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# NMR STUDIES OF MUTANT PROTEINS.

*by*

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A dissertation submitted to the Imperial College of Science and Technology in candidature for the Diploma of Imperial College, and to the University of London in candidature for the degree of Doctor of Philosophy.

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September 1988.

## ABSTRACT.

A combination of nuclear magnetic resonance (NMR) spectroscopy and site directed mutagenesis have been used to study two enzymes, subtilisin BPN' ($M_r$ 27,500) a bacterial protease and barnase a bacterial ribonuclease ($M_r$ 12,400).

The resonances of the histidine C2 protons in the 500 MHz $^1H$ NMR spectrum of subtilisin BPN' have been resolved. The microscopic p$K_a$'s of the histidine residues in subtilisin BPN' have been determined by following the variation of the chemical shift of the C2 proton resonances of these residues with pH. A series of five mutant subtilisins have been constructed using oligonucleotide-directed site-specific mutagenesis in which individual histidine residues were replaced with glutamine. Comparison of the NMR spectra of mutant and wild type proteins has enabled a complete assignment of the histidine C2 protons in the NMR spectrum of subtilisin BPN' to be made.

Signals from the C2 protons of the two histidine residues in barnase have been resolved and the p$K_a$'s of these residues have been determined. These resonances have been assigned using two mutant proteins in which the histidines have been replaced by other amino acids.

**ACKNOWLEDGEMENTS.**

I would like to thank all of my colleagues in the laboratory, in particular those who have worked on the subtilisin and barnase projects. I would also like to thank Richard Sheppard for his help with the NMR spectroscopy. Finally I would like to thank my supervisor Professor Alan R. Fersht.

This work was supported by a grant from the Science and Engineering Research Council.

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# CHAPTER 1: INTRODUCTION.

## 1.1: The role of histidine resonances in the nuclear magnetic resonance spectroscopy of proteins.

In the 30 years since the first experiments on proteins were reported (Saunder *et al.*, 1957), nuclear magnetic resonance spectroscopy has been developed into one of the most powerful spectroscopic techniques for studing protein structure, function and dynamics in solution. These advances have resulted from the introduction of high field spectrometers based on superconducting magnets, and the development of new experimental methods such as Fourier transform NMR (Ernst and Anderson, 1966) and more recently two-dimensional NMR (Ernst *et al.*, 1987). For small proteins of less than 130 amino acids, it is now possible, using two-dimensional NMR techniques, to obtain a complete assignment of the proton spectrum. The individual amino acid spin systems are assigned using 2D methods which give information on through-bond J coupling. Sequence specific assignments are then made using through space interactions via the nuclear Overhauser enhancement (NOE) effect. When a complete assignment is available, a large number of

NOE contacts can be assigned and these can then be analysed using distance geometry algorithms to produce a solution structure for the protein (Wuthrich, 1986). The application of these methods to larger proteins is, however, limited by increasing line widths and spectral overlap. For these proteins, only a limited number of signals can be resolved.

The resonances that are studied in most detail for larger proteins are those arising from histidine C2 protons (Markley, 1975). The chemical shifts of these resonances depend upon the charge state of the imidazole ring of the histidine residue. The difference between the chemical shifts of the protonated and unprotonated forms of the ring is normally much greater than the rate at which they interconvert. The two forms are therefore in fast exchange in the NMR time scale. One signal is consequently observed with an average chemical shift which alters with pH as the relative proportions of the protonated and unprotonated forms change. Microscopic $pK_a$ values for individual histidine residues can be determined by the analysis of the variation of chemical shift with pH. This is particularly useful because histidine residues play a central role in the catalytic mechanisms of many enzymes. The chemical shift of a His C2-H resonance in a protein, at a given pH, depends upon both the perturbation of chemical shift produced by the local environment of the histidine side chain, and on the protonation state of the histidine ring which depends on the $pK_a$ of the residue. The combination of these two factors

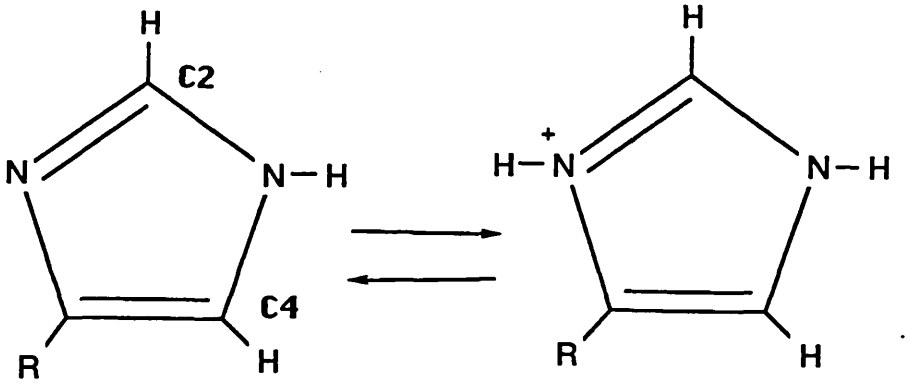



Fig. 1.2: Ionisation of histidine side chains

makes it possible to resolve individual histidine resonances in proteins with relative molecular masses of up to 70,000. Resolved histidine resonances can provide a sensitive probe of the local environment in proteins, and these resonances have been used to study a wide variety of effects such as ligand binding, conformational changes and protein folding (Jardetzky and Roberts, 1981).

The $pK_a$ of the imidazole ring of a histidine residue in a random coil environment is approximately 6.3 (Mathews, 1986). In proteins the $pK_a$ of histidine varies from below 4 to over 8. This wide range of values is produced by a number of factors.

The $pK_a$ of a residue is influenced by the polarity of its surroundings in the protein (Warshel, 1987). In a non-polar environment, the protonated form of the imidazole ring of a histidine side chain will be less stable than in a polar one and so the $pK_a$ of the residue will be lower. The interiors of proteins are generally considered to be non-polar whilst the bulk solvent is polar. It is difficult, however, to classify the environments of individual histidine residues in proteins as polar or non-polar. Most of these residues are exposed to the solvent to some extent and it is not known how inaccessible to solvent a residue must be for an effect on $pK_a$ to be observed (Mathews, 1986).

The $pK_a$ of a histidine residue is also influenced by the electrostatic interactions made by the protonated form of the imidazole ring with charged groups in the protein. Interaction with positive charges destabilises the protonated form and leads to an decrease

in $pK_a$ whilst interaction with negative charges produces an increase in $pK_a$. The magnitude of electrostatic interactions are governed by the dielectric constant of the medium between the two charges. Attempts to model electrostatic interactions in proteins have been hampered by the difficulty in obtaining values for dielectric constants in such a heterogeneous system (Mathews, 1986, Rodgers, 1987). The interaction of histidine side chains with dipoles in proteins also affects the $pK_a$ of these residues. This effect is especially important where dipoles are aligned in α–helices (Hol, 1985).

When assignments for the histidine resonances of proteins with known crystal structures are available, a correlation of the microscopic $pK_a$ of a given histidine residue with its environment in the protein can be made. This can provide an excellent opportunity to test the validity of theoretical models which attempt to predict the electrostatic properties of proteins.

One of the main limitations in the study of histidine residues in proteins by NMR is a lack of sequence-specific resonance assignments. For small proteins 2-dimensional NMR methods can be used. In some cases, however, the β CH to C4-H NOE contacts that are required to produce the sequential assignments are not observed because of an unfavourable side chain conformation. These methods are also not applicable to the majority of proteins because of the molecular mass limitation discussed above. For

proteins for which a crystal structure is available, assignments can be made by correlating spectral parameters, such as chemical shift or line width, to the environment of a particular residue in the structure. NMR parameters can, however, be predicted reliably only in a limited number of cases. The chemical shifts of protons in close proximity to the aromatic rings of amino acid side chains or to heme groups can be calculated with some accuracy , and this method has been widely used to obtain assignments (Perkins & Dwek, 1980). The effect of paramagnetic metals on the line widths and / or chemical shifts of protons can also be estimated with sufficient accuracy for assignments to be made on this basis (Campbell *et al.* 1975b). This method is only applicable, however, if the protein of interest contains an intrinsic metal ion or if a metal ion can bind to a unique site in the molecule. The observation of interactions between protons via the nuclear Overhauser effect has also been used for assignment (Dubs *et al.*1979). NOE effects can be used to identify neighbouring residues of a resolved resonance. An assignment can then be made by comparing this information to the environment of residues in the crystal structure. The use of this method is limited to relatively small proteins by spectral overlap and the effects of spin diffusion.

Histidine C2-H protons exchange slowly for deuterium when incubated in $D_2O$ and this exchange reaction has been used to obtain assignments (Markley, 1975b). The exchange rates for individual histidine residues depend upon their environment in the

protein, and assignments can be made by correlating the rate of disappearance of a proton resonance with the chemically determined rate of incorporation of deuterium into a particular histidine residue. Although this method can give unequivical assignments, the technical difficulty of these experiments have limited the use of this technique.

One promising new approach to the problem of resonance assignment is the use of site-directed mutagenesis. Naturally occurring protein mutants have been used for resonance assignments for many years but this approach had been limited by the availability of suitable proteins (Jardetzky and Roberts, 1981). The advances in recombinant DNA techniques now allow the introduction of any desired mutation into proteins for which the cloned gene is available (Zoller and Smith, 1981). Provided that the mutation does not produce a large conformation change in the protein, the spectra of the mutant and wild type proteins should be identical, except for the absence of the peak of interest. Assignment is therefore straight forward. This work describes the use of site-directed mutagenesis to assign histidine C2-H resonances in two bacterial proteins; subtilisin BPN' and Barnase.

## 1.2: Site-directed mutagenesis.

In recent years, techniques have been developed that allow the introduction of any desired point mutation into a region of cloned DNA (Zoller and Smith, 1983). These methods involve the synthesis of an oligonucleotide that is complementary to the target DNA except for a mismatch which directs the change of sequence required (Fig 1.2). The mutagenic oligonucleotide is anealed to single-stranded target DNA, and is used to prime DNA synthesis to generate a double-stranded hetroduplex product. This is then transfected into an appropriate strain of *E. coli*, where it gives rise to progeny that contain both mutant and wild-type sequences. The mutagenic oligonucleotide will bind preferentially to the mutant sequence and under the appropriate conditions it can be used to identify phage containing the mutated DNA.

A variety of modifications of this basic method have been introduced. Several methods involve protection of the mismatch from 5'→3' exonuclease correction. This can be achieved by using a second primer that binds close to the 5' end of the mutagenic oligonucleotide (Zoller and Smith,1984). During the extension-ligation reaction, DNA synthesis is initiated from this site and continues until the 5' end of the mutagenic primer is reached. Ligation of this DNA to the mutagenic oligonucleotide then protects the mismatch from repair. Similar results can be obtained by constructing a gapped duplex so that there is only a small region of single stranded DNA around the position

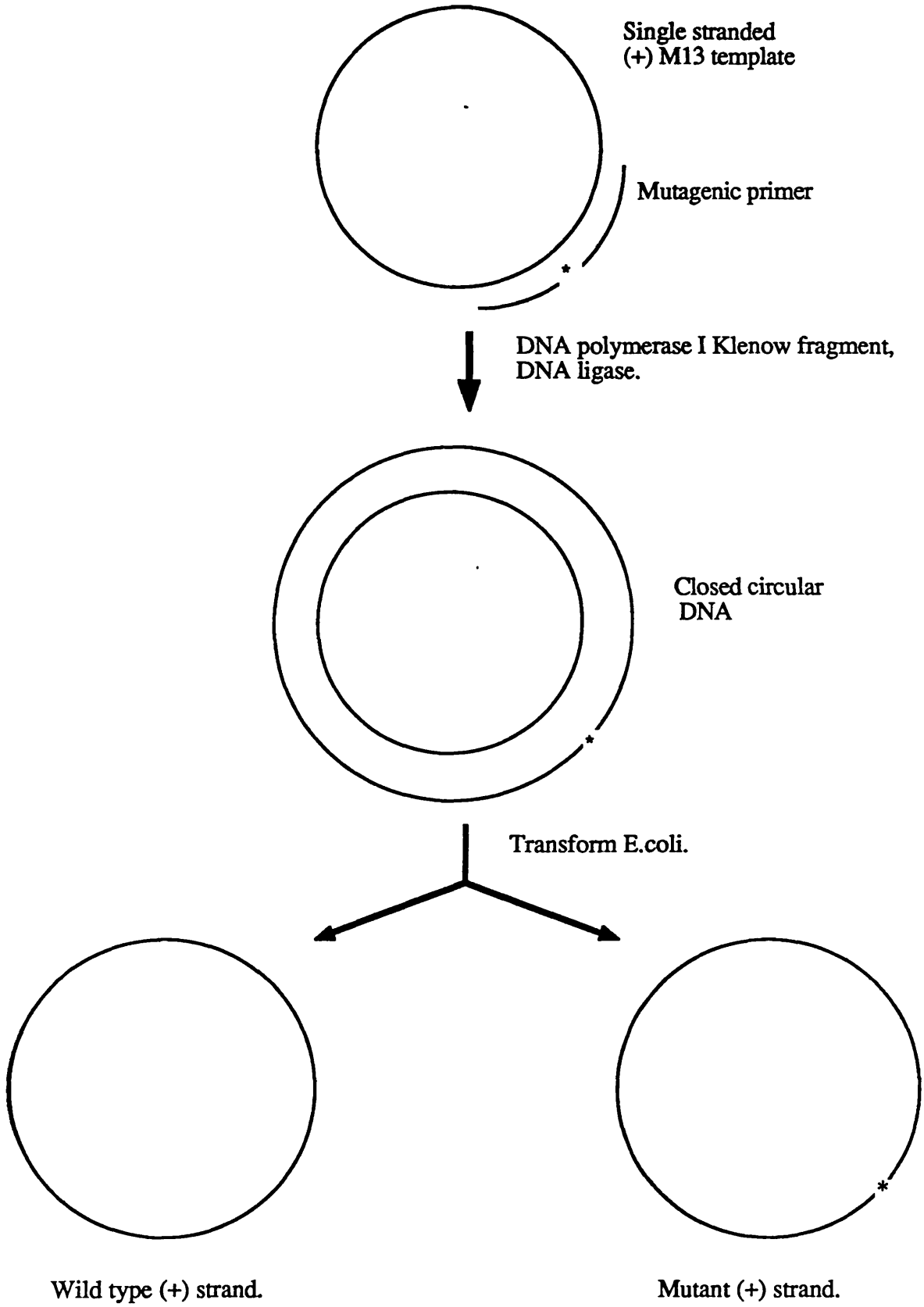



Fig. 1.2: Scheme for oligonucleotide directed site specific mutagenesis

where the mutation is to be made (Kramer *et al.*, 1984). The yields of mutants can also be increased by transfecting the heteroduplex DNA into a repair-deficient strain of *E. coli* that is less likely to repair the mismatch (Kramer *et al.*, 1982). A number of methods have been described that improve the yield of mutants by enabling the *in vivo* selection of phage derived from the newly synthesized strand (Carter *et al.*, 1985, Kunkel *et al.*, 1985).

For site directed mutagenesis to be carried out, it is generally necessary to have the target DNA cloned into a vector which can produce single-stranded DNA. Derivatives of the phage M13 have been widely used for site-directed mutagenesis. However, it has been found that large inserts of DNA are often unstable in this vector which can lead to variable levels of protein expression. Mutations are often made therefore with the gene of interest cloned into an M13-based vector and the mutant proteins are then expressed by subcloning the gene into an expression vector. In recent years, plasmid vectors have been introduced that contain the f1 origin of replication which enables them to produce single-stranded DNA when the host strain is infected with a helper phage (Dente *et al.*, 1985). These vectors do not have the problems that are associated with M13-based vectors and they are increasingly being used for site-directed mutagenesis studies (Lau and Fersht, 1986).

One of the main applications of site-directed mutagenesis has been the production of mutant proteins with altered properties. This technique has been used to study a wide

range of effects in a number of proteins and the this field has been the subject of a number of recent reviews (Leatherbarrow and Fersht 1986; Gerlt, 1987; Knowles, 1987). To be able to interpret correctly the results of a mutagenesis experiment, it is necessary to establish whether or not the mutation has produced a large change in structure of the protein. One approach to this problem has been to analyse a series of mutants and to try to establish general trends in the effects of the mutation of certain types of residues. Mutations that do not follow this trend are more likely to have produced large structural changes. The most sophisticated application of this approach has been the use of linear-free-energy relationships to analyse mutants of the tyrosyl tRNA synthetase (Fersht *et al.*, 1987). A more direct method is to solve the X-ray crystal structure of the mutant proteins (Howell *et al.* 1986; Brown *et al.* 1987). In most cases, the mutant and native structures have been sufficiently similar for difference Fourier methods to be used, but this method is still slow and so relatively few structures have been solved. The structures that are available have, in general, shown that point mutations do not produce large conformational changes, although some local rearrangement in structure has been observed.

For small proteins, NMR appears to offer an attractive method for investigating the structural consequences of mutations. To exploit fully the potential of NMR in this area, it is essential that a large number of assignments are available. At present most of the mutants studied have been of proteins for which only a limited number of

assignments have been made. The most detailed study in this area to date has been on the enzyme staphylococcal nuclease. The mutation of a Glu at the active site of this enzyme has been shown to produce a number of changes in the NMR spectrum but analysis of the spectra of the mutant protein has been hampered by a lack of assignments (Hibler *et al.*, 1987).

## 1.3: The Subtilisins

The Subtilisins are a group of extracellular proteases that are produced during sporolation by a number of species of bacilli (Markland and Smith, 1971). The two enzymes that have been studied in most detail are subtilisin BPN' which is produced by *Bacilus amyloliquefaciens* and subtilisin Carlsberg which is produced by *Bacilus licheniformis*. Subtilisin Carlsberg is an important commercial enzyme and is used extensively in the detergent, food-processing and leather industries.

The crystal structures of subtilisins BPN' and Carlsberg have been solved at high resolution (Drenth and Hol, 1967; Wright *et al.*, 1969, McPhalen *et al.*, 1985). The protein contains eight segments of α–helix all running in the same orientation (Fig. 1.3). The active site consists of a shallow grove and contains a histidine, an aspartate and a serine arranged in a manner similar to the charge relay system of chymotrypsin and the other mammalian serine proteases (Blow *et al.*,1970). There is considerable kinetic and chemical modification evidence that subtilisin, like the mammalian serine proteases, acts via an acyl-enzyme mechanism (Philipp and Bender, 1983) (Fig 1.4). Although both of these groups of enzymes share the same catalytic mechanism, they show no sequence or structural homology and their common mechanism of action appears to be the result of

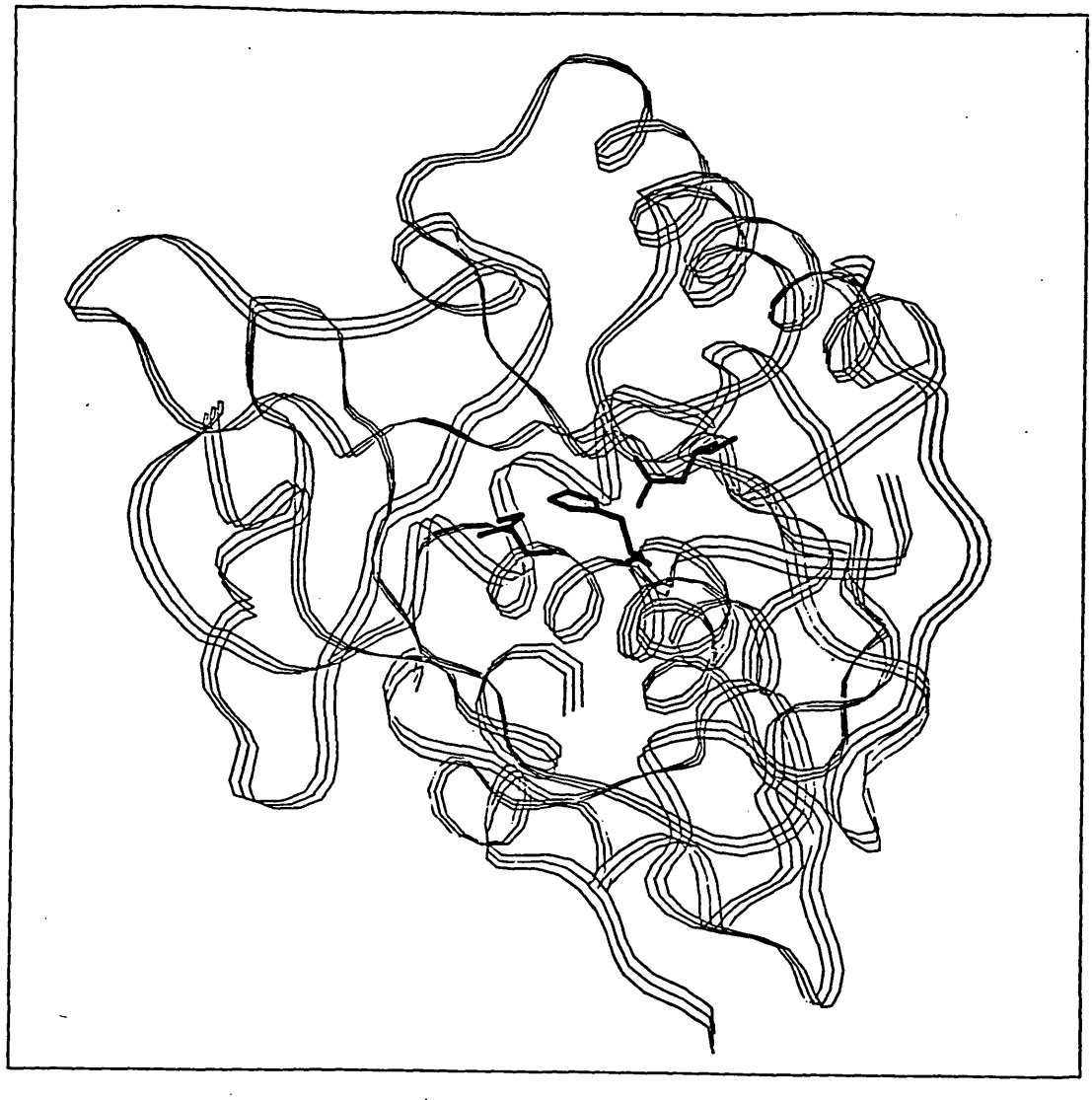


Fig. 1.3 Crystal structure of Subtilisin BPN'. Picture created using the FRODO graphics program (Jones, 1978) on an Evans and Sutherland PS 300. The main chain of the protein is depicted as a ribbon and the side chains of the active site Asp, His and Ser are shown.

