Properties of proton binding kinetics in a new microsomal SR Ca-ATPase preparation, determined with a millisecond time resolution by the usage of caged compounds

Doctoral thesis for obtaining the academic degree
Doctor of Natural Sciences (Dr. rer. nat.)

submitted by
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at the

Universität Konstanz

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Konstanz, 2019
Date of the oral examination: 16\textsuperscript{th} December 2019

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Abstract

The properties of the proton binding kinetics of the SR Ca-ATPase were investigated in this work. The Proteins were reconstituted in lipid vesicles of the sarcoplasmic reticulum (SR) from the rabbit’s psoas muscle. For the analysis new methods were developed, which allowed further in-depth knowledge about the function of cation transport in this protein. Previous work has been limited to the cytosolic access channel of the cation binding sites in the protein, which was accessible in the vesicular preparation of SR Ca-ATPase. The properties of the luminal access channels of SR Ca-ATPase could be determined in previous studies mainly by equilibrium titrations, since the lipid membrane is only permeable to cations. The first step in this work was the development and optimisation of a process for the production of open lipid fragments (Fibich, et al., 2008). The proteins integrated into the lipid fragments should have the same properties as the original SR Ca-ATPase preparations, but allow free access of the cations to the luminal access channel. This goal was achieved by preparation steps with SDS buffers and dialysis procedures. The properties of the SR Ca-ATPase preparation were verified in various tests. Examinations with the electron microscope confirmed the open lipid fragments. The protein activities were investigated and confirmed with the coupled pyruvate kinase/lactate dehydrogenase test and the cation binding properties were confirmed in equilibrium titrations. The result was a direct comparability of the properties of both preparations.

This preparation formed the basis for kinetic studies of proton binding in the $E_2$ state of the so called Post-Albers (pump) cycle of SR Ca-ATPase (Post, et al., 1972), (Albers, 1967). Caged compounds were used for investigations of fast proton binding kinetics with time resolutions in the millisecond range. These caged compounds are physiologically inactive. Thus a homogeneous solution of the SR Ca-ATPase and all relevant substrates could be produced. The molecular structure of all caged compounds in the buffer solution was split simultaneously by a UV light trigger. In addition to commercially available caged compounds, the 2-methoxy-5-nitrophenol sulfate (MNPS.Na) synthesised by Karl Janko in the group of Prof. Dr. Hans-Jürgen Apell was used as caged proton (cg $H^+$) for the first time (Fibich, et al., 2007). Measurements showed the efficacy of cg $H^+$ and allowed its use for further kinetic investigations of proton binding in the SR Ca-ATPase.

The activation of the caged compound and the release of physiologically active substrates caused a change in the state of equilibrium of the SR Ca-ATPase, which was associated with conformational changes in protein structure and cation binding in the protein. These processes of all proteins were synchronised by this measuring technique. It was possible to observe and analyze defined proton binding and structure change steps. Structural protein changes and binding of additional cations in the protein caused electrostatic field changes in the immediate
vicinity of SR Ca-ATPase. In order to detect these changes, electrochromic styryl dyes were used. Due to the chemical structure of the dyes with a hydrophobic part, the bound chromophore and a subsequent hydrophilic part, the dye molecules were positioned in the lipid membrane. The chromophores were located nearby the membrane domain of SR Ca-ATPase, where the electrogenic changes were expected. The original styryl dyes were synthesised in the laboratory of Prof. Dr. Martin by M. Birmes in 1995 at the University of Düsseldorf and made available to the research group of Prof. Dr. Hans-Jürgen Apell. On the basis of these molecular structures, Karl Janko synthesised further styryl dyes in the group of Prof. Dr. Hans-Jürgen Apell (Fibich, et al., 2011). With the support of Anke Friemel and Dr. Heiko Möller, the purity of these dyes was investigated by NMR measurements at the University of Constance. The new styryl dyes were tested for their suitability for further investigations of the proton binding kinetics of the SR Ca-ATPase. The F52 molecule proved to be promising, as it provided comparable results with the vesicular and open lipid fragments of the SR Ca-ATPase preparations and reacted sufficiently electrosensitive to the charge shifts, which occurred during the state changes of the protein.

With these new caged compounds, dyes and methods further investigations of the proton binding in the SR Ca-ATPase in the E₂ state of the Post-Albers cycle were realised. Measurable increases and decreases of the fluorescence levels represented the release or binding of cations in the SR Ca-ATPase as well as the associated conformational structural change of the protein. With time resolutions in the millisecond range, the recorded signals also allowed the investigation of fast kinetic processes. The binding kinetics could be described by the mathematical model of a sum of exponential functions. Using the analytical software Fig.P, the mathematical model of the ion binding kinetics and the signals obtained from the measurements were compared and the parameters of the exponential functions were determined numerically with sufficient accuracy. The characteristic parameters of the proton binding kinetics for the measured signals are the time constants \( \tau_{\text{kin},i} \) and the change in fluorescence \( \Delta F_{\text{kin},i} \). The variable \( i \) reflects the number of exponential functions.

A sum of four exponential functions was required to describe the measured signals numerically with sufficient accuracy using a mathematical model of ion binding kinetics. These were four processes that could be assigned to the structural changes and cation bindings of the SR Ca-ATPase. Analyses of the associated activation energies \( E_A \) allowed a more detailed assignment of diffusion-controlled processes or conformational structural changes of the SR Ca-ATPase as a result of the pH jump. As a result of the pH jumps, generated by the \( \text{cgH}^+ \), a first fast process could be determined, which was described by the exponential function with the two variables \( \tau_{\text{kin},1} = 1.25 \text{ ms} \) and a fluorescence decrease of about \( \Delta F_{\text{kin},1} = 8 \% \), representing a diffusion-controlled charge movement. A proton binding can be assumed to this process, which takes place without any structural change of the SR Ca-
ATPase. In this case, amino acids of the protein were probably protonated, which leads to a change in the electrical field nearby the proteins. The second exponential function with the parameters $\tau_{\text{kin},2}$ and $\Delta F_{\text{kin},2}$ showed a pH-independence. The activation energy $E_{\text{A,2}}$ was determined to be $1.4 \pm 2.0$ kJ/mol. This very low activation energy and the parameters determined in the measurements indicate that this is an artefact caused by the measurement setup, which cannot be assigned to an ion binding process or structural protein changes. The parameters of the third exponential function of the mathematical model showed a significant pH-dependence. From the measurements, a time constant of $\tau_{\text{kin},3} = 208.3 \text{ ms} \pm 82.5 \text{ ms}$ was determined for the third process. The associated activation energy $E_{\text{A,3}} = 94.2$ kJ/mol also indicates a conformational structural change caused by the proton release with simultaneous charge binding of the SR Ca-ATPase. With the fourth and last exponential function, a very slow process was investigated in the measurements. The average time constant $\tau_{\text{kin},4}$ is $3.32 \text{ s} \pm 0.30 \text{ s}$. With an activation energy of $77.3 \text{ kJ/mol}$, the results indicate a change in the protein structure due to the binding of additional protons after their release from the $c$g $H^+$. Considering the already known steps in the Post-Albers cycle of SR Ca-ATPase, this process could represent the transition to the $E_1$ state, which represents a conformational change of the protein from the $E_2 (H_2)$ state to the $E_1 H_2$ state. Previous studies (Fibich, et al., 2007) have shown that this step is very slow.

A second alternative explanation for this extremely slow structural change of the SR Ca-ATPase was also examined in more detail. Thus, under these non-physiological conditions with a buffer composition containing only minor concentrations of cations, it is possible that the protein is converted into a non-physiological unstable state (Toyoshima, et al., 2004 (a)). At high pH values and lacking Ca$^{2+}$ ions, the cation binding sites of the SR Ca-ATPase remain unoccupied. This leads to a conformational structural change, which is necessary to stabilise the protein structure.

With these results on the proton binding kinetics of SR Ca-ATPase in the $E_2$ state, the following refinement and extension of the Post-Albers cycle in the $E_2$ state can be proposed. A fast diffusion controlled process of proton binding takes place. This correlates with the two possible steps $P-E_2 \text{ Ca} \rightarrow P-E_2 \text{ Ca H}$ and $P-E_2 \rightarrow P-E_2 \text{ H}$ (marked blue in the scheme). A further process was found which describes a net charge release from the protein at high proton concentrations (pH 6.6 to pH 7.2) and is associated with a conformational structural change of the protein. This indicates the exchange of Ca$^{2+}$ ions for protons and describes the transition of the two protein states $P-E_2 \text{ Ca H} \rightarrow P-E_2 \text{ H}$ (marked blue in the scheme). The last very slow process is associated with a conformational change of the SR Ca-ATPase. Looking at the measurement results, this can best be explained as a side branch of the Post-Albers cycle $P-E_2 \leftrightarrow P-E_2^{(*)}$, in which the binding sites of the proteins remain free due to the buffer composition.
(marked red in the scheme). The structural change of the SR Ca-ATPase towards the P-E$_2^{(*)}$ state serves to stabilise the protein, while the binding sites remain unoccupied.

