From DEPARTMENT OF BIOSCIENCES AND NUTRITION
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UNDERSTANDING STRUCTURE AND FUNCTION OF MEMBRANE PROTEIN TRANSPORTERS

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Stockholm 2008

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden
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ISBN 978-91-7409-240-0

ABSTRACT

Membrane protein transporters are important proteins in the cell, as they maintain different solutes and metabolites at a stable concentration. Numerous diseases can be linked to malfunctioning transporters, which makes them interesting pharmaceutical targets. To learn more how these proteins are regulated both functional and structural studies are needed. Membrane proteins are challenging since it is not easy to produce large amounts of these proteins and they readily precipitate due to their hydrophobic nature.

In this thesis the function or structure of three different membrane protein transporters were studied; Melibiose permease (MelB), a sugar co-transporter from *Escherichia coli*, a potassium channel (Kch) from *Escherichia coli* and a divalent ion transporter from *Thermotoga maritima* (CorA). Studies of two-dimensional crystals from MelB with Cryo electron microscopy resulted in a three-dimensional structure to 10 Å. The structure revealed an asymmetrical cone shaped molecule that clearly was closed in one end and open in the other. The overall structure resembled that of the Na^+/H^+ antiporter (NhaA) more than that of the lactose permease (LacY). Preliminary results from crystals without substrate show structural differences in the projection map. Also crystals without substrate were obtained, which preliminary show structural differences in the projection map. Single particle studies of Kch indicated that the majority of tetramers of the protein probably dimerize in a way that can explain why the protein has been difficult to reconstitute into liposomes. Functional studies of CorA from *Thermotoga maritima* demonstrated that the substrate may be Co^{2+}, and thus Mg^{2+} may not be the primary substrate of all CorA proteins as previously anticipated. Furthermore fluorescence measurements of tryptophan quenching indicated that there are two binding sites for Co^{2+} with different affinities (K_d values was 28 μM and 750 μM).

During these studies methods to obtain stable protein were investigated. A small-scale approach for a buffer screen with analytical size exclusion chromatography was developed. With this method a protein, that previously precipitated during ultrafiltration was possible to concentrate to amounts suitable for crystallization trials.

Also, the activity of TEV protease was investigated in different detergents. This protease is commonly used to remove affinity tags from proteins. Our results show that the choice of the detergent affects the accessibility of the membrane protein cleavage site and not the activity of the TEV protease.

Taken together; the results presented in this thesis deals with problems of stability of purified membrane proteins and protease activity it detergent solutions. Structures of two membrane proteins (MelB and Kch) were determined with two different electron microscopy methods, and the substrate specificity of CorA was explored and revised.

LIST OF PUBLICATIONS

I. **Lundbäck AK**, van den Berg S, Hebert H, Berglund H and Eshaghi S. 2008. *Exploring the activity of tobacco etch virus protease in detergent solutions.* Anal Biochem. 382:69-71.

II. Martinez Molina D[¤], **Lundbäck AK**[¤], Niegowski D and Eshaghi S. 2008. *Expression and purification of the recombinant membrane protein YidC: A case study for increased stability and solubility.* Protein Expr Purif. 62:49-52.

III. Purhonen P, **Lundbäck AK**, Lemonnier R, Leblanc G and Hebert H. 2005. *Three-dimensional structure of the sugar symporter melibiose permease from cryo-electron microscopy.* J Struct Biol. 152:76-83.

IV. **Lundbäck AK,** Müller S, Engel A and Hebert H. 2008. *3D structure of Kch, a potassium channel in Escherichia coli.* Manuscript

V. **Lundbäck AK,** Nordlund G, Brzezinski P, Hebert H and Eshaghi S. 2008. *The thermostability of the CorA from Thermotoga maritima is substrate specific.* Manuscript

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LIST OF ABBREVIATIONS

2D	Two dimentional
3D	Three dimentional
ATP	Adenosine triphosphate
CMC	Critical micelle concentration
LPR	Lipid to protein ratio
PDB	Protein data bank
PhoA	Alkaline phosphatase
SDS-PAGE	Sodium dodecyl sulfat-polyacrylamid gel electrophoresis
TEM	Transmission electron microscopy
TEV	Tobacco etch virus
WT	Wild type

INTRODUCTION

The lipid bilayer forms a barrier around the cell, and also protects the interior of organelles such as mitochondria, endoplasmic reticulum, thylakoids and the nucleus in eukaryotic cells. Some molecules such as water (osmosis) and oxygen can seep through lipid membranes, but the membranes are impermeable towards small charged molecules and hydrophilic compounds. Therefore there is a set of protein molecules that facilitate the transport and regulate the concentration of these solutes (Fig. 1).

Molecules and ions move spontaneously down their concentration gradient to even out the differences in concentration (*i.e.* diffusion). Uniporters and channels are a set of membrane proteins that let small molecules such as glucose or ions pass by diffusion. Energy will be consumed in order to move a molecule up its concentration gradient and there are two solutions to this problem. A transporter can use the energy from for instance ATP hydrolysis to generate the required energy (primary active transport) or use the energy that is released when one compound is transported down its concentration gradient (secondary active transport). The latter group of transporters are divided into antiporters and symporters, depending on what relative direction the substrates are transported.

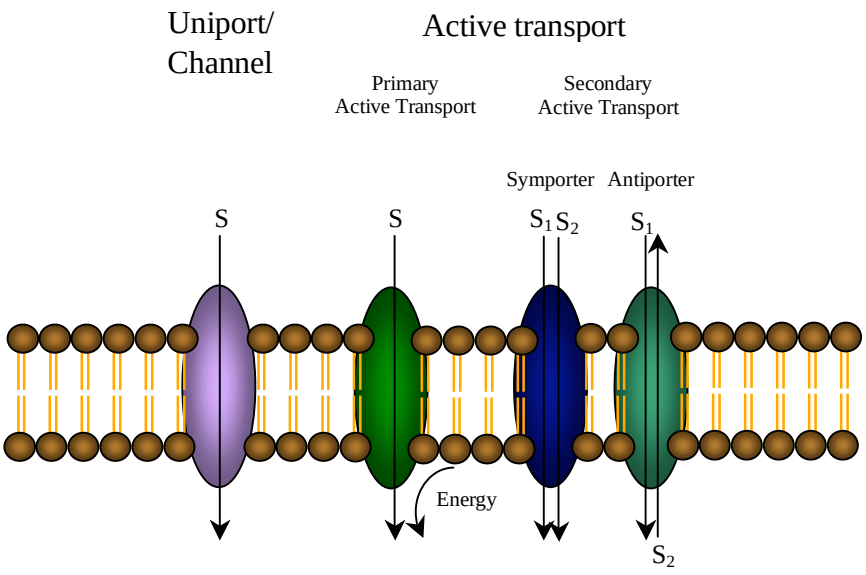


Figure 1 - Schematic picture of different types of transporters. S=Substrate.

The transporters are tightly regulated and respond to internal or external signals. As a result they open and close to release and take up ions or compounds. The concentration of metabolites needs to be balanced within the cell to prevent malfunction or even cell death. Numerous diseases can be linked to transporters and they are important candidates for pharmaceuticals. To understand how these proteins are regulated, in order to find cures for diseases, both structural and functional studies are needed. However, membrane proteins have proven to be a challenge. The amount of protein that can be obtained from natural sources or through recombinant expression is usually modest. Also membrane proteins need to be extracted from the lipid bilayer, which sometimes affect the function and/or the stability of the protein.

It this thesis the structure and/or function of three different membrane protein transporters were explored. Two of the membrane proteins (Melibiose permease and a potassium channel from *Escherichia coli*) were subjected to studies with electron microscopy using two different methods; two-dimensional crystallography and single particle. The function of the third protein, CorA from *Thermotoga maritima*, was explored with support from the previously reported structure [1]. During the course of these studies, methods to obtain stable protein and protease activity in detergent solutions were also investigated.

PURIFICATION OF MEMBRANE PROTEINS

For functional and/or structural studies, membrane proteins usually need to be extracted from the lipid membrane, purified and enriched. In order to get sufficient amounts of protein and to facilitate purification the protein is generally expressed recombinantly in *Escherichia coli* with an affinity tag. Commonly used tags are maltose binding protein (MBP), glutathione S-transferase (GST), antibodies or histidines (His). These tags bind to ligands, which are immobilized on a solid matrix, and in this way contaminants are removed (affinity purification). The protein that is attached through the tag is released by adding a compound that competes for binding. Sometimes tags need to be removed from the protein, since the activity can be affected or crystallization of the target protein can be prohibited [2, 3]. For tag removal, a cleavage site, *i.e.* the specific amino acid sequence that is recognized by a protease, is inserted between the tag and the target protein. Usually affinity purification is used to capture the desired protein and the purity is improved by a subsequent step such as size exclusion chromatography or ion exchange.

During extraction, purification and crystallization, membrane proteins need to be in the presence of detergents, which keeps the protein soluble in buffers. The choice of the detergent is very important, since it can affect the amount of protein that is extracted, the stability and activity of the purified protein and the crystallization.