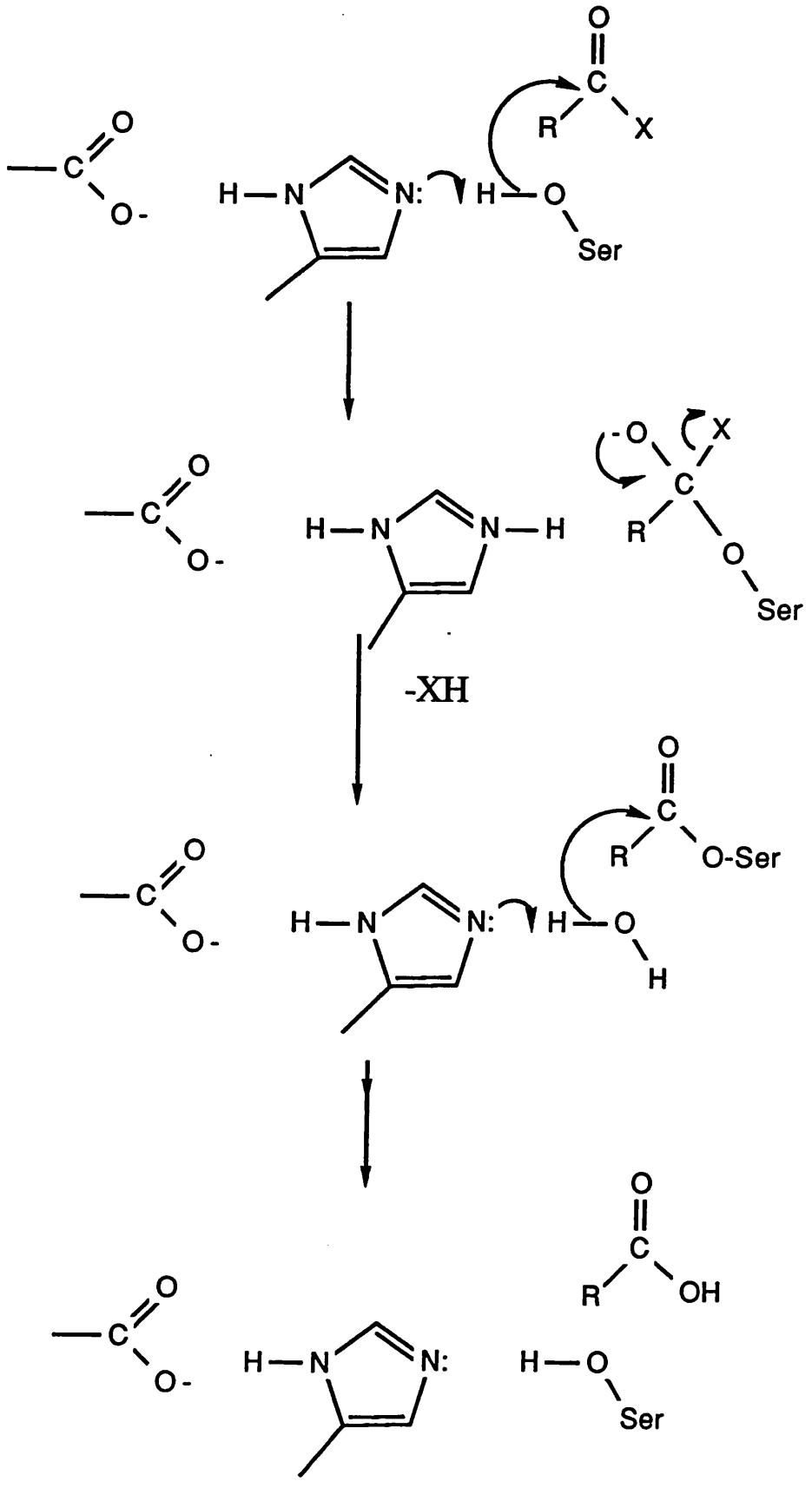
convergent evolution.

The wealth of structural and kinetic information available on the subtilisins together with their commercial importance has prompted a number of groups to study this enzyme using site-directed mutagenesis and this work has been the subject of a recent review (Wells and Estell, 1988). The gene coding for subtilisin BPN' has been cloned in several different laboratories and the protein has been expressed at high level in protease deficient strains of *B. subtiliis*. Several of the amino acids that are believed to be involved in catalysis have been mutated. It has been shown that the deletion of any of the amino acids in the catalytic triad, Asp, His, Ser, results in a substantial reduction in the value of $k$cat, illustrating the highly cooperative nature of the rate enhancement produced by these residues. It has also been shown that substitution of Asn 155, which binds the oxyanion of the tetrahedral transition state, also results in a considerable drop in rate enhancement.

Subtilisins have a broad specificity but preferentially hydrolyse peptide bounds which follow large hydrophobic residues. In the native, enzyme Gly 166 is at the bottom of the substrate binding cleft. This residue has been replaced by all other 19 amino acids in order to study steric and electrostatic effects on substrate binding.

Mutations have also been made in order to alter the physical and chemical properties of subtilisin in order to make it more suitable for commercial use. In the most successful of these experiments the resistance of subtilisin to inactivation by oxidizing agents has been

Fig. 1.4 Mechanism of action of subtilisin



increased. Oxidation of Met 222 in subtilisin to the sulphoxide abolishes enzymic activity. Replacement of Met 222 with an Ala results in only a 50% drop in specific activity but greatly improves the oxidative stability of the enzyme. Attempts have also been made to improve the thermostability of subtilisin by the introduction of disulphide bonds. One of the disulphide bonds introduced produced a small increase in thermostability but the other protein was less stable.

Subtilisin has also served as a model system in which to study electrostatic effects in proteins using site-directed mutagenesis (Thomas *et al.*, 1985; Russell and Fersht, 1987; Russell *et al.*, 1987, Sternberg *et al.*, 1987). Subtilisin is active only when histidine 64 is unprotonated and the catalytic activity varies with pH following the ionisation of this residue. The p$Ka$ of histidine 64 can be obtained with high precision from the analysis of activity vs pH data. The effect on the p$Ka$ of histidine 64 of the mutation of several charged residues has been studied. Mutation of these residues was found to perturb the p$Ka$ in a manner consistent with the removal of an electrostatic interaction between the charged residue and the protonated form of the imidazole ring. Removal of a positive charge or introduction of a negative charge increases the p$Ka$ while addition of a positive charge or removal of a negative charge produces a decrease in p$Ka$. Using this approach, the p$Ka$ of His 64 has been perturbed by up to 1 pH unit. Several attempts have been made to model the changes in p$Ka$ of His 64 in subtilisin using algorithms that calculate

electrostatic interactions in proteins (Sternberg *et al.*, 1987; Gilson and Honig, 1987).

There have been a number of NMR studies of subtilisins. In the initial NMR experiments on subtilisin, attempts were made to observe a low field peak similar to those seen in the $^{1}H$ NMR spectra of the mammalian serine proteases. This resonance had been attributed to the hydrogen located between the Asp and His residues in the active site of these enzymes. Since the arrangements of the Asp, His and Ser residues in the active site of subtilisin is the same as in the mammalian serine proteases it was believed that a similar peak should be present in the spectrum of subtilisin. A low field peak was not found in the spectra of native subtilisin but was observed in an enzyme-phenylboronic acid complex and in a derivative of subtilisin in which the active site Ser had been converted to a Cys by chemically modification (Jordan and Polgar, 1981). The lack of a peak in the native enzyme was attributed to exchange broadening and it was argued that in the derivatives in which it was seen the exchange rates were reduced so that the resonance could be observed.

$^{31}P$ NMR spectra of a diisopropylphosphoryl inhibitor bound to active site serine of subtilisin have been obtained. It was found that the chemical shift of the $^{31}P$ resonance titrated with a p$Ka$ of 7.40. This was assumed to result from a perturbation in the chemical shift of the derivative produced by the ionisation of the active site histidine.

The resolution and pH titration of the histidine C2-H peaks in subtilisins BPN' and Carlsberg has also been reported (Jordan *et al.*, 1986). Since the publication of that work , however, it has been shown that the subtilisin BPN' used was not the authentic enzyme (Russell and Fersht, 1986) and this has cast doubt on a number of the conclusions of that paper.

In the present work the histidine C2 proton resonances from authentic subtilisin BPN' expressed from the cloned gene have been resolved. These resonances have been assigned using site-directed mutagenesis. The availability of these assignment has enable a comparison to be made of the p*Ka*'s of individual histidine residues to their environments in the crystal structure of subtilisin.

## 1.4: Barnase

Barnase is a protein of 110 amino acids and $M_r$ 12,400. It is produced by *B. amyloliquefaciens* and is an extracellular, purine-specific, ribonuclease (Hartley, 1968). The intra-cellular RNA of *B. amyloliquefaciens* is protected from hydrolysis by a small intra-cellular inhibitor of barnase, barstar (Hartley,1968).

The crystal structure of barnase has been solved to 2.5 Å resolution (Maugen *et al.* 1982). The protein consists of a central five-stranded β-pleated sheet and 2 α–helices (Fig. 1.5). Barnase has no structural homology to pancreatic ribonuclease but does share some structural, and sequence homologies with a number of other microbial ribonucleases (Hill *et al.*, 1983). The genes coding for barnase and barstar have been cloned and barnase has been expressed in high levels in *E. coli.* (Hartley, 1988)

Barnase is believed to hydrolyse RNA in a two step process in which a cyclic phosphate intermediate is formed (Takahashi & Moore 1982). In the first step a basic group abstracts a proton from the 2'-OH of the ribose moiety and an acidic group protonates the 5'-OH of the adjacent ribose (Fig. 1.6). In the second step the catalytic roles of these residues are reversed. One of the groups activates the attack of water by general base catalysis while the second group protonates the leaving group. All the microbial ribonucleases which are homologous to barnase have conserved residues at positions equivalent to Glu 73 and His 102 in barnase and it has been suggested that

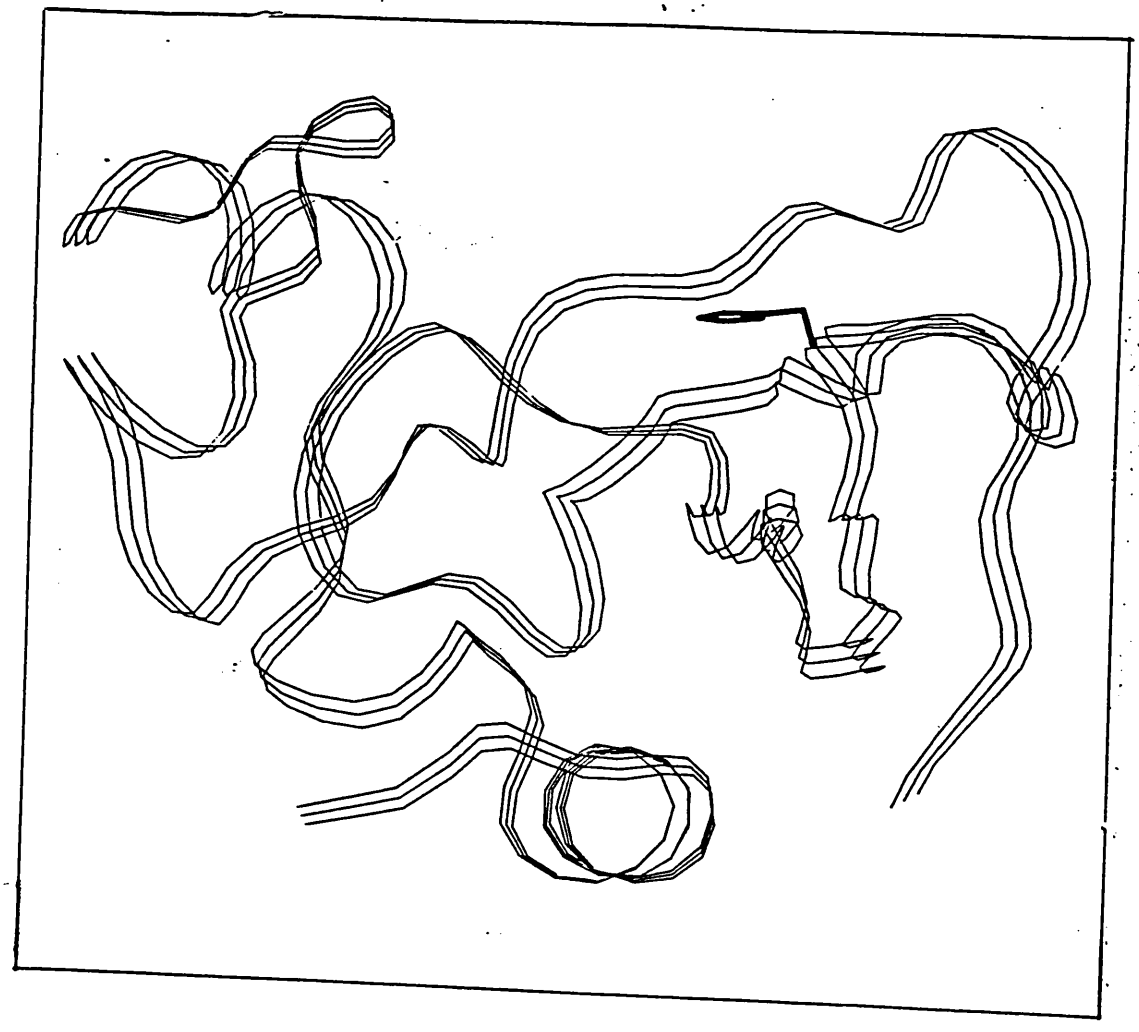
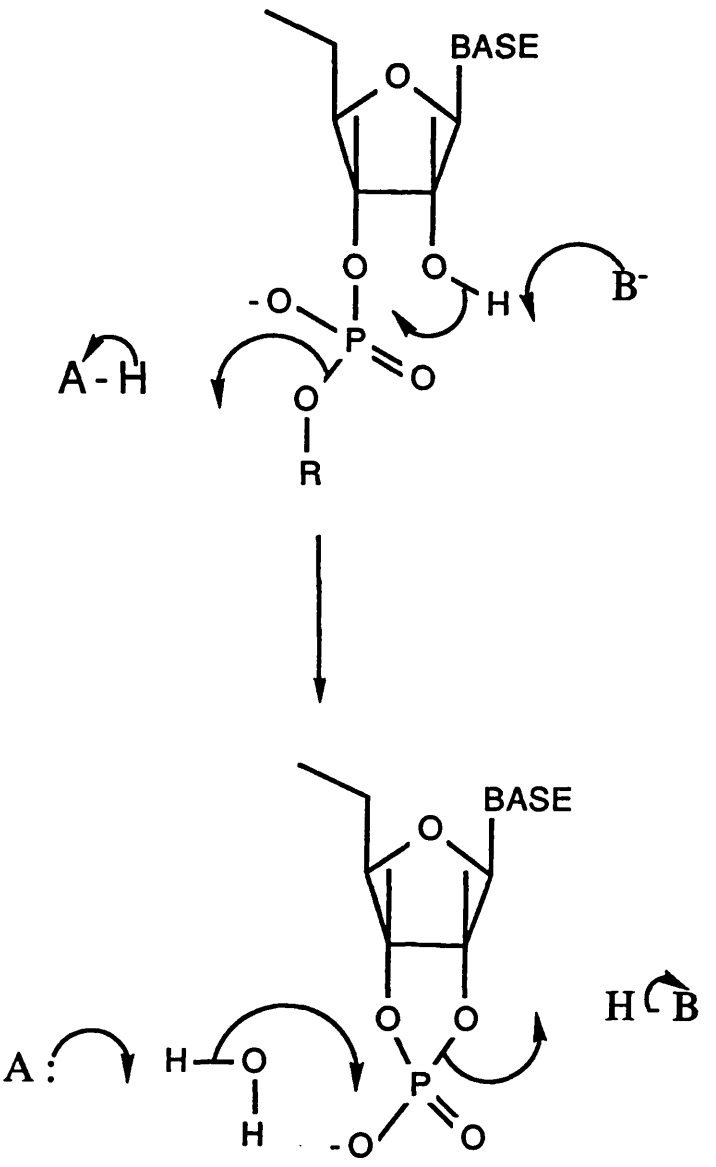


Fig. 1.5: Crystal structure of barnase. The main chain is depicted as a ribbon and the side chain of His 102 is shown.

these residues are the acidic and basic groups involved in catalysis. There is, however, no structure available of a substrate analogue bound to barnase. In the crystal structure of barnase, His 102 and Glu 73 are grouped together in one region of the protein and model building studies, based on enzyme inhibitor structures of other ribonucleases, suggest that these residues can interact with substrates.

Recently, site directed mutagenesis has been used to study the roles of these residues (Mossakowska *et al.*, unpublished data). It has been found that the substitution of His 102 and Glu 73 with other amino acids results in a large drop in activity, confirming that these residues are some how important in catalysis. The effects of a number of other amino acid substitutions in barnase on the reaction rates and substrate binding are also currently under investigation.

Barnase unfolds reversibly with increasing temperatures and concentration of denaturents such as urea and guadinium hydrochloride. By analysing data from denaturation experiments, it is possible to measure the stability of the folded form of the protein (Hartley, 1968). Barnase is currently being used to investigate protein stability using site-directed mutagenesis. A number of residues which are involved in a variety of interactions that are believed to stabilize the folded form of the enzyme, have been mutated and the effects of these mutations on the stability of barnase have been determined. The initial experiments in this area have involved the truncation of hydrophobic residues that are involved in the packing of the $\alpha$–helix, formed by


BASE
O
O
O
-O
P
O
H
B-
A-H
O
R
BASE
O
O
O
H
B
A:
H
O
H
P
-O
O

residues 6-18 onto the central region of β-sheet (Kellis *et al.*, 1988). From the results of these experiments, it has been possible to obtain an estimate of the contribution of hydrophobic interactions to protein stability.

This work reports the results of a preliminary study of barnase using $^1$H NMR. The microscopic p*Ka*'s of the two histidines in barnase have been determined by the titration of their C2 proton resonances. The assignment of these protons has been obtained using mutant proteins in which the histidines have been replaced by other residues. This has enabled the role of histidine 102 in the catalytic mechanism of barnase to be investigated. NMR has also been used to investigate the structural consequences of the mutation of a number of residues in barnase.

# CHAPTER 2: MATERIALS AND METHODS.

## 2.1: DNA Methods.

### Bacterial strains

The genotypes of the bacterial strains used in this study are summarised in Table 2.1. The *E. Coli* strains TG2 and BMH71-18 *mutL* were propagated on MGB1-agar plates to select for the maintenance of the F factor. The *B. subtilis* strain DB104 was propagated on L-agar plates. All strains were stored in 15% glycerol at -70°C.

### Buffers and media.

L-Broth contains 10 g Bacto-typtone (Difco), 5 g yeast extract (Difco), 10 g NaCl, per litre. pH was adjusted to 7.5 with NaOH. L-agar was prepared from L-broth containing 2% bacto-agar (Difco)

2xTY broth contains 16 g Bacto-typtone (Difco), 10 g yeast extract (Difco), 5 g NaCl, per litre.

Minimal agar (MGB1) was prepared by adding 100 ml of 5xMM salts (52.5 g $K_2HPO_4$, 22.5 g $KH_2PO_4$, 5 g $(NH4)_2SO_4$, 2.5 g sodium citrate, per litre) to 400 ml

Table 2.1: Bacterial strains.

| Strain | Genotype | Reference |
|---|---|---|
| *E. coli* TG2 | K12,Δ(lac-proAB),*thi*,*sup*E, *hsd*Δ5,*Rec*A,*sr*1::Tn 10, [F', *tra*D36,*pro*AB,*lac*Iq, *lac*ΔM15] | T. Gibson (1984) |
| *E. coli* BMH71-18 *mut*L | K12,Δ(lac-proAB),*thi*,*sup*E, *hsd*Δ5,*mut*L::Tn 10, [F', ,*pro*AB,*lac*Iq, *lac*ΔM15] | Kramer *et al.*, (1984) |
| *B. subtilis* DB104 | *His*,*npr*R2,*npr*E18,Δ*apr*A3 | Kawamura & Doi, (1984) |

of water containing 7.5 g of molten agar (Difco). After the mixture had cooled to 55°C 0.25 ml of vitamin B1 (1% w/v), 0.5 ml $MgSO_4$ (20% w/v) and 5 ml glucose (20% w/v) were added.

H-top agar contains 10 g Bacto-typtone (Difco), 6 g bacto-agar (Difco), 8 g NaCl, per litre.

Milk plates were prepared from L-agar with the addition of 2% powdered milk. Plates were poured into 90 mM petri-dishes.

Media and buffers were sterilised by autoclaving at 121°C for 20 minutes.

The composition of the buffers used in this work are tabulated in Table 2.2.

Reagents were Analar-grade and were purchased from BDH. Antibotics were purchased from Sigma.

Recombinant plasmid and phage vectors.

M13apr: This is a derivative of M13mp9 in which a 3Kb EcoR1 fragment containing the gene coding for subtilisin has been cloned into the polylinker (Thomas, 1988). The subtilisin BPN' gene is orientated such that the non-coding strand is contiguous with the (+) strand of the viral DNA. Subtilisin is not expressed from this vector. M13aprwt is, like many other derivatives of M13mp vectors containing large DNA inserts, structurally unstable in $recA^+$ strains of *E. coli* due to Rec A mediated recombination. This problem

Table 2.2: Composition of buffers.

| Buffer. | Composition. |
|---|---|
| TE | 10 mM Tris-HCl pH 7.6, 1 mM EDTA. |
| TM | 10 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$ |
| 1xTBE | 90 mM Tris, 90 mM Borate, 2 mM EDTA pH 7.8 |
| 1xSSC | 0.15 M NaCl, 0.015 M sodium citrate. |
| Kinase buffer | 50 mM Tris-HCl *p*H 8.5, 10 mM $MgCl_2$,5mM DDT, 0.1 M spermidine-HCl, 0.1 mM EDTA. |

was overcome by propagating the phage in TG2, a RecA$^-$ *E.coli* strain.

pPT30: This is a derivative of pUB110, a plasmid from *Staphlyococcus aureus* that replicates in *B. subtilis*, that contains the subtilisin gene cloned into the EcoRI site (Thomas, 1988). Subtilisin is expressed off its own promoter in *B. subtilis* during sporulation, and is directed into the culture supernatant by a leader peptide (Power *et al.*, 1986) .

Phenol / chloroform extraction of DNA.

Proteins were removed from solutions of DNA by extraction with a 2:1 mixture of phenol, which had been saturated with TE buffer and chloroform. The DNA solution was mixed with an equal volume of phenol / chloroform in an Eppendorf tube and the contents of the tube were mixed to form an emulsion. The tube was then spun in a microfuge for 3 minutes and the upper aqueous layer was removed with a micropipette. Residual phenol was removed by extraction with diethylether.

Ethanol precipitation of DNA

DNA solutions were adjusted to 0.3 M in sodium chloride or 0.25 M in sodium acetate and 2.5 volumes of 96% ethanol were added. The solution was allowed to precipitate at -20°C for 2 hours and then recovered by centrifugation. The pellet of DNA was washed with chilled 70% ethanol, dried under vacuum and resuspended in an appropriate volume of TE buffer.

Quantification of DNA and oligonucleotides.

DNA concentrations were determined spectrophotometrically using the assumption that an absorbance of 1 at 260 nm corresponds to 50 µg / ml of double stranded DNA and 40 µg / ml for single stranded DNA (Maniatis *et al.*,1982). The concentrations of oligonucleotides were determined using millimolar extinction coefficients calculated by the summation of the contributions of the individual bases using to the values given in Table 2.3 (Sproat & Gait, 1984).

Agarose gel electrophoresis.

DNA samples were analysed by electrophoresis through horizontal, submerged 0.8% agarose gels. 1xTBE was used as the running buffer and the electrophoresis was

Table 2.3 Contributions of individual base to oligonucleotide extinction coefficients.

| Base | Contribution to $\varepsilon_{260}$ ($mM^{-1}cm^{-1}$) |
|---|---|
| A | 8.8 |
| G | 7.3 |
| C | 11.7 |
| T | 15.4 |

carried out at a constant 40 mA. Gel tanks were obtained from BRL (model H4) and agarose was purchased from Pharmacia. DNA samples (5-10 µl) were mixed with an equal volume of 20% glycerol, 2 mM EDTA and 0.04% bromophenol blue and were applied to the gel using a micropipette. Ethidium bromide (1 µg / ml) was including in the gel and the running buffer and DNA was visualised by fluorscence using a 305 nm trans-illuminator. Where appropriate a sample of DNA cut with the restriction enzyme *Hind* III was used as a molecular weight marker.

Restriction enzyme digestion of DNA.

Restriction enzyme digests were performed in either low, medium or high salt digestion buffers (Maniatis *et al.*,1982) depending upon the manufactures instructions. Typical reactions contained 0.2-1 µg of DNA in a volume of 10-20 µl. One unit of restriction enzyme was added in a volume of 1-2 µl, and the digestions were allowed to proceed for 2 hours. The reactions were terminated by phenol / chloroform extraction. The products of the reaction were analysed by agarose gel electrophoresis.

Preparation of M13apr template DNA

Single plaques were picked using a sterile tooth pick into 1.5 ml of 2TY broth containing 15 µl of an overnight culture of *E. Coli* TG2. The culture was incubated for 5-6 hours with vigorous aeration at 37°C and was then transferred to a 1.5 ml Eppendorf tube and the cells were pelleted by centrifugation in a microcentrifuge. The supernatant was then transferred to another Eppendorf tube containing 200 µl of a 20% polyethylene glycol 6000, 1.5 M NaCl solution. This was left at room temperature for 15 minutes and the phage particles were collected by centrifugation for 5 minutes in a microfuge. The supernatant was removed using a drawn-out Pasteur pipette attached to a water pump. The pellet was dissolved in 100 µl of TE buffer and was then phenol / chloroform extracted twice to remove the phage coat proteins. The single-stranded viral DNA was precipitated using ethanol and then redisolved in an appropriate volume of TE buffer.

