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Structural and Functional Characterisation of the Human Vitamin C Transporters

Menebere Woubshete

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Department of Life Sciences

Imperial College London

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Statement of Originality

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Abstract

Vitamin C is an essential micronutrient that functions as an antioxidant and a cofactor for several enzymes. Vitamin C has also shown great potential as a possible therapeutic for treating several diseases such as cancers and neurodegenerative diseases. Humans and other primates are not able to synthesise vitamin C, so they must obtain it exclusively from their diet. The sodium-dependent vitamin C transporters (SVCT1 and SVCT2) are responsible for the uptake of the reduced form of vitamin C, ascorbic acid, into cells. A greater understanding of the molecular mechanisms of the SVCTs would provide insights into their roles and potential with respect to health and disease.

The recent mouse SVCT1 structure provided insights into the molecular determinants of substrate binding and revealed that SVCT1 exists as a homodimer. Based on this structure and that of a related protein, I introduced several individual mutations, such as L65A, F112A, R206A, I439A and L458G, to human SVCT1 (hSVCT1) and explored their effect on expression, membrane trafficking and function of the protein. Results showed that these residues are important in substrate transport with no influence on expression and membrane trafficking. In addition, truncations at the N- and C-terminal ends of the protein confirmed the importance of the C-terminus in membrane trafficking. Correct dimerisation was also found to be essential in both membrane trafficking and function of hSVCT1.

hSVCT2 differs from hSVCT1 in that, in addition to transporting ascorbic acid, it activates the JAK/STAT pathway. However, this transceptor activity is not well understood as the structure of hSVCT2 has not yet been solved. Here, I performed extensive optimisation of hSVCT2 expression and purification, including changes to the expression construct, buffer conditions and purification resin, enabling the production of stable protein for structural studies. This will provide the basis for the future structural characterisation of hSVCT2 using cryo-EM.

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“For the Lord is good and His love endures forever; His faithfulness continues through all generations.” – Psalms 100:5

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Table 4.3. MPD detergent properties

Table 4.4. Solubilisation efficiencies of selected MPD-Q detergents

Abbreviations

2OG	2-oxoglutarate
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AD	Alzheimer's disease
AE1	Anion exchanger 1
AML	Acute myeloid leukaemia
APC	Amino acid polyamine-organocation
Apo-A1	Apolipoprotein A-1
ATP	Adenosine triphosphate
AzgA	Hypoxanthine-adenine-guanine transporter
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMC	Critical micelle concentration
CMV	Cytomegalovirus
CPM	7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin
CRC	Colorectal cancer
CTAB	Cetyltrimethylammonium bromide
CV	Column volume
DDM	n-Dodecyl- β -D-maltoside
DHA	Dehydroascorbic acid
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DPPC	Dipalmitoyl-phosphatidylcholine
EM	Electron microscopy
ENT	Equilibrative nucleoside transporter
ER	Endoplasmic reticulum

FBS	Foetal bovine serum
FC-12	Fos-Choline-12
FRASC	Ferric Reducing/Antioxidant and Ascorbic Acid
FSEC	Fluorescence size exclusion chromatography
FT	Flowthrough
GFP	Green fluorescent protein
GlpT	Glycerol-3-phosphate transporter
GLUT	Glucose transporter
GNG	Glucose neopentyl glycol
GPI	Glycosylphosphatidylinositol
GULO	L-gulonolactone oxidase
HEK	Human embryonic kidney cells
hFSEC	Heated fluorescence size exclusion chromatography
HLB	Hydrophilic-lipophilic balance
HRP	Horseradish peroxidase
HSPCs	Haematopoietic stem/precursor cells
hSVCT	Human sodium-dependent L-ascorbate transporter
JAK	Janus kinase
JHDMs	Jumonji C domain containing histone demethylase
LB	Lysogeny broth
LDAO	Lauryldimethylamine-N-oxide
LeuT	Leucine transporter
LMNG	Lauryl maltose neopentyl glycol
MPD	Multiple pendant detergents
MSP	Membrane scaffold protein
mSVCT	Mouse sodium-dependent L-ascorbate transporter
MW	Molecular weight

NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nanoDSF	Nano differential scanning fluorimetry
NAPols	Glucose-based nonionic amphipols
NAT	Nucleobase ascorbate transporter
NCS	Nucleobase Cation Symporter
NMR	Nuclear magnetic resonance
OG	n-Octyl- β -D-glucoside
P-GNGs	Pendant-bearing glucose-neopentyl glycol
PBS	Phosphate buffered saline
PC-APols	Phosphorylcholine-based amphipols
PCR	Polymerase chain reaction
PD	Parkinson's disease
PEI	Polyethylenimine
PFA	Paraformaldehyde
POI	Protein of interest
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine
RFU	Relative fluorescence units
Rh	Hydrodynamic radius
RMSD	Root mean square deviation
ROS	Reactive oxygen species
SAPols	Sulfonated amphipols
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide
SEC	Size exclusion chromatography
SEM	Standard error of mean
SLC	Solute carrier

SMA	Styrene maleic acid
SMALPs	Styrene maleic acid lipid particles
SNP	Single nucleotide polymorphism
STAT	Signal transduces and activator of transcription
SV40	Simian virus 40
SVCT	Sodium dependent L-ascorbate transporters
TAE	Tris-acetate-EDTA
TCEP	Tris(2-carboxyethyl)phosphine
TET	Ten-eleven translocation enzymes
TEV	Tobacco etch virus
TGP	Thermostabilised green fluorescent protein
TM	Transmembrane
TMG	Tandem malonate-based glucosides
UapA	Uric acid permease A
UraA	Uracil transporter
VPP	Voltage phase plate
WT	Wild-type
XRC	X-ray crystallography
YFP	Yellow fluorescent protein

1. Introduction

1.1. Vitamin C

Vitamin C is an essential micronutrient that plays fundamental roles in a range of biochemical and physiological processes in both plants and animals. All plants and most animals synthesise vitamin C via glucose oxidation (Figure 1.1). However, humans, most other primates, guinea pigs, capybara and some birds and fish are unable to synthesise vitamin C as they lack a functional L-gulonolactone oxidase (GULO), the key enzyme in ascorbate biosynthesis (Chaudhuri and Chatterjee, 1969, Chatterjee, 1973). Thus, these species need to obtain vitamin C exclusively from their diet.

Vitamin C is a highly effective antioxidant which reduces the reactive oxygen species (ROS) produced during cell metabolism. Due to its anti-oxidation effects, vitamin C has been used as a potential therapeutic for treatment of neurodegenerative diseases and several types of cancers (Han et al., 2021). Moreover, vitamin C is an important cofactor to several enzymes including hydroxylases, dioxygenases and histone or DNA demethylases. These enzymes have an effect on neurotransmitter synthesis, epigenetic regulation and the function of the immune system (Aumailley et al., 2022).

Vitamin C exists as the reduced ionisable form, ascorbic acid, and the oxidised non-ionic form, dehydroascorbic acid (DHA) (Rumsey et al., 1997). At physiological pH, ascorbic acid exists in its anionic form of ascorbate. The recommended amount for vitamin C is 75 mg and 90 mg per day for women and men respectively, and vitamin C plasma concentration is usually about 50 $\mu\text{mol/L}$ with $<28 \mu\text{mol/L}$ considered to be deficient (Schleicher et al., 2009, Travica et al., 2019). Deficiency of vitamin C is related to anaemia, scurvy, slow wound healing, capillary haemorrhage, muscle degeneration and neurosis. The vital role of vitamin C for normal bodily functions, together with its potential in various therapies, makes understanding how it is transported into cells imperative.

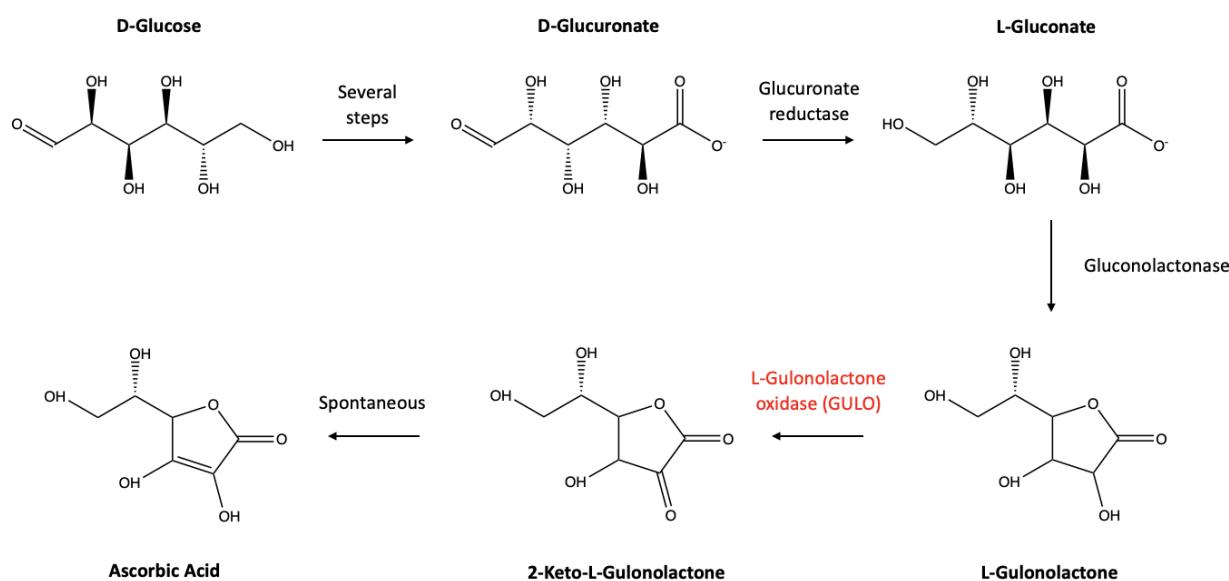


Figure 1.1. Ascorbic acid is synthesised by glucose oxidation. Humans and most other primates lack a functional L-Gulonolactone oxidase enzyme (red) and are therefore unable to synthesise ascorbic acid.

1.1.1. History

Scurvy is a disease caused by the deficiency of vitamin C. Its symptoms include anaemia, swelling, ulceration of the gums and loss of teeth (Léger, 2008). Even though scurvy has been known since ancient Greek and Egyptian times, it wasn't until the 18th century that the cause was discovered. Scurvy was most commonly associated with sailors who had been at sea for at least 10 weeks. Between the 16th and 18th centuries, scurvy killed more than 2 million sailors (Milne, 2012). By 1800, it was discovered that lemons and oranges help in preventing scurvy in sailors, but it was difficult to store them on ship as they would rot. As a stable alternative to lemons and oranges, the British navy introduced the use of sulfuric acid as a dietary supplement, the rationale being that both lemons and oranges are acidic. In 1746, James Lind carried out the first controlled trial in clinical nutrition where he gave 6 different types of treatments to 2 sailors each who had scurvy. The 6 treatments were i) hard apple cider, ii) sulfuric acid, iii) vinegar, iv) sea water, v) lemons and oranges and vi) medicinal paste made of garlic, mustard seed, resin, gum, etc. Out of the 6 treatments lemons and oranges were superior. Even though they were superior, they still couldn't be stored on ships so they started searching for acids other than sulfuric acid that they could introduce to sailors. They decided to try citric acid as this was the acid present in lemons and

oranges, but this acid was also not effective at treating scurvy. By 1795, the British Admiralty agreed to issue lemon juice to sailors which eventually led to the defeat of Napoleon's forces in the Mediterranean who didn't have the same resource. A French medical historical journal later wrote "We were defeated by a lemon."

Although great success came with the introduction of lemons and oranges to sailors' diets in the 18th century, the cause of scurvy was still unknown. In 1845, the Great Potato Famine led to a rise in scurvy in England, Scotland and Ireland. Coincidentally, around the same time of the famine, there was an increase of scurvy in the US navy which was being treated by eating raw potatoes. In hindsight, we know now that the success attributed to oranges, lemons and potatoes was because they are all a good source of vitamin C.

Vitamin C was first accidentally isolated by Albert Szent-Gyorgyi in 1928 while he was attempting to isolate a compound in the adrenal cortex that was a strong reducing agent in biochemical systems. He showed that it had the empirical formula $C_6H_8O_6$ and called it hexuronic acid because it was acidic and had 6 carbon atoms. At this time, other groups were trying to fractionate lemon juice and other anti-scorbutics to obtain the active product responsible for treating scurvy. In 1932, Charles Glen King identified that it was Szent-Gyorgyi's hexuronic acid that was the active product. Szent-Gyorgyi won the Nobel Prize in Physiology or Medicine in 1937 for first isolating vitamin C but it can be argued that Charles Glen King was undeservedly overlooked (Carpenter, 2012).

Nowadays, given ready access to fruits and vegetables scurvy should not still be a problem, but it still occasionally arises in people with mental disorders, unusual eating habits and alcoholics. One particular form is interestingly called 'bachelor' or 'widower' scurvy; which occurs in men who live alone and do not eat any fresh fruits or vegetables (Bernardino et al., 2012).

1.1.2. Metabolism of Vitamin C

Vitamin C is a reducing agent and the electrons it donates are responsible for all its known physiological effects (Padayatty and Levine, 2016). Vitamin C is therefore an antioxidant which reduces ROS produced during cell metabolism (Han et al., 2021). ROS are the product of normal physiological processes with the majority produced by the electron transport chain; during the production of adenosine triphosphate (ATP) in the mitochondria, electrons escape and interact with molecular O₂ to form superoxide radicals. Vitamin C is an effective antioxidant because of both its water solubility and its ability to scavenge a wide range of ROS. ROS can be neutralised or reduced in reactivity via reaction with an antioxidant molecule such as vitamins C or E (Harrison, 2012).

At physiological pH, ascorbic acid loses a proton to exist as an ascorbate anion in a reversible reaction. Ascorbate can then lose electrons one by one; when donating a single electron an ascorbate radical is generated and when losing a second electron and a proton, DHA is generated (Padayatty and Levine, 2016). The ascorbate radical can also be reduced back to ascorbate by NADH- and NADPH-dependent reductases (Wakefield et al., 1986). DHA can then be converted to ascorbate by direct reduction by glutathione or by enzymatic reduction by thiol transferases or NADPH-dependent reductases (Bielski et al., 1981, Fowler, 2024). Alternatively, DHA can also lose its hydrolytic ring to form 2,3-diketogulonic acid in an irreversible reaction (Figure 1.2) (Wells and Xu, 1994, Padayatty and Levine, 2016).

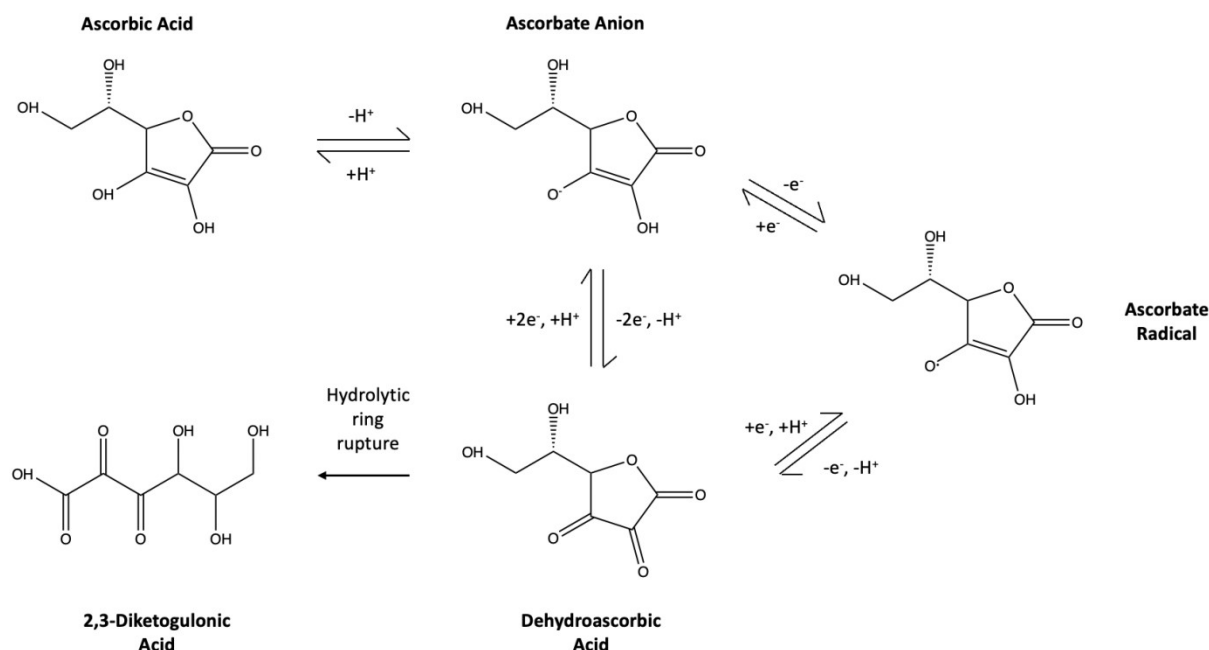


Figure 1.2. The redox states of vitamin C. Ascorbic acid can lose a proton to become ascorbate. Ascorbate can then sequentially lose electrons to first form an ascorbate radical then to form the oxidised form of vitamin C, dehydroascorbic acid. Dehydroascorbic acid can be reduced back to an ascorbate anion or irreversibly converted to 2,3-diketogulonic acid.

In addition to the role of vitamin C as an antioxidant, it can also act as a pro-oxidant. Ascorbate reduces metals such as copper and iron which results in the generation of superoxide and hydrogen peroxide and in turn also generates ROS. The superoxide is converted to H_2O_2 which then gets partially reduced to produce a hydroxide ion, hydroxyl radicals and water (Padayatty and Levine, 2016). At physiological concentrations, ascorbic acid tends to act as an antioxidant, whereas at much higher concentrations it acts as a pro-oxidant (Chen et al., 2007).

1.1.3. Vitamin C plays a role as a cofactor

Vitamin C also plays a role as a cofactor for several enzymes, and even as a co-substrate in some (Chatterjee et al., 1975). Ascorbate plays a role as a reducing agent for fifteen mammalian enzymes and three fungal enzymes. The mammalian enzymes include two monooxygenases, twelve dioxygenases and one amine oxidase (Padayatty and Levine, 2016). These enzymes are involved in several processes such as the synthesis of catecholamines,

carnitine, cholesterol, amino acids and collagen. Due to the importance of vitamin C in these processes, a deficiency can have serious implications. One example is in wound healing.

Collagen is the main protein in the skin, bones, cartilage, tendons and blood vessels (Padayatty and Levine, 2016, Moores, 2013). Collagen is essential in wound healing as it acts as a scaffold to provide the necessary structural strength for regenerating destroyed or damaged tissues (Gajbhiye and Wairkar, 2022). Ascorbic acid plays a role in collagen synthesis which makes it an essential micronutrient for wound healing (Thevi et al., 2024). Procollagen is synthesised in the endoplasmic reticulum and is rich in proline. Ascorbic acid activates the enzymes prolyl hydroxylase and lysyl hydroxylase to convert proline and lysine residues in procollagen to hydroxyproline or hydroxylysine respectively. These hydroxylated residues stabilise the triple-helix α -chains of collagen. A lack of ascorbic acid results in reduced hydroxylase activity and in turn the hydroxyproline residues in the collagen structure are compromised. This leads to a defect in the collagen structure which in turn affects wound healing (Padayatty and Levine, 2016, Baum and Arpey, 2005).

Due to the role of ascorbic acid in wound healing, there is increased turnover of ascorbic acid at wound sites. Inflammation present in wounds can also increase the consumption of ascorbic acid, and there is evidence that vitamin C as a supplement could be helpful in wound healing (Bechara et al., 2022). Thevi et al. (2024) recently reported that preoperative administration of 2 g of vitamin C accelerated wound healing and even reduced pain levels in patients.

1.1.4. Vitamin C plays a role in neurodegenerative diseases

As of 2022, Dementia and Alzheimer's disease (AD) are the biggest causes of death in the UK. Brain tissue has the highest concentration of ascorbate in the body, and low ascorbate levels in the plasma and in the cerebrospinal fluid have been found in patients with AD (Rivière et al., 1998, Schippling et al., 2000). Vitamin C plays an important role in brain development and protection (Harrison, 2012). The brain is susceptible to oxidative stress and free radical damage because of its high metabolic activity (Subramanian et al., 2021).

Oxidative stress occurs when the production of free radicals is greater than their elimination, leading to apoptosis in neurons. Oxidative stress plays a key role in the pathogenesis of AD, possibly due to vitamin C's antioxidant role. A number of studies have shown a positive relationship between ascorbate supplementation and reduced AD incidence (Masaki et al., 2000, Harrison et al., 2009, Murakami et al., 2011, Praticò, 2002). One of the causes of AD is the abnormal buildup of β -amyloid which then forms plaques and disrupts cell function. Low levels of vitamin C play a role in β -amyloid accumulation and deposition in AD. In an Alzheimer's experimental model, ascorbic acid was shown to lead to reduced oxidative stress and lower β -amyloid plaque burden. Moreover, in a study done on SH-SY5Y neuroblastoma cells, vitamin C was shown to prevent β -amyloid-induced apoptosis (Huang and May, 2006).

Oxidative stress also plays a role in the pathogenesis of Parkinson's disease (PD). PD is caused by decreases in dopaminergic signalling in the central nervous system. Dopamine deficiency causes movement disorder which is a common symptom of PD (Kalia and Lang, 2015). Ascorbic acid acts as a co-factor in the synthesis of dopamine and could therefore have therapeutic potential in treating PD. Levodopa, the precursor to dopamine, is thought to be the most effective anti-PD drug. The bioavailability of levodopa can be low in patients with PD. Ascorbic acid has been shown to improve the bioavailability and pharmacokinetics of levodopa (Nagayama et al., 2004).

1.1.5. Vitamin C plays a role in cancer

Cancer cells are more sensitive to oxidative stress because they usually have defective mitochondria. Oxidative stress leads to increased levels of ROS, important in cancer cell proliferation, differentiation and migration. An excess of ROS however can be toxic to cancer cells and result in cell death (Nakamura and Takada, 2021). Because of the importance of ROS in the cellular processes of cancer cells, antioxidant treatments have been investigated as anti-cancer treatments. However, these treatments seemed to promote cancer development instead. For example, the antioxidants N-acetylcysteine and vitamin E were shown to increase lung cancer progression in mice (Sayin et al., 2014). On the other hand,

pro-oxidant treatments, such as radiotherapy and chemotherapies that work by producing excess ROS, have proven to lead to cancer cell death (Mussa et al., 2022).

Plasma vitamin C levels are usually significantly lower in cancer patients, and vitamin C deficiency has been previously shown to improve the invasiveness of cancer cells (Linowiecka et al., 2020). Therefore, several studies have been done to investigate vitamin C as a potential anti-cancer treatment (Park et al., 2021, Lee et al., 2019, Darwiche et al., 2020). Both *in vitro* and *in vivo* studies have shown that vitamin C plays a role in inhibiting the invasion, migration and metastasis of breast cancer cells, melanoma cells, glioblastoma cells and prostate cancer cells (Zeng et al., 2019, Cha et al., 2013, Ryszawy et al., 2019, Bai et al., 2015). It does this by inhibiting the epithelial-mesenchymal transition, which is a feature in which cells gain migratory properties enabling the spread of cancer cells. *In vitro* studies have also shown that vitamin C can contribute to increased chemosensitivity in lymphoma cells (Shenoy et al., 2017). For prostate cancer, studies have shown that parenteral administration of vitamin C can help in tumour suppression, but dietary administration does not decrease the risk or decrease the aggressiveness of prostate cancer (Pollard et al., 2010, Parent et al., 2018). Moreover, there has been contradictory results when it comes to using vitamin C in adjuvant cancer therapy. On one hand, it seems to be effective in preventing the side effects of chemotherapy and radiotherapy but on the other hand for therapies that work by generating excess ROS, vitamin C may eliminate the ROS and diminish the effect of the therapies (Carr et al., 2014, Carr and Cook, 2018).

As discussed in section 1.1.2, vitamin C acts as an antioxidant at physiological concentrations but as a pro-oxidant at higher concentrations. Vitamin C is only able to induce cell death of melanoma tissue at high concentrations of >1 mM; at a low concentration of 100 μ M it can actually promote invasiveness and tumour growth. This indicates that the anti-cancer ability of vitamin C is primarily due to its pro-oxidant properties (Yang et al., 2017). The exact mechanism by which vitamin C attacks cancer cells is not known but it has been suggested that it disrupts their cellular metabolism. One way in which it does this is by formation of hydrogen peroxide (H_2O_2), an intermediate in the generation of ascorbate radical which can be cytotoxic to cancer cells. H_2O_2 can also be generated when vitamin C reacts with Fe^{2+} ions, which are abundant in cancer microenvironments. In addition, Uetaki et al. (2015) suggest

that vitamin C disrupts the metabolism of cancer cells by inhibiting glycolysis, which is the way in which cancer cells produce ATP. The effect of vitamin C on survival may be more exaggerated in cancer cells than in normal cells because of the dependence of cancer cells on glycolysis for ATP production. Moreover, vitamin C can also help boost the immune system and improve sensitivity to chemotherapy (Mussa et al., 2022).

Overall, high dose vitamin C shows great potential as an anti-cancer treatment. The fact that it's cheap, available in nature and has low toxicity makes it an even more ideal anti-cancer treatment candidate.

1.1.6. Vitamin C plays a role in epigenetics

In the mammalian genome, DNA and histone methylation are important epigenetic hallmarks. There are several Fe^{2+} and 2-oxoglutarate (2OG)-dependent dioxygenases that catalyse the hydroxylation of methylated nucleic acids and histones. Some of these enzymes, such as the ten-eleven translocation enzymes (TET) and the Jumonji C domain containing histone demethylase (JHDMs), use ascorbic acid as a cofactor for DNA and histone demethylation processes respectively. The oxidation of 5-methylcytosine (5mC) in DNA to 5-hydroxymethylcytosine (5hmC), an important intermediate in DNA demethylation, is catalysed by TET enzymes using ascorbic acid as a cofactor (Figure 1.3). Vitamin C encourages DNA demethylation by increasing TET-mediated oxidation of 5mC (Yue and Rao, 2020). Minor et al. (2013) reported that the addition of vitamin C to mouse fibroblasts increased 5hmC generation in a dose- and time-dependent manner.

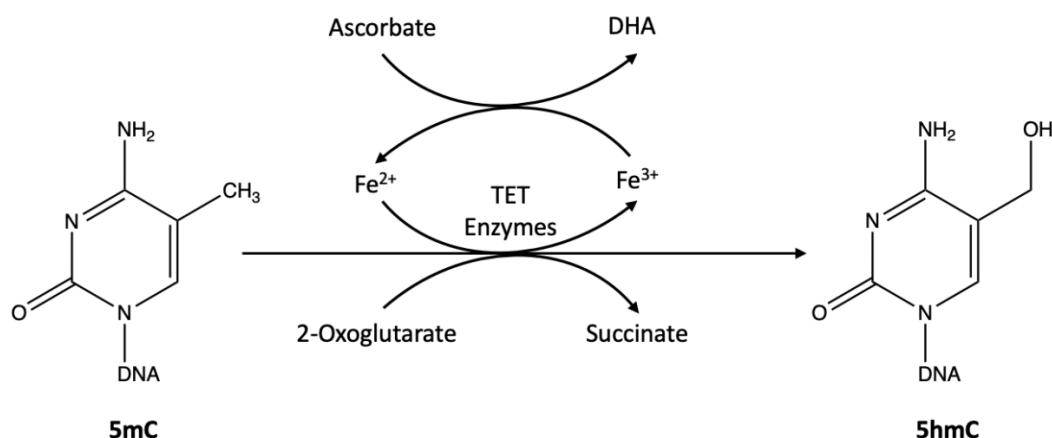


Figure 1.3. Ascorbate plays a role in DNA demethylation. The oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in DNA is catalysed by TET enzymes during the oxidation of ascorbate to dehydroascorbic acid (DHA). The production of 5hmC results in DNA demethylation.

Loss of 5hmC is an epigenetic hallmark of most types of cancers because it affects DNA methylation/demethylation which in turn affects gene expression and can lead to malignancies. Reduction of 5hmC was identified in leukaemia (Young et al., 2015). Mutations in TET genes, specifically TET2, that cause loss of function are common in haematopoietic malignancies, such as acute myeloid leukaemia (AML). The progression of AML is characterised by abnormal self-renewal and differentiation of haematopoietic stem/precursor cells (HSPCs) which is associated with increased DNA methylation. Loss of TET2 means increased DNA methylation and this contributes to the abnormal differentiation of HSPCs leading to the progression of the disease (Agathocleous et al., 2017). Vitamin C supplementation pharmacologically mimicked the role of TET2 and helped in inhibiting myeloid disease progression (Yue and Rao, 2020).

1.2. Membrane proteins

Biological membranes are selectively permeable membranes that separate the interior of all cells from the external environment. Membranes contain different types of lipids, sugars and proteins. There are several types of membrane proteins that play different key roles such as in membrane transport, cell-cell recognition, signal transduction and cell adhesion and mobility. These proteins represent about 25% of most organisms' proteomes and more than

40% of drug targets. Without membrane proteins, the cell membrane would be impenetrable, and the cells would not survive (Watson, 2015, Carpenter et al., 2008).

Membrane proteins can be broadly classified as integral or peripheral membrane proteins (Guo et al., 2019). Integral membrane proteins are firmly anchored to both sides of the membrane and contain one or more transmembrane domains; whereas, peripheral membrane proteins interact with one side of the membrane by hydrophobic interactions or by glycosylphosphatidylinositol (GPI) anchors (Boes et al., 2021). Examples of integral membrane proteins are channels and transporters (Figure 1.4).

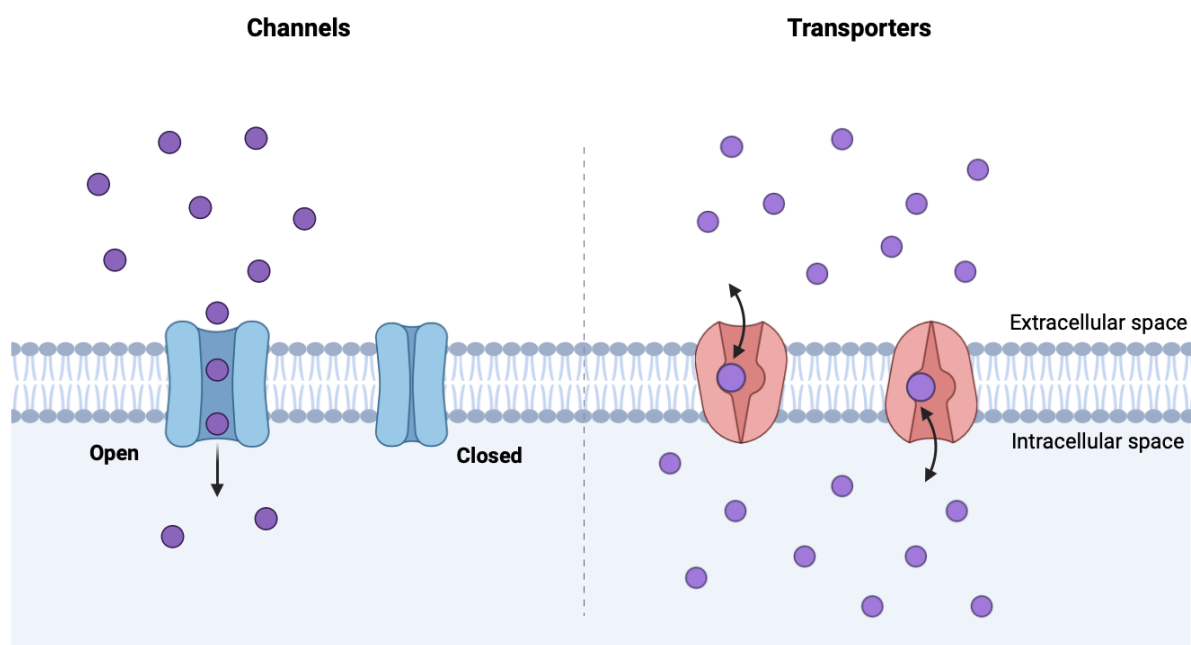


Figure 1.4. Schematic representation of channel proteins (left) and membrane transporters (right). Channel proteins form pores to allow the movement of molecules down their electrochemical gradient. Transporters contain a substrate binding site that upon binding of substrate induces a conformational change to allow transport of substrate up or down their electrochemical gradient.

Channels are membrane proteins that form pores which create a hydrophilic path to allow the very rapid translocation of molecules through the hydrophobic membrane. These channels allow the selective downhill movement of molecules along their electrochemical gradient. The structure of the channel pore differs in size and charge to very specifically select substrate molecules (Watson, 2015). Channels open and close via different gating mechanisms, such as pH, membrane potential, mechanical stress or ligand binding

(Goldschen-Ohm and Chanda, 2017). Movement of substrate is rapid as the substrate molecules form only very transient interactions with the protein. Thus, channels are involved when very rapid responses are required, such as in the voltage-gated sodium channels involved in neuronal excitability (Goldfarb, 2012). On the other hand, transporters contain a relatively high affinity substrate-binding site. Binding of substrate induces a conformational change in the protein that allows the transport of the substrate across the membrane (Drew and Boudker, 2016).

1.2.1. Transporters translocate solutes across biological membranes

Membrane transport proteins translocate solutes, such as ions, nutrients and signalling molecules, across biological membranes. They are essential for cell survival, division and differentiation (David et al., 2019). Defective membrane transporters can cause several diseases such as neurodegenerative diseases, diabetes and cystic fibrosis (Yarwood et al., 2020). Membrane transport proteins are usually multipass transmembrane proteins that allow specific hydrophilic substrates to cross the membrane without coming into contact with the hydrophobic interior of the membrane (Alberts, 2002).

The translocation of solutes can proceed by either passive or active transport mechanisms, which describe the transport of solutes down or against their electrochemical gradient respectively. Facilitated diffusion is the movement of solutes by passive, concentration-dependent transport, without the requirement of any additional energy source, using a highly specific transmembrane protein. Active transport is the movement of solutes against their electrochemical gradient with the requirement of an energy source and results in a nonequilibrium distribution of the solute across the membrane (Quick and Javitch, 2007, Stillwell, 2016). This additional energy can be supplied by ATP hydrolysis, light or the co-transport of a second molecule, or ion, down its electrochemical gradient (Watson, 2015).

Active transporters can be further divided into primary and secondary active transporters. Primary active transporters use ATP hydrolysis to drive the movement of solutes across membranes against their electrochemical gradient. Secondary active transporters, or

cotransporters, couple the downhill movement of an ion along its concentration gradient, to the uphill movement of the solute against its concentration gradient. The movement of the two molecules can either be in the same direction, symport, or in the opposite direction, antiport (Figure 1.5) (Stillwell, 2016). Secondary active transporters typically contain 10-14 hydrophobic transmembrane domains (TMs), and hydrophilic N- or C-terminal regions which are usually cytoplasmic (Mikros and Diallinas, 2019). An example of secondary active transporters are the sodium-dependent L-ascorbate transporters (SVCTs), the main topic of this thesis.

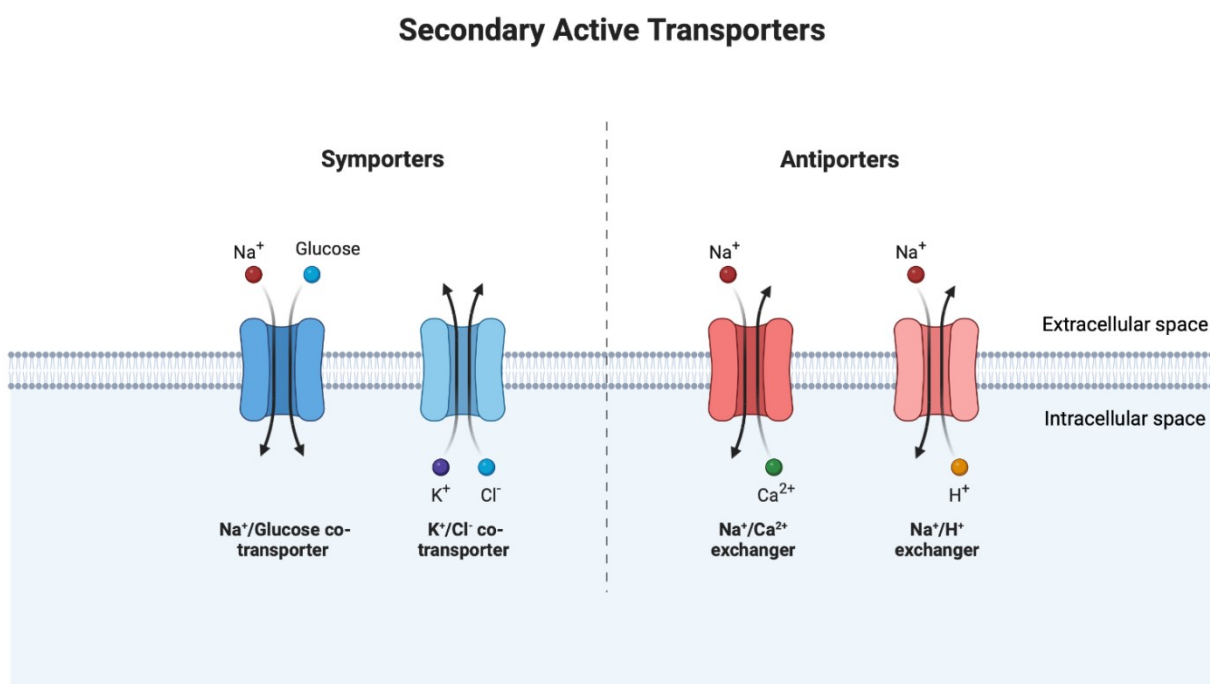


Figure 1.5. Schematic representation of secondary active transporters adapted from “Examples of Secondary Active Transporters” by BioRender.com (2022). On the left are symporters that couple the movement of solutes to ions in the same direction. On the right are antiporters that couple the movement of solutes to the movement of ions in the opposite direction.

1.2.2. Secondary active transporters translocate solutes using the alternating-access mechanism

Secondary active transporters translocate substances using the alternating-access mechanism, in which substrates bind to a high affinity binding site which triggers a conformational change of the transporter resulting in the release of the substrate on the

opposite side of the membrane (Lopez-Redondo et al., 2018). In this alternating-access mechanism, the substrate binding site is alternately accessible to each side of the membrane (Drew et al., 2021). Transporters, specifically symporters, alternate between at least five distinct conformations: i) outward-facing - in which the substrate binds, ii) outward-facing occluded – in which the substrate is oriented in the binding site and the outer gate is closed, iii) fully occluded – in which the binding site is occluded from both sides, iv) inward-facing occluded – in which the substrate binding triggers inward conformation and v) inward-facing open – in which the inner gate opens and the substrate is released (Mikros and Diallinas, 2019). The occluded state is important as it ensures the electrochemical gradients, established by the cell in order to transport the substrate, are maintained (Drew et al., 2021).

In the case of secondary active transporters, there are three types of alternating-access mechanisms: rocker-switch, rocking-bundle and the elevator mechanism (Figure 1.6). In the rocker-switch and rocking-bundle mechanisms; the substrate binds at the interface of the two transporter domains and the domains rearrange themselves around the substrate to allow its translocation. Both domains rearrange themselves in the rocker-switch mechanism whereas only one domain shifts in the rocking-bundle mechanism. The rocker-switch mechanism involves nearly symmetrical movements of two structurally similar bundles around the substrate in order to alternatively expose the substrate binding site to only one side of the membrane at a time. Whereas in the rocking-bundle mechanism, the two bundles are structurally different and do not move symmetrically (Drew et al., 2021). Glycerol-3-phosphate transporter (GlpT) in *Escherichia coli* and the bacterial leucine transporter, LeuT, are examples of proteins that function via the rocker-switch and the rocking-bundle mechanism respectively (Law et al., 2007, Forrest and Rudnick, 2009).

In the elevator mechanism, the substrate-binding site is located entirely within a single domain; this domain, known as the transport or core domain, moves through the membrane against a structurally dissimilar domain, known as the scaffold or gate domain, to translocate the substrate from one side of the membrane to the other (Drew and Boudker, 2016). Most transporters in this class are either dimers or trimers (Dimakis et al., 2022). The protomers interact via their relatively static gate domains, an interaction stabilised by membrane lipids,

leaving the transport domains free to move (Pyle et al., 2018). The elevator mechanism was first reported for the bacterial homologue of glutamate transporters, Glt_{ph}, by comparing the inward- and outward-facing structures of this protein (Reyes et al., 2009).

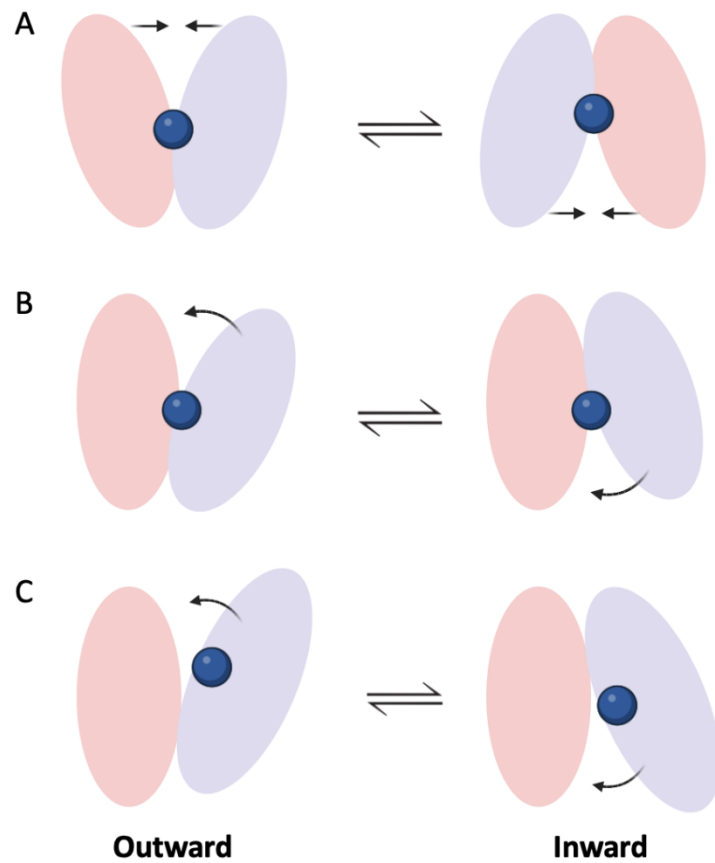


Figure 1.6. Schematic representation of alternating-access mechanisms for membrane proteins: A) Rocker switch, B) Rocking bundle and C) Elevator mechanism.

1.3. Sodium-dependent L-ascorbate transporters (SVCTs)

1.3.1. The nucleobase-ascorbate transporter (NAT) family is responsible for the cellular uptake of nucleobases and ascorbic acid

Specialised transporters that facilitate the movement of nucleobases across the membrane exist in bacteria, algae, fungi, protozoa, plants and mammals (de Koning and Diallinas, 2000). These nucleobase transport systems can be classified into four distinct protein families. These are the Nucleobase Cation Symporter family 1 (NCS1), the Nucleobase-Ascorbate Transporter family (NAT or NCS2), the AzgA-like family and the Equilibrative Nucleoside Transporter family (ENT). The NAT family are responsible for the cellular uptake of metabolites in almost all species (Diallinas and Gournas, 2008). The NAT family consists of proteins that are 414-650 amino acid residues in length and contain 14 mostly α -helical TMs and have an intracellular N- and C-termini. These intracellular N- and C-termini usually control the trafficking of the proteins to the cell membrane (Kourkoulou et al., 2018, Mikros and Diallinas, 2019). NATs have a highly conserved signature sequence motif (Q/E/P)-N-X-G-X-X-X-T-(K/R/G), where X is a nonspecific amino acid, generally found in an amphipathic region vital for function and specificity of the transporters (Diallinas et al., 1998, Koukaki et al., 2005). In addition to this motif, Q-H in TM1 and E-X-X-G in TM8 are also highly conserved and have been shown to be necessary for binding and transport (Pantazopoulou and Diallinas, 2006).

In bacteria, plants and fungi, NATs are specific for nucleobases, whereas in humans they are specific for ascorbic acid. Based on their substrate specificity, the NATs can be classified into three sub-families. The first sub-family is proton-dependent transporters specific for the transport of xanthine and/or uric acid and is found in bacteria, fungi and plants. A well-studied NAT that belongs to this family is the uric acid/xanthine H^+ symporter, UapA from *Aspergillus nidulans* (Gournas et al., 2008). UapA is specific for the purines xanthine and uric acid but is unable to transport hypoxanthine or adenine (Pantazopoulou and Diallinas, 2007). The second sub-family contains proton-dependent transporters specific for the transport of uracil and are only found in bacteria. UraA is a well-studied uracil proton symporter from *Escherichia coli*. UraA contains 14 TMs with intracellular N- and C-termini,

and it forms a dimer which is required for its transport activity (Yu et al., 2017). Uracil can be used in nucleic acid synthesis, whereas xanthine and uric acid cannot be used to make nucleosides but can be used as nitrogen sources (Diallinas and Gournas, 2008). The third sub-family contains the sodium-dependent transporters found in mammals. This sub-family can be further categorised into two groups. The first group, that includes the rat rSNBT1 transporter, is specific for nucleobases such as uracil, thymine, xanthine, hypoxanthine and guanine, whereas the second group is highly specific for ascorbic acid (Yamamoto et al., 2010). The mammalian NATs responsible for transporting ascorbic acid are the sodium-dependent L-ascorbate transporters (SVCT1 and SVCT2), the topic of this thesis. Higher primates, including humans, only express the ascorbate-specific NATs; the genes encoding nucleobase-specific NATs are truncated pseudogenes (Gournas et al., 2008). Given that humans have lost the ability to synthesise ascorbic acid, these ascorbate-specific NATs are important in delivering ascorbic acid to cells. The bacterial and fungal NATs, more specifically UraA and UapA respectively, have been well-studied, however the human NATs, SVCTs, are relatively understudied. It is surprising that the SVCTs transport ascorbic acid rather than purines or pyrimidines, unlike other members of the NAT family. Investigating the structural differences between the SVCTs and other NATs, such as UraA and UapA, could provide insights into the underlying reasons for this functional divergence.

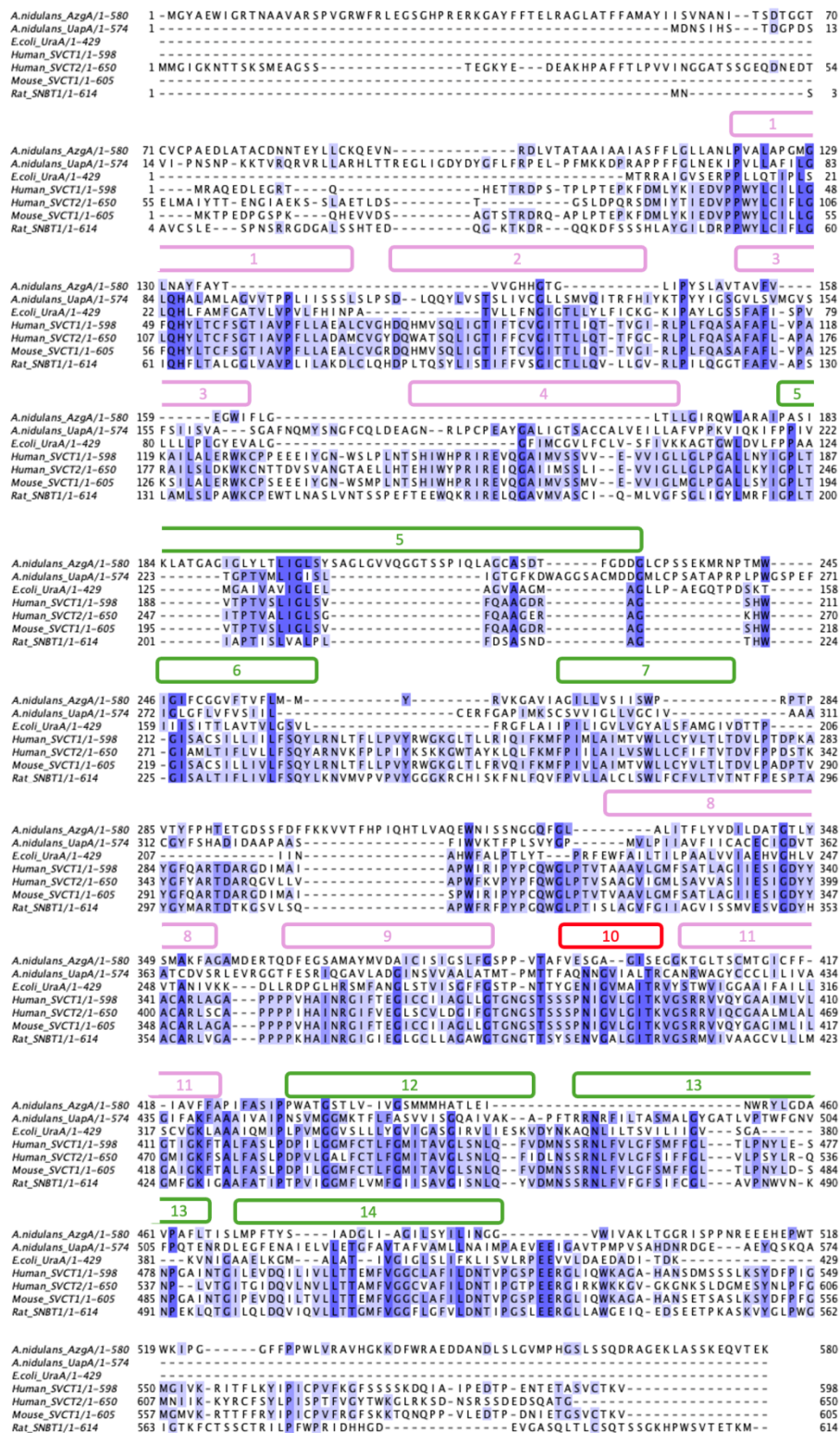


Figure 1.7. Sequence alignment of chosen NAT members. Residues are coloured according to sequence identity with dark blue representing >80%, the lighter blue representing >60% and the lightest blue representing >40% percentage identity. Transmembrane (TM) domains highlighted in pink represent the core domain and those highlighted in green represent the gate domain. TM10 which contains the NAT motif is highlighted in red.

