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TALLINN UNIVERSITY OF TECHNOLOGY

Faculty of Science
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Department of Molecular Biology and Genetics

Structural and functional studies of Ca^{2+}-ATPases

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Master Thesis

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Ca^{2+}-ATPaaside struktuursed ja funktsionaalsed uuringud

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Abbreviations

AMPPCP	Adenosine 5'-(β,γ-methylene)-triphosphate. Non-hydrolysable ATP analogue
BHQ	2,5-di-(*tert*-butyl) hydroquinone
$C_{12}E_8$	Octaethyleneglycol Mono-n-dodecyl Ether
CPA	Cyclopiazonic acid
DDM	n-Dodecyl β-D-maltoside
DMSO	Dimethyl sulphoxide
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
E.coli	*Escherichia coli*
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N.N',N'-tetraacetic acid
IC_{50}	half maximal inhibitory concentration
IMAC	Immobilized metal affinity chromatography
LLCA1	*Lactococcus lactis* Ca^{2+}-ATPase
LMCA1	*Listeria monocytogenes* Ca^{2+}-ATPase
MOPS	3-(N-morpholino)propanesulfonic acid
MR	Molecular replacement
PEG	Poly ethylene glycol
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	Sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SR	Sarcoplasmic reticulum
TEV	Tobacco Etch Virus protease
TG	Thapsigargin
TM	Transmembrane (helix)
Tris	tris(hydroxymethyl)aminomethane
WT	Wild-type
B-ME	β-mercaptoethanol

Introduction

P-type ATPases are a large family of transmembrane proteins which actively transport cations and phospholipids across the membrane and that are of vital importance. These proteins play crucial roles in cell growth, proliferation, removal of toxic ions from cells, muscle contraction, signal transduction. Impaired function of P-type ATPases can lead to various pathological conditions, such as cardiovascular and neurodegenerative diseases. Thus, detailed knowledge of P-type ATPase structure and function is of high importance due to their potential use as drug targets and general understanding of the human physiology.

Ca^{2+}-ATPases belong to the subfamily of P_{II}-type ATPases and have been the most extensively studied P-type ATPases to date. The major representative of this subgroup is the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), which is responsible for removing Ca^{2+} ions from the cytosol and transporting them back into sarcoplasmic reticulum. In mammals, SERCA is encoded by three different genes (1, 2 and 3). At least two isoforms from each gene can be formed as a result of alternative splicing. SERCA is inhibited by nanomolar concentrations of a mycotoxin called cyclopiazonic acid (CPA).

A bacterial homolog of SERCA1a, the *Listeria monocytogenes* Ca^{2+}-ATPase (LMCA1), was functionally characterized in 2011 by our group. Furthermore, the crystal structure of LMCA1 was solved to a resolution of 4.3 Å. However, due to the relatively low resolution of the X-ray diffraction data, the molecular details of the structure could not be seen. Moreover, LMCA1 was shown to be insensitive to the SERCA1a inhibitor CPA, and it was predicted from sequence alignments that the residues important for CPA binding by SERCA1a were absent from LMCA1. This fact made the inhibitor binding pocket in LMCA1 one of the focuses of the current work. To understand the nature of CPA-insensitivity of LMCA1, mutant forms of the pump have been constructed, over-expressed, purified and tested for the activity in the presence of CPA. When residues, responsible for inhibitor binding were identified, the results were confirmed on another bacterial pump from *Lactococcus lactis*. Understanding the general mechanism of CPA inhibition by examining LMCA1 mutants could help to develop inhibitor derivatives that can block the pump in *L. monocytogenes* and in other pathogenic bacteria. Moreover, if

bound, it is predicted that CPA will stabilize the pump and thus improve crystal quality and diffraction resolution. In this way, the molecular details of a bacterial Ca^{2+} pump will finally be revealed. In this study, the importance of two amino acids in CPA binding was underscored with activity studies on LMCA1 mutant forms. The results were confirmed on another Ca^{2+}-ATPase from *Lactococcus lactis.*

Another major focus of this study was the purification of cardiac SERCA2a, which regulates relaxation and contraction of the heart muscle. The activity of SERCA2a is regulated by the small transmembrane protein phospholamban (PLB). A detailed understanding of SERCA2a regulation by PLB is one of the most prominent challenges in the field, and this can be achieved through the determination of a high resolution crystal structure of a SERCA2a-PLB complex. Moreover, crystal structure of SERCA2a alone is of high interest as well. However, the efficient purification that would allow for SERCA2a crystallization trials and a possible structure determination at atomic resolution is required. This would provide valuable functional information that would aid the design of therapeutic agents to improve contractility of the failing heart. In present work, SERCA2a was successfully purified, the presence of PLB in the preparation was identified and preliminary candidate SERCA2a crystals have been obtained.

Theoretical background

P-type ATPases

P-type ATPases are a large group of membrane proteins ubiquitous amongst all life forms from bacteria to mammals. Using energy released from ATP hydrolysis, P-type ATPases transport mainly cations or phospholipids across cellular membranes. They are responsible for many vital processes such as cell growth, cell proliferation, signal transduction, muscle contraction and relaxation, gastric acid secretion, action potential regulation in the brain, removal of toxic heavy metal ions from cells, potassium reabsorption and acid-base regulation in the kidney. Impaired function of these highly important enzymes can lead to different abnormalities and disorders, for instance cardiovascular, metabolic, neurodegenerative and skin diseases, as well as cancer (Bublitz, Morth et al. 2011).

In 1997, the Nobel Prize in Chemistry was awarded to Jens Christian Skou for his discovery of a Na^+/K^+-ATPase (Skou 1957). The enzymes from this large family, with molecular masses of 60-150 kDa and 6-10 transmembrane helices, have several well-conserved sequence features. Of these, arguably the most important is the D-K-T-G motif, which is present in all P-type ATPases. It contains an aspartate residue that is reversibly phosphorylated during functional cycle. The formation of an aspartyl phosphoanhydride intermediate is the signature feature that gave P-type ATPases their name. Another conserved motif in the family is the T-G-E-S loop, which is responsible for catalyzing the subsequent hydrolysis of the aspartyl phosphate intermediate (Palmgren and Nissen 2011).

The transport of ions across the membrane is facilitated by large rigid-body conformational changes in the three cytoplasmic domains of P-type ATPases, which drives a rearrangement of the transmembrane "M" domain. The three soluble domains are known as the "A" (actuator) domain, the "N" (nucleotide binding) domain and the "P" (phosphorylation) domain (Figure 1). During the pump cycle, they move relatively to each other, while remaining relatively unchanged themselves (Toyoshima, Nakasako et al. 2000, Palmgren and Nissen 2011).

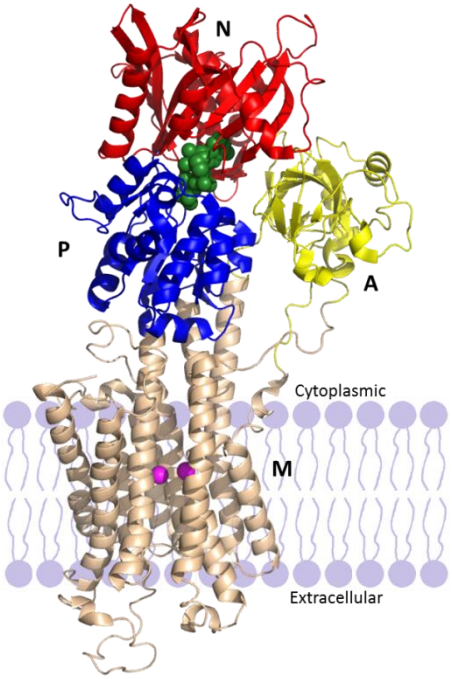

Figure 1. Structure of P-type ATPase. Model of SERCA1a is represented in this figure (PDB entry 1T5S). "A", "N", "P" and "M" domains, intrinsic to all P-type ATPases, are shown in yellow, red, blue and wheat colors respectively. ATP is bound in the "N" domain and is shown in green. Two calcium ions (magenta spheres) are bound on the cytoplasmic side of the membrane and will be transported by the pump to the extracellular side.

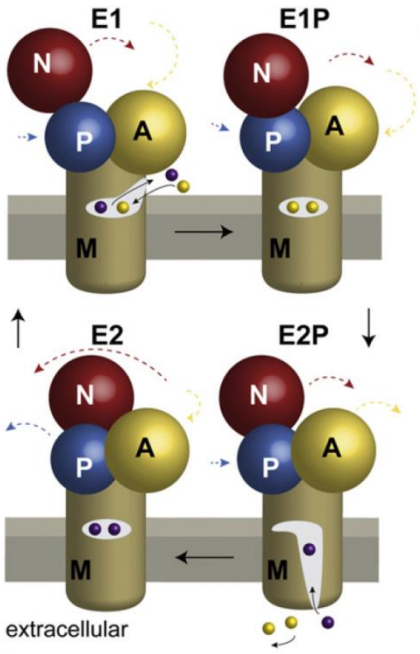

Figure 2. A schematic representation of the P-type ATPase catalytic cycle (**Post-Albers cycle**) is represented in the figure. A detailed description can be found in the main text. (Mattle, Sitsel et al. 2013)

Two major conformational states of P-type ATPases are known, namely E1 and E2 (Figure 2). The first is open towards the cytoplasm/lumen and is characterized by high affinity to exported substrate ions. Ion binding triggers the formation of the high-energy phosphorylated intermediate, where the ion-binding site becomes occluded (E1-P). A rotation of an "A" domain loop harboring the T-G-E-S motif causes dissociation of ADP. The subsequent conversion from the high-energy E1-P state to the low-energy E2-P state is spontaneous. As a result of this, the transmembrane domain opens to the luminal side of the membrane and the ions are released due to disruption of the high-affinity binding site. Counterions (if any) may then bind to a newly-formed high-affinity site, which triggers dephosphorylation of the E2-P state to E2. This is then subsequently converted to E1 by occlusion of the transmembrane domain with subsequent opening towards the cytoplasmic/luminal side and release of counterions. The cycle can then begin anew (Albers 1967, Post, Kume et al. 1969).

Despite common catalytic cycle, similarity in sequence, function and overall structure, P-type ATPases have many differences among the five major subfamilies (P_I-P_V), which they are divided into based on the phylogenetic analysis. This classification was introduced by Palmgren and Axelsen in 1998 (Palmgren and Axelsen 1998).