As a result of the investigations, the following scheme of the Post-Albers cycle for the SR Ca-ATPase in the E$_2$ state can be drawn up.
Zusammenfassung


Die Aktivierung der Caged-Verbindung und die freierdenden, physiologisch wirksamen Substrate verursachten eine Änderung des Gleichgewichtszustandes der SR Ca-ATPase,

Mit diesen neuen Caged-Verbindungen, Farbstoffen und Methoden wurden die kinetischen Untersuchungen der Protonenbindung in der SR Ca-ATPase im E2-Zustand des Post-Albers-Zyklus realisiert. Messbare Anstiege und Abfälle der Fluoreszenzniveaus repräsentierten dabei die Freisetzung beziehungsweise Bindung von Kationen innerhalb der SR Ca-ATPase, sowie die damit einhergehende Strukturänderung des Proteins. Mit Zeitauflosungen im Millisekunden-Bereich erlaubten die aufgenommen Signale auch Untersuchungen schneller kinetischer Prozesse. Die Kinetik der Fluoreszenzänderungen konnten durch das mathematische Modell einer Summe von Exponentialfunktionen beschrieben werden. Mit Hilfe der analytischen Software Fig.P wurde das mathematische Modell der Ionenbindungskinetik und die Messsignale abgeglichen und die offenen Funktionsparameter nummerisch mit hinreichender Genauigkeit bestimmt. Die für die gemessenen Signale charakteristischen Parameter der Protonen-Bindungskinetik sind dabei die Zeitkonstanten $\tau_{kin,i}$ und die Fluoreszenzänderung $\Delta F_{kin,i}$. Hierbei spiegelt die Variable $i$ die Anzahl der Exponentialfunktionen wieder.
Um die gemessenen Signale nummerisch hinreichend genau mit dem mathematischen Modell der Ionenbindungskinetik zu beschreiben war eine Summe von vier Exponentialfunktionen notwendig. Diese stellten vier Prozesse dar, welche den Strukturänderungen und Kationenbindungen der SR Ca-ATPase zugeordnet werden konnten. Analysen der zugehörigen Aktivierungskinetik (Eₐ) erlauben eine genaue Zuordnung von diffusionskontrollierten Prozessen beziehungsweise von Strukturänderungen der SR Ca-ATPase als Folge des pH-Sprungs. Im Ergebnis konnte nach den durch das cg H⁺ erzeugten pH-Sprüngen festgestellt werden, dass der erste schnelle Prozess, beschrieben durch die Exponentialfunktion mit den beiden Variablen \( \tau_{\text{kin},1} = 1,25 \) ms und \( \Delta F_{\text{kin},1} \) mit etwa 8% Fluoreszenzabnahme, eine diffusionskontrollierte Ladungsverschiebung darstellt. Anhderdessen kann hier von einer Protonenbindung ausgegangen werden, welche ohne eine strukturelle Änderung der SR Ca-ATPase stattfindet. Hierbei werden wohl Aminosäure des Proteins protoniert, was zu einer Veränderung des elektrischen Feldes in unmittelbarer Umgebung der Proteine führt. Die zweite Exponentialfunktion mit den Parametern \( \tau_{\text{kin},2} \) und \( \Delta F_{\text{kin},2} \) zeigte eine pH-Abhängigkeit. Die Aktivierungskinetik \( E_{\text{A,2}} \) wurde mit 1,4 ± 2,0 kJ/mol ermittelt. Diese sehr geringe Aktivierungskinetik und die in den Messungen bestimmten Parameter deutet darauf hin, dass es sich dabei um ein durch den Messaufbau verursachtes Artefakt handelt, welches keiner Ionenbindung oder strukturellen Proteinänderung zuzuordnen ist. Die Parameter der dritte Exponentialfunktion des mathematischen Modells zeigten eine signifikante pH-Abhängigkeit. Aus den Messungen wurde für den dritten Prozess eine Zeitkonstante von \( \tau_{\text{kin},3} = 208,3 \) ms ± 82,5 ms bestimmt. Auch die dazu gehörende Aktivierungskinetik \( E_{\text{A,3}} = 94,2 \) kJ/mol deutet auf eine durch die Protonenfrequenz verursachte Strukturänderung mit gleichzeitiger Ladungsbindung der SR Ca-ATPase hin. Mit der vierten und letzten Exponentialfunktion wurde in den Messungen ein sehr langsamer Prozess untersucht. Die durchschnittliche Zeitkonstante \( \tau_{\text{kin},4} \) beträgt 3,32 s ± 0,30 s. Mit einer Aktivierungskinetik von 77,3 kJ/mol deuten die Ergebnisse auf eine Änderung der Proteinstruktur in Folge der Bindung zusätzlicher Protonen nach deren Freisetzung aus dem cg H⁺ hin. Betrachtet man den die bereits bekannten Schritte im Post-Albers Zyklus der SR Ca-ATPase so könnte dieser Prozess den Übergang in den E₁ Zustand darstellen, welcher die Strukturänderung des Protein \( E₂ (H₂) \rightarrow E₁ H₂ \) darstellt. In früheren Studien (Fibich, et al., 2007) wurde gezeigt, dass dieser Schritt sehr langsam abläuft.

Eine zweite alternative Erklärung für diese extrem langsame Strukturänderung der SR Ca-ATPase wurde auch näher beleuchtet. So ist unter diesen nicht-physiologischen Bedingungen der Pufferzusammensetzung die Möglichkeit gegeben, dass das Protein in einen nicht physiologischen instabilen Zustand überführt wird (Toyoshima, et al., 2004 (a)). Bei hohen pH-Werten und fehlenden Ca²⁺-Ionen bleiben die Kationen-Bindestellen der SR Ca-ATPase...
unbesetzt. Dies führt zu einer Strukturänderung, welche zu einer eigenständigen Stabilisierung des Proteins führt.

Mit diesen Ergebnissen zur Protonenbindungskinetik der SR Ca-ATPase im E\textsubscript{2}-Zustand kann folgende Verfeinerung und Erweiterung des Post-Albers-Zyklus im E\textsubscript{2} Zustand vorgeschlagen werden. Ein schneller diffusionskontrollierter Prozess der Protonenbindung findet statt. Das sinkende Fluoreszenzniveau verdeutlicht die zusätzliche Bindung von Ladungen im Protein. Dies korrespondiert mit den beiden möglichen Schritten P-E\textsubscript{2} Ca → P-E\textsubscript{2} Ca H und P-E\textsubscript{2} → P-E\textsubscript{2} H (Im Schema blau markiert). Ein weitere Prozess wurde gefunden, welcher bei hohen Protonenkonzentrationen (pH 6,6 bis pH 7,2) eine Netto-Ladungsfreisetzung aus dem Protein beschreibt und mit einer Strukturänderung des Protein einhergeht. Dies deutet auf den Austausch von Ca\textsuperscript{2+}-Ionen gegen Protonen hin und beschreibt den Übergang der beiden Proteinzustände P-E\textsubscript{2} Ca H → P-E\textsubscript{2} H (Im Schema blau markiert). Der letzte sehr langsame Prozess ist mit einer konformationellen Änderung der SR Ca-ATPase verbunden. Betrachtet man die Messergebnisse, so lässt sich dies am besten als ein Seitenzweig des Post-Albers-Zyklus P-E\textsubscript{2} ↔ P-E\textsubscript{2} (*) erklären, in welchem aufgrund der Pufferzusammensetzung die Bindestellen der Proteine frei bleiben (Im Schema rot markiert). Die Strukturänderung der SR Ca-ATPase hin zum P-E\textsubscript{2} (*)-Zustand dient dabei der Stabilisierung des Proteins, während die Bindestellen unbesetzt bleiben.

