parameters for ordered mixed triple-site phosphorylation models: model 32, model 35 and model 36) (a) Unsteady state property (Oscillation in 3-site mixed systems) Total kinase (K_{tot}) and total phosphatase (P_{tot}) is equivalent to 25 and 5, respectively (i) Model 32: $k_6 = 100$, $k_8 = 0.9$, $k_9 = k_{12} = 3$ and $k_{11} = k_{14} = 100$ (ii) Model 35: $k_8 = 100$, $k_{10} = 0.9$, $k_{11} = k_{14} = 3$ and $k_{13} = k_{16} = 100$ (iii) Model 36: $k_8 = 100$, $k_{10} = 0.9$, $k_{11} = k_{14} = 3$ and $k_{13} = k_{16} = 100$ (b) Steady state property All kinetic parameters and the total amount of phosphatase are the same as (a)

Appendix C

Bridging the gap between the multi-site phosphorylation in isolation and as parts of signalling networks

This section presents some details regarding the parameters employed in chapter 5. Non-dimensionless parameters are used in all cases. Chapter 5 analyses the information processing capabilities of multisite phosphorylation when it functions as a part of signalling networks. Four aspects are considered: (i) The effect of enzyme activation (ii) The effect of retroactivity due to a phosphorylated substrate sequestered in some downstream pathway (iii) The effect of production and degradation of some substrate species and (iv) The effect of embedding a multisite module in some typical network modules. In all the numerical simulation and bifurcation analysis, unless otherwise mentioned, the initial conditions of substrate, also equal to total substrate (A_{tot}), and phosphatase, (equal to total phosphatase, P_{tot}), are 40 and 1, respectively, while those for other variables are zero. Furthermore, kinetic parameters (k) such as binding, unbinding and catalytic constant are equal to 1, unless otherwise mentioned. The ordered double distributive phosphorylation model used here is illustrated in figure 3.2d (in the chapter 3) and model of explicit activation, retroactivity, open systems and feedback and feed-forward module is presented in the chapter 5.

C.1 Explicit activation

Figure 5.5

$K_{\text{tot}} = 17$, $P_{\text{tot}} = 17$, $k_7 = 200$ and $k_{12} = 40$

(b) $S_1 = S_2 = 1$, $k_{a,p} = 0.032$

Figure 5.6

$K_{\text{tot}} = 30$, $P_{\text{tot}} = 17$, $k_1 = 10$, $k_7 = 100$ and $k_{12} = 40$

(b) $S_1 = S_2 = 0.5$, $k_{a,p} = 0.32$ and $k_3 = 0.6$

Figure 5.7

(b) All parameters and the total amount of enzyme are the same as 5.5b

(c) $K_{\text{tot}} = 40$, $k_{a,K} = 0.032$ and other parameters are the same as (b)

Figure 5.8

(a) $S_1 = S_2 = 0.1$ and other parameters are the same as 5.5b

(b) $K_{\text{tot}} = 30$, $P_{\text{tot}} = 1$, $S_1 = 0.0275$, $S_2 = 0.03$, $k_1 = 100$, $k_3 = 0.01$, $k_7 = 100$, $k_9 = 0.9$, $k_{10} = 3$, $k_{12} = 10$, $k_{a,K} = 0.1$ and $k_{a,p} = 0.1$

Figure 5.9

$k_1 = k_6 = 40$, $K_{\text{tot}} = P_{\text{tot}} = 17$, $k_{a,p} = 0.032$

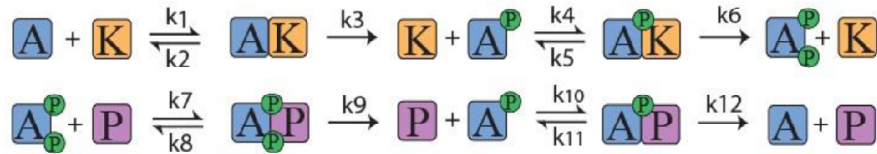
Figure 5.10

$K_{\text{tot}} = 23$, $P_{\text{tot}} = 17$, $k_7 = 100$, $k_{12} = 40$.