The **P_I-type ATPases** are considered to be the most ancient subfamily. They can be further divided into P_{IA}-ATPases and P_{IB}-ATPases. The former are represented by the bacterial potassium ion transport systems that are responsible for turgor pressure regulation. Although they are very minimalistic, having only 6 TMs, these pumps are incapable of transporting ions by themselves and form a large 3-4 subunit complex with other transmembrane proteins to function (Hu, Rice et al. 2008). Pumps from the P_{IB}-subgroup play a crucial role in heavy metal detoxification by removing toxic ions such as Cu^+, Zn^{2+}, Cd^{2+}, Ag^+, Pb^{2+} and Co^{2+} from cells. They also supply metalloproteins with cofactor ions such as Cu^+ (Petris, Strausak et al. 2000). P_{IB}-ATPases often have a subgroup-specific heavy metal binding domain (HMBD) at their N-termini, which seems necessary for the activity of the pump. However, the exact function of the HMBD remains unclear. Additionally to the core six helices, shared by all P-type ATPases, members of P_{IB}-subgroup have two N-terminal TM segments (MA and MB), that possibly play a role in ion transport. Including MA and MB, members of this subgroup have only 8 transmembrane helices (Mattle, Sitsel et al. 2013). Insight into the structural details of P_{IB}-ATPases was gained only recently with the first structure of a copper pump solved by X-ray crystallography in 2011 (Gourdon, Liu et al. 2011). It is well known that mutations in human copper pumps are associated with the severe Menkes (Agertt, Crippa et al. 2007) and Wilson diseases (Ala, Walker et al. 2007).

P_{II}-ATPases are considered to be the most diverse subgroup, which includes a variety of Ca^{2+}-ATPases. The three major subfamilies P_{IIA}, P_{IIB}, and P_{IIC} are described below.

Subfamily P_{IIA} contains the sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs), which will be described later in more detail, as well as the secretory-pathway Ca^{2+}-ATPases (SPCAs). Two SPCA isoforms are known (SPCA1 and SPCA2), of which SPCA1 is a housekeeping enzyme. SPCA2 expression is tissue-specific, and is

predominantly found in the gastrointestinal tract and secretory tissues. SPCAs are found in the Golgi apparatus and secretory compartments. SPCAs have only one ion binding site. In addition to calcium, SPCAs are also able to transport manganese ions, and are responsible for providing cofactors for enzymes and cell detoxification (Vanoevelen, Dode et al. 2005, Vandecaetsbeek, Vangheluwe et al. 2011). Underscoring the importance of SPCAs, mutations of these proteins are the cause of Hailey-Hailey disease, a skin disorder (Missiaen, Raeymaekers et al. 2004).

The P_{IIB} subfamily is represented by calmodulin-regulated plasma membrane Ca^{2+}-ATPases (PMCAs). Among the four PMCA isoforms, PMCA1 and PMCA4 are found in most tissues, while PMCA2 is expressed in the brain, striated muscle, liver, kidney and mammary gland, and PMCA3 in the choroid plexus. Together with calcium channels, these proteins remove cytosolic calcium into extracellular medium. In contrast to SERCA, PMCA has only one calcium biding site; however, the overall structure is predicted to be similar (Brini and Carafoli 2011). Malfunctioning of PMCAs is associated with different pathological conditions including diabetes, atherosclerosis, aging (Brini and Carafoli 2011), as well as Alzheimer disease (Mata, Berrocal et al. 2011) and cancer (Curry, Roberts-Thomson et al. 2011).

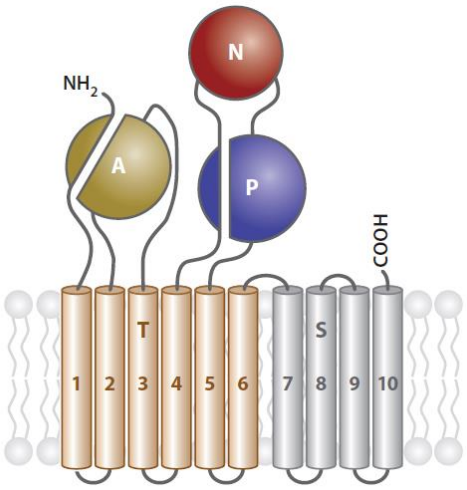

Figure 3. Schematic representation of the SERCA1a structure is shown in Figure 1. All domains are indicated. While six transmembrane helixes are intrinsic to all P-type ATPases, subgroup P_{II} has four additional C-terminal helixes M7-10 represented in grey. "T" (transport) and "S" (class-specific support) domains are part of the transmembrane "M" domain (Palmgren and Nissen 2011).

The type P_{IIC} proteins are hetero-oligomeric and include the well-known and highly important Na^+/K^+ and H^+/K^+-pumps of animals. The former is a housekeeping enzyme, involved in action-potential formation (Jorgensen, Hakansson et al. 2003), proper heart function, signal transduction and regulation of many different cellular processes (Aperia 2007), while the latter is crucial for kidney function (Wingo 1989) and stomach acidification (Sachs, Chang et al. 1976). Both pumps are essential drug targets in humans (Sachs, Shin et al. 2007, Yatime, Buch-Pedersen et al. 2009, Wang and O'Doherty 2012).

While P_{IB}-ATPases have two additional N-terminal TMs, P_{II}-ATPases have four additional C-terminal segments M7-M10 (Figure 3) (Mattle, Sitsel et al. 2013).

The major representatives of subgroup **P_{III}-type ATPases** are the H^+-ATPases in plants and fungi. They maintain electrochemical gradients, and play an important role in nutrient uptake and cell turgor maintenance (Harper, Manney et al. 1990). A high resolution crystal structure of a plant proton pump was solved in 2007, gaining insight into its unique structural features (Pedersen, Buch-Pedersen et al. 2007).

The role of **P_{IV}-type ATPases** is to maintain the phospholipid asymmetry in eukaryotic cell membranes. These are the only P-type ATPases to transport lipids rather than ions. Malfunction of proteins from this subfamily is associated with developmental defects in animals and several disorders in humans (van der Mark, Elferink et al. 2013).

P_V-ATPases are poorly understood, with both their substrate and function being unknown. However, knockouts of plant P_v-ATPases have indicated their crucial function for pollen development (Jakobsen, Poulsen et al. 2005). Moreover, the association of these pumps with neurological diseases has also been shown (Ramirez, Heimbach et al. 2006).

Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA)

Calcium functions as an important intracellular signalling molecule, controlling muscle contraction, gene expression, cell proliferation, differentiation and apoptosis. Thus, the control of calcium regulation is of vital importance. A key protein controlling the very low (nanomolar range) cytosolic calcium levels in the cell is the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) – one of the major representatives of the P_{IIA}-subfamily.

In mammals, SERCA is encoded by three genes that produce different isoforms of the protein: ATP2A1, ATP2A2 and ATP2A3 (or SERCA1,-2,-3). During every catalytic cycle SERCA transports two calcium ions into the sarcoplasmic reticulum and

countertransport two (Yu, Carroll et al. 1993) to three (Stokes and Green 2003) protons per ATP hydrolyzed.

Two isoforms of **SERCA1** are known, SERCA1a and SERCA1b, which are generated by alternative splicing. The latter is expressed in neonatal skeletal muscles together with SERCA2a, however later on it is entirely replaced by SERCA1a (Periasamy and Kalyanasundaram 2007). SERCA1a is expressed in fast-twitch muscles and it pumps calcium ions back into sarcoplasmic reticulum (SR) after their release into the cytosol during muscle contraction. SERCA1a is the best described P-type ATPase, with structures of the majority of its conformational states having been solved (Bublitz, Poulsen et al. 2010). The availability of many high resolution structures allowed insight into the pump, showing in detail the structural changes of the pump during its catalytic cycle. Malfunction of SERCA1a can lead to Brody myopathy (Brody 1969).

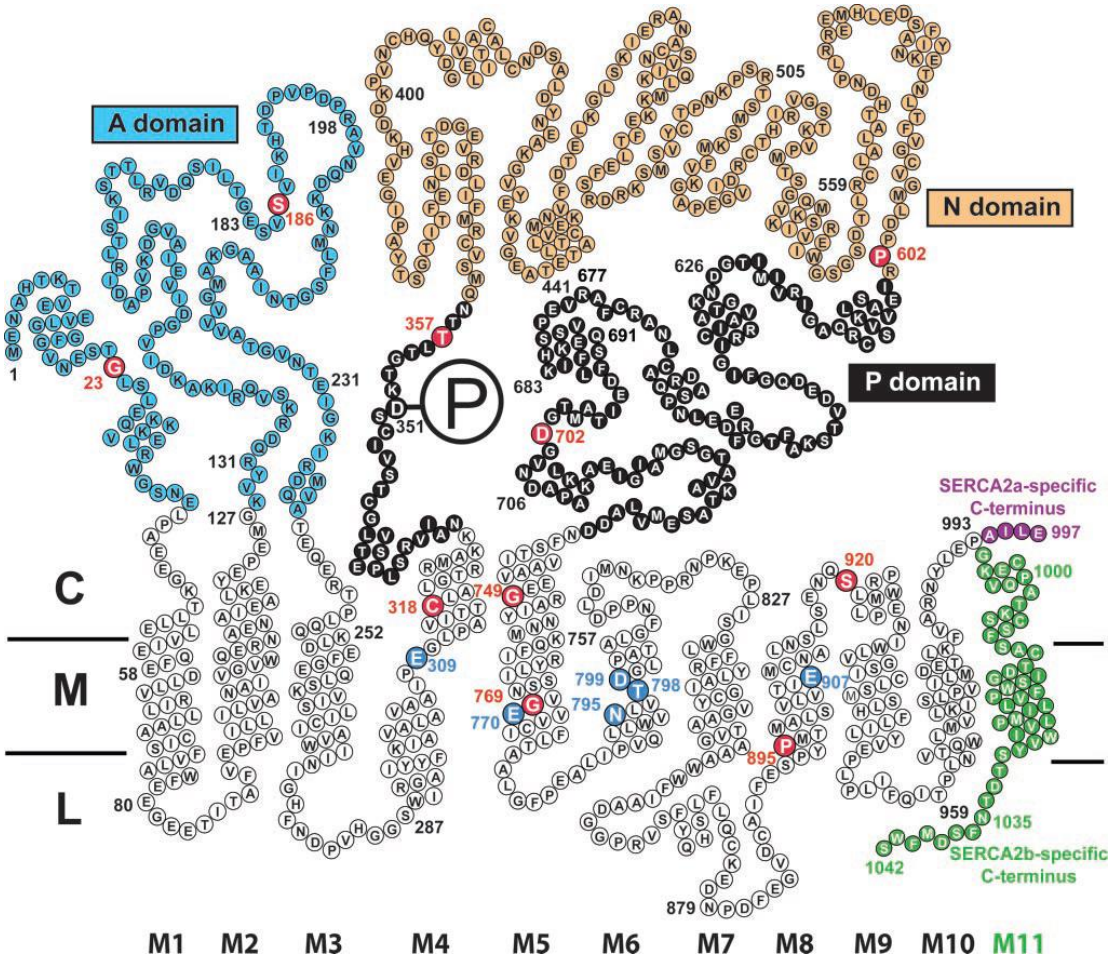

Figure 4. Illustrative scheme of SERCA2 isoforms showing C-terminal differences between SERCA2a and SERCA2b. The topology model is based on the SERCA1a structure. Mutations associated with Darier's disease are highlighted in red (Dode, Andersen et al. 2003).