1.3.2. Uric acid permease A (UapA) harnesses proton flux to import xanthine and uric acid

Structural and functional analyses of the fungal NAT, UapA, have given important insights into the working mechanisms of NATs. UapA transports xanthine and uric acid and can also transport xanthine analogues, such as the drugs allopurinol and oxypurinol (Arst and Scazzocchio, 1975, Gorfinkiel et al., 1993, Diallinas and Scazzocchio, 1989, Christos et al., 2000). UapA was expressed in *Saccharomyces cerevisiae* and the high-resolution crystal structure was solved using an engineered version containing the single-residue substitution, G411V, in the NAT signature motif sequence and missing the N-terminal 11 residues. This mutation locked UapA into the inward-facing conformation which helped stabilise the protein for structural analysis, but resulted in a protein that was transport inactive although still able to bind substrate. The structure revealed that UapA contains 14 TMs arranged into a 7+7 TM fold divided into a core/substrate (TMs 1-4 and 8-11) and a gate/dimerisation (TMs 5-7 and 12-14) domain (Alguel et al., 2016, Koukaki et al., 2005).

Genetic, biochemical and cellular assays as well as structural information have revealed key residues located in TMs 3, 8 and 10 that are crucial for xanthine binding (Diallinas, 2016). Alguel et al. (2016) reported that the xanthine binding site is located in a cleft formed by the half helices of TMs 3 and 10, similar to the uracil binding site in UraA (Lu et al., 2011). Gln408 (TM10), Glu356 (TM8), Ala407 (TM10), Val153 (TM3) and Phe155 (TM3) are all involved in xanthine binding (Figure 1.8C). These residues that are directly interacting with xanthine are all from the core domain. However, residues that are important in substrate specificity can be found in both the core and gate domains. For example, mutation of Gln113 and Ala441, located in hinge regions between the core and gate domains, resulted in variants of the protein that exhibit decreased uptake of xanthine and low uptake of hypoxanthine and adenine, two substrates that wild-type (WT) UapA is unable to transport (Papageorgiou et al., 2008, Kosti et al., 2010). Combining mutations in distinct specificity regions of UapA resulted in the ability to efficiently transport hypoxanthine and adenine. This showed that even though the substrate binding site is located in the core domain, other residues that are not located within the binding site or are even in the gate domain play roles in substrate selectivity.

UapA is a homodimer, with the dimer interface involving TMs 12, 13 and 14 of the gate domain, with TM13 being most closely involved. Dimer formation is important for UapA trafficking and turnover as well as substrate transport. When WT UapA was co-expressed with dominant negative UapA mutants, exhibiting low or no transport of substrate, uptake of xanthine was reduced to a greater extent than would be expected if the WT protein was functional in dimeric arrangements with a non-functional mutant. These results showed that dimer formation with two active protomers is required for UapA function, as reported for UraA (Alguel et al., 2016, Yu et al., 2017).

The Solute Carrier 4 (SLC4) transporter family is homologous to the NAT family. The structure of anion exchanger 1 (AE1), a member of the SLC4 family, was solved in the outward-facing conformation (Arakawa et al., 2015). When looking at the core and gate domains of AE1 and UapA individually, they superimpose well, therefore, justifying the use of AE1 in trying to determine the working mechanisms of UapA. In UapA, a tight interaction was observed between the gate domains at the dimer interface, suggesting that these domains remain static in the cell membrane. Furthermore, when comparing the outward-facing AE1 structure with the inward-facing UapA structure, it was observed that the core domain of AE1 is displaced along TMs 1, 3, 8 and 10 (Alguel et al., 2016).

UapA is thought to function via the elevator mechanism (Alguel et al., 2016). The first piece of evidence that UapA functions with an elevator mechanism is that its substrate-binding site is located within the core domain; a key characteristic of the elevator mechanism is that the substrate-binding site is located entirely within a single domain (Drew and Boudker, 2016). Secondly, most transporters that function with the elevator mechanism are dimers or trimers (Dimakis et al., 2022). The homodimerisation and the static gate domains identified in UapA therefore point to UapA functioning via the elevator mechanism. Lastly, in the elevator mechanism, the gate domains remain static and the core domains move alongside the gate domains to transport the substrate across the membrane. As mentioned above, a comparison between the UapA and AE1 structures showed that the core domain shifts between the outward and inward facing conformations; this gives the final piece of evidence that UapA functions via the elevator mechanism.

1.3.3. Sodium-dependent L-ascorbate transporters (SVCTs) harness sodium flux to import ascorbic acid

Vitamin C exists as the reduced ionisable form, ascorbic acid, and the oxidised non-ionic form, dehydroascorbic acid (DHA) (Subramanian et al., 2021). Ascorbic acid is actively transported in humans via sodium-dependent L-ascorbate transporters (SVCTs) and dehydroascorbic acid is transported via glucose transporters (GLUTs), including GLUT1, GLUT3 and GLUT4, by facilitative diffusion (Godoy et al., 2007). The SVCTs include SVCT1, SVCT2 and two orphan members SVCT3 and SVCT4. SVCT1, encoded by SLC23A1, and SVCT2, encoded by SLC23A2, are active transporters that couple the transport of ascorbic acid against its concentration gradient to the transport of sodium ions along their electrochemical gradient, with a 2:1 sodium ion to ascorbic acid stoichiometry (Godoy et al., 2007). Replacing sodium ions with either potassium or lithium ions almost completely inhibits ascorbic acid uptake (Savini et al., 2008).

SVCT1 has a high capacity and low affinity for ascorbic acid transport with an apparent K_m of $\sim 70 \mu\text{M}$, whereas SVCT2 is the opposite with a low capacity but high affinity for ascorbic acid with an apparent K_m of $\sim 15 \mu\text{M}$ (Han et al., 2021). SVCT1 and SVCT2 are 66% identical at the amino acid sequence level but exert distinct functions because of differential expression; SVCT1 is mainly expressed in epithelial cells to transport ascorbic acid for further distribution, whereas SVCT2 is expressed ubiquitously to concentrate ascorbic acid for cellular processes (Savini et al., 2008, Bürzle and Hediger, 2012, Tsukaguchi et al., 1999a). SVCT1 and SVCT2 function independently of each other because SVCT2 deficiency does not affect the expression of SVCT1 and neither does it affect the ascorbic acid levels in SVCT1-expressing organs (Kuo et al., 2004). SVCT3, encoded by SLC23A3, is predominantly expressed in the kidney and its function is currently unknown. SVCT4, encoded by SLC23A4, transports nucleobases in rats and is predominantly expressed in the small intestine; the human SLC23A4 gene is considered to be a pseudogene (Bürzle and Hediger, 2012).

1.3.3.1. SVCT1 transports ascorbic acid for further distribution

SVCT1 is primarily distributed in epithelial cells (Godoy et al., 2007). It is expressed in lung, liver and kidney cells, and is responsible for absorption of vitamin C from the diet across the apical membrane of enterocytes (Bürzle and Hediger, 2012). SVCT1 is also responsible for maintaining a plasma vitamin C concentration of 50 μ M by regulating gastrointestinal and renal reabsorption (Forman et al., 2020).

Wang et al. (2023) solved the structure of mouse SVCT1 (mSVCT1) in the inward-facing open state using single-particle cryogenic electron microscopy (cryo-EM) in the presence (PDB: 7YTW) and absence (PDB: 7YTY) of vitamin C at resolutions of 3.2 Å and 3.5 Å respectively. Like UapA and UraA, mSVCT1 forms a homodimer, with each protomer containing 14 TMs separated into a core domain (TM1-TM4 and TM8-TM11) and a gate domain (TM5-TM7 and TM12-TM14). The dimeric interface is formed by TM5, TM6 and TM12 from one subunit and TM13 from the neighbouring subunit through hydrophobic interactions and van der Waals contacts.

Similar to the uracil binding site in UraA and the xanthine binding site in UapA, the vitamin C binding site is located between the half helices of TM3 and TM10 in the core domain. In mSVCT1, Thr387-Ser388-Ser389-Ser390-Pro391 directly interact with vitamin C; this binding site is identical in human SVCT1 (Figure 1.8A and 1.8B) (Kobayashi et al., 2024). Similarly, in UapA, the corresponding amino acids Thr404-Thr405-Phe406-Ala407-Gln408 all play a role in xanthine binding, with Thr404 and Thr405 contributing to the architecture of the binding site and Phe406, Ala407 and Gln408 involved in direct binding of xanthine (Figure 1.8C).

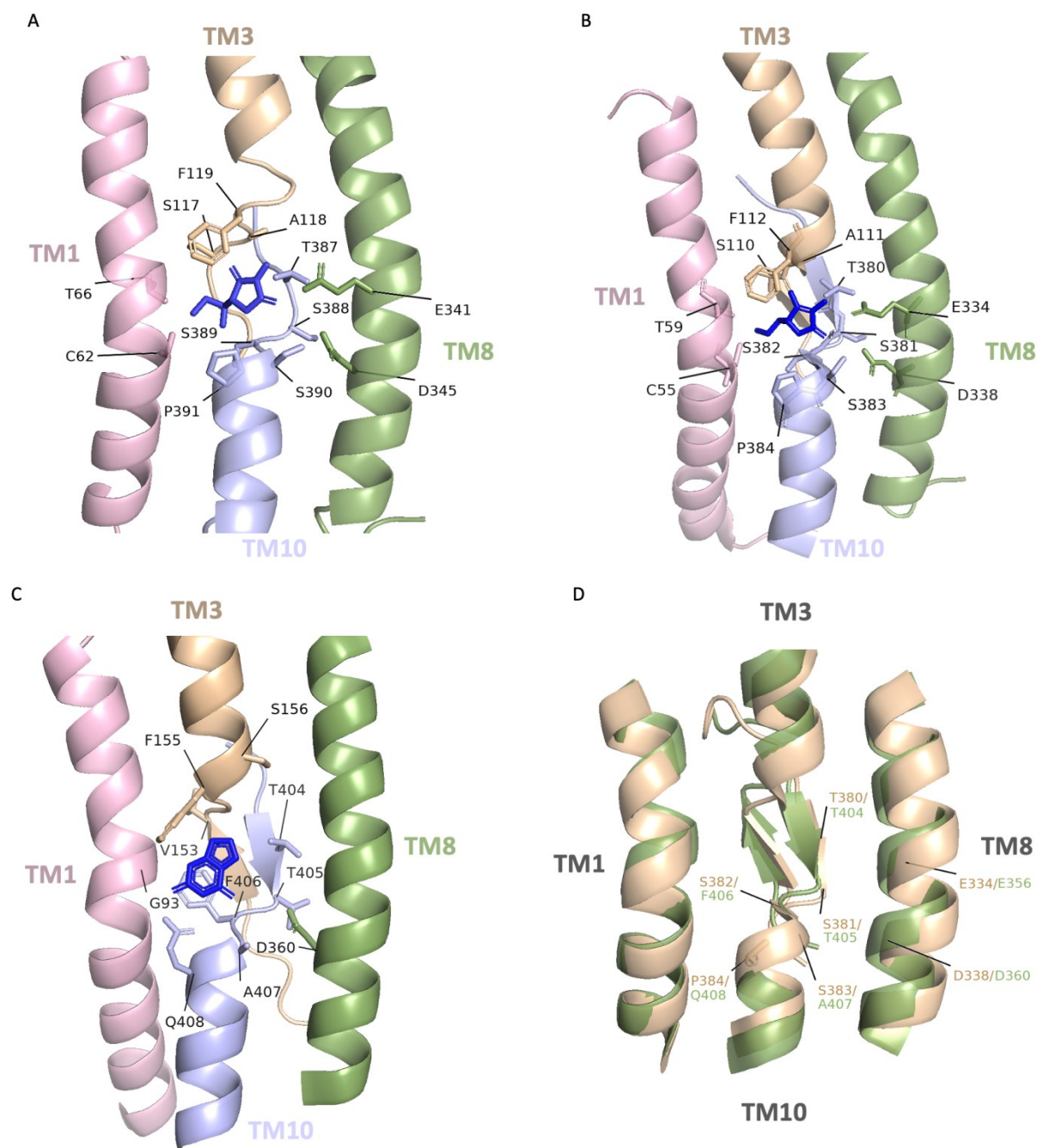


Figure 1.8. Substrate binding sites of mouse SVCT1, human SVCT1 and UapA. A) Vitamin C binding site of mouse SVCT1, B) Vitamin C binding site of human SVCT1, C) Xanthine binding site of UapA and D) Structural alignment of the binding sites of human SVCT1 (pink) and UapA (green).

Interfaces I and II, which are tightly packed extracellular interfaces between the core and gate domains, make up the closed extracellular gate of the inward-open structure of mSVCT1. Leu72 (TM1), Phe119 (TM3), Arg213 (TM5) and Ile446 (TM12) on interface I were shown to play a role in vitamin C transport of mSVCT1 (Figure 1.9). This is consistent with

previous studies on UapA where residues on TM5 and TM12 were shown to play a role in substrate affinity and specificity (Dimakis et al., 2022). Mutations within the xanthine binding site of UapA did not broaden the ability of UapA to transport other substrates such as hypoxanthine and adenine. However, mutations on TM12, TM13 and TM14 of the gate domain allowed the transport of hypoxanthine and adenine, suggesting that these residues are important in substrate specificity. This suggests that similar mutations in the gate domain of SVCT1 could broaden the ability of SVCT1 to transport other substrates.

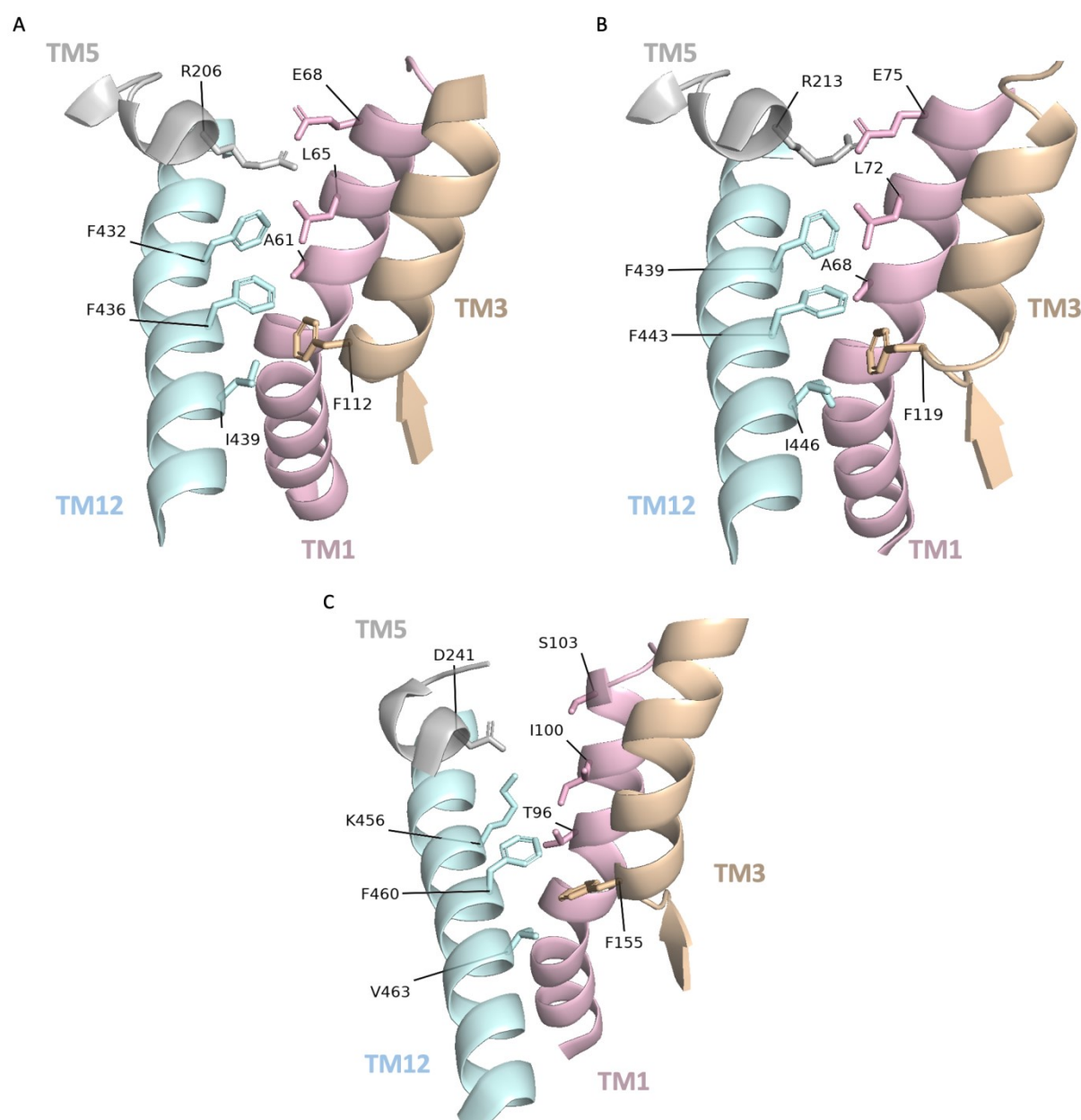


Figure 1.9. The extracellular interface of SVCT1 and UapA are important in substrate transport. Extracellular interface of human SVCT1 (A), mouse SVCT1 (B) and UapA (C).

When comparing the inward-open mSVCT1 structure and occluded UraA structure, the intracellular segments of the core domain in mSVCT1 have undergone an outwards movement relative to the occluded UraA structure which resulted in the opening of the intracellular side of mSVCT1. The vitamin C bound to mSVCT1 also showed a downward shift which agrees with an elevator mechanism. Moreover, the residues that bind vitamin C are all found in the core domain, therefore further supporting the idea that SVCT1 functions by the elevator mechanism. As previously stated, the elevator mechanism has also been suggested for UapA (Alguel et al., 2016, Wang et al., 2023, Yu et al., 2017).

Kobayashi et al. (2024) recently solved the human SVCT1 structure using cryo-EM in the substrate-bound and substrate-free inward-open states (PDB: 8JEW and 8JEZ respectively) and the substrate-free occluded state (PDB: 8JF0) at 2.5-3.5 Å resolutions. The two inward-open structures aligned with a root mean square deviation (RMSD) of 0.28 Å and showed no significant differences except for the substrate binding. Unsurprisingly, hSVCT1 forms a homodimer containing 14 TMs separated into a core domain (TM1-TM4 and TM8-TM11) and a gate domain (TM5-TM7 and TM12-TM14). Similar to UraA, UapA and mSVCT1, the binding site is located between the half-helices of TMs 3 and 10. Subramanian et al. (2008) previously reported the presence of an N-linked glycosylation site at Asn144 which has now been confirmed by the hSVCT1 structure. This N-glycosylation has been shown to be important for substrate transport (Subramanian et al., 2008). An S-acylation at Cys565 was also observed and is thought to contribute to protein stability or membrane trafficking. It was previously reported that SVCTs transport sodium ions and vitamin C in a 2:1 stoichiometry (Godoy et al., 2007) and this was confirmed by the mSVCT1 and hSVCT1 structures (Wang et al., 2023, Kobayashi et al., 2024). The sodium ions are required to neutralise the negatively charged binding site to allow vitamin C binding. The first sodium ion interacts with the sidechains of Glu334, Asp338 and Ser383, the backbone carbonyl of Ser381 and an oxygen atom of vitamin C, which is consistent with the mouse SVCT1. When comparing the substrate-bound and substrate-free structures, it was observed that after substrate release the sidechain of Ser383 is flipped allowing interaction with the sidechain of Asp338. This suggests that after substrate release the substrate binding pocket is rearranged which could then trigger the subsequent conformational change to the outward facing conformation. The second sodium ion interacts with the aromatic sidechain of Phe112 and

forms polar interactions with Ser110 and vitamin C. In mSVCT1, the second sodium ion is surrounded by TMs 3 and 8 on the same side as the first sodium ion but in hSVCT1 the second sodium ion is located opposite the first one (Figure 1.10). In hSVCT1, the lactone ring of vitamin C is stabilised by the core domain through the two sodium ions and three water molecules. Introducing mutations to residues involved in coordinating the sodium ions significantly reduced ascorbic acid uptake (Kobayashi et al., 2024).

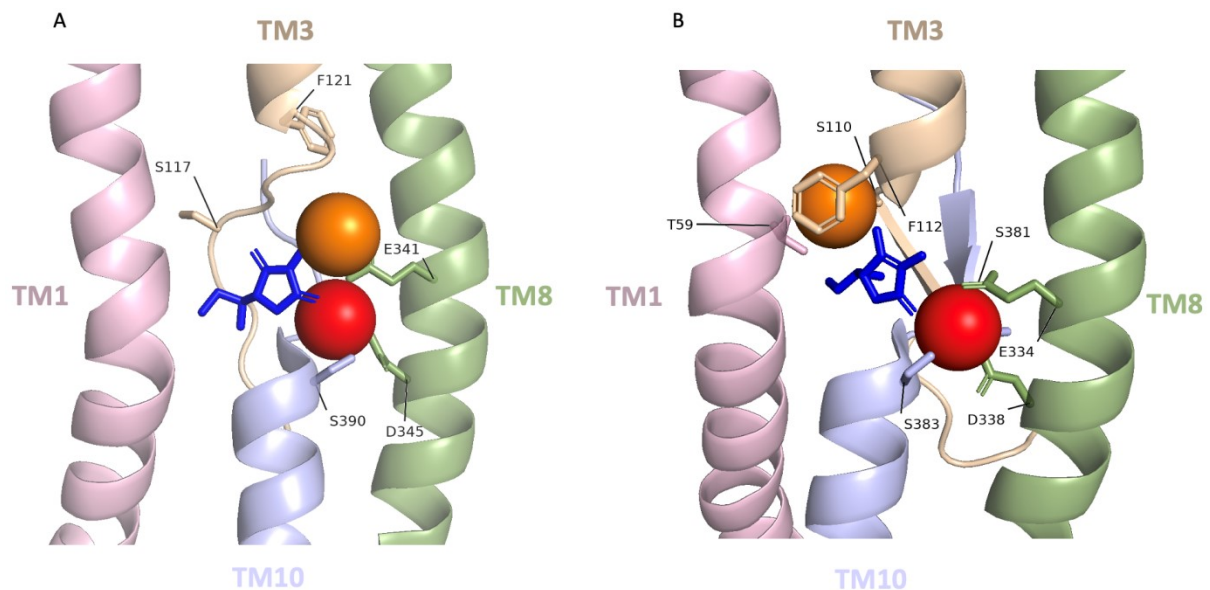


Figure 1.10. Sodium binding sites of mouse SVCT1 (A) and human SVCT1 (B). In mSVCT1, the sodium ions are on the same side whereas in hSVCT1 they are opposite each other. Vitamin C (blue), first sodium ion (red) and second sodium ion (orange).

Purifying hSVCT1 in sodium-free conditions resulted in a substrate-free occluded state structure at 2.8 Å resolution. The superimposition of the core domains of the inward-open and substrate-free occluded structures revealed that in the occluded state there's an upward shift of TM10 towards the extracellular side consistent with an elevator mechanism. Unlike previous reports, TM10 in hSVCT1 also undergoes a horizontal shift toward the gate domain blocking the interior of the protein. Although the upward shift of TM10 is consistent with an elevator mechanism, Kobayashi et al. (2024) also reports that the gate domain tilts towards the cytoplasmic side of the core domain and TMs 6 and 13 outwardly rotate during the transition from the inward-open to the occluded state which is suggestive of a rocking bundle-like mechanism.

1.3.3.2. SVCT2 concentrates ascorbic acid for cellular processes

SVCT2 is expressed ubiquitously either to protect cells from oxidative stress or to deliver vitamin C into cells for enzymatic reactions (Bürzle and Hediger, 2012). In addition to other tissues, SVCT2 is preferentially expressed in the adrenal and pituitary glands, lymphoid tissue, muscle and bone (Tsukaguchi et al., 1999b). More recent studies indicate that SVCT2 is also expressed in the brain, such as in the hippocampus, cerebral cortex, cerebellum and choroid plexus (Subramanian et al., 2021). SVCT2 is important for maintaining vitamin C homeostasis indicated by the death of homozygous SVCT2 knockout mice due to brain haemorrhage and respiratory failure immediately after birth (Park et al., 2021, Rivas et al., 2008).

It has been reported that SVCT2 has two functions: i) transport of vitamin C and ii) a receptor-like function (Figure 1.11). Janus kinases (JAKs) are non-receptor tyrosine kinases that upon binding of cytokines to their receptors, are activated and in turn phosphorylate signal transducer and activator of transcription (STAT) leading to downstream signalling. In addition to transporting vitamin C, SVCT2 plays a JAK-associated receptor-like role by mediating the activation of JAK2/STAT2 signalling pathway. The JAK2/STAT2 pathway is generally involved in the immune response, cell growth and differentiation by mediating the effects of cytokines and regulating gene expression (Villarino et al., 2015). Vitamin C-activated JAK2 plays a role in scavenging ROS, promoting differentiation and in epigenetic regulation, such as DNA demethylation (Han et al., 2021). As mentioned in section 1.1.6, vitamin C plays a role in DNA demethylation by acting as a cofactor for TET enzymes to catalyse the conversion of 5mC to 5hmC in cellular DNA. In addition to this, Han et al. (2021) reports that vitamin C induces DNA demethylation by activating JAK2 which in turn directly phosphorylates TET3 and results in 5mC oxidation. Questions remain on how exactly SVCT2 associates with JAK2/STAT2 to activate this pathway.

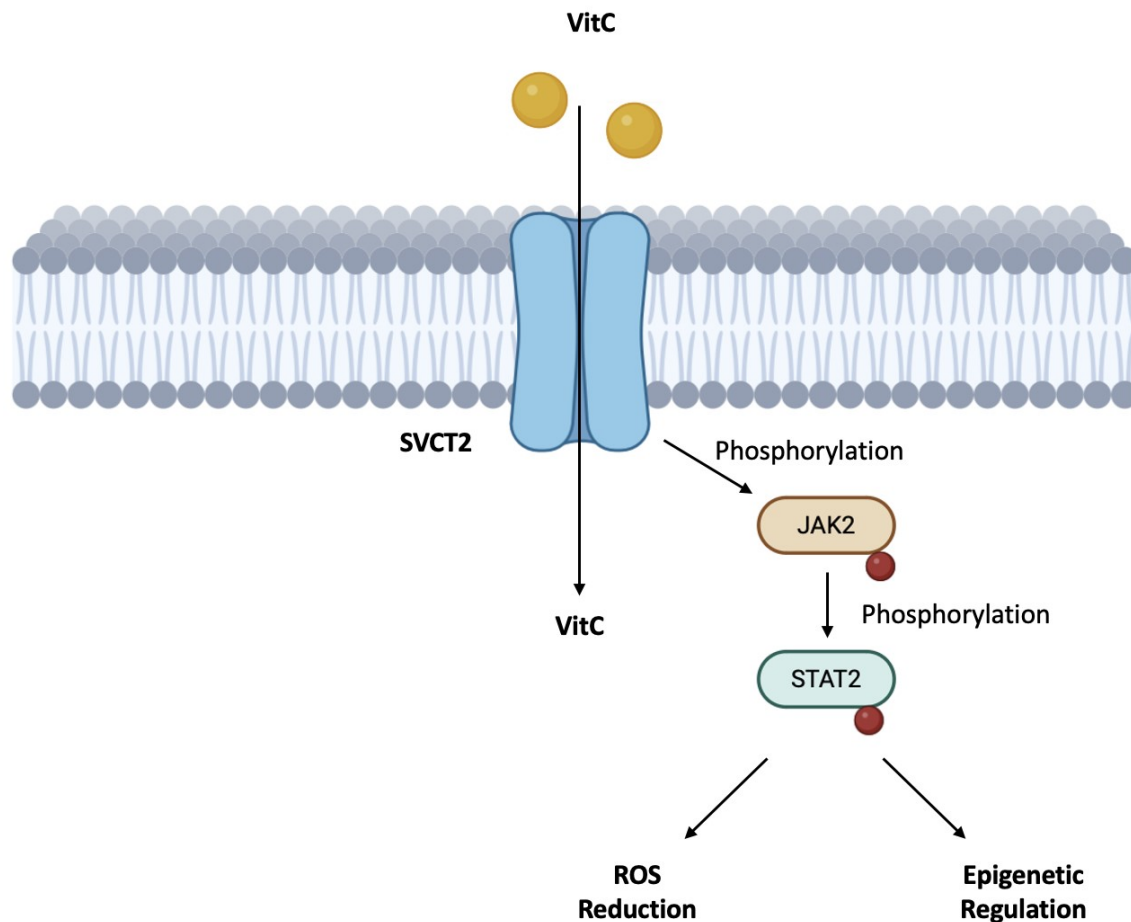


Figure 1.11. SVCT2 plays a role as a transporter and a transceptor. SVCT2 transports ascorbic acid across the cell membrane and also has a receptor-like function in which the transport of ascorbic acid activates the JAK2/STAT2 signalling pathway resulting in ROS reduction and epigenetic regulation.

In sections 1.1.4 and 1.1.5, I discussed the effects of vitamin C deficiency in the progression of neurodegenerative diseases and cancers. These effects are most tightly linked to the transport of vitamin C by SVCT2, even though there is some evidence that SVCT1 can also play a role in the anti-cancer effect of vitamin C. Low vitamin C plasma levels in cancer patients has been linked to low SVCT2 expression (McCarty, 2013). SVCT2 is present in most metabolically active tissues and regulates vitamin C accumulation which may explain its involvement in carcinogenesis. Hong et al. (2013) suggests that SVCT2 is important for ascorbate-induced cancer cell death. Moreover, single nucleotide polymorphisms (SNPs) in the coding regions of SLC23A2 (i.e. the gene encoding SVCT2) have been linked to several types of cancers. For example, SLC23A2 rs6116568 and rs12479919 were correlated with

gastric cancer incidence and SLC23A2 rs1776948 increased the risk of follicular lymphoma by up to 80% (Duell et al., 2013, Skibola et al., 2008).

High SVCT2 expression, and therefore high vitamin C transport, has been associated with good prognosis in cancers, such as liver, pancreatic and urothelial cancers (Lv et al., 2018, Park et al., 2021). In colorectal cancer (CRC), however, high levels of SVCT2 were associated with poor survival; this is a result of SVCT2 being frequently mutated in CRC patients. More specifically, the mutant SVCT2 E264K is expressed in 58.5% of CRC patient tissues and has a high pathogenic score. This mutation was shown to inhibit the cell membrane trafficking of SVCT2 which in turn leads to decreased levels of ascorbic acid uptake and results in disease progression. Moreover, SVCT2 E264K causes upregulation of expression of proteins involved in cell proliferation compared to wild-type SVCT2. Knockdown of SVCT2 E264K suppresses CRC cell proliferation, migration and invasion. This mutation can therefore be a potential biomarker and therapeutic target for treating CRC (Park et al., 2021).

There have been several studies showing that SVCT2 plays a role in neurological disorders, such as epilepsy and Alzheimer's disease (Salazar et al., 2018, Teafatiller et al., 2022, Subramanian et al., 2021). In AD patients, the level of plasma ascorbic acid is significantly lower than in a healthy person. Treating an AD experimental model with ascorbic acid led to lower β -amyloid plaque burden, suggesting that controlling the level of ascorbic acid in the body can help against cognitive decline and AD. As mentioned before, SVCT2 is responsible for vitamin C uptake in the brain. Expression of SVCT2 was found to be lower in the hippocampus of AD patients. Calsyntenin-3 is a synaptogenic adhesion molecule that was identified as an SVCT2 accessory protein that helps regulate vitamin C uptake in the brain. Both SVCT2 and Calsyntenin-3 are downregulated in AD and are therefore thought to be responsible for the low levels of vitamin C in the AD brain (Subramanian et al., 2021).

Low levels of vitamin C also accelerates cognitive decline which increases epileptic seizure activity; therefore, it has been suggested that vitamin C supplementation can help epileptic patients (Teafatiller et al., 2022). Valproic acid, which is a class I histone deacetylase inhibitor, has been used to treat epilepsy and bipolar disorder. Teafatiller et al. (2022)

reports that valproic acid upregulates SVCT2 expression, in turn upregulating vitamin C uptake.

The role that SVCT2 plays in cancer, neurodegenerative diseases and epilepsy, along with its dual transporter/receptor function, makes understanding the structure and working mechanisms of SVCT2 important. The structure of SVCT2 has not yet been solved, however, several mutational and functional experiments have been performed to better understand the role that different residues play in SVCT2 trafficking and function. SVCT2 contains 650 amino acids and, like all other NAT proteins, contains 14 TMs with intracellular N- and C-termini, two extracellular N-linked glycosylation sites and a re-entrant loop between TMs 5 and 6 (Rivas et al., 2008). Asn188 and Asn196 are important for SVCT2 uptake function and expression, however they do not influence membrane targeting of SVCT2 (Subramanian et al., 2008). SVCT2 has 5 histidine residues (His109, His203, His206, His269 and His413) that are regulators of SVCT2 function, important for pH sensitivity, transport kinetics, conformational stability and localisation to the plasma membrane (Ormazabal et al., 2010).

The N- and C-termini of both SVCT1 and SVCT2 are important for proper cell-surface targeting, membrane incorporation and retention (Subramanian et al., 2004, Subramanian et al., 2010). Subramanian et al. (2010) reported that complete truncation of the N- and C-termini of SVCT2 resulted in the loss of correct membrane trafficking and therefore loss of function. According to their results, the N-terminus starting from about the 24th amino acid is important in cell surface expression. In the C-terminus, the removal of the last 8 or 23 amino acids did not affect cell surface expression but resulted in a decrease of ascorbic acid uptake. Moreover, deletion of amino acids 618-621 and 568-648 reduced association of SVCT2 with JAK2. SVCT2 seems to be more tolerant to truncations introduced to the N-terminus than the C-terminus (Subramanian et al., 2010, Han et al., 2021).

In summary, there are several studies on the functional roles of SVCT2; however, many questions regarding its mechanism of action remain unanswered. The structures of UapA and SVCT1 gave important insights on the arrangement of the proteins, their substrate binding sites and their transport mechanisms; this shows the importance of structural analysis in better understanding the function of a protein and therefore makes finding the

structure of SVCT2 important. SVCT1 and SVCT2 share a high sequence similarity of 66% and we would expect the structures of the two proteins to be similar. However, the fact that only SVCT2 is able to activate the JAK/STAT pathway gives us reasons to believe that there are structural differences between SVCT1 and SVCT2 that is worth exploring.

1.4. Working with membrane proteins

1.4.1. Membrane proteins are challenging to work with

Membrane proteins remain challenging to work with for structural biology. Determining the structures of membrane proteins is important in better understanding their mechanism of action. However, the number of high-resolution membrane protein structures is relatively low; less than 2% of solved structures in the Protein Data Bank (PDB) are of membrane proteins (www.rcsb.org and <https://blanco.biomol.uci.edu/mpstruc/>). Membrane proteins are difficult to work with at all stages, such as expression, solubilisation, purification and structural studies. Solving the structures of eukaryotic membrane proteins is even more difficult due to low expression levels of functional protein and their tendency to be unstable during solubilisation and purification (Tate, 2001, Tate, 2010).

Firstly, the design of the expression construct is important and needs to be well thought out. The construct needs to contain the elements necessary for transcription, translation and eventually protein purification. The tags chosen for identification or purification of the protein, such as a fluorescent tag or an affinity tag, need to have minimal effect on the function and structure of the protein. Tag cleavage sites can also be introduced to the expression construct to allow the removal of the tags if their presence is thought to influence the structure of the protein (Kesidis et al., 2020). The type of promoter used is also an important factor to consider when designing expression constructs as it can affect the level of protein expression. Promoters can be constitutive, always active in a cell, or inducible, which are activated on a stimulus. Most commonly used strong constitutive promoters for mammalian cell expression are derived from viral genomes, such as the human cytomegalovirus (CMV) and the simian virus 40 (SV40). Both CMV and SV40 are able

to infect various human cell types (Tate et al., 2003, Andréll and Tate, 2013). For structural studies, mutations could be introduced to the protein coding sequence to increase the stability of the protein or to prevent heterogeneity caused by post-translational modifications (Magnani et al., 2008, Serrano-Vega et al., 2008).

Choosing the correct expression system can also greatly influence the successful production of a stable membrane protein. Membrane proteins can be expressed in bacteria, such as *Escherichia coli* and *Lactococcus lactis*, and in eukaryotic systems: yeast, such as *Pichia pastoris* and *Saccharomyces cerevisiae*, insect cells, such as Sf9, and mammalian cell lines, including human embryonic kidney cells (HEK) (Dilworth et al., 2018, Massotte, 2003, Goehring et al., 2014). Expression in *E. coli* is an easy to use, quick and a relatively cheap system, however, eukaryotic membrane proteins usually need a eukaryotic expression system. The composition of the lipid bilayer varies among the different expression systems, therefore, since the type of lipids can affect the stability and functionality of the protein using a eukaryotic expression system for eukaryotic membrane proteins is best. Moreover, higher eukaryotic cell lines, such as mammalian cell lines, can provide post-translational machinery and the necessary chaperones needed for eukaryotic proteins (Kesidis et al., 2020). Even though yeast systems can provide post-translational modifications, there is a difference in these modifications between higher eukaryote and yeast systems (Jigami, 2008). For example, there is a difference in O-glycosylation patterns such as the linkage between the residues and the composition of the monosaccharides (Lommel and Strahl, 2009). There are insect cell lines that possess mammalian-like cell machineries however, the major downside of using insect cells to express mammalian proteins is that the insect lipid bilayer contains higher amounts of unsaturated lipids and lower amounts of cholesterol that can affect the stability and functionality of the mammalian membrane protein (Marheineke et al., 1998, Massotte, 2003).

The next factor to consider is membrane protein solubilisation. Expressing and purifying human membrane proteins in a stable state for structural studies is challenging because they show loss of activity and function outside of a phospholipid environment (Boggavarapu et al., 2013). Membrane proteins are amphiphilic meaning they have both hydrophobic and hydrophilic characteristics which makes them difficult to maintain in a soluble and stable

state outside a biological membrane. Membrane proteins need to be extracted from the membrane lipid bilayer and this needs to be replaced with an amphiphilic system. Choosing the correct amphiphilic system is essential in maintaining the protein in a stable and functional state. Some examples of amphiphilic systems that can be used are detergents, nanodiscs, amphipols and polymers (Lee et al., 2022).

1.4.2. Detergents are the most widely used membrane mimetic systems

Membrane proteins are embedded in the lipid bilayer, thus for many applications they must be extracted from the host cell membrane in a structurally and functionally relevant state. The most widely used amphiphilic systems for solubilisation of membrane proteins are detergents. Detergents have been successfully used in the structural, biophysical and functional analyses of membrane proteins. About 80% of the membrane protein structures obtained thus far are in detergents (Ehsan et al., 2021). Detergents consist of a polar head group, usually sugar based, and a hydrophobic non-polar tail group (Seddon et al., 2004). In an aqueous environment, detergents self-assemble into micelles in which the polar head groups are exposed on the surface and interact with the aqueous environment, and the hydrophobic tails are buried in the interior of the micelle minimising contact with water. In this micellar state, the detergents can disrupt the interactions that hold the membrane together and effectively shield the hydrophobic surface of the protein, forming protein-detergent complexes that are soluble in aqueous solution (Figure 1.12) (Lee et al., 2022, Anandan and Vrielink, 2016).

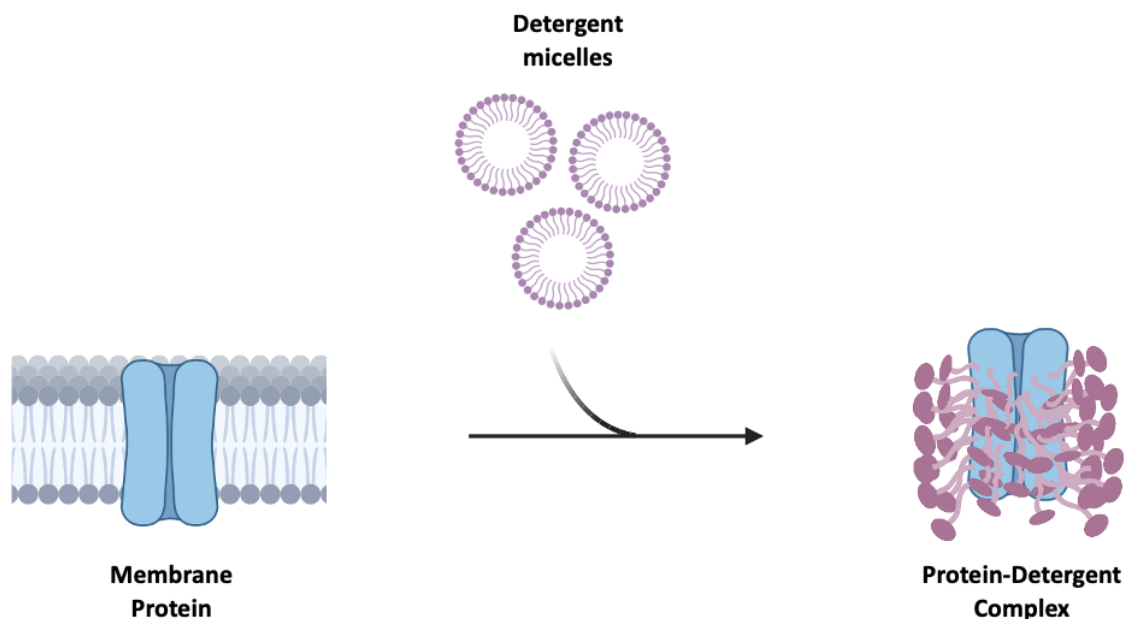


Figure 1.12. Detergents can be used to extract membrane proteins. Detergent molecules form micelles which can disrupt the cell membrane and extract the membrane protein. The micelles shield the hydrophobic surface of the protein from the aqueous environment.

A key parameter determining the formation of detergent micelles is the critical micelle concentration (CMC), specific to each detergent. The CMC is the minimal concentration required for the detergent molecules to self-assemble into micelles. The CMC varies with pH, ionic strength and temperature (Seddon et al., 2004). Another characteristic of a detergent is the aggregation number which is the number of molecules in the micellar particle (Tummino and Gafni, 1993). Both CMC and aggregation number influence the solubilisation ability of the detergent (Tschapek et al., 2015). Detergents tend to be used at concentrations above their CMC in order to act as effective solubilisers. The head group of the detergent strongly influences its interactions with proteins, whereas the length of the alkyl chain affects the detergent CMC and aggregation number. The longer the alkyl chain the gentler the detergent becomes and the lower the solubility of the detergent in aqueous solution (Privé, 2007).

Detergents are classified according to their structure and the main categories are: ionic detergents, zwitterionic detergents and non-ionic detergents (Figure 1.13) (Seddon et al., 2004). Ionic detergents contain positively or negatively charged headgroups. Common

examples of ionic detergents are sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB). These detergents are classified as harsh detergents because they disrupt the hydrophobic interactions within the protein, resulting in protein unfolding, as well as disrupting the interactions holding the membrane together. Moreover, SDS also disrupts the hydrogen bonds in the secondary structure of the protein. Therefore, due to its harsh characteristics, SDS is used for protein denaturation in sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis rather than for membrane protein extraction for downstream analyses (Bhuyan, 2010, Jafari et al., 2018).

Zwitterionic detergents have an overall neutral charge as a result of the presence of both positively and negatively charged groups in their headgroups. These detergents are less harsh than ionic detergents. Common examples of zwitterionic detergents are 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and lauryldimethylamine-N-oxide (LDAO) (Luche et al., 2003).

The most widely used detergent family for membrane proteins are non-ionic detergents. They are generally mild because they disrupt lipid-lipid and lipid-protein interactions rather than interfering with protein-protein interactions. Membrane proteins can therefore be solubilised in these detergents without affecting their structural features (Seddon et al., 2004). Non-ionic detergents can be classified into maltosides, such as n-Dodecyl- β -D-maltoside (DDM), and glucosides, such as n-octyl- β -D-glucoside (OG), based on their headgroups and the lengths of their alkyl chains (Kotov et al., 2019). Recent years have seen increases in the chemical variety and properties of detergents which in turn has opened up the possibility of working with a greater variety of membrane proteins.

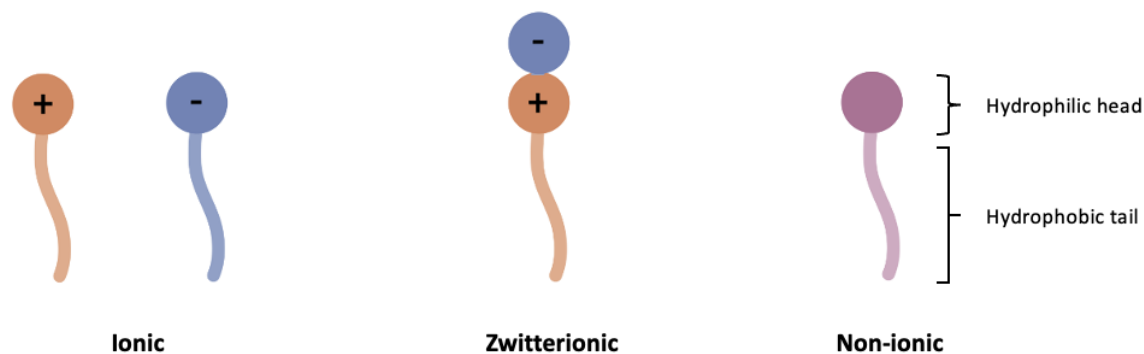


Figure 1.13. There are three main types of detergent families: ionic, zwitterionic and non-ionic. Detergents contain a hydrophilic head group and a hydrophobic tail. Ionic detergents can either have positively or negatively charged head groups. Zwitterionic detergents contain both positively and negatively charged head groups and have an overall neutral charge. Non-ionic detergents are mild detergents and are the most widely used for membrane proteins.

The disadvantage of detergents is that they can remove functionally important phospholipids and can perturb native protein conformations (Young, 2023). Since they poorly mimic the lateral pressure and thickness of the membrane bilayer, this can result in disruptions to the structure of the protein. Moreover, having to use detergents above their CMC can lead to protein complex dissociation, non-physiological oligomerisation and a reduction in protein activity (Kurauskas et al., 2018). Detergent micelles can also make protein structural characterisation more difficult by reducing contrast and increasing noise in techniques such as cryo-electron microscopy (Higgins et al., 2021). Therefore, other membrane mimetics such as nanodiscs and amphipols have been developed to address the issues caused by detergents.

1.4.3. Nanodiscs provide high protein structural stability

Nanodiscs are small, water-soluble discoidal lipid bilayers that are used to provide a native membrane environment and are generally better at preserving the native conformation of membrane proteins compared to detergents (Denisov and Sligar, 2016, Young, 2023). They are made up of modifiable phospholipid molecules and an amphipathic molecule that encircles the lipids to keep the whole complex in solution. Nanodiscs are 8-16 nm in diameter and are stabilised and maintained in a soluble state by two amphipathic helical

protein belts that wrap around the outside of the lipid molecules called membrane scaffold proteins (MSPs). MSPs are typically derived from the human apolipoprotein A-1 (Apo-A1) which is a protein that packages lipids in the bloodstream. With the addition of phospholipids, the MSPs self-assemble into nanodisc particles of homogenous size. Lipids that are most commonly used for nanodiscs are 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and dipalmitoyl-phosphatidylcholine (DPPC) (Denisov and Sligar, 2016, Li et al., 2019).

The length of the MSPs and the stoichiometry of the lipids determines the size of nanodiscs. In order to incorporate different sized membrane proteins into nanodiscs, the size and ratio of MSPs to membrane protein can be modified. MSPs of varying lengths have been developed to allow the customisation of the size of the nanodisc particle. Recently, covalently circularised MSPs have been used to form larger nanodiscs of up to 50 nm in diameter (Young, 2023). To optimise the yield of membrane protein incorporation, the molar stoichiometric ratio of lipid to scaffold protein is also an important factor to consider. Determining the optimal ratio of membrane protein to lipids and MSPs can be difficult to predict and several different mixtures may need to be screened.

Nanodiscs are able to provide higher protein structural stability compared to detergents because they are able to better replicate the natural lipid environment of membrane proteins (Marty, 2020). Nanodiscs are frequently used for structural studies of membrane proteins, such as electron microscopy (EM), because they help in preventing aggregation of the protein and improve sample quality (Denisov and Sligar, 2016). Solving the structures of nanodisc-embedded proteins has helped reveal binding sites for specific lipids which may play a role in protein function (Young, 2023).