DETERGENTS

Detergents are amphiphilic molecules that mimic the lipid environment, which surrounds the protein in the membrane, and also keep the protein soluble in aqueous buffers. These molecules are composed of a tail and a head part, where the head is hydrophilic and the tail is hydrophobic. There are several different types of detergents. These are divided into groups according to the chemical property of the head part; ionic, non-ionic or zwitterionic. The head part of the ionic detergents can be either positive or negative, while zwitterionic detergents have both a positively and negatively charged head group. There is also a fourth group of detergents; bilesalts. Their overall molecular structure is different; they are more bulky and have two sides, one is hydrophilic and the other is hydrophobic, rather than a head and tail.

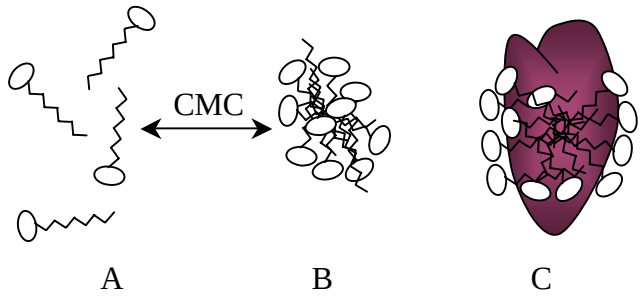


Figure 2 – Detergents. A) Monomers of detergent molecules, below CMC. B) Micelle of detergents, over CMC. C) At CMC a membrane protein can be solubilized. The detergents form a collar around the hydrophobic part of the membrane protein.

Detergents are believed to form a collar around the membrane protein, where the lipid bilayer should be. In aqueous solutions detergents form micelles at a specific concentration, which is called Critical Micelle Concentration (CMC). At this concentration, the detergent molecules are able to aggregate in order to minimize the exposure of the hydrophobic part to the aqueous solution. Below CMC the majority of detergent molecules exist as monomers and are not able to solubilize a membrane protein [4]. This concentration depends on properties of the detergent molecule and is different for all detergents. The CMC is also influenced by environmental parameters such as pH, temperature and salt concentration. The ability of a detergent to keep a membrane protein from precipitating differs from protein to protein. Non-ionic detergents are considered to be mild and non-denaturing, but can fail to keep the membrane protein stable. In addition, the detergent may influence enzymatic assays, which must be considered in functional studies. For instance it was noticed that the enzymatic activity of the tobacco etch virus protease (TEV) was affected in certain detergent solutions. This was explored further in paper I.

The Effect of Detergents on Tobacco Etch Virus Protease Activity

There are several proteases used for removal of an affinity tag such as human rhinovirus 3C, thrombin, factor X and tobacco etch virus (TEV) protease. TEV protease has several advantages; its specificity is rather high and it retains activity in different buffers [5, 6]. Removal of an affinity tag from a membrane protein requires that the protease is active in the detergent solution. One previous report explored the activity of TEV protease in detergent solutions. This report concludes that some detergents completely or partially inhibit the TEV protease activity [7].

In paper I we explored this further since the previous report only studied the removal of the affinity tag from a soluble protein. We believed that it was very important to study the detergent - membrane protein - protease system for a set of detergents that are commonly used in various studies of membrane proteins. There were also some observations in our laboratory that did not concur with the previous publication. We examined the protease activity in the presence of 16 detergents from seven different detergent families using three membrane proteins. As a control we also performed the experiment on a soluble protein; YhaK [8] in the presence of the same detergents.

Possible explanations for unsuccessful digestion of the cleavage site in detergent solutions are either that the detergent inactivates the protease or that the cleavage site is obscured. We observed that in the presence of Fos-choline 10 (FC10), the tag was partially removed from two of the membrane proteins, but not at all from the soluble protein, YhaK (Table 1, Fig. 3). It is possible that the tag was not removed from YhaK because the cleavage site was obscured, however the tag could be removed in the presence of other detergents. Did FC10 induce structural changes in YhaK so that the tag became obscured? Our result showed that the activity of the TEV protease varied for each combination of membrane protein and detergent [9]. We could not observe any clear trends in what detergent would allow for digestion of the cleavage site thus leaving screening as the only choice. The small screen approach used here is fast and

can produce a lot of information from a small amount of protein. With these experiments we have only been able to observe the protease - membrane protein - detergent system as a whole. To really understand if the structure of the TEV protease is affected by the detergent or perhaps if soluble parts of membrane proteins are affected, more experiments are needed (*i.e.* circular dichroism or nuclear magnetic resonance). Detergents may affect the activity of soluble proteins as well as membrane proteins. Therefore it is important to screen several detergents when exploring the activity of a membrane protein in detergent solutions.

Table 1 – Results of the TEV protease activity assay in detergent solution on three membrane proteins. 1=tmCorA, 2=ecCorA, 3=PgeS. I=incomplete, C=complete and N= no digestion.

No	Detergent	4 x CMC (% w/v)[a]	Detergent Family	1	2	3
1	Nonyl maltoside (NM)	1.2	Maltoside	I	I	C
2	Decyl maltoside (DM)	0.40	Maltoside	I	I	C
3	Undecyl maltoside (UDM)	0.12	Maltoside	I	I	C
4	Dodecyl maltoside (DDM)	0.10	Maltoside	I	C	C
5	Fos-choline 10 (FC10)	1.4	Fos-choline	N	I	I
6	Fos-choline 12 (FC12)	0.20	Fos-choline	N	N	N
7	LDAO	0.10	Amine oxide	N	N	N
8	Octyl glucoside (OG)	2.0	Glucoside	X	X/I	I
9	Nonyl glucoside (NG)	0.80	Glucoside	X	X/I	C
10	C_8E_4	1.0	Polyoxyethylene glycols	X	I	C
11	C_8E_6	1.6	Polyoxyethylene glycols	X	I	C
12	Cymal 5	0.50	Maltoside	I	I	C
13	Cymal 6	0.12	Maltoside	I	I	C
14	Cymal 7	0.040	Maltoside	I	I	C
15	CHAPS	2.0	Bile acid based	N	I	I
16	Triton X-100	0.060	Triton	N	I	C

[a]CMC = Critical Micelle Concentration. Values obtained from Anatrace Inc.

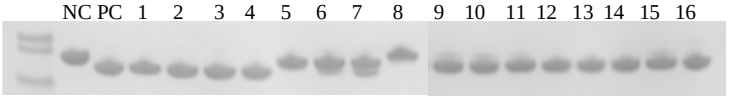


Figure 3 – SDS PAGE of the soluble protein YhaK incubated with TEV protease in the presence of 16 different detergents. The numbers correspond to the numbers in Table 1. NC=Control without TEV protease. PC=Control with TEV protease without detergent.

Stability of Membrane Proteins

Membrane proteins are usually stable in the lipid membrane of the cell but when they are extracted, for biochemical or structural studies, the stability may be affected. The amount of protein obtained from recombinant expression and subsequent purification, can be rather low and the protein must be concentrated. At this stage the protein can become destabilized and precipitate. Membrane proteins precipitate for a number of reasons, for example, extensive delipidation, use of wrong type of detergent, other factors such as buffer composition (pH, salt) or absence of cofactors. Also to discover a condition where the protein is homogeneous is desirable as that enhances the probability of finding a successful crystallization condition. Finding the right parameters can be rather time-consuming and also costly since some of the detergents are expensive.

In paper II we sought to identify a buffer comosition where the YidC protein would be of high purity and remain stable and soluble. The method that was used was fast and consumed little amounts of protein.

YidC was initially purified in a buffer containing 20 mM Tris-HCl, pH 8.0 and 300 mM NaCl. The size exclusion chromatography profile revealed that the protein was inhomogeneous and when the protein was concentrated with ultrafiltration, it precipitated. Three pH were selected in combination with three different NaCl concentrations. The buffer screening revealed that the protein was most instable and rather inhomogeneous at pH 7.5 and a majority of the protein was eluted in the void (Fig. 4). At the higher pH of 9.5 the protein became more homogeneous in the presence of high salt concentration. At pH 5.2, however, the protein was highly stable and homogeneous at both low and high salt concentration. The theoretical isoelectric point of YidC is at pH 7.7. It is plausible that this value corresponds to the actual isoelectric point of the protein, promoting protein aggregation at this pH region due to lack of surface charge. The salt concentration seemed to prevent some of the aggregation.

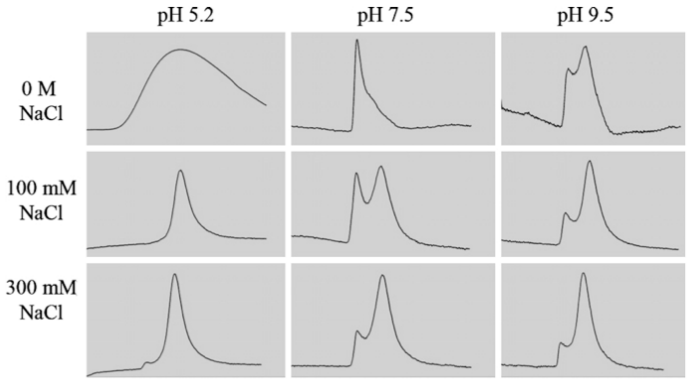


Figure 4 – The profiles from analytic size exclusion chromatography. Nine parameters were tested, and at pH 5.2 in the presence of 300 mM NaCl the protein was homogenous.