Preparation of double-stranded M13apr RF DNA

M13apr replicative form DNA (RF) was prepared from *E.coli* TG2 cells infected with M13apr, using the alkaline lysis procedure (Birnboim and Doly, 1979) followed by density gradient centrifugation. An early-log phase culture of *E.coli* TG2 (500 ml)

was infected with 2 ml of phage stock ($10^9$ pfu per ml). The culture was grown overnight and the cells were pelleted by centrifugation (8000 rpm, 30 min soval SS-34 rotor). The cells were resuspended in 10 ml of lysis buffer (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 5 mg / ml lysozyme) and were incubated on ice for 15 min. Freshly prepared 0.2 M NaOH, 0.1 M SDS (20 ml) was then added and the mixture was then incubated for a further 5 minutes on ice. A cold solution of 5 M potassium acetate pH 4.8 (15 ml) was then added and the resulting precipitate was removed by filtration though glass wool. Chilled ethanol, 96% 2 volumes, was added to the filtrate and this was then left at -20°C for 1 hour. The solution was then centrifuged at 8000 rpm Sorval SS-34 rotor for 30 min and the resulting precipitate was dried under vacuum. The pellet was then dissolved in 6 ml of TE buffer containing 6 g of caesium chloride and 480 µl of 10 mg / ml ethidium bromide. The resulting solution was then transferred to a Beckman ultracentrifugation tube and this was then topped up with liquid paraffin and sealed. The caesium chloride density gradient was formed by centrifugation for 16 hours at 48,000 rpm in a Beckman Ti 70.1 rotor. The banded RF DNA was recovered through the side of the centrifuge tube using a syringe and a hypodermic needle. The ethidium bromide was removed by extraction (3 times) with water saturated butan-1-ol. Residual caesium chloride was removed by dialysis against TE buffer. The DNA was stored frozen at -20°C.

Purification of pUB110 plamid DNA .

pUB110 DNA was purified from 500 ml overnight cultures of the strain DB104 habouring the plasmid grown in L-broth containing 2% glucose and 50 μg / ml kanamycin. DNA was purified as described above except that the lysozyme treatment step of the procedure was carried out at 37°C and for 30 min.

Oligonucleotide synthesis and purification

Oligonucleotides were synthesised by Mr. J. Knill-Jones using an Applied Biosystems Inc. model 380B automated DNA synthesiser by the phosphoramidite method. Typical step-wise yields were greater than 98%. Protecting groups on the bases were removed by incubating for 18 hours at 56°C in a 35% (v/v) ammonia solution. The oligonucleotides were dialysed against water and were then purified by ion exchange chromatography using a Pharmacia FPLC apparatus. The oligonucleotide (3-4 $A_{260}$ units) was applied to a 1 ml mono Q anion exchange column that had been equilibrated in 10 mM NaOH. The column was eluted using a 0.2-1 M NaCl gradient and the elusion was monitored at 254 nM. The fractions containing the largest peak were retained, pooled and dialysed against TE buffer. Oligonucleotides were stored at -20°C until use. Oligonucleotides that were to be used for site-directed mutagenesis were tested for specificity of priming by using them as primers in a dideoxy chain

termination DNA sequencing reaction.

Preparation and transformation of competent cells.

Transformation of *E.coli* with M13apr DNA

*T*ransformation competent *E. coli* were prepared using the method of Hanahan, 1985. 2xTY broth (20 ml) was inoculated with 200 µl of an overnight culture of the required strain and this culture was grown at 37°C until the absorbance at 550 nM was 0.3. The cells were pelleted by centrifugation (8,000 rpm Sorval SS-34 rotor) and were resuspended in 10 ml of ice cold 100 mM $CaCl_2$. The cells where then kept on ice for one hour and then spun down again and resuspended in 2 ml of 100 mM $CaCl_2$.

DNA solution 5 µl was added to 200 µl of competent cells on ice and this mixture was incubated for 40 minutes. The cells were then heat shocked at 42°C for 2 minutes and 200 µl of a fresh culture to act as plating cells was added. Molten H-top agar (3 ml) was then added and the mixture was poured onto H-plates. After the plates had set, they were inverted and incubated at 37°C until plaques became visible.

Transformation of *B. subtilis.* with plasmid DNA

Transformationally competent *B. suibtilis.*was prepared using the method described by Hardy (1985). *B. Subtilis* DB104 was grown overnight at 30°C in 10 ml of SPI medium (0.2% $(NH_4)_2SO_4$, 1.4% $K_2PO_4$, 0.6% $KH_2PO_4$, 0.1% sodium citrate 0.02% $MgSO_4$, 0.02% casamino acids, 0.1% yeast extract, pH 7.2, 0.5% glucose, 50 µg / ml histidine). This culture was used to inoculate 100 ml of the same medium and this was grown at 37°C with vigorous aeration. The $A_{550}$ was determined periodically and, at the end of logarithmic growth, 10 ml of this culture was used to inoculate 90 ml of SPII (as SPI but without histidine). This culture was grown for a further 90 minutes and the cells were then centrifuged for 10 min at 5000 rpm, Sorval SS34 rotor. The cells were resuspended in 10 ml of supernatant in which glycerol had been added to 10 %. The competent cells were stored until use at -70°C.

Frozen competent cells (0.5 ml) were thawed quickly at 37°C and were placed in a 20 ml Sterilin tube. DNA (1-5 µg) was added in 10-20 µl and the mixture was incubated for 1 hour at 37°C with gently agitation. After one hour, 5 ml of L-broth was added and the culture was incubated for a further 1.5 hours with vigorous agitation. Aliquots of the culture were spread on L-agar plates containing kanamycin to select for plasmid-determined resistance.

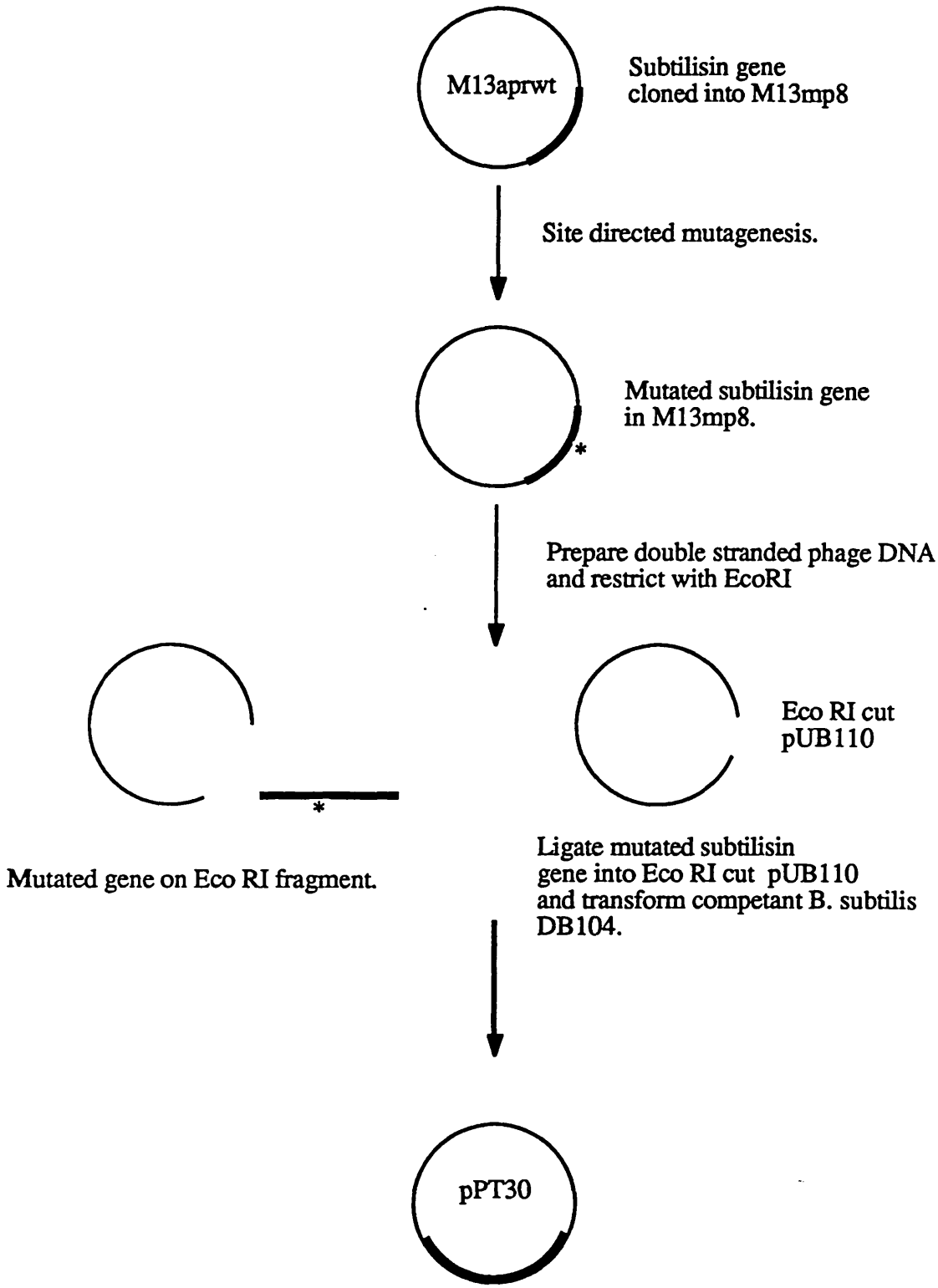



Fig. 2.1: Stratergy for the construction of mutant subtilisins.

Oligonucleotide directed Mutagenesis of the gene coding for subtilisin BPN'.

Strategy of mutagenesis.

The Subtilisin BPN' gene is expressed at high levels only in *B. subtilis*. Mutagenesis was, therefore, carried out in *E. coli* with the subtilisin gene cloned into M13mp8 and the mutated proteins were then expressed in *B. subtilis* (Fig. 2.1). The double-priming mutagenesis method (Zoller and Smith, 1984) was used. Yields of mutants were also increased by transforming the products of the mutagenesis reaction into a repair deficient strain. After the mutagenesis, the mutated genes were ligated into the plasmid vector pUB 110, and this was then used to transform *B. subtilis* DB104. This strain of *B. subtilis* has a lesion in the alkaline protease gene and does not express any subtilisin. There is, therefore, no problem of interference of endogenous enzyme when subtilisin mutants are expressed in this strain.

Design of mutagenic oligonucleotides.

The aim of this study was to replace the histidine residues in subtilisin by other amino acids which would not disrupt the structure of the enzyme. It was decided to replace the His residues by Gln because Gln is known to have some of the hydrogen bounding capacity of Histidine (Lowe *et al.*,1985), and only one change is need to

Table 2.4: Mutagenic oligonucleotides.

| Mutation | Sequence 5'-3' |
|---|---|
| HQ17 | CTTGAGATTGCAGAC |
| HQ39 | AATCAGTTGAGAAG |
| HQ67 | CGGCAACCTGAGTTC |
| HQ226 | CGGCAACTTGCGGAG |
| HQ238 | TCCAGTTCGGGTGCTTAGA |

convert a His codon to that of Gln. A series of five oligonucleotides was therefore designed to direct the alteration of the histidine codons in subtilisin to Gln codons. The histidine residues at positions 17, 67, 226 and 238 in subtilisin BPN' have the codon CAC while the histidine at position 39 is coded for by CAT. The His codons of residues 17, 39, 226 and 238 were mutated to the Gln codon CAC while the codon for His 67 was mutated to CAG.

Phosphorylation of oligonucleotides.

Oligonucleotides (100 pM) were phosphorolated using 0.5 units of T4 polynucleotide kinase (BRL) in a solution of total volume of 20 µl containing 1xkinase buffer 10 mM dithiothreitol and 1 mM rATP. Reactions were carried out for 30 min at 37°C and the polynucleotide kinase was then heat inactivated by incubating the mixture at 70°C for 10 min.

Mutagenesis reactions.

Phosphorylated mutagenic oligonucleotide (10 pmol) and M13 universal primer (10 pmol) were annealed to 1 µg of M13apr template in 10 µl of TM buffer by heating to 80°C and slowly cooling to room temperature. A solution (8 µl) of dATP,dCTP, dGTP

and dTTP at 0.5 mM, 0.5 mM rATP and 10 mM DTT in TM buffer was then added to the anealed mixture. The extension and ligation reactions were started by adding 1 unit of T4 DNA ligase (BRL) and 1 unit of DNA polymerase Klenow fragment and the reaction was allowed to proceed overnight at 15°C. TE buffer was then added to make the solution up to 200 µl and 0.1 , 1 and 10 µl samples were then used to transform competent BMH 71-18 *mut L* cells using TG2 as the plating cell in order to minimise the exposure of the DNA to the mutator strain.

Colony screening.

Colony blotting was adapted from Grunstein and Hogness, 1975. Plaques from the transformation of the products of the mutagenesis reaction (typically 50-100) were gridded out on an H-plate using a sterile tooth pick and grown as infected colonies at 37°C overnight . The colonies were transferred onto nitrocellulose filters (Schleider & Schuell, BA85) and the filters were marked so that they could be orientated with respect to the colonies on the master plate. The filters were transferred to a sheet of Whatman 3MM paper which had been soaked in 0.5 M NaOH and left for three minutes. The filters were neutralised by transferring them to sheets of Whatman 3MM paper soaked in 1M Tris-HCl twice for 1 minute. The filters were then transferred to paper that had been soaked in 0.5 M tris-HCl , pH 7.5, 1.5M NaCl for 5 min. The filters were air dried and then baked between sheets of Whatman 3MM paper at 80°C for 1 hour.

An oligonucleotide probe was prepared by labelling (phosphorylating) the mutagenic oligonucleotide primer with $^{32}P$ using T4 polynucleotide kinase (BRL). 100 pmol of primer was phosphorylated using 0.5 units of polynucleotide kinase with 1mM, 3000 Ci / mmole $\gamma$-$^{32}P$ ATP (New England Nuclear) as the substrate in 30 µl of kinase buffer. The reaction was carried out at 37°C for 30 min and 50 µl of 6xSSC buffer was then added. The labelled primer was purified from unincorporated label by passing the solution through a 1 ml spinning column of Sephadex G-25 equilibrated in 6xSSC (Maniatis *et al.*, 1982). Filters were prehybridised in sealed plastic bags in Denhardt's solution (Denhardt, 1966) containing 100 µg / ml of sonicated salmon sperm DNA (Sigma) and 0.2% SDS for 2 hours at 67°C in sealable plastic bags. The filters were cooled to room temperature and labelled primer was added to a final concentration of 1 pMol / µl. Hybridisations were carried out for 16 hours at room temperature. The filters were then removed from the plastic bags and rinsed with 3x100 ml of 6xSSC buffer at room temperature. The filters were then wrapped in Saranwrap and autoradiographed for 6 hours. The dissociation temperature ($T_d$) for the binding of the mutagenic primer to mutant DNA was calculated using the relationship

$$T_d = 4°C \text{ per GC base pair} + 2°C \text{ per AT base pair}$$

(Suggs *et al.*, 1982). The filters were washed twice for 2 min at a temperature 4°C below the $T_d$ value and autoradiographed overnight. This process was repeated until mutant colonies could be clearly distinguished. The yields of mutants varied between 10

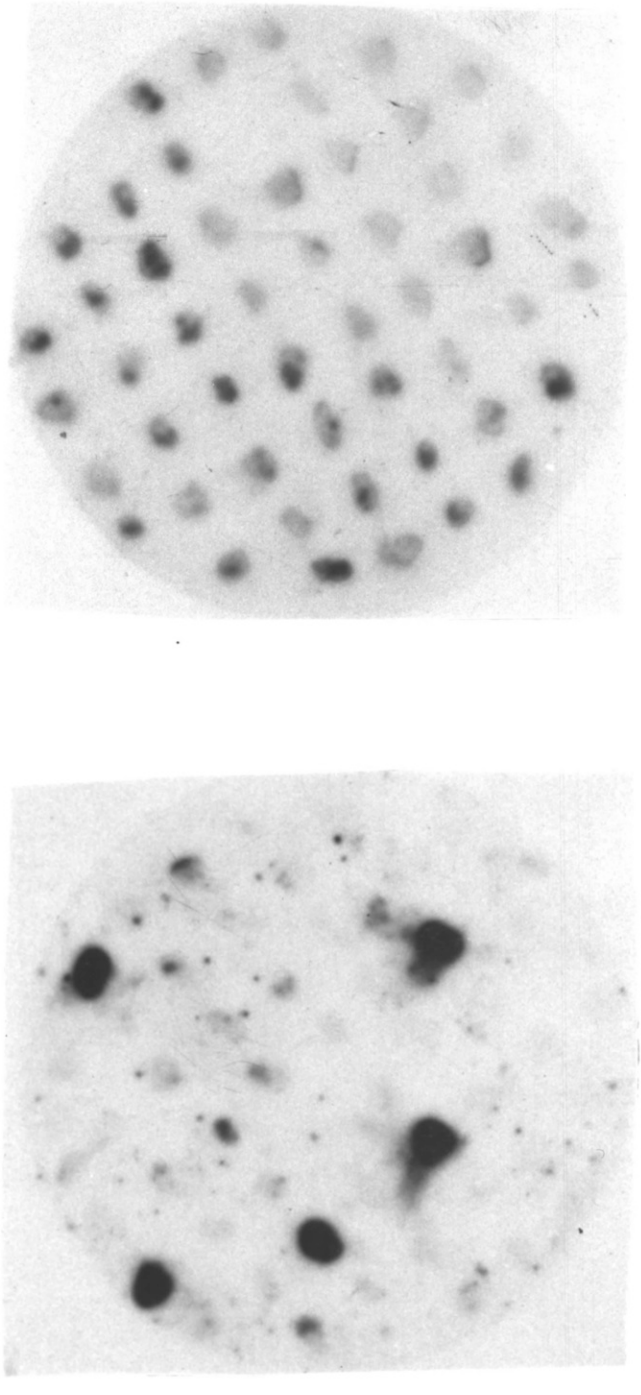


Fig. 2.2: Identification of the His→Gln 67 subtilisin BPN' mutant by colony hybridisation. Top; room temperature wash, bottom; 48°C wash ($T_d$ - 2).

Table 2.5: Subtilisin sequencing primers.

| Primer | Sequence (5'-3') | Priming position |
|---|---|---|
| SP1 | AAGATTAACGCTAAAGC | 485 |
| SP2 | TTTTCAGAAATGACATC | 620 |
| SP3 | CGTACGGATGTGCTACG | 754 |
| SP4 | AGCCGCAACTGTGCCGC | 975 |
| SP5 | TCGCCCACTCGATTCC | 1097 |
| SP6 | CCGGCTGCCGCAACGAC | 1214 |
| SP7 | CAGGTCCTACGCTTGA | 1337 |
| SP8 | GATCAGCCCTTTTCC | 1559 |
| SP9 | GACCGGAAACCGGGAAG | 1769 |

and 20%. The results of a typical colony hybridisation experiment are shown in Fig. 2.2

Verification of mutants by DNA sequencing

Cells from positive colonies were picked into 1 ml of 2xTY broth with a sterile toothpick and the diluted cells were struckout over an H plate using a sterile loop. H-top agar (3 ml) containing 200 µl of an overnight TG2 culture was poured onto the plate. After the agar had set the plate was inverted and incubated overnight at 37°C. Single stranded template DNA was then prepared from six isolated plaques.

The sequences of mutant subtilisin genes were checked by dideoxy chain termination DNA sequencing using a modification of the method described by Bankier and Barrell (1983). The sequencing reactions were carried out using a series of primers distributed thought the subtilisin gene (Table 2.5). Sequencing primer (0.4 pM) was anealed to template DNA (0.25 pM) in 10 µl of TM buffer by heating the to 80°C and then slowly cooling to room temperature. The anealed primer and template was added to 4 capless Eppendorf tubes (2 µl per tube) that had been labelled A,G,C,T. The appropriate deoxynucleotide mixtures (1 µl of mixtures listed in Table 2.6) and dideoxynucleotides (1 µl of a 0.1 mM solution except for ddTTP where a 0.4 mM solution was used ) were added to each tube. The sequencing reaction was started by adding 2 µl of a mixture containing 2.5 µCi of [$\alpha$-$^{32}$P] dATP (Amersham International 410 Ci / mMol) and 0.25

Table 2.6: DNA sequencing reagents

| dNTP | A tube | G tube | C tube | T tube |
|---|---|---|---|---|
| 0.5 mM GTP | 20 µl | 1 µl | 20 µl | 20 µl |
| 0.5 mM CTP | 20 µl | 20 µl | 1 µl | 20 µl |
| 0.5 mM TTP | 20 µl | 20 µl | 20 µl | 1 µl |
| TE buffer | 5 µl | 5 µl | 5 µl | 5 µl |

units of the Klenow fragment of DNA polymerase I ( BRL) in TM buffer. The reaction was allowed to proceed at room temperature for 15 minutes. A chase mixture (2 µl) containing all four deoxynucleotides at 0.5 mM was then added and the reactions were incubated for a further 15 min. The reactions were terminated by addition of a dye mixture (2 µl) consisting of bromophenol blue (0.1% w/v) and xylene cyanol (0.1 % w/v) in formamide containing 10 mM EDTA. The products of the sequencing reactions were resolved on 360 x 180 x 0.4 mM 6% polyacrylamide gels containing 8 M urea and 1x TBE buffer. Prior to loading onto the gel the samples were boiled for 2 minutes. Electrophoresis was carried out at 40 Watts constant power using 1xTBE as the running buffer. After electrophoresis the the gel was fixed in 10 % acetic acid for 15 minutes and then transferred to a sheet of Whatman 3 MM paper. The gel was dried under vacuum for 1 hour at 80°C and then autogradiographed overnight using Fuji RX X-ray film. The result of a typical DNA sequencing experiment is shown in Fig. 2.3

Expression of mutated subtilisins in *B. subtilis*.

Double stranded RF M13apr DNA of the mutant gene and pUB110 DNA, prepared using the methods described, were cleaved with Eco RI restriction enzyme (BRL). Equal quantities of pUB110 and M13apr DNA (typically 5 µg) were ligated together using 1 unit of T4 DNA ligase (BRL) in a total volume of 20 µl of TM buffer containing

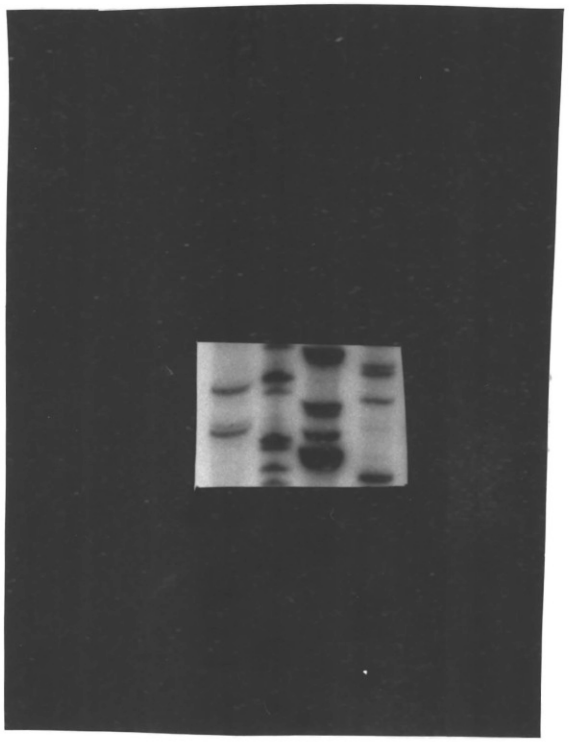


Fig. 2.3: DNA sequencing gel showing the sequence of the His-Gln 67 mutation. The gel is loaded from left to right, AGCT. The sequence reads (top to bottom) GTGCCGGCAACC*TGAGTTCCG. In the native sequence the base marked with a * is a G.