Of the three mammalian SERCA isoforms, **SERCA2** is the most ubiquitous, maintaining internal calcium stores in most cells, while the others are specific to certain tissues. Three SERCA2 splice variants can be distinguished: SERCA2a, b and c.

SERCA2a is the predominant SERCA protein in the heart, and is also found in slow-twitch skeletal muscles and smooth muscles. It has 84% protein sequence similarity to SERCA1a, however, a small C-terminal extension of four amino acids (AILE) is present (Figure 4) (Dode, Andersen et al. 2003). SERCA2a initiates cardiac relaxation by pumping calcium ions back into the SR after their release into the cytosol during heart contraction. SERCA2a also controls the amount of Ca^{2+} taken up into the cardiac SR and thereby also the amount of Ca^{2+} that can be released during the next heart contraction. Thus, SERCA2a controls both cardiac muscle contraction and relaxation. Tight control of SERCA2a activity is therefore crucial for cardiac function. In pathological conditions such as heart failure, the major cause of death in the Western world, SERCA2a expression and activity are chronically reduced, which at least partly underlies the poor contractile properties of the failing heart (Vandecaetsbeek, Raeymaekers et al. 2009, Locatelli, de Assis et al. 2013). Thus, a high-resolution crystal structure of SERCA2a poses one of the most challenging and attractive goals in the field.

Another isoform, SERCA2b, is ubiquitously expressed. The defining difference between SERCA2a and SERCA2b is the presence of a 49 amino acid C-terminal extension in SERCA2b, which encompasses an additional transmembrane helix (2b-tail) (Figure 4). The 2b-tail is responsible for the higher calcium affinity but lower turnover rate of SERCA2b (Vandecaetsbeek, Trekels et al. 2009), as compared to the shorter SERCA2a variant. Although it remains unclear how exactly the 2b-tail affects the properties of the Ca^{2+} pump, it is without doubt of significant interest also from a therapeutical point of view to develop Ca^{2+} pump modulators. Mutations in the SERCA2 gene are mainly manifested as a skin disorder known as Darier's disease (Dhitavat, Dode et al. 2003), which is related to an impaired SERCA2b activity. Furthermore, SERCA2b was also found to be extensively expressed in Purkinje neurons and the pyramidal cell layer of the hippocampus (Baba-Aissa, Raeymaekers et al. 1996). It was shown that increased expression of SERCA2b in the brain resulted in high amyloid beta levels, thus contributing to Alzheimer's disease (Green, Demuro et al. 2008).

Another SERCA isoform, SERCA2c, was found in the human normal and failing heart (Dally, Corvazier et al. 2010). It has more restricted expression area than SERCA2a and SERCA2b, localizing in subsarcolemmal region of cardyomyocites. Also, SERCA2c showed lower affinity for cytosolic calcium than SERCA2a and SERCA2b (Dally, Bredoux et al. 2006).

Six different **SERCA3** isoforms (SERCA3a-f) can be translated from alternatively spliced mRNA transcripts. SERCA3 is about 75% similar with other isoforms, but has much lower affinity for calcium compared to other SERCAs. SERCA3 is mainly found in the intestine and lymphatic tissue, but also in platelets (Wuytack, Papp et al. 1994) and Purkinje neurons (Baba-Aissa, Raeymaekers et al. 1996). Together with SERCA2c, SERCA3a, 3d and 3f were recently found in the normal and failing heart tissue of humans (Dally, Corvazier et al. 2010). SERCA3 was shown to be always expressed together with SERCA2b (Xu, Gou et al. 2012). Unlike other SERCAs, SERCA3 has a different pH optimum (pH 7.2–7.4 compared to pH 6.8 –7.0), and it is not inhibited by phospholamban (see below) (Periasamy and Kalyanasundaram 2007). Altered expression levels of SERCA3 are associated with different abnormalities, including diabetes and cancer (Xu, Gou et al. 2012).

Although all SERCA isoforms possess high sequence similarity, the tissue-specific and development stage-specific expression suggest functional differences among SERCAs, which address the particular calcium homeostasis requirements in different tissues or cell type (Periasamy and Kalyanasundaram 2007).

Phospholamban and Sarcolipin – regulators of SERCA

It is well known that SERCA is regulated by the small transmembrane proteins phospholamban (PLB) and sarcolipin (SLN). These proteins of 52 and 31 amino acids respectively belong to the same family. PLB is mostly regulating SERCA2a isoform in the heart. At the same time SLN is mostly responsible for SERCA1a regulation and is expressed in fast-twitch muscles. In addition to that, SLN can also be found in heart, but its expression is restricted to atria (Vandecaetsbeek, Raeymaekers et al. 2009). Both PLB and SLN have three domains. However, PLB includes two cytoplasmic domains (IA and

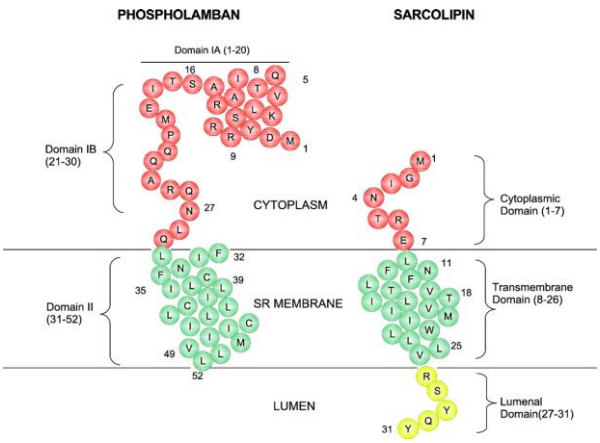

Figure 5. Schematic representation of phospholamban (PLB) and sarcolipin (SLN) structures. Three domains of PLB and SLN are represented in this figure. PLB has two cytoplasmic domains (IA and IB) and one transmembrane domain. SLN had cytoplasmic, transmembrane and luminal domains. Highly conserved SLN specific luminal extension of five amino acids of SLN is shown in yellow (Bhupathy, Babu et al. 2007).

IB) and one transmembrane (II) domains, while SLN has a cytoplasmic, transmembrane and luminal domains, as represented in Figure 5. The luminal domain of SLN consists of five highly conserved among different species amino acids (RSYQY) (Bhupathy, Babu et al. 2007). Under normal conditions in the heart, PLB binding inhibits SERCA2a by lowering its calcium affinity and stabilizing the pump in the E2 conformational state. Phosphorylation of serine 16 and threonine 17 on the IB cytoplasmic region of PLB during beta-adrenergic stimulation relieves this inhibition and restores SERCA2a function, which significantly enhances cardiac contractility (Bhupathy, Babu et al. 2007). Moreover, it was shown that *in vitro* PLB together with SLN can form heterodimers and then increase their effect, behaving as superinhibitor for SERCA2a. The occurrence of the superinhibition effect under physiological conditions remains unclear (Vandecaetsbeek, Raeymaekers et al. 2009).

Despite similarity between PLB and SLN and the same effect on SERCA, the regulation mechanism of the inhibition is considered to be different, probably due to the presence of luminal domain in SLN (Bhupathy, Babu et al. 2007). Also, the high concentrations of cytosolic calcium can release the inhibitory effect of PLB, but not effect of SLN. New important insight on the regulation of SERCA function was recently obtained with a recent crystal structure determination of SERCA1a in complex with SLN (Toyoshima, Iwasawa et al. 2013, Winther, Bublitz et al. 2013). As mentioned, the effect of SLN and PLB on SERCA function is different in many respects, and the exact mechanism of SERCA2a regulation by PLB remains unclear.

One of the most interesting and medically relevant complexes is that of phospholamban and cardiac SERCA2a. The PLB/SERCA2a ratio is chronically increased in heart failure

and the phosphorylation of PLB is reduced, making PLB a stronger inhibitor of the cardiac Ca^{2+} pump, which further contributes to the impaired contractility. Elevating SERCA2a activity by diminishing the PLB-SERCA2a interaction is currently explored as possible therapeutic strategy for heart failure treatment.

SERCA inhibitors

In addition to SERCA's abovementioned regulators, many different compounds have been shown to inhibit SERCA in its different conformational states. ATPase inhibitors are useful for investigating everything from the molecular properties of the pump to medical applications in disease treatment. Also, certain inhibitors have been used to stabilize P-type ATPases in particular conformational states and thus improve the crystallization process. This, in turn, made it possible to determine the structures of the pumps. The major inhibitors of SERCA in low calcium affinity E2 conformational states are Thapsigargin (TG), 2,5-di(tert-butyl) hydroquinone (BHQ) and cyclopiazonic acid (CPA) that will be discussed below in more detail (Michelangeli and East 2011). The chemical structures of TG, BHQ and CPA are represented in the Figure 6.

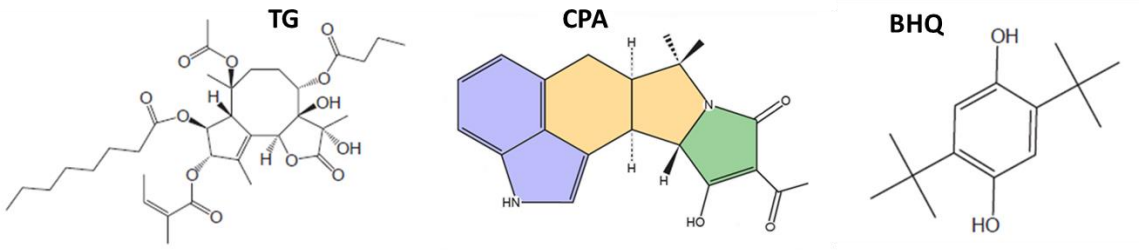

Figure 6. Chemical structures of Ca^{2+}-ATPase specific inhibitors TG, BHQ and CPA are shown in this figure. The indole moiety of CPA is colored in blue, hydrindane moiety in yellow and tetramic acid moiety in green.