The condition where the analytical size exclusion profile showed the most non-aggregated and homogeneous protein (at pH 5.2 and 100 mM NaCl) was then concentrated to 10 mg/ml and protein did not precipitate. The profiles from analytical size exclusion chromatography reveal whether the protein is heterogeneous. For many proteins this can indicate that the protein is destabilized and will aggregate over time. This method can be used to find the conditions where the protein is homogeneous and, therefore stable.

METHODS FOR STRUCTURAL STUDIES

What theoretically limits the smallest detail that can be resolved in a light microscope is the wavelength of visible light (400-780 nm). With today's technique we can observe objects such as organelles in a cell with an ordinary light microscope. To examine even smaller objects there are a few methods that can be used: Nuclear Magnetic Resonansce (NMR), X-ray crystallography or electron microscopy. NMR is a powerful method to study the protein dynamics in solution, but is restricted to smaller proteins. X-ray crystallography has so far generated the most protein structures at highest resolution. The proteins need to be crystallized, which limits the states a protein can be studied in. With electron microscopy it is possible to study the protein both in a crystalline state (two-dimensional crystals) or in solution (single particle). The resolution may be poorer with these techniques than with X-ray crystallography, but can still provide important information.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Electrons move as waves, just like light and the wavelength depends on the applied voltage. In an electron microscope operated at 200 kV would produce electrons with the wavelength 0.025 Å. Unfortunately, it is not possible to get this kind of resolution of the object because of limitations of the microscope and how it affects the specimen.

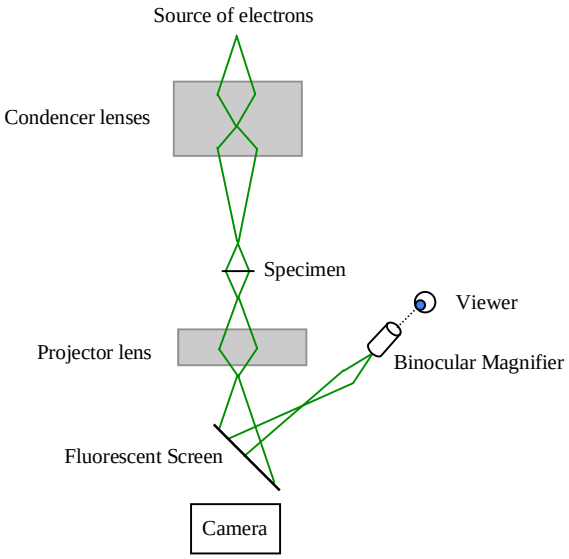


Figure 5 – Section through a transmission electron microscope.

Ruska and Knoll built the first electron microscope in 1931 and it could resolve smaller details than a light microscope, but the magnification was only about 100x [10]. They used the fact that diffracted electrons can be focused by electromagnetic lenses into an enlarged image. Electrons can be emitted from a heated lanthanum-hexaborite or a tungsten filament. Modern high resolution electron microscopes are equipped with a field emission gun (FEG), which produces a more coherent beam. The emitted

electrons are guided through a set of condenser lenses, which determines the size and intensity of the electron beam that will hit the specimen. The electron can pass through the sample without interacting with the specimen, transfer energy (inelastic scattering), which cause radiation damage, or interact without energy loss (elastic scattering). The elastic scattered electrons carry structural information and are either focused to an image or collected as diffraction pattern (two-dimensional crystals) [11]. In this way the phases that are needed to calculate the structure can be derived, which is a problem in X-ray crystallography. The image of the object that is produced in electron microscopy depicts the electron potential of the molecules.

Data Collection

The electrons that have travelled through the sample form an image, a projection, which is two-dimensional. In order to build a three dimensional model, the sample is tilted and several images are collected at different tilt angles. The images are then merged to calculate the three dimensional structure. Limitations in the microscope prevents the specimen to be tilted to 90° and this will produce a lack of data, *i.e* the missing cone. Therefore the resolution is often poorer in the z-axis than in the x-y plane [12].

Two-Dimensional (2D) Crystallography

The strong interaction of electrons and matter limits the sample thickness to about about 100 Å in order to avoid secodary scattering effects. to avoid secondary scattering. 2D crystals are too small for X-ray crystallography, but thin enough to be observed by electron microscopy. There are a few known membrane proteins that form 2D crystals naturally which have been studied with electron microscope [13, 14]. Since these are really rare, methods to produce 2D crystals hava been explored.

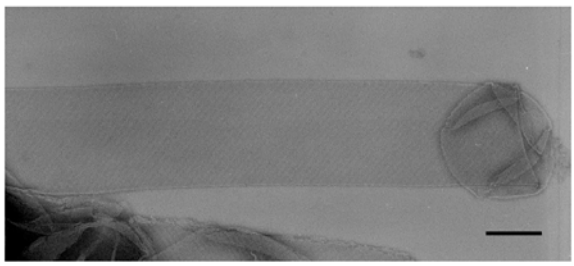


Figure 6 – Tubular 2D crystal of melibiose permease from *Escherichia coli* formed in the presence of melibiose and sodium. Scale bar is 200 Å.

The basics behind 2D crystallization of membrane protein is based on reconstitution of solubilized protein with lipids in a controlled way so that the protein arranges in a crystalline manner [15]. This is accomplished by mixing the solubilized protein with solubilized lipids, and then removing the detergent. The removal can be done with dilution, adsorption to bio-beads or dialysis. Bio-beads are synthetic beads that absorb detergents but not lipids [16]. Factors that are important for 2D crystallization formation are the choice of lipid, lipid to protein ratio (LPR), detergent, the rate of detergent removal, temperature, type of buffer, salts, pH and also co-factors.

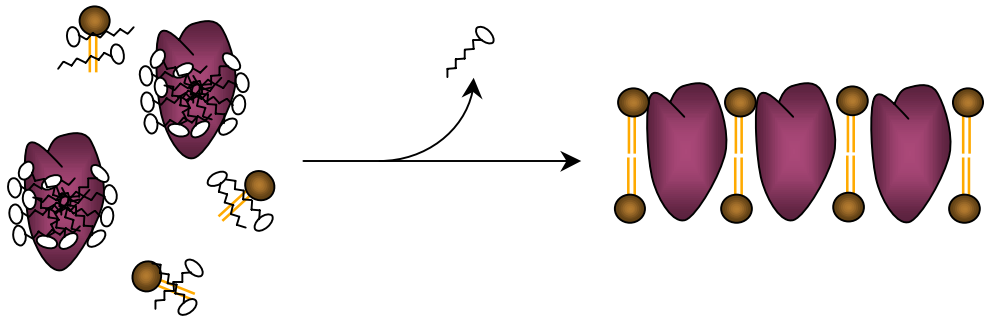


Figure 7 – Formation of 2D crystals of a membrane protein. Solublized protein and lipids are mixed. The detergent is removed and the protein molecules are arranged together with lipids in a crystalline manner.

There are different types of 2D crystals: tubular, sheets or vesicles. Tubular crystals usually collaps in the microscope and therefore can generate two crystal lattices. Typically sheets are preferred, because they are larger, which allows electron diffraction to be collected. Sheets can be single layer or multilayered [15].

The Single Particle Method

As production of 2D crystals can be difficult, another approach may be tried. If the protein or protein complex is large enough, it is possible to take images of the particles in solution [17]. The molecules will end up in several orientations on a grid and these images are then classified according to this orientation (Fig. 8). The particles representing a certain orientation are then averaged in a class and these classes are used to calculate the three dimensional reconstruction. The single particle method is very suitable for larger complexes and viruses and can give important information of how a protein complex is assembled.

The reasons for limited resolution of a single particle reconstruction may be several but one that contributes is heterogeneity of samples. Protein molecules can exist in several states in an aqueous solution. With this method it is possible to distinguish and obtaining populations of theses different states [18].