Als Ergebnis der Untersuchungen, kann abschließend folgendes Schema des Post-Albers-Zyklus für die SR Ca-ATPase im E\textsubscript{2}-Zustand aufgestellt werden.

\[
\begin{align*}
P - E_{2}^{(*)} & \\
\uparrow & \\
\cdots \rightarrow P - E_{2} Ca_{2} & \leftrightarrow P - E_{2} Ca & \leftrightarrow P - E_{2} \\
\downarrow & \uparrow & \uparrow \\
P - E_{2} Ca H & \leftrightarrow P - E_{2} H & \leftrightarrow P - E_{2} H_{2} & \rightarrow \cdots
\end{align*}
\]
1 Introduction

1.1 P-type ATPases

The P-type ATPases are a huge family of transmembrane proteins. They are common in cells in the animal kingdom and the plant kingdom, too (Kühlbrandt, 2004). These proteins are located in the plasma membrane, as well as the membranes of inner compartments of the cells. They transport ions across the membranes from one compartment with lower ion concentration to another compartment with higher ion concentration. Whereby the compartments could be the cytosolic and the extracellular sections, as well as the cytosolic and luminal sections of intracellular compartments. During this process an ion gradient is generated, which results in an electro-chemical membrane potential. Transport of ions against this gradient consumes energy, which is provided by the cell in form of adenosine triphosphate (ATP). Hydrolysis of ATP by cleavage of the γ-phosphate provides the energy to drive ion transport by all P-type ATPases. In this energy-providing step the enzyme is phosphorylated by γ-phosphate. This phosphorylated intermediate of the enzyme gives the whole family its name (James, et al., 1987).

There are various groups of P-Type ATPases, which differ in the ion species transported by each ATPase. P-Type ATPases exist which transport Na⁺/K⁺ ions, K⁺/H⁺ ions and Ca²⁺/H⁺ ions (Skou, 1957). All these transports are counter-transport processes of both ion species. Each kind of P-Type ATPases is expressed in specific tissues, where the function is relevant for survival of each cell type and the whole tissue or organism. Although the Na⁺/K⁺-ATPase is a crucial house-keeping enzyme in virtually all animal cells there are tissues in which it is present in very high density. Examples for the Na⁺/K⁺-ATPases are renal and the neuronal cells, in which ATPases are responsible for ion resorption or provide the gradients of Na and K ions across the cell membrane for action potentials. The stoichiometry of this transport process was determined to be 3 Na⁺/2 K⁺ per one hydrolysed ATP molecule (Apell, 2003). The gastric K⁺/H⁺-ATPases can be found in the parietal cells of the gastric mucosa. In this transport process a stoichiometry of 2 H⁺/2 K⁺ per one molecule ATP was found (Diller, et al., 2005) (Munson, et al., 2007). In the sarcoplasmic reticulum (SR) of muscle cells a dominant representative of the P-type ATPases is the SR Ca-ATPase. The name of the SR Ca-ATPase doesn't explicitly show the fact of an ion exchange but this protein provides the counter-transport, too. The SR Ca-ATPase performs the exchange of Ca²⁺ ions and H⁺ with the stoichiometry of 2 Ca²⁺/2-3 H⁺ per hydrolysed ATP molecule (Møller, et al., 2005). For the SR Ca-ATPase a protein density of 31-34,000 pumps/µm² was found in the membrane of the SR from rabbit skeletal muscles (Franzini-Armstrong, et al., 1985)
The transport mechanism of the P-type ATPases can be characterised as a cyclic ping-pong mechanism with distinguishable steps of ion binding, enzyme phosphorylation/dephosphorylation, conformation changes of the protein and the ion release. This so-called Post-Albers cycle was defined and introduced by Post and Albers in studies of the Na\(^+\)/K\(^+\)-ATPase (Albers, 1967), (Post, et al., 1972). It is valid for all P-type ATPases and is based on two fundamental conformations of the protein structure. These so-called E\(_1\) and E\(_2\) states are conformations of the enzyme in which the ion binding sites are accessible either from the cytosolic or from the luminal/extracellular side of the membrane, respectively. In the transition between both conformations so-called occluded states are intercalated in which the binding sites are accessible from neither side (Glynn, et al., 1990), (Läuger, 1991). This mechanism is necessary to protect the cells from short-circuiting the membrane potential. Ions in the aqueous solutions have access to the binding sites only from one side at a time.

![Diagram of the Post-Albers cycle of the SR Ca-ATPase](image)

*Figure 1: Post-Albers cycle of the SR Ca-ATPase with physiological states. The ion binding steps at the cytoplasmic and luminal sides are shown. Different structurally relaxed states of the enzyme are marked with *, ~, % and #: Phosphorylation a dephosphorylation leads to the occlusion of the bound ions and the transition from E\(_1\) to E\(_2\) state and vice versa. (Fibich, BioDraw Ultra)*
sites in the enzyme. Also shown are some intermediate states of the cycle. P-type ATPases which transport more than one ion in one direction are assumed to bind these ions in a sequential order. Binding or release of the first ion results in a conformational relaxation of the enzyme. This small rearrangement of the enzyme structure allows the second ion to bind to or dissociate from the second binding site in the P-type ATPase. These reaction steps are reversible and depend on the equilibrium with the ion concentration in the solution. In the case of the SR Ca-ATPase the binding affinity for one kind of transported ions in the $E_1$ differs strongly from the binding affinity of the same kind of ions in the $P-E_2$ state (Ikemoto, 1975). For the transported counter ions the binding affinity in both states is reversed to that of the first ion's species. This fact is caused by a smooth relaxation of the enzyme structure near the binding sites. Ion complexing amino acid side chains change their distance to the bound ion and speed up their exchange (Tanford, 1982) (Läuger, 1991). The transition from $E_1$ to the phosphorylated $P-E_2$ state is only possible if two $Ca^{2+}$ ions are bound in the enzyme. During this phosphorylation step and the dephosphorylation step at the transition from $P-E_2$ to $E_1$ state, the ions are bound in the enzyme and both access channels between the aqueous phases and the binding sites are closed. Both intermediate states were called "occluded states". In these states no ion exchange is possible between the solvent and the enzyme. This mechanism is verified by a number of studies (Apell, 2003), (Clausen, et al., 2003), (Fibich, et al., 2011), (Toyoshima, et al., 2004 (a)), (Toyoshima, et al., 2004 (c)), (Vilsen, et al., 1988) and (Xu, et al., 2002).

The X-ray crystallography structures of the SR Ca-ATPase were meanwhile determined in several conformations (see below). They show molecular characteristics of this protein and provide a detailed model to explain the ion transport mechanism. But the exact biophysical and biochemical ion binding and release properties are widely unidentified. Therefore, it was an appropriate model for the further mechanistic studies on ion binding properties with fluorescence methods and the use of caged compounds (Ellis-Davies, 2007), (Fibich, et al., 2007), (Janko, et al., 1987).

### 1.2 Amino acid sequence of the SR Ca-ATPase

The first amino acid sequence of the SR Ca-ATPase was determined in 1985 by the group of MacLennan (MacLennan, et al., 1985). The SR Ca-ATPase has a molecular mass of about 110 kDa and consists of a single amino acid strand, which is built of 994 amino acids. With the amino acid sequence it was possible to propose the first structure predictions by known sequence-secondary structure relations and hydrophobicity plots. These predictions allowed the first presentation of a mechanical model of this ion pump by MacLennan and others (Glynn, et al., 1990), (James, et al., 1987), (Stürmer, et al., 1989).
The secondary structure analysis showed three separated cytosolic domains with combinations of β-strands and amphipathic α-helices. The amino acid sequences of these parts brought out the homology to other well-known structure motifs. The first cytosolic domain was named transduction domain. Later it was called the actuator domain (A-domain). The other domains were identified as structures containing the phosphorylation site (P-domain) and the nucleotide binding site (N-domain). Between these three domains are regions of hydrophobic α-helices. These helices form the transmembrane part of the enzyme, which contains the cation binding sites. By amino acid exchange experiments amino acid residues were identified, which are crucial for the ion binding sites. The hydrophobic character of these regions indicates the transmembrane part of the protein.

1.3 Structure of the SR Ca-ATPase

The first highly resolved structure of a P-type ATPase was the crystal structure of the SR Ca-ATPase determined by X-ray crystallography in the year 2000 (Toyoshima, et al., 2000). These results revealed an estimated structure of the P-type ATPases. Further investigations and new crystal structures during the following years led to higher resolutions and different conformational structures of the SR Ca-ATPase with different bound ligands (Toyoshima, et al., 2002; Xu, et al., 2002; Sørensen, et al., 2006; Toyoshima, et al., 2004 (a); Toyoshima, et al., 2004 (b); Obara, et al., 2005; Jensen, et al., 2006; Olesen, et al., 2007; Takahashi, et al., 2007; Toyoshima, et al., 2007). These structures consist of three large cytosolic domains and a transmembrane domain of the protein. Every domain is associated with a special function in the whole transport cycle of the protein, as predicted by the amino acid sequence from 1985 (MacLennan, et al., 1985). The three well separated cytosolic domains are classified by their functions.