Although nanodiscs have helped in stabilising membrane proteins for structural studies, a major downfall of nanodiscs is their inability to extract the protein from the membrane. Proteins need to be extracted by detergents and then reconstituted into nanodiscs by incubating the protein with detergent-solubilised phospholipids and MSPs (Figure 1.14). The detergent is stripped away by dialysis or by incubation with BioBeads and the nanodisc-protein complex is separated from empty nanodiscs by size-exclusion chromatography

(Ritchie et al., 2009). Moreover, determining the optimal ratio of membrane protein to lipids and MSPs can also be a time-consuming process. Alternatives to MSP-based nanodiscs have therefore been developed. Examples are i) Saposin-A based nanoparticles which are able to accommodate different sized membrane proteins (Frauenfeld et al., 2016), and ii) peptidiscs which are formed from an engineered version of an Apo-A1 derived nanodisc scaffold peptide and better allow membrane protein manipulation (Carlson et al., 2018). A modified version of peptidiscs, by the addition of a His tag, have been developed that allow the efficient extraction of membrane proteins from the cell membrane (Young et al., 2020).

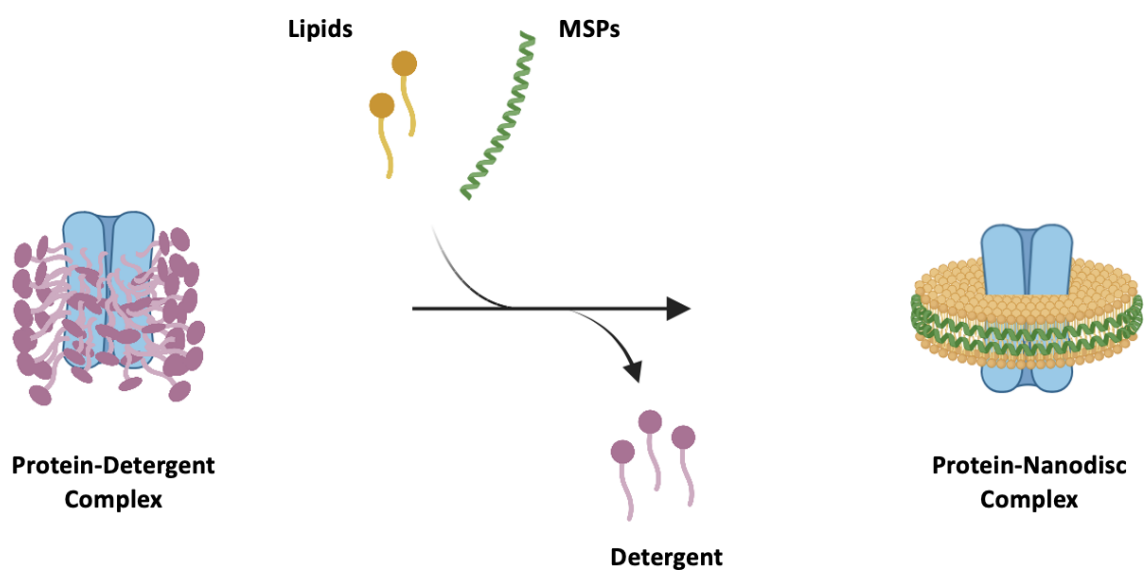


Figure 1.14. The reconstitution of protein-detergent complexes into nanodiscs. The membrane protein is incubated with lipids and membrane scaffold proteins (MSPs) in order to remove the detergent and reconstitute the protein into nanodiscs.

1.4.4. Amphipols form stable complexes with membrane proteins

Amphipols are short, flexible amphipathic polymers with a short hydrophilic backbone and hydrophobic chains that vary in number and size. They form more stable complexes with membrane proteins because they are able to interact with hydrophobic surfaces at several points along their length (Champeil et al., 2000). Amphipols have a slow dissociation rate from the membrane protein which increases the stability of the protein in solution but also makes amphipols inefficient at solubilising membranes (Calabrese et al., 2015). To determine

the optimal protein/amphipol ratio, a range of amphipol concentrations must be tested. Low amphipol concentration can lead to protein aggregation and high amphipol concentration can affect the stability of the protein, therefore finding the ideal amphipol concentration is important (Popot et al., 2011).

There are several types of amphipols, such as A8-35, phosphorylcholine-based amphipols (PC-APols), glucose-based nonionic amphipols (NAPols) and sulfonated amphipols (SAPols) (Popot et al., 2011). A8-35 is the most extensively studied amphipol and it is a polyacrylic acid polymer that has been modified with octylamine and isopropylamine side chains (Figure 1.16) (Higgins et al., 2021). Amphipols are more advantageous for the preparation of cryo-EM grids than detergents as they produce less background and more optimal ice thickness (Michon et al., 2023). The first high-resolution single-particle cryo-EM structure of a membrane protein, TRPV1, was solved using A8-35 (Higgins et al., 2021).

Most amphipols are unable to extract proteins from the membrane so the proteins need to be extracted by detergents and then exchanged into amphipols (Figure 1.15). Concentrated amphipols are added to detergent-protein complexes and during incubation the amphipols mix with the detergent in solution and at the protein's hydrophobic transmembrane surface. Biobeads or dialysis is then used to remove the detergent molecules. However, more recent amphipol derivatives, such as the cycloalkane amphipols, allowed the direct extraction of *E. coli* AcrB from the membrane for structural analysis (Higgins et al., 2021).

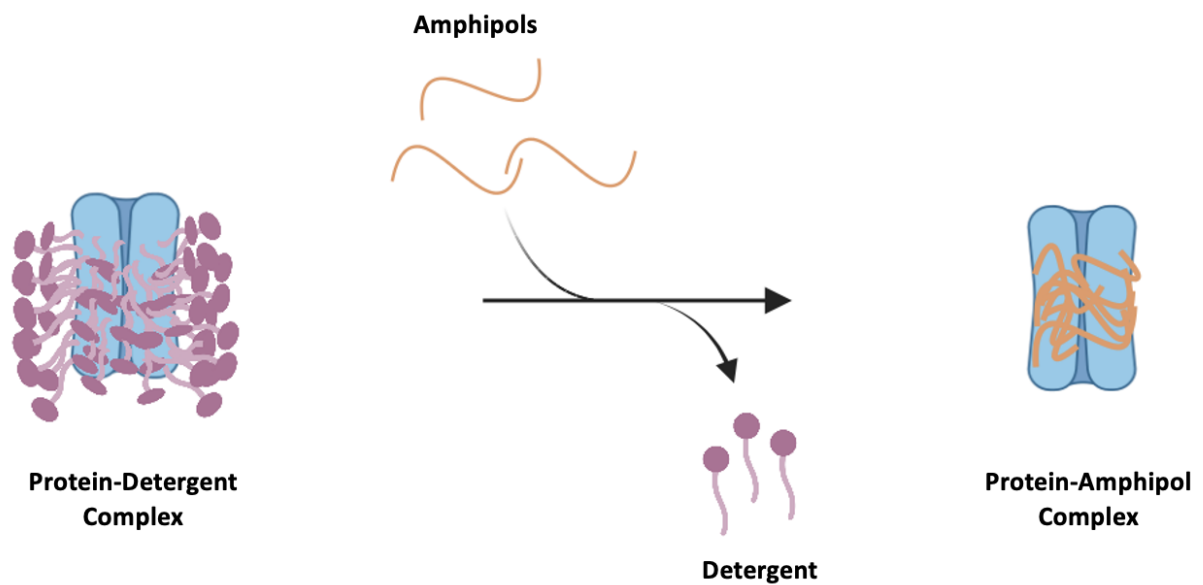


Figure 1.15. The reconstitution of protein-detergent complexes into amphipols. The membrane protein is incubated with amphipols in order to remove the detergent and exchange it for the amphipols.

1.4.5. Polymers provide a native membrane environment

The membrane mimetics discussed above all remove the native lipids that surround the membrane protein. To address this, polymers, such as styrene maleic acid (SMA), have been developed to allow the extraction of proteins from the membrane (Marconnet et al., 2020). SMAs consist of an alternating arrangement of hydrophobic styrene and hydrophilic maleic acid groups which can form disc-like arrangements in lipid membranes in a pH-dependent manner. SMAs are able to enclose the membrane protein with its associated native lipid molecules resulting in SMA-lipid particles (SMALPs) (Jamshad et al., 2011). SMAs therefore have an advantage over the aforementioned membrane mimetics because they provide the protein with a more physiological environment.

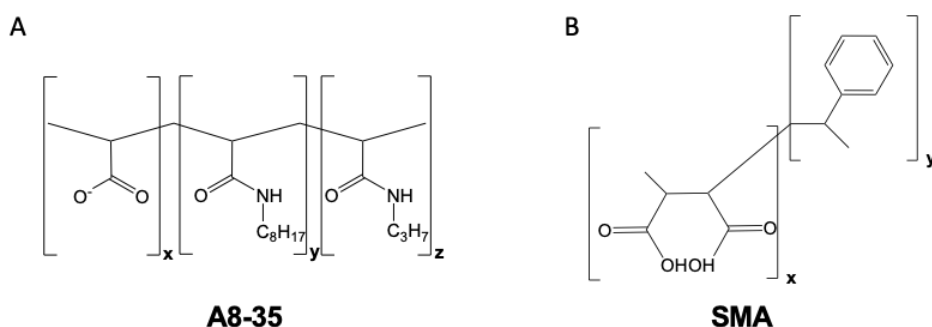


Figure 1.16. Chemical structures of membrane mimetics. A) Example of amphipols and B) example of polymers.

1.4.6. Electron microscopy (EM) can be used for membrane protein characterisation

Cryo-electron microscopy (cryo-EM) is a technique that uses transmission electron microscopy to determine the 3D structures of biological macromolecules. The macromolecules are frozen and fixed in ice to then obtain several 2D images. 3D reconstruction is then used to convert the 2D images into a 3D structure. High vacuum is needed for electron microscopy and the electrons can also cause radiation damage to the sample; the process of vitrification reduces radiation damage (Benjin and Ling, 2020).

Before using cryo-EM to image biomacromolecules, negative stain electron microscopy can be used for sample characterisation. Negative stain EM is a rapid and cheap way to screen for sample quality prior to sample preparation for cryo-EM (Chua et al., 2022). Sample purity, concentration and heterogeneity can be assessed using negative stain EM. In negative stain EM, the protein sample is adsorbed onto an EM grid and stained with an electron dense compound, usually uranyl acetate. The sample particles have low electron scattering power and therefore appear lighter than the surrounding stain which has a high electron scattering power and appears darker. Once sample quality is verified, the sample can be taken forward for cryo-EM analysis (Scarff et al., 2018).

Cryo-EM has several advantages over other structural biology techniques such as X-ray crystallography (XRC) and nuclear magnetic resonance (NMR). Cryo-EM does not require crystals, it can be used for large proteins and their complexes, it can maintain the functional

state of the protein and several conformational states can be obtained in a single experiment (Vénien-Bryan et al., 2017). Cryo-EM is especially advantageous for the structural characterisation of membrane proteins. This is because the need for membrane proteins to be embedded in a membrane mimetic makes them challenging for techniques like XRC (Chua et al., 2022). On the other hand, a disadvantage of cryo-EM is that it can be difficult to find small proteins under the electron microscope. Overall, cryo-EM has revolutionised protein structural characterisation, especially the characterisation of membrane proteins (Callaway, 2020).

1.5. Aims

Vitamin C is an essential micronutrient that plays a role in several cellular processes and is a potential therapeutic for treatment of various diseases. It is therefore important to understand the mechanism by which vitamin C is transported into cells by its transporters, SVCT1 and SVCT2. Structural information is important in understanding the working mechanisms of the SVCTs.

The first aim of this PhD project was to explore the molecular determinants that contribute to the making of a correctly functioning hSVCT1 protein. I accomplished this by investigating the effects of several mutations and N- and C-termini truncations on the expression, localisation and function of hSVCT1. By mutating substrate specificity residues of hSVCT1, its ability in transporting other substrates, such as xanthine, was investigated. As previously mentioned in section 1.3.2, dimerisation is essential for the function of UapA (Alguel et al., 2016), therefore the importance of dimerisation on hSVCT1 function was also investigated.

The second aim of this project was to structurally characterise hSVCT2. I expressed hSVCT2 in mammalian cells to obtain functioning and correctly trafficking protein. Extensive optimisation of hSVCT2 expression and purification were performed to improve sample quality before submission to structural characterisation using cryo-EM.

Membrane proteins must be extracted from the host cell membrane by the addition of detergents but choosing an optimal detergent to maintain the protein in a stable and functional state can be challenging. Given this difficulty, the final aim of this project was to investigate the ability of novel detergents in extracting and stabilising the membrane protein, UapA-G411V. This aim was carried out in collaboration with Professor Pil Seok Chae's group at Hanyang University, South Korea.

2. Investigations into the molecular mechanism of hSVCT1

The results presented in this chapter are included in the following publication:

WOUBSHETE, M., CHAN, L. I., DIALLINAS, G. & BYRNE, B. 2024a. The dimer of human SVCT1 is key for transport function. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1866, 184390.

2.1. Introduction

hSVCT1 is a low affinity and high-capacity transporter, highly specific for ascorbic acid and unable to transport nucleobases (Tsukaguchi et al., 1999b). The structure of mSVCT1 gave important insights into the structural arrangement of the protein, the ascorbic acid binding site and also highlighted some residues that are important in the substrate specificity of mSVCT1 (Wang et al., 2023). At the time that the work described in this chapter was conducted, only the mSVCT1 structure, along with the structures of the fungal and bacterial homologues, UapA and UraA respectively, were available. Based on these structures, I investigated the effect of introducing mutations to the ascorbic acid binding site and the substrate translocation channel of hSVCT1.

The xanthine binding site in UapA is ⁴⁰⁴TFAQ⁴⁰⁸ whereas in mSVCT1, the ascorbic acid binding site is ³⁸⁷TSSSP³⁹¹ (³⁸⁰TSSSP³⁸⁴ in hSVCT1); the TFAQ sequence in UapA is replaced with SSSP in SVCT1 (Alguet et al., 2016, Wang et al., 2023). As SVCT1 is unable to transport nucleobases, we wanted to investigate if changing the SSSP sequence in hSVCT1 to the TFAQ sequence of the UapA xanthine binding site would enable xanthine transport in hSVCT1. To this end, a double mutation replacing ³⁸¹SS³⁸² of hSVCT1 with TF of UapA, generating the double mutant ³⁸¹SS³⁸²TF, and a quadruple mutation replacing ³⁸¹SSSP³⁸⁴ with TFAQ, generating ³⁸¹SSSP³⁸⁴TFAQ, was introduced (Figure 2.1).

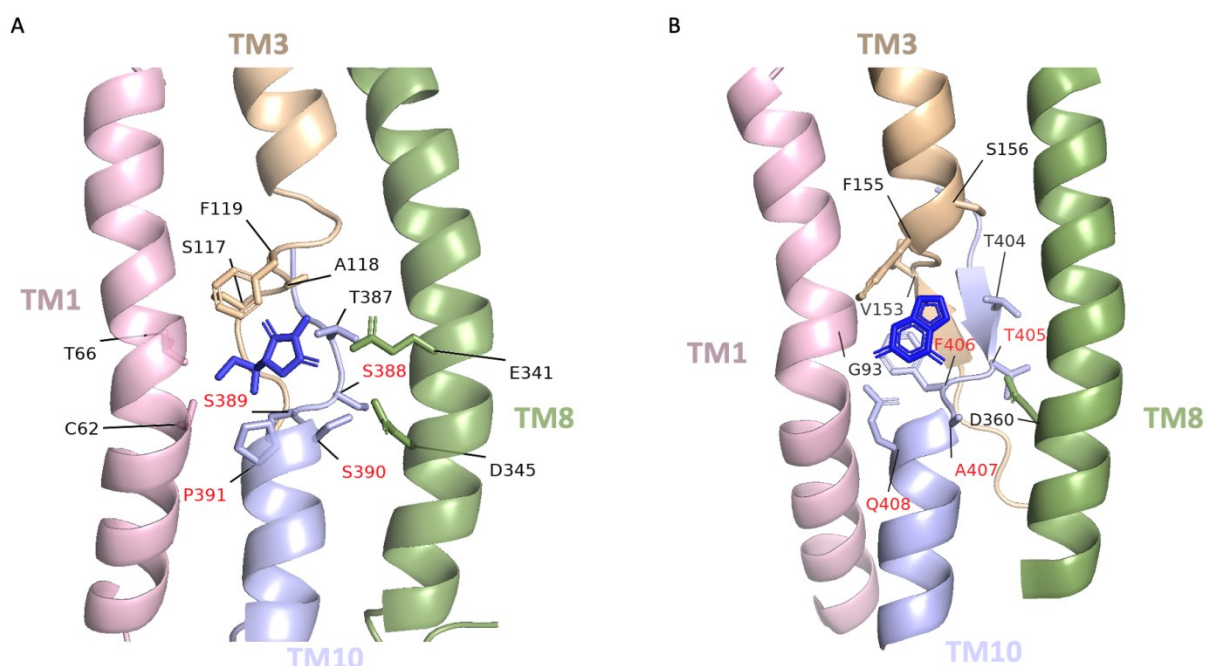


Figure 2.1. Substrate binding sites of (A) mouse SVCT1 and (B) UapA. A) Vitamin C binding site of mouse SVCT1. B) Xanthine binding site of UapA. Residues in the hSVCT1 binding site were mutated to residues highlighted in (B) to investigate if hSVCT1 can be used to transport xanthine.

In mSVCT1 and UapA, the residues that determine substrate transport are not only located in the substrate binding site but also in the extracellular substrate translocation channel (Wang et al., 2023, Kourkoulou et al., 2021, Kosti et al., 2010). Residues L72, F119, R213 and I446 in the extracellular translocation channel of mSVCT1 all influence the transport of ascorbic acid, with mutations of these individual residues to alanine resulting in decreased ascorbic acid transport (Wang et al., 2023). Here, the equivalent residues in hSVCT1, L65, F112, R206 and I439, were each mutated to alanine residues to investigate whether they have the same effect as in mSVCT1 (Figure 2.2). Interestingly, when mutating F155 and V463 in UapA, the equivalent residues to F112 and I439 respectively, F155A led to the transport of non-native substrates and V463I led to increased xanthine transport rate (Amillis et al., 2011, Kosti et al., 2010).

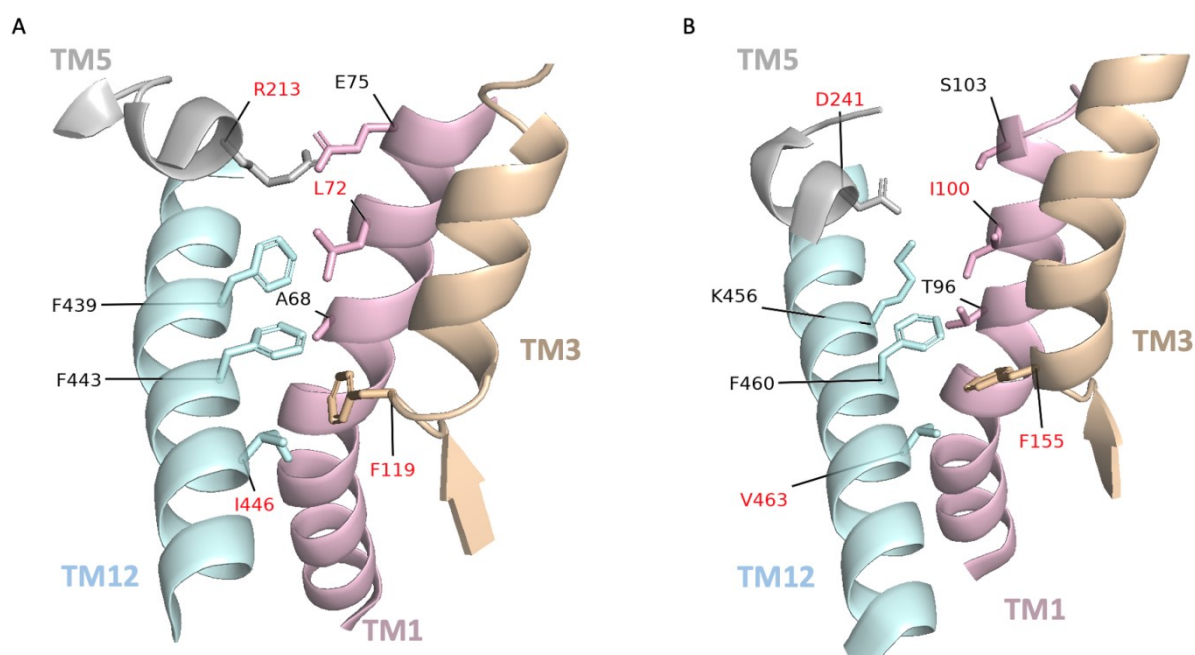


Figure 2.2. The extracellular interface of mSVCT1 and UapA are important in substrate transport. Extracellular interfaces of (A) mouse SVCT1 and (B) UapA. A) Residues highlighted in red were mutated in hSVCT1 to investigate their effect on substrate transport. B) Residues highlighted in red are the UapA equivalent to the residues highlighted in mSVCT1.

Residues in the intracellular translocation channel of UapA have also been found to play a role in substrate specificity. R481 in TM13 of the UapA gate domain extends into the intracellular substrate translocation channel of the opposite protomer. This residue is thought to act as the last checkpoint in ensuring correct substrate transport. Introducing a glycine mutation to R481 broadened the transport ability of UapA by allowing it to transport almost all purines and uracil (Kourkoulou et al., 2021). In order to explore the effects of the equivalent residue in hSVCT1, L458, on substrate profile, I mutated this residue to a glycine.

Overall, in this chapter, I investigated the residues that determine the specificity of hSVCT1 for ascorbic acid. This was accomplished by using results from the mSVCT1 structure, UapA structure and UapA functional analyses, to introduce a range of mutations to hSVCT1. Additionally, I explored the effect of truncating highly disordered N- and C-terminus regions of hSVCT1 on the expression, membrane trafficking and function of the protein.

In addition, I explored the role of dimerisation in hSVCT1. The importance of oligomerisation in the transport activity of several NAT transporters, such as UraA and UapA, has been reported (Yu et al., 2017, Alguel et al., 2016). In detergent, UraA exists in equilibrium between monomer and dimer. The UraA dimer showed ~70% higher activity than that of WT UraA (contains both monomeric and dimeric UraA). Likewise, mutational and functional analysis revealed that dimerisation is important for UapA function (Alguel et al., 2016, Pyle et al., 2018). Despite the similarities in the structures of UapA and SVCT1, it is not clear if dimerisation plays the same role in hSVCT1 as it does in UapA. Therefore, in this chapter, I investigated the importance of dimerisation on the correct membrane trafficking and function of hSVCT1.

2.2. Methods

2.2.1. Infusion cloning

2.2.1.1. PCR reactions

The full-length hSVCT1 gene was obtained from GeneArt. Polymerase chain reaction (PCR) primers for the addition of a His₈ tag and a Tobacco Etch Virus (TEV) cleavage site to a pcDNA3.1-yellow fluorescent protein (YFP) vector, for the amplification of full-length hSVCT1 and for the generation of N- and C-terminal truncations of hSVCT1 were designed and purchased from Eurofins Genomics (Supplementary Figure 1). A pcDNA3.1-YFP-His vector was constructed by cloning in a PCR amplified fragment containing a YFP and a C-terminal His₈ tag into a pcDNA3.1 vector and then hSVCT1 was cloned into the pcDNA3.1-YFP-His₈ vector using the steps described below.

PCR reactions were performed using a Phusion High-Fidelity kit (New England Biolabs). The PCR reactions contained 1x Phusion HF Buffer, 200 µM dNTPs, 1-unit Phusion DNA Polymerase, 1 µM forward primers, 1 µM reverse primer and 100 ng template DNA. 1 µg of the PCR products was digested using BamHI-HF (20 units) and 1x rCutSmart Buffer.

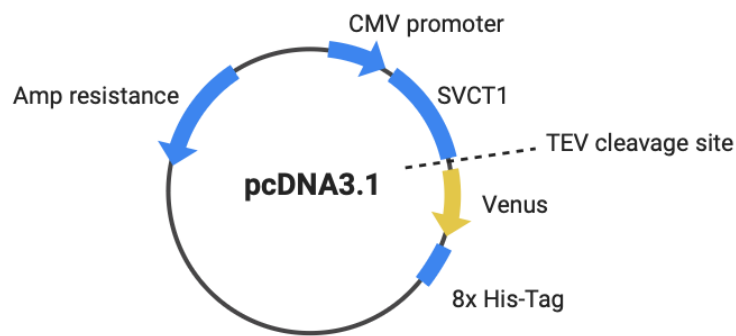


Figure 2.3. *hSVCT1 construct. The plasmid DNA contains a yellow fluorescent protein, Venus, and a C-terminal His-tag for determining expression levels and for protein purification respectively. The plasmid also contains an Ampicillin (Amp) resistant selection gene.*

2.2.1.2. Agarose gel electrophoresis

PCR products were analysed on 0.01% SYBR Safe stained, 1% w/v UltraPure Agarose, Tris-acetate-EDTA (TAE) gels. The PCR products (10 µl DNA + 2.5 µl loading buffer) were separated alongside the Invitrogen 1kb Plus DNA Ladder (3 µl ladder + 1 µl loading buffer) in TAE buffer (90V for 35 minutes). PCR products of the correct size were extracted using the Qiagen QIAquick Gel Extraction Kit.

2.2.1.3. Cloning

The In-Fusion HD Cloning kit (Takara Bio USA) was used for cloning reactions. The cloning reaction, containing 5x In-Fusion HD Enzyme Premix, purified PCR product and vector at an insert to vector ratio of 2:1 in a 20 µl total volume, was incubated for 15 minutes. A 2.5 µl aliquot of the In-Fusion reaction was transformed into *E. coli* NEB 10 competent cells and selected on Lysogeny broth (LB) agar plates containing ampicillin. Single colonies were inoculated in 10 ml LB media containing 100 µg/ml ampicillin and incubated at 37°C for 24 hours. The plasmid was extracted using the GeneJET Plasmid Miniprep Kit according to the manufacturer's instructions and successful cloning was verified by sequencing (Eurofins Genomics). To investigate the role of the N- and C-termini of hSVCT1, hSVCT1_{Δ1-10}, hSVCT1_{Δ1-20}, hSVCT1_{Δ1-26}, hSVCT1_{Δ529-598} and hSVCT1_{Δ567-598} constructs were generated as above.

2.2.2. hSVCT1 mutagenesis

Mutagenic primers for the introduction of mutations to the full length hSVCT1 were designed and purchased from Eurofins Genomics (Supplementary Figure 1). Mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis kit (Agilent). The PCR reactions contained PfuUltra High-Fidelity DNA polymerase (2.5U/ μ l), 1 μ l dNTPs, 10x reaction buffer, 125 ng forward primer, 125 ng reverse primer, 25 ng template DNA with 50 μ l total volume. The amplification products were digested with Dpn I restriction enzyme (10 U/ μ l) and transformed into XL1-Blue supercompetent cells. Transformants were validated by sequencing (Eurofins Genomics).

2.2.3. Cell culture and transfection

Human-derived embryonic kidney (HEK-293) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal bovine serum (FBS) and 0.5% Penicillin-Streptomycin in T25-cm² flasks. The cells were incubated at 37°C, 5% CO₂ and 95% O₂, and were passaged every 3-4 days.

Cells for transfection were plated in T25-cm² flasks and grown to 70-90% confluency. They were transfected with 10 μ g of plasmid DNA and 30 μ g Polyethylenimine (PEI) MAX (Polysciences) according to manufacturer's instructions and were assayed 48 hours later. Protein expression was determined by measuring fluorescence emission using a SpectraMax M2 (Molecular Devices) with an excitation wavelength of 485 nm and emission wavelength of 540 nm.

2.2.4. Confocal microscopy

In order to visualise correct trafficking of the protein to the membrane, transfected cells were fixed with 4% Paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 20 minutes at room temperature. Cells were stained using Fluoromount-G and visualised at 63x magnification on a Confocal 6 – Leica SP8 Inverted microscope using the 488 nm line from an Argon-Ion laser.

2.2.5. Ascorbic acid uptake assay

The Ascorbic Acid Assay Kit II (Sigma-Aldrich) or the Ascorbic Acid Assay Kit (Biological Samples) (Abcam) were used to measure the uptake of ascorbic acid by the different hSVCT1 constructs following the published protocol (Wang et al., 2023). Initially, HEK-293 cells were transfected as described above. After 48 hours, the media was changed to fresh media with 0.1 mM ascorbic acid and the cells incubated for 30 minutes at 37°C. After incubation, cells were harvested and homogenised in Ferric Reducing/Antioxidant and Ascorbic Acid (FRASC) lysis buffer. Insoluble material was removed by centrifuging at 13,000 x g for 10 min at 4°C. The lysates were added to a 96-well plate and incubated with a reaction mix, containing FRASC buffer, ascorbic acid probe and iron chloride solution, for 2-3 minutes. If ascorbic acid was present, then Fe^{3+} was reduced to Fe^{2+} which resulted in a colorimetric product. A blank control was set simultaneously for each sample, in which ascorbic oxidase was added to oxidise the ascorbic acid, and its reading was subtracted from the sample reading to obtain the corrected measurement. A series of ascorbic acid standards were also set up with 0 (blank), 2, 4, 6, 8 and 10 nmol/well to set up a standard curve. The absorbance at 593 nm was measured for each sample and the amount of ascorbic acid was determined from the standard curve.

To investigate if hSVCT1 could transport non-native substrates, a competition assay was carried out. 48 hours post-transfection, the media was changed to fresh media with 0.1 mM ascorbic acid and 0.4 mM xanthine or 3-benzyl xanthine, and the cells incubated for 30 minutes at 37°C. The ascorbic acid uptake assay was then carried out as above.

2.2.6. Xanthine uptake assay

The Xanthine/Hypoxanthine Assay Kit (Abcam) was used to measure the uptake of xanthine by several hSVCT1 constructs according to manufacturer's instructions. Initially, HEK-293 cells were transfected as described above. Wildtype UapA was used as a positive control. UapA was transformed into the *S. cerevisiae* FGY217 strain and positive transformants were selected on URA plates. Positive transformants were grown in URA media supplemented with 2% glucose at 30°C with shaking. The expression of UapA was induced by addition of

2% galactose. The UapA expressing cells and the hSVCT1 expressing cells (48 hours post-transfection) were incubated with 0.4 mM xanthine for 30 minutes at 37°C. After incubation, cells were harvested and homogenised in Assay buffer for 10 minutes. The lysates were added to a 96-well plate and incubated with a reaction mix, containing Assay buffer, Developer probe, Development Enzyme Mix and Developer solution, for 30 minutes. The xanthine is oxidised by the Development Enzyme to form an intermediate product that reacts with the Developer solution/Developer probe to form a colorimetric product. The absorbance at 570 nm was measured to determine the amount of xanthine present in each sample.

2.2.7. Statistical analysis

Significance was tested using a two-tailed unpaired *t*-test. All graphs were plotted and statistical analysis carried out via GraphPad Prism 6.0.

2.3. Results

2.3.1. The C-terminus of hSVCT1 is important for membrane trafficking

To investigate the effect of truncations on hSVCT1 protein expression, I expressed WT hSVCT1 and N- and C-terminus truncations of hSVCT1 as a fusion protein with a YFP in HEK-293 cells. YFP fluorescence was measured in Relative Fluorescence Units (RFU) to quantify expression levels of the different constructs (Figure 2.4).

There was an increase in fluorescence for all of the constructs compared to the negative control. All data are shown as a percentage of WT hSVCT1 expression. Compared to WT hSVCT1, all of the truncations showed similar levels of expression with no significant difference in fluorescence. This showed that truncating the N- and C-terminus of hSVCT1 had no effect on protein expression.

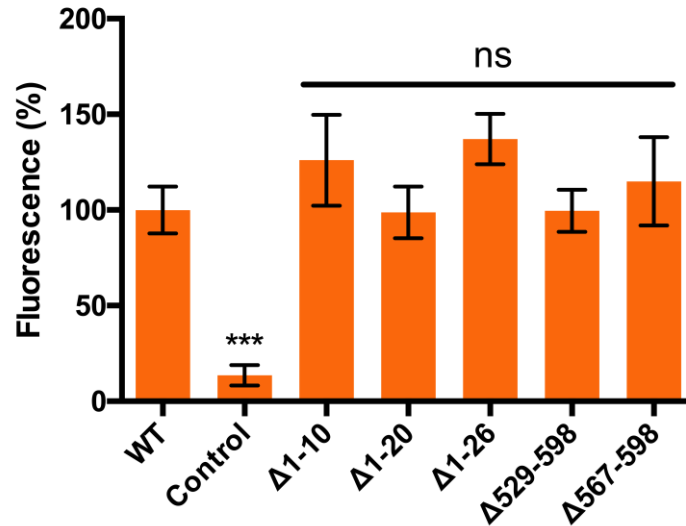


Figure 2.4. Introducing N- and C-terminus truncations to hSVCT1 had no effect on protein expression. To quantify protein expression, YFP fluorescence was measured in relative fluorescence units (RFU). All data are shown as a percentage of WT hSVCT1 expression. Data is shown as an average $\pm$ SEM ($n=4$). Student's t-test was performed between WT and truncations. *** = $p < 0.001$, ns = not significant.

Previously, Subramanian et al. (2004) reported that the N- and C-terminus of hSVCT1 are important for cell-surface targeting and membrane incorporation. Therefore, I investigated the effects of truncating the N- and C-terminus of SVCT1 on correct trafficking to the membrane, using confocal microscopy to visualise the transfected cells (Figure 2.5). WT hSVCT1 was present at the cell membrane but it could also be seen in intracellular vesicles. This result is consistent with results reported by Rashid et al. (2023), where hSVCT1 was observed in both the cell membrane and in intracellular structures. All the N-terminal truncations correctly traffic to the membrane, suggesting that the N-terminus does not play a role in membrane trafficking. Deleting the whole soluble C-terminal domain, $\Delta 529-598$, resulted in exclusively intracellularly localised protein. Moreover, deleting about half of the C-terminal domain, $\Delta 567-598$, showed a large amount of protein in intracellular structures with some fluorescence observed in the cell membrane. These results indicate that the C-terminal domain, and not the N-terminal domain, plays a key role in correct membrane trafficking of hSVCT1. These results were consistent with previous published results (Subramanian et al., 2004, Varma et al., 2009).

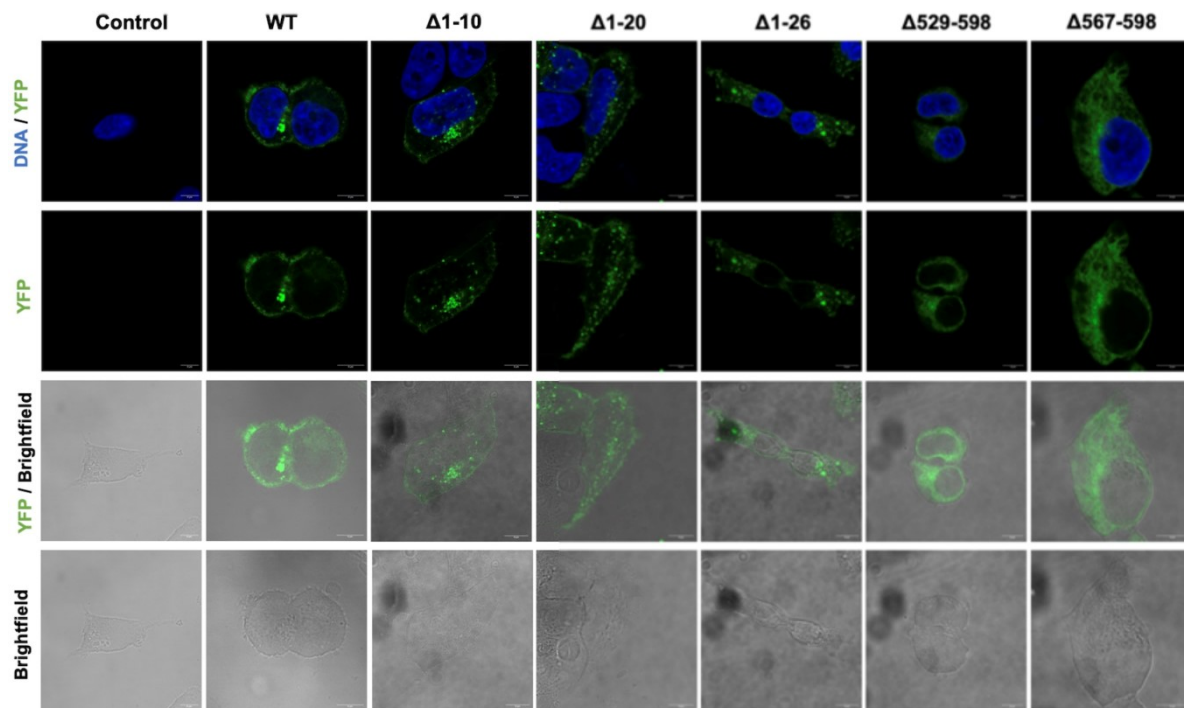


Figure 2.5. The C-terminus is important for hSVCT1 localisation. The first panel shows an overlay of YFP fluorescence and the DAPI stained nuclei, the second panel shows the YFP fluorescence only, the third panel shows an overlay of the YFP fluorescence and the brightfield image and the fourth panel shows the brightfield images only.

hSVCT1 transports ascorbic acid across the cell membrane. An ascorbic acid transport assay was therefore used to assess functionality of the hSVCT1 constructs (Figure 2.6). This assay was based on the production of a colorimetric product in the presence of ascorbic acid which was then quantified by measuring absorbance levels.

Cells expressing WT hSVCT1 showed uptake of ascorbic acid which proved that the expressed protein was functional. The N-terminus truncations also showed similar activity to the WT with no significant difference in ascorbic acid uptake, suggesting that the N-terminus does not play a role in the function of the protein. Consistent with the localisation data indicating little or no trafficking to the membrane, the hSVCT1 $_{\Delta 529-598}$ construct did not show uptake of ascorbic acid (Figure 2.6). The deletion of about half of the C-terminal domain, $\Delta 567-598$, also showed a significant ($p = 0.0017$) decrease in ascorbic acid uptake compared to WT hSVCT1, in line with the microscopy results indicating that membrane localisation was impaired, although not blocked for this construct. This suggested that the C-terminal domain

is important for membrane trafficking but maybe not as important for protein function as the transporter which did get to the membrane was transport active.

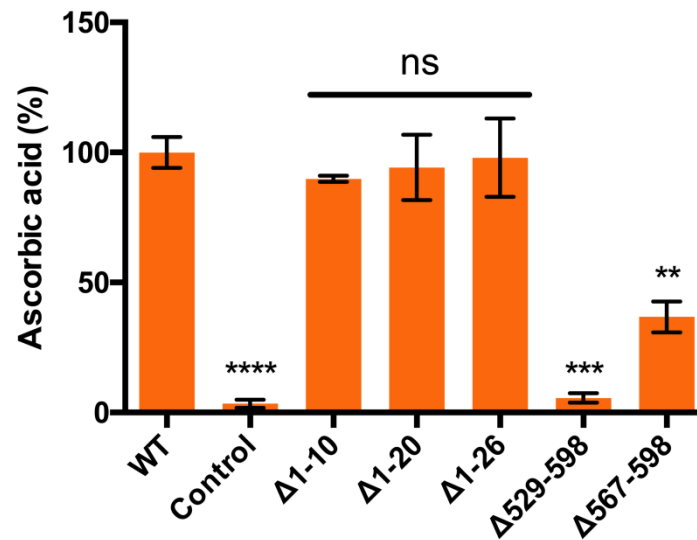


Figure 2.6. C-terminus truncations of hSVCT1 showed reduced transport activity. All data are shown as a percentage of WT hSVCT1 activity. The raw data was first normalised to the expression levels detected in each case so that uptake amount shows the levels of ascorbic acid being taken up per RFU of hSVCT1. Data is shown as an average $\pm$ SEM ($n = 3$). Student's *t*-test was performed between WT and truncations. ****, ***, ** = $p < 0.0001$, $p < 0.001$, $p < 0.01$ respectively, ns = not significant.

2.3.2. Translocation channel residues are important for hSVCT1 function

Several residues along the mSVCT1 and UapA translocation channel were shown to play a role in substrate transport (Wang et al., 2023, Kosti et al., 2010, Kourkoulou et al., 2021). Therefore, I mutated the equivalent residues in hSVCT1, L65, F112, R206, I439 in the extracellular translocation channel and L458 in the intracellular translocation channel to either alanine or glycine residues to investigate their effect on protein expression, membrane trafficking and substrate transport (Table 2.1).

Table 2.1. Summary of mutations introduced to hSVCT1 and their UapA equivalents.

hSVCT1 mutant	UapA equivalent	Location	Rationale
L65A	I100	TM1	Part of the extracellular substrate translocation channel in mSVCT1. L72A mutation in mSVCT1 reduced transport activity.
F112A	F155	TM3	Conserved residue of the substrate binding site in core domain. F155A substitution enlarges specificity of UapA for nucleobases. F119A mutation in mSVCT1 reduced transport activity.
R206A	D241	TM5	Residue of the gate domain at interface with core domain of UapA and mSVCT1. R213A mutation in mSVCT1 reduced transport activity.
I439A	V463	TM12	Gate domain residue at interface with core domain. V463I substitution enlarges specificity for nucleobases in UapA. I446A mutation in mSVCT1 reduced transport activity.
L458G	R481	TM13	Gate domain residue located at cytoplasmic end of the substrate translocation trajectory of the associated monomer. R481G substitution in UapA enlarges specificity for nucleobases

Compared to WT hSVCT1, there was no significant difference in expression for all the mutations (Figure 2.7). The significant difference shown here for F112A ($p = 0.0054$) is most likely not reflective of a true significant difference. Wang et al. (2023) reported that the mSVCT1 equivalents of L65A, F112A, R206A and I439A had no significant effect on expression levels; this is in broad agreement with the results reported here.

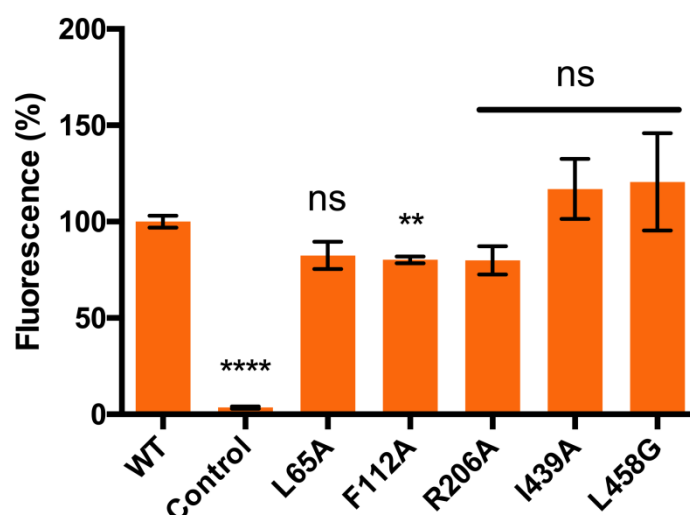


Figure 2.7. Translocation channel mutations had no effect on hSVCT1 expression. To quantify protein expression, YFP fluorescence was measured in relative fluorescence units (RFU). All data are shown as a percentage of WT hSVCT1 expression. Data is shown as an average $\pm$ SEM ($n=3$). Student's t-test was performed between WT and mutants. ****, $p < 0.0001$, $p < 0.01$ respectively, ns = not significant.

Microscopy results showed that all the constructs correctly traffic to the membrane (Figure 2.8). Some protein could be seen in intracellular structures in all the images, but most fluorescence can be observed in the cell membrane. This is consistent with the results reported by Wang et al. (2023), where all the equivalent mSVCT1 constructs investigated (L72A, F119A, R213A and I446A) were shown to correctly traffic to the membrane. The UapA equivalents to F112A and L458G, F155A and R481G, also showed correct trafficking to the membrane (Amillis et al., 2011, Kourkoulou et al., 2021).

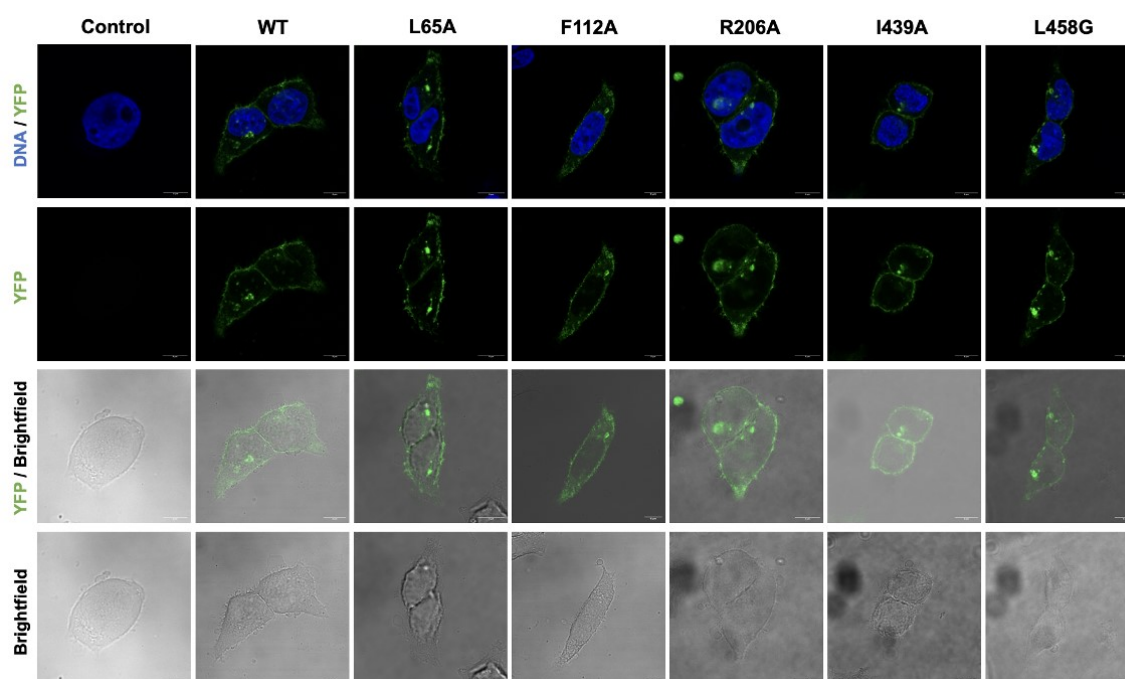


Figure 2.8. Translocation channel mutations had no effect on hSVCT1 localisation. The uppermost set of panels show an overlay of YFP fluorescence and the DAPI stained nuclei, the second set of panels show the YFP fluorescence only, the third set of panels shows an overlay of the YFP fluorescence and the brightfield images and the fourth set of panels show the brightfield images only.

Even though the translocation channel mutants did not have an effect on localisation of hSVCT1, they did have an effect on the function of the protein. The L65A, F112A and R206A mutants resulted in an almost total loss of ascorbic acid uptake ($p < 0.001$). I439A and L458G also showed significant decrease ($p < 0.01$ and $p < 0.05$ respectively) in ascorbic acid uptake compared to WT hSVCT1 but both exhibited much higher activity than the L65A, F112A and R206A mutants (Figure 2.9). These results showed that all 5 residues play a role in hSVCT1

substrate transport. These results were in broad agreement with mSVCT1 in which the alanine substitution of L72, F119, R213 and I446 substantially decreased the transport activity of mSVCT1 ($p < 0.0001$) (Wang et al., 2023). Mutations introduced to the UapA equivalents of I439 and L458, V463I and R481G respectively, resulted in slightly reduced xanthine uptake (Kosti et al., 2010, Kourkoulou et al., 2021). The UapA equivalent to F112A, F155A, also showed slightly reduced xanthine uptake (Alguel et al., 2016). This agreement with the UapA results emphasises the importance of these residues in substrate transport.

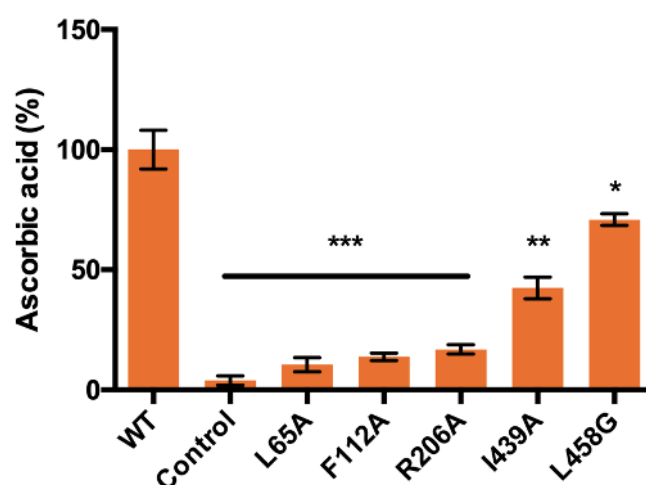


Figure 2.9. Translocation channel mutations reduced the transport activity of hSVCT1. All data are shown as a percentage of WT hSVCT1 activity. The raw data was first normalised to the expression levels detected in each case so that uptake amount shows the levels of ascorbic acid being taken up per RFU of hSVCT1. Data is shown as an average $\pm$ SEM ($n = 3$). Student's t-test was performed between WT and mutants. ***, **, * = $p < 0.001$, $p < 0.01$, $p < 0.05$ respectively.