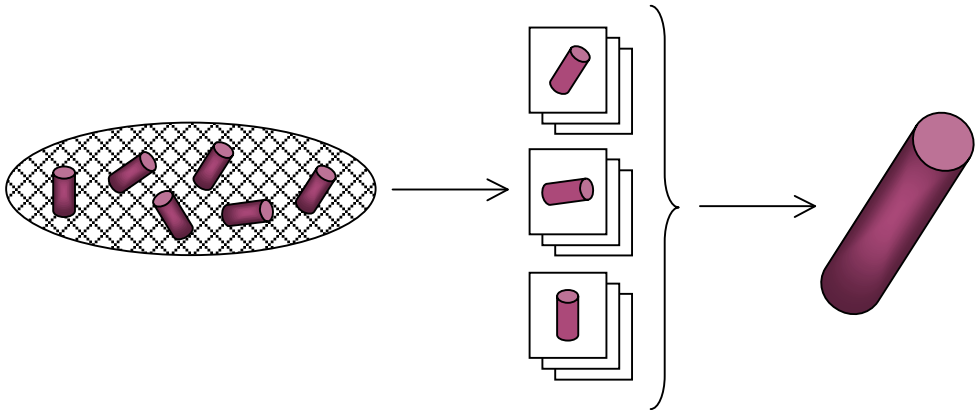


Figure 8 – Single particle method. Molecules are spread on the grid in different orientations. Images are collected and particles are selected. Particles are then sorted in classes and averaged. A 3D structure is calculated from these classes.

Specimen Preparation

The pioneers that wanted to study biological samples with electron microscopy had to contend with several problems. Even as little as 1 $e^-/Å^2$ causes radiation damage to biological samples [19]. One way to deal with radiation damage is to use heavy metal salt such as uranyl acetate or phosphor tungsten, so called negative stain. The stain makes a crust around the specimen, allowing no observation of the interior details. However, the resolution is limited to about 20 Å, although there are results suggesting that it is possible to achieve 4 Å resolution with some stains [20]. Furthermore, since the electrons are charged, the inside of the microscope must be evacuated otherwise the electrons will interact with air molecules. The high vacuum in the microscope induces loss of the three dimensional structure of the biological specimen. To overcome the problems with the radiation damage and vacuum Unwin and Henderson added sugars such as glucose, which are non-volatile to the specimen and recorded data at a low dose [21]. However, this was applicable only on large crystals with a large number of unit cells. Later trehalose has been used successfully, since glucose reduces the contrast of the images [22]. Dubochet and co-workers extended the method by developing low temperature electron microscopy, or Cryo-electron microscopy. The low temperature at -180°C reduces beam damage and instead of freeze-drying the sample, the method for vitrified sample preparation was developed [23]. Vitrification basically means to freeze the specimen in an aqueous buffer, very rapidly so that no ice crystals are being formed [24]. The ice becomes amorphous and does not scatter electrons and therefore does not interfere with the data collection of the sample. Ideally no distortion will occur.

In order to collect data for near atomic resolution structures the carbon that coats the grid, which the specimen attaches to, needs to be absolutely flat. This can be accomplished by being careful when evaporating the carbon [25]. Another important factor is charging caused by electrons absorbed in the grid or the specimen. This causes distortion in the images and diffraction patterns and can be avoided by pre-radiate the carbon grid. Charging also becomes severe when the sample is tilted, but can be reduced by using the sandwich technique [26]. In this approach, the sample is put between two thin carbon layers and this can also help stabilizing 2D crystals during data collection.

This method development in electron microscopy have so far resulted in several a membrane protein structures near atomic resolution; Microsomal Glutathione S-Transferase 1 (3.2 Å) [27], Microsomal Prostaglandin E Synthase (3.5 Å) [28], Aquaporin 0 (1.9 Å) [29], Aquaporin 1 (3.7 Å) [30], Aquaporin 4 (3.2 Å) [31], Light Harvesting Complex II (3.4 Å) [32], Acetyl cholin receptor (4.7 Å) [33, 34] and Bacteriorhodopsin (3.0 Å) [35].

MELIBIOSE PERMEASE FROM *ESCHERICHIA COLI*

Most bacteria use several different carbohydrates as carbon and energy sources, and accordingly contain several different sugar transporters. The melibiose permease transporter (MelB) from *Escherichia coli* was first described in 1965 [36]. It was noticed that there was a distinctly different system from the lactose carrier. This protein was identified by competition studies with thiomethyl-galactoside (TMG) and was first called TMG transferase II and later melibiose permease. The protein has been extensively studied and determined to be a sugar co-transporter that can transport galactosides with the aid of Na^+, Li^+ or H^+ ion gradients [37]. MelB recognizes a broad range of α- and β-galactosides, but α-galactosides are transported in conjugation with Na^+, H^+ or Li^+ while β-galactosides are transported with either Na^+ or H^+. There is a common binding site for all the cations and 1 mole of cation is required for transporting 1 mole of sugar [38, 39]. The binding of sugar is influenced by the membrane potential rather than the specific cat ion [40]. When the chromosomal gene is expressed under normal conditions, about 10 000 MelB carriers are situated in the cell membrane. This is about the same amount of carriers that are expressed from the chromosomal lacY gene [38, 41].

Previously it was believed that prokaryotes solely used the H^+ gradient, where eukaryotes used Na^+. However later it was discovered that MelB and also the TMG permease from *Salmonella typhimurium* coupled Na^+ to sugar transport. Perhaps this feature was first developed in prokaryotes and then selected over H^+ coupled transport in eukaryotic carriers [42].

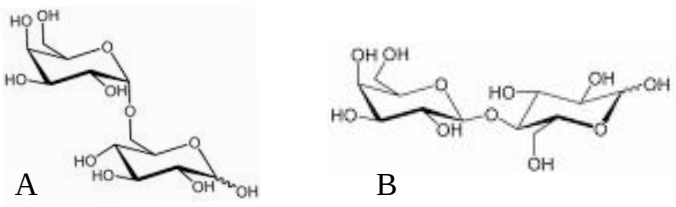


Figure 9 – A) Melibiose (α-galactoside) and B) Lactose (β-galactoside).

MelB belongs to the glycoside (or galactoside)-pentoside-hexuronide (GPH) family, which is distinct from major facilitator superfamily (MFS). There are about 50 members from prokaryotes and eukaryotes, and the proteins of this family they all seem to consist of 12 α-helices. The amino acid sequence of some of the members, including MelB, also contains the characteristic MFS motif (RK)XG(RK) [43, 44]. There are no atomic structures solved for this family of porters yet. The MelB protein consists of 473 amino acids and the topology has been determined experimentally with PhoA fusion analysis which revealed that the protein consists of

12 α-helices [45]. Fourier transform infrared spectroscopy suggests that the protein contains up to 50 % α-helices and about 20 % β-sheets [46].

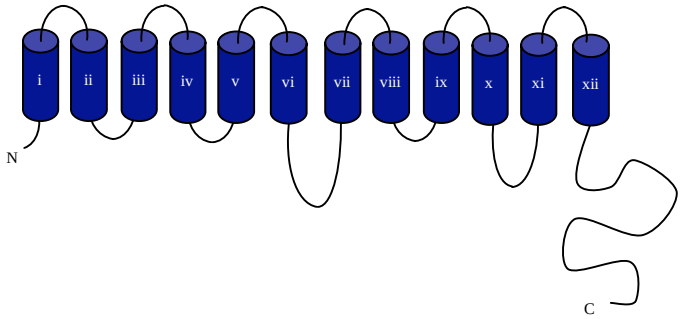


Figure 10 – Topology model of MelB adapted from [45].

SUGAR AND CATION BINDING

Site directed-mutagenesis combined with reconstitution of the protein into liposmes to measure transport have demonstrated that the cation binding is situated in the N-terminal part of the protein. There are four asparagines, which are involved in the coordination of the cat ion; D19 (helix i), D55, D59 (helix ii), and D124 (helix iv) [47-49]. The proline at position 122 is important for H^+ binding. This mutant could transport sugar in the presence of Li^+ and Na^+, but transport was abolished in the presence of H^+ [50]. Furthermore, asparagine 58 and arginine 52 in helix ii are important for the Na^+ recognition [51, 52]. These three helices are also in the proximity of each other forming saltbridges.

The sugar binding sites are proposed to be situated in the C-terminal part of the protein. Several amino acid residues situated in the loop between helix x and xi are proposed to be involved in the sugar binding [53]. Cystein scanning of this loop concluded that it is probably folded inside the aquous channel forming a re-entrant loop, which participates in the transport of sugar [53]. Other amino acid residues indirectly involved, in the sugar binding are tryptophan 299 from helix ix and 342 from helix x [54]. Arginine 141 (in the loop between helix iv and v) is involved in transport mechanism and arginine 149 (in the loop between helix iv and v) and serine 153 (in helix v) lye directly in the sugar binding site [55, 56]. Furthermore, it is likely that the sugar binding and cation sites are overlaping, beacause of the high number of mutations, which simultaneously alters the sugar and cation recognition [57]. It is possible that helix iv is involved in both sugar and cat ion recognition and is connecting the two sites [58].

CONFORMATION CHANGES UPON BINDING

A proposed model for sugar transport is the alternating access, where the protein is open towards one side, binds the substrate, closes and then opens the other side to release the substrates [59]. This model has been proposed for the lactose transporter (LacY) and glycerol 6-phosphate transporter (GlpT) from *Escherichia coli* [60]. The 3D structures revealed molecules that are closed in the side facing the periplasm and open towards the cytoplasm [61, 62]. Also, OxlT from *Oxalobacter formigenes* has been crystallized in a form that is believed to be in the intermediate closed state [63]. The structure of the Na^+/H^+ antiporter (NhaA) from *Escherichia coli*, was crystallized in an inactive state [64]. This protein has a high transport rate and therefore the alternating access model is not thought apply to this protein and more subtle structural changes would occur during transport [64].