The P-domain is localised in the centre of the cytosolic headpiece (Figure 2 A). It is the phosphorylation domain with the residue Asp 351, which is phosphorylated during an active transport cycle (McIntosh, et al., 2003). In the amino acid sequence this domain consists of two separated parts. The first part is in the region between Asn 330 and Asn 359 and is adjoining the transmembrane helix M 4. The second part of the P-domain is located in the region of Lys 605 to Asp 737 which is connected to the helix M 5. The secondary structure of this domain is composed of a seven-stranded parallel β-sheet and eight small helices. That is a typical motif called Rossman fold, with all the characteristics necessary for the hydrolysis of an ATP molecule. The structure in the P-domain of the SR Ca-ATPase as well as its amino acid sequence show essential homology to the core domain of the L-2-haloacid dehalogenase, which catalyses the reaction to a separated halide. Amino acids around the Asp 315 form a highly negatively charged area, which is accessible to solvent.
The N-domain is the largest of the three cytosolic domains (Figure 2 B). It is composed of the amino acids starting at Gln 360 and ending with Arg 604. In the crystal structure of the SR Ca-ATPase it could be shown that this domain binds the ATP analogue TNP-AMP. An important residue engaged in ATP binding is the Phe 487. Adjoining this residue are Lys 515 and Lys 492 which are involved in the binding of the nucleotide, too (Claussen, et al., 2003). So this region forms the nucleotide binding pocket of the ATPase and in contrast to the phosphorylation site of the P-domain this area accommodates positive charges to bind the nucleotide. The structure of this domain is a set of seven-stranded antiparallel β-sheets and two helix bundles.

The smallest cytosolic domain of the SR Ca-ATPase is the A-domain (Figure 2 C). It consists of about 150 amino acids, divided into two parts. The first part consists of the residues 1 to 40, the N-terminal region of the SR Ca-ATPase. The second part of the A-domain is formed by about 110 amino acids between the transmembrane helices M2 and M3. The connection of the domain to the transmembrane helices is built by two very long loops. Additional experiments with proteinase K and tryptic digestion showed, that this domain moves in a wide area during the transport cycle of the enzyme. Different conformations with bound Ca$^{2+}$ ions, other substrates, inhibitors, or the phosphorylated enzyme enable or disable the cleavage of this domain by proteinase K and trypsin. This fact and studies with cyan-fluorescence protein labels (Winters, et al., 2008) indicates the great movements of this domain. It seems to be the actuator domain of the SR Ca-ATPase which is important for the transport cycle and it guides the Ca$^{2+}$ ions to their binding sites in the transmembrane region.
The M-domain is the transmembrane part of the SR Ca-ATPase (Figure 3). It consists of ten hydrophobic α helices (M1-M10), which are localised in the phospholipid bilayer. Most of the helices are longer than the bilayer is thick. Helices M2 and M5 have an overall length of about 60 Å. The major part of the long helices, which is not located in the hydrophobic membrane, stick out into the cytoplasm headpiece and link the enzymatic domains P, N and A to the transmembrane domain. Helix M5 has a direct connection to the P-domain and seems to be the centre stalk of the enzyme. M2 and M3 are also two of the long helices, whereas these helices are not localised in the middle of the SR Ca-ATPase. A special group of transmembrane helices are the shorter but more interesting ones: M4, M6 and the helix M10. Helix M4 and M6 are unwound in the centre region of the membrane and they are presenting the polar amino acid residues which are involved in ion coordination. M10 is kinked in its centre region. These amino acid sequences and the secondary structure motifs represent the binding sites of the transported ions. Very important in the rearrangement of the enzyme during a transport cycle is the extremely long cytoplasmic loop L67 between the transmembrane helices M6 and M7. This loop undergoes extended movements during the ion binding and release steps and is thought to be connected to the M3 helix by a hydrogen bond. M1, M2, and M3 are rather separated from the other helices, and show only a few hydrogen bonds with the other transmembrane helices. However, they are tighter bound to the A-domain and may transfer the movements to the ion binding sites. Loop L78 between helix M7 and M8 is the largest loop on the luminal side, and seems to play a role in guiding the ions to the binding sites.
In the X-ray crystallographic structures the SR Ca-ATPase inhibitor, Thapsigargin (TG), was included. The molecule binds in a cavity near the luminal side. It seems to interact with the helices M3 and M4, and the cytoplasm loop L67, which supports the assumption that this region is important for ion binding and the transport process.

1.4 Further studies

With the knowledge about the Post-Albers cycle for the P-type ATPases, known amino acid residues of the binding sites and the structural model of the SR Ca-ATPase from the X-ray analysis, it is possible to upgrade the whole model with biophysical data of each step in the transport cycle. Questions about the counter ions to the transported Ca\(^{2+}\) ions, the stoichiometry and the exchange of both species in the binding sites have to be answered, as well as on the data about the energy for each rearrangement of the enzyme structure during these steps.

To study these characteristics of the SR Ca-ATPase, time-resolved kinetic experiments were performed with time resolutions in the order of milliseconds and sub-milliseconds. To detect charge movements in the enzyme special electrochromic styryl dyes can be used (Grinvald, et al., 1982; Ephart, et al., 1993; Birmes, 1995). These styryl dyes respond to proximate electron charges, and report modifications with fast changes in the emitted light intensity (Apell, et al., 1985). The fluorescence emission has a very fast time response in the order of \(10^{-9}\) s to \(10^{-7}\) s (Lakowicz, 2006) so that is a very good reporter for high time resolution measurements.

Within these short time intervals it is not possible to use methods like the stopped-flow technique. Electrophysiological methods are inappropriate with vesicular preparations, and of

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*Figure 3: The red marked helices constitute the transmembrane part of the SR Ca-ATPase. The upper part of the long helices poke out of the membrane (yellow). In the lower part there are the ion binding sites, near the centre of the membrane. (Structure ISU4 (Toyoshima, et al., 2000), figures created with Delano PyMol, membrane supplemented by Fibich)*
course, for experiments with open SR membrane preparations containing the SR Ca-ATPase. To produce by stirring a homogeneous mixture of two components, which is necessary to activate a defined enzyme reaction, needs a few seconds. And measurements with a stopped-flow apparatus reach a time resolution of about 5-10 ms. The alternative way, which was used in the following study, is the use of caged compounds e.g. caged proton (Janko, et al., 1987; Barth, et al., 2002; Fibich, et al., 2007) and caged ATP (Kaplan, et al., 1978; McCray, et al., 1980; Sokolov, et al., 1998; Peinelt, et al., 2004). For a number of enzymatic substrates inactive precursors are available which can be used to prepare homogeneous mixtures (Ellis-Davies, 2007). To measure their binding to the enzyme with a high time resolution a trigger pulse in the form of an UV flash releases the desired substrate from the covalent bound protection group.

The combination of both methods, styryl dyes as detection probes and caged compounds for synchronised start of an enzymatic reaction, allow advanced investigations of ion binding to and ion exchange steps in the SR Ca-ATPase. Kinetics properties of the structural relaxations and conformational changes following the ion movements may be determined as well as the dependence of the kinetics on environment parameters such as ionic strength, buffer solution and competitive, non-transported ions.
2 Materials and Methods

2.1 Chemical compounds, buffers, etc.

All chemical compounds used were of the highest grade commercially available (Table 1). Solid compounds were solved in double-distilled water or apolar solvents according to their solubility. These stock solutions with defined concentrations were stored in the fridge or freezer as described in the datasheets provided by the distributors.

Table 1: Chemical compounds and their distributors.