2.3.3. Correct dimerisation is important for hSVCT1 function and membrane trafficking

Similar to UapA and UraA, SVCT1 forms a dimer (Alguel et al., 2016, Yu et al., 2017, Wang et al., 2023). Dimerisation is essential for the function of UapA; therefore, I investigated the importance of dimerisation on hSVCT1 function using a similar experiment to the one reported for UapA (Alguel et al., 2016). I carried out the co-expression of two copies of WT hSVCT1, by doubling the amount of DNA used during transfection; this roughly doubled the overall hSVCT1 activity ($\sim 200\%$ activity). Additionally, I co-expressed WT hSVCT1 with an

hSVCT1 mutant, hSVCT1-G387V, that exhibited undetectable ascorbic acid transport activity; hSVCT1-G387V (equivalent to UapA-G411V) is thought to lock the protein in the inward-facing conformation therefore inhibiting function. This co-expression results in the formation of four dimer populations: WT/WT, WT/Mutant, Mutant/WT and Mutant/Mutant (Figure 2.10). If dimerisation is important then only the WT/WT dimer would be transporting ascorbic acid, resulting in ~50% activity i.e. 25% activity of the WT/WT co-expression. However, if dimerisation is not important and each monomer of the dimer can function independently then the WT/WT, WT/Mutant and Mutant/WT dimers would be transporting ascorbic acid, so 100% activity would be observed i.e. 50% activity of the WT/WT co-expression. In addition, I co-expressed WT hSVCT1 with the non-trafficking construct, hSVCT1 Δ 529-598, to investigate the role of dimerisation in membrane trafficking.

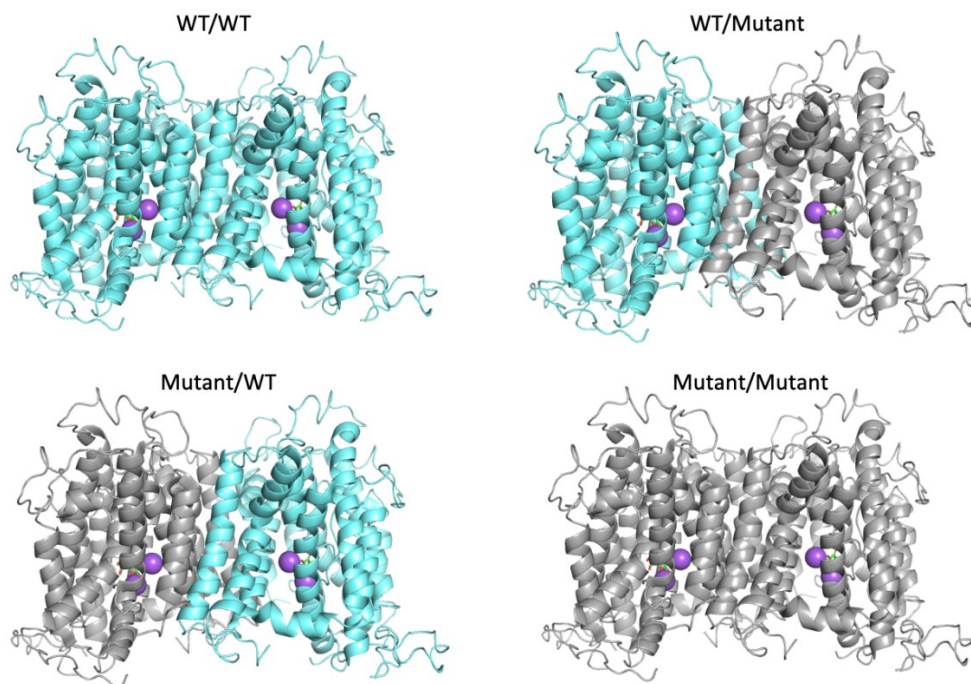


Figure 2.10. Schematic representation of the different variations of dimers that form when WT protein is co-expressed with a mutant protein.

Similar levels of expression were seen between WT hSVCT1, hSVCT1-G387V and hSVCT1 Δ 529-598. A significant increase in expression was observed when co-expressing two copies of WT hSVCT1 (WT/WT), WT/G387V and WT/ Δ 529-598 (Figure 2.11).

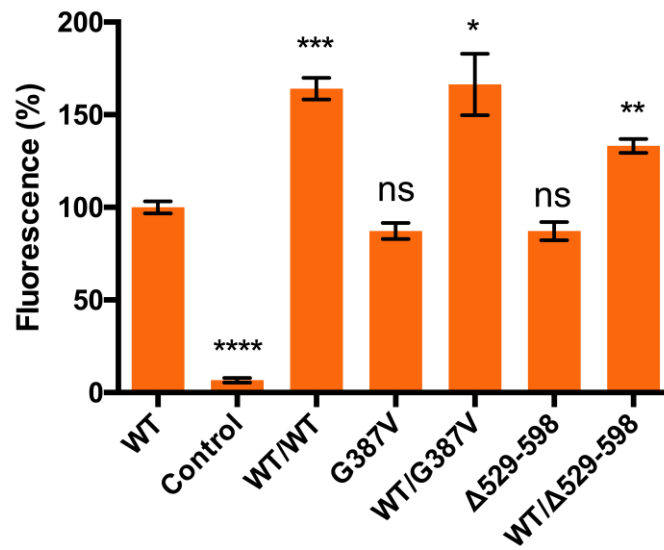


Figure 2.11. Co-expression of two copies of hSVCT1 increased protein expression. To quantify protein expression, YFP fluorescence was measured in relative fluorescence units (RFU). All data are shown as a percentage of WT hSVCT1 expression. Data is shown as an average $\pm$ SEM ($n=3$). Student's t-test was performed between WT and mutants/dimers. ****, ***, **, * = $p < 0.0001$, $p < 0.001$, $p < 0.01$, $p < 0.05$ respectively, ns = not significant.

The WT/WT co-expression doubled hSVCT1 ascorbic acid uptake activity compared to the expression of a single copy of WT hSVCT1 (Figure 2.12). hSVCT1-G387V and hSVCT1 $_{\Delta 529-598}$ showed no uptake of ascorbic acid. When co-expressing WT hSVCT1 and hSVCT1-G387V, ascorbic acid uptake decreased to about half that of the single amount of WT hSVCT1 i.e. a quarter of the WT/WT co-expression. This result showed that both protomers within the dimer need to be functional in order for the whole dimer to be functional, therefore proving that dimerisation is important for hSVCT1 function. In other words, the hSVCT1-G387V is a dominant negative mutant. Similar results were seen in the co-expression of WT hSVCT1 with the non-membrane trafficking construct hSVCT1 $_{\Delta 529-598}$. This showed that dimerisation is also important for the proper trafficking of hSVCT1 to the membrane.

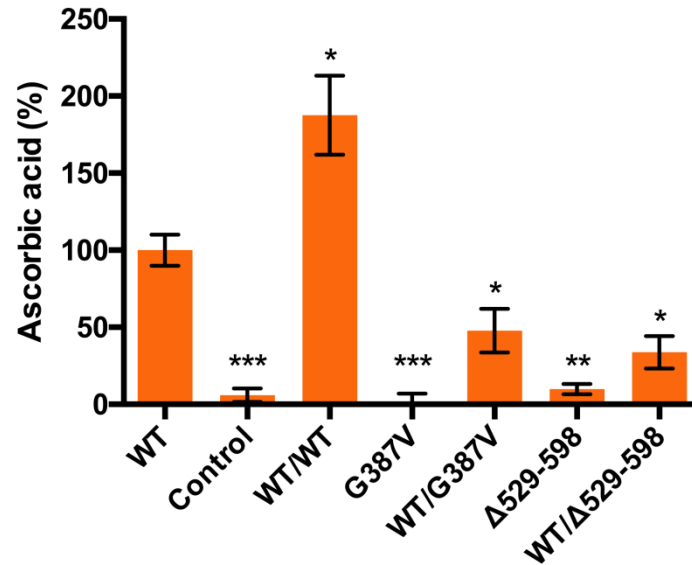


Figure 2.12. Co-expression of WT hSVCT1 with dominant negative mutants reduced hSVCT1 transport activity. All data are shown as a percentage of WT hSVCT1 activity. The raw data was first normalised to the expression levels detected in each case so that uptake amount shows the levels of ascorbic acid being taken up per RFU of hSVCT1. Data is shown as an average $\pm$ SEM ($n=3$). Student's t-test was performed between WT and mutants/dimers. ***, **, * = $p < 0.001$, $p < 0.01$, $p < 0.05$ respectively, ns = not significant.

The importance of dimerisation for correct membrane trafficking was further supported by confocal microscopy analysis (Figure 2.13). WT hSVCT1 showed correct membrane trafficking whereas deleting the C-terminus, hSVCT1 $_{\Delta 529-598}$, inhibited correct trafficking (see also section 2.3.1). The WT/ $\Delta 529-598$ co-expression showed the majority of protein in intracellular structures with some fluorescence observed in the cell membrane. This suggested that both protomers need to have the correct targeting signals for proper membrane trafficking. The G387V point mutation did not affect hSVCT1 membrane trafficking as the microscopy images showed fluorescence within the cell membrane. The WT/G387V co-expression also showed correct trafficking to the membrane proving that the decrease in ascorbic acid uptake was due to the dominant negative effect of the G387V mutant and not because of incorrect membrane trafficking.

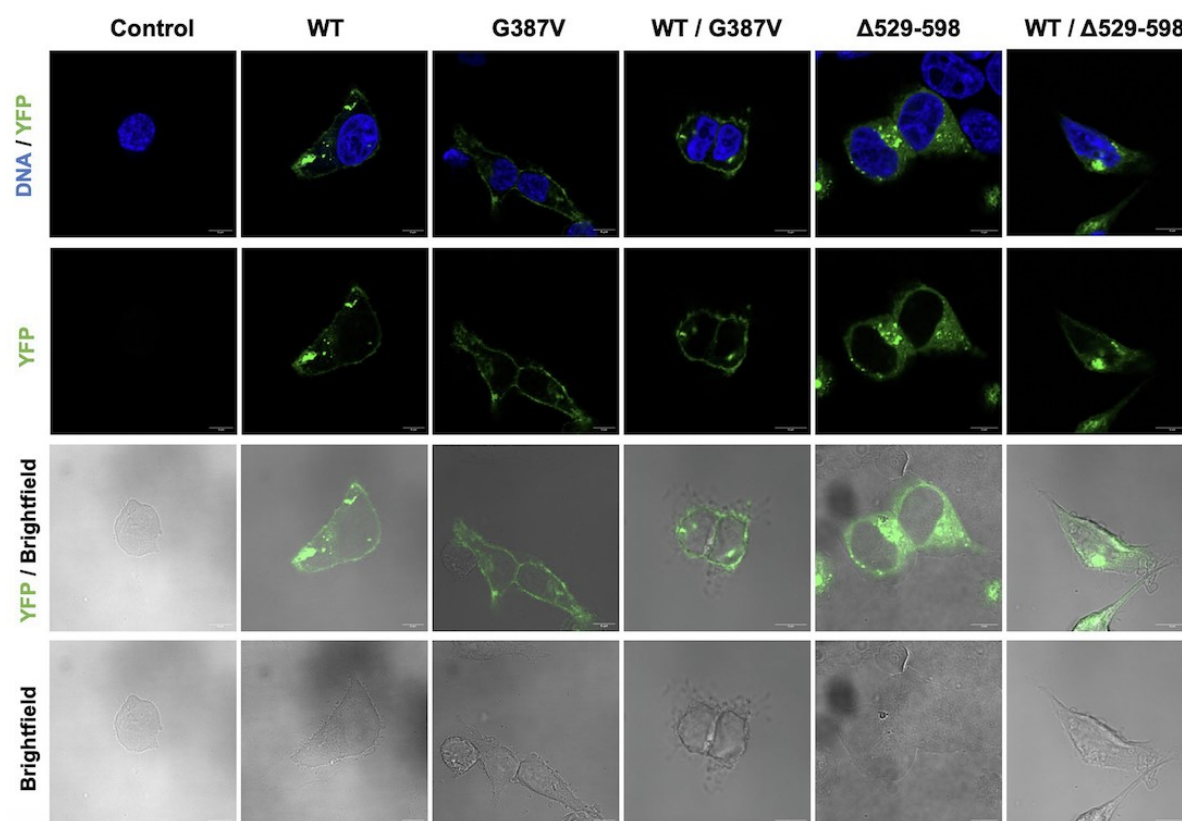


Figure 2.13. Co-expression of WT hSVCT1 with a non-trafficking hSVCT1 mutant reduced hSVCT1 localisation. The first panel shows an overlay of YFP fluorescence and the DAPI stained nuclei, the second panel shows the YFP fluorescence only, the third panel shows an overlay of the YFP fluorescence and the brightfield image and the fourth panel shows the brightfield images only.

2.3.4. Xanthine and 3-benzylxanthine do not inhibit the transport of ascorbic acid by hSVCT1

In UapA, mutating some residues along the translocation channel, V463I and R481G, broadened its ability to transport non-native substrates, such as hypoxanthine and adenine (Kosti et al., 2010, Kourkoulou et al., 2021). Therefore, similar mutations were introduced in the hSVCT1 equivalents of V463 and R481, I439 and L458 respectively, to investigate if this would allow the binding/transport of non-native substrates. A competition assay was carried out to investigate if the non-native substrates xanthine and 3-benzyl xanthine would inhibit the transport of ascorbic acid in hSVCT1-I439A and hSVCT1-L458G. hSVCT1-I439A and hSVCT1-L458G were chosen to carry out this competition assay because according to Figure 2.9, these two were the ones that showed significantly reduced but not abolished ascorbic

acid uptake activity compared to WT hSVCT1, therefore if there was competition by the non-native substrate then a decrease in ascorbic acid uptake activity would be observed.

According to the results (Figure 2.14), xanthine or 3-benzyl xanthine did not compete with the transport of ascorbic acid; there was no significant difference in ascorbic acid uptake with the addition of xanthine or 3-benzyl xanthine. These results suggested that unlike in UapA, these point mutations were unable to broaden the transport ability of hSVCT1.

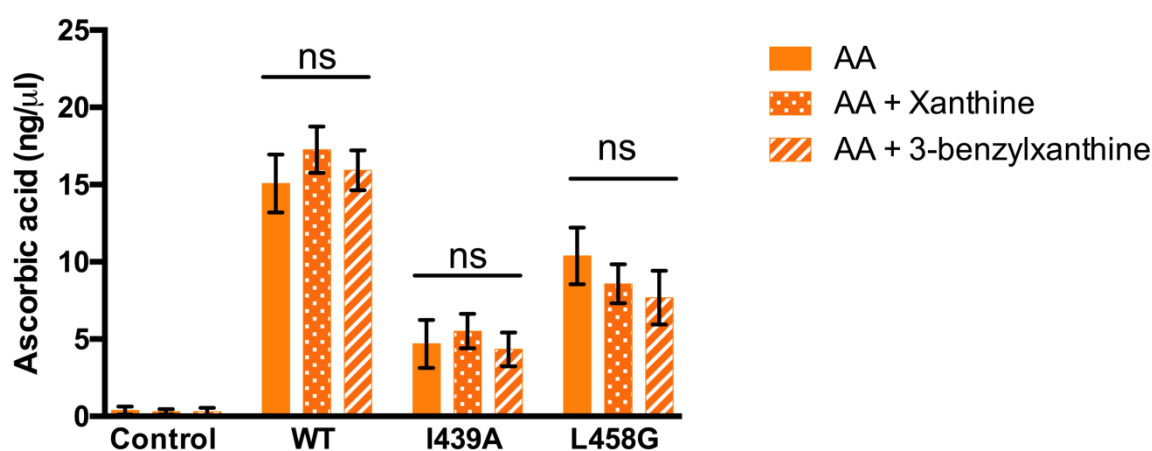


Figure 2.14. Xanthine and 3-benzylxanthine did not inhibit the transport of ascorbic acid (AA) by hSVCT1 constructs. Data is shown as an average $\pm$ SEM ($n=3-5$). Student's *t*-test was performed between ascorbic acid treated cells and xanthine or 3-benzyl xanthine treated cells. ns = not significant.

2.3.5. hSVCT1 is not able to transport non-native substrates

A member of our lab, Lok Chan, generated binding site mutants in which the ascorbic acid binding site of hSVCT1 (³⁸¹SSSP³⁸⁴) was mutated to the xanthine binding site of UapA (⁴⁰⁵TFAQ⁴⁰⁸). Additionally, residue T526 in UapA, located in the gate domain, had been shown to broaden the transport ability of UapA by allowing it to transport all purines and uracil at increased rates (Papageorgiou et al., 2008); therefore, the equivalent residue in hSVCT1, T499, was mutated to an alanine residue to investigate if it could broaden the

transport of ability of hSVCT1 and allow the transport of xanthine. SS381-382TF, SSSP381-384TFAQ and T499A were all shown to inhibit ascorbic acid uptake by Lok Chan (Woubshete et al., 2024a).

Even though the competition assay (section 2.3.4) could tell us if xanthine was competing for transport with ascorbic acid, for constructs that already have their transport ability completely inhibited (i.e. SS381-382TF, SSSP381-384TFAQ) it would be impossible to tell if xanthine is competing with ascorbic acid using a competition assay. Moreover, the competition assay can only suggest that xanthine is binding to the substrate binding site and it cannot verify if xanthine is actually being transported through the protein. Therefore, a xanthine uptake assay was performed to investigate if changing the hSVCT1 binding site to the UapA xanthine binding site would allow the transport of xanthine. Additionally, the xanthine transport ability of combining the binding site mutation with gate domain mutations (T499A and L458G), of residues that allowed UapA to transport non-native substrates, was investigated.

No significant increase in xanthine uptake activity was seen with the hSVCT1 constructs (Figure 2.15). WT UapA showed uptake of xanthine and was therefore used as a positive control. From the competition assay (section 2.3.4), it was already concluded that I439A and L458G did not allow xanthine transport (Figure 2.14), and this was further proved by the xanthine uptake assay (Figure 2.15). Changing the ascorbic acid binding site of hSVCT1 to the xanthine binding site of UapA, as well as combining this mutation with the gate domain mutations, did not allow the transport of xanthine. This result showed that hSVCT1 is highly specific for ascorbic acid and its specificity is not only dictated by its substrate binding site but also by other residues outside the binding site.

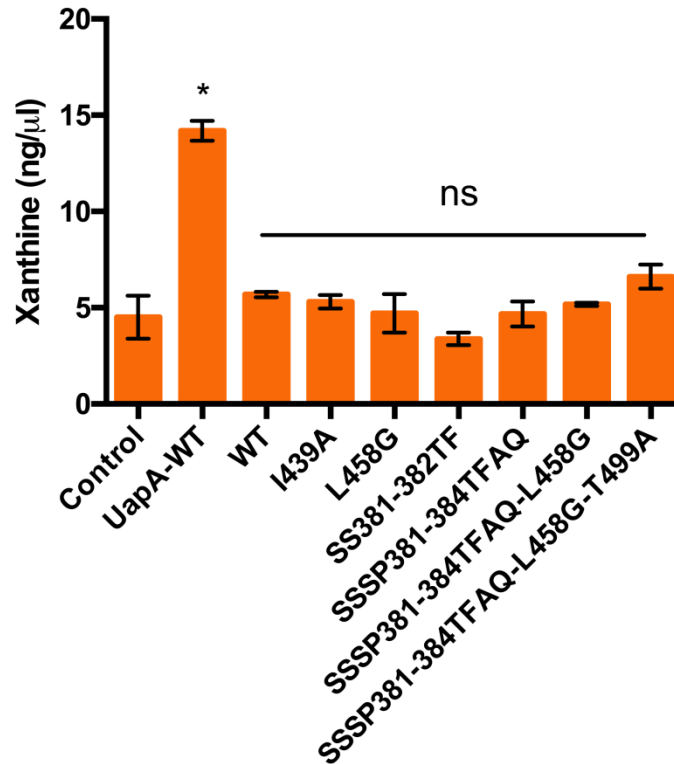


Figure 2.15. Xanthine uptake analysis of cells expressing either UapA or a range of different hSVCT1 constructs. hSVCT1 constructs were not able to transport xanthine. Data is shown as an average $\pm$ SEM ($n=2/3$). Student's *t*-test was performed between the different constructs and the mock-transfected control. * = $p < 0.05$, ns = not significant.

2.4. Discussion

This chapter describes the analysis of the structure-function relationship of hSVCT1. A range of amino acid residues, within the extracellular and intracellular translocation channels of hSVCT1, that play a role in substrate specificity and transport were identified. Moreover, the importance of the C-terminus in correct membrane trafficking was highlighted. Correct hSVCT1 dimerisation was also found to be essential for proper membrane trafficking and for substrate transport.

Mutating residues in the hSVCT1 extracellular translocation channel, L65A, F112A, R206A, I439A, and in the intracellular translocation channel, L458G, inhibited or reduced ascorbic acid uptake. This showed that substrate specificity could be determined by residues within the whole substrate translocation channel and not just the substrate binding site. These

results were in broad agreement with the results for mSVCT1, where the extracellular translocation channel mutations led to reduced ascorbic acid uptake (Wang et al., 2023). The UapA equivalents to F112A, I439A and L458G showed slightly reduced xanthine uptake and also allowed the transport of non-native substrates (Alguel et al., 2016, Kosti et al., 2010, Kourkoulou et al., 2021). Moreover, the UraA equivalent of F112A, F73A, also showed a slight reduction in uracil transport and allowed the transport of the non-native substrate, thymine (Lu et al., 2011). In general, SVCT1 seems to be more sensitive to mutations in the translocation channel than UapA, with the reduction of substrate uptake being more dramatic in SVCT1 than in UapA. This suggests that there might be differences in the working mechanisms of SVCT1 and UapA even though they have very similar structural architecture.

The SVCTs are highly specific for ascorbic acid. Substituting the hSVCT1 ascorbic acid binding site (³⁸¹SSSP³⁸⁴) to the UapA xanthine binding site (⁴⁰⁵TFAQ⁴⁰⁸) did not result in xanthine uptake (Figure 2.15). Similarly introducing residues found in SVCTs to UapA, such as from TFAQ to TFAP, TFSP, **SSSQ** or **TSSP** (changes in bold), did not result in ascorbic acid uptake (Kourkoulou et al., 2018). Nucleobases, such as xanthine, are planar low solubility molecules whereas ascorbic acid is non-planar and soluble. This suggests that the binding site and substrate translocation channel of nucleobase transporters vs ascorbic acid transporters are different in architecture in order to accommodate the difference in substrate. This could explain why the mutations explored in this chapter are insufficient in changing hSVCT1's substrate specificity. However, Toyoda et al. (2023) recently reported that SVCT1 also functions as a sodium-dependent urate transporter. This suggested that SVCT1 could have retained some aspects of its ancestral proteins which were most likely nucleobase transporters. Overall, further studies of co-evolving amino-acid combinations are needed to better understand substrate specificity within the NAT family.

The C-terminus of hSVCT1 is important for proper cell-surface targeting, membrane incorporation and retention (Subramanian et al., 2004, Varma et al., 2009). Results reported in this chapter showed that truncating the hSVCT1 N-terminus did not have an effect on the protein whereas truncating the C-terminus inhibited proper membrane trafficking and in turn affected protein function. These results are consistent with previous studies conducted on the N- and C-terminus of hSVCT1. Varma et al. (2009) reported that deleting up to 30

amino acids from the hSVCT1 N-terminus had no effect on localisation and did not affect transport activity. Subramanian et al. (2004) reported that hSVCT1 Δ 511-598, hSVCT1 Δ 538-598 and hSVCT1 Δ 554-598 exhibited loss of cell surface targeting and hSVCT1 Δ 567-598 led to an intermediate phenotype where some membrane targeting, and some intracellular fluorescence was observed. This is consistent with the results in this report as hSVCT1 Δ 529-598 showed no uptake of ascorbic acid and hSVCT1 Δ 567-598 showed reduced uptake of ascorbic acid compared to WT hSVCT1. On the other hand, Subramanian et al. (2004) reports that hSVCT1 Δ 562-598 and hSVCT1 Δ 566-598 showed reduced protein expression; this disagrees with the expression results presented in this chapter which showed that the N- and C-terminus truncations did not have an effect on protein expression (Figure 2.4). Overall, the importance of the C-terminus in membrane trafficking suggests that the C-terminus may contain a signalling sequence responsible for membrane trafficking. Subramanian et al. (2004) suggests that this signalling sequence could be ⁵⁶³PICPVFKGFS⁵⁷².

The use of dominant negative mutants showed that the dimerisation of two functional protomers is important in hSVCT1 function and membrane trafficking (Figures 2.12 and 2.13). There seems to be functional crosstalk between the protomers within the dimer that influences the activity of the protein as a whole. Both protomers also need to be competent for membrane trafficking to allow the trafficking of the dimer as a whole. This importance in dimerisation in the function and trafficking of UapA has also been previously reported (Alguel et al., 2016, Martzoukou et al., 2015). The endoplasmic reticulum (ER) is responsible for assembling transporters in eukaryotes. After this assembly, the transporters are trafficked through COPII vesicles to the membrane (Springer et al., 2014). The best explanation for why oligomers need to form in the ER is that oligomer formation requires the protomers to be correctly folded, therefore this acts as a quality control to allow only correctly folded protein to be trafficked to the membrane (Martzoukou et al., 2015). The loss of the C-terminus in hSVCT1 could result in misfolded protein and this could explain why hSVCT1 Δ 529-598 is a dominant negative mutant that blocks correct membrane trafficking of any associated WT monomer.

Subsequent to the work described in this chapter, the structure of hSVCT1 was solved and it confirmed that dimer formation is necessary for transport function in hSVCT1 (Kobayashi et al., 2024). In the inward-open state of hSVCT1, TMs 6, 12 and 13 form the dimer interface and TM13 is inserted into an intracellular cleft formed by TMs 6 and 12 of the other protomer. In the substrate-free occluded state, this TM13 is removed from the cleft and is instead exposed to the plasma membrane. When introducing mutations to the residues involved in dimer formation, the transport of vitamin C decreased but there was no effect on membrane trafficking of the protein.

The main difference between the hSVCT1 dimer reported and other NAT dimers is that in hSVCT1 the gate domain undergoes a rotational shift and opens the cytosolic side of the dimer interface between the dimers. Kobayashi et al. (2024) suggests that when transitioning from the substrate-bound occluded state to the inward-open state, the core domain moves against the gate domain as in an elevator-like mechanism. Once the substrate is released, the transition from the inward-open state to the substrate-free occluded state occurs by the gate domains undergoing a rotational shift. They suggest that it is the dimer interface reassembly that allows this motion of the gate domain which is why a correlation between dimer formation and transport activity is seen.

Overall, results presented in this chapter have given insight into the working mechanisms of hSVCT1. Further studies in this area are needed to better understand the molecular determinants of hSVCT1 substrate specificity. The role of lipids in dimer formation and function of the protein would also be an interesting area to explore. Additional protein structures, specifically an outward-facing hSVCT1 structure, can also help in understanding how hSVCT1 transports its substrate.

3. hSVCT2 optimisation for structural studies

3.1. Introduction

hSVCT2 is an ascorbic acid transporter implicated in several diseases, including cancers and neurodegenerative disorders (McCarty, 2013, Salazar et al., 2018), and as such is a potential drug target. Although there are several functional studies on hSVCT2 (Han et al., 2021, Hong et al., 2013, Salazar et al., 2023), the structure of the protein has not yet been solved. The protein structure would help in understanding the transport mechanism of the protein and facilitate rational drug design. In order to solve the structure of the protein, hSVCT2 has to first be expressed and purified in a stable and functional state.

To obtain protein for structural studies, I first successfully cloned WT hSVCT2 into a pcDNA3.1-YFP-His vector. The expression of this construct however was unsuccessful. Therefore, this chapter explains how several variations of the pOPIN vector, differing in the type of fluorescent tags, purification tags and cleavage sites contained, were tested to see if the change in vector could help with protein expression.

As discussed in section 1.3.3.2, the N- and C-terminus of hSVCT2 was shown to be important in correct membrane trafficking and function of the protein (Subramanian et al., 2010). The hSVCT2 N-terminus, starting from residue E24, was found to be important in membrane trafficking (Subramanian et al., 2010, Han et al., 2021). The removal of disordered N- and C-terminal regions can promote expression and increase stability of proteins (Oldfield et al., 2005). Therefore, I introduced an N-terminal truncation, $\Delta 1-23$, to help increase protein stability.

In mouse SVCT2 (mSVCT2), the residue Y626 (Y628 in hSVCT2) was found to be important in the activation of the JAK2/STAT2 pathway and also for vitamin C transport. A Y626F mutation in mSVCT2 decreased ascorbic acid uptake and inhibited JAK2 phosphorylation activation (Han et al., 2021). In this chapter, the effect of the hSVCT2 equivalent of Y626F, Y628F, on

the expression and function of the protein was investigated. This mutation was introduced for the possible future investigation of the JAK2/STAT2 pathway.

A number of attempts were required to obtain hSVCT2 expression. Once this was successful it was then necessary to optimise each of the steps used in protein purification. Obtaining pure and stable membrane proteins during purification can be influenced by several factors, such as type of membrane mimetic used, presence of endogenous/exogenous lipid and buffer composition (Gamage et al., 2023). In this chapter, the optimisation of protein purification conditions to obtain relatively pure and stable hSVCT2 for structural studies using cryo-EM is reported.

3.2. Methods

3.2.1. Cloning into pcDNA3.1-YFP-His vector

The hSVCT2-TEV gene, obtained as a synthetic gene (GeneArt), was cloned into the pcDNA3.1-YFP-His vector using methods described in section 2.2.1.

3.2.2. Mutagenesis

A Y628F mutation was introduced to hSVCT2 using methods described in section 2.2.2.

3.2.3. High throughput cloning

PCR reactions were performed using a PhusionTM Flash master mix from ThermoScientific (F-548L). The PCR reactions contained 0.6 μ M forward primers, 0.6 μ M reverse primers and 10 ng template DNA. Primers used were ones that allowed the cloning of hSVCT2 into different vectors and ones that also allowed the N-terminal truncation, Δ 1-23, of hSVCT2 (Supplementary Figure 2). The template used was WT hSVCT2 in pcDNA3.1 (section 3.2.1) or hSVCT2-Y628F in pcDNA3.1 (section 3.2.2). A 5 μ l aliquot of the PCR product was run on an E-gel with SYBR-safe from Invitrogen along with a HyperLadder 1kb molecular weight marker

(BioCat GmbH) to verify successful PCR amplification. The PCR products were digested with 5 µl of 5 units DpnI (10U/µl) in 1x CutSmart buffer from New England Biolabs.

The ClonExpress II One Stem Cloning Kit from Stratech was used for cloning reactions. The cloning reactions contained 2 µl of 5x CEII buffer, 1 µl Exnase II, 2 µl PCR product and 100 ng of linearised pOPIN vector with 10 µl total volume. After incubation at 37°C for 30 minutes, 2 µl of the cloning reactions were transformed into *E coli* TOP10 competent cells and selected on LB agar plates containing carbenicillin. Single colonies were inoculated in 10 ml LB media containing 100 µg/ml carbenicillin and incubated at 37°C for 12 hours. The plasmid was extracted using the Wizard SV96 purification system according to the manufacturer's instructions and successful cloning was verified by PCR screening. These experiments were performed with the help of James Birch from the Membrane Protein Laboratory (MPL) Group at Diamond Light Source funded by INSTRUCT.

3.2.4. Adherent HEK-293 cell culture and transfection

See section 2.2.3.

3.2.5. Expi-293 cell culture

Expi-293 cells were cultured in Expi293TM Expression Medium (Thermo Fisher Scientific) at 20 ml volume in 125 ml Erlenmeyer flasks. The cells were incubated at 37°C with a humidified atmosphere of 8% CO₂ on an orbital shaker at 125 RPM, and the cells were split every 3-4 days.

3.2.6. Micro-scale test purification

The following experiments were performed at the MPL funded by INSTRUCT.

3.2.6.1. Expression

Expi-293 cells for transfection were grown to $1.5\text{--}2 \times 10^6$ /ml and $\geq 95\%$ viability in 3 ml volume. Cells were transfected with 3 µg of plasmid DNA and 16 µg PEI MAX (Polysciences). After 24 hours, 5 mM valproic acid, 6.5 mM sodium propionate and 0.9% glucose were

added to the cells and temperature of the incubator decreased to 30°C. Cells were harvested 72 hours post-transfection.

3.2.6.2. Solubilisation and purification

Harvested cells were resuspended in 1 ml lysis buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 10 mM imidazole pH 7.5 and protease inhibitor cocktail tablet). The cells were lysed by sonication for 2 min 30 s (5 s on/ 10 s off) at 20% amplitude using a 500W sonicator. The membranes were solubilised by adding either 1% DDM + 0.1% CHS or 1% Fos-Choline-12 (FC-12) for 1 hour at 4°C. Unsolubilised material was removed by centrifugation for 30 minutes at 3500 x g and 4°C. The supernatant was incubated with TALON resin, suspended in lysis buffer, for 1 hour at 4°C. The resin was added to a 96-well filter plate (Crawford Scientific) and was washed with 500 µl wash buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 25 mM imidazole pH 7.5 and 0.03% DDM + 0.003% CHS or 0.03% FC-12). The protein was eluted with 50 µl elution buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 300 mM imidazole pH 7.5 and 0.03% DDM + 0.003% CHS or 0.03% FC-12). 10 µl sample was analysed by fluorescence size exclusion chromatography (FSEC) and 10 µl by SDS-PAGE as described in sections 3.2.11. and 3.2.12. respectively.

3.2.7. Screening for the optimal membrane mimetic system

The following experiments were performed at the MPL funded by INSTRUCT.

3.2.7.1. Expression

Expi-293 cells for transfection were grown to $1.5\text{--}2 \times 10^6/\text{ml}$ and $\geq 95\%$ viability in 30 ml volume. Cells were transfected with 30 µg of plasmid DNA and 160 µg PEI MAX (Polysciences) as described in section 3.2.6.1.

3.2.7.2. Membrane preparation

Harvested cells were resuspended in 70 ml buffer containing 1x PBS, 1 mM MgCl_2 and EDTA-free protease inhibitor cocktail tablet (SIGMAFAST™, Sigma-Aldrich). The cells were lysed under compression at 2x 15kPSI. Unbroken cells were pelleted (4°C, 10000 x g, 10 minutes)

and discarded. The membranes were centrifuged at 100000 x g for 1 hour at 4°C and resuspended in 12 ml buffer containing 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol and protease inhibitor cocktail tablet.

3.2.7.3. Detergent/polymer screen

Membrane samples, obtained from section 3.2.7.2, were aliquoted into a 96-deep-well block (900 µl each) and solubilised by adding the following detergents: 1% DDM, 1% DDM + 0.1% CHS, 1% LMNG, 1% LMNG + 0.1% CHS, 1% Digitonin, 1% GDN, 1% FC-12 or 2.5% SMA for 1 hour at 4°C. Unsolubilised material was pelleted (4°C, 3500 x g, 30 minutes) and discarded. Purification was carried out as described in section 3.2.6.2. using the respective detergents for the buffers. No detergent was added for the SMA sample. 40 µl sample was analysed by FSEC and 10 µl by SDS-PAGE as described in sections 3.2.11. and 3.2.12. respectively.

3.2.7.4. Amphipol screen

Membrane samples, obtained from section 3.2.7.2, were aliquoted into a 96-deep-well block (900 µl each) and solubilised by adding 1% DDM for 1 hour at 4°C. Unsolubilised material was pelleted (4°C, 3500 x g, 30 minutes) and discarded. The amphipols that were screened were Amphipol A18, Amphipol A8-35, NAPol and PMAL C12. The solubilised samples screened with Amphipol A18, Amphipol A8-35 and NAPol were purified using TALON resin and the sample with PMAL C12 was purified with Ni-NTA resin using the steps described in section 3.2.6.2, up to the point of washing the column. After all the wash steps, 1 mg/ml Amphipol A18, 1 mg/ml Amphipol A8-35, 0.1 mg/ml NAPol and 1 mg/ml PMAL C12 supplemented wash buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol and 25 mM imidazole pH 7.5) were added to individual resin samples containing bound hSVCT2 and incubated for 1 hour at 4°C. Biobeads were then added and incubated at 4°C overnight. The resin was washed twice with 1 ml wash buffer and the protein eluted with 50 µl elution buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol, 300 mM imidazole pH 7.5) (Figure 3.1). 40 µl sample was analysed by FSEC and 10 µl by SDS-PAGE as described in sections 3.2.11. and 3.2.12. respectively.

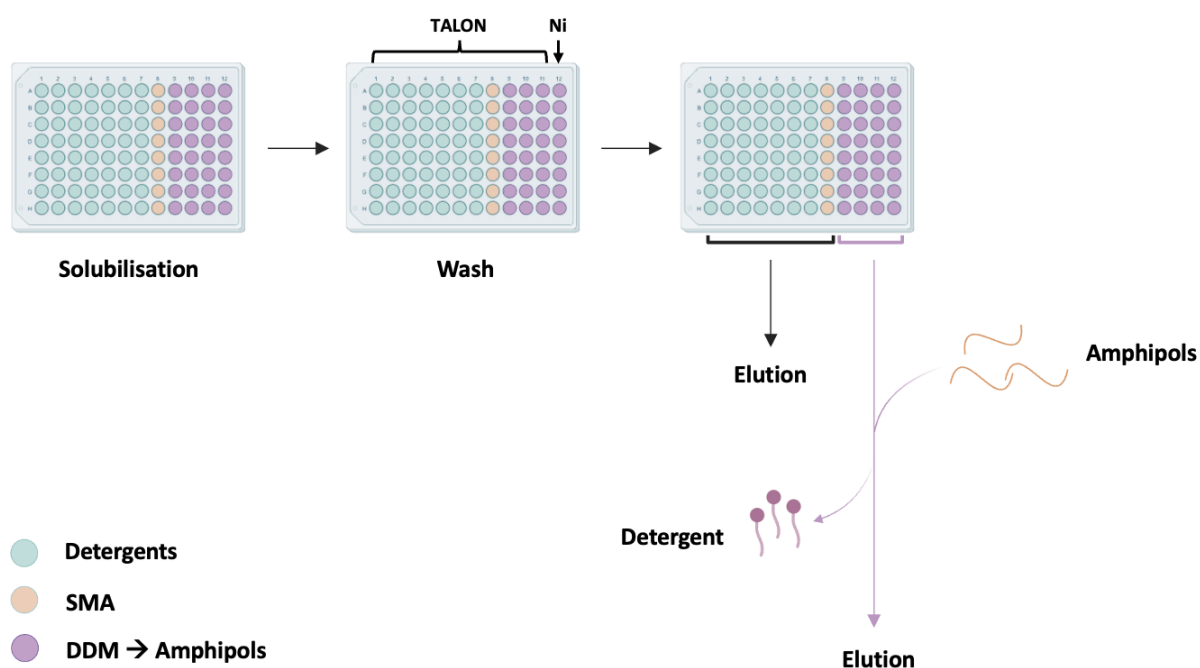


Figure 3.1. Screening for the optimal membrane mimetic system. Membrane samples were solubilised in different detergents (green and purple) or SMA (orange). Samples were purified using TALON or Ni resin. After all the wash steps, the protein samples purified in detergents (green) and SMA (orange) were eluted. The protein samples purified in DDM (purple) were incubated with amphipols and biobeads to exchange the DDM for the different amphipols and then eluted. Column 1 = DDM, 2 = DDM + CHS, 3 = LMNG, 4 = LMNG + CHS, 5 = Digitonin, 6 = GDN, 7 = FC-12, 8 = SMA, 9 = DDM → Amphipol A18, 10 = DDM → Amphipol A8-35, 11 = DDM → NAPol and 12 = DDM → PMAL C12.

3.2.8. His-tag purifications

3.2.8.1. Expression

Expi-293 cells for transfection were grown to 1.5×10^6 /ml and $\geq 95\%$ viability in 100 ml media in a 1 L flask. The cells were transfected with 100 μ g of plasmid DNA and 533 μ g PEI MAX (Polysciences). The amounts of DNA and PEI used were adjusted for larger culture volumes accordingly. 24 hours post-transfection, expression enhancers, valproic acid and sodium butyrate were added to final concentrations of 5 mM and 10 mM respectively and cells harvested 48 hours post-transfection by centrifugation (4°C, 700 x g, 20 minutes).

3.2.8.2. Membrane preparation

See section 3.2.7.2.

3.2.8.3. Solubilisation and purification

Membranes were thawed and complete protease inhibitor was added (1 tablet per 50 ml). The membranes were solubilised using gentle stirring for 1 hour at 4°C in 100 ml solubilisation buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol, 1% DDM, +/- 10 mM imidazole depending on experiment). Unsolubilised material was discarded by centrifugation (1 hour, 100000 x g, 4°C). The supernatant was loaded onto a 1 ml HisTrap HP column that had been equilibrated with Buffer A (50 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol, 25 mM imidazole, 0.03% DDM) and the column connected to the AKTA Pure protein purification system. The column was washed with 10 ml Buffer A then with 10 ml of 5% Buffer B (50 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol, 500 mM imidazole, 0.03% DDM). Protein was eluted with 15 ml of Buffer B applied over a linear gradient of 5% to 100%. Based on the chromatogram, SDS-PAGE gel electrophoresis was performed on those fractions appearing to contain protein (see section 3.2.12.). In order to cleave the thermostabilised green fluorescent protein (TGP) and His-tag, TEV protease was added at a molar ratio of 1:1 between TEV and TGP (only performed for the first purification). The mixture was dialysed with 2000 ml dialysis buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol, 0.03% DDM) in 12kDa MWCO dialysis tubing for 16 hours. Aggregates were pelleted (3000 x g, 4°C, 5 minutes) and the supernatant was filtered through a 25 mm, 0.2 mm low protein binding syringe filter. The sample was loaded onto a 1 ml HisTrap column that had been equilibrated with Buffer A. SDS-PAGE analysis was used to check for efficient cleavage. The flow through containing untagged hSVCT2 was concentrated to 0.5 ml using 100 kDa MWCO concentrators and injected onto a Superdex 200 Increase 10/300 GL column that had been equilibrated with size-exclusion chromatography (SEC) buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 2.5% glycerol, 0.03% DDM).

3.2.9. Strep-tag purifications

3.2.9.1. Expression

Expi-293 cells for transfection were grown to $1.5\text{--}2 \times 10^6/\text{ml}$ and $\geq 95\%$ viability in 200 ml media in a 1 L flask. The cells were transfected with 200 μg of plasmid DNA and 1066 μg PEI MAX (Polysciences). The amounts of DNA and PEI used were adjusted for larger culture volumes accordingly. 24 hours post-transfection, either 5 mM valproic acid only or with the addition of 10 mM sodium butyrate were added to cells. Cells were harvested 48 hours post-transfection by centrifugation (4°C , 700 x g, 20 minutes) and frozen at -70°C until further use.

3.2.9.2. Membrane preparation

Membranes were prepared using methods described in section 3.2.7.2. and buffers listed in Table 3.1.

3.2.9.3. Solubilisation and purification

See Table 3.1 for all buffer details.

Solubilisation was carried out as described in section 3.2.8.3. Affinity chromatography was carried out in one of two ways: i) The soluble supernatant was loaded onto a 1 ml StrepTrap HP column that had been equilibrated with Buffer A and the column was connected to the AKTA Pure. The column was washed with 20 ml Buffer A and protein was eluted with 10 ml of Buffer B applied over a linear gradient of 5% to 100%. Based on the chromatogram, SDS-PAGE gel electrophoresis was performed (see section 3.2.12). ii) The soluble supernatant was incubated with Strep-Tactin® XT 4Flow® resin equilibrated in Buffer A. The resin was added to a gravity column and washed 4 or 5 times with 1x column volume (CV) of Buffer A and the protein was eluted 3-7 times with 0.5 CV elution buffer. SDS-PAGE gel electrophoresis was then performed on all samples.

In both cases, protein samples were pooled and concentrated using 100kDa MWCO concentrators. The concentrated sample was injected onto a Superdex 200 Increase 10/300 GL column that had been equilibrated with SEC buffer.

Table 3.1. Summary of hSVCT2 Strep-tag purification buffer conditions. All changes made from the preceding method are highlighted in red.

	Culture (ml)	Cell Resuspension Buffer	Membrane Resuspension Buffer	Solubilisation Buffer	Buffer A	Buffer B	SEC Buffer
1	200	50mM HEPES pH 7.8, 1mM MgCl ₂ , Protease inhibitor	50mM HEPES pH 7.8, 300mM NaCl, 5% glycerol, Protease inhibitor	50mM HEPES pH 7.8, 300mM NaCl, 5% glycerol, 1% DDM	50mM HEPES pH 7.8, 300mM NaCl, 5% glycerol, 0.03% DDM, 5mM Desthiobiotin	50mM HEPES pH 7.8, 300mM NaCl, 5% glycerol, 0.03% DDM, 5mM Desthiobiotin	-
2	200	50mM HEPES pH 7.8, 1mM MgCl ₂ , Protease inhibitor	50mM HEPES pH 7.8, 300mM NaCl, 5% glycerol, Protease inhibitor	50mM HEPES pH 7.8, 300mM NaCl, 5% glycerol, 1% DDM, 1mM TCEP	50mM HEPES pH 7.8, 300mM NaCl, 5% glycerol, 0.03% DDM, 1mM TCEP, 50mM Biotin	50mM HEPES pH 7.8, 300mM NaCl, 5% glycerol, 0.03% DDM, 1mM TCEP, 50mM Biotin	25mM HEPES pH 7.8, 300mM NaCl, 2.5% glycerol, 0.03% DDM, 0.6mM TCEP
3	200	50mM HEPES pH 7.8, 1mM MgCl ₂ , Protease inhibitor, 2mM TCEP, 500mM Trehalose	50mM HEPES pH 7.8, Protease inhibitor, 500mM NaCl, 2mM TCEP, 500mM Trehalose, (Removed glycerol)	50mM HEPES pH 7.8, 5% glycerol, 500mM NaCl, 1% LMING, 0.1% CHS, 20mM glutamic acid, 2mM TCEP	50mM HEPES pH 7.8, 5% glycerol, 500mM NaCl, 0.01% LMING, 0.001% CHS, 20mM glutamic acid, 2mM TCEP	50mM HEPES pH 7.8, 5% glycerol, 50mM Biotin, 500mM NaCl, 0.01% LMING, 0.001% CHS, 2% glycerol, 0.01% LMING, 20mM glutamic acid, 2mM TCEP	25mM HEPES pH 7.8, 0.6mM TCEP, 500mM NaCl, 2% glycerol, 0.01% LMING, 0.001% CHS
4	400	50mM HEPES pH 7.8, 1mM MgCl ₂ , Protease inhibitor, 2mM TCEP, 500mM Trehalose	50mM HEPES pH 7.8, Protease inhibitor, 500mM NaCl, 2mM TCEP, 500mM Trehalose	50mM HEPES pH 7.8, 5% glycerol, 500mM NaCl, 1% LMING, 0.1% CHS, 20mM glutamic acid, 2mM TCEP, 2mM TCEP, 0.2mM ascorbic acid	50mM HEPES pH 7.8, 5% glycerol, 500mM NaCl, 0.01% LMING, 0.001% CHS, 20mM glutamic acid, 2mM TCEP, 0.2mM ascorbic acid	50mM HEPES pH 7.8, 5% glycerol, 50mM Biotin, 500mM NaCl, 0.01% LMING, 0.001% CHS, 20mM glutamic acid, 2mM TCEP, 0.2mM ascorbic acid	25mM HEPES pH 7.8, 0.6mM TCEP, 500mM NaCl, 2% glycerol, 0.01% LMING, 0.001% CHS, 20mM glutamic acid, 0.2mM ascorbic acid
5	1000	50mM HEPES pH 7.8, 1mM MgCl ₂ , Protease inhibitor, 2mM TCEP, 500mM Trehalose	50mM HEPES pH 7.8, Protease inhibitor, 500mM NaCl, 2mM TCEP, 500mM Trehalose	50mM HEPES pH 7.8, 5% glycerol, 500mM NaCl, 1% LMING, 0.1% CHS, 20mM glutamic acid, 2mM TCEP, 2mM TCEP, 0.2mM ascorbic acid	50mM HEPES pH 7.8, 5% glycerol, 500mM NaCl, 0.01% LMING, 0.001% CHS, 20mM glutamic acid, 2mM TCEP, 0.2mM ascorbic acid	50mM HEPES pH 7.8, 5% glycerol, 50mM Biotin, 500mM NaCl, 0.01% LMING, 0.001% CHS, 20mM glutamic acid, 2mM TCEP, 0.2mM ascorbic acid	25mM HEPES pH 7.8, 0.6mM TCEP, 500mM NaCl, 2% glycerol, 0.01% LMING, 20mM glutamic acid, 0.2mM ascorbic acid, (Removed CHS)

3.2.10. Fluorescence measurements

Fluorescence measurements of harvested cells were taken to quantify protein expression. The fluorescence emission was measured using a SpectraMax M2 (Molecular Devices) with an excitation wavelength of 485 nm and emission wavelength of 507 nm.

3.2.11. Fluorescence size-exclusion chromatography (FSEC)

10 µl or 40 µl of each protein sample was injected onto a 20 ml SRT-C-300 HPLC column connected to a 3 ml SRT-C 300 guard column, including a 1 in 10 diluted gel filtration standard. The samples were run in FSEC buffer (50 mM HEPES pH 7.5, 500 mM NaCl and 0.03% DDM + 0.003% CHS or 0.03% FC-12). GFP fluorescence (Ex = 485 nm, Em = 507 nm) and tryptophan fluorescence (Ex = 280 nm, Em = 350 nm) were measured. This work was conducted using the technology available at the MPL and funded by INSTRUCT.