The translocation model for MelB is proposed to be a six state process (Fig. 11). The cation binds (1), then sugar (2). The protein alters conformation and releases the sugar (3 and 4) and finally the cation (5). The empty channel alters conformations so that another cycle can take place (6). The rate-limiting step is the release of the cation. Lately this model has been expanded with an intermediate step between 3 and 4 [65]. Fluorescence measurements of tryptophans and H/D exchange have showed that the structural changes occur in the N-terminal part upon Na^+ binding and probably in the loop between helix iv and v [66-68]. Binding of sugar causes further structural changes in the loop between helix iv and v and also in the re-entrant loop between helix x and xi [53, 65, 66]. Also the sugar and ion binding causes an overall compactness of the molecule, which could indicate a closed state [67].

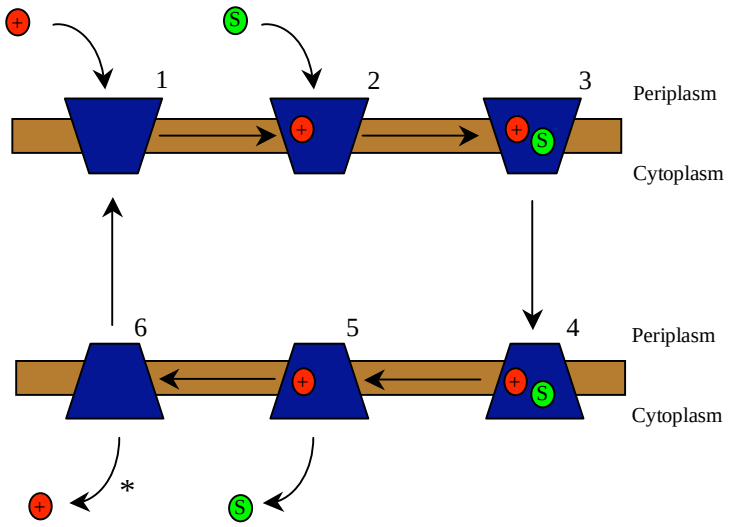


Figure 11 – The transport cycle of MelB. The step marked with a star (*) is rate-limiting. Picture adapted from [39].

THE STRUCTURE OF MELB

In order to study the helical arrangement of MelB, the protein was subjected to 2D crystallization. The break-through was that to grow crystals at a slightly acidic pH (pH 6.0) and at this pH the protein is still active [69, 70]. The protein was crystallized in the presence of melibiose and Na^+ and the projection map was calculated to 8 Å [71]. This map revealed that the protein consists of 12 α-helices organized in two domains, one elongated (helices A-E) and another dense bundle of helices (helices F-L). These domains were arranged asymmetrically around a water filled cavity. This asymmetrical arrangement of the helices is similar to that of the Na+/H+ antiporter (Fig. 12).

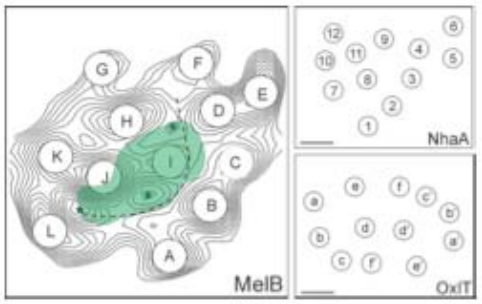


Figure 12 – Projection map of MelB to 8 Å and comparison of the helical arrangement of two other transporters NhaA and OxlT [71].

In paper III the 3D structure of MelB from 2D crystals with cryo electron microscopy was determined to 10 Å. The crystals were formed in the presence of both the substates melibiose and Na^+. The structure revealed a molecule, which was closed in one end and open in the other end, much like the structure of LacY and GlpT (Fig. 13). Docking with NhaA showed an overall fit that was better than with LacY or GlpT (Fig. 14). Considering the six state model, it is surprising that the molecule is in an open conformation, since both substrates are available. Neither LacY or GlpT have crystallized in any other conformation than the one being open towards the cytoplasm. Perhaps the high salt concentration (300 mM) of the crystallization buffer drives the MelB into this conformation.

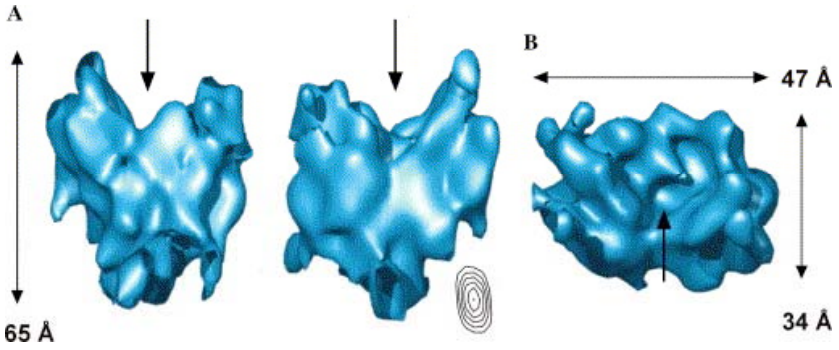


Figure 13 – 3D structure of MelB to 10 Å. The arrow points to the opening of the molecule. The point-spread function shows the resolution in z axis (17 Å) [72].

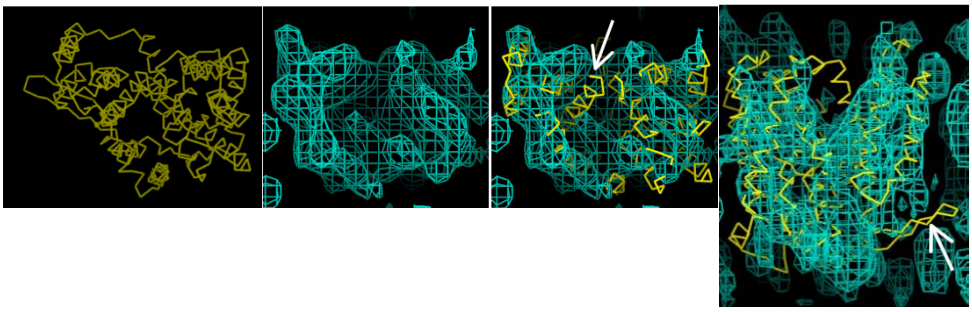


Figure 14 – Docking of NhaA (yellow) and MelB (turquoise). The parts that do not fit well are indicated with white arrows.

Furthermore, we were able to produce crystals without melibiose, but in the presence of Na^+ (unpublished data). The projection map from these crystals showed differences in the structure (Fig. 15). The molecule appears to be more dense, but since no sugar is available, it seems unlikely that it is in the intermediate closed state. The molecule in the 3D structure of MelB can be in either of two states; facing the periplasm or the state where the molecule has flipped facing the cytoplasm. Since the projection map without melibiose is different, it is possible that the 3D model is in a conformation where the sugar and Na^+ are bound. Also, these conformational changes are induced by the sugar binding. No crystals have so far been formed in the absence of both Na^+ and melibiose.

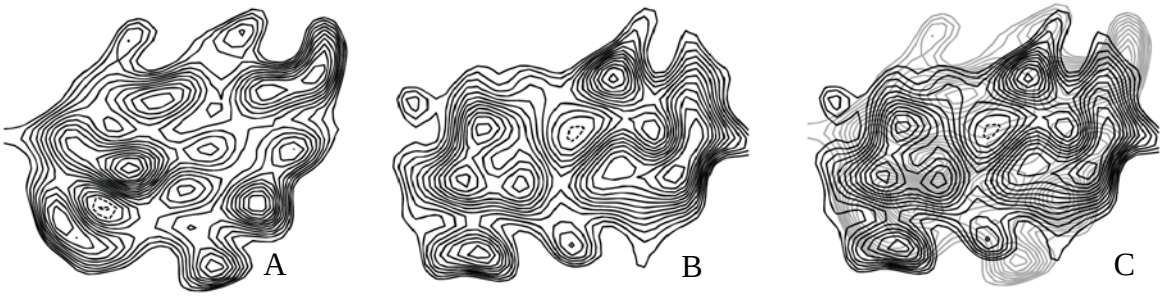


Figure 15 – Projection structures of MelB. A) with melibiose. B) without melibiose and C) Superposition of MelB with meibiose (gray) and MelB without melibiose (black).

KCH, A POTASSIUM CHANNEL IN *ESCHERICHIA COLI*

Potassium is one of the most abundant cations in *Escherichia coli*. The ion is involved in many functions such as control of cellular osmolarity, activating enzymes, maintaining pH or being a secondary messenger [73]. The exchange of potassium occurs in different ways through pumps and symporters. So far, influx systems such as Kdp, Trk, Kup (found in some bacteria) and efflux systems such as Kef been identified and studied [73, 74]. There is also another protein that carries the typical features of a potassium channel, Kch.