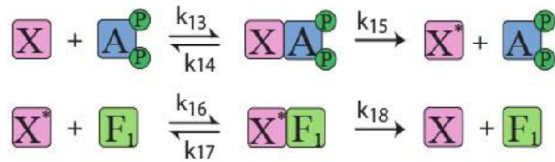
(b) $S_1 = 1$, $S_2 = 1$, $k_{i,K} = 0.01$ and $k_{a,p} = 0.5$

C2 Retroactivity

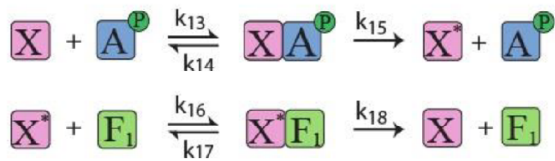
(a) distributive multisite phosphorylation (module in isolation)



(b) Model R1 (A_{pp} sequestered to downstream system)



(c) Model R2 (A_p sequestered to downstream system)



(d) Model R3 (A_{pp} and A_p sequestered to downstream systems)

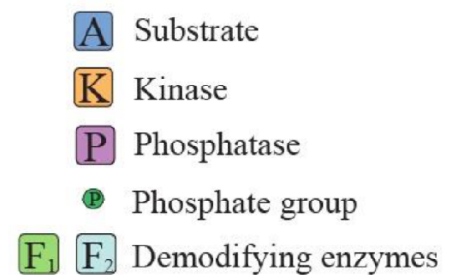
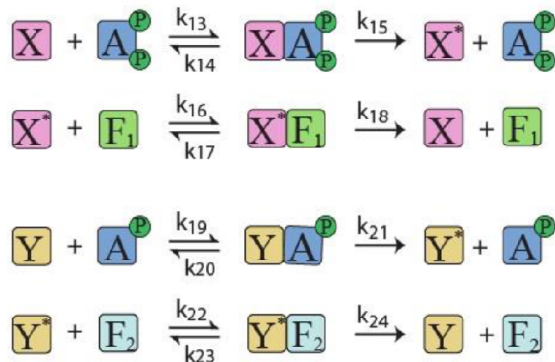


Figure C1 Pictorial depiction of retroactive models.

Figure 5.11

(a)

(i) Isolated system (ordered double distributive phosphorylation)

$$A_{\text{tot}} = 10, P_{\text{tot}} = 0.1, k_1 = 0.1$$

(ii) Retroactivity (Model R1)

Covalent modification cycle (CMC): $k_{13} = 10, k_{16} = 100, F_{\text{tot}} = 7$ X_{tot} were varied, taking values 0, 5, 10 and 15

(b) the same as (a)

Figure 5.12

(a) Isolated system (ordered double phosphorylation)

$$A_{\text{tot}} = 10, P_{\text{tot}} = 0.1, k_1 = k_4 = 100, k_3 = k_6 = 0.1, k_7 = 50$$

(b-d) Retroactivity (Model R1)

Covalent modification cycle (CMC): $k_{13} = 10, k_{15} = 0.1, k_{16} = 100, F_{\text{tot}} = 7$ (b) $X_{\text{tot}} = 10$, (c) $X_{\text{tot}} = 20$ and (d) $X_{\text{tot}} = 30$.

(e) Retroactivity (Model R1)

Covalent modification cycle (CMC): $k_{13} = 100, k_{16} = 100, F_{\text{tot}} = 7, X_{\text{tot}} = 150$ **Figure 5.13**

(a) Isolated system (ordered double phosphorylation)

$$A_{\text{tot}} = 10, P_{\text{tot}} = 0.1, k_1 = k_4 = 100, k_3 = 0.1, k_6 = 10, k_7 = 50$$

(b-c) Retroactivity (Model R1)

Covalent modification cycle (CMC): $k_{13} = k_{16} = 100, F_{\text{tot}} = 7$ (b) X_{tot} were varied, taking values 0, 10 and 20(c) $X_{\text{tot}} = 30$.**Figure 5.14**

(a)

(i) Isolated system (ordered double phosphorylation)

$$A_{\text{tot}} = 30, P_{\text{tot}} = 1, k_6 = 0.01.$$

(ii) Retroactivity

Covalent modification cycle (CMC): $k_{13} = 100$, $k_{15} = 0.1$, $k_{18} = 10$ and $F_{\text{tot}} = 1$

X_{tot} were varied, taking values 0, 10, 20 and 35.