One of the most widely used SERCA inhibitors, TG, was isolated from the Mediterranean plant *Thapsia garganica*. TG can penetrate biological membranes and inhibit Ca^{2+}-pumps at nanomolar concentrations. Interestingly, all SERCA isoforms have been shown to be TG-sensitive; however, TG has no effect on the Na^{+}/K^{+}-ATPase, PMCAs or SPCAs (Periasamy and Kalyanasundaram 2007, Michelangeli and East 2011). It has also been demonstrated that TG inhibits different SERCA isoforms by differing magnitudes (for example, it inhibits SERCA1 60 times stronger than for SERCA3). The structure of a SERCA1a-TG complex was solved in 2002, showing that TM helices M3, M5 and M7

are interacting to keep TG bound to the pump (Toyoshima and Nomura 2002). Derivatives of TG are considered to be a promising treatment for prostate cancer (Yatime, Buch-Pedersen et al. 2009).

BHQ inhibits SERCA in micromolar concentrations by preventing entry of the Ca^{2+} into the pump (Michelangeli and East 2011). BHQ blocks the protein in the same E2 conformational state as TG, and initially the two inhibitors were suggested to have the same binding site. However, the crystal structures of SERCA-inhibitor complexes showed the separate binding pockets. Van der Waals forces keep BHQ bound between M1-M4 transmembrane helices of SERCA (Obara, Miyashita et al. 2005).

CPA is a mycotoxin first isolated from *Penicillium cyclopium*, and later from *Aspergillus* species (Holzapfel 1968). CPA was shown to bind SERCA with nanomolar affinity. In 2009 the structure of a SERCA1a-CPA complex was solved by our group (Laursen, Bublitz et al. 2009). As for BHQ, CPA was demonstrated to bind SERCA1a between four transmembrane helices M1-M4. The structure of CPA has indole, hydrindane and tetramic acid moieties, which are shown in color in the Figure 6, in blue, yellow and green respectively. The CPA binding pocket in SERCA1a is shown in Figure 7. Interacting with tetramic acid moiety of CPA, a divalent ion was shown to be crucial for the binding of the inhibitor. Moreover, glutamine 56 and three water molecules are involved in coordination of the cation. Helices M3 and M4 participate in the formation of hydrophobic pocket, where the indole moiety of CPA is bound.

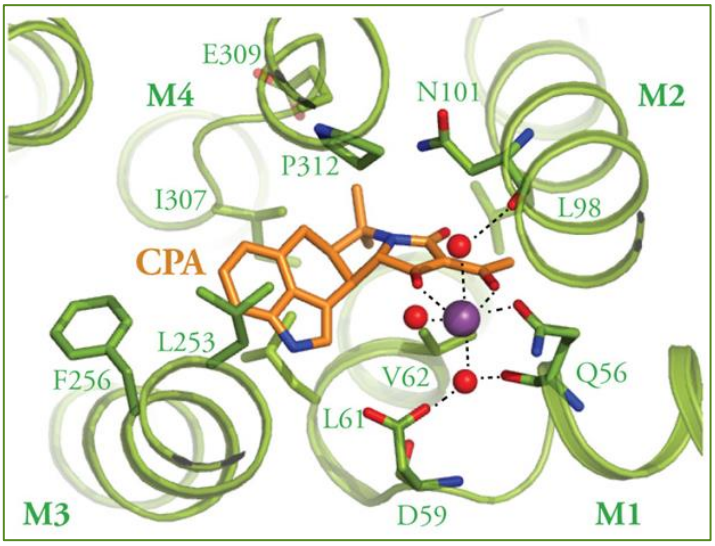

Figure 7. CPA binding pocket. CPA is bound to SERCA1a between M1-M4 helices. Divalent ion (purple sphere) stabilizes inhibitor in the binding pocket by interacting with Q56. Three water molecules (red spheres) are also involved in this coordination. (made by Andersen, J.L.)

Listeria monocytogenes Ca^{2+}-ATPase

Listeria monocytogenes is a foodborne pathogenic bacterium, widely spread in plants, soil, and surface water samples, as well as silage, sewage, slaughterhouse waste, cow milk, and human and animal feces. It is a gram-positive, rod-shaped, non-spore forming facultative anaerobe, which thrives in a -0.4 to 50°C temperature range (Farber and Peterkin 1991, Barbuddhe and Chakraborty 2009). *L. monocytogenes* can cause listeriosis in immunodeficient patients, newborn children and pregnant women, as well as gastroenteritis in all individuals. In order to cause the infection, the pathogen has to overcome numerous barriers and survive under extreme conditions and such as acidity in the stomach, increased osmolarity and the presence of bile salts in the small intestine (Barbuddhe and Chakraborty 2009). Moreover, *L. monocytogenes* was shown to adapt to the high external Ca^{2+} concentrations during host entry, and alkaline pH often used during food processing (Giotis, Muthaiyan et al. 2008).

In 2011 Faxén, Andersen et al. characterized a Ca^{2+}-ATPase from *Listeria monocytogenes* that they named LMCA1 (Faxen, Andersen et al. 2011). During every transport cycle, LMCA1 pumps one Ca^{2+} out of the cell and one H^{+} into the cell per ATP molecule hydrolyzed. The pH optimum for the pump was shown to be around 9. Furthermore, it was shown that LMCA1 is upregulated during bacterial adaptation to alkaline pH, suggesting that LMCA1 might be important for bacterial survival (Giotis, Muthaiyan et al. 2008). LMCA1 is a homolog of SERCA1a and thus belongs to the P_{IIA}-subfamily of P-type ATPases. In contrast to SERCA1a, LMCA1 has only one Ca^{2+} binding site. Moreover, despite 38% sequence similarity of these two proteins, it was shown that the SERCA1a inhibitor cyclopiazonic acid does not have an effect on LMCA1. The low resolution structure of LMCA1 was solved by Andersen, J.L. in 2011 (Andersen, Gourdon et al. 2011), however more structural information is needed to gain molecular insight into detailed features of the bacterial pump.

Objectives of the present work

Recently, the first steps towards understanding the nature of LMCA1 insensitivity towards calcium pump inhibitors were performed. The studies revealed that when two amino acids of the pump were mutated to match those in SERCA1a, a gain of function in

CPA-sensitivity was observed (Kotsubei, Gorgel et al. 2013). The aim of this project was to validate these results by investigating several other mutants and to confirm our conclusions by testing mutants of a CPA-insensitive bacterial Ca^{2+}-ATPase from *Lactococcus lactis*. This would help obtain a general understanding of the CPA insensitivity mechanism of some bacterial Ca^{2+}-ATPases.

Furthermore, the most CPA-sensitive mutant was used in crystallization trials of the LMCA1-inhibitor complex. Structural characterization of this complex will reveal the molecular details of the binding pocket and the unique features of a bacterial Ca^{2+}-ATPase.

Moreover, one of the focuses in this study was the purification of native SERCA2a. This is an important first step towards the eventual crystal structure determination of the cardiac sarco(endo)plasmic reticulum Ca^{2+}-ATPase. Once an appropriate purification scheme for SERCA2a has been established, the long-term goal is to crystallize SERCA2a both alone and in complex the protein phospholamban (PLB), its natural regular. Therefore, western blotting experiments were undertaken as part of the current work in order to determine whether PLB copurifies with SERCA2a.

Methods

Expression of wild type and mutant forms of bacterial ATPases

Genes encoding LMCA1 WT and LCCA1 WT were ligated into pET-22b vectors. The constructs were prepared and provided by Andersen, J.L. (Aarhus University). The plasmid was transformed into C43 competent cells which were grown on lysogeny broth (LB) agar plates containing selection agent ampicillin in a final concentration 100 μg/ml. Afterwards 5-10 colonies were transferred into liquid LB media and incubated at 37°C until A_{600nm} reached 0.6-0.8. Protein expression was induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells were grown overnight at 20°C.

Lactococcus lactis and *L.monocytogenes* Ca^{2+}-ATPase mutagenesis

Mutations in LLCA1 WT and LMCA1 WT constructs were introduced using QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. All mutant forms of LMCA1 and LLCA1 were treated the same way as LMCA1 WT and LLCA1 WT.

Membrane isolation from bacterial cells

Bacterial cells were isolated from the media by centrifugation for 20 minutes at 7000 × g at 4°C, then resuspended in buffer (50 mM TrisHCl, 200 mM KCl, 20% v/v glycerol, pH=7.6) to 20g/100 ml. After the addition of 1 mM phenylmethylsulfonyl fluoride, DNAse I (10 μg/ml) and Protease inhibitor cocktail tablets (SigmaFAST™) were added, bacterial cells were lysed using a High Pressure Homogenizer (HPH; C3, Avestin) at 15000 psi. Membranes were isolated from lysed cells by two-step centrifugation, at first for 45 minutes at 27000 × g at 4°C and then the supernatant for another 2 hours at 235000 × g at 4°C. The pellet/sedimented membranes from the second centrifugation step were resuspended in buffer (20 mM TrisHCl, 200 mM KCl, 20% v/v glycerol, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM beta-mercaptoethanol [β-ME], pH=7.6) using the Potter-Elvehjem homogenizer.

Purification of bacterial Ca^{2+}-ATPases

Isolated membranes were solubilized with 1% n-Dodecyl β-D-Maltoside (DDM) for 1 hour at 4°C. Solubilized membranes were centrifuged for at 235000 x g for 2h at 4°C. The filtrated supernatant (0.2 µm membrane) was loaded onto Ni^{2+}-NTA beads (QIAGEN) and incubated for 1 h in the presence of 5 mM imidazole and 500 mM KCl. The mixture was then packed into a column XK-16 column (GE Healthcare) and washed with at least 3 CV of buffer [20 mM Tris, 200 mM KCl, 20% v/v glycerol, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.25 mg/ml $C_{12}E_8$, 5 mM β-ME] and then eluted with 0.5 CV of the same buffer with 500mM imidazole. The purity of the protein was analyzed by SDS-PAGE and fractions containing the Ca^{2+}-ATPase were pooled and concentrated. In order to further improve the purity of the LMCA1 double mutant (DM) and in order to exchange the buffer for [100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH=6.8, 80 mM KCl, 20% v/v glycerol, 1 mM $MgCl_2$, 0.15 mg/ml $C_{12}E_8$, 5 mM β-ME], the protein was applied to a G3000SW size-exclusion chromatography column.

Crystallization of LMCA1 double mutant

Prior to crystallization protein was re-lipidated according to the HiLiDe method (Gourdon, Andersen et al. 2011). In order to eliminate all aggregates after the re-lipidation procedure, the protein was centrifuged for 15 minutes at 200 000 × g at 4°C. Protein was then mixed with 2 mM EGTA, 1 mM CPA, 1 mM $MnCl_2$, 1 mM $AlCl_3$ and 10 mM NaF ($AlCl_3$ and NaF were preliminary mixed), 1 mM AMPPCP and incubated for 1 h at 4°C to allow CPA bind. The mixture was centrifuged for 5 minutes at 19000 × g just before setting up trays. Crystallization trials were performed using the vapor-diffusion hanging-drop method (0.8 µl of 10 mg/ml protein + 0.8 µl of 1 × or 1.5 × reservoir solution). The 1.5 × reservoir solution was used when the crystallization conditions were designed to contain detergent or additives, in order to avoid dilution of compounds. Crystallization trays were stored at 19°C. Usually crystals appeared after 4-5 days.