3.2.12. SDS-PAGE gel electrophoresis and in-gel fluorescence

A 10 µl aliquot of each protein sample was combined with 10 µl SDS loading buffer. The samples were separated on a NuPage™ 4-12% Bis-Tris SDS-PAGE Gel (35 minutes, 200V, 120mA), alongside a SeeBlue Plus2 Pre-stained Protein Standard or on a Mini-PROTEAN TGX Precast Gel, alongside a Novex Sharp Pre-Stained Protein Standard or a Precision Plus Protein Dual Xtra Pre-Stained Protein Standard. In-gel fluorescence was visualised using a FLA-5000 imaging system (473 nm). The gels were then stained with Coomassie and visualised on the GelDoc imaging system (BioRad).

3.2.13. Western Blot

The protein bands separated by SDS-PAGE were transferred from the gel to a nitrocellulose membrane using an iBlot2 from Invitrogen (20 V, 7 minutes). The membrane was incubated with blocking buffer (1x PBS, 0.2% TWEEN 20, 5% skim milk) for 1 hour at room temperature with gentle rocking. The membrane was then incubated with an anti-His primary antibody (mouse monoclonal IgG2b, abm), diluted to 1:1000, overnight at 4°C with gentle rocking.

The membrane was washed (1x PBS, 0.2% TWEEN 20) and incubated with a horseradish peroxidase (HRP)-linked secondary antibody (anti-mIgG2, Cell Signaling Technology), diluted to 1:3000, for 1 hour at room temperature with gentle rocking. The membrane was washed further, and the signal was developed using Clarity Western ECL substrates (BioRad) according to manufacturer's instructions.

3.2.14. Long-term stability assessment

Purified hSVCT2 was concentrated to 0.8 mg/ml using a 100kDa MWCO concentrator and incubated at 4°C or 20°C for 7 days. Protein samples were collected on alternating days and mixed 1:1 with SDS-PAGE loading buffer and then frozen. At the end of the incubation period all samples were separated on an SDS-PAGE gel (see section 3.2.12.).

3.2.15. Negative stain electron microscopy

Purified hSVCT2 was diluted to 0.01 mg/ml or 0.005 mg/ml and used to prepare negative stain grids. Carbon-coated copper grids (Carbon Film 300 Mesh Cu, Agar Scientific) were plasma cleaned for 60 seconds. 3 µl protein was applied to the grids for 15 seconds. Grids were washed with 10 µl water, stained with 10 µl of 2% uranyl acetate for 40 seconds and then dried. The grids were screened on a 120 kV Tecnai 12 Spirit electron microscope with images collected using an FEI 2K eagle camera.

3.2.16. Cryo-electron microscopy

The peak fraction from the SEC purification was used to prepare cryo-EM grids (~0.07 mg/ml or ~0.2 mg/ml). Ultrathin Carbon on Lacey 400 mesh Cu (Ted Pella Inc) or Quantifoil R1.2/1.3 + 2nm C Cu 300 (Agar Scientific) were plasma cleaned for 30 seconds. 3 µl protein was applied to the grids, blotted, then plunged into liquid ethane using a Vitrobot mark IV (Thermo Fisher Scientific). This was performed at 4°C, 100% humidity and 0 blot force. 8 seconds, 11 seconds and 3x 5 seconds blotting times were used. The grids were then screened on a 120 kV Tecnai 12 Twin electron microscope with images collected using a CCD camera with the help of Paul Simpson and Yassin Ben-Khoud.

3.2.17. Confocal microscopy

See section 2.2.4.

3.2.18. Ascorbic acid uptake assay

See section 2.2.5.

3.2.19. Statistical analysis

For the uptake assay data, significance was tested using a two-tailed unpaired *t*-test between the hSVCT2 constructs the mock-transfected control. The graph was plotted and statistical analysis was carried out on GraphPad Prism 6.0.

3.3. Results

3.3.1. Expression of hSVCT2 in pcDNA3.1 was unsuccessful

A WT hSVCT2 construct in pcDNA3.1-YFP-His was successfully generated. Although this was the same construct as the one used to express hSVCT1 (see Chapter 2), the expression of hSVCT2 was unsuccessful. Different concentrations of hSVCT2 DNA and different transfection agents, such as PEI and Lipofectamine 2000, were trialled to help express hSVCT2 but they all failed (data not shown). There was neither any detectable functional or correctly localised protein as assessed by ascorbic acid uptake analysis, confocal microscopy or YFP expression analysis. Previous studies have reported the expression of hSVCT2 in HEK-293 cells using a different expression vector to pcDNA3.1 (Subramanian et al., 2010, Subramanian et al., 2021). Moreover, as discussed in section 1.4.1, the design of the expression construct is important in expressing functional protein (Kesidis et al., 2020). Therefore, it was hypothesised that using a different expression vector could help in the expression of hSVCT2.

3.3.2. Expression of hSVCT2 in several pOPIN vectors was successful

Following the unsuccessful attempts of expressing hSVCT2 in pcDNA3.1, hSVCT2 constructs (WT, Y628F and Δ 1-23) were cloned into various pOPIN vectors (Table 3.2) and expressed in 3 ml Expi-293 cells. Protein was extracted in DDM+CHS or FC-12 and purified using TALON resin. SDS-PAGE analysis of the isolated proteins suggested the successful expression of hSVCT2 in all the different vectors (Figure 3.2). Bands were present near the various molecular weights (MW) we would expect for the different constructs; however, it was difficult to identify which bands exactly correspond to the protein of interest.

Table 3.2. List of hSVCT2 constructs cloned into multiple vectors. GFP = green fluorescent protein, TGP = thermostabilised green fluorescent protein, 2Strep = twin strep tag, POI = protein of interest, Sig. peptide = HA signal peptide.

Gene	Vector	N or C	Protease	Tag 1	Tag 2	Tag 3
hSVCT2 WT	pOPINF	N	3C	6His	-	-
hSVCT2-Y628F						
hSVCT2 WT	pOPINEneo-3C-GFP-His	C	3C	GFP	8His	-
hSVCT2 Δ 1-23						
hSVCT2 WT	pOPINEneo-TEV-TGP-His	C	TEV	TGP	8His	-
hSVCT2 Δ 1-23						
hSVCT2 WT	pOPINEneo-TGP-2Strep-His	C	3C	TGP	2Strep	8His
hSVCT2 Δ 1-23						
hSVCT2 WT	pOPINEneo-HA-FLAG-TEV-POI-TGP	N/C	Sig. peptide	FLAG	TGP	8His
hSVCT2 Δ 1-23						
hSVCT2 WT	pOPINEneo-FLAG	C	C	3C	8His	FLAG
hSVCT2 Δ 1-23						
hSVCT2 WT	pOPINEneo-2Strep	C	C	3C	2Strep	8His
hSVCT2 Δ 1-23						

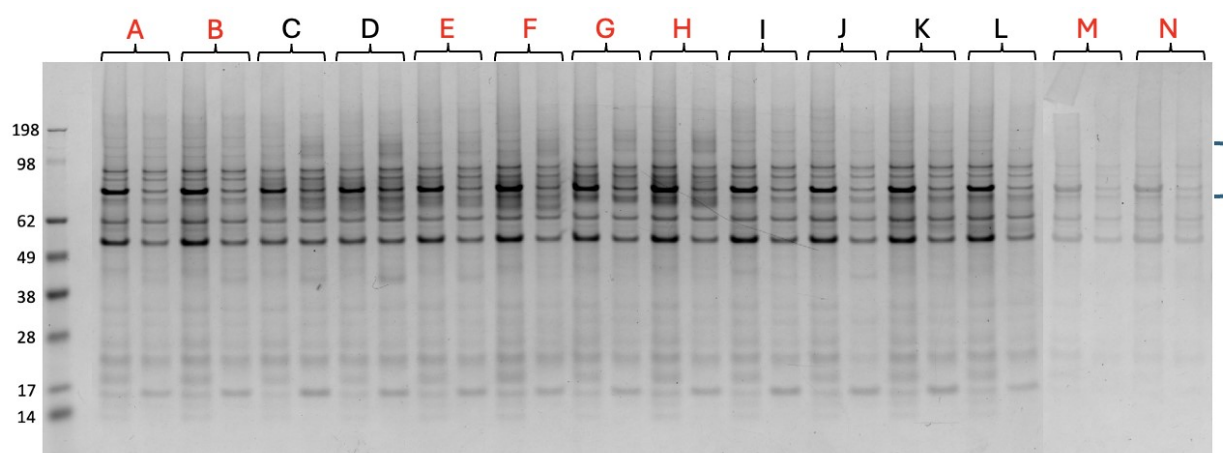


Figure 3.2. SDS-PAGE gel for hSVCT2 test purification showed protein expression. Fractions from test purification were run on a NuPage™ 4-12% Bis-Tris SDS-PAGE Gel, alongside a SeeBlue Plus2 Pre-stained Protein Standard. A = hSVCT2 WT and B = hSVCT2-Y628F in pOPINF, C = hSVCT2 WT and D = hSVCT2 Δ 1-23 in pOPINeNeo-3C-GFP-His, E = hSVCT2 WT and F = hSVCT2 Δ 1-23 in pOPINeNeo-TEV-TGP-His, G = hSVCT2 WT and H = hSVCT2 Δ 1-23 in pOPINeNeo-TGP-2Strep-His, I = hSVCT2 WT and J = hSVCT2 Δ 1-23 in pOPINeNeo-HA-FLAG-TEV-POI-TGP, K = hSVCT2 WT and L = hSVCT2 Δ 1-23 in pOPINeNeo-FLAG, and M = hSVCT2 WT and N = hSVCT2 Δ 1-23 in pOPINeNeo-2Strep. The first lanes for each construct represent protein solubilised with 1% DDM + 0.1% CHS and the second lanes represent those solubilised with 1% FC-12. Bands that could represent hSVCT2 have been marked in blue. Constructs highlighted in red were taken forward to a membrane mimetic screen based on subsequent FSEC experiments.

FSEC analysis was performed on the purified hSVCT2 constructs. Both GFP and Tryptophan fluorescence were assessed for the fluorescent protein-containing constructs and only Tryptophan fluorescence was assessed for the constructs that did not contain a fluorescent protein. The FSEC results showed that the protein eluted between 7-10 ml and also showed smaller peaks between 10-11 ml, which could be the result of protein degradation (Figures 3.3 and 3.4). According to the FSEC results for the fluorescent constructs, hSVCT2 Δ 1-23 in pOPINeNeo-TEV-TGP-His and hSVCT2 Δ 1-23 in pOPINeNeo-TGP-2Strep-His showed the highest monodisperse peaks (Figure 3.3). There did not seem to be protein present for hSVCT2 Δ 1-23 in pOPINeNeo-HA-FLAG-TEV-POI-TGP as no peak can be seen when measuring TGP fluorescence (Figure 3.3A). A peak is present for this construct when measuring Trp fluorescence, but this could be from contaminant proteins present in the sample (Figure 3.3B). hSVCT2 WT in pOPINeNeo-HA-FLAG-TEV-POI-TGP also did not have a high protein

peak compared to the other constructs. In general, hSVCT2 did not seem to express as well in the pOPINeNeo-HA-FLAG-TEV-POI-TGP vector so these constructs were not taken forward. Overall, the N-terminal truncation constructs seemed to express better than their full-length counterparts.

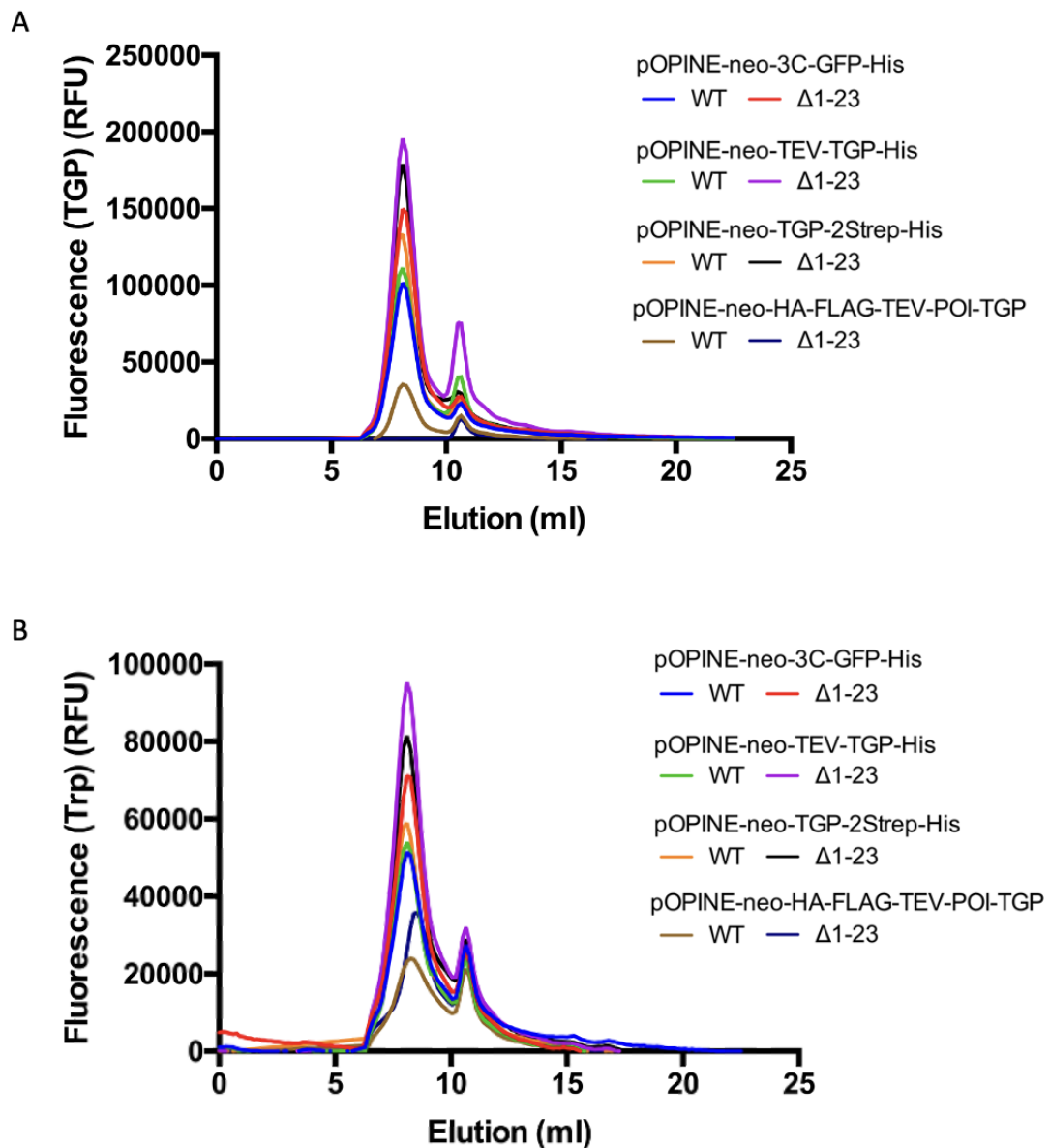


Figure 3.3. FSEC chromatograms for the test purification of fluorescent protein-containing hSVCT2 constructs. Protein was extracted in 1% DDM + 0.1% CHS. A) GFP/TGP fluorescence measured at Ex = 485 nm and Em = 507 nm. B) Tryptophan fluorescence measured at Ex = 280 nm, Em = 350 nm.

For the non-fluorescent constructs, Trp fluorescence was measured (Figure 3.4). Most of the constructs had similar monodisperse peaks with the highest one being hSVCT2 Δ 1-23 in pOPINeNeo-2strep. hSVCT2 WT and hSVCT2 Y628F in pOPINF had the lowest peaks but were comparable with the Trp fluorescence of the GFP constructs hSVCT2 WT in pOPINeNeo-3C-GFP-His, in pOPINeNeo-TEV-TGP-His and in pOPINeNeo-TGP-2Strep-His.

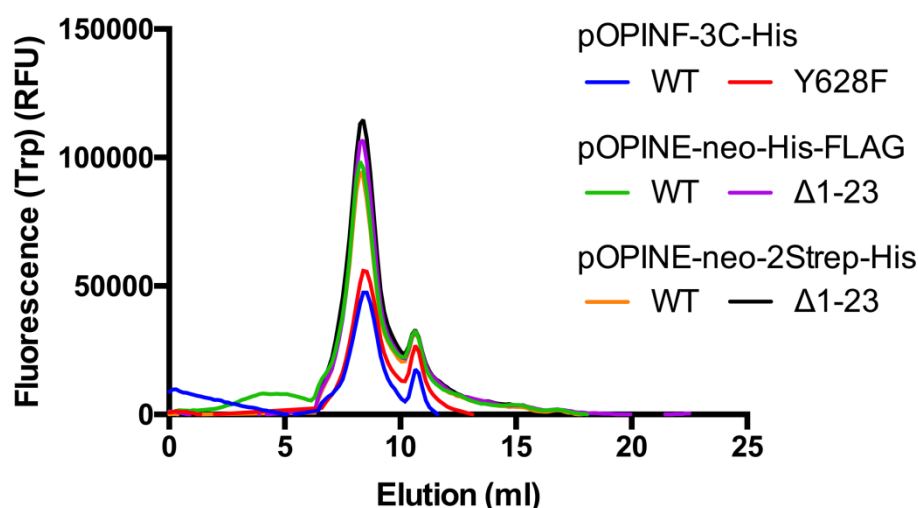


Figure 3.4. FSEC chromatograms for the test purification of non-fluorescent protein-containing hSVCT2 constructs. Tryptophan fluorescence measured at Ex = 280 nm, Em = 350 nm.

The hSVCT2 constructs containing GFP seemed to be eluting around 8 ml and the ones without GFP were eluting just after 8 ml. According to the standard that was run, γ -globulin (MW – 158 kDa) elutes just after 8 ml (Figure 3.5). The hSVCT2 dimer has a predicted MW of about 140 kDa in its unglycosylated form so its elution volume is somewhat consistent with that of the standard. The addition of GFP increases the MW by around 25-27 kDa which resulted in the elution of the GFP constructs before the non-GFP constructs.

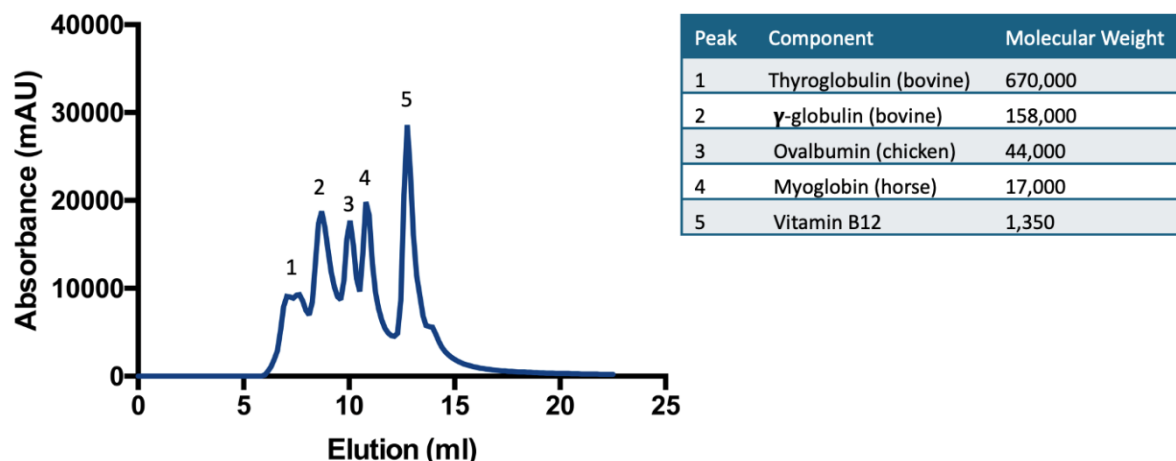


Figure 3.5. SEC chromatogram for a protein standard. A 1 in 10 diluted gel filtration standard was run through the SEC column in order to compare to hSVCT2 results.

Overall, changing to the pOPIN expression vectors, instead of using the previously tried pcDNA3.1 vector, resulted in the expression of hSVCT2. All the constructs, except for those in the pOPIN_{Eneo}-HA-FLAG-TEV-POI-TGP vector, showed good enough expression to be taken forward to a membrane mimetic screen. Therefore, 8 constructs that differed in vector, length and containing a mutation were chosen for the screen (Table 3.3). The pOPIN_{Eneo}-TEV-TGP-His and pOPIN_{Eneo}-TGP-2Strep-His showed the highest monodisperse peaks in the FSEC results so these were taken forward. The pOPIN_F constructs showed comparable results to the previously mentioned vectors so these were also taken forward. Although the pOPIN_{Eneo}-3C-GFP-His constructs also showed good results, these were not taken forward because this construct was similar to the pOPIN_{Eneo}-TEV-TGP-His but the latter had a thermostable GFP instead which made it more appealing. pOPIN_{Eneo}-FLAG and pOPIN_{Eneo}-2Strep showed similar results and showed good enough expression to be taken forward but FLAG tags tend to require more expensive, and lower capacity resin for purification compared to the Strep tags so the latter construct was chosen instead (Lichty et al., 2005).

Table 3.3. List of hSVCT2 constructs cloned into multiple vectors. Constructs highlighted in red were not taken forward to a membrane mimetic screen. GFP = green fluorescent protein, TGP = thermostabilised green fluorescent protein, 2Strep = twin strep tag, POI = protein of interest, Sig. peptide = HA signal peptide

Gene	Vector	N or C	Protease	Tag 1	Tag 2	Tag 3
hSVCT2 WT	pOPINF	N	3C	6His	-	-
hSVCT2-Y628F						
hSVCT2 WT	pOPINEneo-3C-GFP-His	C	3C	GFP	8His	-
hSVCT2 _{Δ1-23}						
hSVCT2 WT	pOPINEneo-TEV-TGP-His	C	TEV	TGP	8His	-
hSVCT2 _{Δ1-23}						
hSVCT2 WT	pOPINEneo-TGP-2Strep-His	C	3C	TGP	2Strep	8His
hSVCT2 _{Δ1-23}						
hSVCT2 WT	pOPINEneo-HA-FLAG-TEV-POI-TGP	N/C	Sig. peptide	FLAG	TGP	8His
hSVCT2 _{Δ1-23}						
hSVCT2 WT	pOPINEneo-FLAG	C	C	3C	8His	FLAG
hSVCT2 _{Δ1-23}						
hSVCT2 WT	pOPINEneo-2Strep	C	C	3C	2Strep	8His
hSVCT2 _{Δ1-23}						

3.3.3. Detergents are the optimal membrane mimetic system for hSVCT2

As discussed in section 1.4, there are several types of membrane mimetic systems, such as detergents, amphipols and polymers, that help keep the membrane protein in a stable and functional state. Here, a series of detergents, amphipols and a polymer were screened to help identify the most stabilising system for hSVCT2. Following success in an initial purification attempt using pOPIN vectors, 8 constructs were chosen to carry out the membrane mimetic screen (Table 3.4).

Table 3.4. List of chosen hSVCT2 constructs for membrane mimetic screening. TGP = thermostabilised green fluorescent protein, 2Strep = twin strep tag.

Gene	Vector	N or C	Protease	Tag 1	Tag 2	Tag 3
hSVCT2 WT	pOPINF	N	3C	6His	-	-
hSVCT2-Y628F						
hSVCT2 WT	pOPINeNeo-TEV-TGP-His	C	TEV	TGP	8His	-
hSVCT2 Δ 1-23						
hSVCT2 WT	pOPINeNeo-TGP-2Strep-His	C	3C	TGP	2Strep	8His
hSVCT2 Δ 1-23						
hSVCT2 WT	pOPINeNeo-2Strep	C	C	3C	2Strep	8His
hSVCT2 Δ 1-23						

The hSVCT2 constructs were expressed in 30 ml Expi-293 cells. Protein was extracted and purified using TALON or Ni-NTA resin, and SDS-PAGE and FSEC analysis were used to assess the stability of the protein in the different membrane mimetic systems. The chosen constructs were analysed in 6 different detergents (DDM, DDM-CHS, LMNG, LMNG-CHS, Digitonin, GDN, FC-12), 1 polymer (SMA) and 4 amphipols (A-18, A8-35, NAPol, PMAL-12). The protein samples analysed in detergent were extracted and purified in the specific detergents, whereas the samples analysed in amphipols were extracted in DDM and then reconstituted into the different amphipols. For the polymer sample, SMA was used to directly extract the protein from the membrane.

Overall, the SDS-PAGE analysis showed that detergents were more effective at solubilising hSVCT2 compared to the amphipols and SMA (Figure 3.6). Protein bands were present for most of the detergent samples (lanes 1-7) whereas there were no or very faint bands for the polymer and amphipol samples (lanes 8-12). In general, Y628F in pOPINF, Δ 1-23 in pOPINeNeo-TEV-TGP-His and both WT and Δ 1-23 in pOPINeNeo-TGP-2Strep-His and pOPINeNeo-2Strep showed the strongest protein bands.

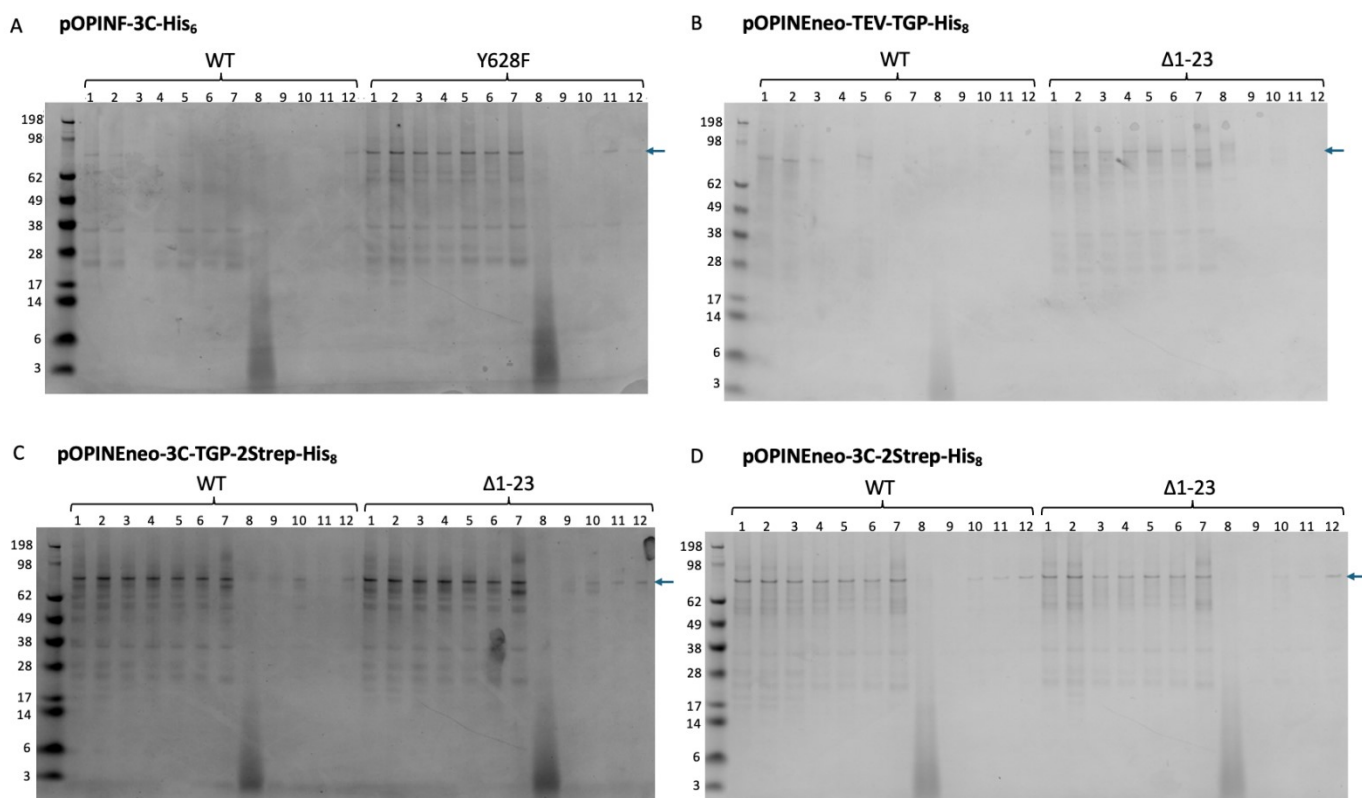


Figure 3.6. SDS-PAGE gel for hSVCT2 membrane mimetic screen showed detergents were effective at solubilising hSVCT2. Fractions from purification were run on a NuPage™ 4-12% Bis-Tris SDS-PAGE Gel, alongside a SeeBlue Plus2 Pre-stained Protein Standard. A) hSVCT2 WT and hSVCT2-Y628F in pOPINF, B) hSVCT2 WT and hSVCT2 $_{\Delta 1-23}$ in pOPINeNeo-TEV-TGP-His, C) hSVCT2 WT and hSVCT2 $_{\Delta 1-23}$ in pOPINeNeo-TGP-2Strep-His, D) hSVCT2 WT and hSVCT2 $_{\Delta 1-23}$ in pOPINeNeo-2Strep. hSVCT2 purified in different membrane mimetics were run on different lanes: 1) DDM, 2) DDM-CHS, 3) LMNG, 4) LMNG-CHS, 5) Digitonin, 6) GDN, 7) FC-12, 8) SMA, 9) A-18, 10) A8-35, 11) NAPol, 12) PMAL-12. Blue arrows correspond to the predicted molecular weight of the hSVCT2 monomer.

FSEC analysis was performed on the purified hSVCT2 constructs. Both TGP and Trp fluorescence were assessed for the fluorescent tag-containing constructs and only Trp fluorescence was measured for the constructs that did not contain a fluorescent tag. The TGP FSEC results showed that in general the detergents resulted in higher monodisperse peaks than the polymer or amphipols (Figures 3.7 and 3.8). The full length and truncation constructs in the vector pOPINeNeo-TEV-TGP-His were most stable in DDM and DDM+CHS (Figure 3.7). The constructs in the vector pOPINeNeo-TGP-2Strep-His were most stable in DDM, DDM+CHS and LMNG+CHS, with no clear difference in stability between these

detergents (Figure 3.8). When comparing the fluorescence values, WT hSVCT2 in pOPINeNeo-TGP-2Strep-His showed the lowest levels of expression. There seems to be some protein present in the SMA sample of the truncation in pOPINeNeo-TEV-TGP-His which can also slightly be seen in the SDS-PAGE gel (Figure 3.6B).

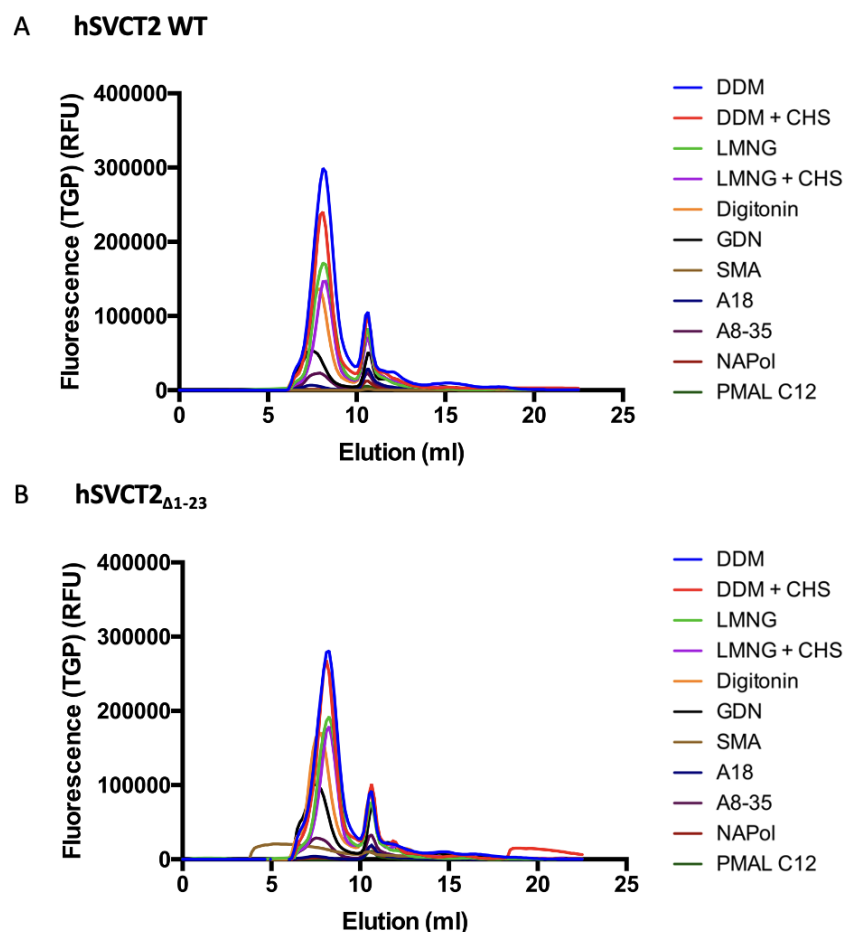


Figure 3.7. FSEC chromatograms for the membrane mimetic screen of TGP-containing hSVCT2 in pOPINeNeo-TEV-TGP-His. TGP fluorescence was measured at Ex = 485 nm and Em = 507 nm. A) hSVCT2 WT and B) hSVCT2_{Δ1-23}.

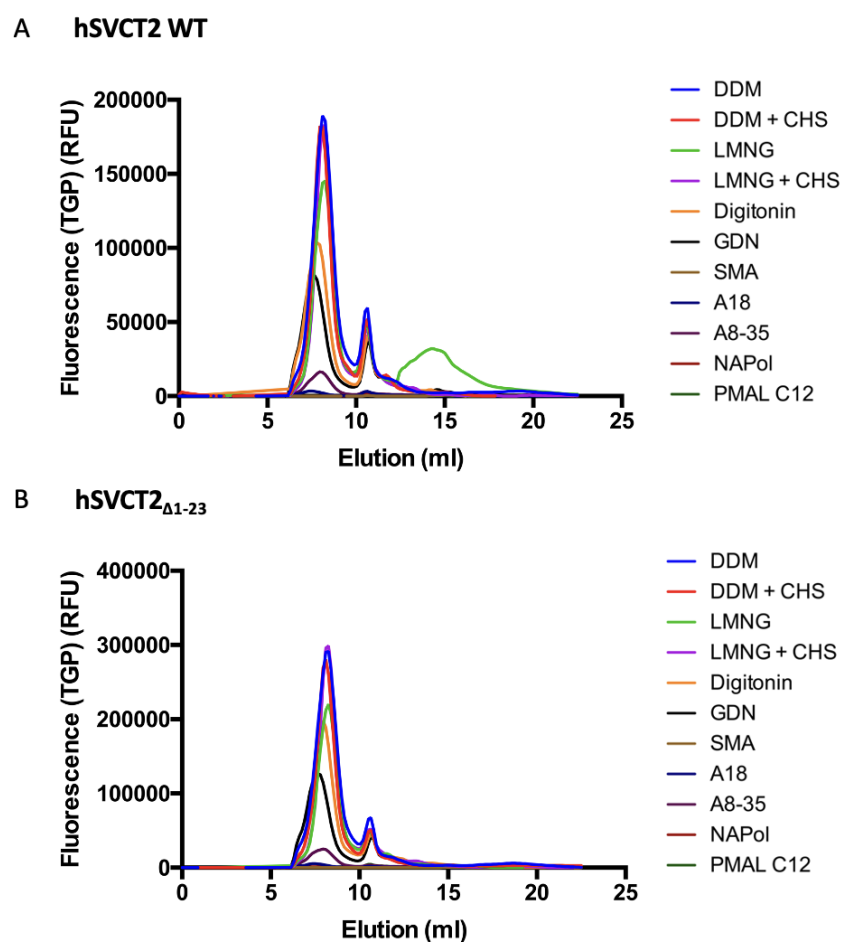


Figure 3.8. FSEC chromatograms for the membrane mimetic screen of TGP-containing hSVCT2 in pOPINNeo-TGP-2Strep-His. TGP fluorescence was measured at Ex = 485 nm and Em = 507 nm. A) hSVCT2 WT and B) hSVCT2 Δ 1-23.

Trp fluorescence was measured for all the constructs (Figure 3.9). All the constructs showed similar amounts of degradation (eluting at 10-11 ml), however unlike all the other constructs, the pOPINF constructs had higher degradation peaks than protein peaks (~8 ml). The truncation in pOPINF had higher protein peaks than the full-length construct. Overall, the same trends as the TGP FSECs (Figures 3.7 and 3.8) and SDS-PAGE gels (Figure 3.6) were seen where the detergents, specifically DDM and DDM+CHS, performed better than the amphipols and polymer. The Trp FSEC results however need to be taken with a grain of salt as the presence of contaminants can alter the fluorescence reading. It is unclear however if the protein was unhappy in the amphipols or if the reconstitution into amphipols was unsuccessful.

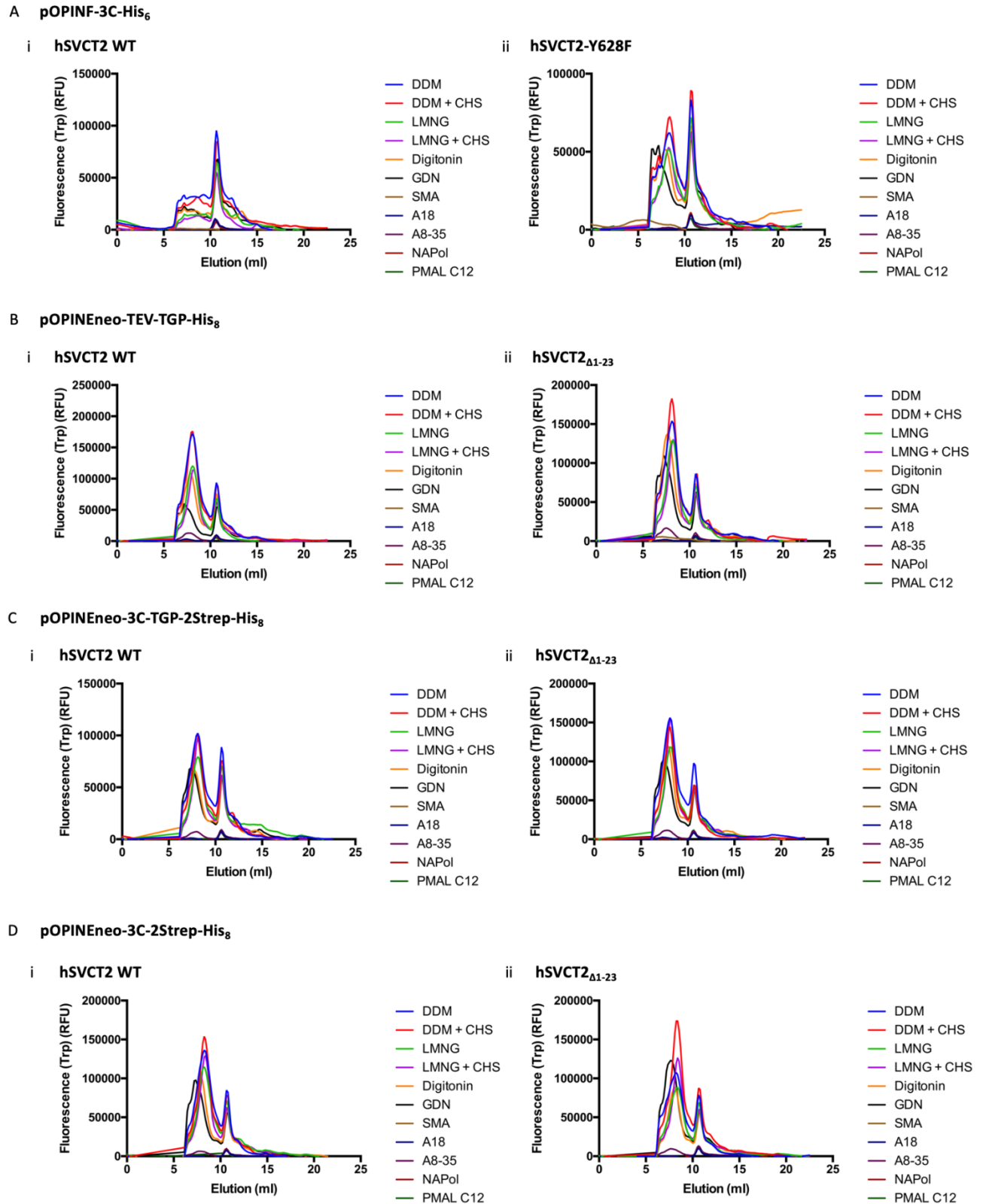


Figure 3.9. FSEC chromatograms for the membrane mimetic screen of hSVCT2 constructs. Tryptophan fluorescence measured at Ex = 280 nm, Em = 350 nm. A) i) hSVCT2 WT and ii) hSVCT2-Y628F in pOPINF, B) i) hSVCT2 WT and ii) hSVCT2_{Δ1-23} in pOPINeNeo-TEV-TGP-His, C) i) hSVCT2 WT and ii) hSVCT2_{Δ1-23} in pOPINeNeo-TGP-2Strep-His, D) i) hSVCT2 WT and ii) hSVCT2_{Δ1-23} in pOPINeNeo-2Strep.

3.3.4. hSVCT2 constructs correctly traffic to the cell membrane

To check for correct cell surface trafficking of hSVCT2, confocal microscopy was performed on hSVCT2 constructs expressed in adherent HEK-293 cells. Microscopy was performed on only the constructs that contained a fluorescent tag. The four constructs imaged showed correct trafficking to the cell membrane with some intracellular fluorescence visible (Figure 3.10).

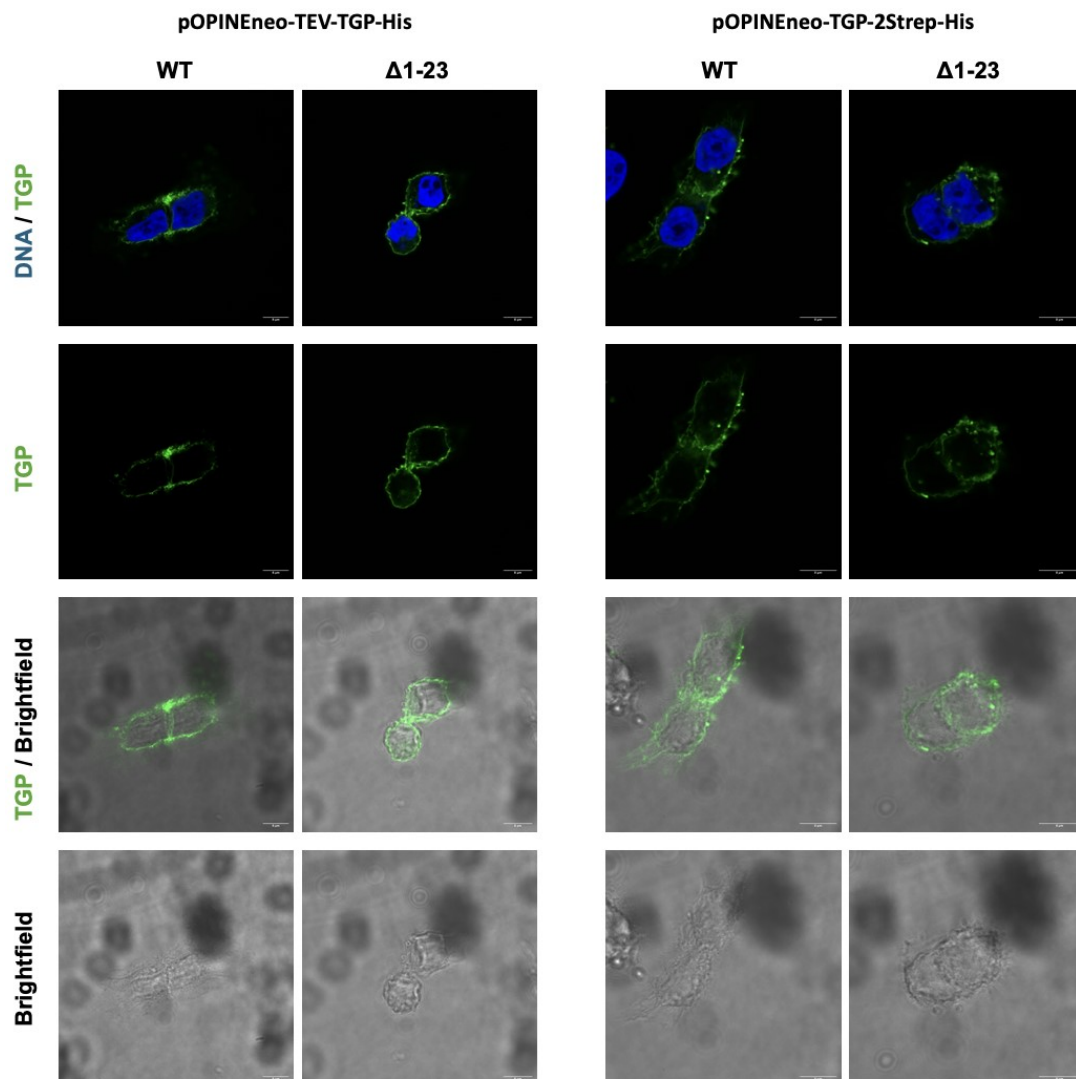


Figure 3.10. Fluorescent protein-containing hSVCT2 constructs showed correct membrane trafficking. The uppermost set of panels show an overlay of TGP fluorescence and the DAPI stained nuclei, the second set of panels show the TGP fluorescence only, the third set of panels shows an overlay of the TGP fluorescence and the brightfield image and the fourth set of panels show the brightfield images only.

3.3.5. hSVCT2 constructs showed ascorbic acid transport activity

An ascorbic acid uptake assay was performed on all 8 constructs, expressed in adherent HEK-293 cells, to ensure correct function. All the constructs were able to transport ascorbic acid and showed similar levels of activity, except for the proteins expressed in the pOPINF-His vector that exhibited lower uptake activity than the rest of the constructs (Figure 3.11). It is possible that the presence of the His tag at the N-terminus of the protein reduces correct folding and/or trafficking of the protein to the plasma membrane. It was not possible to explore how effective this construct was localising to the membrane relative to the other proteins since it lacks a fluorescent fusion partner. Further work could involve exploration of localisation using immunofluorescence and hSVCT2 specific antibodies.

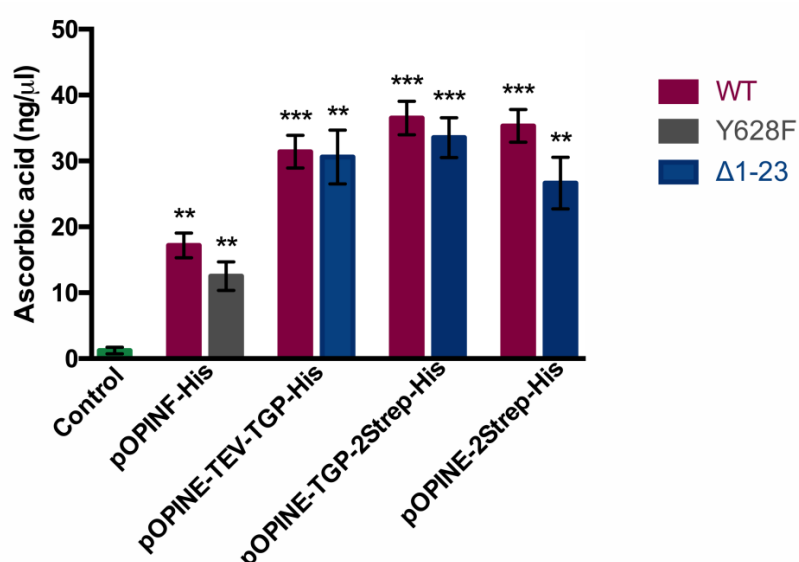


Figure 3.11. hSVCT2 constructs were able to transport ascorbic acid. Data is shown as an average $\pm$ SEM ($n = 3$). Student's t -test was performed between the different hSVCT2 constructs and the mock-transfected control. ***, ** = $p < 0.001$, $p < 0.01$ respectively.

3.3.6. Expression enhancers increased hSVCT2 protein expression

The protein expression experiments reported above were performed according to the methods developed in Gamage et al. (2023); however, this is a general protocol and may not necessarily be optimal for hSVCT2. Thus, I carried out further experiments exploring the effects of adding the expression enhancers valproic acid and sodium butyrate on hSVCT2

expression. Both valproic acid and sodium butyrate are histone deacetylase inhibitors which have been shown to increase protein expression (Wulhfard et al., 2010, Palermo et al., 1991). These enhancers were specifically chosen for hSVCT2 protein expression because Teafatiller et al. (2022) showed that valproic acid upregulates SVCT2 expression, and Wang et al. (2023) and Kobayashi et al. (2024) used sodium butyrate to increase SVCT1 protein expression.

hSVCT2 WT in pOPINeNeo-TEV-TGP-His was expressed in 60 ml Expi-293 cells. 24 hours post-transfection, the cells were split into 3 and to the first sample no enhancers were added, to the second valproic acid to a final concentration of 5 mM was added and to the last one valproic acid and sodium butyrate was added to final concentrations of 5 mM and 10 mM respectively. Aliquots of the three different samples were taken at 24, 48 and 72 hours and protein expression were measured using fluorescence measurements. Cells harvested at 48 hours that were enhanced with both valproic acid and sodium butyrate showed the highest level of protein expression (Figure 3.12). Therefore, these set of conditions were chosen for future experiments.