0.5 mM rATP and 1 mM dithiothreitol. Ligation mixture was incubated overnight at 15°C. Equal proportions of the two DNA substrates in the ligation reaction were used in order to favor the formation of concatemeric DNA which is required to transform *B. subtilis* (Hardy, 1985). At the end of the ligation reaction, a sample of DNA was analysed on agarose gel electrophoresis to check for the formation of the required product. The ligated DNA was used to transform competent *B. subtilis* DB104 using the method described. Aliquots of the transformation mixture were spread onto milk plates containing kanamycin. This allowed for the identification of recombinates containing the subtilisin gene ligated into pUB110, as colonies containing this plasmid are surrounded by clearing zones where the subtilisin produced has digested the milk in the agar. During a transformation, competent *B. subtillus* cells can take up more than one plasmid (Hardy, 1985). Subtilisin-producing colonies were, therefore, colony-purified by streaking out a single colony on milk plates 3 times.

## 1.2: Protein Methods

### Electrophoresis

### SDS-polyacrylamide electrophoresis.

SDS-polyacrylamide electrophoresis was carried out using a modification of the method of Laemmli (1970). A mixture of polyacrylamide (10%), 0.3% N,N-bis-methylene acrylamide in 0.1M tris-glycine buffer *p*H 8.3 containing 0.1% SDS was prepared. Polymerisation was initiated by the addition of tetramethyl-ethylene-diamine (0.25% V/V) and ammonium persulphate (0.02% w/v). The gel was cast in 1 mM thick 83x40 mM moulds. Protein samples (0.1-1.0 µg) were mixed with an equal volume of loading buffer (10 mM Tris-Glycine, *p*H 8.3 0.02% bromophenol blue, 2% SDS 10% 2-mercapto ethanol and 25% glycerol ) and boiled for 3 minutes. PMSF was added to subtilisin samples prior to boiling to prevent autolysis. Samples of 10 µl were loaded onto the gel which was run in 0.1 M tris glycine (pH 8.5) at a constant 75V. The gel was stained in (0.1%) Commassi blue for five minutes and was destained until clear with 50 % methanol 10% acetic acid.

Isoelectric focussing gel electrophoresis.

Isoelectric focussing gel electrophoresis was carried out using Ampholine PAG plates pH range 3.5-9.5 (LKB) using a Multiphor electrophoresis apparatus. Samples (0.5 mg / ml, 15 µl ) were applied to the gel using a micropipette. Electrophoresis was carried out at 1500 V for 2 hours, with 1M $H_3PO_4$ as the anode buffer and 1M NaOH as the cathode buffer. The gel was fixed in 10% TCA containing sulphosalicylic acid (35g / L) for half an hour and was then stained with Commassie blue for 10 minutes at 65°C. The gel was then destained in 25% ethanol / 8% acetic acid until the protein bands became visible.

Purification of subtilisin BPN'.

Wild-type and mutant subtilisins were purified from the supernatants of stationary phase cultures of *B. subtilis* DB104 containing the plasmid pPT30 or a mutated derivative thereof. Cultures (10L) were grown in L-broth containing kanamycin (25 µg / ml), glucose (0.2%) and 2% $CaCl_2$, in a MBR fermentor at 37°C for 24 hours. Prior to harvesting, the culture was chilled to 10°C and the cells were removed by micro-filtration using a millipore HVLP000c5 filter cassette. The supernatant was concentrated to 500ml using a PTGC00005 membrane filter, which has a molecular weight cut-off of 10,0000.

The concentrated supernatant was diluted to 10 L with 0.01 M TES *p*H 6.2, in order to reduce the ionic strength, and was reconcentrated to 500 ml. The concentrate was then loaded onto a 30 ml CM-TrisAcryl (LKB) column equilibrated in 0.01 M TES *p*H 6.2 (start buffer). The column was washed with three volumes of start buffer and subtilisin was eluted using start buffer containing 100 mM NaCl. Subtilisin was found to be greater than 90% pure as judged by active site titration and SDS polyacrylamide (Fig. 2.4) and isoelectric focusing gel electrophoresis. Yields of subtilisin varied between 50 to 100 mg per litre of culture.

Kinetic analysis of mutant subtilisins.

Active site titration

The concentration of subtilisin was determined by active site titration using N-*trans*-cinnamoylimidazole (Bender *et al.*, 1966). N-*trans*-cinnamoylimidazole (NCI) reacts with subtilisin to form a relatively stable cinnamoyl-subtilisin adduct (Fig. 2.5). The subtilisin concentration can be calculated from the resulting decrease in absorbance at 335 nM. The reaction was carried out at 25°C in 25 mM phosphate buffer pH 7.14 and was monitored at 335 nM using a Gilford 2600 spectrophotometer. NCI (Aldrich), 25 µl, 0.66 mM in dry acetonitrile was added to 2.35 ml of buffer in a 3 ml quartz cuvette and the



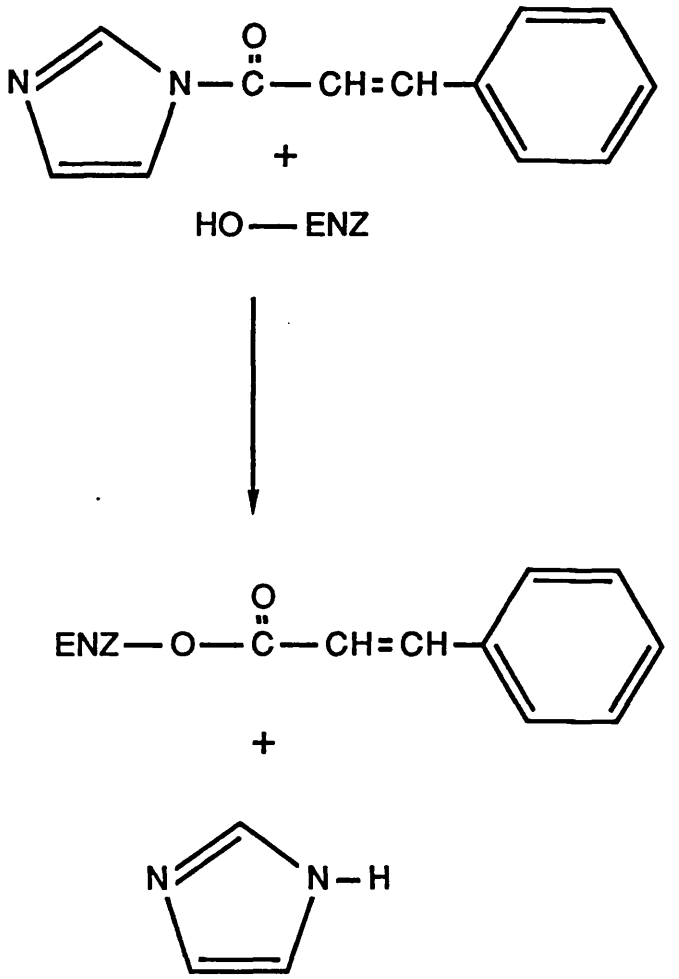
Fig. 2.4: SDS polyacrylamide gel of purified subtilisin BPN'. Marker proteins (lane 2) are bovine albumin (M.W. 66,000), egg albumin, (M.W. 45,000) carbonic anhydrase (M.W. 29,000) and barnase (M.W. 12,500).

spontaneous hydrolysis rate of NCI was determined. Subtilisin (200 µl of an approximately 10 mg / ml solution) was quickly added and the rapid decrease in absorbance was recorded. The concentration of subtilisin was then calculated from the decrease in absorbance at 335 nM using the known extinction coefficient of the cinnamoyl-subtilisin adduct.

Determination of $k_{cat}$ and $K_m$ for the hydrolysis of succinyl-L-Ala-L-Ala-L-Pro-L-Phe *para*-nitroanilide (suAAPFpN).

The hydrolysis of suAAPFpN by subtilisin was followed by observing the increase of absorbance at 412 nM as a result of the release of *p*-nitroanalide. A series of solutions of suAAPFpN in 0.1M Tris-HCl *p*H 8.6 were prepared at concentrations covering a range 1/5 to 5 times the expected $K_M$ value. The reactions were started by addition of 10 µl of enzyme solution (10-20 µM as determined by active site titration) to 3 ml of buffered substrate which had been equilibrated at 25°C. The increase in absorbance at 412 nM was followed using a Gilford 2600 spectrophotometer and the initial rate of reaction at each substrate concentration was calculated. Values of $k_{cat}$ and $K_m$ were obtained by fitting the initial rate vs substrate concentration data to the Michelis Menten equation using a data

Fig. 2.5: Scheme for the active site titration of subtilisin.



fitting computer program (Leatherbarrow, 1986) .

Determination of the pH dependence of $k_{cat} / K_M$ for the hydrolysis of suAAPFpN by subtilisin BPN' in $D_2O$.

The variation of $k_{cat} / K_M$ with pH reflects the ionization of groups on the free enzyme provided the substrate does not ionise within the range of pH being studied (Fersht, 1985). The p$Ka$ of the active site histidine in unligated subtilisin can be determined from the variation of $k_{cat} / K_M$ for the hydrolysis of suAAPFpN with pH. When the experiment is performed under similar conditions to the NMR experiments, the values obtained for the p$Ka$ of the active site histidine by the two methods should be comparable.

A series of buffers were prepared in the pH range 6.0-8.0 containing 10 mM TES and 250 mM KCl in $D_2O$. For each pH value enzyme (10 µl 10-20 µM) was added to 3 ml of buffered substrate (8 µM) maintained at 25°C in a cuvette and the increase in absorbance at 412 nM was followed to completion. Under these conditions the substrate concentration is very much lower than $K_M$ and the rate of reaction is given by

$$v = (k_{cat} / K_M) [E_o] [S]$$

Values for $k_{cat} / K_M$ for each pH were obtained from fitting the substrate concentration vs time data to this equation using a data analysis program. The p$Ka$ for the active site

histidine was obtained by fitting the $k_{cat} / K_m$ vs pH data to a single ionization equation

$$(k_{cat} / K_M) = (k_{cat} / K_M)_{Max}( K_a / ( [H^+] + K_a))$$

using a least square data analysis program.

Purification of barnase.

Barnase was purified from cultures of *E. coli* JM101 containing the plasmid pMT410 (Paddon and Hartley, 1987). This plasmid is a derivative of pUC9 and contains the genes of barnase and its intracellular inhibitor barstar. The expression of the barnase gene is under the control of the promoter of the E. coli alkaline phosphatase (pho A) promoter which is repressed by high levels of phosphate. In order to produce barnase using pMT 410, bacteria containing the plasmid were initially grown in a rich medium and were then transferred to a low phosphate media to induce expression. In pMT 410, the barnase gene is downsteam of DNA coding for the alkaline phosphatase leader peptide which directs the protein to the periplasmic space. Barnase can released from the periplasmic space by addition of acetic acid which disrupts the outer cell membrane but does not lyse the cells. The mutants His→Ala 102, Glu→Ala 60, Lys→Ala 27, Leu-→Val 96 and Leu→Val 88 were constructed by K. Nyborg whilst the mutants His→Gln 18 and Phe→Leu 7 were

constructed by D. Sali.

Starter cultures were grown overnight at 37°C in L-broth. The overnight cultures were used to inoculate MOPS media (Serpersu *et al.* 1987) (1 in 100 dilution) and the bacteria were grown overnight at 37°C. The cultures were then acidified by adding glacial acetic acid (55 ml / l) and the cells were removed by centrifugation (8,000 RPM in a Sorval SS-34 rotor). SP-Tris-acryl (LKB) was added to the supernatant (6 ml / l) and the mixture was stirred for 2 hours at 4°C. The resin was allowed to settle and the liquid was decanted off. The resin was then packed into a column and washed with 3 volumes of 50 mM sodium acetate pH 5.2. Barnase was eluted using 50 mM ammonium acetate pH 5.2 containing 1M sodium chloride. Fractions were assayed for barnase activity by spotting a sample (5 µl) onto a plate containing tRNA (2 mg / ml yeast torula RNA , 0.1M Tris-HCl pH 8.5, 2% agar). The plate was then incubated at 37°C for 15 min. The plate was developed by addition of 15% trichloroacitic acid. Fractions containing ribonuclease activity give clear zones. The barnase containing fractions were then pooled and dialysed against 10 mM sodium acetate pH 5.2 using Spectrapore 3500 MW cutoff dialysis tubing. The protein was then loaded onto a 5 mL Pharmacia FPLC Mono S column. A 0-400 mM gradient of sodium chloride in 10 mM sodium acetate pH 5.2 was used to develop the column. Barnase was eluted from the column at approximately 200 mM NaCl. The protein was

homogeneous as judged by SDS-polyacrylamide electrophoresis and gave a single band on an isoelectric focusing gel at a pI of approximately 8.6 (Fig. 2.6). The yields of wild type barnase and the mutants His→Ala 102, Ile→Val 96, Ile→Val 88, Lys→Ala 27 and Glu-Ala 60 were between 15-20 mg per litre of culture. The mutants His-Gln 18 and Phe-Leu 7 gave only 1-2 mg per litre of culture, presumably because of increased susceptibility to protolysis.

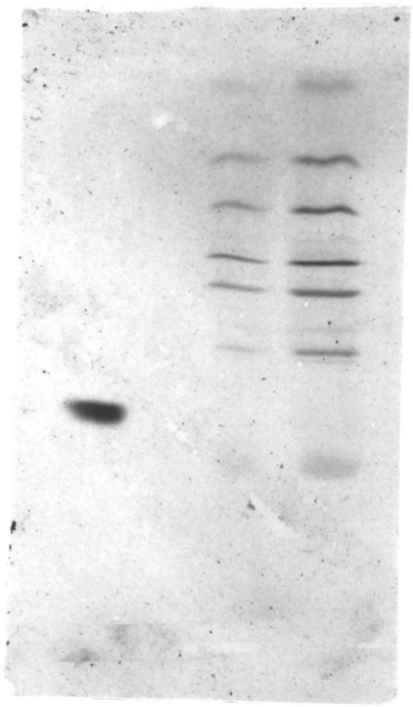


Fig. 2.6: Isoelectric focusing gel of purified barnase (lane 1). Lanes 2 and 3 I.E.F.marker proteins (Sigma), PI's 7.16, 6.76, 6.57, 5.85, 5.13, 4.55.

## 2.3: N.M.R. Methods

$^1H$ N.M.R. spectra were recorded on a Bruker AM-500 spectrometer linked to an Aspect 3000 computer using a dedicated proton probe or a proton / carbon dual probe. The sample volume was between 0.4-0.5 ml of solution and was contained in a 5 mM diameter tube (Aldrich, gold label). Chemical shifts were calculated relative to 4,4-diamethyl-silapentane-1-sulponate (DSS) at 0 ppm as an external standard. The residual HOD signals were suppressed by gated decoupling during the relaxation delay.

For subtilisin, the lyophilized protein was dissolved in 0.5 mL $D_2O$ containing 250 mM KCl and 5 mM EDTA to give a 1-1.5 mM solution based on absorbance at 280 nM ($A_{280}$=1.17 for a 1 mg / ml solution). Spectra were recorded using a Carr-Purcell-Meiboom-Gill spin echo pulse sequence produced by modification of the CPMG pulse sequence provided in the manufacturer's DISNMR program. Sixty four transients were average for each spectrum using 8 K data points with a sweep width of 6200 Hz and a total recycle time of 2.5 seconds. Prior to Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz.

For barnase, lyophilized protein was dissolved in 0.5 mL $D_2O$ to give a 1-2 mM solution based on absorbance at 280 nM ($A_{280}$ for a 1 mg / ml solution of barnase = 2). Before the spectra were recorded, amide backbone N-H protons were exchanged by incubating the enzyme for 16 hours at room temperature at pH 9. Spectra were obtained with a 75° observation pulse in 16 K data points with a spectral width of 6000 Hz. The total recycle time between pulses of 2.6 S. 128 transients were averaged for each spectrum.

Protein solutions were titrated by adding microlitre aliquots of NaOD or DCl (Sigma). pH measurements were made using a Radiometer PHM64 pH meter and a micro-electrode (Aldrich) directly in the NMR tube. The pH meter was calibrated using pH 4.01 and pH 7.00 standards in $H_2O$ (Radiometer). pH readings were uncorrected for the deuterium isotope effect on the hydrogen electrode and are denoted by pH*. Titration data was fitting to the equation

$$\delta_{obs} = \delta_A + (\delta_{AH} - \delta_A)\,[H^+] / (K_a + [H^+])$$

using a least squares data analysis program (Leatherbarrow, 1987). A minimum of 10 points were analysed for each pH* titration. All pH* titrations were reversible.

# CHAPTER 3: HISTIDINE C2-H RESONANCES IN THE $^1$H 500 MHz SPECTRUM OF SUBTILISIN BPN' AND CARLSBERG.

## 3.1: Resolution of Histidine C2 proton resonances in subtilisins.

The aromatic region of the 500 MHz $^1$H NMR spectrum of subtilisin BPN' is shown in Fig 3.1. The line widths in the spectrum appear to be consistent with a monomeric protein of molecular weight 27,500.

The histidine C2-H region of freeze-dried subtilisin which had just been dissolved in $D_2O$ is shown in Fig 3.2a. The sharp lines from the histidine protons can be seen, but are obscured from signals from undeuterated backbone N-H protons. When His C2-H resonances are being studied in a protein the signals from backbone N-H protons are normally removed by carrying out N-H to N-D exchange. The exchange rate for this process is very dependent on pH, with the maximum rates occurring at alkaline pHs. This causes a problem when proteases which have alkali pH optima are being studied as incubation under these conditions results in considerable loss of enzyme through autolysis. For the enzyme chymotrypsin, this problem was overcome by carrying out N-H to N-D exchange on the inactive proenzyme and then cleaving the exchange proenzyme to give

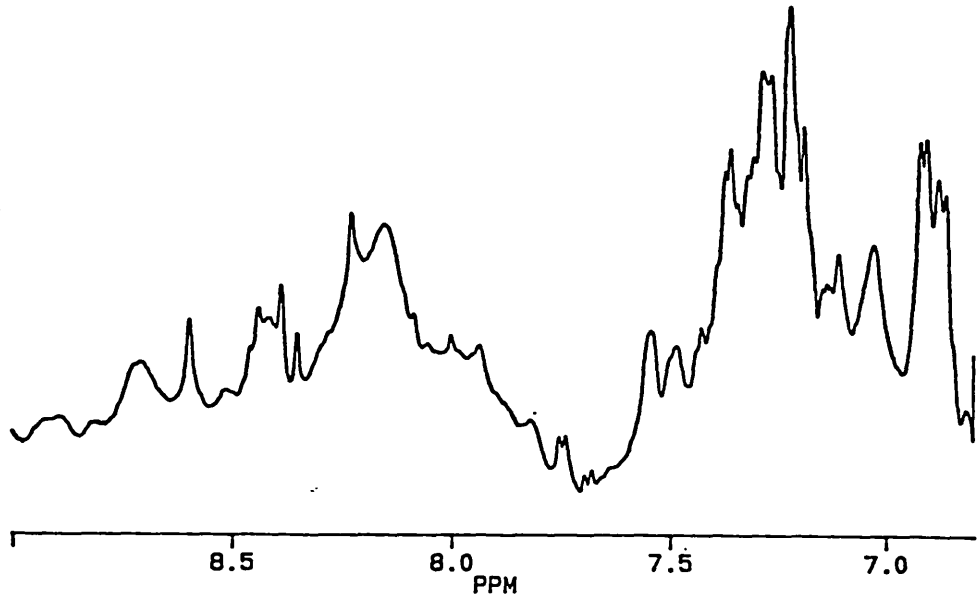



Fig. 3.1: 500 MHz $^{1}H$ NMR spectrum of subtilisin BPN' (25°C, pH* 6.50, 1mM protein 3 mM EDTA, 250 mM KCl).

active enzyme (Markley and Ibanez, 1978). The approach is not applicable to subtilisin as a stable proenzyme does not exist (Powers *et al.*, 1986). In previous studies on subtilisin, N-H to N-D exchange was carried out at high pH and the resulting proteolytic fragments were removed by gel filtration (Jordan *et al.*,1985) . Although this method appeared to give an exchanged and homogeneous preparation on the bases of the published spectrum the procedure result in the loss of 70-80% of the protein. To try and circumvent this, I attempted to suppress the N-H signals using a spin echo pulse sequence as described by Campbell *et al.* (1975). This method exploits the difference in relaxation time between the histidine protons which are attached to carbons and the amide protons that are attached to nitrogens, which relax more rapidly as a result of interactions with quadropolar $^{14}N$ nuclei. A $90_x$-($\tau$-$180_x$-$\tau$-)$_2$ acquire pulse sequence was used to produce the suppression. This is a modification of the Carr-Purcell-Meiboom-Gill spin echo pulse sequence used that is used to measure $T_2$ relaxation times. For suppression to be obtained, the time $\tau$ must be adjusted to a value where the N-H signals are fully relaxed but there is still a signal from the His C2-H protons. By systematic variation of the $\tau$ value it was found that for subtilisin, under the conditions used, 25 ms gave the best results. The suppression obtained is shown in Fig 3.2b. Since the spin echo method does not require a prexchange step this technique represents a considerable improvement over the previously reported method for resolving the histidine

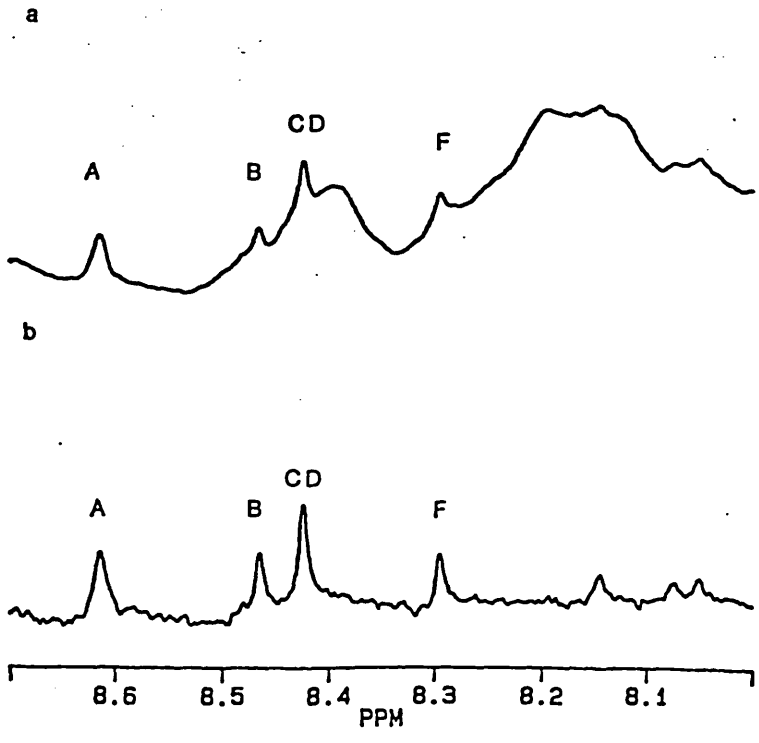



Fig. 3.2: Suppression of N-H resonances using a spin echo pulse sequence (Campbell *et al.*, 1975). a) 75° observation pulse, b) spin echo pulse sequence. The labelling of the peaks is discussed in the legend to Fig. 3.3

C2-H proton resonances in subtilisin.

## 3.2: pH titration of histidine C2-H protons in subtilisin BPN'.

The C2-H region of the NMR spectrum of subtilisin BPN' at five different values of $pH^*$ is shown in Fig 3.3. Below pH 7.0 five peaks were were observed to titrate with pH. Above this pH, one of the peaks splits into two peaks that persist throughout the alkaline titration. These resonances are labeled A to F in Fig 3.3. The variation of chemical shift with pH for all of these peaks fit a single ionization curve (Fig. 3.4) with $pK_a$ values and high and low pH plateau chemical shifts that were within the limits found for histidines in other proteins (Table 3.1). It therefore appears that these resonances correspond to the C2 protons of the six histidines in subtilisin BPN'.