Activity measurements

Activity studies were conducted using the Baginski method, a colorimetric assay in which the amount of inorganic phosphate released during the reaction is measured. The reaction

mixture of 45 µl was prepared first: reaction buffer (20 mM MOPS, pH=7.4, 20% glycerol, 200 mM KCl, 3 mM $MgCl_2$, 5 mM β-ME, 5 mM NaN_3, 0.2 mM $NaMoO_4$, 50 mM KNO_3, 0.25 mg/ml $C_{12}E_8$ and 1 mM EGTA), 5 µg of enzyme and DMSO/CPA in concentrations [5, 12.5, 25, 50, 125, 250, 375, 625, 1250 and 1750 µM]. To initiate the reaction, 5 µl of 30 mM of ATP and 12 mM of $CaCl_2$ were added (resulting in a final concentration of 3 mM and 1.2 mM respectively). Samples were incubated at room temperature for 10 minutes and then the reaction was stopped by addition of 50 µl of ascorbic acid solution (A. 0.75 g ascorbic acid was dissolved in 25 ml 0.5 M HCl, 250 µl of 10% SDS was added; B. 0.175 g ammonium heptamolybdate was dissolved in 5 ml of ddH_2O. Solutions A and B were kept on ice for 1 h and then mixed just before use). Samples together with ascorbic acid solutions were incubated for 10 minutes at RT, and then 75 µl of arsenic acid (150 mM sodium arsenate, 70 mM sodium citrate and 350 mM acetic acid) was added to stabilize the color. The absorbance was measured at 860 nm wavelength using VICTOR^3 equipment (Perkin Elmer). A time course with time points from 1 to 30 minutes for every protein was performed prior to CPA-sensitivity measurements.

Isolation of eukaryotic SERCA2a from a pig heart/microsome isolation

Ventricle tissue from the pig heart was minced and homogenized with 10 mM $NaHCO_3$ buffer (50 g of tissue for 100 ml of buffer). The sample was then centrifuged at 14000 × g for 20 minutes and then the supernatant was centrifuged again with same parameters. This allowed for the removal of nuclei, cell debris and mitochondria. The supernatant was further ultracentrifuged for 45000 × g for 30 minutes. The pellet from this step was re-suspended in buffer (0.6 M KCl, 20 mM Tris pH=6.8) and kept for 1 h at 4°C with magnetic stirring that allowed extraction of contractile proteins. The next centrifugation step was performed for 30 minutes at 45000 x g. The pellet containing crude sarcoplasmic reticulum vesicles was re-suspended in the final buffer (50 mM Tris pH=7, 20% glycerol, 10 mM $CaCl_2$, 1 mM $MgCl_2$,) and flash-frozen in liquid nitrogen.

Purification and crystallization of eukaryotic SERCA2a by Reactive Dye chromatography

Prior to purification, reactive blue beads (Sigma-Aldrich) were washed with 5 CV boric acid and NaCl solution (0.1 M boric acid pH=9.8, 1 M NaCl), then with 5 CV boric acid

solution without salt, then with 5 CV water and finally, equilibrated with buffer (20% (v/v) glycerol, 50 mM MOPS pH=7, 8 mM $CaCl_2$, 8 mg/ml $C_{12}E_8$). Microsomes isolated from the pig heart were solubilized in $C_{12}E_8$ at a w/w ratio 1:3. Once solubilized, the samples were centrifuged for 15 minutes at 200 000 × g at 4°C. The supernatant was afterwards loaded onto Reactive Blue beads and incubated for 1 h. Beads were washed with 5 CV of wash buffer (20% (v/v) glycerol, 20 mM MOPS pH=7, 1 mM $CaCl_2$, 1 mg/ml $C_{12}E_8$). Finally, protein was eluted with 0.5 CV elution buffer (washing buffer with 50 mM NaCl and 4 mM ADP). Protein concentration was measured using the colorimetric Bradford method (Bradford 1976), as the ADP in the buffer absorbed strongly in the UV region and interfered with the protein absorbance at 280 nm. SERCA2a was concentrated to 5 mg/ml using a Vivaspin centrifugal concentrator with a molecular weight cutoff of 30 kDa. The protein was re-lipidated using the HiLiDe method (Gourdon, Andersen et al. 2011). 10 mM Ca^{2+} and Mg^{2+} were added prior to crystallization trials. The initial crystal screening was performed using the inhouse KING screen or commercial MEMGOLD screen (Molecular Dimensions), in which 1 µl of protein (5 mg/ml) was mixed with 1 µl reservoir solution on glass cover slips and equilibrated against 450 µl reservoir solution using vapor-diffusion hanging-drop method. Crystals appeared in 7-10 days.

Detection of PLB in the SERCA2a prep by Western Blot

Samples of SERCA2a were first analyzed by 18% SDS-PAGE and then transfered to a PVDF membrane (Millipore), which was activated with methanol. Transfer was performed in the running buffer (25 mM Tris, 192 mM glycine, 10% (v/v) methanol), at 100 V for 1 h. Afterwards the membrane was blocked by 5% Skim-milk powder (Sigma) dissolved in PBS-Tween (0.1%) for 1 h and then incubated with primary antibody (anti-PLB MA3-922, 1:10000 dilution in PBS-Tween) for 1 h, washed 6 × 10 minutes with PBS-Tween and then incubated with secondary antibody (poly α-mouse peroxidase, 1:5000 dilution in PBS-Tween). Finally, the membrane was washed 6 × 10 minutes. The blot was then developed with a hypercasette and chemiluminescent substrate from Thermo scientific.

Results

Understanding the nature of CPA-insensitivity of bacterial Ca^{2+}-ATPases

In 2009, the structure of the SERCA1a-CPA complex was solved in our laboratory (Laursen, Bublitz et al. 2009). Despite 38% sequence similarity to SERCA1a, LMCA1 is not inhibited by cyclopiazonic acid (CPA). In order to understand the nature of this insensitivity, a multiple sequence alignment of CPA-sensitive and CPA-insensitive Ca^{2+}-ATPases within the 5Å region surrounding the CPA molecule bound in SERCA1a structure, was previously performed. During these studies, four positions potentially responsible for CPA binding in LMCA1 (T54, M59, S240, S295) were identified. Amino acids in these four positions were mutated to match those in SERCA1a (T54Q, M59L, S240G, S295P). Many mutant forms of LMCA1 with different mutational combinations were constructed, including single, double, triple and quadruple mutants. It has been shown previously that some single mutations partially restored CPA sensitivity of the protein. For instance, the single T54Q mutant restored the CPA-sensitivity of the protein twofold. A single mutation in position 59 (M59L) did not have any significant effect. At the same time, a promising triple mutant (T54Q, M59L, S240G) was found, which showed significant sensitivity towards CPA. Moreover, a double mutant (DM) (T54Q, S295P) was shown to be even more promising, revealing remarkable CPA-sensitivity compared to the wild type LMCA1. The restoration of all four amino acids (T54Q, M59L, S240G, S295P) did not reduce the IC_{50} value, obtained for abovementioned double mutant (Figure 8). These observations provided a starting point for the current work, and were recently published along with the results described in this thesis (Kotsubei, Gorgel et al. 2013).

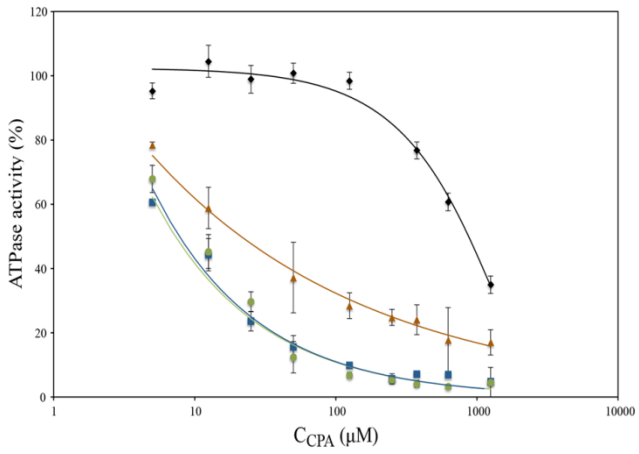

Figure 8. The activity of LMCA1 WT and mutant forms in the presence of CPA is represented in this figure. LMCA1 WT is shown in black circles, LMCA1 DM (T54Q, S295P) in blue squares, LMCA1 triple mutant (T54Q, M59L, S240G) in orange triangles and LMCA1 quadruple mutant (T54Q, M59L, S240G, S295P) in green circles. (Kotsubei, Gorgel et al. 2013)

In this study, the aim was to confirm the results obtained previously by introducing the same mutations to another bacterial Ca^{2+}-ATPase from *Lactococcus lactis* (LLCA1) and testing these mutants for CPA sensitivity. The important mutations were introduced and two single (T54Q; S295P), one double (T54Q, S295P) and one triple mutant (T54Q, M59L, S295P) were constructed. It is important to mention that LLCA1 originally had glycine in the position 240 and no mutation in this position was necessary. Moreover, in order to highlight the importance of the restored glutamine in position 54 (T54Q) obtained from previous studies, the triple mutant of LMCA1 with all other mutations present was designed (M59L, S240G, S295). In order to build up the whole picture, additional single mutants (LMCA1 S240G and S295P) were constructed and tested.

The His-tagged LMCA1 triple mutant (TM) and LLCA1 WT and mutants were expressed in *E. coli* and purified by immobilized metal affinity chromatography (IMAC), the same way as LMCA1 DM (Figure 9B). A single IMAC step was performed for the LLCA1 WT and LLCA1 mutant forms due to the lack of a TEV-cleavage site after the His-tag (Figure 10). It is worth mentioning that the original purification protocol was optimized by increasing the concentration of imidazole and KCl (final concentrations 5mM and 500mM respectively) while binding solubilized protein to the beads. This was done due to the insufficient purity of the protein in the initial purification trial (Figure 9A). Purified LLCA1 WT and mutant forms as well as the LMCA1 TM were tested for activity using the Baginski assay in the absence and presence of cyclopiazonic acid. Unfortunately, the LMCA1 TM (M59L, S240G, S295P) was found to be completely inactive even without addition of CPA.