Figure 5.15

(a)

(i) Isolated system (ordered double phosphorylation): the same parameters as figure 5.11a(i)

(ii) Retroactivity (Model R2)

Covalent modification cycle (CMC): the same kinetic parameters as figure 5.11a(ii)

X_{tot} were varied, taking values 0, 30, 60 and 90.

(b) the same as (a)

Figure 5.16

(a)

(i) Isolated system (ordered double phosphorylation): the same parameters as figure 5.12a

(ii) Retroactivity (Model R2)

Covalent modification cycle (CMC): the same kinetic parameters as figure 5.12(e)

X_{tot} were varied, taking values 0, 30, 60 and 90.

(b)

(i) Isolated system (ordered double phosphorylation): the same parameters as figure 5.13a

(ii) Retroactivity (Model R2)

Covalent modification cycle (CMC): the same kinetic parameters as figure 5.13b

X_{tot} were varied, taking values 0, 30, 60 and 90.

Figure 5.17

(a) Isolated system (ordered double phosphorylation)

$A_{\text{tot}} = 35$, $P_{\text{tot}} = 2$, $k_6 = 0.01$.

(b) Retroactivity

Covalent modification cycle (CMC): $k_{13} = 100$, $k_{15} = 0.1$, $k_{16} = 200$ and $F_{\text{tot}} = 2$

X_{tot} were varied, taking values 0, 10, 20 and 30.

Figure 5.18

(a) Isolated system (ordered double phosphorylation): the same as figure 5.12a.

(b-j) Retroactivity (Model R3)

Covalent modification cycle of X: $k_{13} = 10$, $k_{15} = 0.1$, $k_{16} = 100$, $F_{1,\text{tot}} = 7$

Covalent modification cycle of Y: $k_{19} = 10$, $k_{21} = 0.1$, $k_{22} = 100$, $F_{2,\text{tot}} = 7$

C.3 Open systems

Figure 5.19

Model S1: $k_0 = 0.01$, $k_d = 0.1$

Figure 5.20

All parameters and the total amount of enzyme are the same as figure 5.19

(a) P_{tot} were varied, taking values 0.04, 0.06 and 0.10

(b) k_d were varied, taking values 0.06, 0.08 and 0.10

Figure 5.21

Ordered double distributive phosphorylation:

(a) $k_1 = 100$ and $k_3 = 0.01$

(ii) open system (model S1): $k_0 = 0.01$, $k_d = 0.1$

(b) $k_6 = 0.01$

(ii) open system (model S1): $k_0 = 0.01$, $k_d = 0.1$

Figure 5.22

Ordered double distributive phosphorylation:

(a) Closed system: $k_3 = 0.01$, $k_6 = 0.04$, $k_7 = k_{10} = 100$

(b) Open system (model S1): $k_3 = 0.01$, $k_6 = 0.04$, $k_7 = k_{10} = 100$, $k_0 = 0.01$, $k_d = 0.1$.

Figure 5.23

Open 2-site system (model S2)

(a) $k_0 = 0.01$, $k_d = 0.1$.

(b) $k_1 = k_3 = 0.07$, $k_6 = 0.4$, $k_7 = k_{10} = 100$, $k_0 = 0.01$, $k_d = 0.1$, $P_{\text{tot}} = 1$

Figure 5.24

Open 2-site system (model S3)

(a) (ii) Model S3: $k_0 = 0.01$, $k_d = 0.1$.

(b) (ii) Model S3: $k_0 = 0.01$, $k_d = 0.1$

Figure 5.25

(a) (ii) Model S3: $k_0 = 0.01$, $k_d = 0.1$.

(b) $k_1 = 100$, $k_3 = 0.01$

(ii) Model S3: $k_0 = 0.01$, $k_d = 0.1$

(c) $k_3 = 0.05$, $k_6 = 0.1$, $k_7 = 10$, $k_{10} = 20$, $P_{\text{tot}} = 1.6$

(ii) Model S3: $k_0 = 0.01$ and all $k_d = 0.0025$.