In higher organisms potassium channels have been given a lot of attention. These proteins are involved in many different cellular functions such as neurological signalling, muscle contractions and even cell motility [75, 76]. Much emphasise is given to study the function of eukaryotic potassium channels since these are attractive pharmaceutical targets. Structures of prokaryotic potassium channels such as KcsA from *Streptomyces lividans* and MthK from *Methanobacterium thermoautotrophicum* and also the voltage gated channels KvAP from *Aeropyrum pernix* and Kv1.2 from *Rattus norvegicus* have provided new insights of the regulation and also how the selectivity filter coordinates the potassium ion during transport [77-80]. The prokaryotic potassium channels are probably ancestors to eukaryotic, however the function in prokaryotes is not clear [81].

SELECTIVITY AND GATING

Probably all potassium channels have a tetrameric assembly of monomers and the pore region, containing the TMxTVGYG motif, from the four monomers form the selectivity filter [82]. Potassium channels are highly selective for potassium over sodium. It is believed that the potassium ions need to be dehydrated to pass the filter, and the carbonyl group in the conserved filter sequence stabilizes the ions through the passage. A dehydrated sodium ion is too small (ionic radii is 0.95 Å compared to 1.33 Å for potassium) and cannot be stabilized and therefore would not pass through the channel [79].

Potassium channels can be gated by voltage or ligands. Most voltage gated channels contain six tansmembrane helices and the voltage sensing domain is usually located in helix four, where a typical pattern of basic amino acids are situated [83]. Ligand gated channels, such as MthK, have a large cytosolic domain which is referred to as RCK (Regulator of K^+ Conductance) [84]. These domains bind a substrate, which can be for example NAD^+ or Ca^{2+}, in order to open the passage for potassium ions

KCH

The gene for Kch was identified by Milkman in 1994 [85]. Before this discovery it was not known that prokaryotes contained potassium channels. From sequence alignments it was concluded that the *kch* gene was not recently imported into the *Escherichia coli* genome and that this protein is rather ancestor to eukaryotic potassium channels [85]. The amino acid sequence of Kch contains the characteristic motif that functions as the selectivity filter in many other potassium channels and the functional unit is believed to be a tetramer [86]. The monomer of the protein consists of six transmembrane helices and a hydrophobic pore region between transmembrane helix 5 and 6 followed by an RCK domain. However, the typical voltage sensing pattern is missing in helix 4 [85, 87].

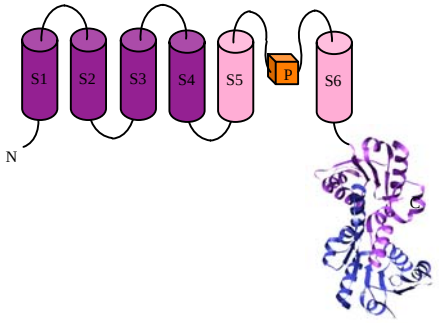


Figure 16 – A topology model of Kch helix S1-S6 adapted from [87]. Helix 5 and 6 (pink) probably form the pore which contains the selectivity filter (orange). The crystals structure of RCK is a dimer, one is marked purple and the other is blue. (PDB code 1d1l [84]).

There is an ATG codon before the start of the nucleotide sequence that codes for the RCK domain. This is observed in other prokaryotic potassium channels such as MthK [88]. As a result of the internal ATG codon there are two proteins produced from the same gene; the full-length protein (46 kDa) and the RCK domain (19 kD). It is believed that the arrangement of soluble RCK domains associated to the full-length protein is common for many potassium channels [89, 90]. Although, the relevance of the soluble RCK domain from the *kch* gene from *Escherichia coli* has been questioned [91].

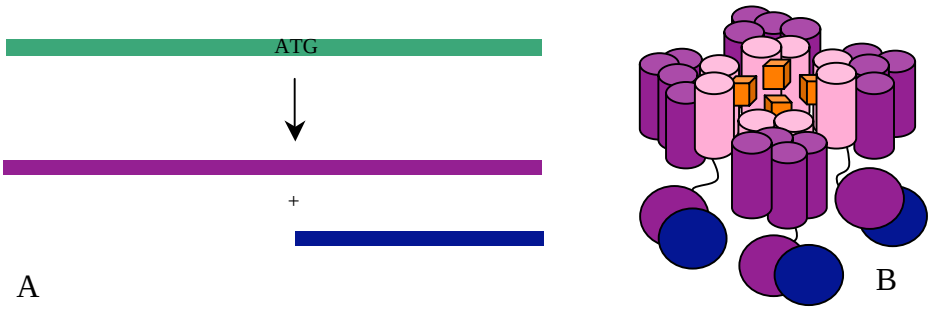


Figure 17 –A) The *kch* gene (green) contains a internal ATG codon. There are two proteins expressed from the gene; the full-length Kch (purple) and the cytosolic C-terminal domain (RCK) (blue). **B)** A possible arrangement of the Kch tetramer and additional RCK domains. The orange cubes represent the selectivity filter that sits between helix 5 and 6 (pink), which form the pore of the channel.

In order to study the structure of Kch we initially wanted to do 2D crystals of the protein. To evaluate whether the protein molecules were aggregated or started to precipitate, the purified protein was analysed with electron microscopy of negatively stained samples. The same shape of molecules was observed in this analysis as in an earlier publication [86]. The conclusion from their observation was that the protein had assembled as trimers of tetramers. However, with closer inspection of their results and ours, we reasoned that there would be another kind of assembly. In paper IV we therefore decided to apply the single particle method to resolve the structural arrangement of this complex.

From the 3D reconstruction it was clear that two tetramers of Kch was dimerised through their RCK domain (Fig. 18). Also, with result from scanning transmission electron microscopy (STEM) it was supported that the size of the majority of the molecules in the purified sample corresponded to dimers of tetramers. The previous structure of the soluble RCK domain showed that the asymmetric unit in the crystal was a dimer, and also pointed out the importance of the hydrophobic interfaces between these [84]. When mutations were performed at the interfaces, the expression of the potassium channel was affected.

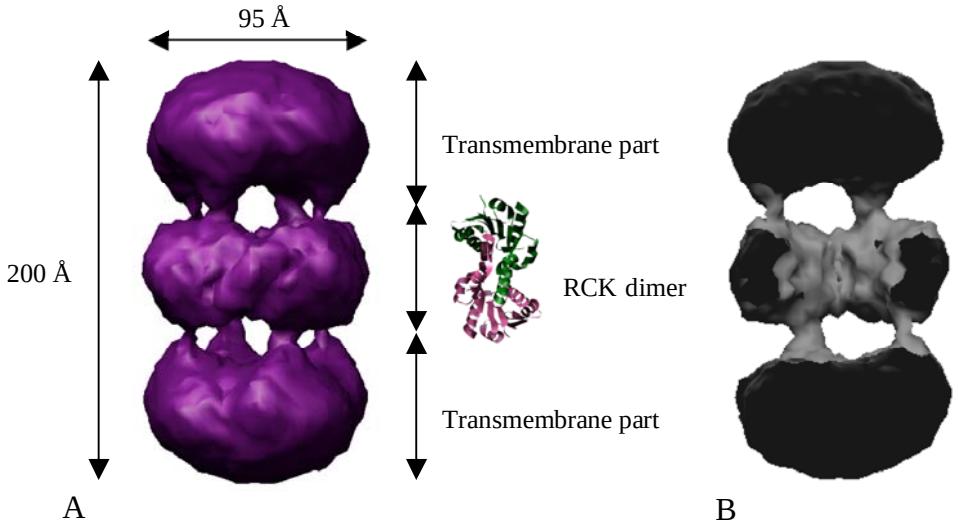


Figure 18 – A) 3D structure of Kch to 25 Å. The RCK dimer PDB code 1idi [84]. B) Section through the structure.

The conclusion from our result is that the functional unit of the RCK domain is a dimer. The soluble RCK domain, which is also expressed from the *kch* gene, probably assembles with the RCK domain of the full-length protein. However, during over-expression of the gene or purification, the full-length protein and the soluble RCK domain form homodimers instead.

We also tested 2D crystallization of Kch, and there were many lamellar aggregates formed in these preparations, regardless of LPR. A tempting interpretation of these aggregates is that the dimer of the Kch tetramer is reconstituted into two lipid bilayers. These kind of lamellar aggregates have been observed in 2D crystallization samples of MelB, however not to this extent.