<table>
<thead>
<tr>
<th>Chemical compounds</th>
<th>Manufacturer / Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-(N-morpholino)propanesulfonic acid, MOPS (209.3 g/mol)</td>
<td>Sigma-Aldrich Steinheim, Germany</td>
</tr>
<tr>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, HEPES (238.3 g/mol)</td>
<td>Sigma-Aldrich Steinheim, Germany</td>
</tr>
<tr>
<td>adenosine 5’-(tetrahydrogen triphosphate), ATP, (605.2 g/mol)</td>
<td>Boehringer-Ingelheim Ingelheim, Germany</td>
</tr>
<tr>
<td>Apyrase, (A-6410) Grade VI, (1.4 units/ml)</td>
<td>Sigma-Aldrich Steinheim, Germany</td>
</tr>
<tr>
<td>D(+)Sucrose (342.30 g/mol)</td>
<td>Roth Karlsruhe, Germany</td>
</tr>
<tr>
<td>Dimethylsulfoxid, DMSO Uvasol, (78.13 g/mol)</td>
<td>Merck Darmstadt, Germany</td>
</tr>
<tr>
<td>EDTA Titriplex II (292.25 g/mol)</td>
<td>Merck Darmstadt, Germany</td>
</tr>
<tr>
<td>Ethanol Absolute, EtOH Uvasol</td>
<td>Merck Darmstadt, Germany</td>
</tr>
<tr>
<td>N-(Tri(hydroxy-methyl) methyl)glycin, Tricine, (179.2 g/mol)</td>
<td>Boehringer-Ingelheim Ingelheim, Germany</td>
</tr>
<tr>
<td>N,N’-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-(carboxymethyl)] tetra-sodium salt, BAPTA, (564.37 g/mol)</td>
<td>Fluka Buchs, Switzerland</td>
</tr>
<tr>
<td>nicotinamide adenine dinucleotide, NADH, (663.4 g/mol)</td>
<td>Boehringer-Ingelheim Ingelheim, Germany</td>
</tr>
<tr>
<td>Phosphoenolpyruvate, PEP, (168.04 g/mol)</td>
<td>Boehringer-Ingelheim Ingelheim, Germany</td>
</tr>
<tr>
<td>Potassium chloride suprapur quality (74.56 g/mol)</td>
<td>Merck Darmstadt, Germany</td>
</tr>
<tr>
<td>Pyruvate kinase/lactat dehydrogenase, PK/LDH solution</td>
<td>Boehringer-Ingelheim Ingelheim, Germany</td>
</tr>
<tr>
<td>Chemical compounds</td>
<td>Manufacturer / Distributor</td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Sodium chloride suprapur quality (58.4 g/mol)</td>
<td>Merck Darmstadt, Germany</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate, SDS, (288.38 g/mol)</td>
<td>Pierce Rockford (Ill.) USA</td>
</tr>
<tr>
<td>Tris(hydroxymethyl)aminomethane maleate salt, Trizma maleate (237.2 g/mol)</td>
<td>Sigma-Aldrich Steinheim, Germany</td>
</tr>
</tbody>
</table>

### 2.2 SR Ca-ATPase specific compounds

The specific SR Ca-ATPase inhibitor Thapsigargin (TG) was obtained from Alomones, Jerusalem Israel. 0.65 mg of the crystalline powder (650.76 g/mol) were dissolved in 100 µl DMSO to obtain a 10 mM stock solution. The solution was divided in small aliquots of 10 µl and stored at -30 °C. The chemical structure of the TG is shown in Figure 4. It is a huge steric molecule, which binds to the F256 Thapsigargin-binding site in the transmembrane region of the SR Ca-ATPase, fixes the enzyme in a form analogous to the E₂ state and blocks the access channel for the transported ions (Toyoshima, et al., 2002).

![Chemical structure of the inhibitor Thapsigargin (TG). (Fibich, BioDraw Ultra)](image)

To allow the Ca²⁺ ions to access the luminal side of the vesicular SR Ca-ATPase the calcium ionophore A23187 was used (Reed, et al., 1972; Pressman, 1976). It was bought from Boehringer Mannheim, Germany. With the molecular weight of 523.6 g/mol a stock solution in absolute Ethanol) was prepared. The concentration of this solution was 12.5 mM. Aliquots with the stock solution were frozen and stored at -30°C.


2.3 Caged Compounds

The NPE-caged ATP (adenosine 5'-triphosphate, P$_3$-(1-(2-nitrophenyl)ethyl) ester) was purchased as disodium salt from Molecular Probes, Eugene, Oregon USA. The molecular weight of the caged ATP salt was 700.3 g/mol. 5 mg were dissolved in a total volume of 714 µl bi-distilled water to achieve a 10 mM stock solution. NPE-ATP in aqueous solution undergoes lysis and released ATP and ADP exist in the stock solution (Thrilwell, et al., 1994).

To reduce the concentration of free nucleotides, 0.014 units of the adenosine-nucleotidase hydrolysing enzyme apyrase, which was purified from potato, was added during the preparation of the stock solution. The apyrase was obtained from Sigma-Aldrich (A-6410) in Grade VI. It hydrolyses ATP and ADP and prevents, thus, side effects of the nucleotides in the experiments performed. 1.32 mM MgCl$_2$ was added to the stock solution to enable the enzymatic activity of the apyrase. The entire volume of 714 µl was portioned in aliquots of 50 µl, put into UV impermeable boxes and stored at -30°C. The molecular ATP-releasing process of the NPE-ATP is presented in Figure 5. The protecting 1-(2-nitrophenyl)ethyl group absorbs the UV light energy and this is sufficient to split the photo-labile bond.

![Figure 5: The photo-cleavage process of the caged NPE-ATP (Fibich, BioDraw Ultra)](image)

The caged proton, 2-methoxy-5-nitrophenyl sulfate (MNPS.Na) was synthesised by Karl Janko in our group at the University of Konstanz (Fibich, et al., 2007). It was prepared as sodium salt with a molecular weight of 272 g/mol and is soluble in water. Stock solutions with concentrations of 50 mM and 25 mM were prepared. The aqueous solution of the cg H$^+$ has acidic characteristics. To avoid significant changes of the pH in the experimental solutions by adding this compound and to stabilise the cg H$^+$, the pH of the stock solution was increased to pH 6-7 by addition of small amounts of 1 M NaOH. The aliquots of 100 µl were stored like the other caged compounds in an UV protected container and in the freezer at -30 °C. By an UV flash sulfuric acid is released from the 2-methoxy-5-nitrophenyl compound.
which is a completely dissociated, strong acid, and produces thus a pH jump. The mechanism is shown in Figure 6.

![Chemical structure](image)

Figure 6: Photo-cleavage of the cg $H^+$. For this reaction a water molecule is necessary. (Fibich, BioDraw Ultra)

### 2.4 Electrochromic styryl dyes

A wide array of different electrochromic styryl dyes is available to detect charge movements in transmembrane proteins. The styryl dyes were synthesised and provided to us by the group of Prof. Dr. Martin from the University of Düsseldorf, Germany (Birmes, 1995). Only a few of all these styryl dyes were useful in the experiments with the SR Ca-ATPase. They were dissolved in absolute ethanol, and the stock solution with concentrations between 1.8 mM and about 25 mM were stored in the fridge at +4 °C. The exact concentration was determined by absorption measurements. Using the Lambert-Beer law (Equation 1) and the known absorption coefficient of each styryl dye, the concentration of each solution was calculated by Equation 2.

**Equation 1:**

$$A = e_{482\,nm} \times c_{dye} \times d_{cuvette}$$

**Equation 2:**

$$c_{dye} = \frac{A}{(e_{482\,nm} \times d_{cuvette})}$$
For experiments with the SR Ca-ATPase, a solution with a concentration of 0.2 mM in absolute ethanol was prepared of each dye by diluting from the stock solutions. These solutions were stored in the freezer at -30 °C to prevent evaporation and, therewith, an increase of the concentration. In all fluorescence experiments a final concentrations was chosen between 200 nM and 800 nM.

The styryl dyes synthesised in Düsseldorf followed a special nomenclature. In Figure 7 the structures of three dyes are shown. The whole set of dyes comprised styryl dyes of the group called nXITC. The chromophore in the molecule mid-section of all dyes was the same and each dye consisted of a polar head piece with the isothiocyanate group. Letters 'n' and 'X' describe the number of C-atoms in the alkyl chains (n) and the spacer chain between chromophore and the isothiocyanate group (X), respectively. The application of these dyes to detect charge movements in the membrane is extensively discussed in (Pedersen, et al., 2002). Best results with the strongest signals and the best signal-to-noise ratio (S/N) in preparations of the SR Ca-ATPase were achieved with the styryl dyes 2BITC (Figure 7 A) and 2HITC (Figure 7 B). In the fluorescence measurements with the SR Ca-ATPase a couple of new synthesised styryl dyes were tested, too. These dyes were produced by Karl Janko in our group at the University of Konstanz. One of these new styryl dyes, F52, is shown in Figure 7 C. Table 2 shows the molecular formula and the molecular weight of these dyes.