Figure 5.26

Open 3-site system

$k_4 = 100$ and $k_6 = 0.01$. For open systems (model S4): $k_0 = 0.01$ and $k_d = 0.1$

Figure 5.27

Open 3-site system

Model S4 and Model S5: $k_0 = 0.01$, $k_d = 0.1$, $k_3 = 0.01$, $k_6 = 0.04$, $k_9 = 0.02$, $k_{10} = k_{13} = k_{16} =$

100 , $P_{\text{tot}} = 1.8$, $K_{\text{tot}} = 500$

Figure 5.28

Model E1: $k_0 = 0.01$, $k_d = 0.1$

Figure 5.29

(a) $k_6 = 0.01$

Model E1: $k_0 = 0.01$, $k_d = 0.1$

(b) $k_1 = 10$, $k_3 = 0.1$, $k_6 = 100$

Model E1: $k_0 = 0.01$, $k_d = 0.1$

Figure 5.30

$k_1 = 30$, $k_3 = 0.2$, $k_4 = 20$, $k_6 = 0.3$

(b) Model E1: $k_0 = 0.01$, $k_d = 0.1$

Figure 5.31

(a) $k_3 = 0.01$, $k_6 = 0.1$, $K_{\text{tot}} = 29$, $P_{\text{tot}} = 1$

(ii) Model E2: $k_0 = 0.01$, $k_d = 0.1$

(b) $k_3 = 0.01$, $k_6 = 0.04$, $k_7 = 40$, $k_9 = 0.1$, $k_{10} = 20$, $k_{12} = 0.3$, $K_{\text{tot}} = 100$, $P_{\text{tot}} = 5$

(ii) Model E2: $k_{0,K} = k_{0,P} = 0.01$, $k_{d,K} = 0.0001$, $k_{d,P} = 0.1$

Figure 5.32

(a) $k_3 = 0.01$, $k_6 = 0.1$, $K_{\text{tot}} = 29$, $P_{\text{tot}} = 1$

(ii) Model ES: $k_0 = 0.01$ (for both enzyme and substrate), $k_{d,P} = 0.1$ and other degradation constants for Kinase and substrates = 0.0008

(b) $k_3 = 0.01$, $k_6 = 0.04$, $k_7 = 100$, $k_9 = 0.1$, $k_{10} = 50$, $k_{12} = 0.3$, $K_{\text{tot}} = 100$, $P_{\text{tot}} = 5$

(ii) Model ES: $k_0 = 0.01$ (for both enzyme and substrate), $k_{d,P} = 0.1$ and other degradation constants for Kinase and substrates = 0.0008

C.4 Positive feedback

Ordered double distributive phosphorylation

Figure 5.33

$K_{\text{tot}} = 60$, $X_{\text{tot}} = 10$. k_{PFB} was varied, taking values: (b) 0.00002, 0.00003, 0.00004, (c) 0.00005, (d) 0.0001 and (e) 0.00015.

Figure 5.34

$K_{\text{tot}} = 60$, $X_{\text{tot}} = 5$, $k_6 = 0.01$ and $k_9 = 10$. k_{PFB} was varied, taking values: (b) 0.0001, 0.001 and (c) 0.002.

Figure 5.35

(a) $K_{\text{tot}} = 70$, $X_{\text{tot}} = 5$, $k_1 = 112.1$, $k_3 = 0.01$, $k_6 = 11$, $k_f = 200$ and $k_{\text{PFB}} = 0.001$ for a(ii).
 (b) $K_{\text{tot}} = 70$, $X_{\text{tot}} = 5$, $k_1 = 40$, $k_3 = 0.01$, $k_6 = 11$, $k_f = 200$ and $k_{\text{PFB}} = 0.0001$ for b(ii).

Figure 5.36

(a) $K_{\text{tot}} = 70$, $X_{\text{tot}} = 5$, $k_1 = 112.1$, $k_3 = 0.01$, $k_6 = 11$, $k_f = 200$ and $k_{\text{PFB}} = 0.0001$ for (b-d).

Figure 5.37

$K_{\text{tot}} = 70$, $X_{\text{tot}} = 5$, $k_1 = 40$, $k_2 = 0.05$, $k_6 = 11$, $k_f = 200$. (b-c) $k_{\text{PFB}} = 0.000005$, (d-e) $k_{\text{PFB}} = 0.000025$ and (f-g) $k_{\text{PFB}} = 0.000045$.

C.4 Negative feedback

Figure 5.38

$K_{\text{tot}} = 70$, $X_{\text{tot}} = 50$, $k_1 = 30$, $k_3 = 0.5$, $k_f = 200$.

(b) k_{NFB} was varied, taking values 0, 0.001, 0.01 and 0.1.

(d) all parameters are the same as (b).