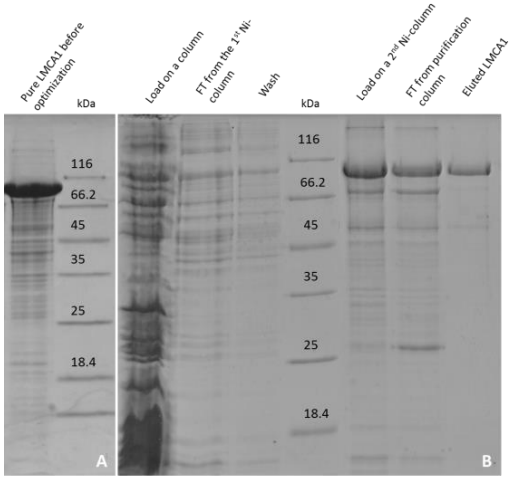

Figure 9. Purification of LMCA1 double mutant. A. Purified LMCA1 before optimization. B. LMCA1 was purified by IMAC. Fractions from loaded solubilized membranes to pure eluted protein are shown.

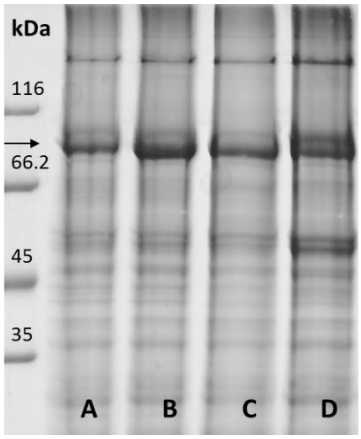

Figure 10. Purified LLCA1. One step of IMAC was performed for LLCA1 WT (not shown) and mutant forms (A: LLCA1 (T54Q); B: LLCA1 (T54Q, M59L, S295P); C: LLCA1 (S295P); D: LLCA1 (T54Q, S295P). LLCA1 is about 95 kDa (indicated by arrow).

The amount of protein and reaction time was then increased 3 times, but the result remained unchanged (Figure 11). However, restoration of the single proline in the position 295 (S295P) resulted in 7-fold reduction of IC_{50} value of the pump in the presence of CPA compared to the LMCA1 wild type. The same as in mutant M59L, single mutation in position 240 (S240G) did not have any significant effect.

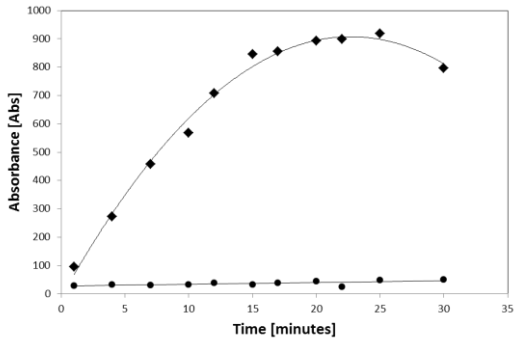

Figure 11. The activity of LMCA1 triple mutant (M59L, S240G, S295P). The activity of constructed LMCA1 triple mutant was tested using Baginski assay. LMCA1 WT is represented in rhombs, LMCA1 triple mutant in circles. No inorganic phosphate was released after 30' of incubation time.

The activity of the wild-type and mutant forms of the *Lactococcus lactis* Ca^{2+}-ATPase in the absence and presence of CPA was also analyzed by Baginski assay. The experiments revealed that restoration of glutamine or proline (single mutants T54Q; S295P) reduced the IC_{50} value of LLCA1 by three times, with the IC_{50} value for LLCA1 T54Q mutant being 343μM, and for LLCA1 S295P being 327μM, compared to 1012μM for wild-type LLCA1. The double mutant (T54Q, S295P) revealed remarkable CPA-sensitivity, with an IC_{50} value of 60μM. The IC_{50} value for the triple mutant, where all amino acids corresponded to those of SERCA1a (T54Q, M59L, S295P and G240), was calculated to be 7μM. Table 1 and Figure 12 summarize these results.

Enzyme	Mutation	IC_{50} (CPA)
LLCA1	WT	1012 ± 61
LLCA1	T54Q	343 ± 44
LLCA1	S295P	327 ± 17
LLCA1	T54Q, S295P	60 ± 9
LLCA1	T54Q, M59L, S295P	7 ± 1

Table 1. IC_{50} values for *Lactococcus lactis* Ca^{2+}-ATPase WT and mutant forms were calculated and are represented in this table. CPA concentration is in µM.

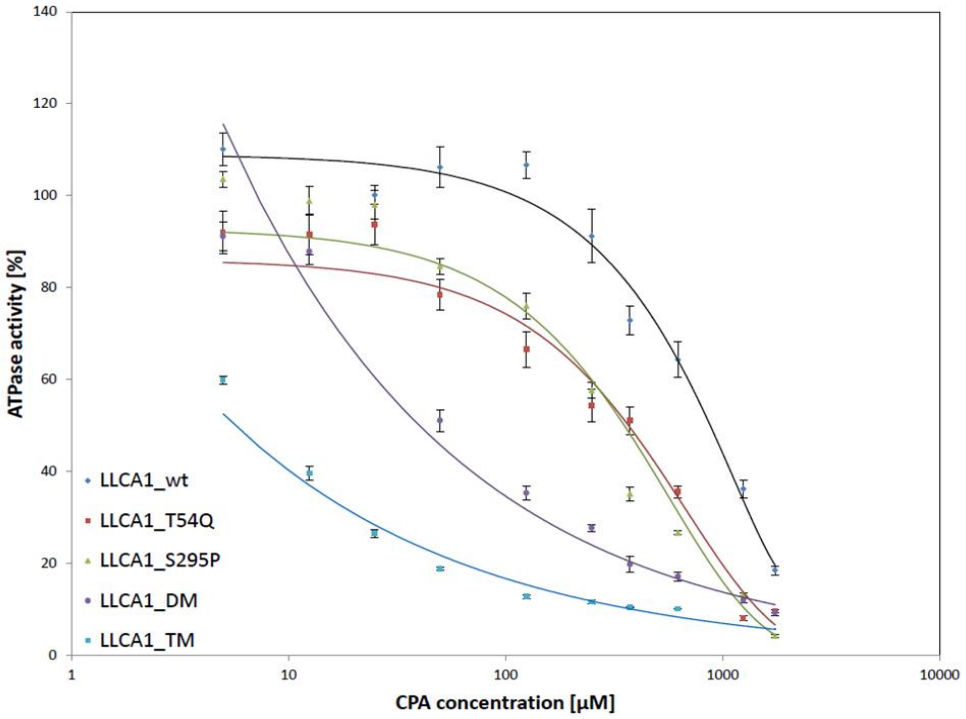

Figure 12. Activity measurements for LLCA1. The activity of LLCA1 WT and mutant forms in the presence of different CPA concentrations is present in this figure. LLCA1 WT is shown in dark blue color, single mutants LLCA1 T54Q and LLCA1 S295P in red and green respectively, double mutant of LLCA1 (T54Q, S295P) is in purple, and LLCA1 triple mutant in light blue. Error bars are represented as standart errors.

Crystallization of LMCA1 double mutant

The LMCA1 double mutant (DM) (T54Q, S295P), previously shown to be inhibited by CPA, was further used in crystallization studies. Prior to that, an additional size-exclusion chromatography purification step was performed that allowed us to improve the purity of

the LMCA1 DM and to exchange the buffer. The purified protein was re-lipidated in order to restore a native-like environment using the HiLiDe technique (Gourdon, Andersen et al. 2011). This was then mixed with EGTA, CPA, AMPPCP and $MnCl_2$ and used for crystallization trials. Initial crystals were obtained in the condition: 16% PEG6000, 12% (v/v) glycerol, 3% t-BuOH, 100 mM $MnCl_2$, 5 mM βME (data not shown). The condition was slightly optimized by screening salt and PEG6000 concentrations. Afterwards, the best looking crystals in condition (18% PEG6000, 12% glycerol, 3% t-BuOH, 140 mM $MnCl_2$, 5 mM βME) were optimized using Detergent and Additive screens (Hampton Research). The screens revealed some good hits, including 100mM L-proline and the following detergents: 32 mM n-nonyl-β-D-thiomaltoside, 43 mM DDMAB, 4 mM ZWITTERGENT 3-14, 144 mM sodium dodecanoyl sarcosine (sarcosyl) (Figure 13). The crystals were tested at the MAX-Lab synchrotron, however only poor diffraction resolution (20 Å) was observed so far. After that, the optimization of LMCA1 DM crystals was continued by combining the best condition with L-proline and second detergents – DDMAB, n-nonyl-β-D-thiomaltoside, ZWITTERGENT 3-14, sodium dodecanoyl sarcosine (sarcosyl). Unfortunately no improvement in crystal quality and diffraction was observed.

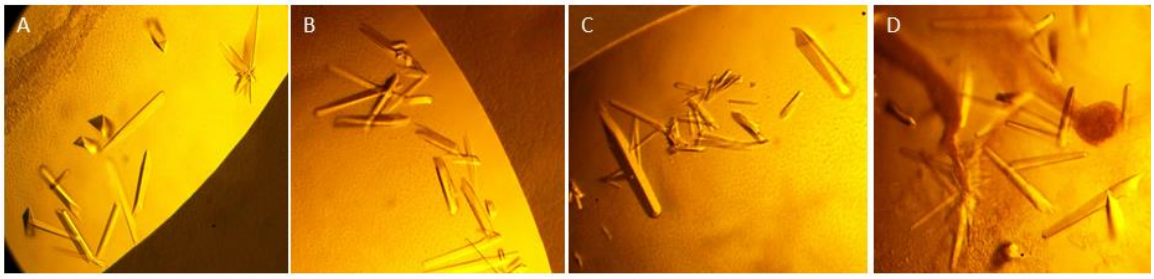

Figure 13. Crystallization of LMCA1 double mutant. Crystals of LMCA1 double mutant are represented in this figure. Crystals appeared in the condition containing 18% PEG6000, 12% glycerol, 3% t-BuOH, 140 mM $MnCl_2$, 5 mM βME and second detergent: A. 144 mM sodium dodecanoyl sarcosine (sarcosyl), B. 32 mM n-Nonyl-β-D-thiomaltoside, C. 4 mM ZWITTERGENT 3-14, D. 43 mM DDMAB.

Towards the crystal structure of SERCA2a

The native cardiac SERCA2a was isolated from the pig heart. First, microsomes were obtained through multiple centrifugation steps. Afterwards, SERCA2a was purified from solubilized microsomes by Reactive Dye chromatography. Reactive Blue and Reactive Green beads were tested, as well as an ADP and AMPPCP/ADP elution mixture.

Figure 15. SERCA2a crystallization. The observed fluorescence under UV-light seen in this figure suggests protein origin of the potential SERCA2a crystal.