Figure 7: Chemical structure of the styryl dyes 2BITC (A) and 2HITC (B) which were synthesised by Martin Birmes at the University of Düsseldorf and a new styryl dye F52 (C) synthesised by Karl Janko in our group at the University of Konstanz. (Fibich, BioDraw Ultra)
Table 2: Names, molecular formula and molecular weight of applied styryl dyes.

<table>
<thead>
<tr>
<th>Name of the electrochromic styryl dye</th>
<th>Molecular formula</th>
<th>Molecular weight g/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>2BITC</td>
<td>C\textsubscript{22}H\textsubscript{28}N\textsubscript{3}BrS</td>
<td>446.45</td>
</tr>
<tr>
<td>2HITC</td>
<td>C\textsubscript{24}H\textsubscript{32}N\textsubscript{3}BrS</td>
<td>474.50</td>
</tr>
<tr>
<td>F52 (syn. F52-H\textsubscript{3})</td>
<td>C\textsubscript{22}H\textsubscript{31}N\textsubscript{2}Br</td>
<td>403.40</td>
</tr>
</tbody>
</table>

The group of new synthesised electrochromic styryl dyes consists of compounds related to the nXITC dyes. Differences are the exchange of the isothiocyanate group with a methyl group or a carboxylic acid. Also, the length of the spacer between the head-group and the chromophore was varied. The stock solutions as well as the diluted solutions for the experiments were handled in the same way as described above. Table 3 shows an overview of the new synthesised styryl dyes.

Table 3: Overview of further new synthesised electrochromic styryl dyes by Karl Janko.

<table>
<thead>
<tr>
<th>Name of the electrochromic styryl dye</th>
<th>Molecular formula</th>
<th>Molecular weight g/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>F42 (F42-H\textsubscript{3})</td>
<td>C\textsubscript{21}H\textsubscript{29}N\textsubscript{2}Br</td>
<td>389.37</td>
</tr>
<tr>
<td>F52 (F52-H\textsubscript{3})</td>
<td>C\textsubscript{22}H\textsubscript{31}N\textsubscript{2}Br</td>
<td>403.40</td>
</tr>
<tr>
<td>F62 (F62-H\textsubscript{3})</td>
<td>C\textsubscript{23}H\textsubscript{33}N\textsubscript{2}Br</td>
<td>417.43</td>
</tr>
<tr>
<td>F82 (F82-H\textsubscript{3})</td>
<td>C\textsubscript{25}H\textsubscript{37}N\textsubscript{2}Br</td>
<td>445.48</td>
</tr>
<tr>
<td>F52-O\textsubscript{2}H</td>
<td>C\textsubscript{22}H\textsubscript{29}O\textsubscript{2}N\textsubscript{2}Br</td>
<td>433.38</td>
</tr>
<tr>
<td>F62-O\textsubscript{2}H</td>
<td>C\textsubscript{23}H\textsubscript{31}O\textsubscript{2}N#Br</td>
<td>447.41</td>
</tr>
</tbody>
</table>

The chemical structures of the new synthesised styryl dyes are shown in Figure 8. All new dyes have the same length of the alkyl chains and the chromophore system is identical in all compounds. The differences between these dyes consists in the length of the spacer chain which varies between four (Figure 8 A), five (Figure 8 C and Figure 8 D), six (Figure 8 B and E) and eight (Figure 8 C) C atoms and the exchanged head-group with methyl (Figure 8 A, B, and C) and carboxylic acid (Figure 8 D and E).

The structures of all these styryl dyes, the new ones as well as the nXITC dyes, show an amphiphilic characteristic of the molecules. In aqueous solutions with membrane preparations the molecules are integrated into these lipid bilayers. The orientation of the molecules is defined by their hydrophobic and hydrophilic chains. Alkyl chains are placed in the membrane and the polar head groups are aligned to the aqueous phase (Pedersen, et al., 2002).
Cations bound in the enzyme binding sites and the membrane potential generate an electric field across the membrane. The parallel alignment of the electrochromic styryl dyes to the electric field allows the detection of changes in the field strength and, therefore, the corresponding charge movements of the Ca\textsuperscript{2+} ions and protons.

![Molecular structures of new synthesised electrochromic styryl dyes produced by Karl Janko. The ITC head-group was exchanged and the spacer length varied. (Fibich, BioDraw Ultra)](image)

### 2.5 Fluorescence mechanism of the electrochromic styryl dyes

The chromophore of these styryl dyes consists of a quaternary pyridine. This ring is connected via a butylene group to a dialkylaniline ring. The pyridine functions as an acceptor and the dialkylaniline as a donor of electrons. In the ground state of the styryl dye, the lowest unoccupied molecular orbital state (LUMO), the positive charge is located in the pyridine. The excitation of the molecule results in a movement of the positive charge directed to the dialkylaniline, the highest occupied molecular orbital state (HOMO), as described in (Loew, et al., 1978). For an excitation of the chromophore a distinct energy is necessary which can be provided by a (laser) light source. The release of the energy from the HOMO to the LUMO
state of the molecule results in an emission of energy in form of fluorescence light with a longer wavelength than the excitation wavelength.

The fluorescence of the styryl dyes can be utilized for electric field studies, if they are integrated in the lipid bilayer of a cell membrane. The amphiphilic characteristic causes a parallel orientation of all dye molecules between the lipid molecules (Figure 9). Across the membrane exists an external electric field as a result of the membrane potential $\Phi_m = \Phi^+ - \Phi^-$. Inner membrane charges, $\Phi_{\text{ion}}$, like bound ions in transmembrane proteins, affect this electrical field, too. A potential gradient across a bilayer is shown in Figure 9. In case of an external electric field and a parallel orientation of all dye molecules the necessary excitation energy is dependent on the external conditions. During the excitation of the styryl dye molecules the positive charge of the pyridine, have to be moved to the dialkylaniline (LUMO $\rightarrow$ HOMO) against the gradient of the external field (Figure 10).

Figure 9: Model of the lipid bilayer (Orange) with integrated protein (Blue). Styryl Dyes (red) aligned in a parallel manner to the electric field. The luminal $\Phi^+$ and the cytosolic $\Phi^-$ potentials are shown with the inner membrane potential $\Phi_{\text{ion}}$ (Fibich, BioDraw Ultra)
To perform this excitation of the dye in Figure 10 a higher energy is required. Equation 3 shows the energy which is needed to move an elementary charge \( q \) against the electric field \( F \) along a distance \( r \). This additional energy is equivalent to a difference of the wavelength \( \Delta \nu \) of the excitation light. With the wavelength and the Planck constant \( h \) the energy can be calculated by the following function:

**Equation 3:**

\[
E = h \cdot \Delta \nu = -q \cdot \vec{r} \cdot \vec{F}
\]

In measurements with and without external electrical fields the additional energy can be observed by the shift of the excitation spectra to shorter wavelengths in case of a higher potential gradient. These characteristics offer the usage of styryl dyes as electrosensitive probes in lipid membrane preparations.

The shift of the excitation wavelength in the spectrum is small. Figure 11 represents two schematic spectra of the excitation wavelengths in Figure 11A and of the emission wavelength in Figure 11B. For high yields in the detection of electric field changes the exciting HeNe-laser was chosen with a wavelength of 543 nm at the red edge of the absorption spectrum of the styryl dye. Small shifts \( (h \cdot \Delta \nu) \) in the excitation spectrum result in a much higher corresponding change of the emission intensity \( \Delta em = f(h \cdot \Delta \nu) \). The photomultiplier detects only the intensity of the fluorescence light band (bandwidth 10 nm) which is related to the changed electric field characteristic near the styryl dye molecule. This detection is as sensitive as possible to probe the movement of an ion which generates an ion potential \( \Phi_{ion} \) in the binding sites of a protein.

![Figure 10: The Chromophore of styryl dyes and the atomic charge shift of positive charges during excitation (green) and emission (red) of the molecule. An external electric field which affects the charge movements is shown with light grey, dotted arrows, from the right to the left. (Fibich, BioDraw Ultra)](image-url)
For the identification and analysis of proteins, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used as described in (Laemmli, 1970). The gel was composed of a 4 % SDS-polyacrylamide stacking gel and a resolving gel with a concentration of 10 % SDS-polyacrylamide. The cathode buffer comprised 0.1 M TRIS, 0.1 M Tricine and 0.1 M SDS at pH 8.25. At the beginning of the gel electrophoresis 0.1 mM thioglycolate was added to the cathode buffer. The anode buffer was a solution of 0.2 M TRIS at pH 8.9. The loading buffer was a mixture of 0.313 M TRIS, 10 % SDS, 50 % sucrose and 0.025 % bromophenol blue. 50 mM glutathione was added to the loading buffer and the protein was mixed with the loading buffer.