## 3.3: pH-titration of histidine C2-H protons in subtilisin Carlsberg.

The previously reported NMR investigation of the histidine protons of subtilisins was carried out using a 200 MHz spectrometer on commercial preparations of subtilisins BPN' and Carlsberg (Jordan *et al.*, 1985). Since the publication of that study, it has been

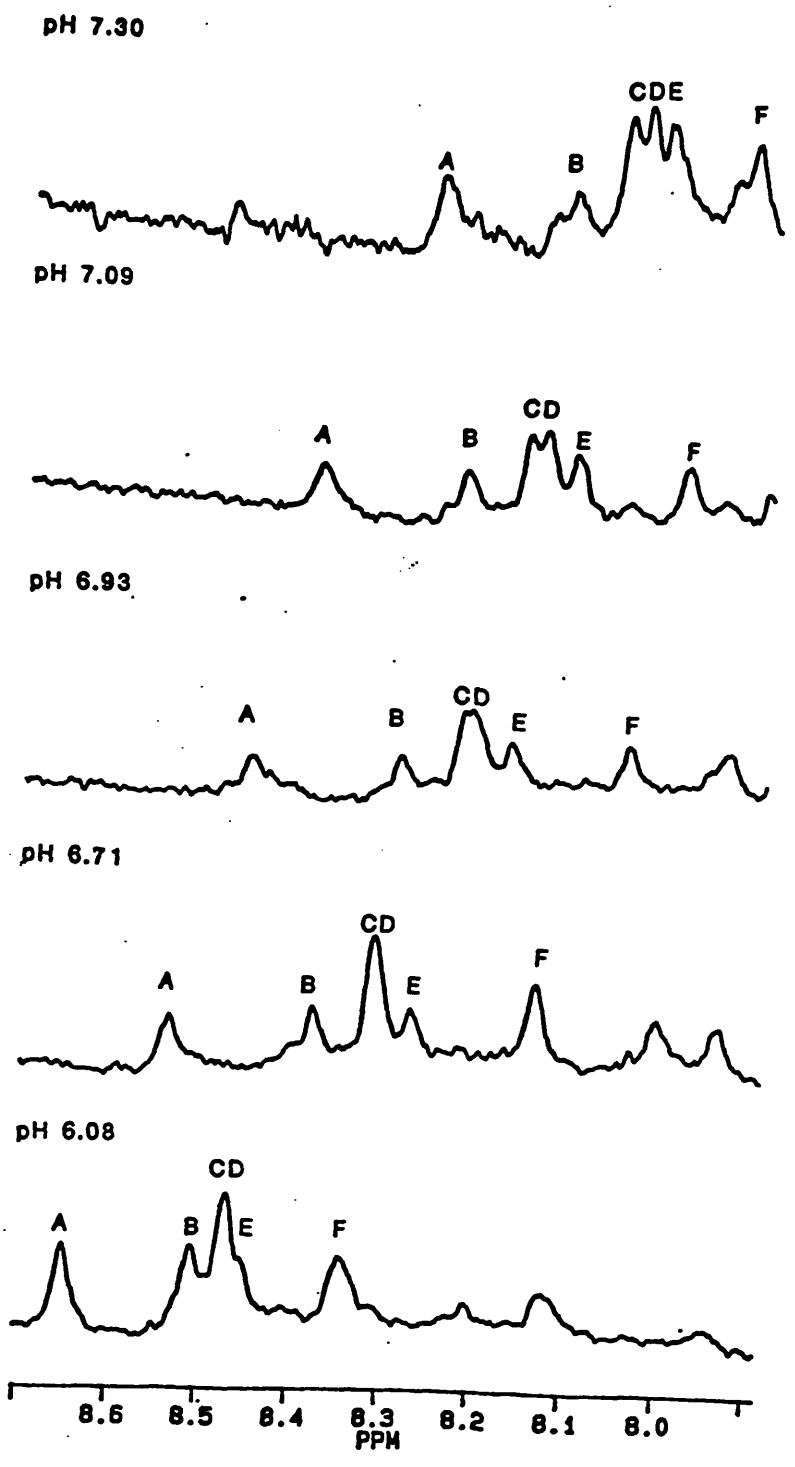



Fig. 3.3: Titration of the His C2 proton resonances in the 500 MHz $^{1}H$ NMR spectrum of subtilisin BPN'. The six peaks that titrate with the characteristics of histidine resonances are denoted A-F.

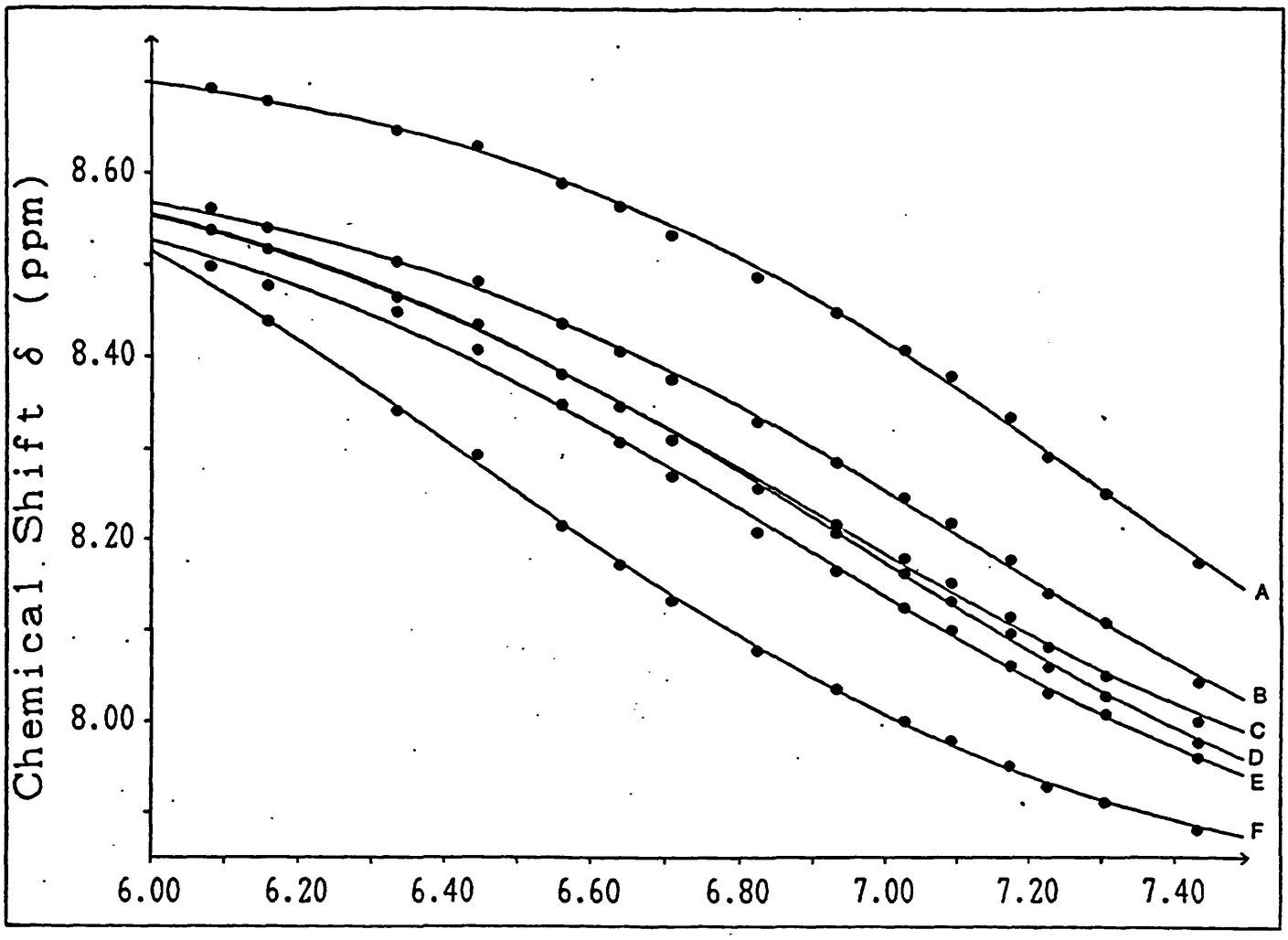



Fig. 3.4: $^{1}H$ NMR monitored titration of the histidine residues in subtilisin.

The solid lines are theoretical curves to the experimental points.

Table 3.1: $^1$H NMR titration parameters for the histidine residues in subtilisin BPN' (25°C, 250 mM KCl).

| Histidine peak[a] | p$K_a$ | low pH chemical shift | high pH chemical shift |
|---|---|---|---|
| A | 7.45 | 8.73 | 7.73 |
| B | 7.23 | 8.61 | 7.66 |
| C | 7.12 | 8.60 | 7.71 |
| D | 7.04 | 8.61 | 7.71 |
| E | 6.95 | 8.57 | 7.71 |
| F | 6.72 | 8.61 | 7.74 |

Notes: [a]The labels refer to the peak designation in Fig. 3.3

Uncertainty in all determinations less than ± 0.05.

shown that the subtilisin BPN' used was not the authentic enzyme, but was probably subtilisin Carlsberg (Russell and Fersht, 1986). The authors of that paper claimed that differences in the spectra of the two enzymes could be seen which were consistent with the fact that subtilisins Carlsberg and BPN' differ in the number of histidines (6 in BPN' vs 5 in Carlsberg). The titration experiments on subtilisin Carlsberg were therefore repeated at 500 MHz. The histidine C2-H region of subtilisin Carlsberg at three different $pH^*$ values is shown in Fig 3.5. Five resonances titrate and this is consistent with the five histidines known to be present in this enzyme. Analysis of the pH vs chemical shift data gives values for the $pK_a$ 's of these residues that are very similar to those reported by Jordan *et al.* (1985). In the 500 MHz spectrum of subtilisin Carlsberg, the peak labelled a in Fig. 3.5 is split into two, indicating that this residues exists in two environments in this protein. This appears to have produced the mistaken interpretation in the previous study of subtilisin Carlsberg, as this splitting is not as apparent at the lower frequency.

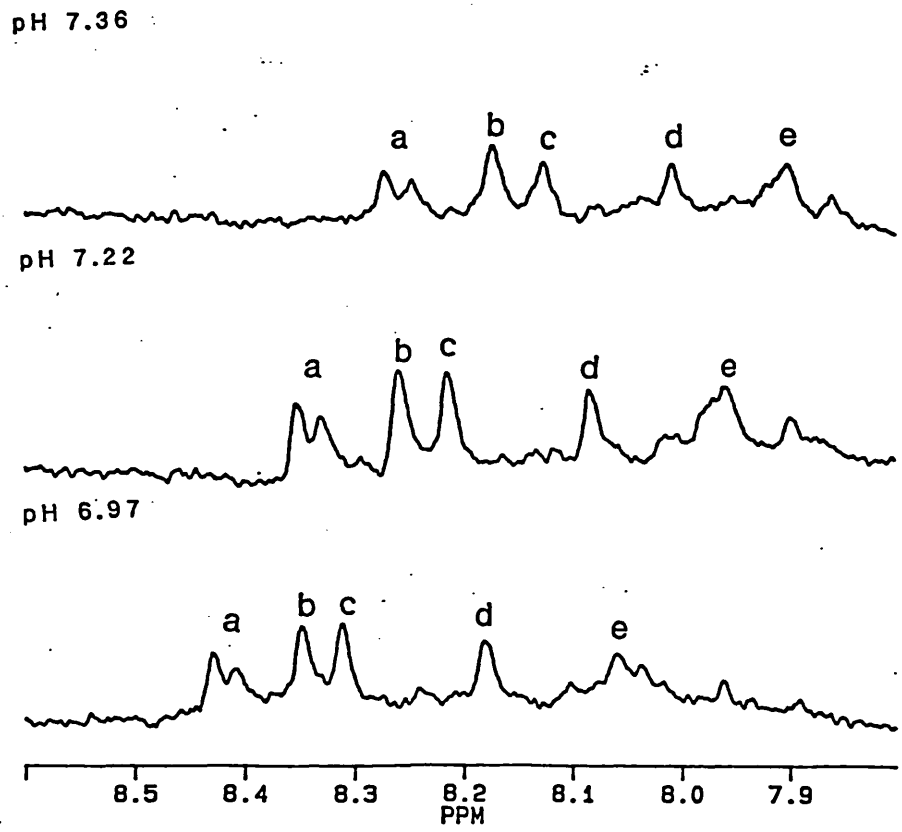



Fig. 3.5: Titration of the His C2 proton resonances in the 500 MHz $^{1}H$ NMR spectrum of subtilisin Carlsberg. The peaks that titrate with the characteristics of histidine resonances are denoted a-e.

# CHAPTER 4: NMR SPECTRA OF MUTANT SUBTILISINS.

## 4.1: Assignment of histidine resonances in subtilisin BPN'.

The histidine C2-H regions of the $^1H$ NMR spectra of the subtilisin mutants His→Gln 17, His→Gln 39, His→Gln 226 and His→Gln 238 are shown in Figs. 4.1 and 4.2. A spectrum of each mutant is presented together with a spectrum of the native enzyme at a similar pH*. Although only one representative spectrum of each protein is shown, full titrations were performed for each mutant. Inspection of the Fig. 4.1 clearly shows that substitution of His 39 by Gln results in the disappearance of peak E while the rest of the spectrum is unchanged. Resonance F is, therefore, that of the C2 proton of His 39. Inspection of this figure similarly shows that when His 238 is replaced with Gln peak A disappears. This leads to the assignment of peak A to the His C2 proton of residue 238. Spectra of the His→Gln 17 and His→Gln226 mutants are shown in Fig. 4.2. For both of these mutants only one resonance is observed to titrate with a p$Ka$ between 7.00 and 7.15, whereas for the native enzyme both peaks C and D titrate in this region. The p$Ka$'s obtained for the titration of the histidine resonances of the His→Gln 17 mutant are shown in Table 4.1. Comparison of the p$Ka$ values for the resonances present in His→Gln 17 to those for the native enzyme shows that peak D is absent in the spectrum of this mutant. The peak that disappears on substitution of His 226 is therefore

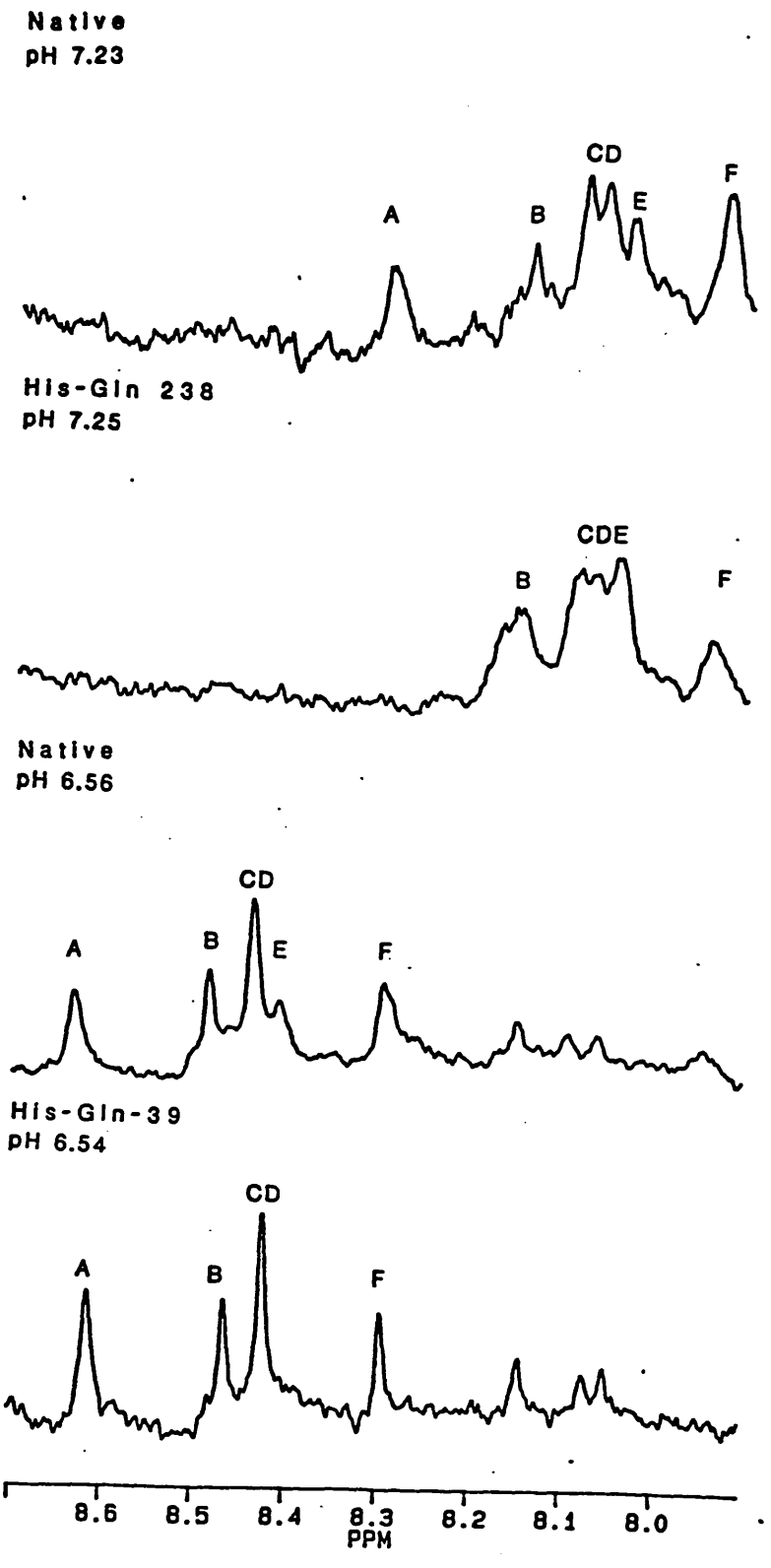



Fig. 4.1: 500 MHz $^{1}$NMR spectra of the histidine C-2 proton resonances of the subtilisin mutants His-Gln 238 and His-Gln 39. The labelling of the histidine resonances is the same as in Fig. 3.3.

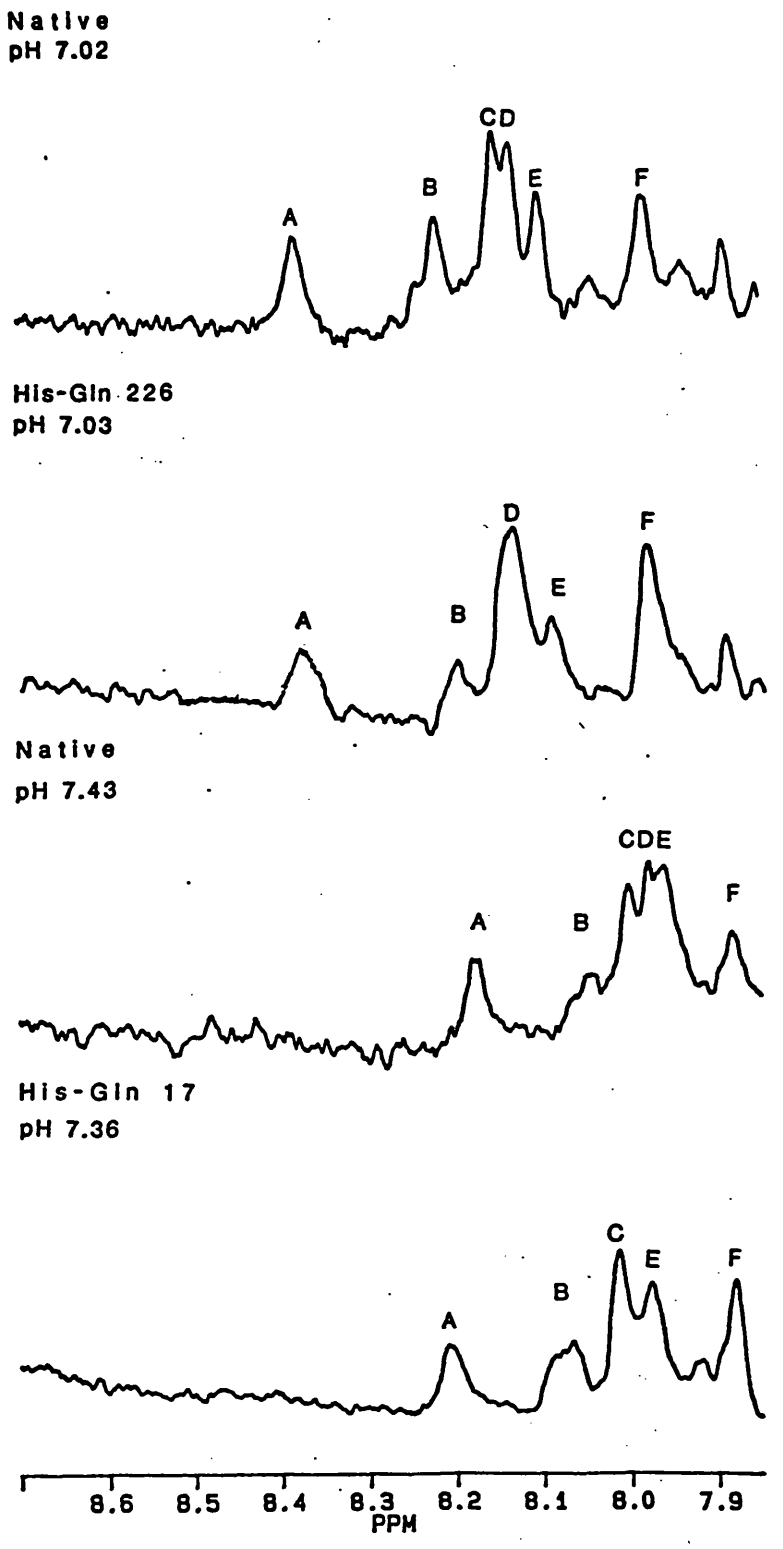



Fig. 4.2: 500 MHz $^{1}$NMR spectra of the histidine C-2 proton resonances of the mutants His-Gln 226 and His-Gln 17. The labelling of the histidine resonances is the same as in Fig. 3.3.

resonance C.

It was not possible obtain spectra of a mutant in which the active site histidine in subtilisin (His 64) has been substituted, because mutation of this residue has been shown to result in a large reduction in the level of protein expression (Carter & Wells, 1987), and it would not be feasible to obtain sufficient material for NMR studies. A value for the p*Ka* of this residue can however be obtained from kinetic measurements. The pH dependence of $k_{cat}$ / $K_M$ for the hydrolysis of suAAPFpNA by subtilisin gives a p*Ka* value that corresponds to the p*Ka* of the active site histidine in the free enzyme. If kinetic measurements are made under similar conditions to the NMR titrations, then the p*Ka* values from the two methods may be compared. Analysis of the pH dependence of $k_{cat}$ / $K_M$ for the hydrolysis of suAAPFpNA in $D_2O$ containing 250 mM KCl gives a p*Ka* value of 7.20 ±0.02. Of the unassigned resonances, only resonance B titrates with a similar p*Ka* and this may therefore be tentatively assigned to histidine 64. This assignment of the resonance of His 64 agrees with the assignment made, based on chemical modification evidence, for the active site residue in an homologous enzyme, subtilisin Carlsberg (Jordan *et al.*, 1986).

Only resonance F remains unassigned, and by a process of elimination it must correspond to histidine 67. The histidine C2-H region of the $^1H$ NMR spectrum of the His-Gln-67 mutant is shown in Fig. 4.3 Simple inspection of this spectrum shows that this mutation has produced substantial changes in this region. Analyses of the titration

Table 4.1: Comparison of the p$K_a$'s of the histidine residues native subtilisin BPN' and the mutant His-Gln 17 .

| Histidine resonance | p$K_a$ native | His-Gln-17 |
|---|---|---|
| A | 7.45 | 7.47 |
| B | 7.23 | 7.25 |
| C | 7.12 | 7.13 |
| D | 7.04 | ---- |
| E | 6.95 | 6.94 |
| F | 6.72 | 6.76 |

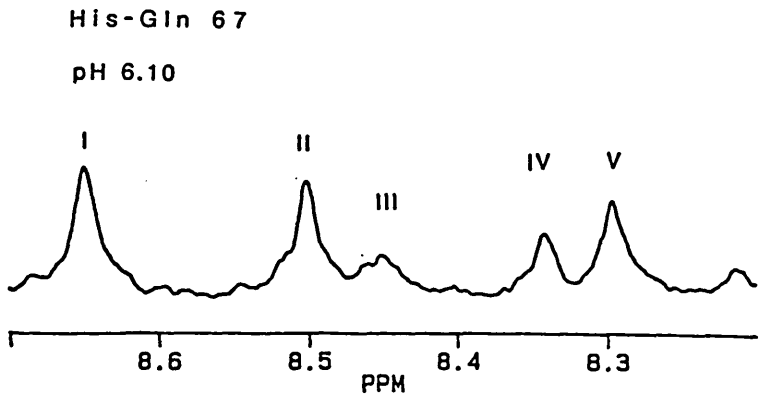



Fig. 4.3: 500 MHz $^{1}$NMR spectra of the histidine C-2 proton resonances of the mutant His-Gln 67. The peaks that titrate with the characteristics of histidine resonances are labelled.

data for this mutant gives p$K_a$ values of 7.48, 7.34, 7.11, 6.81, 6.64. Comparison of these values to those obtained for the native enzyme shows that the p$K_a$'s of histidines 238 and 39 appear to be relatively unaffected by the mutation, but that the p$K_a$'s of the histidines closest to the mutated residue (64, 226) have been perturbed.