Figure 5.39

$K_{\text{tot}} = 60$, $X_{\text{tot}} = 5$, $k_6 = 0.01$, $k_9 = 10$, $k_f = 0.1$.

(b) k_{NFB} was varied, taking values 0, 0.1, 1 and 10.

(c) k_{NFB} was varied, taking values 10, 20 and 30.

Figure 5.40

$K_{\text{tot}} = 20$, $X_{\text{tot}} = 10$, $k_7 = 200$, $k_9 = 0.9$, $k_{10} = 3$, $k_{12} = 100$ and $k_f = 200$.

(b) $k_{\text{NFB}} = 2$

Figure 5.41

$K_{\text{tot}} = 70$, $X_{\text{tot}} = 10$, $k_1 = 40$, $k_3 = 0.1$, $k_f = 200$, $k_a = 0.9$

(b) $k_{\text{NFB}} = 0.02$. (c) $k_{\text{NFB}} = 0.04$. (d) $k_{\text{NFB}} = 0.02$

Appendix D

Multi-site substrate modification through different kinases and phosphatases

This section presents some details regarding the parameters employed in chapter 6. Non-dimensionless parameters are used in all cases. Chapter 6 analyses the information processing capabilities of multisite phosphorylation with multiple enzyme systems (substrate is in excess over enzyme). In all the numerical simulation and bifurcation analysis, unless otherwise mentioned, the initial conditions of substrate, also equal to total substrate (A_{tot}), and phosphatase, (equal to total phosphatase, P_{tot}), are 40 and 1, respectively, while those for other variables are zero. Furthermore, kinetic parameters (k) such as binding, unbinding and catalytic constant are equal to 1, unless otherwise mentioned. The common double distributive phosphorylation model is illustrated in figure 3.2d (in the chapter 3) and the molecular barcode models (Model MC1 – MC6) are shown in figure 6.2 (in the chapter 6).

Figure 6.3

(b) Model MC1: $P_{1,\text{tot}} = P_{2,\text{tot}} = 0.5$. $K_{2,\text{tot}}$ were varied, taking values 0.50, 0.75 and 1.0

(c) Model MC1: $P_{1,\text{tot}} = P_{2,\text{tot}} = 0.5$. $K_{1,\text{tot}}$ were varied, taking values 0.50, 0.75 and 1.0

(e) the same as (b).

(f) the same as (c).

Figure 6.4

- (a) Common ordered double phosphorylation model: $k_6 = 100$
- (b) Model MC1: $P_{1,tot} = P_{2,tot} = 0.5$, $k_6 = 100$. $K_{2,tot}$ were varied, taking values 0.50, 0.75 and 1.0
- (c) Model MC1: $P_{1,tot} = P_{2,tot} = 0.5$, $k_6 = 100$. $K_{1,tot}$ were varied, taking values 0.50, 0.75 and 1.0
- (d) Common ordered double phosphorylation model: $k_6 = 0.01$
- (e) the same as (b).
- (f) the same as (c).

Figure 6.5

- (a) Model MC2: $K_{2,tot}$ were varied, taking values 0 and 0.50.
- (b) the same as (a)
- (c) Model MC2: $K_{1,tot}$ were varied, taking values 0 and 0.50
- (d) the same as (c)

Figure 6.6

- (b) Model MC2: $K_{2,tot}$ were varied, taking values 0.30, 0.60 and 0.9
- (c) Model MC2: $K_{1,tot}$ were varied, taking values 0.30, 0.60 and 0.9
- (d) Common ordered double phosphorylation model: $k_1 = 100$ and $k_3 = 0.01$
- (e) Model MC2: $k_1 = 100$ and $k_3 = 0.01$ $K_{2,tot}$ were varied, taking values 0.30, 0.60 and 0.9
- (f) Model MC2: $k_1 = 100$ and $k_3 = 0.01$ $K_{2,tot}$ were varied, taking values 0.30, 0.60 and 0.9

Figure 6.7

- (a) Common ordered double phosphorylation model: $k_6 = 0.01$
- (b) Model MC2: $k_6 = 0.01$. $K_{2,tot}$ were varied, taking values 0.01, 0.55 and 0.10
- (c) Model MC2: $k_6 = 0.01$. $K_{1,tot}$ were varied, taking values 0.30, 0.60 and 0.9

Figure 6.8

Model MC2 with explicit activation

(b) Model MC2: $K_{1,tot} = K_{2,tot} = P_{tot} = 17$, $k_4 = 0.1$, $k_6 = 10$, $k_7 = 200$, $k_{12} = 40$. Activation constants: $k_{a1} = k_{a2} = 0.5$ and $k_{a,p} = 0.032$.