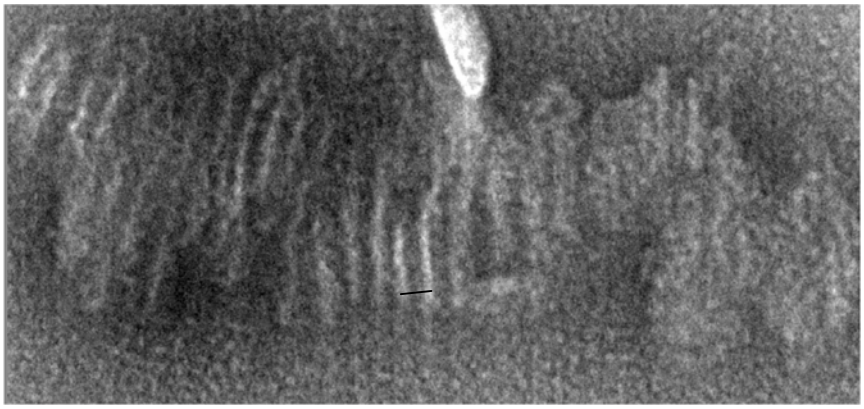


Figure 19 – Negative stain of 2D crystallization attempt of Kch. Image recorded at 40000x magnification. Molar lipid to protein ratio is 40. Numerous aggregates like these were observed. The distances indicated by the black line corresponds to 200 Å.

There is evidence that the protein can transport potassium *in vivo* but no specific biological function has been discovered yet for Kch [91, 92]. Knock-outs of the *kch* gene did not produce a phenotype, however, the gene is well preserved in many different wild-type *Escherichia coli* strains, which indicates that the protein is significant [85, 86]. Attempts to over-express the gene in *Xenopus* oocytes and also reconstitute purified protein into liposomes to measure activity have been made, but no recordable signal has been detected yet [86, 91, 92]. It is possible that the strong dimeristaion effect of the RCK domains is a problem when reconstituting the protein into liposomes. Perhaps more attention needs to be addressed to either over-expression or the purification of Kch to accomplish a functional reconstituted channel.

CORA FROM *THERMOTOGA MARITIMA*

Magnesium is one of the most abundant ions in the cell and is essential for many biological functions such as cofactors for enzymes, DNA transcription and replication and membrane stability [93]. It is interesting to know how this ion is transported and how the concentration is regulated in the cell, since there are diseases connected with malfunctioning magnesium transporters [94]. The first transport system in *Escherichia coli* was reported in 1969 [95]. Initial studies showed that *Escherichia coli, Aerobacter aerogenes, Torulopsis utilis and Aspergillus nigur* could accumulate trace elements such as Ni^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+} and Mn^{2+}, but these had toxic effect on the organisms [96]. This toxic effect also became aggravated at lower concentrations of magnesium. Later a study concluded that there is a transport system in *Escherichia coli* for Mg^{2+} that Co^{2+} competes for, however only at concentrations considered to be non-physiological. [97]. By exposing cells to a mutagen it was possible to isolate a cobalt resistant phenotype [98]. Characterization of this mutant demonstrated that probably two magnesium-linked transport systems (I and II) exists. System I transports Mg^{2+} and Co^{2+}. System II only transports Mg^{2+}, but is repressed at high concentrations of Mg^{2+}. Additional studies concluded that the mutations that contributed to the Co^{2+} resistant phenotype were located in two genes; *corA* and *corB* (for *co*balt *r*esistant), and believed to affect system I. Furthermore, another mutation that abolished system II were identified in the gene *mgt* [99]. The same genes were later discovered in *Salmonella typhimurium.* The *corA* gene was cloned and restored the toxic effect of Co^{2+}, when it was expressed in the Co^{2+} resistant strain [100]. Later the genes involved in Mg^{2+} transport were elucidated to be *corA, mgtE,* and *mgtA/B.* The family of *mgtE* and *corA* genes are found in both prokaryotes and eukaryotes and both are believed to be housekeeping genes [101]. The CorA or MgtE proteins are said to be responsible for the magnesium uptake in prokaryotes and eukaryotes [102]. Only a minority of organisms carry both genes [103]. The gene for *mgtA/B* is found primary in eukaryotes and belongs to the P-ATPase superfamily [101].

The familiy of *corA* genes are distributed in both prokaryotes and eukaryotes and are classified into the 2-TM-GxN group [104]. The monomer of these proteins consists of two trans membrane helices and a larger N-terminal domain. The sequence is not entirely conserved between different organisms. It is most conserved in the C-terminal region, where a typical motif (YGMNF) can be found in the loop between the two transmembrane helices. This motif is believed to be important for accumulation of ions or could be a selectivity filter [105]. The YGMNF motif is less preserved in eukaryotes and is shortened to GxN [98]. Sequence alignments in combination with structural information from CorA of *Thermotoga maritima* have distinguished two subgroups, A and B where CorA of *Escherichia coli* belongs to subgroup B and CorA of *Thermotoga maritima* belongs to A [93].

STRUCTURE OF CORA FROM *THERMOTOGA MARITIMA*

The structure of CorA from *Thermotoga maritima* has been solved by three groups independently [1, 105, 106]. The structure, which is described below, is from Eshaghi *et al*. 2006. The CorA monomer is composed of two transmembrane helices and a large N-terminal domain, were arranged as a pentamer in the crystal structure. The large N-terminal domains form a funnel where two metal binding sites were found in proximity of each other (M1 and M2). The crystals were obtained in the presence of 0.1 M of Mg^{2+} and crystals soaked with Co^{2+} at a concentration of 0.02 M showed that the metal sites were then occupied with Co^{2+}. Aspartate 277 from each monomer forms a ring in the pentamer, where also, most likely, a Mg^{2+} ion is bound asymmetrically. Unfortunately, the loop sequence that contains the conserved motif was not resolved in any of the structures. CorA is proposed to be in a closed state and there are results which conclude that the protein changes conformation to the closed state in the presence of Mg^{2+}, Co^{2+}, Ca^{2+}, Ni^{2+} and cobalt(III)hexamine (CoHex) [106].

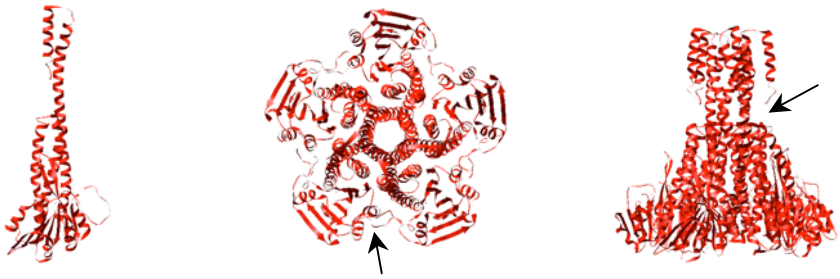


Figure 20 – Structure of CorA from *Thermotoga martitima* to 2.9 Å. PDB code 2IUB [1]. A) Monomer with the two trans membrane helices and the large N-terminal domain. B) Pentamer viewed from the trans membrane helix side. Arrow indicates the metal binding sites. C) Side view of pentamer Arrow indicates the aspartate ring.

The structure of MgtE from *Thermus thermophilus* was recently solved in the presence of Mg^{2+} and the cytosolic domain was solved in both presence and absence of Mg^{2+} [107]. From these structures it was possible to build a hypothesis how the protein changes conformation upon binding of magnesium. At low intracellular concentration of Mg^{2+} it is believed that the ion dissociates from the binding sites and the soluble domains changes conformation, which releases transmembrane helices and opens the pore. It is possible that the same mechanism will apply to CorA also.

FUNCTION OF CORA

Most of the functional studies have been made on CorA from *Escherichia coli*, *Salmonella typhimurium* and *Haemophilus influenza*. From these studies CorA is belived to be the major magnesium transport system for Bacteria and Archea. The protein can transport Co^{2+} and Ni^{2+}, however at higher K_d values (20-30 μM and 200 μM respectively) compared to Mg^{2+} (10-20 μM) and are considered not be the main substrates [101, 108]. There are only few reports on functional studies of CorA from

Thermotoga maritima (tmCorA) [106, 109]. In one study, the protein was reconstituted into liposomes and it was demonstrated that the protein selectively transports magnesium, although the concentration that was required was much higher than for other CorA proteins (1-2.5 mM) [109]. Based on sequence alignments showing that tmCorA belongs to another group of transporters than *Escherichia coli* and that crystals soaked with Co^{2+} could compete with Mg^{2+} for the binding sites, we speculated that perhaps tmCorA has other substrates than magnesium.

Substrate Specifity

In paper V the substrate specificity of tmCorA was explored with two methods, thermostability and tryptophan fluorescence quenching in the presence of different metals. These measurements showed that the protein was stable up to 65°C without addition of metals. In the presence of 1 mM Co^{2+} or Ni^{2+} the protein was stable up to 95°C, but only about 80 % of the protein was stable in 1 mM Mg^{2+} at 85°C, and precipitated completely at 95°C (Fig. 22). As little as 200 µM of Co^{2+} could keep about 85% of the protein stable at 85°C. Thermostability measurements were also made on CorA from *Escherichia coli*, to exclude that this effect was an artefact from unspecific binding of Co^{2+} or Ni^{2+} (Fig. 21). In this experiment it appears as Co^{2+} and Ni^{2+} destabilize the protein and perhaps the binding is different in these two proteins.