However, the best result was observed using Reactive Blue beads and ADP in the elution buffer (Figure 14). The identity of the major eluted protein as SERCA2a was confirmed by mass-spectroscopy analysis. The Ca^{2+}-dependent activity and stability of the protein (presence of activity after being frozen and thawed) was indicated by our collaborators from KU Leuven, Belgium (Vandecaetsbeek, I., data not shown). The protein was concentrated, mixed with Ca^{2+} and Mg^{2+} ions and then used in crystallization trials. The commercial MEMGOLD (Molecular Dimensions) and the inhouse KING screen were used for the initial screening. A crystal was obtained in one of the KING screen conditions and tested under UV-light in order to identify the protein nature of crystal. Fluorescence was observed when the crystal was tested under UV-light, indicating it was likely to be composed of protein (Figure 15). Moreover, one of the goals was to identify the presence of the SERCA2a regulator phospholamban (PLB) in the preparation, therefore a Western-blot was performed. In this experiment, a GST-PLB fusion protein (kindly provided by Drachmann, N.) was used as a positive control. The performed experiment suggested that PLB can indeed be found in the prep (Figure 16).

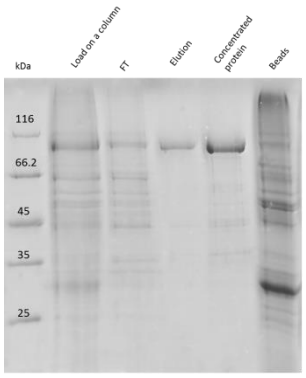

Figure 14. SERCA2a purification steps are shown in this figure: a sample of a column load, the flow-through of the column, elution, concentrated protein and sample of beads. Molecular mass of SERCA2a is 110 kDa, corresponding to the band between 116 and 66.2 kDa of marker. A rich band of SERCA2a can be seen in the elution and concentrated elution fractions with only small contaminants present, indicating high purify of the protein. Moreover, no protein corresponding to SERCA2a molecular mass was left on the beads.

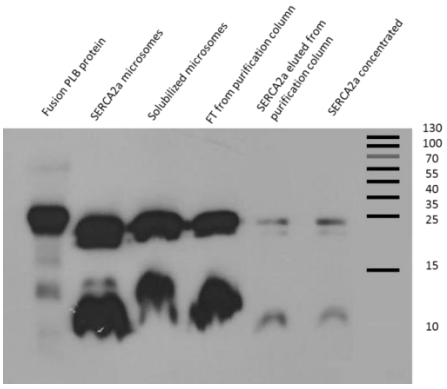

Figure 16. Detection of PLB in SERCA2a samples. Fusion PLB was used as a positive control, the expected mass is 35kDa. In all fractions (microsomes, solubilized microsomes, FT from purification column, and in the eluted and concentrated protein fractions) PLB has been detected. PLB monomer can be seen at 11 kDa, the bands at 25 kDa are considered PLB pentamers.

Discussion

Cyclopiazonic acid (CPA) is a mycotoxin produced by *Penicillum cyclopium* and *Aspergillus*. The toxicity of CPA for animals comes from its potential to inhibit the SR Ca^{2+}-ATPase. However, some bacterial Ca^{2+}-ATPases (*Listeria, Lactococcus and Streptococcus*) were shown to be insensitive to CPA. To determine the molecular basis for this insensitivity, the equivalent CPA binding site residues identified in SERCA1a were investigated in *L. monocytogenes* Ca^{2+}-ATPase (LMCA1). Four amino acids in LMCA1 potentially responsible for inhibitor binding were mutated to match the ones in the SERCA1a (T54Q, M59L, S240G, S295P). Mutant forms with different combinations of mutations were constructed and tested for the activity in the presence of inhibitor. It was shown that restoration of two amino acids (T54Q, S295P) can increase the sensitivity of the enzyme for CPA 100-fold (Kotsubei, Gorgel et al. 2013). Further, some additional LMCA1 mutants (single mutants S240G and S295P and triple mutant M59L, S240G, S295P) were tested in order to validate earlier results and to confirm these results on another bacterial pump from *Lactococcus lactis.* Combinations of three mutations (T54Q, M59L, S295P) were introduced. Wild type and mutant forms of *L. lactis* Ca^{2+}-ATPase (LLCA1) together with some LMCA1 mutants were expressed in *E. coli* and purified using one-step IMAC.

The initial purification protocol for LMCA1 (Faxen, Andersen et al. 2011) was optimized by addition of 5 mM imidazole and 500 mM KCl salt during protein binding to the Ni^{2+} beads. This reduced contaminant binding and thus improved the purity of the protein.

The ATPase activity of purified LMCA1 mutants and LLCA1 wild type and mutant forms were tested in the presence of CPA and compared to wild type activity. As for the LMCA1 mutants, there was no alteration in the IC_{50} value for LLCA1 with the restoration of a single glycine (S240G), while restoration of the proline (S295P) reduced the IC_{50} value 7-fold. The same inhibitory effect was previously seen with an LMCA1 triple mutant (T54Q, M59L, S240G) and LMCA1 double mutant (T54Q, S295P), suggesting gain of function when either interaction with indole or hydrindane moieties is restored (Figure 17). Noticeably, restored glutamine (T54Q) was present in both abovementioned mutants. From the previously solved structure of SERCA1a-CPA complex (Laursen,

Bublitz et al. 2009) the importance of glutamine in the position 56 in SERCA1a was shown, as it is responsible for divalent ion coordination during CPA binding. Q56 in SERCA1a corresponds to T54 in LMCA1 (as well as in LLCA1), and our results suggest that, indeed, the restoration of glutamine in position 54 is one of the most important for obtaining CPA sensitivity in a bacterial pump. This explains why glutamine restoration alone triggers a two fold reduction of the IC_{50} value (Kotsubei, Gorgel et al. 2013). A triple mutant with all mutations present except glutamine (M59L, S240G, S295P) was completely inactive without inhibitor present.

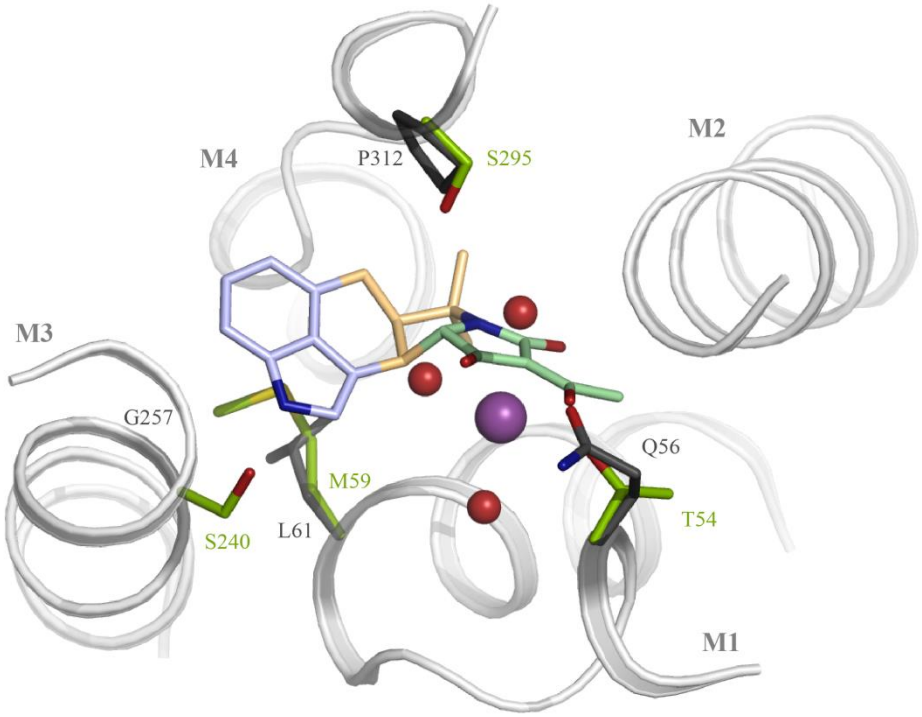

Figure 17. CPA binding pocket in LMCA1. Homology model of LMCA1 based on SERCA1a. Amino acids of LMCA1 are shown in green, SERCA1a amino acids in grey. Indole moiety of CPA (blue) interacts with leucine and glycine in positions 61 and 257 respectively (corresponds to M59 and S240 in LMCA1). Glutamine 56 (T54 in LMCA1) is involved in Mg^{2+} ion (purple sphere) coordination, which is required for CPA binding in the pump. Also three water molecules (red spheres) are involved in this coordination. Proline P312 (S295 in LMCA1) interacts with hydrindane moiety of CPA, and together with another proline (not shown) in M4 helix forms hydrophobic pocket (made by Andersen, J.L)

For the *Lactococcus lactis* ATPase, a very high reduction in IC_{50} was also observed with the double mutations present (T54Q, S295P), which echoes the results obtained for the *Listeria* pump. Both single restorations T54Q or S295P in the *L. lactis* pump turned out to be important for the CPA binding. Interaction of proline with hydrindane moiety of CPA takes place due to formation of a hydrophobic pocket between the inhibitor and two prolines (P308 and P312 in SERCA1a) on the M4 helix. By coordinating the divalent ion

and interacting with a hydrindane moiety of the inhibitor, the T54Q and S295P mutations were able to reduce the IC_{50} value three fold each. Moreover, the mutant with all three amino acids restored showed a significant (almost 150-fold) reduction in IC_{50} value. The IC_{50} value for all mutations present in LLCA1 is lower than for LMCA1, suggesting stronger interaction of CPA with *L. lactis* pump. This might be explained by the presence of an endogenous glycine (G240) in LLCA1, which could affect interactions and alter CPA binding. Thus, the importance of the identified residues potentially responsible for CPA binding in the pump was confirmed. Both lactic acid bacteria and CPA-producing fungi may be found coexisting in environments such as cheeses. We hypothesize that the CPA insensitivity of the *Lactococcus* calcium pumps may be a direct result of such cohabitation (Kotsubei, Gorgel et al. 2013).