(c) the same as (b)

Figure 6.9

Model MC1: $P_{1,tot} = P_{2,tot} = 0.5$, $k_1 = k_4 = k_7 = k_{10} = 10$ and $K_{1,tot} = K_{2,tot} = 1$

Model MC3: $P_{1,tot} = P_{2,tot} = 0.5$, $k_1 = k_4 = k_7 = k_{10} = k_{13} = k_{16} = k_{19} = k_{22} = 10$ and $K_{1,tot} = K_{2,tot} = 1$

Figure 6.10

(a) Model MC1: $k_1 = 0.1$ and other parameters are the same as figure 6.9 (model MC1)

(b) Model MC3: $k_3 = k_9 = 0.1$ and other parameters are the same as figure 6.9 (model MC3)

(c) the same as (a)

(d) the same as (b)

Figure 6.11

Biphasic and oscillatory behaviour in Model MC3

(a) $k_3 = 0.1$, $k_6 = 0.01$, $k_7 = 0.1$, $P_{1,tot} = P_{2,tot} = 0.5$ and $K_{2,tot} = 10$.

(b) $k_3 = 0.1$, $k_6 = 0.01$, $k_7 = 0.1$, $P_{1,tot} = P_{2,tot} = 0.5$ and $K_{1,tot} = 10$.

(c) $k_{13} = 10$, $k_{14} = k_{15} = k_{17} = k_{18} = 0.1$, $k_{19} = k_{22} = 80$, $k_{21} = 0.9$, $k_{24} = 30$, $K_{1,tot} = K_{2,tot} = 9$, $P_{1,tot} = 8$ and $P_{2,tot} = 6$

(d) the same as (c)

(e) the same as (c)

Figure 6.12

Complex behaviour in Model MC3

(a) $k_1 = 100$, $k_3 = 0.01 = k_6 = 0.04$, $k_{16} = 0.01$, $k_{18} = 0.3$, $k_{21} = 20$, $K_{2,tot} = 3.5$, $P_{1,tot} = 0.4$ and $A_{tot} = 60$.

(b) $k_{13} = 10$, $k_{14} = k_{15} = k_{17} = k_{18} = 0.1$, $k_{19} = k_{22} = 80$, $k_{21} = 0.9$, $k_{24} = 30$, $K_{2,tot} = 13$, $P_{1,tot} = 8$ and $P_{2,tot} = 6$

Figure 6.13

(a) Model MC2: $k_1 = 10$, $k_3 = 0.1$, $k_6 = 10$, $K_{2,tot} = 1$

(b) Model MC4: $k_1 = k_7 = 10$, $k_3 = k_9 = 0.1$, $k_6 = k_{12} = 10$, $K_{2,tot} = 1$

(c) Model MC4: $k_1 = 10$, $k_4 = 10 = k_7 = 10$, $k_{10} = 10$, $k_{13} = k_{14} = k_{15} = k_{16} = k_{17} = k_{18} = 0.01$, $k_{19} = 200$, $k_{21} = 0.9$, $k_{22} = 2$, $k_{24} = 30$, $K_{2,tot} = 7$, $K_{2,tot} = 9$, $P_{tot} = 9$.

(d) the same as (c).

Figure 6.14

Model MC4

$k_3 = 10$, $k_4 = 100$, $k_6 = 0.01$, $k_{12} = 0.2$, $k_{19} = k_{22} = 0.001$, $K_{1,tot} = 1.5$, $P_{tot} = 2$ and $A_{tot} = 70$.

(a) $k_1 = 3$; (b) $k_1 = 2$; (c) $k_1 = 1$; (d) $k_1 = 0.5$; (e) $k_1 = 0.01$

Figure 6.15

(b) Model MC5: $K_{2,tot} = 2$

(c) Model MC6: $K_{2,tot} = 2$

Figure 6.16

(a) $k_9 = 100$ (Model MC5 and MC6)

(b) $k_9 = 0.01$ (Model MC5 and MC6)

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