The kinetic constants obtained for the hydrolysis of suAAPFpNA by mutant subtilisins are summarised in Table 4.2

For the mutants His→Gln 17, His→Gln 39, His→Gln 226 and His→Gln 238 the values of $k_{cat}$ and $K_M$ are within the experimental error limits of the values for the native enzyme. All of these residues are greater than 10 Å away from the active site residues. Mutation of His 64, however, results in a large increase in $K_m$ and a halving of $k_{cat}$. The changes in the NMR spectrum together with the observation of changes in the values of $k_{cat}$ and $K_m$ for the His→Gln 67 mutant suggest that mutation of this residue produces a conformational change in the enzyme.

The use of site-directed mutagenesis has enabled all the histidine C-2 proton resonances in subtilisin BPN' to be assigned and the assignments made are summerised in Table 4.3. The assignment of these residues by any other method would have been been difficult, and these results demonstrate the power of this technique for resonance assignment. Four out of five of the mutants studied give spectra that were identical to those of the native protein except for the absence of one of the histidine resonances. The similarity of the mutant and wild type spectra suggest that the mutation of these residues

Table 4.2: Kinetic properties of mutant subtilisins for the hydroysis of suAAPFpN (0.1M Tris-HCl, *p*H 8.6).

| Protein | Km/mM | kcat/s-1 |
|---|---|---|
| Wild type | 0.15 | 57 |
| HQ 17 | 0.16 | 53 |
| HQ 39 | 0.15 | 65 |
| HQ 67 | 1.16 | 25 |
| HQ 226 | 0.20 | 40 |
| HQ 238 | 0.15 | 51 |

Notes: Errors are ±10% in $k_{cat}$, ± 5% in $K_m$

Table 4.3 Summary of histidine assignments in subtilisin BPN'.

| Histidine resonance | residue number |
|---|---|
| A | 238 |
| B | 64 |
| C | 226 |
| D | 17 |
| E | 39 |
| F | 67 |

does not produce any large conformational change. These results are consistent with the findings of a number of X-ray crystalographic studies of mutant proteins that have indicated that point mutations do not produce major perturbations in protein structure (Howell *et al.*, 1986, Brown *et al.*, 1987). In contrast to the other mutants, mutation of His 67 does produce a number of changes in the His C-2 H region of the spectrum, in addition to the removal of one of the peaks, suggesting that a conformational change has been produced by the mutation of this residue. Assignment of a histidine resonance based on this mutant was, accordingly, difficult. Since in this study a series of mutants were analysed, it is still possible to obtain a complete assignment of the histidines in subtilisin BPN'. This results does, however, indicate that problems may be encountered using site-directed mutagenesis to assign structurally important residues.

## 4.2: Assignment of histidine resonances in subtilisin Carlsberg.

The amino acid sequences of subtilisins BPN' and Carlsberg are approximately 70% homologous and the crystal structure of the two enzymes are very similar (McPhalen *et al.*,1985). Both enzymes have histidines at positions 39, 64, 67, 226 and 238. Subtilisin Carlsberg, however, has a Gln at position 17 instead of a histidine. A comparison of the titration data for the histidines in subtilisins BPN' and Carlsberg is shown in Table 4.4. Four of the five conserved histidines appear to titrate with essentially identical p$K_a$ values in the two enzymes. The resonance that has been

Table 4.4: p$K_a$'s of histidine residues in subtilisins BPN' and Carlsberg (25°C, 250 mM KCl).

| Histidine | p$K_a$ in BPN' | [a]p$K_a$ in Carlsberg |
|---|---|---|
| 17 | 7.04 | absent |
| 39 | 6.95 | 6.94 |
| 64 | 7.23 | 7.22 |
| 67 | 6.72 | 6.80 |
| 226 | 7.12 | 7.16 |
| 238 | 7.45 | 6.36 |

[a]Data from Jordan *et al.* (1986).

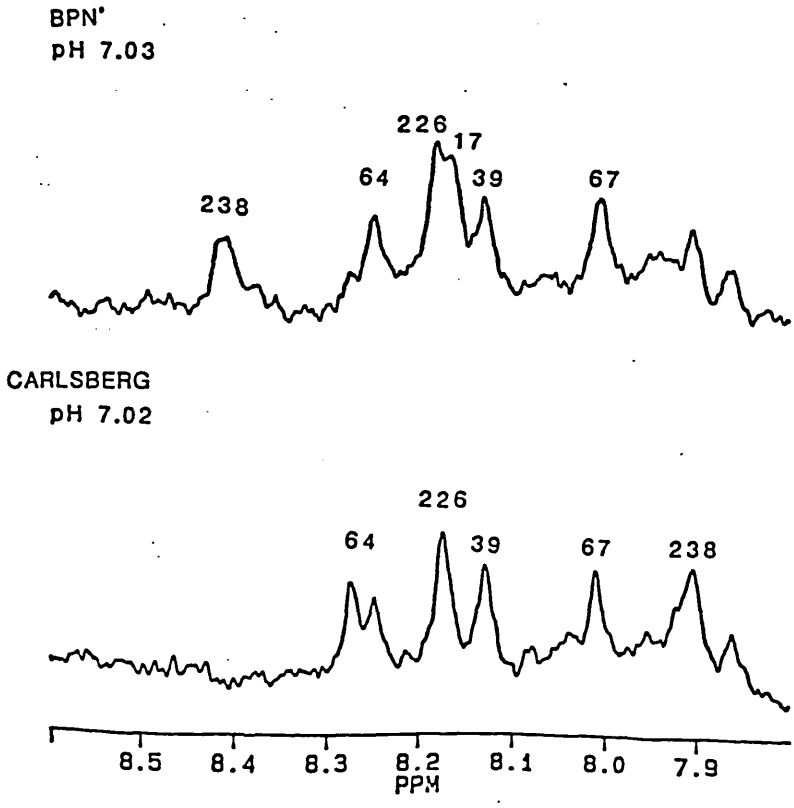



Fig. 4.4: Comparison of the C-2 proton resonances of subtilisins Carlsberg and BPN'. The labels refer to the assignments discussed in the text.

assigned to His 17 in subtilisin BPN' is absent in the spectrum of subtilisin Carlsberg, thus confirming the assignment made by mutagenesis. It appears therefore that for four out of the five conserved histidines the assignments made for these residues in subtilisin BPN' may be transferred to subtilisin Carlsberg. This would lead to the assignment of histidines 39, 64, 67, and 226 in subtilisin Carlsberg and the remaining unassigned peak would correspond to His 238 (Fig. 4.4).

## 4.3: Structural interpretation of the observed histidine p*Ka*'s in subtilisins BPN' and Carlsberg

The availability of assignments for the histidine C2-H resonances in subtilisin BPN' enables a comparison to be made of the p*Ka*'s of these residues to their environment in the crystal structure. The positions of the histidine residues in crystal structure of subtilisin BPN' are shown in Figs. 4.5 and 4.6.

In subtilisin BPN' histidines 64 and 238 have p*Ka*'s that are significantly perturbed from the random coil value (0.9 and 1.1 pH units respectively).

The perturbation of the p*Ka* of the active site histidine probably results from the interaction of this residue with Asp 32. This interaction would be expected to increase the p*Ka* of His 64 by stabilising the protonated form of the imidazole ring through an

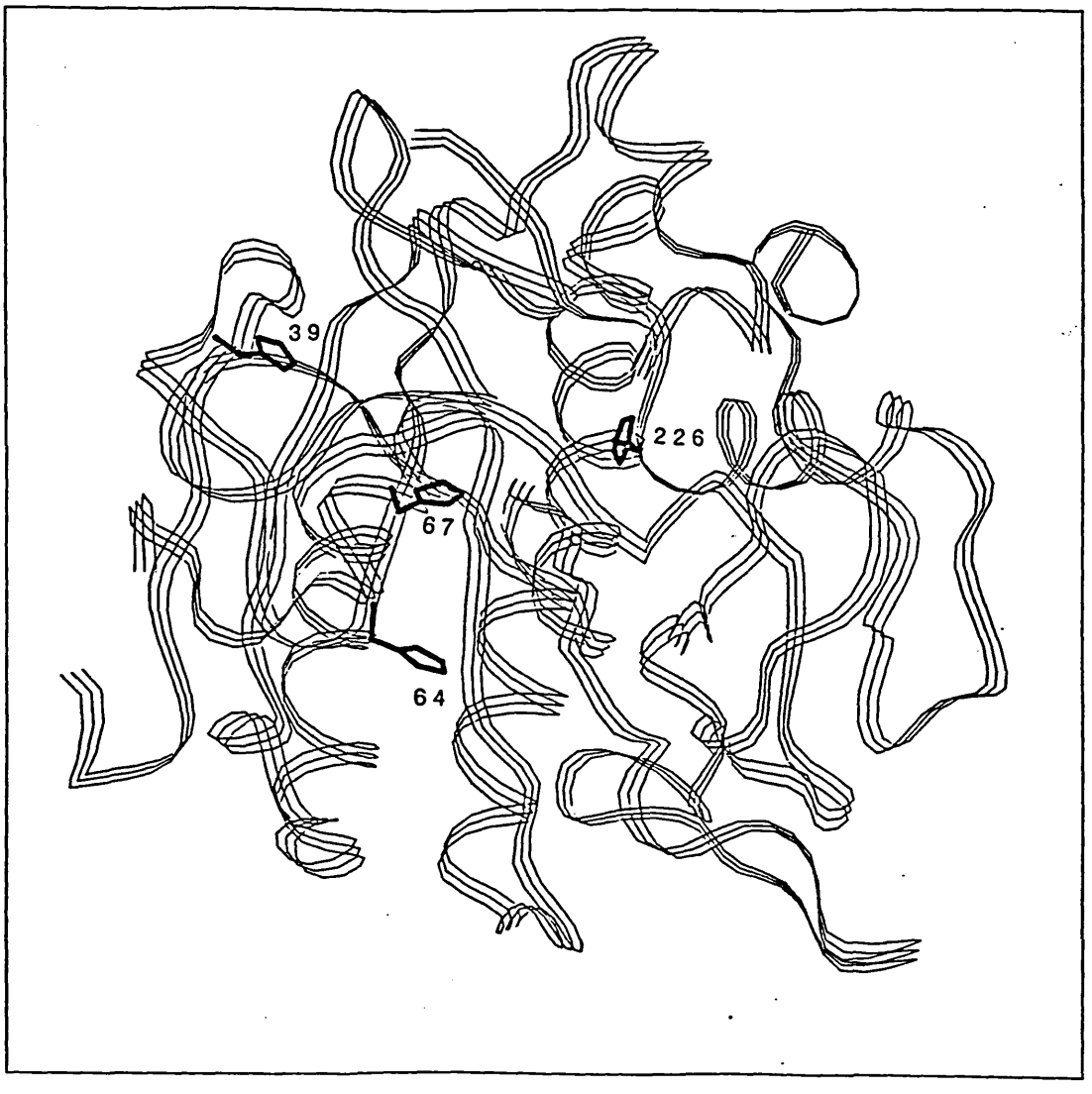



Fig. 4.5: Environments of the histidine residues 39, 64, 67 and 226 in the crystal structure of subtilisin BPN'. The main chain of the protein is depicted as a ribbon and only the side chains of the histidine residues are shown.

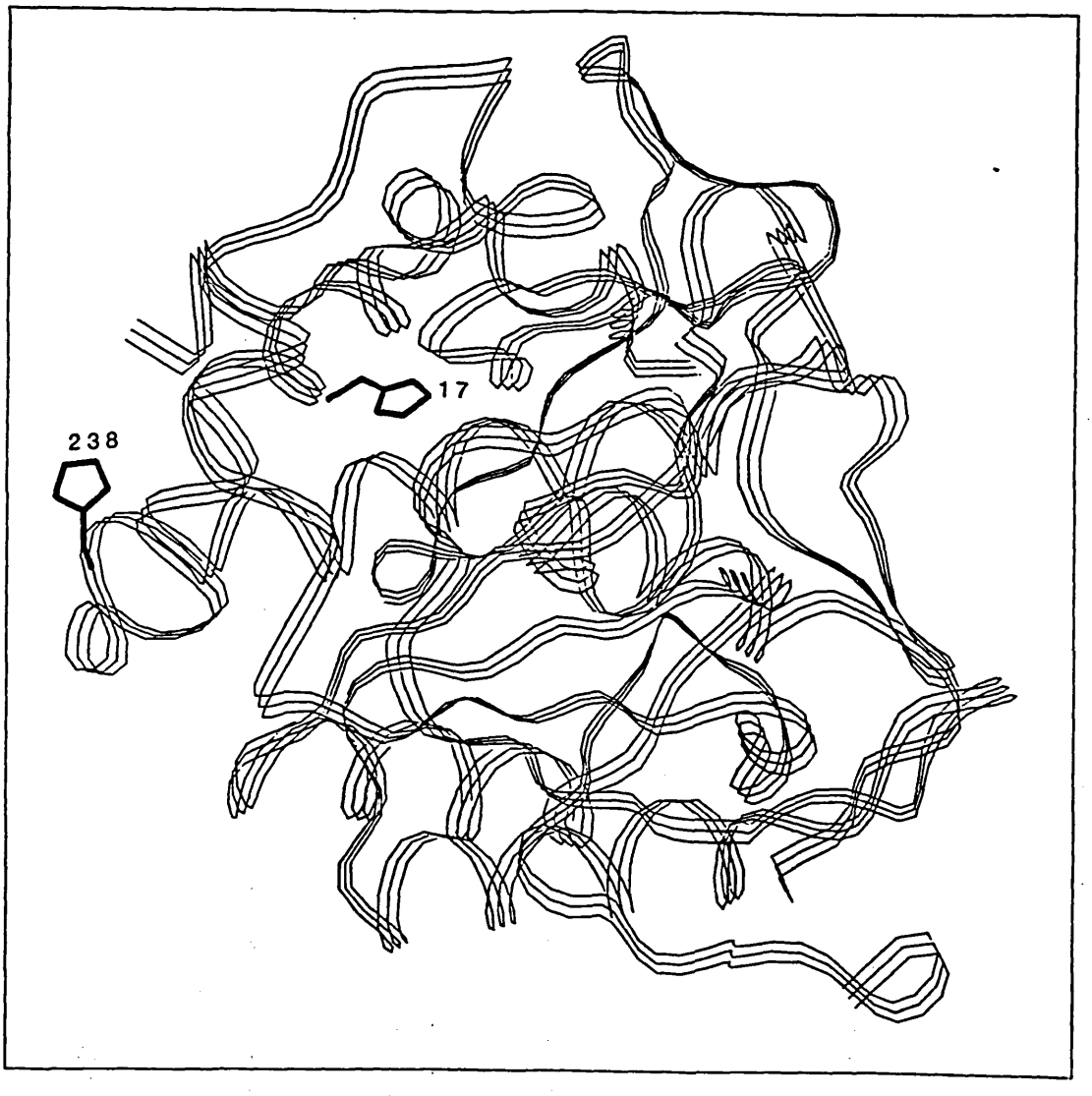



Fig. 4.6: Environments of the histidine residues 17 and 238 in the crystal structure of subtilisin BPN'. The main chain of the protein is depicted as a ribbon and only the side chains of the histidine residues are shown.

electrostatic interaction. In other proteins, histidines in close proximity to a negatively charged residues have been found to have p*Ka*'s of around 8.0 (Perutz *et al.*,1985). The p*Ka* of this residue is therefore considerably lower than would be expected if the interaction with Asp 36 was the only determining factor. His 64 is close to the N-terminal end of the α-helix formed by residues 221-238. The alinement of the peptide dipole moments within an α-helix is believed to produce an overall α-helix dipole with a partial positive charge being associated with the N-terminal end and a partial negative charge associated with the C-terminal end (Hol, 1985). The effect of the Asp 36 on the p*Ka* of His 64 may therefore be offset by the effect of the α–helix dipole.

Histidine 238 has the most perturbed p*Ka* of the histidine residues in subtilisin BPN'. Examination of the environment of this residue in the crystal structure shows that this residue is 5 Å from the C terminal carboxyl group of the protein. The electrostatic interaction between this group and His 238 would be expected to produce an increase in p*Ka*. His 238 is also at the C-terminal end of an α-helix, and the interaction of this residue with the α-helical dipole will also produce an increase its p*Ka*.

Histidines 39, 67 and 17 in subtilisin BPN' are all on the surface of the subtilisin, and do not have significantly perturbed p*Ka*'s.

Histidine 226 has the lowest solvent accessibility of the histidine residues in subtilisin. The imidazole ring of His 226 is also surrounded by the side chains of non-polar amino acids, and there are no charged amino acids with in 12 Å of this residue

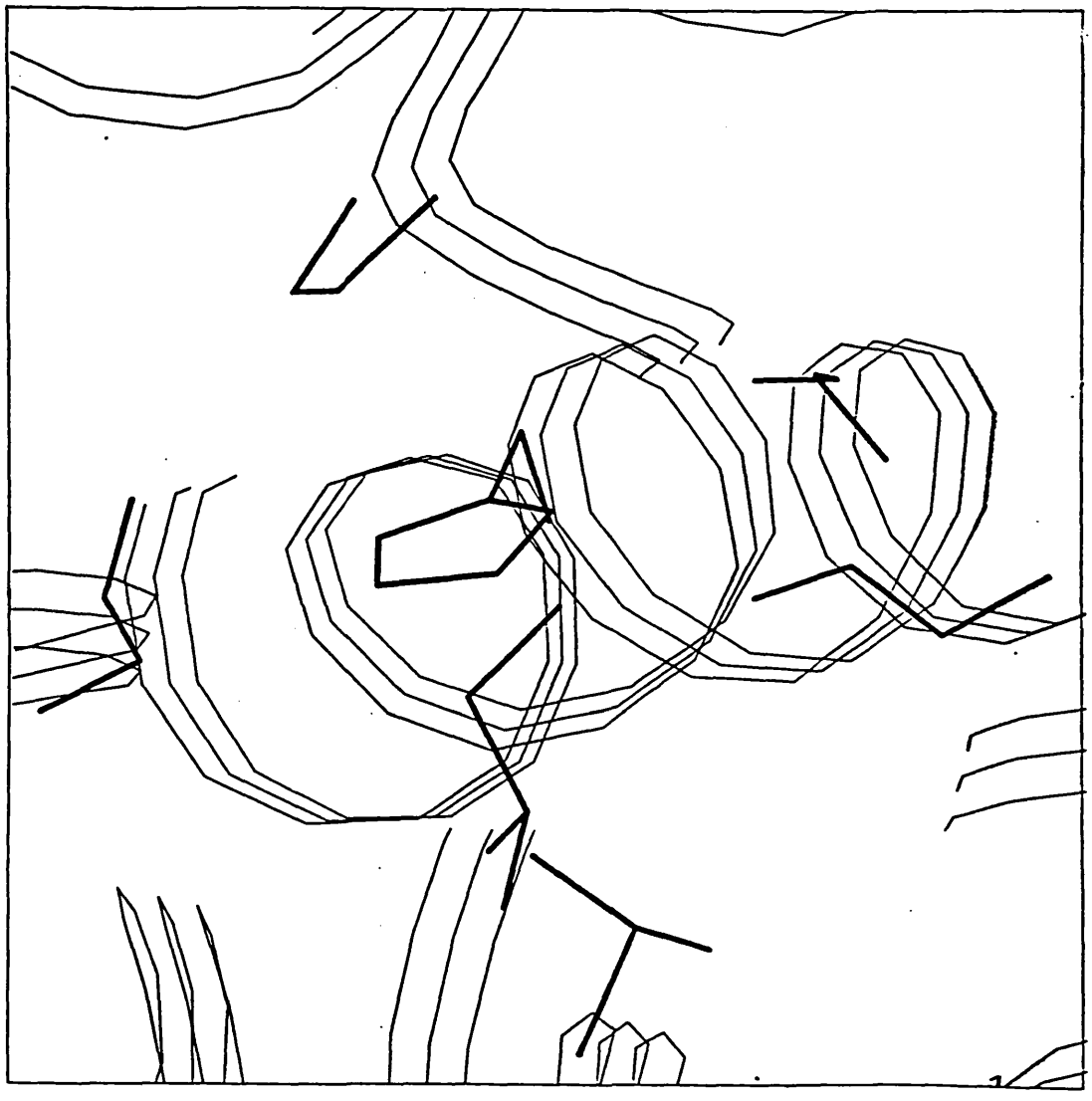


Fig. 4.7: Environment of His 226 in the crystal structure of subtilisin BPN'.The main chain is depicted as a ribbon and the side chains of Pro 201, Ile 205 and Thr 71 are shown. In addition the backbone atoms of residues 7, 222 and 223.

(Fig. 4.7). A nonpolar environment destabilises the protonated form of the imidazole ring of histidine residues and results in a reduction in p*Ka* (Warshel, 1987). The p*Ka* of His 226 is 7.04, which is in fact higher than the random coil value for the p*Ka* of histidine residues. Inspection of the environment of this residue in the crystal structure shows that this residue is 3.4 Å from the carbonyl oxygen of residue 223, 2.8 Å from the carbonyl oxygen of residue 222 and 2.5 Å from the carbonyl oxygen of residue 7. It has been proposed that charged residues in proteins can be stabilised by the dipoles of peptide bonds (Quiocho *et al.* 1987). The close proximity of two carbonyl oxygens to the imidazole ring of His 226 therefore, appears to offset the effect of the surrounding non polar amino acids on the p*Ka* of this residue.

In this study, assignments have also been obtained for the histidine residues in subtilisin Carlsberg by comparison of the p*Ka* of these residues with the values for the histidine residues in subtilisin BPN'. In the crystal structures of the two enzymes, the environments of the histidine residues that have the same p*Ka* are very similar. Any differences in the sequences that involve charged amino acids produce changes that are greater than 15 Å away from these residues. The p*Ka*'s in both enzymes have been determined in the presence of 250 mM KCl. At this ionic strength, these long range electrostatic interactions that could potentially perturb the p*Ka*'s of the histidine residues will be masked by counter ions.

The histidine at positions 238 does, however, have substantially different p*Ka*'s in

the two enzymes. This residue has a p$K_a$ of 7.4 in subtilisin BPN' and a p$K_a$ of 6.3 in subtilisin Carlsberg. Comparison of the crystal structures of the two enzymes shows that the only significant difference in the environment of His 238 results from the substitution of the Trp at 241 in subtilisin BPN' by a Lys in subtilisin Carlsberg. This introduces a positive charge within a few Å of histidine 238. This substitution would be expected to produce the observed decrease in the p$K_a$ of His 238 because of electrostatic destabilisation of the protonated form of the imidazole ring.

## 4.4: NMR studies of subtilisins containing mutations involving charged amino acids.

Subtilisin has been used as a model system in which to study electrostatic effects in proteins using site directed mutagenesis (Russell and Fersht, 1987). In these studies the effects of the mutation of charged residues on the kinetically determined p$K_a$ of the active site histidine have been investigated. These experiments are based on the assumption that the p$K_a$ of the active site histidine obtained from the kinetic measurements reflects the true microscopic p$K_a$ of this residue. The close agreement of the kinetic p$K_a$ of His 64 to that obtained from the NMR measurements reaffirms that this model is correct. The use of NMR spectroscopy to determine the p$K_a$ of histidine residues in subtilisin BPN' provides an alternative means of determining histidine p$K_a$'s in mutants in which the surface charge has been modified. In the kinetic studies on the mutant subtilisins, the largest effects on p$K_a$ were observed at low ionic

strength.