Protein samples and a molecular weight marker were added to different sample slots of the gel mounted in the electrophoresis chamber. A sample volume containing about 10 µg of protein, but not more than 15 µl were filled in the slot of each lane. The gel ran at a voltage of about 120 V and a current in the order of 40 mA over a period of 1 h to 1 ½ h.

When a protein run was finished, the gel was removed from the chamber and put into a fixation bath consisting of 50 % methanol, 10 % acetic acid and glycol, and was gently shaken for 45 min. After that time the gel was transferred from the fixation bath to a solution with a mixture of 10 % acetic acid and 0.025 % Coomassie Blue to stain the proteins in the gel. This staining procedure required about 30-60 min, during which the dish with the gel was shaken to get a homogeneous staining. Thereafter the staining solution was decanted and excess Coomassie Blue was removed by maintaining the gel in a 10 % acetic acid solution. This discoloring procedure needed a few hours and is not critical in terms of time. Finally the
gel was placed between two cellophane layers and dried for prolonged conservation and easier handling.

As protein standard the BMA ProSieve Marker set 5-225 kDa (830547) was used which was obtained from Biozym Scientific GmbH, Hessisch Oldendorf, Germany. This marker set provides standard proteins with molecular weights of 5, 10, 15, 25, 35, 50, 75, 100, 150 and 225 kDa. It was applied in a separate lane to allow the calibration of the proteins run on the gel.

2.7 Preparation of SR Ca-ATPase from rabbit muscles

SR Ca-ATPase was prepared in form of a vesicular membrane fraction from the psoas muscles of rabbits. For the preparation the method of (Heilmann, et al., 1977) was adopted with minor changes to obtain better enzyme activities and to reduce the contamination of the SR Ca-ATPases with debris from the muscle cells.

The psoas muscles were taken from the rabbits immediately after killing of the animal and stored in ice cold buffer of 0.25 M sucrose and 5 mM HEPES. The pH of the buffer was adjusted by NaOH to pH 7.5. In this buffer the muscles could be stored at -70 °C for several months. All the following preparation steps were accomplished on ice or at a maximum temperature of 4 °C.

The muscle tissue was cut into small pieces of about 5 mm in size. About 15 g of the tissue were put into an Erlenmeyer flask filled with 60 ml of the forecited buffer. The tissue in the buffer was chopped five to six times with a Polytron PTDA 3012-2/S mixer at highest speed (about 21700 rpm). After 15 seconds of mixing, the flask was put into ice for at least 1 minute before the next mixing step. After this treatment of the muscle cells a homogeneous suspension was obtained in the flask with pink colour fading into light brown.

In the next preparation steps the cell debris was removed from the Ca-ATPase containing membrane vesicles in the suspension by centrifugation for 15 min at 3000 rpm (~1500 g), at 4 °C in a Sorvall RC5Cplus centrifuge with rotor GSA. The supernatant was filtered two times through an absorbent gauze and centrifuged a second time in the GSA rotor for 20 min at 7000 rpm (~8000 g) and 4 °C.

Then the supernatant was filled into tubes for the 45Ti-rotor and centrifuged in a Beckman L-60 ultracentrifuge for 40 min at 36000 rpm (~150000 g) and at 4 °C. The pellet was homogenised in a buffer containing 0.6 mM KCl and 20 mM MOPS. A pH of 7.5 was adjusted by KOH. The suspension was decanted into a beaker on ice and put on a magnetic stirrer in the refrigerator for 45 min. After that incubation the suspension was centrifuged in the 45Ti-rotor for 40 min at 36000 rpm (~150000 g) and 4 °C. The pellet was resuspended in
the above-mentioned sucrose/HEPES buffer and centrifuged again at 36000 rpm, 4°C for 40 min in the 45Ti-rotor.

For the purification of the SR Ca-ATPase preparation the last pellet was resuspended in a small amount of sucrose/HEPES buffer and overlaid on a continuous sucrose density gradient with concentrations of 25 % to 45 % sucrose. The bottom of the tube was filled with a 70 % sucrose cushion. The gradient centrifugation was performed in a TV850 rotor at 40000 rpm (~151000 g) and 4 °C in the Beckman L-60 ultracentrifuge overnight (about 16 hours).

An opalescent band about 5 mm above the 70 % cushion contained the purified SR Ca-ATPase in native SR phospholipid vesicles and could be extracted by suction with Pasteur pipettes. All the obtained SR Ca-ATPase samples were pooled and apportioned in aliquots of 1000 µl or 100 µl. The aliquots were rapidly frozen in liquid nitrogen and stored at -70 °C.

The method of (Markwell, et al., 1978) for membrane proteins was used to determine the enzyme concentration in these preparations. The protein activity was checked by the coupled pyruvate kinase/lactate dehydrogenase assay according to (Schwartz, et al., 1971). By adding the Ca\(^{2+}\) ionophore A23187 to the vesicular SR Ca-ATPase preparation the enzyme activity could be raised to approximately threefold of the starting activity. The reason for this increase was the induced high permeability of the SR membrane for Ca\(^{2+}\) ions, which prevented an inhibitory Ca\(^{2+}\) gradient across the vesicle membrane. The SR Ca-ATPase activity was much higher in the absence of a counterforce against the Ca\(^{2+}\) transport. To check the purity of the SR Ca-ATPase in the preparations, the specific Ca-ATPase inhibitor TG was added during the enzyme activity test. The remaining, unspecific activity showed typically only a small contamination by other ATPase activity.

2.8 Utilised vesicular SR Ca-ATPase preparations

The yield of the purification method described above, was between 60 mg and 92 mg SR Ca-ATPase per 100 g Psoas muscle tissue in the various preparations. The protein preparations were stored at -70 °C and could be thawed and used for the experiments still after a long period of a couple of months or years, without a significant loss in the enzyme activity. Each charge of the frozen enzyme was tested before starting a series of experiments with this SR Ca-ATPase preparation.

All experiments were performed with the charges of the SR Ca-ATPase obtained from the preparations of 30.10.2002, 08.05.2003, 10.07.2007 (fraction 'A' and 'B'), 08.05.2008, and 10.11.2008 by Milena Roudna and Andreas Fibich in our group at the University of Konstanz. These charges were also used as raw material for the preparation of open membrane fragments. To compare SR Ca-ATPase in vesicle preparations and in the open membrane fragment experiments were performed with preparation of the same date to assure their
comparability. Enzyme properties of these charges were quite similar and are shown in Table 4 in the Results section, chapter 3.1 Kinetics studies of the vesicular SR Ca-ATPase, page 33.

2.9 Preparation of protein-free phospholipid vesicles

For the validation of the results obtained from the enzyme-kinetics experiments a reference zero value was necessary. These control preparations were protein-free phospholipid vesicles.

Two kinds of these preparations were used to confirm the results from the SR Ca-ATPase measurements. The first type of protein-free vesicle was a preparation from phosphatidyl choline, PC 18:1. A stock solution with 10 mg/ml PC 18:1 and cholic acid in a buffer containing 25 mM histidine, and 0.5 M EDTA was provided by Milena Roudna in our group at the University of Konstanz. The pH of this stock solution was set to pH 7.1. To obtain vesicular phospholipid structures, this stock solution was filled in a Visking dialysis tube type 8/32 (diameter 6 mm with a cut-off size of 14 kDa). About 200 µl of this stock solution was dialysed for two days in a buffer volume of 200 ml (dilution ratio 1000:1). The composition of the dialysis buffer corresponded to the buffer used in the SR Ca-ATPase experiments. After dialysis the suspension of the protein-free lipid vesicles was transferred into Eppendorf tubes and kept on ice. The end volume was determined and compared to the starting volume of 200 µl, to calculate the dilution factor and concentration of phospholipids. The lipid vesicles had to rest for a minimum of 2 h at temperatures below +4 °C for stabilisation and sealing of the membrane. After that procedure the protein-free lipid vesicles were ready to use and were stored in the fridge at +4 °C.