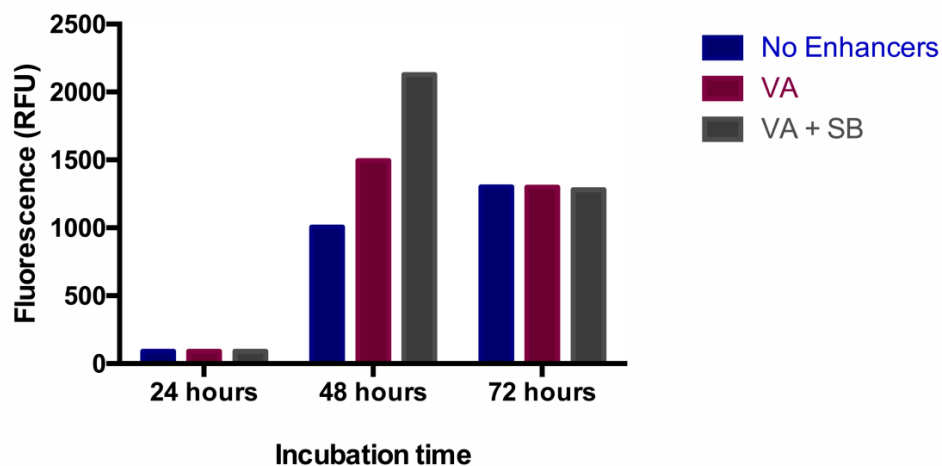


Figure 3.12. The addition of expression enhancers increased hSVCT2 expression. hSVCT2 WT in pOPINeNeo-TEV-TGP-His was used to optimise incubation time. Aliquots of cells were taken at 24 hours, 48 hours and 72 hours, and fluorescence was measured to quantify protein expression. To investigate the effect of adding expression enhancers, valproic acid (VA) or VA and sodium butyrate (SB) were added to cells 24 hours post-transfection. All data was normalised to cell count ($n=1$).

3.3.7. His-tag purifications

3.3.7.1. Cleavage of fluorescent tag resulted in protein loss

Using knowledge gained from the expression and purification results reported above, a larger scale purification was attempted. WT hSVCT2 in a pOPINeNeo-TEV-TGP-His vector was expressed in 100 ml Expi-293 cells, and valproic acid and sodium butyrate were added to enhance protein expression. The protein was purified in DDM using a HisTrap HP column followed by treatment with TEV protease to cleave off the TGP-His tag. SEC was then used to further purify the protein. DDM was the detergent of choice as it showed promising results in the FSEC experiments performed in section 3.3.3.

The affinity chromatogram suggested that more protein was eluting with increasing concentrations of Buffer B (Figure 3.13A). The SDS-PAGE gel showed that the protein mostly eluted between ~20% - 80% Buffer B with a protein yield of 273 μ g (Figure 3.13B). The long elution in imidazole indicated that there was heterogeneity in binding. The elution sample of the post-TEV cleavage/dialysis SDS-PAGE gel showed a bright band at 30 kDa which represented the cleaved tag. There were a few faint bands between 60 – 80 kDa which could represent the protein (Figure 3.13C).

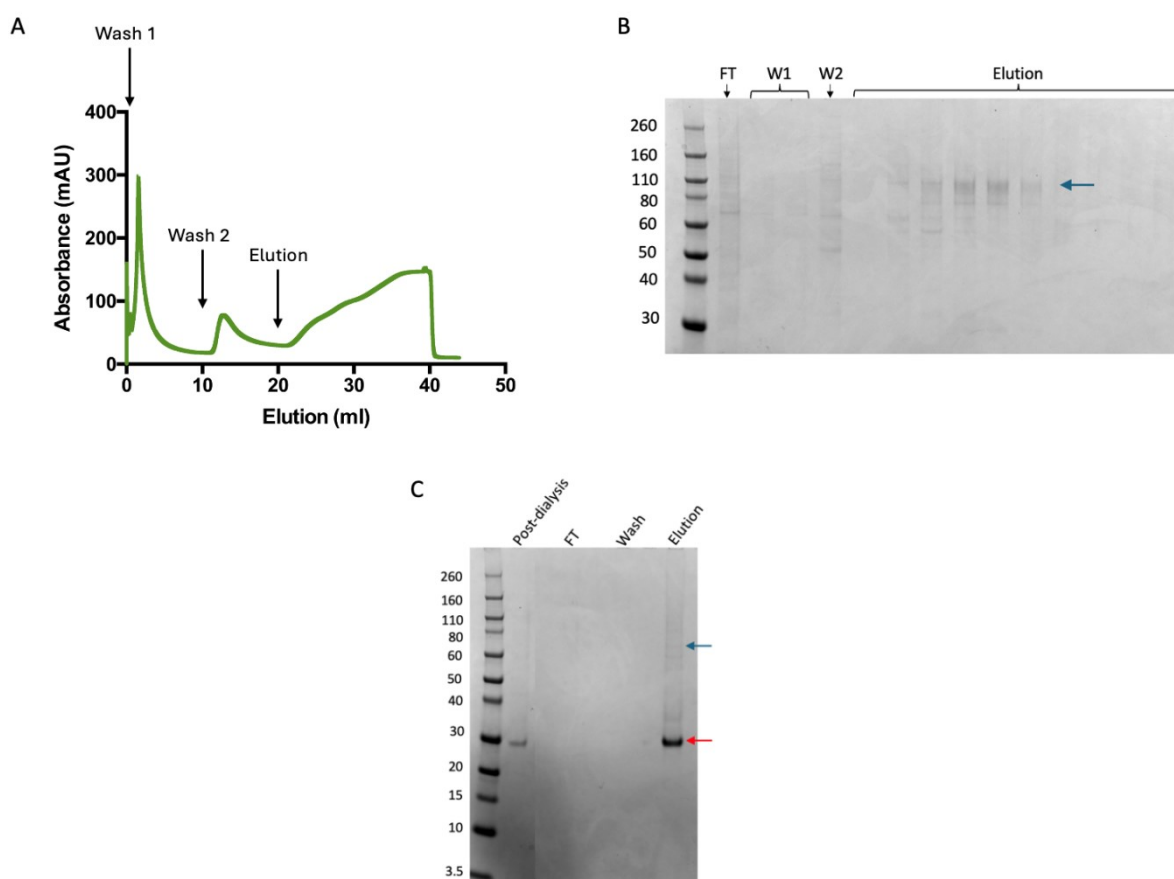


Figure 3.13. *hSVCT2* WT His-tag purification with TEV cleavage. *hSVCT2* WT in pOPINeNeo-TEV-TGP-His was expressed in 100 ml Expi-293 cells and expression was enhanced with valproic acid and sodium butyrate. A) UV absorbance spectra of *hSVCT2* WT purified in DDM on a HisTrap HP column. B) Flowthrough (FT), Wash 1 (W1), Wash 2 (W2) and elution samples from HisTrap purification were run on a NuPageTM 4-12% Bis-Tris SDS-PAGE Gel, alongside a Novex Sharp Pre-stained Protein Standard. Blue arrow corresponds to the predicted molecular weight of the *hSVCT2* monomer (~71kDa) and TGP-His (~28kDa). C) SDS-PAGE gel of post-TEV cleavage samples. Lanes show a post-dialysis sample and the FT, Wash and Elution samples from the reverse His-Trap affinity step. Blue arrow corresponds to cleaved *hSVCT2* and red arrow corresponds to cleaved TGP-His.

The SEC chromatogram showed multiple peaks, and these were all run on an SDS-PAGE gel (Figure 3.14). The peak shown in red could represent the monomeric protein as faint bands are visible on the gel. Very faint bands can also be seen for the peak shown in yellow around 40 kDa which could be degraded protein. The absorbance values for the SEC chromatogram were also very low. The starter Expi-293 culture was small but most protein seems to have been lost at the TEV cleavage step. Therefore, for the following purification the volume of

culture was increased and for all subsequent purifications the TEV cleavage step was omitted.

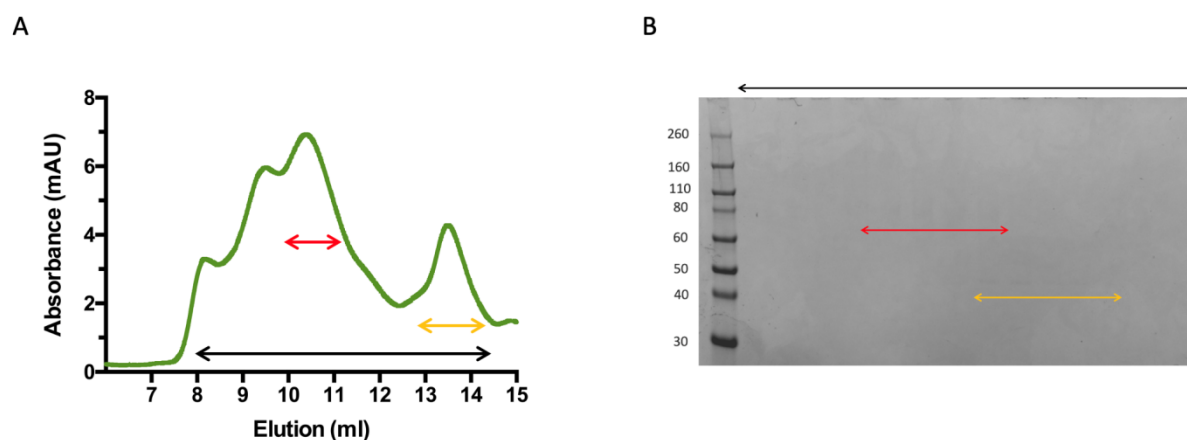


Figure 3.14. SEC of hSVCT2 WT His-tag purification with TEV cleavage. A) SEC UV absorbance spectra of cleaved hSVCT2 purified in DDM after affinity chromatography. B) SEC fractions were run on a NuPageTM 4-12% Bis-Tris SDS-PAGE Gel, alongside a Novex Sharp Pre-stained Protein Standard. Main protein peak is indicated by red arrows on both the chromatogram (eluting around 10 – 11ml) and the SDS-PAGE gel (faint bands seen at <80kDa). Yellow arrow shows faint bands around 40kDa which could be protein degradation.

3.3.7.2. HisTrap purification resulted in high amounts of contamination

WT hSVCT2 in a pOPINeNeo-TEV-TGP-His vector was expressed in 400 ml Expi-293 cells, and valproic acid and sodium butyrate were added to enhance protein expression. 10mM imidazole was added to the solubilisation buffer to help improve protein purity through limiting non-specific binding of proteins during the affinity chromatography step. The protein was purified in DDM using a HisTrap HP column followed by SEC.

The affinity chromatogram (Figure 3.15A) and the SDS-PAGE gel (Figure 3.15B) showed that the protein was eluting at around ~80% Buffer B. The SDS-PAGE gel showed the presence of a lot of contaminants, and it was difficult to identify the correct protein band. Protein yield at this point was 3 mg, however the presence of large amounts of contaminants could have interfered with the concentration measurements. The SEC chromatogram showed two major peaks (Figure 3.15C). The first peak, showed in black, was thought to represent the

aggregation peak and the second peak, showed in red, was thought to be the protein peak. Using both the SEC chromatogram (Figure 3.15C) and the gel (Figure 3.15D), the peak highlighted in red represents both the hSVCT2 protein and protein degradation: fractions with retention volumes between 10-12 ml contain the protein and between 12 ml and 13 ml represents degradation. Even though there was an increase in the absorbance values compared to the previous purification, most of the protein had aggregated and the SDS-PAGE gel still showed the presence of contaminants.

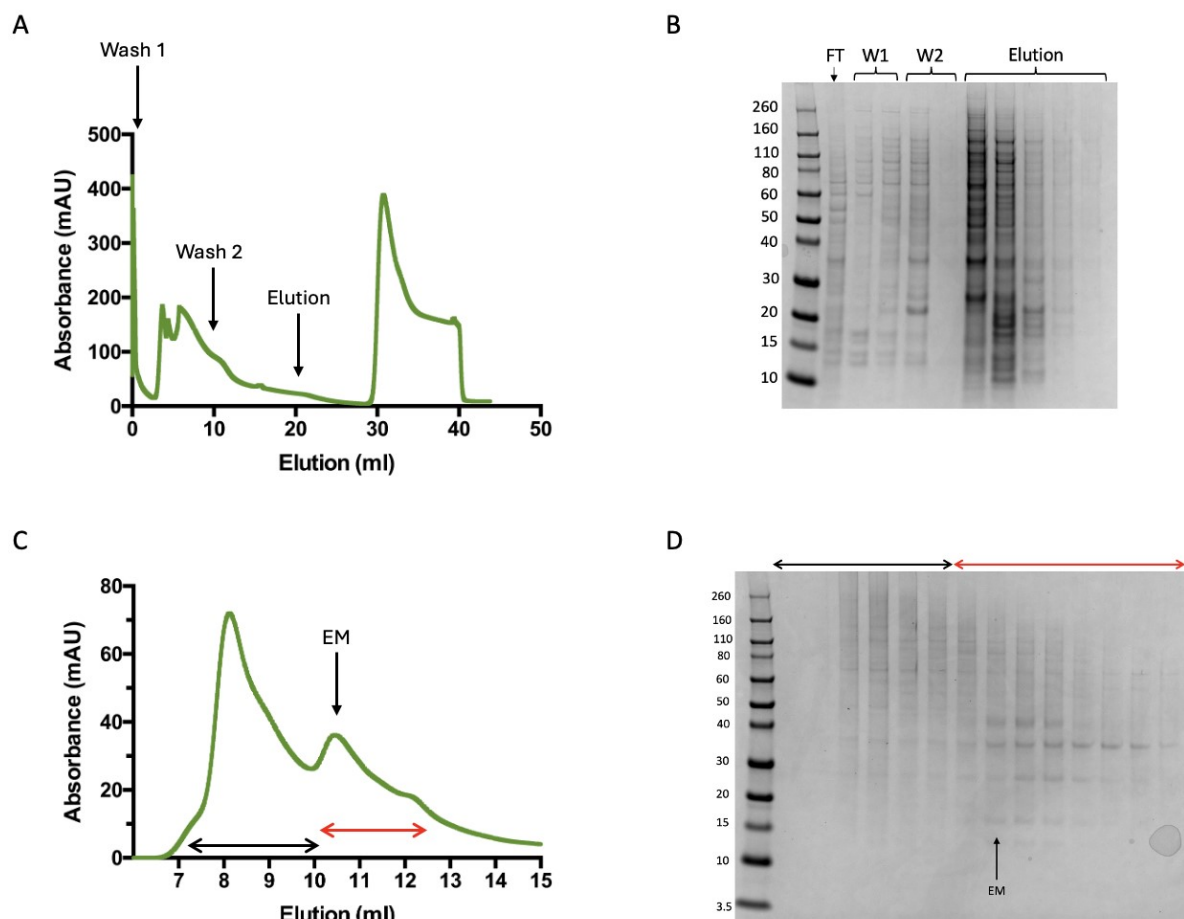


Figure 3.15. hSVCT2 WT His-tag purification without TEV cleavage resulted in high amounts of contamination. hSVCT2 WT in pOPINeNeo-TEV-TGP-His was expressed in 400 ml Expi-293 cells and expression was enhanced with valproic acid and sodium butyrate. To improve protein purity, 10 mM imidazole was added to the solubilisation buffer A) UV absorbance spectra of hSVCT2 WT purified in DDM on a HisTrap HP column. B) Flowthrough (FT), Wash 1 (W1), Wash 2 (W2) and elution samples from HisTrap purification were run on a NuPageTM 4-12% Bis-Tris SDS-PAGE Gel, alongside a Novex Sharp Pre-stained Protein Standard. C) SEC UV absorbance spectra of hSVCT2 purified in DDM. D) SDS-PAGE gel of SEC fractions. Black arrows on both SEC chromatogram and SEC gel correspond to protein aggregation. Main protein peak is indicated by red arrows on both the chromatogram and the SDS-PAGE gel. The gel showed the presence of a lot of contaminants and made it difficult to identify correct protein band. EM – fraction taken for negative stain electron microscopy.

0.01 mg/ml of the peak fraction ('EM' in Figure 3.15) was used to prepare negative stain grids. The grids were stained for 1 minute in 2% uranyl acetate. The micrograph showed a lot of burning that suggested that the staining time was too long (Figure 3.16). Therefore, for all subsequent negative stain grid preparations, the staining time was reduced to 40 seconds.

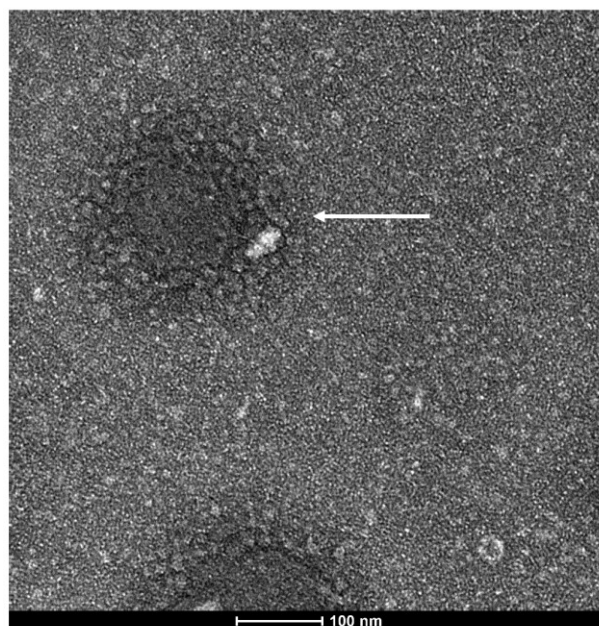


Figure 3.16. Negative stain micrograph of hSVCT2 WT purified in DDM. 0.01 mg/ml of SEC peak fraction was applied to the grid and stained for 1 minute in 2% uranyl acetate. The staining time was too long as the sample kept burning during imaging (represented by white arrow).

3.3.7.3. Mass spectrometry analysis confirmed the presence of hSVCT2

WT hSVCT2 in a pOPINNeo-TEV-TGP-His vector was expressed in 500 ml Expi-293 cells, and valproic acid and sodium butyrate were added to enhance protein expression. 10 mM imidazole was kept in the solubilisation buffer to help improve protein purity. The protein was purified in DDM using a HisTrap HP column followed by SEC.

The affinity chromatography SDS-PAGE gel showed that the protein was eluting with 100 mM to 300 mM imidazole with the strongest bands seen with 200 mM imidazole (Figure 3.17A). Due to the HisTrap HP column breaking, this affinity chromatography had to be run using a peristaltic pump instead of the AKTA Pure which is why a chromatogram is not shown. The SDS-PAGE gel showed the presence of less contaminants compared to previous purifications. An in-gel fluorescence was also run that showed bands that represented the monomeric and dimeric forms of the protein (Figure 3.17B). The protein yield at this step was 442 µg. The protein yield with the 100 ml culture purification in section 3.3.7.1. was 273 µg. Since the culture for this purification was 5 times larger, a protein yield of about 1.4 mg would have been expected.

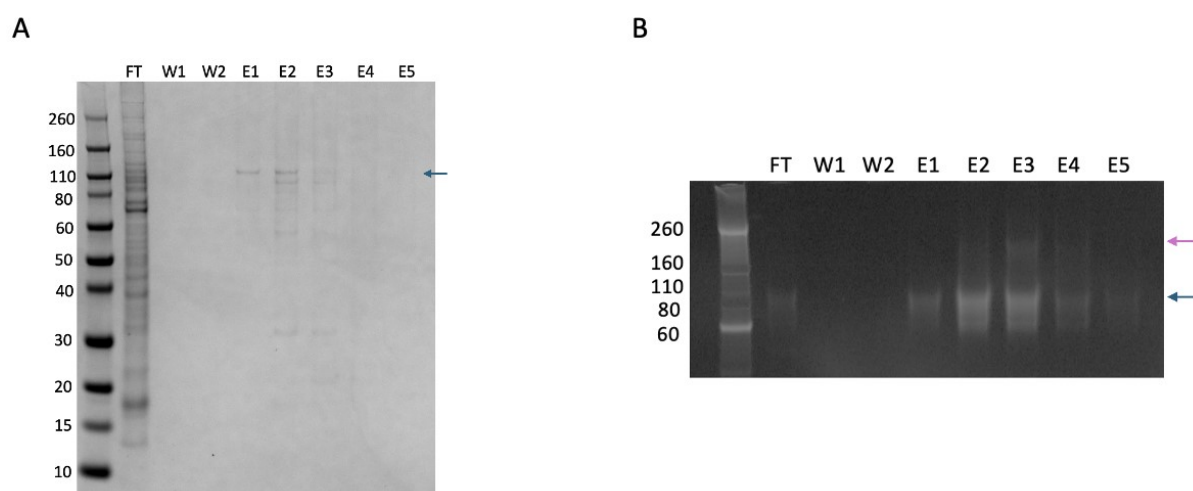


Figure 3.17. hSVCT2 WT His-tag purification showed the presence of protein bands. hSVCT2 WT in pOPINeNeo-TEV-TGP-His was expressed in 500 ml Expi-293 cells and expression was enhanced with valproic acid and sodium butyrate. hSVCT2 WT was purified in DDM on a HisTrap HP column. A) Coomassie-stained SDS-PAGE gel of flowthrough (FT), Wash samples (W1, W2), and elution samples (E1 – 100 mM imidazole, E2 - 200 mM imidazole, E3 - 300 mM imidazole, E4 – 400 mM imidazole and E5 - 500 mM imidazole) from His-Trap purification. Samples were run on a NuPage™ 4-12% Bis-Tris SDS-PAGE Gel, alongside a Novex Sharp Pre-stained Protein Standard. Blue arrow corresponds to the predicted molecular weight of the hSVCT2 monomer (~71kDa) and TGP-His (~28kDa). B) In-gel fluorescence of FT, Wash and Elution samples from His-Trap purification. Blue arrow corresponds to the MW of hSVCT2 monomer and pink arrow corresponds to the MW of hSVCT2 dimer.

In this SEC chromatogram (Figure 3.18A), the aggregation and protein peaks are not as well separated as in the previous purification. Nevertheless, the peak highlighted in black represents the aggregation peak as suggested by the elution volume and by the smearing that can be seen on the SDS-PAGE gel (Figure 3.18B). The peak highlighted in red represented the hSVCT2 protein peak therefore this peak was concentrated, a gel was run and the band shown with a blue arrow was sent for mass spectrometry analysis at the University of St. Andrews (Figure 3.19). The mass spectrometry analysis confirmed that this band was in fact hSVCT2. Even though this purification was done with an increased volume of 500 ml Expi-293 culture, the absorbance values were lower than that of the previous purification (400 ml Expi-293 culture). This suggested that the protein expression was not as good in this purification or protein was lost during prior purification steps.

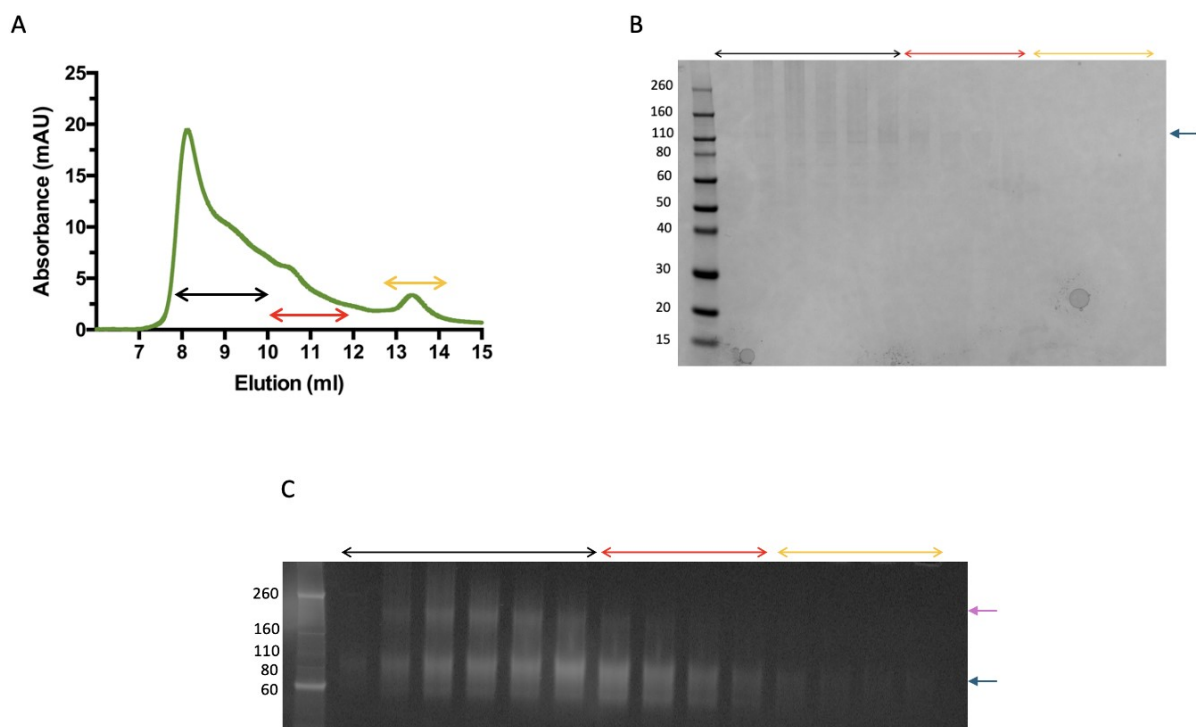


Figure 3.18. SEC of hSVCT2 WT His-tag purification without TEV cleavage. A) SEC UV absorbance spectra of hSVCT2 purified in DDM after affinity chromatography. B) Coomassie-stained SDS-PAGE gel of SEC fractions. C) In-gel fluorescence of SEC fractions. Samples were run on a NuPage™ 4-12% Bis-Tris SDS-PAGE Gel, alongside a Novex Sharp Pre-stained Protein Standard. Black arrows on both SEC chromatogram and gels correspond to protein aggregation. Main protein peak is indicated by red arrows on both the chromatogram and the gels. Yellow arrows could represent protein degradation. Blue arrow corresponds to hSVCT2 monomer and pink arrow corresponds to the dimer.

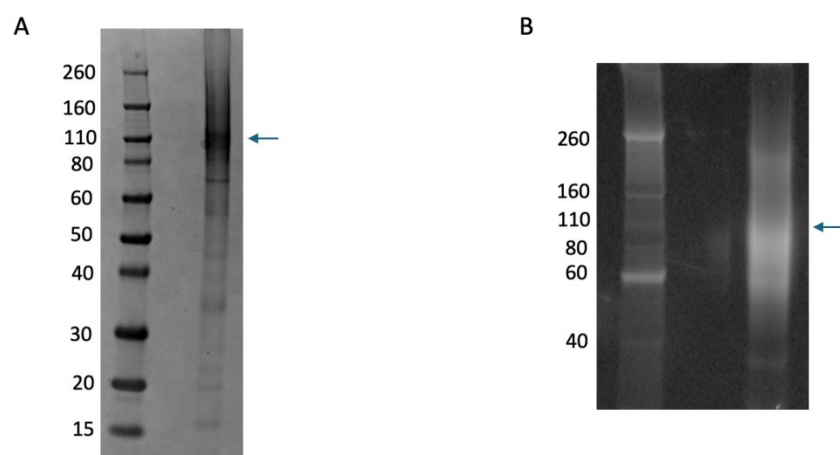


Figure 3.19. Concentrated hSVCT2 WT protein sample was submitted to mass spectrometry analysis. The main protein peak from Figure 3.18 was concentrated and ran on a NuPage™ 4-12% Bis-Tris SDS-PAGE Gel, alongside a Novex Sharp Pre-stained Protein Standard. A) Coomassie-stained SDS-PAGE gel. B) In-gel fluorescence. Blue arrow indicates hSVCT2 monomer which was confirmed by mass spectrometry analysis.

Even though this purification and mass spectrometry analysis proved that hSVCT2 elutes between 10 ml and 11 ml, it was concluded that a HisTrap HP purification was probably not best because the SDS-PAGE gel still showed the presence of contaminants. Mammalian proteins contain a high percentage of His residues which could bind to the nickel resin, and this could explain the presence of contaminants. A Strep-tag on the other hand specifically binds to streptavidin and is less likely to occur naturally compared to a His-tag (Kimple et al., 2013, Schmidt and Skerra, 2007). Therefore, to improve protein purity, Strep-tag purifications were undertaken.

3.3.8. Strep-tag purifications

3.3.8.1. StrepTrap HP purification resulted in protein loss

WT hSVCT2 in a pOPINeNeo-TGP-2Strep-His vector was expressed in 200 ml Expi-293 cells, and valproic acid and sodium butyrate were added to enhance protein expression. After the addition of enhancers, the cells were incubated at 30°C instead of 37°C as this increases protein yields for some membrane proteins (Gamage et al., 2023, Wulhfard et al., 2008). The 1x PBS used in the cell resuspension buffer for the His-tag purifications was replaced with 50 mM HEPES pH 7.8 to see if keeping HEPES consistent in all the buffers would help in stabilising the protein. The protein was purified in DDM using a StrepTrap HP column (prepacked with StrepTactin Sepharose resin) and desthiobiotin was used to elute the protein.

The chromatogram and gel images, specifically the in-gel fluorescence, showed that the protein did not bind to the column and was instead removed in the flow through and wash fractions (Figure 3.20). The type of Strep-tag on the vector used was a Twin-Strep-tag which consists of two Strep-tag II motifs (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) connected by a linker. Twin-Strep-tag has a nM affinity to StrepTactin whereas it has a pM affinity to StrepTactinXT (Schmidt et al., 2021). Therefore, for following purifications, StrepXT loose resin was used instead of the StrepTrap HP column. Additionally, a member of our lab, Lok Chan, completed an experiment on hSVCT1 investigating the difference in protein expression when incubating the cells at 30°C or at 37°C after the addition of enhancers. Her results showed decreased protein expression when incubating the cells at 30°C so it was decided to keep the cells at 37°C for the hSVCT2 purifications reported here.

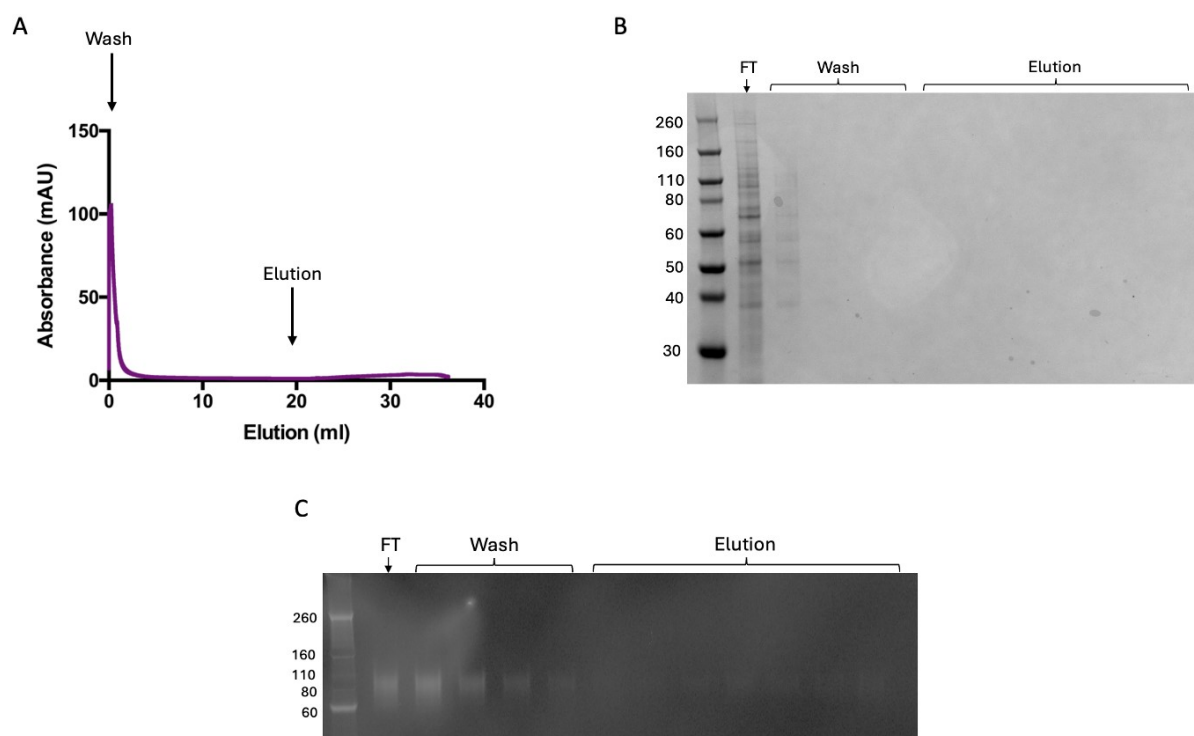


Figure 3.20. hSVCT2 WT Strep-tag purification on a Strep-HP column resulted in protein loss. hSVCT2 WT in pOPINeNeo-TGP-2Strep-His was expressed in 200 ml Expi-293 cells and expression was enhanced with valproic acid and sodium butyrate at 30 °C. A) UV absorbance spectra of hSVCT2 WT purified in DDM on a StrepTrap HP column. B) Coomassie-stained SDS-PAGE gel of flowthrough (FT), wash and elution samples from StrepTrap purification. C) In-gel fluorescence of flowthrough (FT), wash and elution samples from StrepTrap purification. Samples were run on a NuPage™ 4-12% Bis-Tris SDS-PAGE Gel, alongside a Novex Sharp Pre-stained Protein Standard. The protein did not bind to the StrepTrap HP column and was eluted in the FT and wash fractions.

3.3.8.2. StrepXT purification resulted in relatively pure hSVCT2 protein

Truncated hSVCT2, $\Delta 1-23$, in a pOPINeNeo-TGP-2Strep-His vector was expressed in 200 ml Expi-293 cells. The truncated protein was used instead of the full-length protein that had been used thus far because from the FSEC results in section 3.3.3 (Figure 3.8), the truncated protein seemed to express better. From this point forward, only valproic acid was added to enhance protein expression. The addition of sodium butyrate was omitted because it was thought that the overloading of the protein production machinery in the cells could have been resulting in misfolded protein and aggregation. The protein was purified in DDM using StrepXT resin and gravity flow chromatography. Tris(2-carboxyethyl)phosphine (TCEP) was

added to all purification buffers, to reduce disulphide bonds and decrease protein aggregation, and biotin was used to elute the protein.

The gel images showed that the protein bound well to the resin and the sample was relatively pure (Figure 3.21). This showed that using a StrepTag purification was better at ensuring specific protein binding than the HisTag purifications. The StrepXT was also more effective at binding the protein than StrepHP because of its stronger binding affinity to the twin strep tag. The protein yield at this point was 100 µg, which is much lower than the His-tag purifications, but the protein sample was a lot purer in this Strep purification. The SEC gels showed stronger protein bands than previous SEC gels, however, the chromatogram showed that all the protein had aggregated (Figure 3.22).

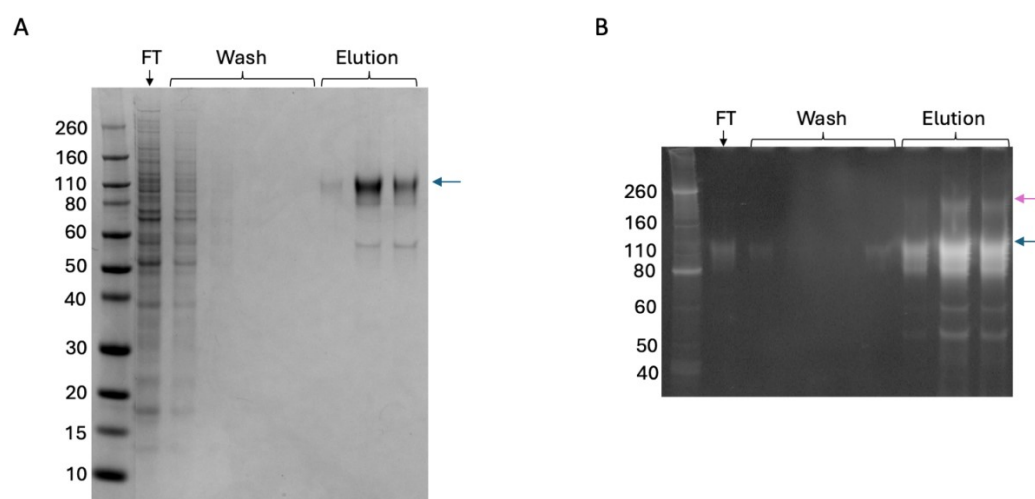


Figure 3.21. *hSVCT2_{Δ1-23} Strep-tag purification using Strep-XT resin resulted in relatively pure protein. hSVCT2_{Δ1-23} in pOPINeNeo-TGP-2Strep-His was expressed in 200 ml Expi-293 cells and expression was enhanced with valproic acid only. TCEP was added to all purification buffers to decrease aggregation. A) Coomassie-stained SDS-PAGE gel of flowthrough (FT), wash and elution samples from StrepXT purification. B) In-gel fluorescence of flowthrough (FT), wash and elution samples from StrepXT purification. Samples were run on a NuPageTM 4-12% Bis-Tris SDS-PAGE Gel, alongside a Novex Sharp Pre-stained Protein Standard. Blue arrow corresponds to the MW of hSVCT2 monomer and pink arrow corresponds to the MW of hSVCT2 dimer.*

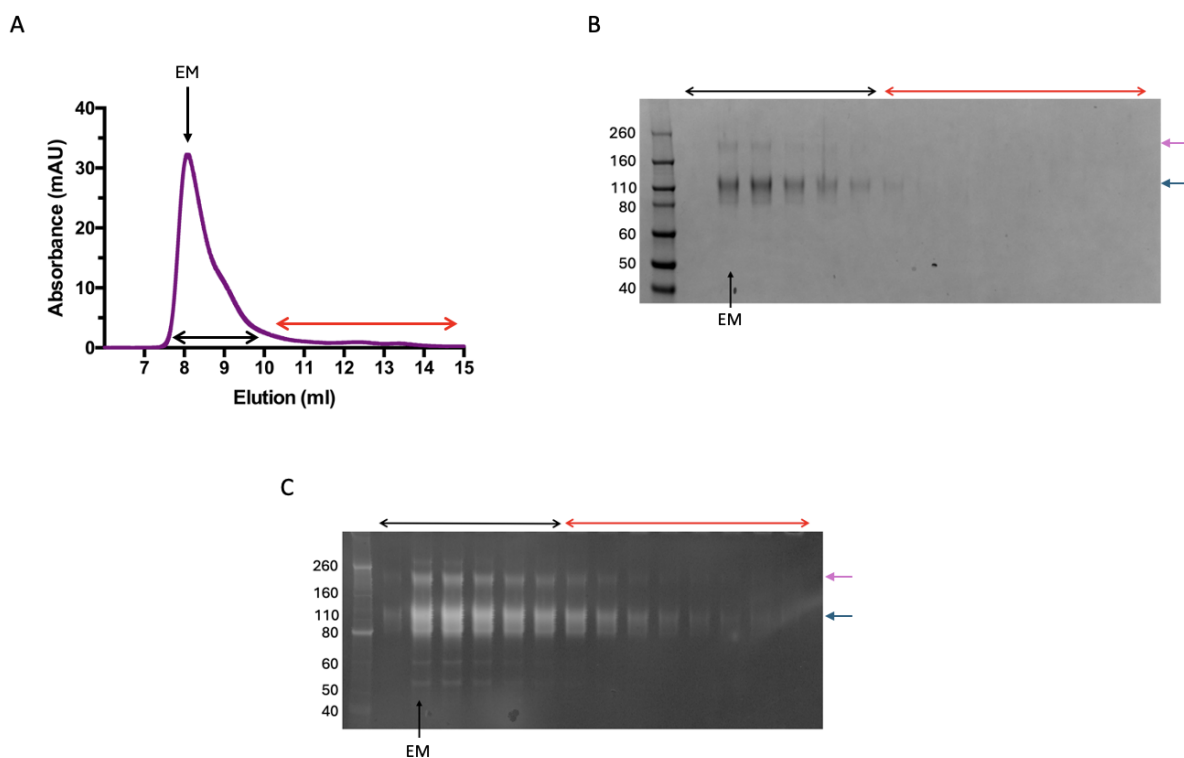


Figure 3.22. SEC of hSVCT2_{Δ1-23} Strep-tag purification using Strep-XT resin showed protein aggregation. A) SEC UV absorbance spectra of hSVCT2_{Δ1-23} purified in DDM after affinity chromatography. B) Coomassie-stained SDS-PAGE gel of SEC fractions. C) In-gel fluorescence of SEC fractions. Samples were run on a NuPageTM 4-12% Bis-Tris SDS-PAGE Gel, alongside a Novex Sharp Pre-stained Protein Standard. Black arrows on both SEC chromatogram and gels correspond to protein aggregation. Blue arrow corresponds to the MW of hSVCT2 monomer and pink arrow corresponds to the MW of hSVCT2 dimer. EM – fraction taken for negative stain electron microscopy.

0.01 mg/ml of protein sample after the StrepXT purification step (Figure 3.21) and 0.01 mg/ml of the peak SEC fraction (Figure 3.22) were used to make negative stain grids. The peak SEC fraction was used for negative stain EM to confirm that that peak was in fact the aggregation peak and the post-StrepXT sample was used to check if aggregation happened during the SEC step or prior to that. Both micrographs showed the presence of aggregation, suggesting that aggregation occurred before the SEC step (Figure 3.23).

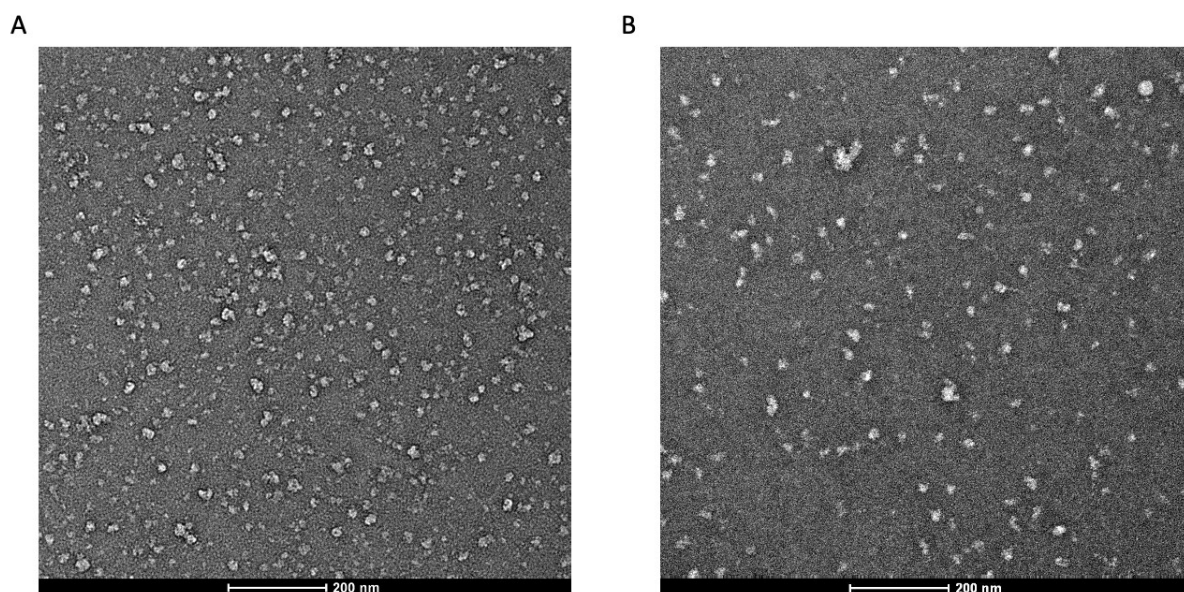


Figure 3.23. Negative stain micrographs of hSVCT2 $_{\Delta 1-23}$ purified in DDM showed the presence of large and mostly irregular particles which can be assumed is aggregation. A) 0.01 mg/ml of post-affinity chromatography protein sample and B) 0.01 mg/ml of SEC peak fraction was applied to the grid and stained for 40 seconds in 2% uranyl acetate.

With this purification, the purity of the protein was significantly improved. However, protein aggregation was still a problem that needed to be solved. Therefore, for the following purification several changes were introduced to buffer components to help improve protein stability.

3.3.8.3. Changes to buffer components increased hSVCT2 stability

hSVCT2 $_{\Delta 1-23}$ in a pOPINeNeo-TGP-2Strep-His vector was expressed in 200 ml Expi-293 cells. Several changes were introduced to buffer components to help decrease protein aggregation. I added trehalose to the cell and membrane resuspension buffers because it was found to be a great stabiliser for several proteins (Ahlgren et al., 2023, Sola-Penna and Meyer-Fernandes, 1998). I also increased the concentrations of NaCl and TCEP to see if that could help with protein stability. I added glutamic acid to the solubilisation buffer and the affinity buffers because glutamic acid could help improve protein stability and decrease aggregation (Arakawa et al., 2007). Instead of using DDM, I purified the protein in LMNG and CHS. Kobayashi et al. (2024) reported that the dimeric form of hSVCT1 was mainly present

when the protein was solubilised in LMNG-CHS. Due to the importance of dimer formation in hSVCT1 function as well as the advantages of higher molecular weights and symmetry that dimers provide, I purified hSVCT2 in LMNG-CHS.

The affinity gel showed even purer protein bands than the previous purifications, although the bands were fainter (Figure 3.24A). The protein yield at this point was 282 μ g, which was about 3 times more than the previous purification. The SEC chromatogram showed a much lower aggregation peak (elutes about 8.5 ml), suggesting that the changes made to the buffers helped to reduce protein aggregation. There also seems to be more distinct separation of two hSVCT2 peaks, likely the dimeric (10 ml, EM1) and monomeric forms (11 ml, EM2) of the protein (Figure 3.24B). Peak fractions from these two peaks were therefore used to prepare negative stain grids. Unfortunately, the SDS-PAGE gel for this purification was lost and is therefore not shown.

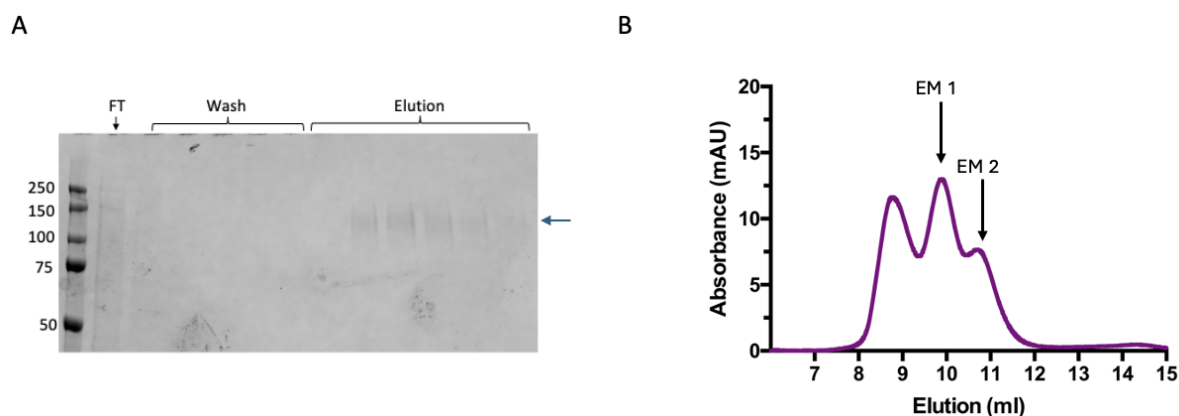


Figure 3.24. *hSVCT2_{Δ1-23} Strep-tag purification in LMNG-CHS. hSVCT2_{Δ1-23} in pOPINeNeo-TGP-2Strep-His was expressed in 200 ml Expi-293 cells and expression was enhanced with valproic acid only. Trehalose, glutamic acid and higher concentrations of NaCl and TCEP were added to purification buffers to improve protein quality. A) Flowthrough (FT), wash and elution samples from StrepXT purification were run on a Mini-PROTEAN TGX Precast Gel, alongside a Precision Plus Protein Dual Xtra Pre-Stained Protein Standard. Blue arrow corresponds to hSVCT2 monomer. B) SEC UV absorbance spectra of hSVCT2_{Δ1-23} purified in LMNG-CHS after affinity chromatography. EM 1/2 – fractions taken for negative stain electron microscopy.*

The negative stain micrograph for the first peak (EM 1) showed a heterogeneous sample that could be a mixture of the hSVCT2 dimer and aggregation (Figure 3.25A). The micrograph for the second peak (EM 2) at 0.01 mg/ml was very crowded (Figure 3.25B) so another grid at a lower concentration of 0.005 mg/ml was prepared (Figure 3.25C). The lower concentration micrograph showed particles that could be hSVCT2 protein. This grid however was prepared after freeze-thawing the protein so the dramatic decrease in particle number could suggest that hSVCT2 is intolerant to freeze-thawing.