Unlike their eukaryotic counterparts, not much is known about bacterial Ca^{2+}-ATPases in general. However, a Ca^{2+}-ATPase from *Streptococcus pneumonia* (CaxP) is required for the bacterial entry into the host calcium enriched surroundings (Rosch, Sublett et al. 2008). Moreover, as was mentioned earlier, LMCA1 was shown to be up-regulated during bacterial adaptation to the environment, thus suggesting its importance for the bacterial survival. A high resolution crystal structure of mutant LMCA1 with CPA will help to elucidate the difference between the bacterial and eukaryotic inhibitor binding pocket. This could be used to develop inhibitor derivatives that can block bacterial pump. Usually, high purity of the protein is required for crystallization. Therefore, prior to crystallization trials, an additional purification step of size exclusion chromatography for LMCA1 DM (T54Q, S295P) was performed. The initial crystals of LMCA1 DM were obtained using the HiLiDe method (Gourdon, Andersen et al. 2011) and the best hit was then optimized using commercial detergent and additive screens. Well-formed, three dimensional single crystals were harvested and exposed to synchrotron X-ray radiation. Unfortunately, only poor diffraction was observed. This could be caused by several factors. A more obvious one might be the lack of additional condition optimization, however, one further step of combining conditions with the best crystal hits from detergent and additive screens did not reveal any improvement of diffraction. Also, problems with cryoprotection or dehydration could be the case. Moreover, it was the condition in which the best looking first microcrystals appeared which was further optimized. Yet it is well known that there need not always be a correlation between best looking and best diffracting crystals. The best diffracting crystals can be identified only

empirically, however it is possible not to see any protein diffraction if the crystal is too small. Thus, the lack of diffraction potential of the initial hit optimized can also be one of the explanations, in which case obtaining better diffraction is a matter of further screening.

An atomic structure of SERCA2a is of high medical interest due to its potential as a drug target for a number of pathological heart conditions. Crystal structure determination is a long and difficult process in general, particularly when transmembrane proteins are concerned. However, the first step towards that aim was performed in this study. Native SERCA2a was isolated from the pig heart and purified using Reactive Blue chromatography. The presence of SERCA2a was confirmed by mass-spectroscopy analysis. The Ca^{2+}-dependent activity in purified sample and stability of the protein was shown by our collaborators from KU Leuven (Vandecaetsbeek, I.). Moreover, natively isolated microsomes from the pig heart contain many other membrane proteins, such as phospholamban (PLB). It is hypothesized that PLB binds SERCA2a the similar way as sarcolipin binds SERCA1a, also due to similarities between SERCA1a and SERCA2a, and homology of PLB and SLN. Recently, the structure of SERCA1a-SLN complex was solved in our laboratory (Winther, Bublitz et al. 2013) and also by Toyoshima group in Japan (Toyoshima, Iwasawa et al. 2013). It can be clearly seen that SLN binds SERCA1a in the groove between M2, M6 and M9 region. It can be assumed that PLB might be bound to SERCA2a during its purification from microsomes. Therefore, it was interesting to determine whether PLB is present in the purification preparation. Also, as both crystal structures of SERCA2a alone and in complex with PLB are of high interest, the detection of PLB in the preparation is required for further crystallization trials. Using Western-blot analysis it was demonstrated that phospholamban was indeed copurified with the enzyme and remained in the solution even after protein concentration procedures. Even though phospholamban is a 52 amino acid protein with a molecular mass of 6 kDa, many experiments showed that it can be found on SDS-PAGE gels and WB experiments at the mass of 11 kDa as a monomer and 25 kDa, in form of pentamer (Wegener and Jones 1984). Therefore, we believe that both bands represented in the each sample in Figure 16 correspond to phospholamban. Moreover, preliminary crystallization experiments revealed potential initial SERCA2a protein crystals that gave fluorescence under UV-light. Protein crystals are known to fluoresce under UV-light due to the tryptophans in amino acid compositions. However, false-negative as well as false-positive cases of

obtained fluorescence are known (Desbois, Seabrook et al. 2013), so protein diffraction data is required to confirm that result. There is no doubt that crystallization experiments will be continued.

In the current work, various functional and structural aspects of two Ca^{2+}-ATPases have been investigated. Being responsible for many cellular processes, P-type ATPases in general are highly important for cell survival. The pumps studied in this project, LMCA1 and SERCA2a, are not an exception. The former was shown to be up-regulated during bacterial adaptation to the environment, while the latter is crucial for proper heart function. Detailed knowledge of these pumps is an advancement in fundamental and applied sciences and will allow us better understand the nature of their operational mechanisms with the view to exploit them for medical purposes.

Summary

Calcium homeostasis is orchestrated and maintained by a complex system involving calcium channels, calcium binding proteins and active transporters. A key protein in generating steep ion gradients across biomembranes is the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), which belongs to the large family of P-type ATPases. P-type ATPases can be found among all domains of life and are of vital importance for cell survival and proliferation. The majority of the today's knowledge about P-type ATPases comes from the well-studied SERCA1a.

Cyclopiazonic acid (CPA), an inhibitor of SERCA, is a mycotoxin produced by *Penicillum cyclopium* and *Aspergillus*. SERCA1a was shown to bind CPA at nanomolar range. However, despite high sequence similarity, a bacterial homolog of SERCA1a, *Listeria monocytogenes* Ca^{2+}-ATPase (LMCA1) was shown to be insensitive to the inhibitor. To examine the reasons behind the CPA insensitivity of the bacterial pump, different mutant forms of LMCA1 were studied. Several mutant forms of LMCA1 were constructed, over-expressed, purified and tested for the activity in the presence of CPA. The experiments revealed that the restoration of two amino acids rendered the pump sensitive to the inhibitor. When residues, responsible for CPA binding in LMCA1 were found, the results have been confirmed on another bacterial pump from *Lactococcus lactis.* The difference between eukaryotic CPA-sensitive and prokaryotic CPA-insensitive binding pockets could be a potential basis for specific drug development against bacteria. These results have been published in an international peer-reviewed journal (Kotsubei, Gorgel et al. 2013).

The characterization and low-resolution structure determination of LMCA1 was previously done in our lab (Andersen, Gourdon et al. 2011, Faxen, Andersen et al. 2011). To improve crystal quality and diffraction resolution, it was attempted to crystallize a complex of CPA-sensitive LMCA1 double mutant and inhibitor, which was meant to stabilize the pump. Well-formed, single, three dimensional crystals were obtained in this study. However, so far only poor diffraction was observed after testing crystals under X-ray source and further optimization needs to be continued.

Another isoform of SERCA, cardiac SERCA2a, was also investigated in this study. Regulation of SERCA2a by phospholamban (PLB) is responsible for proper heart function; however, the exact mechanism of SERCA2a regulation by PLB remains unclear. A detailed understanding of this regulation is one of the most prominent challenges in the field, and the determination of an atomic structure of SERCA2a alone and in complex with PLB, would be a major milestone towards the design of therapeutic agents to improve contractility of the failing heart. In this study native SERCA2a was isolated and purified from the pig heart. In order to continue with crystallization trials, the presence of phospholamban in the preparation was identified. Moreover, the possible initial crystals have been obtained.

Kokkuvõte

Kaltsiumi homeostaas rakkudes on tagatud keerukate süsteemidega, milles osalevad nii erinevad kaltsiumi kanalid, kaltsiumi siduvad valgud kui ka aktiivsed transporterid. Kõige olulisemaks valguks, mis tagab kõrge kaltsiumi gradiendi bioloogilistel membraanidel, on tsütoplasmavõrgustiku Ca^{2+}-ATPaas (SERCA), mis kuulub suure P-tüüpi ATPaaside pere hulka. Transmembraansed P-tüüpi ATPaasid pumpavad erinevaid ioone ja fosfolipiide läbi biomembraani, kasutades ATP hüdrolüüsi käigus vabanevat energiat. Neid on leitud kõikides eludomeenides ja nad on ülimalt tähtsad raku elus püsimiseks ja paljunemiseks. Talitlushäired P-tüüp ATPaasides võivad põhjustada erinevaid kardiovaskulaarseid, metaboolseid ja neurodegeneratiivseid haigusi, mis tingib vajaduse nende ekstensiivseks uurimiseks potentsiaalsete ravimite sihtmärkidena.

Üks SERCA inhibiitoritest on mükotoksiin CPA, mida produtseeritakse *Penicillum cyclopium'i* ja mitmete *Aspergillus* perekonda kuuluvate seente poolt. Vaatamata 38% valgu järjestuse sarnasusele, ei olnud SERCA1a homoloog, *Listeria monocytogenes'e* Ca^{2+}-ATPaas (LMCA1), CPA suhtes tundlik. Eesmärgiga uurida põhjuseid, mis tingivad CPA mittetundlikuse, konstrueeriti, ekspresseeriti ning puhastati erinevad LMCA1 mutandid ning määrati nende aktiivsus CPA juuresolekul. Eksperimendid näitasid, et kahe amino happe asendamine vastavate jääkidega SERCA1a järjestusest, muutub pump CPA tundlikuks. Pärast LMCA1's CPA sidumise eest vastutavate aminohapete jääkide kindlaks tegemist kontrolliti tulemusi ühe teise bakteriaalse pumba peal, mis oli pärit *Lactococcus lactis'*est. Erinevus eukarüootsete CPA-tundlikute ja prokarüootsete CPA-mittetundlike kaltsiumi pumpade sidumisalade vahel võib olla spetsiifiliste bakterite vastaste derivaatide arendamise aluseks. Saadud tulemused avaldati ajakirjas FEBS Journal (Kotsubei, Gorgel et al. 2013).

Hiljuti iseloomustati LMCA1 ja määrati tema madala resolutsiooniga struktuur (Andersen, Gourdon et al. 2011, Faxen, Andersen et al. 2011). Kristallide kvaliteedi ja difraktsiooni resolutsiooni parandamiseks otsustati kasutada CPA-tundlikku LMCA1 kaksikmutanti, mida prooviti kristalliseerida kompleksis inhibiitoriga, mis võiks stabiliseerida selle struktuuri. Uurimustöö käigus saadi üksikud hästi formeerunud, ruumilised LMCA1 kaksikmutandi kristallid, kuid nende kristallide sünkrotronil

mõõdetud difraktsioon oli suhteliselt nõrk ja korraliku lahutuvuse saamiseks on plaanis kristalliseerimise tingimuste optimiseerimist jätkata.

Käesolevas töös uuriti ka teist SERCA isovormi, südame SERCA2a-d. SERCA2a, mida reguleerib fosfolambani nimeline valk (PLB) vastutab korraliku südame töö eest. Kõrge resolutsiooniga SERCA2a kristallstruktuur nii üksinda kui ka kompleksis PLB'ga pakub hetkel suurt huvi antud valdkonnas, kuna ta on südameveresoonkonna haiguste potentsiaalsete ravimite sihtmärgiks. Käesolevas töös eraldati ja puhastati natiivne SERCA2a sea südamest. Määrati kindlaks, et puhastatud preparaat sisaldab PLB'i ja saadi ka esialgsed SERCA2a kristallid, mis fluorestseerisid UV-valguse käes.

Käesolevas töös läbiviidud valgu ekspressiooni, solubilisatsiooni, puhastamise, re-lipidatsiooni ja kristallisatsiooni etappe tuleb vaadata kui esimesi olulisi samme uuritud valkude kristallstruktuuri määramise suunas.

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