Subtilisins are known to aggregate in the absence of salt (Jordan *et al.*, 1985) and all the spectra presented in this work were obtained in solutions containing 250 mM KCl. At this ionic strength the changes in p$K_a$ produced by many of these mutations are masked by the effects of counter ions. Titration of the histidines in the mutants Asn→Asp 62 and Asp→Ser 99 gives p$K_a$'s for the histidines in these proteins which are the similar to those in the wild-type enzyme at the ionic strength used. These results suggest that these mutations do not disrupt the structure of the enzyme and that the changes in p$K_a$ observed at low ionic strength in the kinetic studies do not result from a structural perturbation . The kinetic measurements on mutant subtilisins indicate that, at the ionic strengths used in the NMR studies, changes in the p$K_a$ of the active-site histidine would only be observed in mutants which contain multiple changes of charged residues. The spectrum of a mutant subtilisin in which the Glu at position 156 and the Asp at position 99 have both been replaced by Lys is presented in Fig. 4.8. Analysis of the spectrum shows that there is no peak titrating with the same p$K_a$ as the active-site peak in the native enzyme, and a new peak that titrates with a p$K_a$ of 6.82 is visible. The kinetic analysis of this mutant indicates that at this ionic strength the active site histidine would be expected to have a p$K_a$ of approximately 6.8 (Russell and Fersht, 1987). This resonance can, therefore, be tentatively assigned to the active site histidine but an unequivocal assignment would require the construction of mutants of this protein.

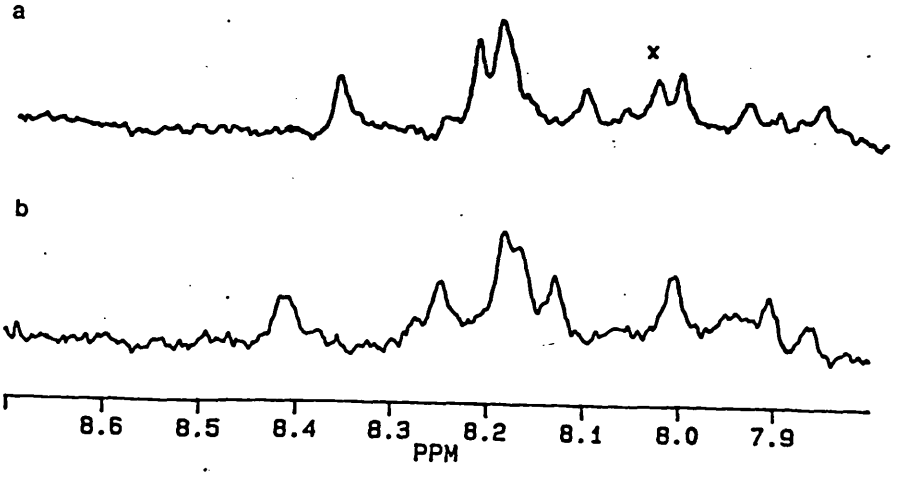



Fig. 4.8: Comparison of the C-2 proton resonances of native subtilisin and the double mutant Glu-Lys 156, Asp-Lys 99. The peak tentatively assigned to the active site histidine in the mutant enzyme is indicated.

# CHAPTER 5: NMR STUDIES OF BARNASE.

## 5.1: $^1$H NMR spectrum of barnase.

The 500 $^1$H NMR MHz spectrum of barnase is shown in Fig. 5.1 The spectrum contains a number of well-resolved resonances, and the line widths are consistent with a monomeric protein of 12,400 relative molecular mass.

## 5.2: Histidine C2 proton resonances in barnase.

The histidine C2-H region of the spectrum is shown in Fig. 5.3 a and b The spectrum in Fig. 5.3a is of a sample of barnase freshly dissolved in $D_2O$. The resonances of a number of amide protons are present and they obscure the other resonances in this region. It was found that the amide protons could be exchanged with deuterium by incubating the protein overnight in $D_2O$ at pH* 9.0, 25°C. A spectrum of an exchanged sample of barnase is shown if Fig 5.3b.

The His C2 H region of the $^1$H NMR spectrum of barnase at 4 different pH* values is shown in Fig 5.4. Two peaks, labelled A and B in Fig. 5.4, decrease in chemical shift as pH increases. The titration curves for these peaks are shown in Fig. 5.5 and the parameters obtained from fitting the titration data to an ionisation equation are

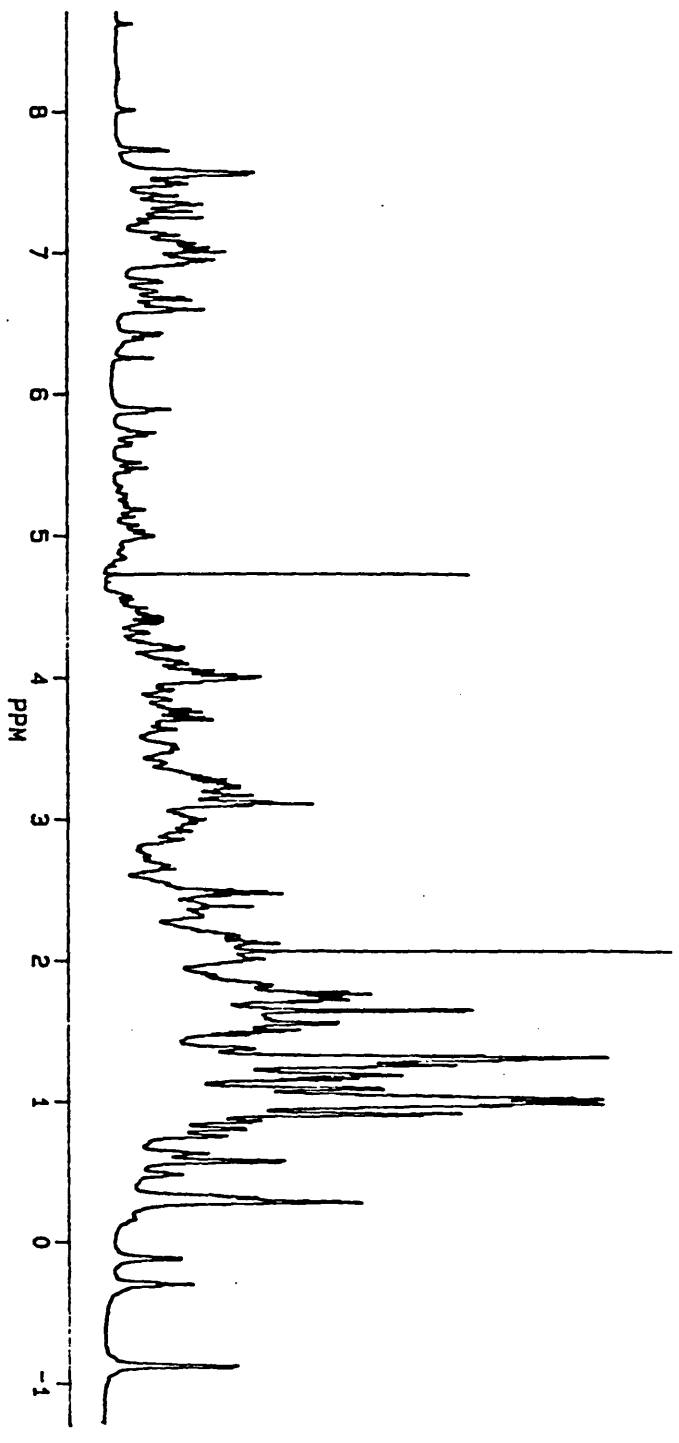



Fig. 5.1: 500 MHz $^{1}H$ NMR spectrum of barnase (pH 4.5, 2 mM protein, 25°C).

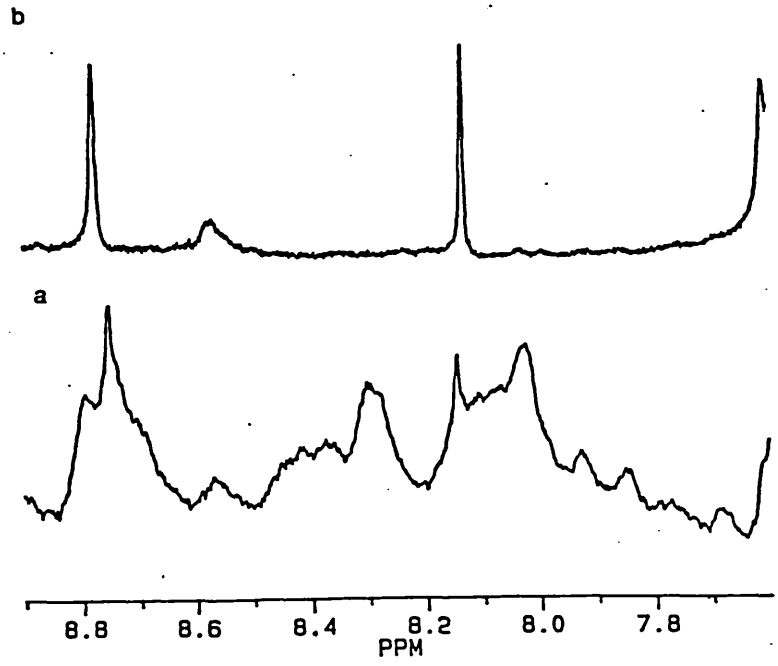



Fig. 5.2: Histidine C2 proton region of the $^{1}H$ NMR spectrum of barnase (pH 4.5, 2 mM protein, 25°C).

summarised in Table 5.1. For both the peaks, the calculated p$Ka$ values and differences in the high pH and low pH plateau chemical shifts obtained are similar to those found for other histidine residues in proteins. It appears therefore that these two peaks correspond to the C2 protons of the two histidines in barnase. The chemical shift of peak B, however, is approximately 0.5 PPM upfield from the normally observed value.

The line width of peak A increases substantially as the pH is raised from pH 4.0. The broadening goes though a maximum and then decreases as the peak stops titrating. The behaviour, which has been observed for a number of other histidine residues in proteins (Sudmeier *et al.*, 1980), results from an exchange effect. The protonated and unprotonated forms of the imidazole ring of histidine residues are normally in fast exchange on the NMR time scale. If these rate constants are reduced, fast exchange line broadening is produced.

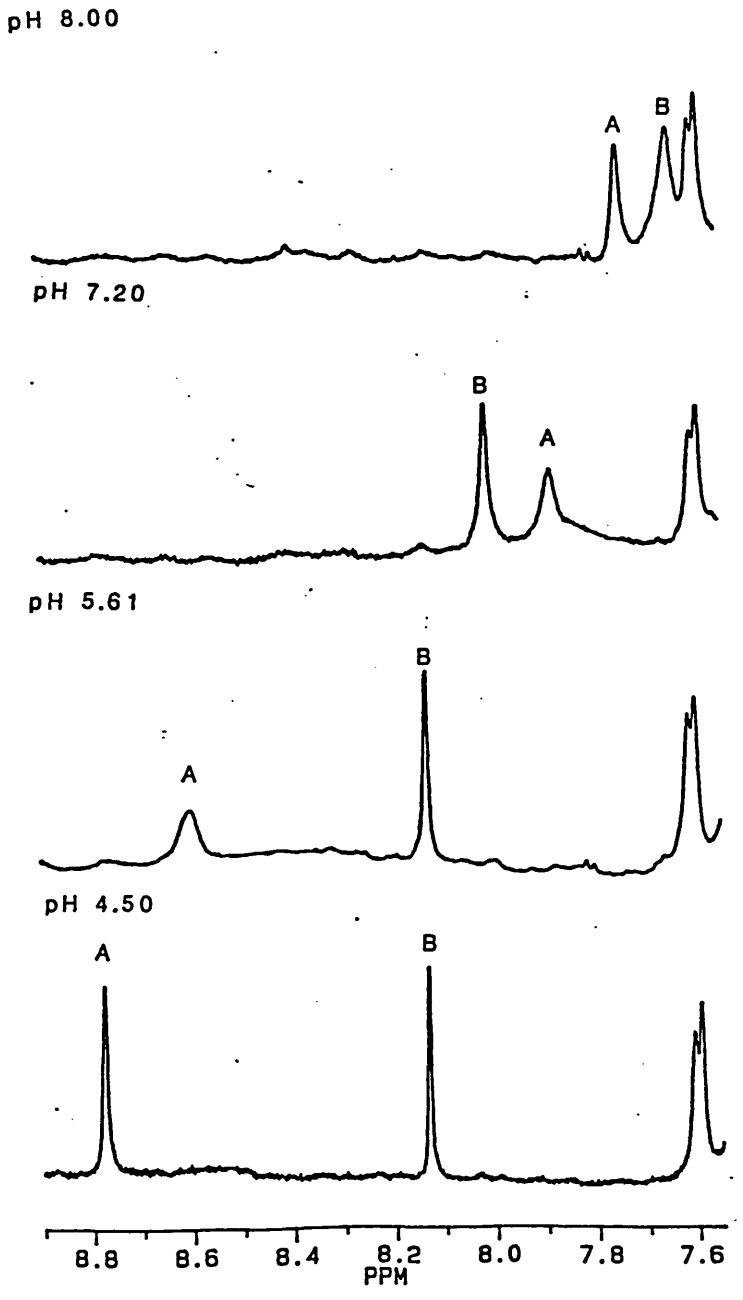



Fig. 5.3: Titration of the histidine C2 protons in the NMR spectrum of barnase (2 mM protein, 25°C).

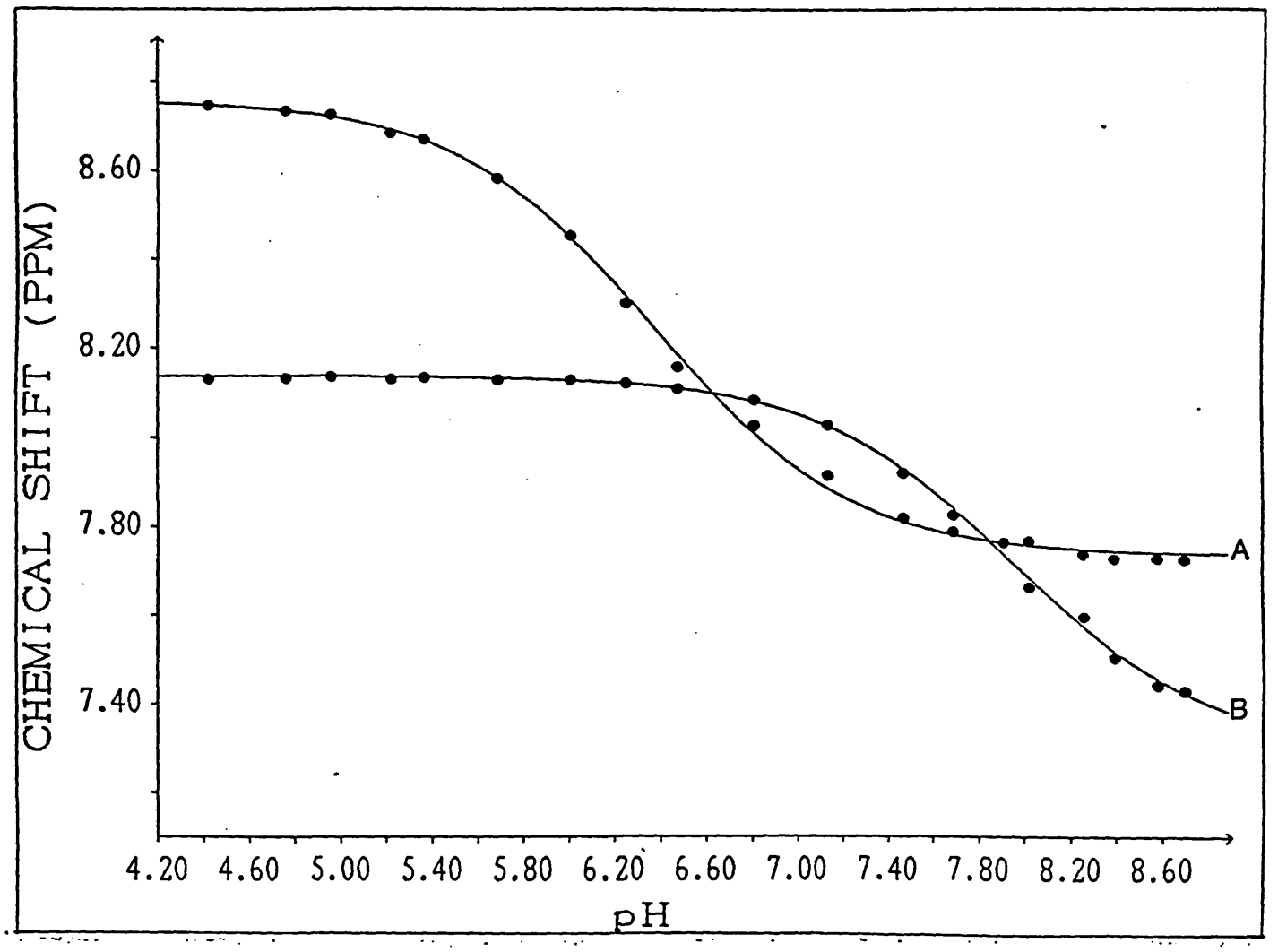



Fig. 5.3: Titration of the histidine C2 H resonances in the 500 MHz $^{1}H$ NMR spectrum of barnase (2 mM protein, 25°C).

Table 5.1: $^1$H NMR titration parameters for the histidine residues in barnase (25°C)

| peak[a] | $pK_a$[b] | High pH chemical shift | Low pH chemical shift |
|---|---|---|---|
| A | 6.37 (0.02) | 8.76 | 7.73 |
| B | 7.95 (0.03) | 8.14 | 7.29 |

[a] Labels refer to the peak designation in Fig. 5.3. [b]Figures in parentheses are standard errors. Errors for the chemical shifts are all less than 0.03.

5.3: Assignment of the histidine C2 proton resonances in barnase.

The histidine C2 proton region of the $^1$H NMR spectrum of the barnase mutant His→Ala 102 is shown in Fig. 5.5. Inspection of this figure clearly shows that substitution of His 102 results in the disappearance of peak A. Resonance A may therefore, be assigned to the C2 proton of His 102. The histidine C2 proton region of the $^1$H NMR spectrum of the mutant His→Gln 18 is also shown in Fig. 5.5. From this figure it can be seen that replacement of His 18 by a Gln results in the disappearance of peak B, and this resonance may therefore be assigned to the C2 proton of His 18.

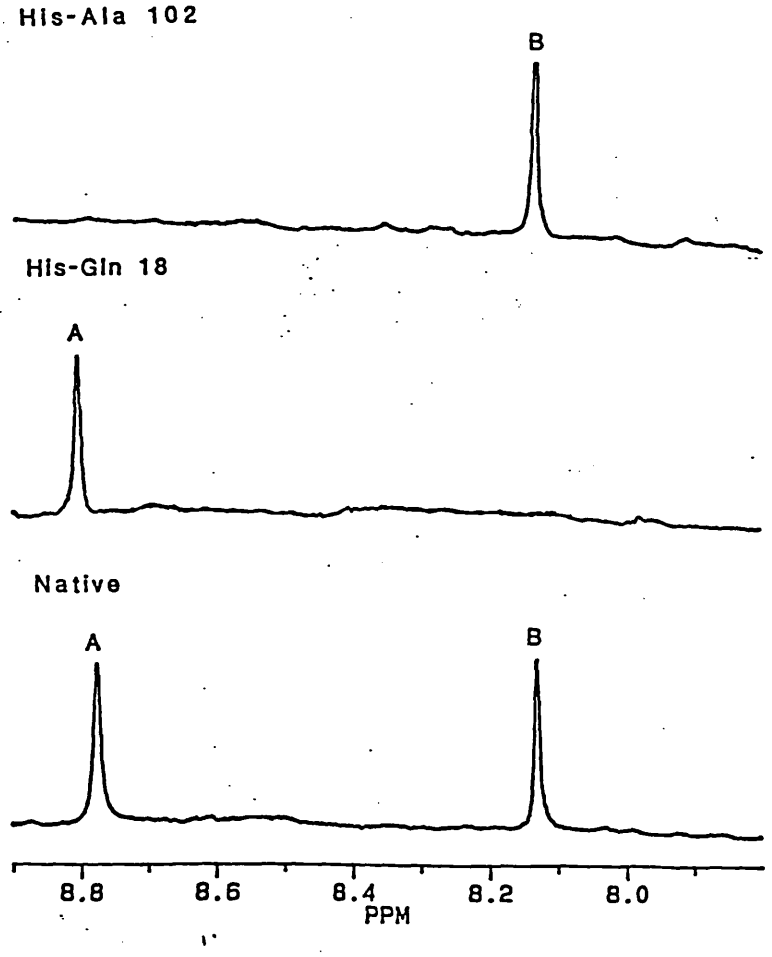



Fig. 5.5: $^{1}H$ NMR spectra of the barnase mutants His-Ala 102 and His-Gln 18 (pH 4.5, 2 mM protein, 25°C).

## 5.4: Role of active site histidine in barnase.

Site-directed mutagenesis experiments have demonstrated that substitution of His 102 in barnase by either Glu or Ala results in a substantial decrease in the activity of the enzyme. The 1D spectra of native barnase and the His→Ala 102 mutant are shown in Fig. 5.6. The mutant and wild-type spectra are essentially identical except for the absence of the histidine C-2 and C-4 protons. It appears, therefore, that the decrease in activity that accompanies the substitution of His 102 is not produced by a large conformation change in the enzyme. Ribonucleases such as barnase are believed to hydrolyse RNA in a two step process in which a cyclic phosphate intermediate is formed (Takahashi & Moore, 1982). In the first step, a basic group abstracts a proton from the 2'-OH of the ribose moiety and an acidic group protonates the 5'-OH of the adjacent ribose. The pH dependence of $k_{cat} / K_M$ for the hydrolysis of dinucleotides by barnase is a narrow bell-shaped curve with a maximum at approximately pH 5.8 (Mossakowska *et al.*,unpublished results) which presumably reflects the ionisation of the acidic and basic groups. The ascending limb of this curve corresponds to the ionization of an acid with a p$Ka$ similar to that obtained for His 102 from the NMR titration data. This evidence suggests that, to be catalytically active, His 102 must be protonated. This data is consistent with a mechanism of catalysis for barnase in which His 102 acts as a general acid.

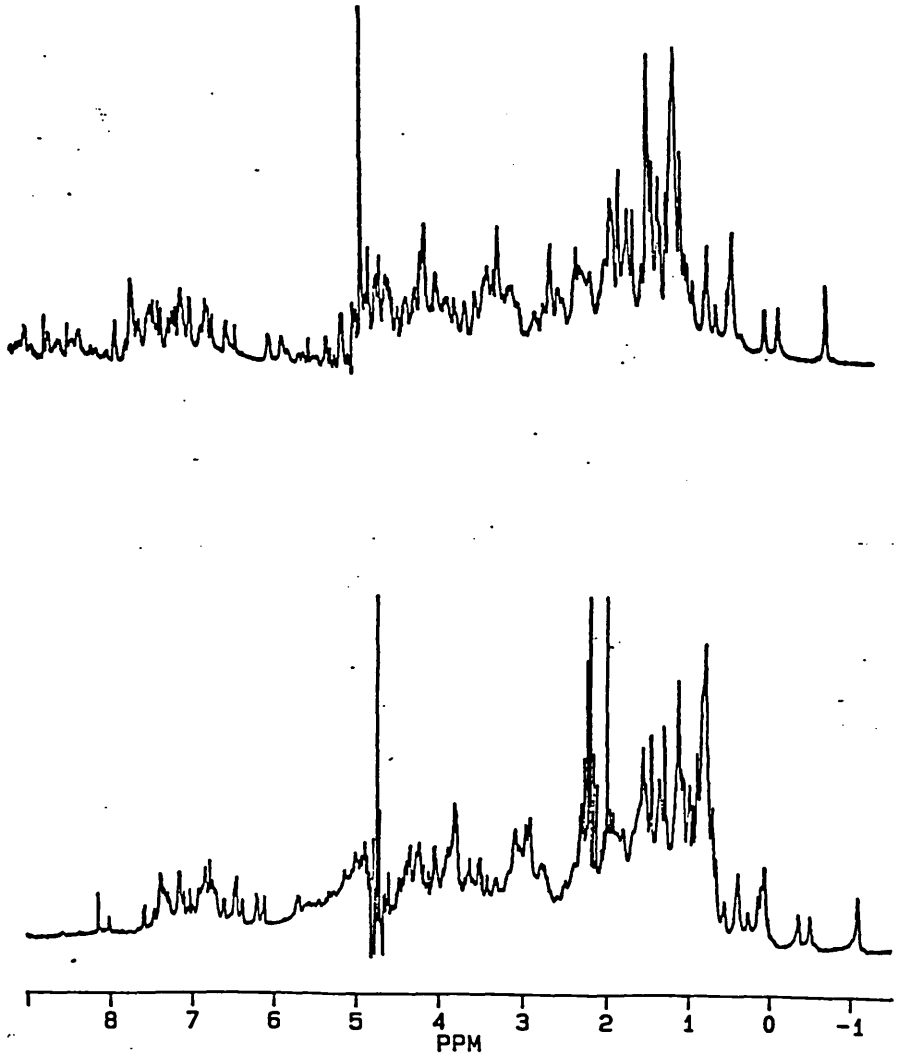



Fig. 5.6: Comparison of the $^{1}H$ NMR spectra of native barnase and the mutant His-Ala 102 (pH 4.5, 2 mM protein, 25°C).