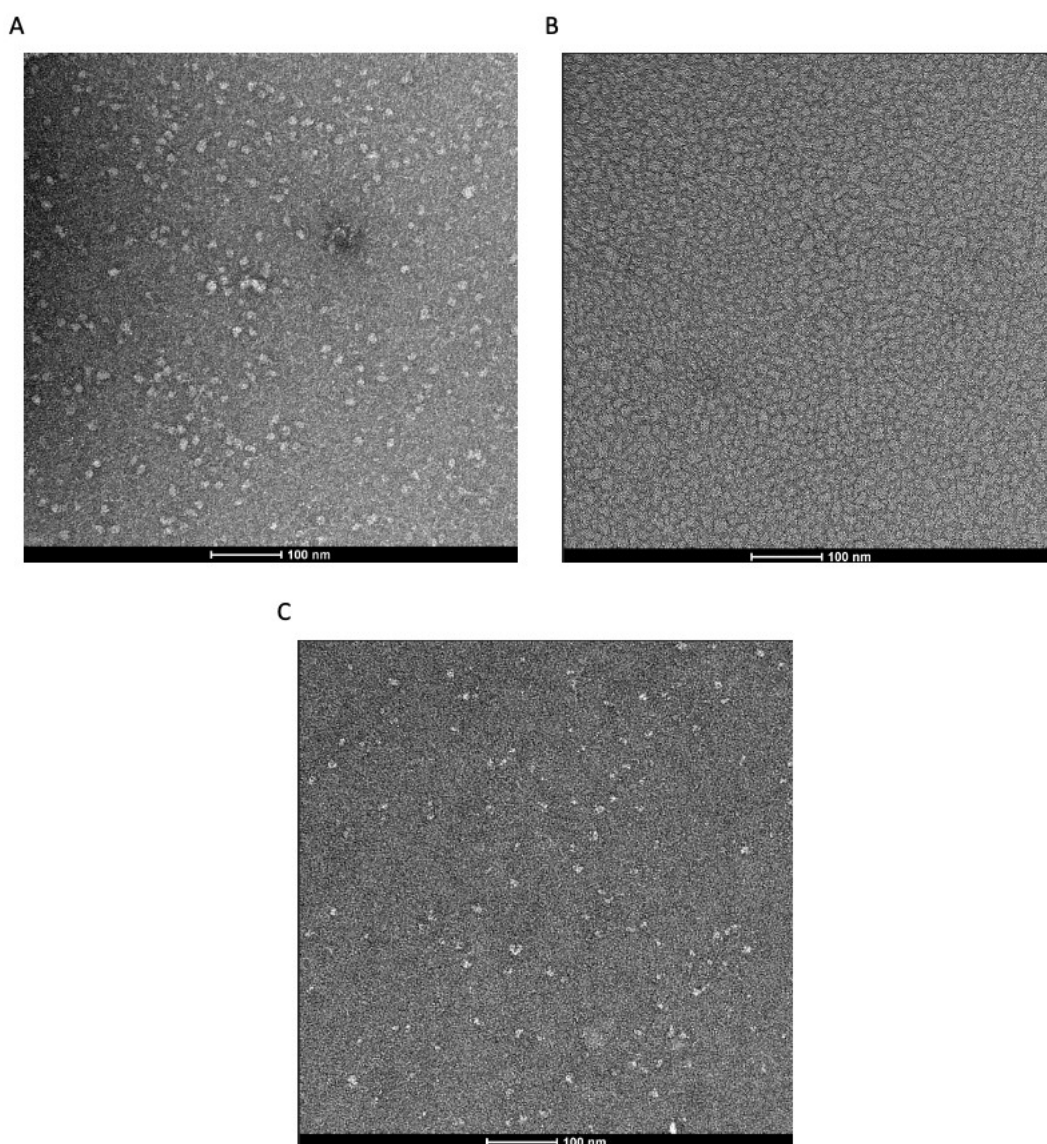


Figure 3.25. Negative stain micrographs of hSVCT2_{Δ1-23} purified in LMNG and CHS showed a heterogenous sample which could be due to different orientations of the protein. A) 0.01 mg/ml of first SEC peak fraction (EM 1), B) 0.01 mg/ml of second SEC peak fraction (EM 2) and C) 0.005 mg/ml of second SEC peak fraction (EM 2) was applied to the grids and stained for 40 seconds in 2% uranyl acetate. A) First micrograph showed particles that could have been the dimeric form of the protein or aggregation. B) Second image was very crowded suggesting that the concentration used was too high. C) In the last micrograph, particles that could be hSVCT2 were visible.

Overall, changes made in this purification greatly improved protein yield and stability. The negative stain micrographs suggested that this protein sample could be taken forward to cryo-EM. Therefore, for the following purification, the volume of culture was increased in order to obtain higher concentrations of protein for cryo-EM. Additional changes to buffer

components were also introduced to further increase protein stability and decrease aggregation.

3.3.8.4. Protein yield was increased for initial cryo-EM analysis

hSVCT2_{Δ1-23} in a pOPINeNeo-TGP-2Strep-His vector was expressed in 400 ml Expi-293 cells. Culture volume was increased to obtain higher protein concentrations for cryo-EM. Ascorbic acid and glutamic acid were added to all purification buffers to further improve protein stability.

The affinity gel showed slightly stronger protein bands than the previous purification, suggesting that protein yield had increased (Figure 3.26). Protein yield at this point was 540 µg, about double the previous purification. The Coomassie-stained SDS-PAGE gel showed bands around 100kDa which represents the monomeric form of the protein and in-gel fluorescence of the same samples showed bands that represented both the monomeric and dimeric forms of hSVCT2 (Figure 3.26B).

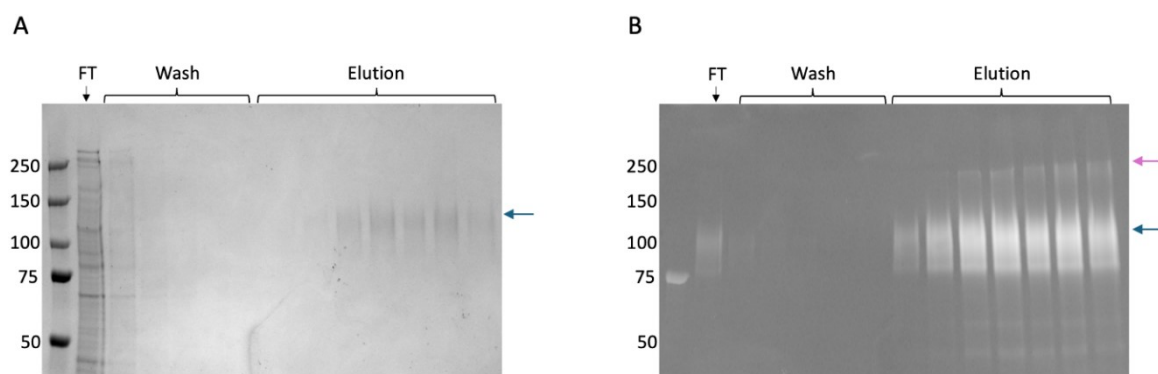


Figure 3.26. hSVCT2_{Δ1-23} Strep-tag purification with ascorbic acid. hSVCT2_{Δ1-23} in pOPINeNeo-TGP-2Strep-His was expressed in 400 ml Expi-293 cells and expression was enhanced with valproic acid only. Ascorbic acid was added to all purification buffers to improve protein stability. A) Coomassie-stained SDS-PAGE gel of flowthrough (FT), wash and elution samples from StrepXT purification. B) In-gel fluorescence of flowthrough (FT), wash and elution samples from StrepXT purification. Samples were run on a Mini-PROTEAN TGX Precast Gel, alongside a Precision Plus Protein Dual Xtra Pre-Stained Protein Standard. Blue arrow corresponds to hSVCT2 monomer and pink arrow corresponds to the dimer.

The SEC chromatogram showed a much higher protein peak than aggregation peak, suggesting that the addition of ascorbic acid and glutamic acid to the SEC buffer helped in stabilising the protein (Figure 3.27A). There was also only one protein peak (~10 ml) unlike previous purifications where two clear peaks (probably representing the dimeric and monomeric forms of the protein) were evident. This suggested that the addition of ascorbic acid stabilises the hSVCT2 dimer. However, the protein peak remains quite broad suggesting that some heterogeneity remains. In addition, the whole SEC chromatogram was noisy suggesting that ascorbic acid has an effect on UV absorbance. The Coomassie-stained SDS-PAGE analysis of the SEC fractions showed faint bands around 100 kDa for the protein peak (shown in red) which represents the monomeric form of the protein and in-gel fluorescence showed bands that represented both the monomeric and dimeric forms of hSVCT2 (Figure 3.27B and C).

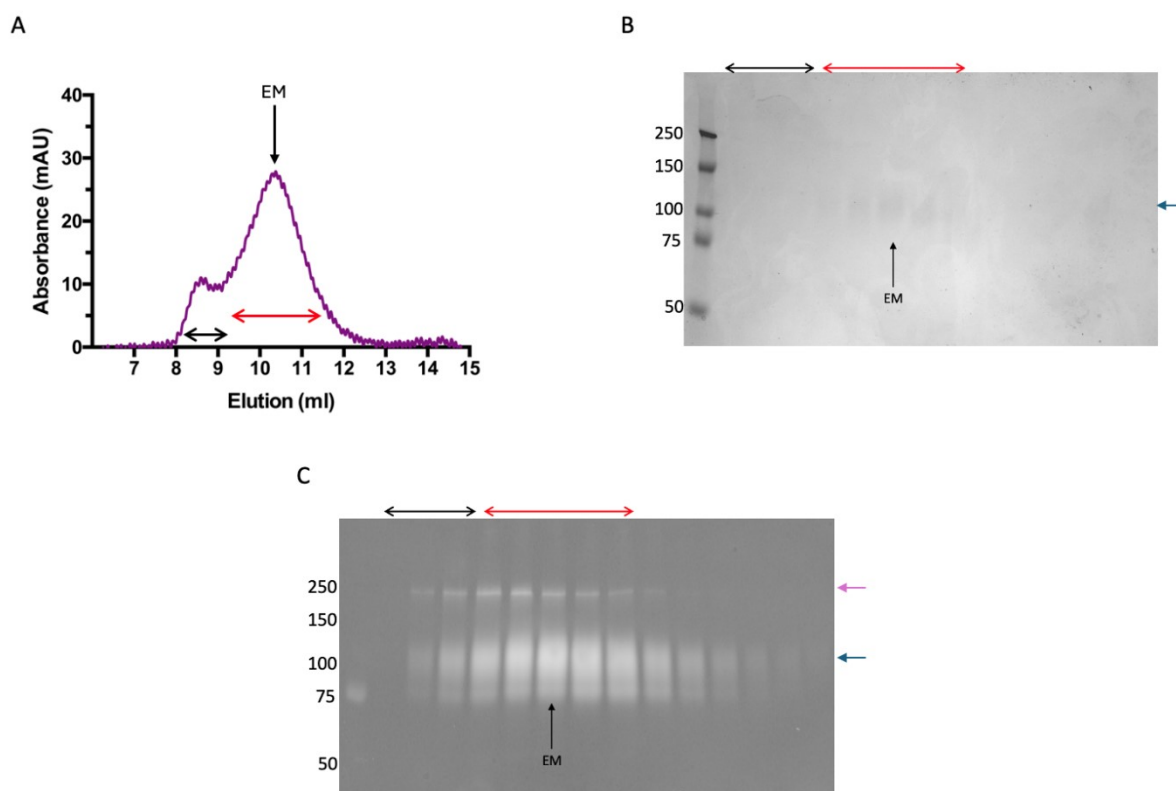


Figure 3.27. SEC of hSVCT2 $_{\Delta 1-23}$ Strep-tag purification with ascorbic acid showed a larger protein peak. A) SEC UV absorbance spectra of hSVCT2 $_{\Delta 1-23}$ purified in LMNG-CHS after affinity chromatography. B) Coomassie-stained SDS-PAGE gel of SEC fractions. C) In-gel fluorescence of SEC fractions. Samples were run on a Mini-PROTEAN TGX Precast Gel, alongside a Precision Plus Protein Dual Xtra Pre-Stained Protein Standard. Black arrows on both SEC chromatogram and gels correspond to protein aggregation. Red arrows correspond to main protein peak. Blue arrow corresponds to hSVCT2 monomer and pink arrow corresponds to the dimer. EM – fraction taken for negative stain electron microscopy and cryo-electron microscopy.

The peak fraction ('EM' in Figure 3.27) was used to prepare negative stain and cryo-EM grids. The negative stain micrographs showed particles of different sizes and shapes which could represent different orientations of the protein and also some degradation (Figure 3.28). The cryo-EM micrograph showed some particles (white arrows) that could be showing hSVCT2 (Figure 3.29). However, the concentration of protein used (0.07 mg/ml) was far too low for cryo-EM to be able to see much. The ice layer was also too thin so the blotting time used of 11 seconds needed to be decreased to obtain correct ice thickness. Therefore, for the following purification an even higher volume of Expi-293 culture was used to increase

protein yield for cryo-EM, without the need of pooling and concentrating the protein, and blotting times were optimised.

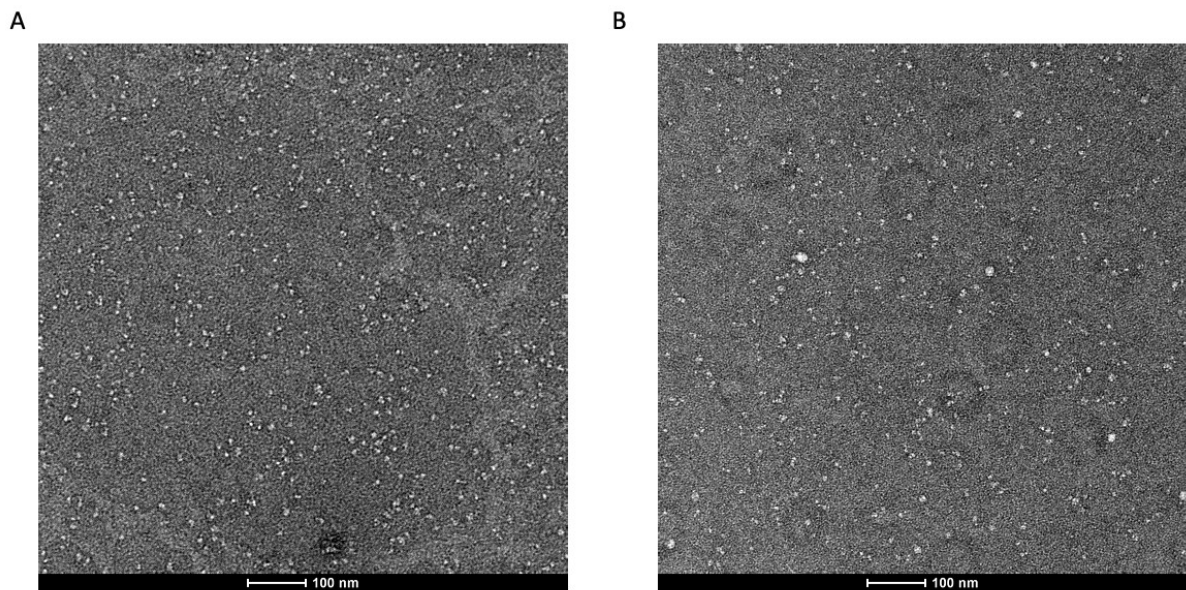


Figure 3.28. Negative stain micrographs of hSVCT2 purified in LMNG and CHS showed particles of different sizes and shapes. A) 0.01 mg/ml and B) 0.005 mg/ml of SEC peak fraction was applied to the grid and stained for 40 seconds in 2% uranyl acetate.

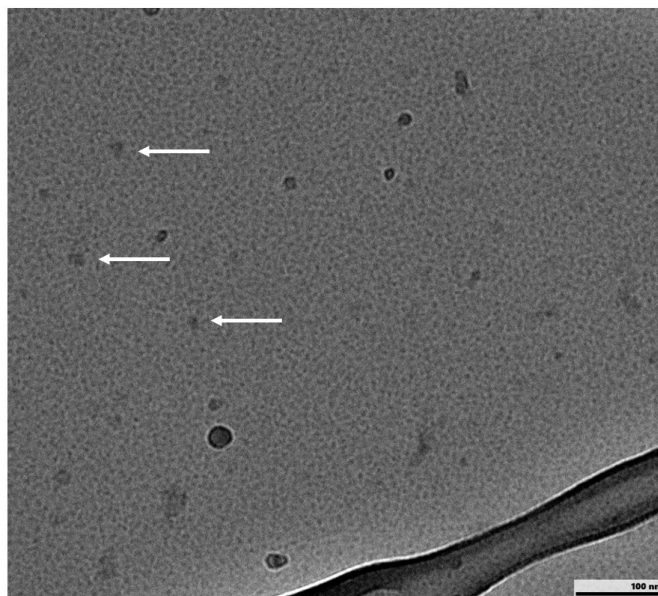


Figure 3.29. Cryo-electron micrograph of hSVCT2 purified in LMNG and CHS showed possible protein particles. 0.07 mg/ml of SEC peak fraction was applied to a UC-A on Lacey 400 mesh Cu grid. Grid was blotted for 11 seconds at 0 blot force. Image was taken on the 120 kV Tecnai 12 Twin electron microscope, using a CCD camera. White arrows show possible protein particles, but protein concentration was too low.

To investigate stability of the isolated hSVCT2, a time- and temperature-dependent stability experiment was completed. The peak protein fractions were pooled and concentrated to 0.8 mg/ml and incubated at 4°C or 20°C for 7 days. An SDS-PAGE gel was then run with samples taken at several time points within the 7 days (Figure 3.30). The protein sample incubated at 4°C remained relatively stable. Degradation bands present between 25 kDa and 37 kDa got stronger with increasing time. At 20°C, the degradation of the protein was more evident with the protein band almost completely disappearing by day 7.

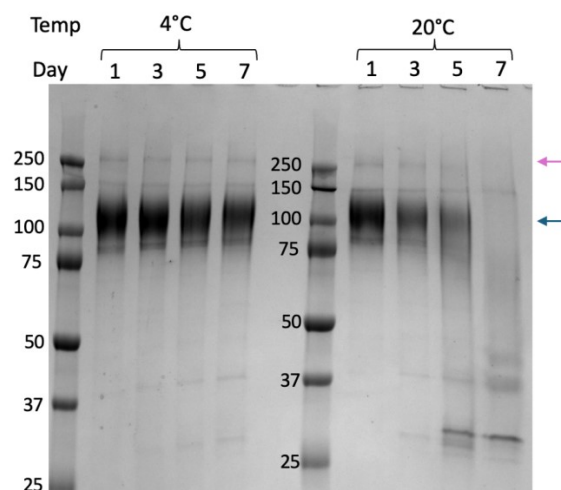


Figure 3.30. *hSVCT2 thermostability assessment showed that the protein is stable at 4 °C for up to 7 days. Samples taken at different timepoints were run on a Mini-PROTEAN TGX Precast Gel, alongside a Precision Plus Protein Dual Xtra Pre-Stained Protein Standard. Protein sample incubated at 4 °C remained relatively stable whereas sample incubated at 20 °C had completely degraded by day 7. Blue arrow corresponds to hSVCT2 monomer and pink arrow corresponds to the dimer.*

3.3.8.5. Large-scale purification was performed for cryo-EM analysis

hSVCT2_{Δ1-23} in a pOPINeNeo-TGP-2Strep-His vector was expressed in 1 L Expi-293 cells to increase protein yield. The only difference between this purification and the previous one was that CHS was removed from the SEC buffer as it was causing clogging of the AKTA Pure inlet valve and the SEC column.

The SDS-PAGE analysis of the fractions obtained from the affinity purification step showed slightly stronger protein bands than the previous purification (Figure 3.31). However, the increase in concentration did not mirror the increase in culture volume from 400 ml to 1 L (540 µg to 500 µg protein yield respectively). It is unclear why the protein yield was lower. A possible explanation can be that the cells used were older so they did not produce as much protein as cells with different passage numbers may respond differently to transfection conditions (Fus-Kujawa et al., 2021).

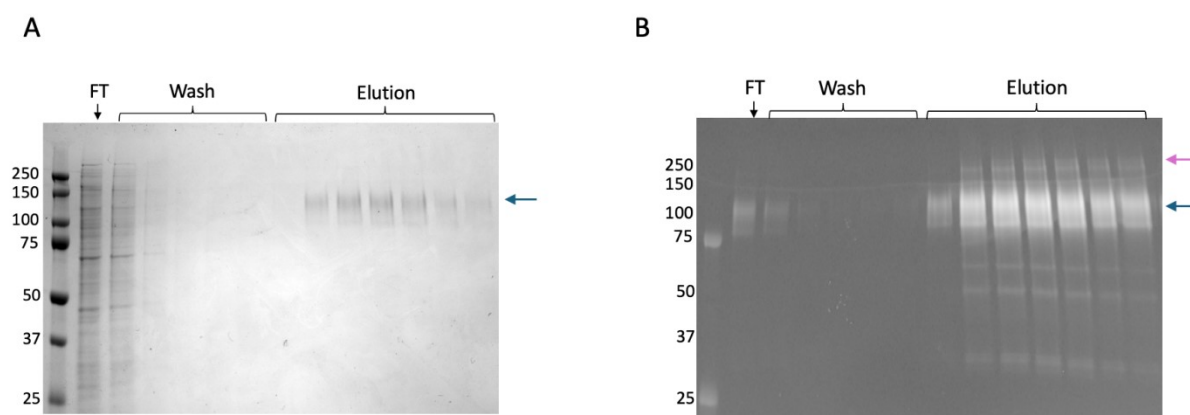


Figure 3.31. Large-scale hSVCT2 $_{\Delta 1-23}$ Strep-tag purification with ascorbic acid. hSVCT2 $_{\Delta 1-23}$ in pOPINeNeo-TGP-2Strep-His was expressed in 1 L Expi-293 cells and expression was enhanced with valproic acid only. Samples were run on a Mini-PROTEAN TGX Precast Gel, alongside a Precision Plus Protein Dual Xtra Pre-Stained Protein Standard. A) Coomassie-stained SDS-PAGE gel of flowthrough (FT), wash and elution samples from StrepXT purification. B) In-gel fluorescence of flowthrough (FT), wash and elution samples from StrepXT purification. Blue arrow corresponds to hSVCT2 monomer and pink arrow corresponds to the dimer.

The SEC chromatogram showed a slightly increased protein peak which again did not mirror the increase in culture volume (Figure 3.32A). The aggregation peak was almost double of the previous purification which could have been a result of removing CHS from the SEC buffer. The chromatogram was also noisy in this purification like the previous one further supporting the hypothesis that ascorbic acid has an effect on UV absorbance. The SDS-PAGE gel of fractions obtained from the SEC column showed stronger bands around 100 kDa for the protein peak (shown in red) which represents the monomeric form of the protein (Figure 3.32B). These bands were confirmed by Western blot analysis (Figure 3.32D).

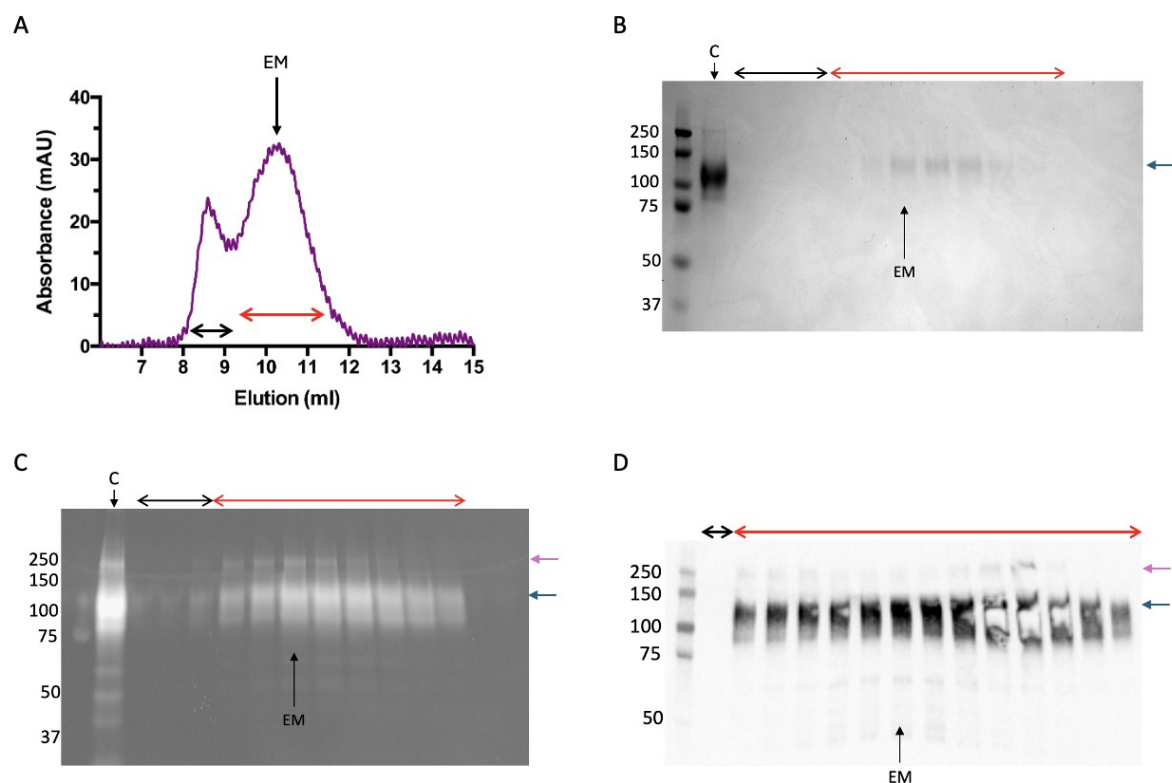


Figure 3.32. SEC of large-scale hSVCT2 $\Delta 1-23$ Strep-tag purification with ascorbic acid. A) SEC UV absorbance spectra of hSVCT2 $\Delta 1-23$ purified in LMNG after affinity chromatography. B) Coomassie-stained SDS-PAGE gel of SEC fractions. C) In-gel fluorescence of SEC fractions. D) Western blot of SEC fractions. Samples were run on a Mini-PROTEAN TGX Precast Gel, alongside a Precision Plus Protein Dual Xtra Pre-Stained Protein Standard. Black arrows on both SEC chromatogram and gels correspond to protein aggregation. Red arrows correspond to main protein peak. C – concentrated post-affinity chromatography sample, EM – fraction taken for negative stain electron microscopy and cryo-electron microscopy.

The peak fraction ('EM' in Figure 3.32) was used to prepare negative stain and cryo-EM grids. The negative stain micrographs showed the presence of aggregation which may be due to the removal of CHS (Figure 3.33). Cryo-EM grids blotted for 8 seconds all resulted in black ice (not shown) suggesting that this blotting time was too short, therefore, a different condition of blotting 3 times for 5 seconds was tried. This blotting time resulted in better ice thickness but further optimisations are still needed (Figure 3.34).

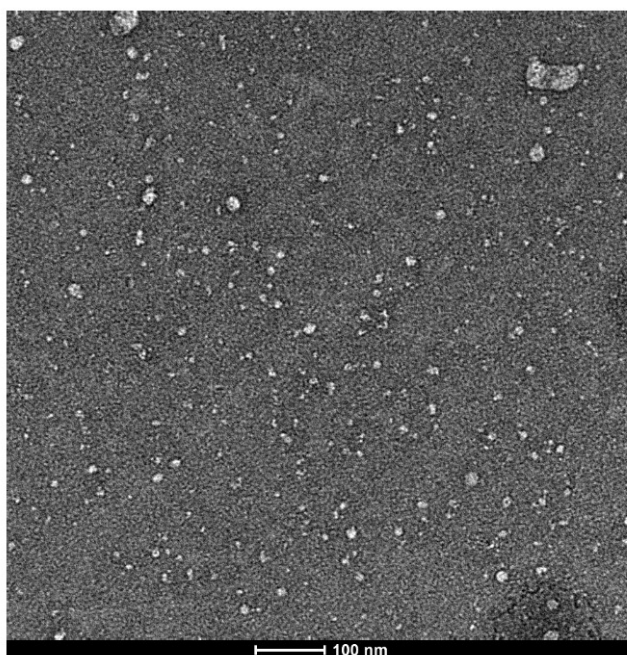


Figure 3.33. Negative stain micrograph of hSVCT2 purified in LMNG showed the presence of aggregation. 0.01 mg/ml of SEC peak fraction was applied to the grid and stained for 40 seconds in 2% uranyl acetate. The presence of aggregation suggested that the removal of CHS from the SEC step might have made the protein more prone to aggregation.

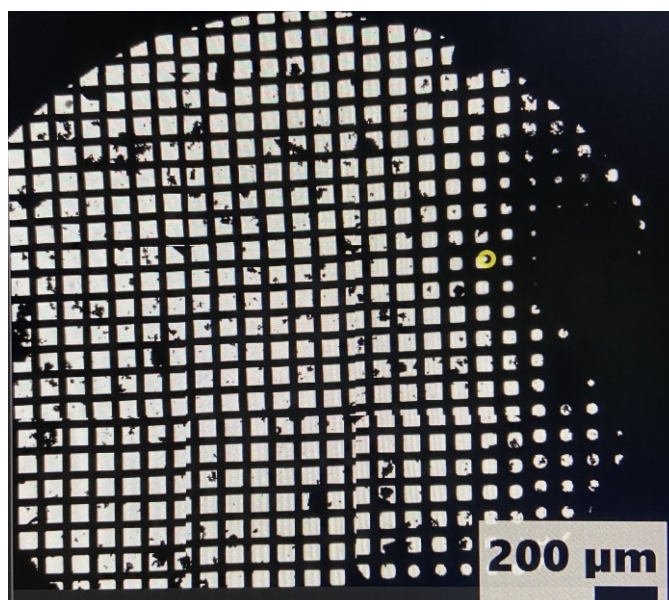


Figure 3.34. Cryo-electron micrograph of hSVCT2 purified in LMNG. 0.2 mg/ml of SEC peak fraction was applied to a Quantifoil R1.2/1.3 + 2nm C Cu 300 grid. Grid was blotted 3 times for 5 seconds at 0 blot force. Grid was screened on the 120 kV Tecnai 12 Twin electron microscope, but unfortunately the camera could not be used to take an image due to drifting.

3.4. Discussion

In this chapter, extensive optimisation of the expression and purification conditions for obtaining stable hSVCT2 for structural studies has been described. The successful expression and purification of hSVCT2 in pOPIN vectors has been reported. Negative stain electron microscopy has been used to assess sample purity and heterogeneity, and preliminary structural studies, using cryo-EM, has also been initiated.

Protein sample optimisation is crucial before embarking on cryo-EM. Vitrified samples are prone to aggregation or denaturation, therefore ensuring that the sample is homogeneous before grid preparation is important (Weissenberger et al., 2021). Main factors that needed addressing to produce appropriate protein samples for structural studies were yield, purity and stability of hSVCT2. According to nanodrop measurements, the His tag purifications were giving much higher yield than the Strep tag purifications. However, the SDS-PAGE gels showed that the samples contained high amounts of contaminants which could have affected nanodrop measurements. The shift to Strep tag purifications resulted in drastic improvements in the purity of the protein samples. Next, to increase protein yield, additional changes were introduced to buffer components during purification. The most dramatic improvement in yield was seen between purifications reported in section 3.3.8.2 and section 3.3.8.3, where trehalose, glutamic acid, higher concentrations of TCEP and NaCl were added, and DDM was replaced with LMNG-CHS. The protein yields pre-SEC increased from 100 µg to 282 µg with the same culture volume of 200 ml. Ideally these changes to buffer conditions would have been introduced sequentially so that the exact change that is resulting in increased protein yield would have been identified. However, due to time constraints all these changes were introduced simultaneously. In the future, these conditions can be introduced one by one to see their effect on the protein and to identify a more effective, and possibly stripped back, list of buffer conditions. The addition of ascorbic acid to the buffers also appeared to stabilise the dimeric, physiological form of the protein. In terms of structural studies, the dimeric form of the protein is beneficial as it has a higher molecular weight and symmetry than the monomeric form (Kobayashi et al., 2024).

In order to obtain higher concentrations of protein for cryo-EM, further optimisations will be required. Adding ascorbic acid to the cells could increase protein yield. Covarrubias-Pinto et al. (2018) reported that culturing cells in the absence of ascorbic acid resulted in hSVCT2 mainly located in intracellular compartments, whereas culturing in the presence of ascorbic acid resulted in a concentration dependent increase in hSVCT2 localisation at the membrane. This suggested that hSVCT2 has an ER regulated mechanism in which cell surface trafficking is substrate dependent (Acuna et al., 2013). Performing small scale expressions to optimise DNA:PEI ratio as well as optimising enhancer concentrations can also help in increasing protein expression (Longo et al., 2013). The simultaneous addition of glutamic acid and arginine could also be explored as this has been shown to increase the solubility of proteins up to 4-8 times. The charged side chains of arginine and glutamic acid can interact with oppositely charged residues on the protein surface and the hydrophobic parts of the amino acids can shield neighbouring exposed hydrophobic residues therefore inhibiting protein aggregation (Golovanov et al., 2004).

In addition to negative stain EM, biophysical characterisations can be performed to assess sample purity, homogeneity and stability before cryo-EM. Dynamic light scattering (DLS) and nano differential scanning fluorimetry (nanoDSF) are techniques that can be used for sample characterisation. DLS can help determine the polydispersity of the protein and nanoDSF can help determine the stability of the protein in different buffers and detergents. These techniques can therefore help in optimising buffer and detergent conditions as well as decreasing aggregation (Niebling et al., 2022). Additionally, a thermostability assay using a 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) dye can also be performed to assess the stability of the protein in different detergents (Alexandrov et al., 2008). The advantage of nanoDSF over a CPM assay is that nano-DSF needs much lower concentrations of protein.

Conditions for cryo-EM grid preparation need to be optimised in order to continue with hSVCT2 structural studies. An ideal cryo-EM grid should have a good concentration of pure and evenly distributed protein particles fixed as a monolayer in thin ice. Forming a thin layer of liquid in the holes of the foil of the grid is important because thinner ice decreases background noise and can result in higher-resolution reconstructions (Glaeser et al., 2016).

Layer thickness can be optimised by changing blotting conditions, such as blot time and blot force, or by adding a thin film of carbon or graphene to the surface of the foil. For the hSVCT2 grids, a blot time of 8 seconds was too short as the images showed black ice and blot times of 11 seconds and 3 x 5 seconds were too long as the sample was dry. Finding a midpoint between these conditions can help obtain an optimal layer of ice. Using different grids, such as ones with a layer of carbon or graphene, can also help with ice thickness as well as helping with low sample concentrations because these layers can induce the adsorption of particles across the holes. The amount of detergent in the sample can also be decreased as detergent can make the sample more sensitive to any evaporation that may occur between blotting and freezing. Evaporation can change the pH, salt and particle concentration leading to protein conformational changes (Passmore and Russo, 2016, Weissenberger et al., 2021). Additionally, as discussed in section 1.4.3, hSVCT2 can be reconstituted into nanodiscs as they are generally better at preventing aggregation and improving sample quality of membrane proteins compared to detergents (Denisov and Sligar, 2016).

Overall, this chapter reports significant improvements in the production of hSVCT2 for structural studies. Several areas of improvement have been identified, such as ways to increase protein yield and stability, quality control experiments and cryo-EM grid optimisations, that could all be used to eventually solve the structure of this protein.

4. Screening of novel detergents using UapA-G411V

Some of the results presented in this chapter are included in the following publication:

YOON, S., BAE, H. E., HARIHARAN, P., NYGAARD, A., LAN, B., WOUBSHETE, M., SADAF, A., LIU, X., LOLAND, C. J., BYRNE, B., GUAN, L. & CHAE, P. S. 2024. Rational Approach to Improve Detergent Efficacy for Membrane Protein Stabilization. *Bioconjug Chem*, 35, 223-231.

4.1. Introduction

As already discussed in section 1.4, membrane proteins need to be extracted from the membrane lipid bilayer and this needs to be replaced with an amphiphilic system in order to maintain the protein in a functional and folded state in aqueous solution. Detergents are the most commonly used amphiphilic system to solubilise membrane proteins. At their CMC, detergents are able to form micelles that disrupt the interactions that hold the membrane together. They form hydrophobic interactions with the hydrophobic regions of the proteins and lipids, forming detergent-protein-lipid, detergent-protein and detergent-lipid complexes. These detergent micelles are therefore able to stabilise the membrane protein outside its natural lipid bilayer environment (Lee et al., 2022, Orwick-Rydmark et al., 2016, le Maire et al., 2000).

Even though detergents are the most-widely used tool for membrane protein extraction, proteins solubilised in popular detergents, such as DDM and OG, tend to lose their function over the course of sample preparation (Hussain et al., 2017, Lee et al., 2022). This is either due to denaturation or aggregation of the membrane protein (Bae et al., 2020). In order to increase the efficacy of detergents, there are a few factors to take into consideration. Detergents are amphipathic molecules that consist of a polar head group and a hydrophobic non-polar tail group (Seddon et al., 2004). If the hydrophobic tail is too short then the detergent is not as efficient in stabilising the protein and if it is too long then its ability to solubilise the protein is reduced (Urner et al., 2023). The hydrophilic-lipophilic balance (HLB) of detergents is also important in increasing membrane protein stability (Bae et al., 2020). If the detergent is too hydrophilic then it binds weakly to the protein which can result in

aggregation and if the detergent is too hydrophobic it interacts with the protein too strongly potentially destabilising its structure (Chae et al., 2013). The size of the micelles is also an important factor to consider. For example, DDM forms relatively large micelles which effectively covers the hydrophobic regions of the protein, however, this can be disadvantageous for structural studies, such as XRC, as it can shield regions of the protein that are important for crystal lattice formation. On the other hand, OG forms smaller micelles which is beneficial for XRC but the exposure of more of the protein, including hydrophobic regions, can lead to protein aggregation and precipitation (Yoon et al., 2024, Ostermeier and Michel, 1997).

Conventional detergents are limited in their ability to stabilise membrane proteins because they typically contain a single alkyl chain and a single head group (Hussain et al., 2017). DDM contains a maltose head group and a C12 alkyl chain whereas OG contains a glucose head group with a C8 alkyl chain (Figure 4.1) (Bae et al., 2020). They therefore do not account for the different functions and 3D structures of membrane proteins. Thus, it is important to develop new detergents that have enhanced efficacy towards membrane proteins. Several improvements have been introduced to conventional detergents, such as DDM and OG, to increase their efficacy at stabilising membrane proteins. Second-generation detergents, such as lauryl maltose neopentyl glycol (LMNG) and glucose neopentyl glycol (GNG), dimers of DDM and OG respectively linked by a quaternary carbon atom, were shown to be as efficient as OG and DDM at extracting protein and even better at stabilising them (Chae et al., 2010). These second-generation detergents have contributed to the determination of a substantial number of membrane structures.

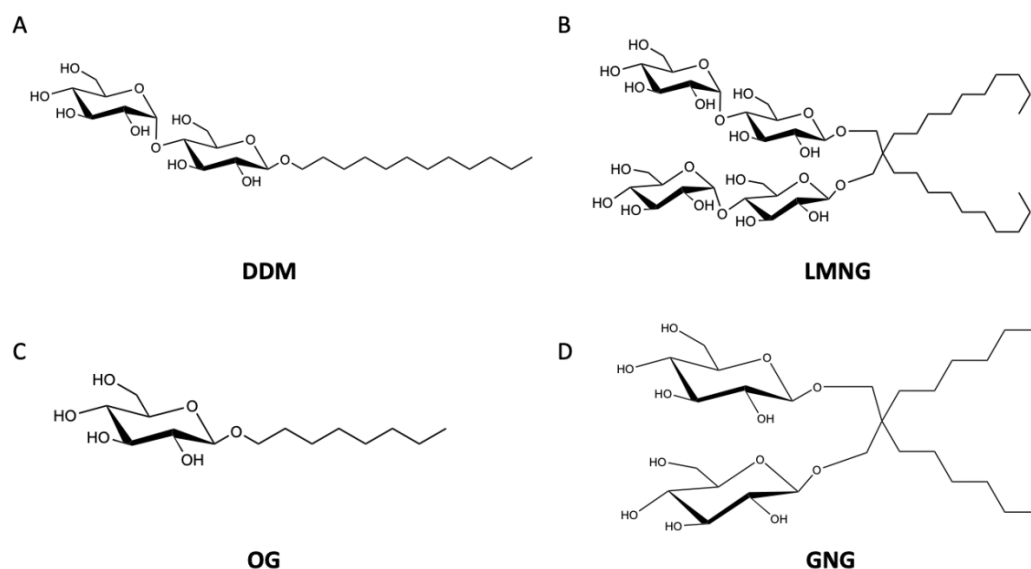


Figure 4.1. Chemical structures of A) *n*-Dodecyl- β -D-maltoside (DDM), B) lauryl maltose neopentyl glycol (LMNG), C) *n*-octyl- β -D-glucoside (OG) and D) glucose neopentyl glycol (GNG) (Woubshete et al., 2024b).

A series of detergents are usually tested to determine the detergent that extracts the largest quantity of soluble, active and stable protein (Carpenter et al., 2008). A thermal denaturation assay, using CPM dye, and fluorescence size-exclusion chromatography (FSEC) are some ways in which detergents can be screened to find the best detergent for a specific protein. The former is based on the accessibility of native cysteine residues available upon protein unfolding. CPM is a highly reactive thiol-specific fluorochrome which is nonfluorescent in its unbound form but fluoresces upon binding to cysteine residues (Alexandrov et al., 2008). Upon heating, the protein starts to unfold making more cysteine residues available to bind to the CPM dye and produce a fluorescent signal; this fluorescence signal can be monitored to determine the stability of the protein in different detergents. FSEC is a technique used for fluorescent-tagged proteins to gain information on the expression level, monodispersity and approximate molecular mass of the protein (Kawate and Gouaux, 2006). The advantage of FSEC is that large-scale protein expression or protein purification are not needed, which makes it a rapid process to screen the stability of proteins in different detergents.

In this chapter, the ability of two novel glucoside families of detergents, designed and synthesised in the laboratory of Professor Pil Seok Chae (Hanyang University, South Korea), to both stabilise and effectively extract membrane protein was investigated. The tandem malonate-based glucosides (TMGs) and the multiple pendant detergents (MPDs) were assessed to see if they were effective at stabilising UapA-G411V using the CPM assay and FSECs.

4.1.1. TMG Detergents

Tandem malonate-based glucosides (TMGs) are novel glucosides synthesised by connecting two malonate-based core units. They contain two branched diglucosides and two alkyl chains as head and tail groups respectively. Previously, TMG-As and TMG-Ts were designed and tested (Hussain et al., 2017). These molecules contain two malonate units connected at their hydrophilic-lipophilic interfaces with either a propylene or dimethyl sulfide linker in the TMG-As and TMG-Ts respectively (Figure 4.2). TMG-As/Ts were tested with several membrane proteins and they showed greater protein stability than DDM. TMG-A13 was shown to be generally effective at stabilising membrane proteins (Hussain et al., 2017). However, in these sets of detergents there was a large gap between the two alkyl chains; consequently, the micelles formed by these detergents had suboptimal alkyl chain density which limited the efficacy of these detergents for membrane protein stabilisation.

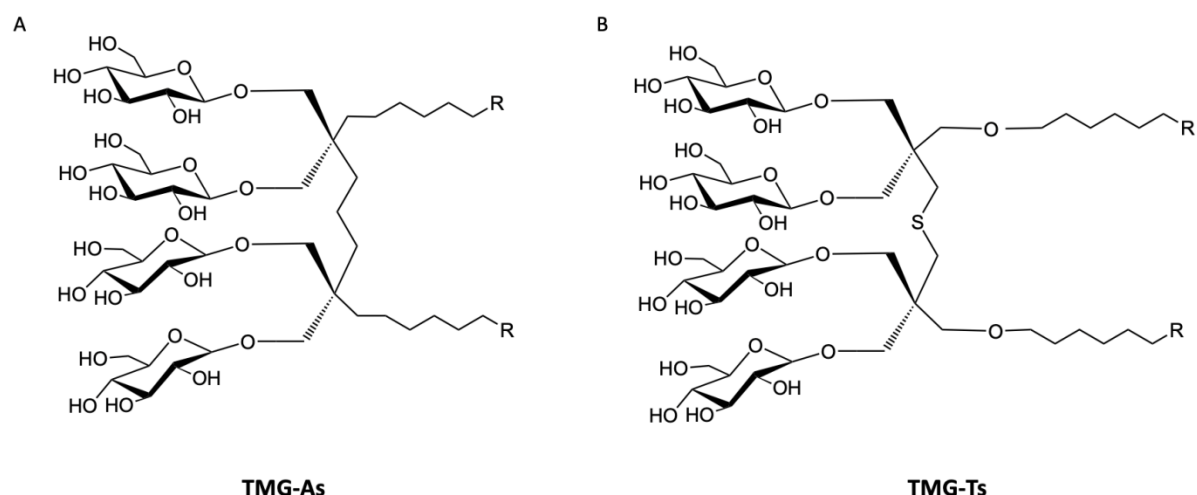


Figure 4.2. Chemical structures of the tandem malonate-based glucosides, TMG-As/Ts. These TMGs contain two branched di-glucosides connected with a propylene link in TMG-As (A) and a dimethyl sulfide link in TMG-Ts (B) (Hussain et al., 2017).

In order to increase the ability of the TMG detergents to stabilise membrane proteins in aqueous solution, the TMG-Ms and TMG-Ps were designed by Professor Pil Seok Chae's laboratory. In the TMG-Ms, the two alkyl chains were connected by a methylene spacer instead of a propylene spacer as in TMG-As. In TMG-Ps, an additional alkyl chain was added between the two alkyl chains of the TMGs using an ether linkage (Figure 4.3) (Yoon et al., 2024). TMG-Ms have a shorter inter-alkyl chain distance and TMG-Ps have an additional alkyl chain which in both cases increases the alkyl chain density. It was hypothesised that this increase in alkyl chain density within the detergent micelle should make the TMG-Ms/Ps more effective at stabilising membrane proteins than the TMG-As/Ts. In this chapter, I compared the ability of TMG-Ms/Ps at stabilising UapA-G411V to the conventional detergents, DDM and LMNG, and to the previously tested TMG-A13. To find an optimal HLB, crucial for membrane protein stability, the alkyl chain length was varied from decyl (C10) to tetradecyl (C14) in TMG-Ms. In the TMG-Ps, the two outer alkyl chains were varied from octyl (C8) to undecyl (C11) and the newly introduced inner alkyl chain was varied from hexyl (C6) to nonyl (C9) (Yoon et al., 2024).

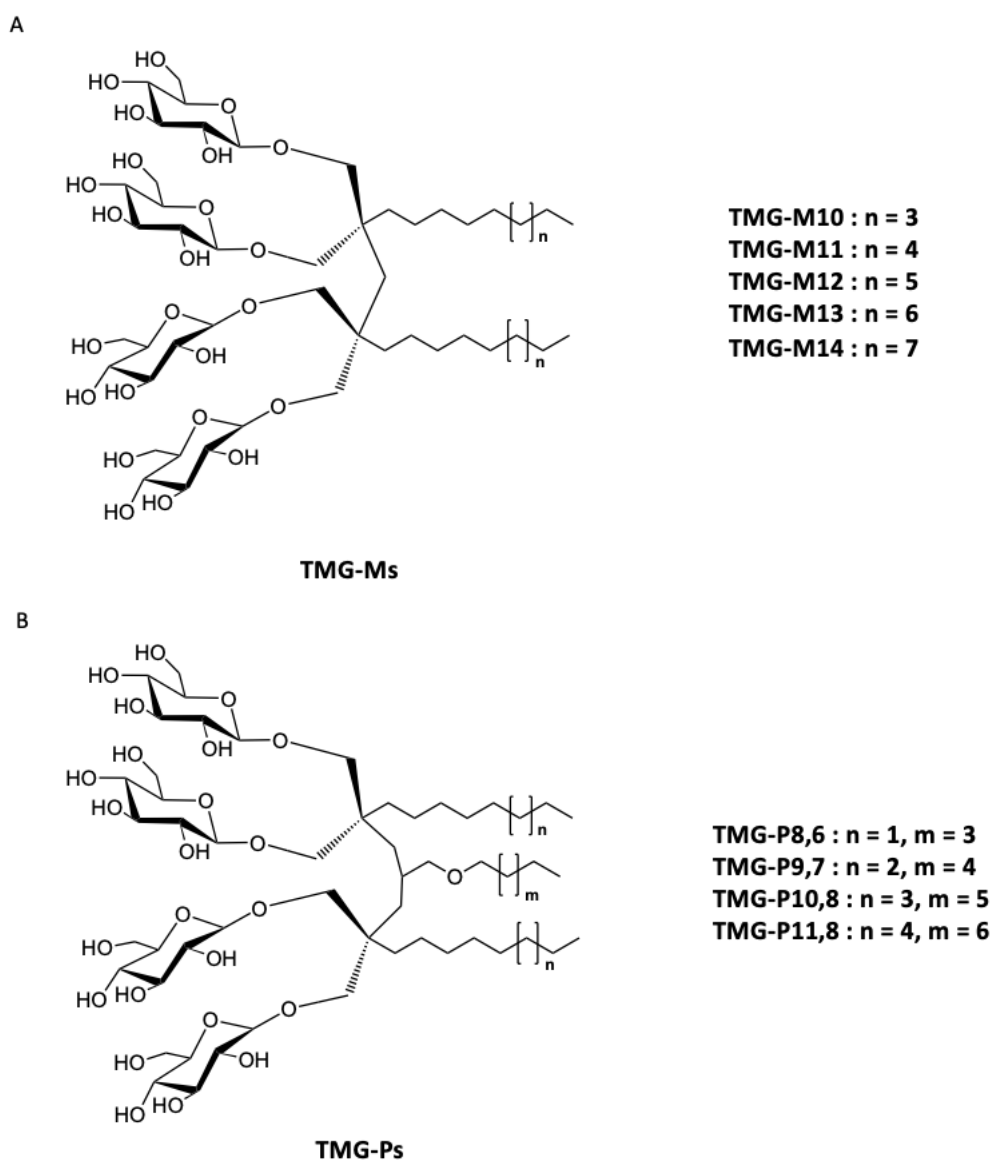


Figure 4.3. Chemical structures of the tandem malonate-based glucosides, TMG-Ms/Ps. These TMGs contain two branched di-glucosides connected with by a methylene space in TMG-Ms (A) and an alkyl chain in TMG-Ps (B) (Yoon et al., 2024).