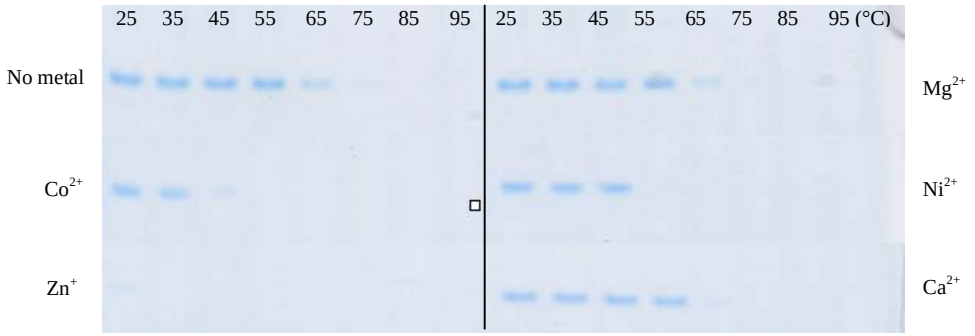


Figure 21 – Thermostabiliy assay of CorA from *Escherichia coli* at 25-95°C in the presence of 1 mM of different metals.

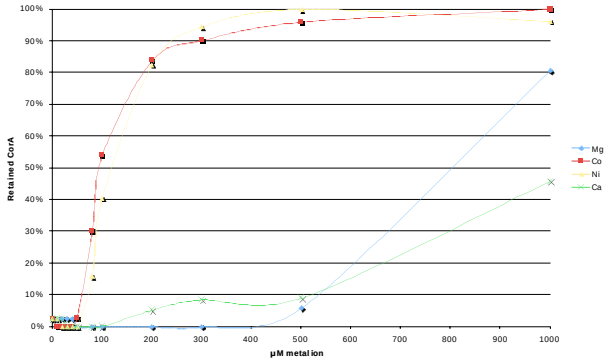


Figure 22 - Stability of tmCorA at 85°C in the presence of Mg^{2+}, Co^{2+}, Ni^{2+} and Ca^{2} at different ion concentrations. The amount of protein is estimated in relation to unheated protein (100%).

Fluorescence quenching of tryptophan measurements gave indications of structural changes upon binding of Co^{2+} and Ni^{2+}. From these measurements we could see stronger quenching in the presence of Co^{2+} at a lower concentration than Ni^{2+}. No quenching was observed from Mg^{2+} even at 5 mM. From these experiment we concluded that Co^{2+} possibly binds to two different sites, which has different affinity for the ion. The K_d for binding site one is 28 μM and the other is 750 μM. There was probably only one binding site for Ni^{2+} with a K_d value of 34 μM. This is interesting since previous measurements of CorA from other organisms report K_d values of Ni^{2+} in the range of 200 μM [101]. *Thermotoga maritima* is a thermofile, that can grow in temperatures between 55 and 90°C, and optimally at 80°C. These studies were made on the protein in detergent solution at room temperature and therefore these K_d values may not be definite. These results suggests that tmCorA may have Co^{2+} as a primary substrate.

CONCLUSIONS AND FUTURE PERSPECTIVES

Structural studies together with functional studies are really important to shed more light on how a particular protein works. A structure alone is not enough to draw all conclusions and usually generates more questions, and functional studies are sometimes not sufficient to completely understand how a protein is regulated.

Paper I – In this study the detergent effect on the TEV protease activity was examined. It was concluded that there are no trends for how a detergent will affect the removal of an affinity tag. This can also be expanded to other activity assays of membrane proteins that are performed in the presence of detergents.

Paper II – The stability screen of YidC yielded a more stable protein and it can be used for other membrane proteins in the future. There are other factors that can be screened in order to obtain a more stable protein, such as co-factors, addition of lipids and type of detergents used.

Paper III –2D crystals of MelB has generated a 3D structure and the resolution of the structure from melibiose permease is at this stage only to 10 Å. This makes it difficult to resolve any particular features of the ion or sugar binding. The protein has to this point only formed rather small tubular crystals, which has made it impossible to collect diffraction data. The 2D crystals without melibiose, which initially show differences in the projection maps, are indeed interesting and promising.

Attempts to do 3D crystallization of MelB have been made. Crystals have been obtained, although it appears to be difficult to solve the structure from these (Personal communication with G. Leblanc). Perhaps 2D crystals are at the moment the most hopeful approach to get better structural understanding how the protein changes conformation upon binding of sugar and/or sodium.

Paper IV – The structure of Kch provided insight in a complex that is formed during over-expression or purification. Two tetramers dimerises through their RCK domains, and this is unlikely the physiological relevant form. We established that the gene for *kch* expresses two proteins; the full-length and the soluble RCK domain. It is plausible that these protein associate to form the functional unit.

Paper V – The next step to explore the transport activity of tmCorA will be to reconstitute the protein into liposomes to compare Co^{2+} and Mg^{2+} transport. Also, now that we have established a system for monitoring changes in the structure by fluorescence quenching of tryptophans, mutants can be investigated. These measurements will be performed at higher temperatures to simulate the optimal growth condition of *Thermotoga martitima*.

ACKNOWLEDGEMENTS

I am very grateful for being surrounded by nice people that have shared ups and downs and always been there for me in my life and during the time as a PhD student. There are a few people I especially would like to thank:

Hans Hebert for accepting me as a PhD student in your group and introducing me to the EM field. I am really thankful for you kindness and I really appreciate that you always supported and believed in me.

Said – I am so happy for being your bi-doktorand! You really made the projects so inspiring and you have been such a fantastic support!

Pär Nordlund for welcoming me into your group and letting me be a part of it!

Ralf Morgenstern for being a very nice support and always taking time for my science problems!

Past and present members of the EM group especially; Kimberley –Thank you for always being there and for all the fun trips we made together! It's been really nice having you as a friend during this time! Caroline – for being a really nice roomy and for all the chats about science and other things! Pasi – Thank you for taking care of me as a rooky in the group and with your patience with all my questions. It's been really nice working together on the MelB project! Ulrika and Peter for all the good times in Lund and Stockholm! Priyo for you calm always being so nice! Philip for help with single particle and processing problems! Urban for all the data support!

The people at Novum; Sofia, Boel, Katarina and Linda for ranting and fika! Clara and Sassa for good times especially not in the lab, the legendary Nov2k party! Kristina for always listening and taking care of us PhD students! Erik for everything computer related! The Inger's: for helping me with all sorts of things and for always being in such good moods!

Yoshi for welcoming us in your laboratory! You took very good care of us! Especially thanks to Tanaka-san, Chris, Nishisawa and Imai-san who helped us a lot and made the stay pleasant and fruitful!

Shirley for good collaboration on the Kch paper!

Gustav och Peter för bra samarbete på CorA!

Gérard for all the help with MelB and Raymonde for helping me purfify it!

Lars och Lars – För att ni var jättebra ex-jobb handledare och gav mig en bra grund att stå på i mitt doktorerande!

Past and present people in PN group especially: my roomies which made the PhD life much more enjoyable! Daniel G – det har varit så otroligt roligt att dela rum med dig! Tack för din sköna humor, bra pep-talks emellanåt och för Yhak klonen! Damian dessutom för all hjälp med CorA! Var försiktig på motorcykeln i framtiden! Karin för att du varit hjälpsam och bra fika kompis. Henrik – en skön norrlänning! Tack för att du stått ut med oss!

Hanna och Maria för afterworks och fika och ett jättefint bröllop! Pelle – det var roligt att jobba med dig i Mempipe 1, back in the good old days! Daniel MM för roligt chit-chat och en grym resa till Grenoble! Ella, Elle l'la! Och tack för att du läste igenom avhandlingen! Agnes för tips och trix med kristalliseringarna! Marina för en bra lab-granne! Marie för trevligt prat och för att vara expert på Greta! All the new Post-docs! Anna, för all hjälp under sommaren med odlingarna och reningarna! People at SGC especially: Helena och Susanne för jättebra samarbete på TEV. Martina, Andreas, Tobias och Ida för luncher, fika, träning och afterwork.

Lola – for all the good times in and outside the lab, the skvaller and fika!

Audur! För att du är så otroligt rolig och juste! Och för att du står ut med våra dåliga islandsskämt! Tackur!

Ulrika! U som i Ullig, L som i Labrador…tack för att du är en så bra vän och att jag alltid kan älta med dig!

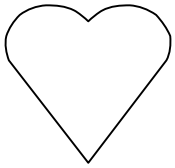
Jasmine! Tack för att du är en så fin vän! For all the good times and coming ones!!

Min fina familj i norrland!

Sue-Li för allt allt allt! För att du är världens bästa vän genom vått och torrt, genom studier, kontinenter och allt! Och Tobias som är världens bästa man till världens bästa vän!

John – Min kära storebror! Som alltid finns för mig och vill lyssna på mig om allt möjligt!

Mamma och Pappa – Tack så mycket för att ni alltid finns där för mig och att ni stödjer mig i allt och att ni tror på mig när jag själv tappar den!



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