5.5: Effects of amino acid substitutions on the p$K_a$ of His 102 in barnase.

Titration of the C2 protons of histidine 102 in the mutants Glu→Ala 60 and Lys→Ala 27 gives values for the p$K_a$ of this residue of 6.21 and 6.50 respectively. Comparison with the p$K_a$ of His 102 in the native enzyme shows that the mutation of these residues has produced a shift in p$K_a$ of -0.15 units for Glu→Ala 60 and +0.14 units for Lys→Ala 27. Although these values are small, they are beyond the error limits for the p$K_a$ determinations (±0.04). The changes in p$K_a$ are in the directions expected for the removal of electrostatic interactions with positively charged form of His 64. Lys 27 and Glu 60 are 18 Å and 14 Å away from the imidazole ring of His 64. The magnitude of the p$K_a$ shifts can be expressed in terms of an effective dielectric constant $D_{eff}$ for the interaction using the equation

$$D_{eff} = 244 / \Delta pK_a.\Delta q.r$$

where $\Delta q$ is the difference in charge and r is the distance between the charges (Russell *et al.*, 1987). This gives a values for the effective dielectric constant of 110 for the Glu 60, His 102 interaction and 96 for the interaction of Lys 27 and His 102. Both these values are close to the dielectric constant of water. Glu 60 and Lys 27 are both within the active site cleft of barnase (Fig 5.7) and these results demonstrate that the local dielectric environment in this region of the protein is similar to that of water.

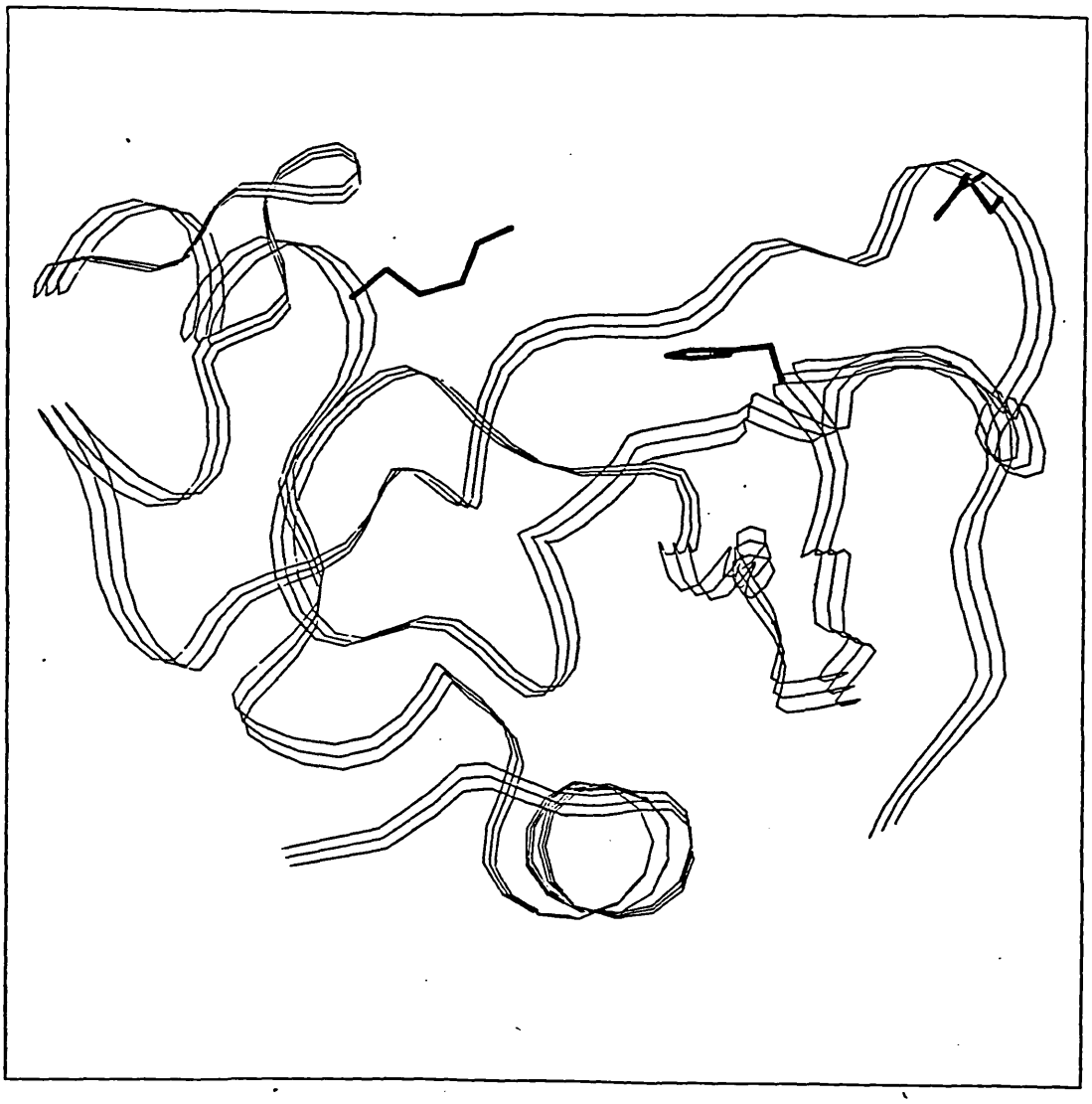


Fig. 5.7: The active site region of the crystal structure of barnase. The main chain is depicted as a ribbon and only the side chains of His 102, Glu 60 and Lys 27 are shown.

5.6: The anomalous p$Ka$ of histidine 18 in barnase.

The intrinsic p$Ka$ of the imidazole ring of histidine residues in proteins is generally taken to be about 6.3 (Matthews, 1986). The titration of the C2-H proton of histidine 18 in the presence of 5 M urea in which the protein is unfolded, gives a p$Ka$ value of 6.36. The p$Ka$ of His 18 in the folded form of barnase is therefore approximately 1.7 pH units above the random coil value. Normally only histidines that are involved in salt bridges with negatively charged residues have p$Ka$'s as high as this. There are, however, no negatively charged residues with in 12 Å of His 18 in the crystal structure of barnase. His 18 is at the C-terminal end of the α–helix formed by residues 6-18 (Fig. 5.8). α-Helices are believed to posses an overall dipole moment with a partial positive charge being associated with the N-terminal end, and a partial negative charge associated with the C-terminal end (Hol *et al.*, 1978). It appears therefore that the perturbation of the p$Ka$ of His-18 results from the interaction of this residue with the dipole associated with the end of the α-helix. It has been suggested that the field of an α-helix can be approximated by the field produced by placing a charge of 0.75 at either end of the helix (Hol, 1985), and the magnitude of the shift of the p$Ka$ of histidine 18 is consistent with this model. Titration of the histidine C2 proton resonances in barnase in the presence of 1 M KCl shows that, under these conditions, the p$Ka$ of His 102 rises to

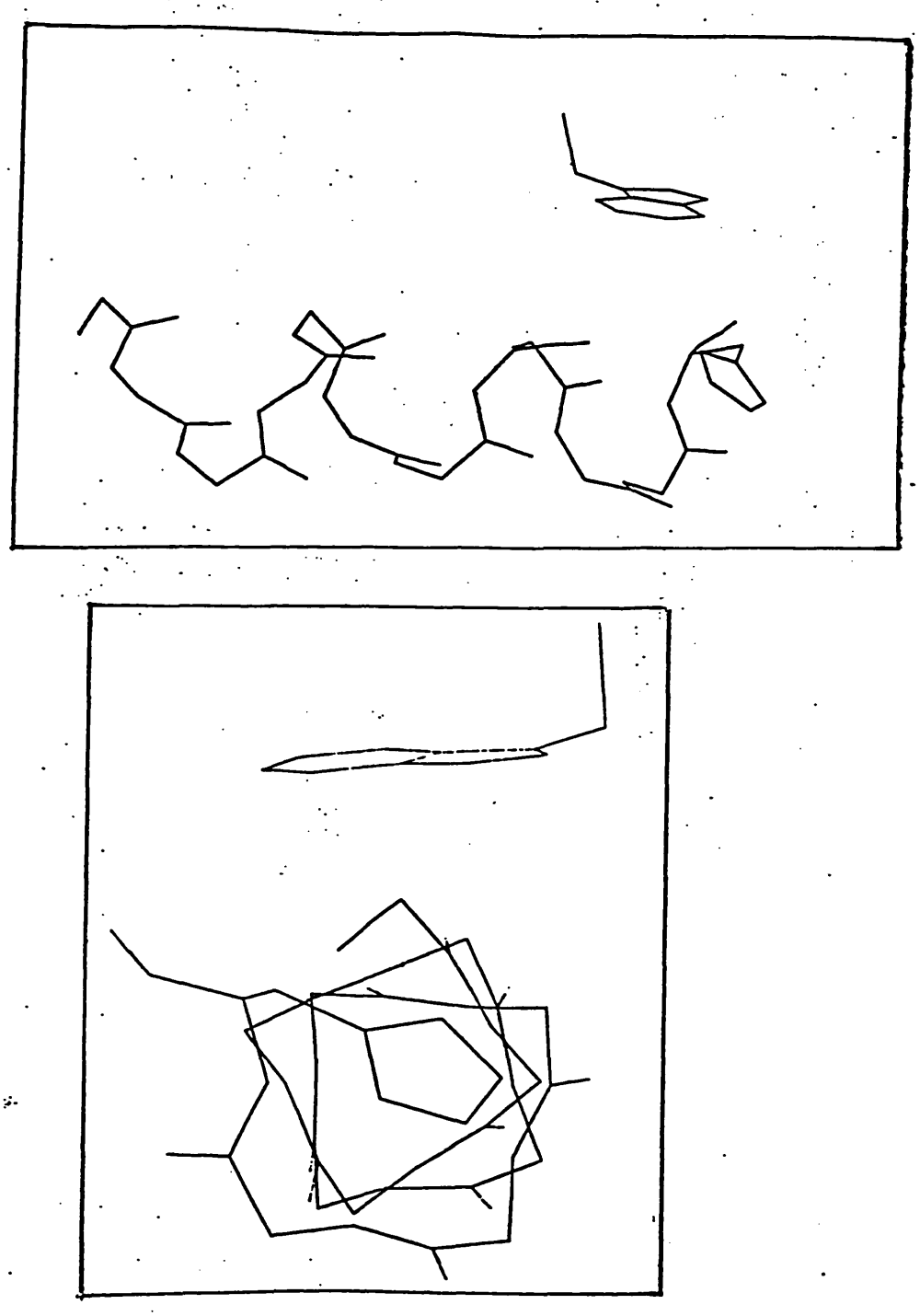


Fig. 5.8: Environment of Histidine 18 in the crystal structure of barnase. The main chain of the α-helix formed by residues 6-18 is shown together with the side chains of His 18 and Trp 94

6.5 whilst the p$K_a$ of His 18 is essentially the same as at zero ionic strength. At this ionic strength most charge / charge electrostatic interactions will be masked and this result demonstrates that the anomalous p$K_a$ of His 18 in barnase does not result from this type of interaction. The insensitivity of the p$K_a$ of His 18 to ionic strength is consistent with theoretical work that predict that this type of interaction should be less susceptible to shielding by counter-ions than charge / charge interactions (Gilson and Honig, 1988).

5.7: The chemical shift of the C2 proton of histidine 18 in native and mutated barnase.

The high and low pH chemical shifts of the histidine 18 in barnase are perturbed approximately 0.5 PPM up field from their random coil values. In the crystal structure (Maugen *et al.*, 1982) this proton is close to the ring Trp 94. Ring current shift calculations (Perkins and Dwek, 1980) predict that the proximity of this residue to the C2-H proton of His-18 would produce an up field shift of 0.3 ppm. (The ring current shift calculations were kindly carried out by Dr. R.J. Leatherbarrow, Department of Chemistry, Imperial College.) Ring current shift calculations are very sensitive to the position of the proton involved. The discrepancy between the observed and calculated values for the chemical shift of the C2 proton of His 18 may reflect some small difference in the solution and crystal structures or may be the result of the limited resolution of the crystal structure.

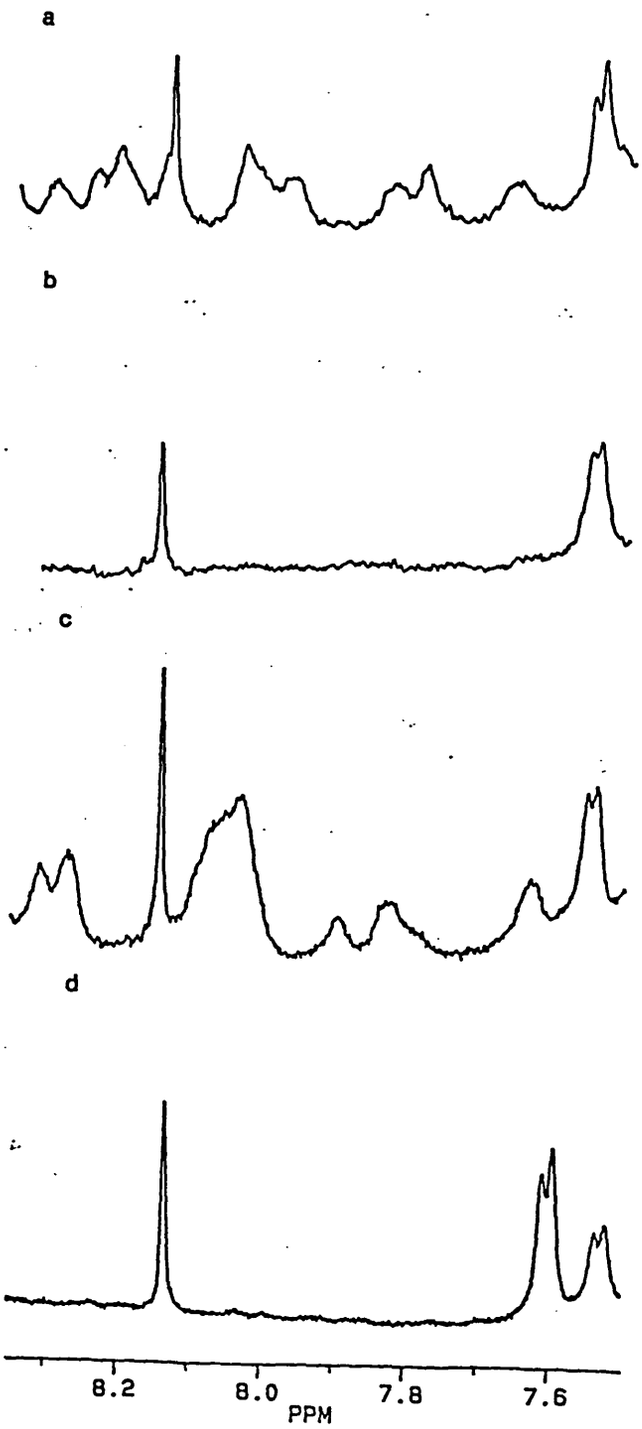



Fig. 5.9: $^{1}H$ NMR spectra of the barnase mutants Ile-Val 96 (a), Ile-Val 88 (b), c) Phe-Leu 7 (c) and native barnase (d) (25°C,1-2 mM protein).

Histidine 18 lies at the end of the α–helix formed by residues 6-18 whilst Trp 94 lies on the end of a region of β–sheet. The chemical shift of the C2 protons of His 18 should be sensitive to the relative orientations of these pieces of secondary structure. Residues 7, 96 and 88 in barnase are involved in the packing of the α–helix formed by residues 6-18 onto the central region of β-sheet. Mutations at these positions have been made to study the role of these residues on the stability of barnase (Kellis *et al.*,1988). Spectra of these mutants are shown in Fig. 5.9. Inspection of this figure shows that these mutations have not affected the chemical shift, or the p$K_a$ of His 18. This demonstrates that these mutations have not produced a large change in the structure of this region of the protein. The destabilisation of the folded form of the protein produced by the mutation of these residues must, therefore, result solely from the removal of hydrophobic interactions.

# CHAPTER 6: CONCLUSIONS.

This work has described the use of site directed mutagenesis to assign the histidine C2 proton resonances of subtilisin BPN' and Barnase. The assignment of these resonances would have been difficult to achieve by any other method and these results have demonstrated the potential of this technique for resonance assignment in protein NMR spectroscopy.

This method does have a number of limitations. The construction of mutant proteins requires that the gene coding for the enzyme is cloned and expressed at high levels. Many eukaryotic protein, however, do not express at high levels in bacteria. The construction of mutant proteins requires some effort and expense and in practice it will only be practical to obtain a fairly limited number of assignments using this method. For some proteins it may be possible to use assignments made by mutagenesis as starting points for sequential assignments using 2-D NMR methods and preliminary work in this area has been reported (Alexandrescu *et al.*, 1988).

One of the main factors that will limit the use of site directed mutagenesis for resonance assignment is the potential disruptive effect of mutations on proteins structure. If the mutation produces a conformational change in the protein, such as in the His-Gln 67 mutant of subtilisin, the spectra of the mutant and native enzymes will be too

dissimilar to enable an assignment to be made. The mutation of a residue can also, whilst leaving the structure of the enzyme relatively unaffected, produce a substantial reduction in the level of protein expression. This can make it difficult to obtain sufficient material for NMR studies. The mutation of His 18 in barnase, for example, leads to a 50 fold decrease in the level of protein expression even though the spectra of the mutant and wild type proteins are not significantly different.

In this study the resonances that have been assigned have been in well resolved regions of the spectra and it has been easy to identifie the peak that has disappeared as a result of the mutation. The assignment of resonances in more crowded regions of the NMR spectrum by mutagenesis may well prove more difficult and will have to rely on difference spectroscopy or 2-D NMR.

In spite of the limitations discussed above, for many protein site directed mutagenesis will be the only practical method for obtaining resonance assignments, and it is likely that this technique will be used increasingly in protein NMR spectroscopy.

The availability of assignments for the histidine residues in barnase has enabled the role of His 102 in the mechanism of action of barnase to be elucidated using a combination of NMR and kinetic data. Catalytically important histidine residues have been studied by NMR for many years (Markley, 1975). The availability of unequivocal assignments based on mutant proteins will increase the potential of this technique.

Electrostatic effects are important in every aspect of protein function (Perutz, 1978). There is much interest in gaining a quantitive knowledge of these interactions (Rodgers, 1986). Experimental data on the magnitude of these effects is, however, limited. The experiments carried out by Russell, Thomas and Fersht on mutant subtilisins with altered surface charge (Thomas *et al.*, 1985, Russell and Fersht, 1987) have provided both a rational method for altering the pH activity profiles of enzymes and a set of experimental data to test theoretical models of electrostatic effects in proteins. These experiments have produced considerable interest and several groups have attempted to model the effects of these mutations using a verity of algorithms (Sternberg et al., 1987, Gilson and Honig, 1987). In the present work NMR has been used to determine the p$K_a$ of the active site histidine in native subtilisin and in mutants containing alterations in surface charge. The determination of the p$K_a$ of the active site histidine in subtilisin using NMR has demonstrated that the p$K_a$ value obtained from kinetic data does reflect the microscopic p$K_a$ of this residue. In addition, NMR has been used to confirm the shifts in the p$K_a$ of the active site histidine observed in the kinetic studies.

In this work the experimental approach used on subtilisin has been applied to barnase. It has been shown using NMR that the mutation of charged residues on the surface of barnase produces perturbations in the p$K_a$ of His 102. In addition, from the magnitude of the changes in p$K_a$ it has been shown that the effective dielectric constant in the active site of barnase is, like in the active cleft of subtilisin, close to that of water.

The comparison of the p$K_a$'s of the histidine residues in subtilisin and barnase to their environments in the crystal structures of these enzymes has provided a number of incites into the factors that determine the ionisation constants of these residues in proteins. At the ionic strength used for the work on subtilisin, which is close to physiological ionic strength, it has been found that many long range ( >10 Å) electrostatic interactions are masked by counter ions. Shorter range electrostatic interactions, however, have been shown to still be important. The role of the dipoles in the electrostatic properties of proteins has been clearly demonstrated by the results described in this work. The dipole associated with α-helices has proved to be especially important in determining the p$K_a$'s of the histidines in subtilisin and barnase. The α-helix dipole is believed to have a role in many of the properties of proteins including, participation in catalysis, ion binding and protein stability (Hol, 1985). Information on the magnitude of the dipole moment is, however, limited. In this work it has been shown that the p$K_a$ of one of the histidines in barnase is perturbed as a result of the interaction of the residue with a α-helix dipole. This has allowed an estimation to be made of the magnitude of the electrostatic interaction of this residue with the dipole. Studies on the stability of the His→Gln 18 mutant by D. Sali (Sali *et al.*, 1988) have shown that this mutant is less stable than the native enzyme at pH values where His 18 is protonated and this has demonstrated that α-helix dipoles have a role in protein stability. These results suggest the effects of peptide bond dipoles must be taken into

account when modelling the electrostatic properties of proteins.

In order to interpret the results of a site directed mutagenesis experiment, it is necessary to establish if the mutation has produce a large change in the conformation of the protein molecule. NMR spectroscopy offers a rapid means of establishing the structural consequences of mutations. X-ray crystallography can also be used to study the structures of mutant proteins. Although the structural information available from X-ray crystallography will in general be more detailed than that available from NMR, NMR spectra can be obtained more quickly, and can potentially provide information on ionisation equilibria and protein dynamics that cannot be obtained from a crystal structure. In this study NMR has been used to study the effects of mutations in barnase. Even with the limited number of assignments that are currently available it has still been possible to gain detailed information on the structure of mutant proteins.

For small proteins it is clear that NMR has considerable potential to establish if a mutant protein is structurally unchanged and is likely that this method will be used extensively in this area.

## ABBREVIATIONS

| | |
|---|---|
| ATP | adenosine 5'-triphosphate. |
| Bis-Tris | Bis [2-hydroxyethyl]amino-tris[hydroxymetyl]methane. |
| dNTP | deoxynucleotide 5'-Triphosphate. |
| ddNTP | dideoxynucleotide 5'-Triphosphate. |
| DTT | dithiothreitol. |
| EDTA | ethylene diamine tetra-acetic acid. |
| MOPS | 3-[N-Morpholino]propanasulfonic acid. |
| NCI | N-*trans*-cinnamoylimidazole. |
| NMR | nuclear magnetic resonance. |
| PFU | plaque forming units. |
| PMSF | phenylmethane sulphonyl fluoride. |
| RPM | revolutions per minute. |
| SDS | sodium dodecyl sulphate. |
| suAAPFpN | succinyl-L-Ala-L-Ala-L-Pro-L-Phe *para*-nitroanilide. |
| Tris | 2 amino-2-(hydroxymethyl)propane-1,3-diol. |

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