4.1.2. MPD Detergents

Glucoside detergents are typically not as effective at stabilising membrane proteins as maltoside detergents, such as DDM, because they form smaller micelles leaving more of the protein exposed; therefore, designing new glucoside detergents that provide increased protein stability is important. To this end, OGNG (dimer of OG) variants named pendant-bearing glucose-neopentyl glycol (P-GNGs) were previously designed and tested on some membrane proteins. These P-GNGs contain a branched glucoside headgroup, a main alkyl

chain and a short pendant chain located at the hydrophobic-hydrophilic interface (Figure 4.4). The presence and location of the pendant chain increases the alkyl chain density in the detergent micelles resulting in increased micellar stability because of the strengthened hydrophobic interactions between the detergent alkyl chains. The P-GNGs were efficient at extracting and stabilising all the membrane proteins tested compared to DDM (Bae et al., 2020).

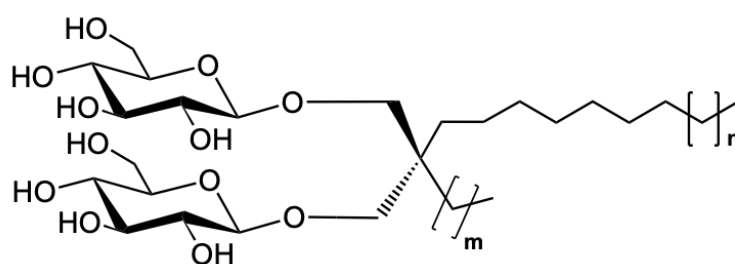


Figure 4.4. Chemical structure of the pendant-bearing glucose-neopentyl glycol detergents (P-GNGs). P-GNGs contain a branched diglucoside group, a long alkyl chain ($n=3-6$) and a short pendant chain ($m=0-2$) (Bae et al., 2020).

As previously stated, the pendant chain is located in the empty spaces present in the hydrophobic-hydrophilic interfaces of detergent micelles. It is difficult to predict the size of these empty spaces in the interfaces, therefore, to effectively increase the alkyl chain density in the detergent micelles, multiple pendant detergents (MPDs) were designed with varying number of pendants (di-pendant and quad-pendant) or varying pendant chain length (methyl to propyl, C1 to C3). The main alkyl chain length was also varied from a decyl to a dodecyl group (C10 to C12) (Figure 4.5). In this chapter, the efficacy of these MPDs at stabilising UapA-G411V was tested and compared to DDM.

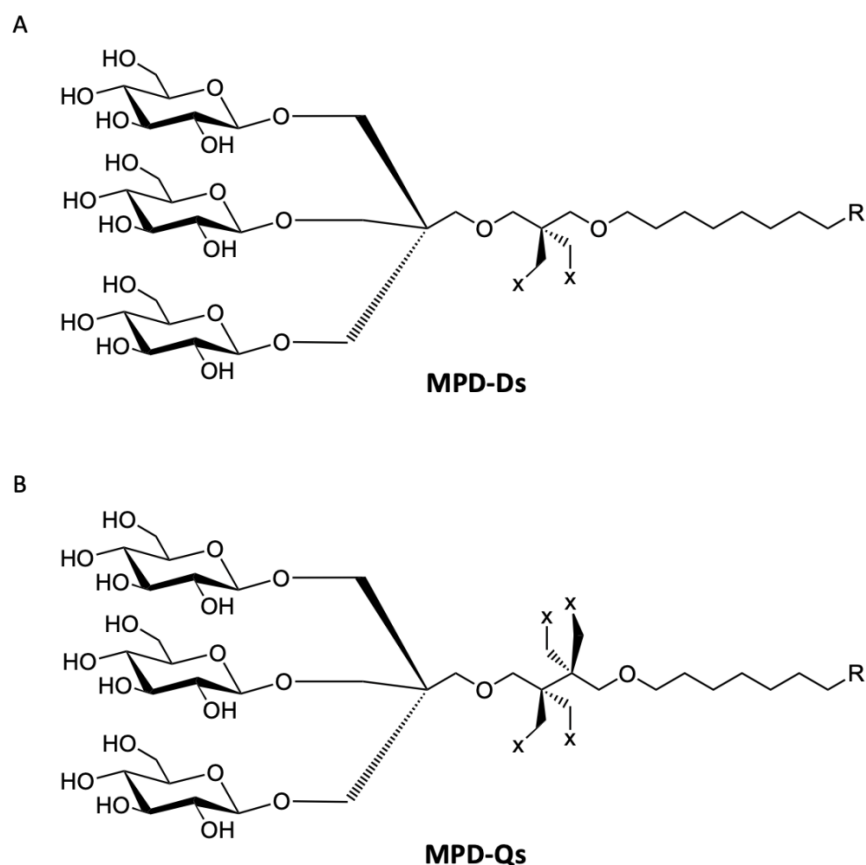


Figure 4.5. Chemical structures of the multiple pendant detergents (MPDs). MPDs contain branched glucoside groups, a long alkyl chain (C10 to C12) and two (MPD-Ds) or four (MPD-Ps) short alkyl chains denoted by x (C1 to C3) (Structures were obtained from Prof Pil Seok Chae laboratory Hanyang University, South Korea).

4.2. Materials and Methods

4.2.1. Expression of UapA-G411V in *S. cerevisiae*

UapA-G411V was grown in 350 ml URA medium, supplemented with 2% glucose at 30°C and 250 RPM, and then diluted in URA medium to $OD_{600} = 0.16$ (6x 1 L; 0.1% glucose). The cultures were incubated at 30°C and 250 RPM until $OD_{600} = 0.6$. Expression was then induced with the addition of 2% galactose. The culture was incubated overnight (30°C, 250 RPM, 22 hours), cells were pelleted (4°C, 1500 x g, 10 minutes), resuspended in cell resuspension buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.6 M sorbitol) and stored at -80°C.

4.2.2. Cell lysis and UapA-G411V purification

Cells were thawed and 1 complete EDTA- free protease inhibitor (SIGMAFAST™, Sigma-Aldrich) was added per 50 ml of sample. The cells were lysed under sequential compression (at 25, 30, 33, 36 kPSI) through a Constant systems cell disruption system. Unbroken cells were pelleted (4°C, 10000 x g, 10 minutes) and discarded. The membranes were pelleted (4°C, 100000 x g, 2 hours) and homogenised in buffer containing 20 mM Tris pH 7.5, 0.3 M sucrose and 0.1 mM CaCl₂. These were either used immediately or stored at -80°C for later use.

Membrane solubilisation was achieved using gentle stirring for 1 hour at 4°C in 200 ml solubilisation buffer (1x PBS, 100 mM NaCl, 10% glycerol, 1% DDM), supplemented with complete protease inhibitor tablets. The sample was centrifuged at 100000 x g and 4°C for 45 minutes. Imidazole at a final concentration of 10 mM was added to the soluble supernatant, and the solution incubated with Ni-NTA resin equilibrated in affinity buffer (1x PBS, 100 mM NaCl, 10 mM imidazole pH 7.6, 10% glycerol and 0.03% DDM). The resin was added to a gravity column and washed with 100 ml affinity buffer and 300 ml wash buffer (1x PBS, 100 mM NaCl, 30 mM imidazole pH 7.6, 10% glycerol and 0.03% DDM). The protein was eluted with 50 ml elution buffer (1x PBS, 150 mM NaCl, 10% glycerol, 310 mM imidazole, 0.03% DDM). In order to cleave the GFP and His-tag, TEV protease was added at a molar ratio of 1:1 between TEV and GFP. The mixture was dialysed with 1500 ml dialysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol and 0.03% DDM) in 12 kDa MWCO dialysis tubing for 16 hours. Aggregates were pelleted (3000 x g, 4°C, 5 minutes) and the supernatant was filtered through a 25 mm 0.2 mm low protein binding syringe filter. Imidazole was added to a final concentration of 12 mM, and the sample loaded onto a 5 ml HisTrap column that had been equilibrated with equilibration buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.03% DDM and 15 mM imidazole). The flow through containing untagged UapA was concentrated to ~2 ml using 100 kDa MWCO concentrators and was injected onto a Superdex 200 Increase 10/300 GL column equilibrated with SEC buffer (20 mM Tris pH 7.5, 150 mM NaCl and 0.03% DDM).

4.2.3. SDS-PAGE gel electrophoresis

See section 3.2.12.

4.2.4. UapA-G411V thermostability assay by CPM assay

The assay solution was prepared in a 96-well black, clear bottom plate with each detergent at CMC+0.04% or CMC+0.2% in 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 μ L of 10 mg/ml UapA-G411V in a total volume of 150 μ L. CPM dye was dissolved at 4 mg/ml in DMSO and diluted 1:100 in 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.03% DDM. 3 μ L of CPM dye was added to each well in the dark and the plate was incubated at 40°C for 120 minutes. The fluorescence emission was monitored every 5 minutes using a SpectraMax M2 (Molecular Devices) with an excitation wavelength of 387 nm and emission wavelength of 463 nm. The raw fluorescence was normalised to the most unstable condition to calculate the percentage of folded protein.

4.2.5. FSEC and hFSEC of UapA-G411V

Membranes were prepared as described in section 4.2.2. Novel detergents were added to 1 ml membranes to a final concentration of 1% detergent and incubated for 1 hour at 4°C. The individual solubilisation samples were centrifuged for 1 hour at 20000 x g and 4°C. The fluorescence emission was measured before and after centrifugation using a SpectraMax M2 (Molecular Devices) with an excitation wavelength of 488 nm and emission wavelength of 512 nm in order to calculate the solubilisation efficiencies.

The solubilised protein samples obtained in each detergent were individually injected onto a Superose 6 Increase 10/300 GL column pre-equilibrated with FSEC buffer (20 mM Tris pH 7.5, 150 mM NaCl and 0.03% DDM). For heated FSEC (hFSEC), the samples were heated for 10 minutes at 45°C prior to injection. The eluate was collected in 200 μ L fractions in a 96-well black/clear bottom plate and fluorescence was measured as above.

4.3. TMG results

4.3.1. TMG detergents had lower CMC values than DDM

The following results were obtained by the laboratory of Professor Pil Seok Chae (Hanyang, University, South Korea). The CMCs of the TMG detergents ranged from 4 – 20 μM , markedly lower than DDM, 170 μM . For both the TMG-Ms and TMG-Ps, the CMCs decreased as the alkyl chain lengths increased. In general, the hydrodynamic radius (R_h) values for the TMG-Ps were higher than the TMG-Ms, showing that the TMG-Ps form larger micelles due to the additional alkyl chain increasing the volume of the hydrophobic groups. All the TMGs were ~10% water-soluble except for TMG-P11,9 which formed a gel at ~5%; this low water solubility may explain why TMG-P11,9 forms the largest micelles of ~8.7 nm (Table 4.1) (Yoon et al., 2024).

Table 4.1. TMG detergent properties: molecular weight (MW), critical micelle concentration (CMC), hydrodynamic radius (R_h) and water solubility (results obtained in Professor Pil Seok Chae laboratory, Hanyang University, South Korea).

Detergent	MW (g/mol)	CMC (μM)	CMC (wt%)	R_h (nm)	Solubility (wt%)
TMG-M10	1093.31	~10	~0.0011	2.6 ± 0.1	~10
TMG-M11	1121.36	~9	~0.0010	2.7 ± 0.2	~10
TMG-M12	1149.41	~7	~0.0008	2.9 ± 0.1	~10
TMG-M13	1177.47	~6	~0.0007	3.2 ± 0.3	~10
TMG-M14	1205.52	~4	~0.0005	3.3 ± 0.4	~10
TMG-P8,6	1179.44	~20	~0.0024	2.9 ± 0.1	~10
TMG-P9,7	1221.52	~15	~0.0018	3.8 ± 0.8	~10
TMG-P10,8	1263.60	~10	~0.0013	8.7 ± 2.8	~10
TMG-P11,9	1305.68	~6	~0.0008	-	~5
TMG-A13	1205.52	~6	~0.0007	3.6 ± 0.2	~10
DDM	510.62	170	0.0087	3.4 ± 0.1	~10

4.3.2. TMG detergents were better at stabilising UapA-G411V than DDM

To determine the efficacy of the TMG detergents to stabilise UapA-G411V, a thermostability assay, using CPM dye, was performed. The protein was mixed with the different detergents at two different concentrations of CMC+0.04% and CMC+0.2% and the unfolding of the protein was monitored over time.

The results showed that all of the detergents were better at stabilising the protein than DDM and LMNG (Figures 4.6 and 4.7). At CMC+0.04%, TMG-M14 was the least stabilising detergent and at CMC+0.2%, TMG-P11,9 was the least stabilising detergent although they were both still more stabilising than DDM. At both concentrations of detergents, TMG-M11 and TMG-P8,6 were the most stabilising detergents with TMG-M11 being the better of the two. TMG-A13, TMG-M13, TMG-P9,7 and TMG-P10,8 all had similar stabilising effects on UapA. Therefore, TMG-M11, TMG-M13, TMG-P8,6, TMG-P9,7 and TMG-P10,8 were chosen for solubilisation efficiency assessment and FSEC analysis.

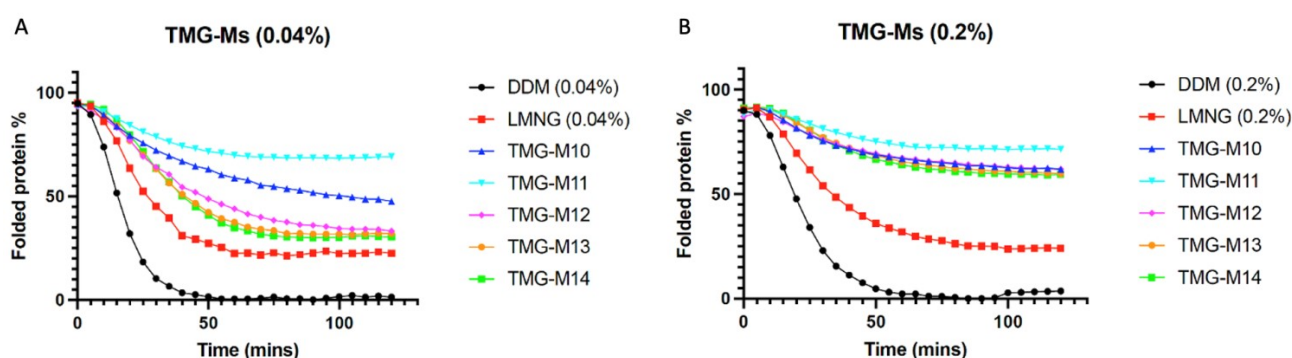


Figure 4.6. Thermostability assay of UapA-G411V in DDM, LMNG and novel TMG-M detergents showed that all TMG-Ms were more stabilising than DDM and LMNG. The detergent concentrations were at A) CMC +0.04% and B) CMC +0.2%. The samples were incubated at 40 °C and fluorescence emission was monitored every 5 minutes. Representative data of n=2 independent experiments.

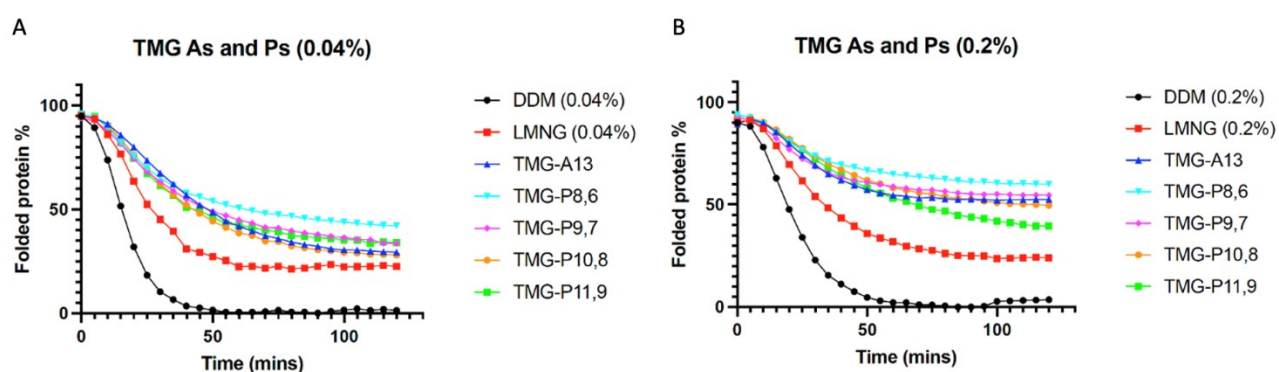


Figure 4.7. Thermostability assay of UapA-G411V in DDM, LMNG and novel TMG-A/P detergents showed that all TMG-A/Ps were more stabilising than DDM and LMNG. The detergent concentrations were at A) CMC +0.04% and B) CMC +0.2%. The samples were incubated at 40 °C and fluorescence emission was monitored every 5 minutes. Representative data of n=2 independent experiments.

4.3.3. TMG-M13 was the most stabilising detergent for UapA-G411V

The most stabilising detergents chosen from the results of the CPM assay were further characterised by determining their solubilisation efficiencies and by FSEC at 4°C and hFSEC at 45°C. All of the TMG detergents had lower solubilisation efficiencies than DDM which showed that they were less effective at extracting UapA from the membrane compared to DDM. Of the novel detergents, TMG-P10,8 had the highest solubilisation efficiency of 93.5% although it was still lower than DDM. TMG-P9,7 had the lowest solubilisation efficiency of 59.2% (Table 4.2).

FSEC analysis showed that UapA eluted as a monodispersed peak but with varying yields. TMG-P8,6 yielded the largest aggregation peak showing that UapA is more prone to aggregate in this detergent, indicating that this detergent is not suitable for extraction and isolation of UapA. The monodispersed peaks for TMG-M11, TMG-M13 and TMG-P10,8 were comparable to that of DDM (Figure 4.8A). hFSEC analysis showed that TMG-M13 had the largest monodispersed peak and one of the lowest aggregated peaks (Figure 4.8B). Therefore, from the novel detergents, TMG-M13 was the most stabilising detergent for UapA.

Table 4.2. Solubilisation efficiencies of selected TMG detergents. Representative data of n=2 independent experiments.

DDM	LMNG	TMG-A13	TMG-M11	TMG-M13	TMG-P8,6	TMG-P9,7	TMG-P10,8
96.4%	95.7%	73.8%	81.0%	85.7%	77.2%	59.2%	93.5%

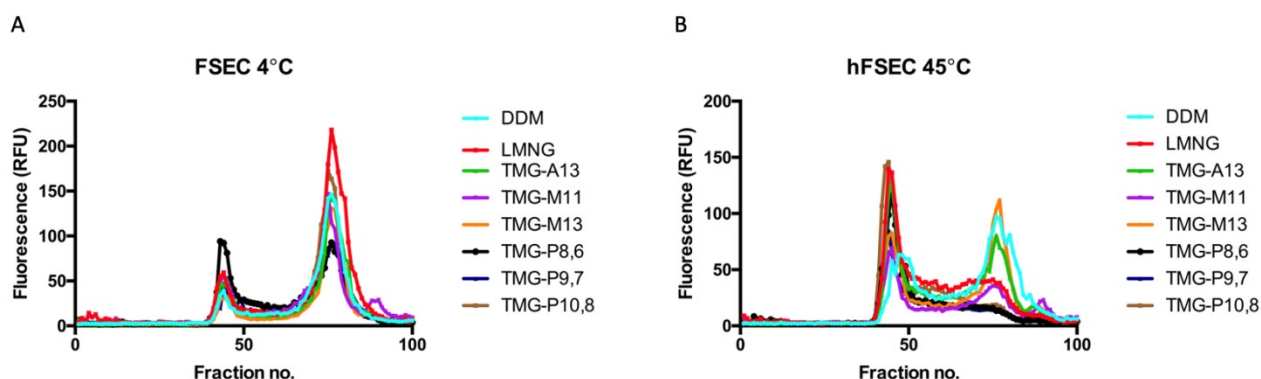


Figure 4.8. A) Fluorescence size-exclusion chromatography at 4 °C and B) heated fluorescence size-exclusion chromatography at 45 °C of UapA-G411V in the presence of DDM, LMNG and novel TMG-A/M/P detergents showed that TMG-M13 was the most stabilising detergent. Representative data of $n=2$ independent experiments.

4.4. MPD results

4.4.1. MPD detergents had lower CMC values than DDM

The following results were obtained by the laboratory of Professor Pil Seok Chae (Hanyang, University, South Korea). The CMC values of the MPD detergents ranged from 6 – 150 μM . The MPD detergents had lower CMCs than that of DDM, 170 μM . MP-D2-C10 had a relatively comparable CMC of 150 μM to DDM. The MPD-Ds had higher CMC values than their MPD-Q equivalents. For example, MP-D2-C10 had a higher CMC value of ~ 150 μM than its MPD-Q equivalent MP-Q2-C10 (~ 30 μM). As the length of the pendant chain increases the CMC values decrease and, similarly, as the length of the main alkyl chain increases the CMC values decrease. All the MPDs were $\sim 10\%$ water-soluble except for MP-Q4-C11 and MP-Q4-C12 which were $\sim 7\%$ and $\sim 5\%$ respectively (Table 4.3).

Table 4.3. MPD detergent properties: molecular weight (MW), critical micelle concentration (CMC) and water solubility (results obtained in Professor Pil Seok Chae laboratory, Hanyang University, South Korea).

Detergents	MW (g/mol)	CMC (μ M)	CMC (wt%)	Solubility (wt%)
MP-D2-C10	877.02	~150	~0.0132	~10
MP-D2-C11	891.05	~60	~0.0053	~10
MP-D2-C12	905.08	~45	~0.0041	~10
MP-D3-C10	905.08	~65	~0.0059	~10
MP-D3-C11	919.10	~35	~0.0032	~10
MP-D3-C12	933.13	~20	~0.0019	~10
MP-D4-C10	933.13	~50	~0.0047	~10
MP-D4-C11	947.16	~25	~0.0024	~10
MP-D4-C12	961.19	~18	~0.0017	~10
MP-Q2-C10	947.16	~30	~0.0028	~10
MP-Q2-C11	961.19	~25	~0.0024	~10
MP-Q2-C12	975.21	~20	~0.0020	~10
MP-Q3-C10	1003.27	~25	~0.0025	~10
MP-Q3-C11	1017.29	~20	~0.0020	~10
MP-Q3-C12	1031.32	~10	~0.0010	~10
MP-Q4-C10	1059.37	~10	~0.0011	~10
MP-Q4-C11	1073.46	~8	~0.0009	~7
MP-Q4-C12	1086.43	~6	~0.0007	~5
DDM	510.62	170	0.0087	10

4.4.2. At higher concentrations MPD detergents were better at stabilising UapA-G411V than DDM

Similar to the TMG detergents, a thermostability assay, using CPM, was used to determine the efficacy of the MPD detergents at stabilising UapA-G411V. At CMC+0.2%, all of the novel detergents were better at stabilising UapA than DDM. The results of the lower concentration of CMC+0.04% were highly variable suggesting that these results were unreliable.

At CMC+0.2%, the MPD quad-pendant detergents (MPD-Qs) were more stabilising than the MPD di-pendant detergents (MPD-Ds) (Figures 4.9A and 4.10A). This is not surprising because having four pendant chains versus two increases the alkyl chain density in the detergent micelles resulting in increased micellar stability and increased stabilisation of the protein. MP-D3-C10 was the least stabilising detergent with a similar stabilising effect to LMNG. MP-D2-C10, MP-D2-C11, MP-D2-C12, MP-D3-C11, MP-D4-C10 and MP-D4-C12 showed similar stabilising effects (Figure 4.10). With the MPD-Qs, increasing lengths of the

pendant and main alkyl chains resulted in increasing stabilisation effects (Figure 4.9). Therefore, MP-Q3-C11, MP-Q3-C12, MP-Q4-C10, MP-Q4-C11 and MP-Q4-C12 were chosen for solubilisation efficiency assessment and FSEC analysis.

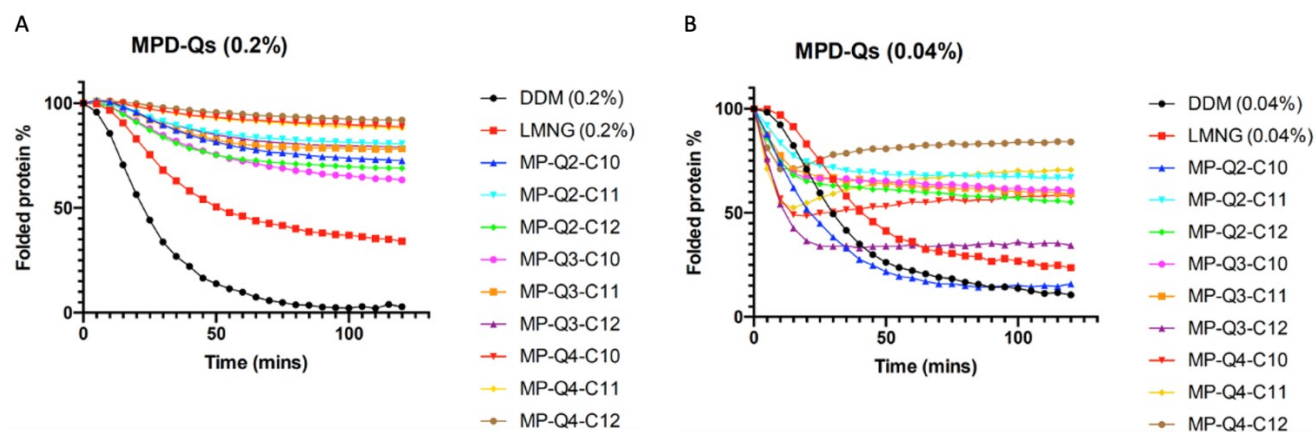


Figure 4.9. Thermostability assay of UapA-G411V in DDM, LMNG and novel MPD-Q detergents showed that at higher concentrations all MPD-Qs were more stabilising than DDM and LMNG. The detergent concentrations were at A) CMC +0.2% and B) CMC +0.04%. The samples were incubated at 40 °C and fluorescence emission was monitored every 5 minutes. Representative data of n=2 independent experiments.

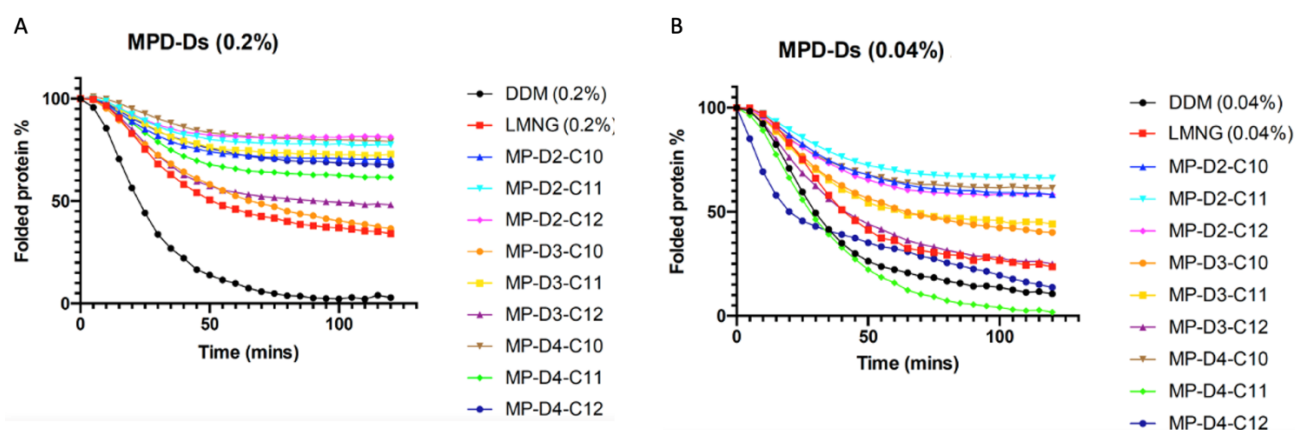


Figure 4.10. Thermostability assay of UapA-G411V in DDM, LMNG and novel MPD-D detergents showed that at higher concentrations all MPD-Ds were more stabilising than DDM and LMNG. The detergent concentrations were at A) CMC +0.2% and B) CMC +0.04%. The samples were incubated at 40 °C and fluorescence emission was monitored every 5 minutes. Representative data of n=2 independent experiments.

4.4.3. MPD detergents were not effective at extracting UapA-G411V

Like the TMG detergents, all of the MPD detergents had lower solubilisation efficiencies than DDM which showed that they were less effective at extracting UapA from the membrane (Table 4.4). MP-Q3-C11 had the highest solubilisation efficiency of the novel detergents of 78%. In the CPM assays, MP-Q4-C12 was the most stabilising detergent but it had the lowest solubilisation efficiency of 51.3%, suggesting that it was effective at stabilising UapA but not so effective at extracting the protein from the membrane compared to DDM.

FSEC analysis showed that UapA solubilised in most of these novel MPD detergents was not monodispersed with virtually all the protein in the void peak (8 ml) (Figure 4.11). For MP-Q3-C11 and MP-Q3-C12, small, monodispersed peaks were present, although even in these detergents the aggregation peaks were much larger. Upon heating, hFSEC analysis showed that the protein had completely aggregated in all of the novel detergents. Even though the CPM assay showed promising results for MP-Q3-C11, MP-Q3-C12, MP-Q4-C10, MP-Q4-C11, MP-Q4-C12, the solubilisation efficiency assessment, FSEC and hFSEC analysis showed that these detergents were not efficient at extracting UapA in a stable state.

Table 4.4. Solubilisation efficiencies of selected MPD-Q detergents. Representative data of $n=2$ independent experiments.

DDM	LMNG	MP-Q3-C11	MP-Q3-C12	MP-Q4-C10	MP-Q4-C11	MP-Q4-C12
96.8%	79.5%	78.0%	69.6%	73.1%	66.9%	51.3%

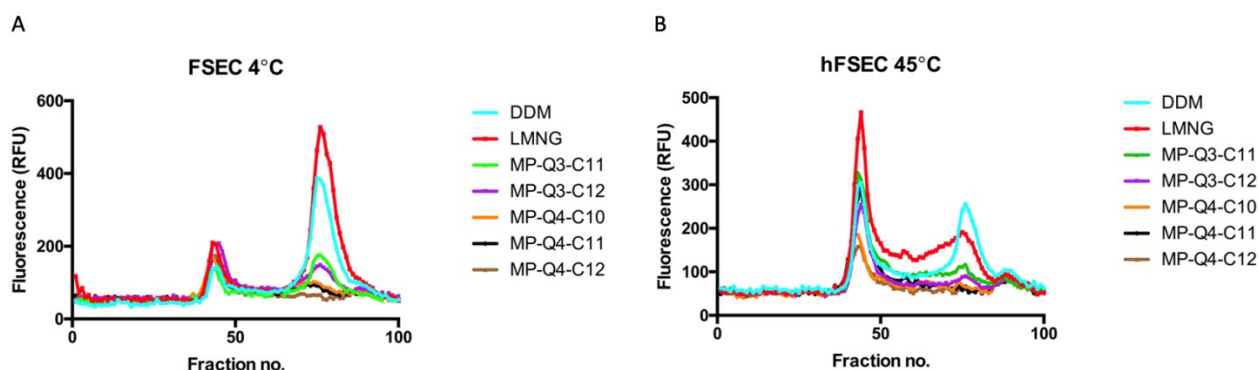


Figure 4.11. A) Fluorescence size-exclusion chromatography at 4 °C and B) heated fluorescence size-exclusion chromatography at 45 °C of UapA-G411V in the presence of DDM, LMNG and novel MPD-Q detergents showed that the protein had aggregated in all the novel detergents. Representative data of n=2 independent experiments.

4.5. Discussion

4.5.1. TMGs

TMG-Ms/Ps were designed based on the results of TMG-Ts/As (Hussain et al., 2017). TMG Ts/As had two alkyl chains that were relatively distant which resulted in suboptimal alkyl chain density. Therefore, to increase alkyl chain density the TMG-Ms and TMG-Ps were designed in which the two alkyl chains were connected by a methylene spacer or an alkyl chain respectively (Figure 4.3). To investigate the effects of these chemical changes, CPM assays and FSECs using UapA-G411V, a thermostabilised version of UapA, were carried out.

The CPM assays generally showed positive results for all of the novel detergents except for TMG-P11,9 (Figures 4.6 and 4.7). At CMC+0.04%, TMG-P11,9 was comparable to TMG-A13 but at CMC+0.2%, TMG-P11,9 seemed to be slightly less stabilising. In contrast, when the efficacy of these detergents in stabilising human β_2 AR, a G-protein coupled receptor, was investigated, TMG-P11,9 was one of the most stabilising detergents (Yoon et al., 2024). The CPM assays also showed that both the TMG-Ms and TMG-Ps were more stabilising at a higher concentration of CMC+0.2% than at CMC+0.04%. Hussain et al. (2017) reports that increased detergent concentration tends to be detrimental for protein stability which is not consistent with the results in this study.

The FSEC results showed that TMG-M11, TMG-M13 and TMG-P10,8 were as good at stabilising UapA as DDM and LMNG. The CPM and h/FSEC results collectively showed that TMG-M13 was the most stabilising detergent for UapA. These results were in partial agreement with those obtained for β_2 AR which showed that TMG-M12, TMG-M13, TMG-P10,8 and TMG-P11,9 were the most stabilising detergents, with TMG-P10,8 being the best at maintaining long-term stability (Yoon et al., 2024). TMG-P10,8 was also the most effective at maintaining long-term stability of the leucine transporter, LeuT, from the bacterium *Aquifex aeolicus*.

In general, the TMG-M/Ps were at least as good as TMG-A13 at stabilising UapA. As per the hypothesis that increasing the alkyl chain density would make the detergent more stabilising, TMG-M13 should have been better than TMG-A13, however, a similar level of protein stability was observed in the CPM assays. It is possible that this is due to the shorter alkyl spacer resulting in TMG-M13 being more hydrophilic than TMG-A13 and leading to an altered HLB. The hFSEC results did show that TMG-M13 is the most stabilising detergent for UapA but because these experiments were not performed on TMG-A13, it is difficult to conclude that TMG-M13 is better at stabilising UapA overall than TMG-A13. With the TMG-Ps, the additional alkyl chain increased the alkyl chain density without increasing their hydrophilicity which should theoretically make them better detergents than the TMG-Ms. This is seen for LeuT, where the TMG-Ps were more stabilising than the TMG-Ms, however for UapA, the TMG-Ms were generally better.

DDM and LMNG were best at extracting the protein from the membrane with a solubilisation efficiency of 96.4% and 95.7% respectively. All the other TMGs tested were less efficient than DDM and LMNG but more efficient than TMG-A13, except for TMG-P9,7 which was the least efficient at extracting UapA from the membrane (Table 4.2). TMG-P10,8 was best at extraction and was comparable to DDM and LMNG. Yoon et al. (2024) tested the efficacy of these detergents in the extraction of a prokaryotic transporter, melibiose permease from *Salmonella typhimurium* (MelB_{St}). They reported that the TMG-Ms yielded 50-60% soluble MelB_{St} and the TMG-Ps yielded 90-100% soluble MelB_{St}. The TMG-Ms were more efficient at extracting UapA than they were MelB_{St}, whereas TMG-P10,8 was efficient at extracting both UapA and MelB_{St}.

In summary, consistent with previous studies on TMG-Ts/As, all of the novel TMG-M/P detergents were better at stabilising UapA than DDM, however, DDM was the best at extracting the protein from the membrane. This could suggest that using DDM for protein extraction and then exchanging into a TMG to keep the protein stable for purification/analysis might be ideal. In conclusion, TMG-M13 seems to be the best at stabilising UapA but when looking at all of the different membrane proteins discussed, TMG-P10,8 seems to be the best at extracting and stabilising the different proteins across the board. In conclusion, these TMG detergents showed great potential for membrane protein research.

4.5.2. MPDs

The MPD detergents were designed based on the results of the P-GNGs (Bae et al., 2020). P-GNGs contain pendant alkyl chains that are most likely accommodated at the empty spaces in the hydrophobic-hydrophilic interface of the detergent micelle resulting in more stable micelles. The location of these pendant chains also means that they are unlikely to interact too strongly with the protein which is advantageous for aggregation-prone proteins (Lee et al., 2022). In order to better understand the size of these empty spaces, detergents with varying number and length of pendants were designed. The stability of UapA in these MPD detergents was then investigated by a thermostability assay and by FSEC and hFSEC analysis.

The results obtained for the MPD detergents were inconsistent. The CPM assays showed that the MPD detergents were efficient at stabilising UapA compared to DDM, with the most stabilising detergents being MP-Q3-C11, MP-Q3-C12, MP-Q4-C10, MP-Q4-C11 and MP-Q4-C12. The observation that these detergents were the most stabilising also indicated that four propyl or butyl chains were an optimal fit in the empty spaces of the detergent micelles. On the other hand, the MPD detergents were not efficient at UapA extraction which resulted in poor recovery of the protein into FSEC. The monodisperse peaks on the FSEC results were very low if at all existent. The small amount of protein that had gone into solution seemed to have mostly aggregated.

Overall, even though the CPM analysis showed some potential of these novel MPD detergents at stabilising UapA, the FSEC and hFSEC results seemed to show otherwise. A possible explanation for this inconsistency is that during extraction, the MPD detergents could be removing lipids that are normally present during DDM extraction, which in turn destabilises the protein and leads to aggregation.

4.5.3. Conclusion

The ability of the TMG and MPD detergents in stabilising UapA-G411V was investigated. Overall, some of the TMG detergents, such as TMG-M13 and TMG-P10,8, showed promising results in terms of stabilising UapA. However, the MPD detergents were not efficient at extracting UapA and stabilising the protein. Conventional detergents, such as DDM and LMNG, were shown to be better at extracting UapA from the membrane but less efficient at keeping the protein stable in solution. In conclusion, there is a lot of variability in the best detergent for the different membrane proteins discussed above which shows that detergent efficacy is protein specific. This highlights the difficulty in solubilising and purifying stable membrane proteins as there is no single detergent that is suitable for all membrane proteins.

5. Final Discussion

Vitamin C plays an essential role in various cellular processes and is a possible therapeutic for treating a range of conditions including cancer and neurodegenerative diseases. The SVCTs are highly specific ascorbic acid transporters and understanding their structure and working mechanism is crucial in identifying the way in which vitamin C can be used as a therapeutic. In this thesis, various molecular determinants that contribute to the making of a correctly functioning hSVCT1 protein were explored; this helped in identifying several residues that are important in ascorbic acid transport. Additionally, significant steps were taken to express and purify hSVCT2 in an appropriate state for structural studies.

In this thesis, several residues crucial for hSVCT1 substrate transport were identified. hSVCT1 was shown to be highly specific for ascorbic acid with even single point mutations in the substrate translocation channel, such as L65A, F112A, R206A, I439A and L458G, resulting in significant decreases of substrate transport. Moreover, mutating residues in the ascorbic acid binding site of hSVCT1 to mirror the residues in the xanthine binding site of UapA did not allow the transport of xanthine. These results suggest that even though UapA and hSVCT1 share a very similar structural architecture, there are subtle differences in their working mechanisms. This is a positive result when it comes to developing anti-fungals. UapA is a uric acid/xanthine transporter from *Aspergillus nidulans* (Section 1.3.2). *Aspergillus* is responsible for life-threatening fungal infections, such as aspergillosis (Brown et al., 2012). It has been suggested that the development of novel antimicrobial drugs that specifically target nucleobase-transporting NATs, such as UapA, could be advantageous as nucleobase-specific transporter homologs are absent in humans (Frillingos, 2012). Consequently, previous work in our lab has attempted to use UapA as a potential drug carrier to transport cytotoxic small molecules into fungal cells (Deacon-Smith, 2023). The clear differences in the mechanisms of UapA and hSVCT1 reported in the present work further alleviates concerns that an antifungal treatment against *Aspergillus* that targets UapA will also be transported by the SVCTs. This is despite the marked similarities in their structures and general mode of transport, via the elevator mechanism.

In order to understand the precise differences between the working mechanisms of UapA and hSVCT1, additional structures of different conformational states are required. Specifically, the outward-facing conformations of both proteins would be useful as all structures available of NATs to date are in the inward or occluded conformations. The outward conformation would allow identification of the conformational rearrangements associated with the switch between the inward and outward conformations. The residues important in the conformational rearrangements could also be identified. This can not only help in understanding the mechanism of substrate transport in hSVCT1 but of other NATs as well. Identifying mutations and/or inhibitor combinations that can lock hSVCT1 in the outward conformation is ongoing within the group.

Knowing both the structure of the SVCTs and the specific residues responsible for substrate recognition can help in using these transporters as drug targets or drug carriers. One way in which the SVCTs could be used as drug targets is by developing compounds that specifically stimulate SVCTs to increase the transport of ascorbic acid, enhancing intracellular availability. In addition, research into understanding how to use transporters in the epithelial cells of the intestine, liver and kidney to absorb, distribute and eliminate drugs has been reported (Giacomini et al., 2010). As SVCT1 is mainly expressed in epithelial cells to transport ascorbic acid for further distribution, it could potentially be used for drug absorption, distribution and elimination. SVCTs can also be used to transport prodrugs conjugated to ascorbic acid. Luo et al. (2011) reported that an ascorbic acid-conjugated prodrug of saquinavir, a protease inhibitor used for the treatment of HIV infected patients, targeting SVCT increased the oral absorption and bioavailability of the drug. Therefore, the identification of residues important in substrate specificity reported in this thesis can help in the targeted design of drugs and drug conjugates.

In this thesis, the importance of dimerisation in the function of hSVCT1 was also demonstrated. In the hSVCT1 structure, the presence of lipid molecules at the dimer interface was identified (Kobayashi et al., 2024). The authors suggested that these lipid molecules may play a role in the hSVCT1 transport cycle by moving into the dimer interface during the transition from the inward open to the occluded state therefore driving this conformational change and stabilising the occluded state. In addition, the stabilisation effect

of lipids on the UapA dimer has been reported. Pyle et al. (2018) suggested that lipids such as phosphatidylinositol and phosphatidylethanolamine have a stabilising effect on the UapA dimer. This could suggest that lipids also play a role in stabilising the hSVCT1 dimer. However, the role of lipids remains speculative and investigating the precise role that lipids play in hSVCT1 would be an interesting area for further research.

In order to obtain stable protein for structural studies of hSVCT2, extensive optimisation of hSVCT2 expression and purification has been performed. hSVCT2 has been successfully expressed and purified, and preliminary structural studies has also been commenced. Several areas for further optimisations have been identified, including ways to increase protein yield and stability and ways to obtain optimal cryo-EM grids. The hSVCT2 structure would not only be beneficial for understanding the protein's working mechanism and for drug design but also for understanding SVCT2's transceptor activity. As discussed in section 1.3.3.2, SVCT2 plays a receptor-like role and activates the JAK/STAT pathway. A number of questions still remain on how exactly SVCT2 associates with JAK/STAT and activates the pathway. The hSVCT2 structure could help reveal the way in which hSVCT2 associates with the pathway and it could also help in understanding why only hSVCT2, and not hSVCT1, is able to activate this pathway.

Lastly, members of the NAT family can provide a useful tool in finding ways to alleviate the difficulties of expressing and purifying membrane proteins for structural studies. In this thesis, the ability of novel detergents in stabilising UapA was investigated in order to develop effective detergents for maintaining membrane proteins in a stable state. Additionally, NATs can also be used to develop lipid screens to identify lipids that stabilise membrane proteins in detergent-based solutions; this is ongoing research within our group.

Overall, this project has given invaluable insight into the working mechanism of hSVCT1 and has set up the groundwork for the future structural characterisation of hSVCT2, both of which potentially contribute to the future development of therapeutics. Furthermore, results in this thesis have contributed to addressing the overarching challenge of maintaining membrane proteins in a stable state for downstream analysis.

6. Bibliography

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7. Appendix

7.1. hSVCT1 primer sequences

Supplementary Table 1. Forward (F) and Reverse (R) primers for the generation of different hSVCT1 constructs.

Constructs	Primer Sequences (5'-3')	
Addition of His₈ tag	F	TACCGAGCTCGGATCCACCATGGTGAGCAAGGGCGAGG
	R	GATATCTGCAGAATTCTTAGTGATGATGGTGGTGATGGTGTGCTTGTACAGCTCGTCCAT
WT	F	TACCGAGCTCGGATCCATGAGGGCCAGGAGGAC
	R	TGCTCACCATGGTGGATCCAAATTGACCTTGAAAATATAAATTTTCGACCTGGTGCACACAGATGC
Δ1-10	F	TACCGAGCTCGGATCCATGACACAGCATGAAACCACCAG
	R	TGCTCACCATGGTGGATCCAAATTGACCTTGAAAATATAAATTTTCGACCTGGTGCACACAGATGC
Δ1-20	F	TACCGAGCTCGGATCCATGACCCCGCTACCCACAGAGCC
	R	TGCTCACCATGGTGGATCCAAATTGACCTTGAAAATATAAATTTTCGACCTGGTGCACACAGATGC
Δ1-26	F	TACCGAGCTCGGATCCATGCCTAAGTTTGACATGTTGTAC
	R	TGCTCACCATGGTGGATCCAAATTGACCTTGAAAATATAAATTTTCGACCTGGTGCACACAGATGC
Δ529-598	F	TACCGAGCTCGGATCCATGAGGGCCAGGAGGAC
	R	TGCTCACCATGGTGGATCCAAATTGACCTTGAAAATATAAATTTCTGGGCAGATAGGAATGTATTTCAG
Δ567-598	F	TACCGAGCTCGGATCCATGAGGGCCAGGAGGAC
	R	TGCTCACCATGGTGGATCCAAATTGACCTTGAAAATATAAATTTCTGGGCAGATAGGAATGTATTTCAG
L65A	F	CGCCGTGCCCTTCGCGCTGGCTGAGGCG
	R	CGCCTCAGCCAGCGCGAAGGGCAGGCG
F112A	F	CCAGGCCAGTGCCGCTGCATTTCTGGTTCCAG
	R	CTGGAACCAGAAATGCAGCGGCACTGGCCTGG
R206A	F	AGCTGCTGGCGACGCAGCTGGCTCCAC
	R	GTGGGAGCCAGCTGCGTCGCCAGCAGCT
G387V	F	GTCCAGTCCCAACATTGTCGTCCTGGGAATTACC
	R	GGTAATCCCAGGACGACAATGTTGGGACTGGAC
I439A	F	GCACTCTCTTGGCATGGCTACAGCTGTGGGGCTGT
	R	ACAGCCCCACAGCTGTAGCCATGCCAAAGAGAGTGC
L458G	F	GAACTCCTCTCGCAACGGCTTCGTGCTGGG
	R	CCCAGCACGAAGCCGTTGCGAGAGGAGTTC

7.2. hSVCT2 primer sequences

Supplementary Table 2. Forward (F) and Reverse (R) primer sequences for cloning hSVCT2 into different pOPIN vectors.

Vector	Gene	Primer Sequences (5'-3')	
pOPINF	WT	F	AAGTTCTGTTTCAGGGCCCGATGATGGGTATTGGTAAGAATACCACATC CAAATC
		R	CTGGTCTAGAAAAGCTTTATCCCGTGGCCTGGGAGTCTTCATC
	Y628F	F	AAGTTCTGTTTCAGGGCCCGATGGGTATTGGTAAGAATACCACATCCA AATC
		R	CTGGTCTAGAAAAGCTTTATCCCGTGGCCTGGGAGTCTTCATC
pOPIN ^{Eneo} -3C- GFP-His	WT	F	AGGAGATATACCATGATGGGTATTGGTAAGAATACCACATCCAAATC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC
	Δ 1-23	F	AGGAGATATACCATGGAAGACGAGGCAAAGCACCCAGC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC
pOPIN ^{Eneo} -TEV- TGP-His	WT	F	AGGAGATATACCATGATGGGTATTGGTAAGAATACCACATCCAAATC
		R	TACAGGTTCTCGTTTCCTCCCGTGGCCTGGGAGTCTTCATC
	Δ 1-23	F	AGGAGATATACCATGGAAGACGAGGCAAAGCACCCAGC
		R	TACAGGTTCTCGTTTCCTCCCGTGGCCTGGGAGTCTTCATC
pOPIN ^{Eneo} -TGP- 2Strep-His	WT	F	AGGAGATATACCATGATGGGTATTGGTAAGAATACCACATCCAAATC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC
	Δ 1-23	F	AGGAGATATACCATGGAAGACGAGGCAAAGCACCCAGC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC
pOPIN ^{Eneo} -HA- FLAG-TEV-POI- TGP	WT	F	GTACTTCCAGGGATCCATGATGGGTATTGGTAAGAATACCACATCCAAA TC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC
	Δ 1-23	F	GTACTTCCAGGGATCCGAAGACGAGGCAAAGCACCCAGC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC
pOPIN ^{Eneo} -FLAG	WT	F	AGGAGATATACCATGATGGGTATTGGTAAGAATACCACATCCAAATC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC
	Δ 1-23	F	AGGAGATATACCATGGAAGACGAGGCAAAGCACCCAGC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC
pOPIN ^{Eneo} - 2strep	WT	F	AGGAGATATACCATGATGGGTATTGGTAAGAATACCACATCCAAATC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC
	Δ 1-23	F	AGGAGATATACCATGGAAGACGAGGCAAAGCACCCAGC
		R	CAGAACTTCCAGTTTTCCCGTGGCCTGGGAGTCTTCATC

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