The second type of protein-free vesicles was a preparation with a mixture of different phospholipids that correspond to the composition of the native SR membrane. The preparation procedure was the same as described for the PC 18:1 vesicles. Data about the composition of native SR membranes in rabbit muscles were found in (Krainev, et al., 1995). Protein-free vesicles with mixed lipids were made of a combination with 70 % (w/w) phosphatidyl choline (PC 18:1), 20 % (w/w) phosphatidyl-ethanolamine (PE), 8 % (w/w) phosphatidyl serine (PS) and 2 % (w/w) phosphatidic acid (PA). The lipids were dissolved in a methanolic cholic-acid solution (28.7 mg Na-cholate in 1 ml methanol). After dialysis, the concentration of the mixed vesicles was determined and they were stored at +4 °C in the fridge for usage in experiments.

2.10 Preparation of open membrane fragments with SR Ca-ATPase

The well-established SR Ca-ATPase preparation by (Heilmann, et al., 1977) lead to vesicular membrane structures with inserted SR Ca-ATPases. In this preparation the cytosolic domain of the Ca-ATPases is oriented to the outside, the luminal domains are present at the inside of the vesicles. These vesicular structures are composed of native SR phospholipids and they have the characteristics of the genuine SR membranes. The membranes are impermeable for
divalent cations, especially \( \text{Ca}^{2+} \) ions. In contrast, small monovalent cations, such as the \( \text{H}^+ \) and \( \text{K}^+ \) ions, can permeate the membrane. This fact is a handicap for the study and analysis of ion binding to the luminal binding sites. For the analysis of the luminal binding properties of the Ca-ATPase a membrane preparation had to be introduced in which the luminal side of the membrane becomes directly accessible. The approach was based on the method developed by (Jørgensen, 1974) for the Na,K-ATPase.

Starting with the Ca-ATPase preparation in SR vesicles described above, the Ca-ATPase preparations were incubated with SDS. The incubation buffer, called buffer B contained 50 mM imidazole and 2 mM EDTA. The buffer pH was adjusted to pH 7.5 by NaOH. 20 ml of buffer B were supplemented by \( \text{Na}_2\text{ATP} \) to a final concentration of 3 mM ATP. In 10 ml of this buffer SDS was dissolved to a final concentration of 19 mM.

The optimum concentration ratio of SR Ca-ATPase : SDS was determined to be 2.2 mg/ml protein : 1.9 mM SDS (Fibich, et al., 2008). The exact volume of Ca-ATPase solution varied, depending on the enzyme concentration of the used SR Ca-ATPase charge, mentioned in chapter 1.8 and chapter Results-X.1. Buffer B was added to complement the aliquots to a final volume of 410 µl. The addition of buffer B happened under continuous stirring on a magnetic stirrer at highest speed. The recipe of buffer B contained sufficient ATP and SDS to open the lipid bilayer membrane of the SR Ca-ATPase vesicles undergoing the 15 min incubation step. Subsequently, the aliquots were incubated for 15 min in a thermostated heating block or water bath at 25 °C. After incubation the aliquots were transferred into the ice bath to stop or reduce the denaturing effect of the SDS.

For fast removal of the SDS from the incubated SR Ca-ATPase, the solutions were filled into dialysis tubes and underwent a dialysis for 1 hour. The tubes were from Visking type 8/32 (diameter 6 mm with a cut off size of 14 kDa), which were first boiled in a buffer with 1 mM EDTA/Tris for 1 h to remove possible contaminations by heavy-metal ions. The aliquots of the incubated SR Ca-ATPase were split in samples of 100 µl volume before the dialysis to improve the volume-surface-ratio. 9x100 µl of the protein solution were dialysed in 500 ml volume of buffer. Dialysis buffer was an ice cold buffer of 20 mM Trizma maleate and 3 % D(+)-sucrose. pH was set to pH 7.0 by adding KOH. Dialysis was performed in an ice bath on a magnetic stirrer on medium speed.

After one hour of dialysis the contents of the tubes were pooled in a cold beaker. To obtain a greater stock of membrane fragments containing SR Ca-ATPase another incubation and dialysis was performed. The beaker containing the first preparation was covered by parafilm and stored on ice. In the end both preparations were pooled. The whole volume was apportioned in aliquots of 150 µl and rapidly frozen in liquid nitrogen. Stored at -70 °C, the
protein could be used for the experiments for a couple of years without a loss of its characteristic properties.

The first tested method was a dialysis for three days. In this procedure the incubated SR Ca-ATPase was dialysed as described above, but the beaker was kept in an ice bath for three days. During this dialysis the buffer was stirred with a magnetic stirrer at medium speed. This method had some unfavourable effects on the enzyme activity, which decreased to 50.6 % of the original activity, and enzyme concentration, which decreased to 67.3 %. Both effects will be shown in chapter 3.2.3, page 46.

The protein concentration was determined by the method of (Markwell, et al., 1978) and the enzyme activity by the coupled pyruvate kinase/lactate dehydrogenase assay of (Schwartz, et al., 1971). In the activity test the Ca\(^{2+}\) ionophore A23187 and the inhibitor TG were added. The activity did not increase when the ionophore was added, contrarily to the vesicle preparations. This observation indicated the formation of open membrane fragments which do not act as Ca\(^{2+}\) ion storages. TG acted, however, as inhibitor comparable to its action in the vesicle preparation.

### 2.11 Technical equipment

#### 2.11.1 pH meter and pH probe

pH was determined with two pH meters, GPH014 from Greisinger Electronic, Regenstauf Germany, and CG817T purchased from Schott Geräte, Mainz Germany. Both pH meters were used with a combination electrode from Laseranalytik GmbH, Germany type TR/CMAWL/S7/TB which is a micro-electrode with 5 mm in diameter, and the diaphragm is located near the tip of the probe. The storage buffer for the electrodes was a pH 7.00 calibration buffer bought from Riedel-de Haën Seelze, Germany with 3 M KCl.

The pH meters were calibrated with two of three calibration buffers of pH 4.00, pH 7.00 and pH 9.00 (Riedel-de Haën Seelze, Germany), dependent on the desired pH range of the measurements. The stability of the calibrated electrode was high, and it was not often necessary to perform a recalibration. Most measurements on a single day were accomplished with the same calibration.

#### 2.11.2 Commercial fluorospectrometer and spectrophotometer

For standard fluorescence measurements the fluorospectrometer LS 50B from Perkin Elmer Hamburg, Germany was used. Figure 12 displays the light pathway of the fluorospectrometer. The light flashes, produced by a high-pressure xenon flash lamp, pass the tuneable excitation monochromator and is limited in its bandwidth with an adjustable slit-aperture. The selected light excites the styryl dyes in a half-micro cuvette with a volume of 1 ml. The cuvettes are
placed in a thermostated cuvette holder with a built-in magnetic stirrer. The emitted light is filtered by an additional cut off filter at 430 nm, to reduce background effects of harmonics of the wavelength selected by the subsequent monochromator. The output of the emission monochromator is detected by a photomultiplier. The measurements were performed and saved with the Perkin Elmer FL-WinLab Software Version 4.00.02 running under Windows on a standard PC.

The spectrophotometer for all absorption measurements was the Perkin Elmer LAMBDA 40 UV/VIS photometer. The light of either a halogen or a deuterium lamp was selected according to the chosen wavelength and passes the monochromator and a slit-aperture to produce 'monochromatic' light with a defined half-width. Both, the wavelength and the bandwidth of the light are adjustable by external control. The beam is split into two beams and passes two thermostated cuvettes in the measurement chamber. This method allows difference spectra between two solutions. For normal measurements a reference cuvette filled with buffer solution only is placed into the reference beam to correct for background effects. The optical pathway, which the light has to pass through these cuvettes, is 1 cm. After passing the cuvettes the light beams are detected by photodiodes which detect the remaining light intensity in both pathways. The cuvettes were made of optical glass for visible wavelength or quartz glass for measurements in the UV range.

All data were recorded and saved by the UV-WinLab Software Version 2.85.04 from Perkin Elmer. This software package has supplemental features. It allows recordings of time drives at constant wavelength, recordings of absorption spectra and it provides an arithmetical package. With the arithmetical functions minima, maxima and slopes of the increasing or decreasing absorption trends can be determined.
2.11.3 Special laser fluorimeters

Two non-commercial laser fluorimeter types were used to investigate ion binding and release of the SR Ca-ATPase by styryl dyes. Both instruments were developed and built in the mechanical workshop of the University of Konstanz (Stürmer, et al., 1989).

The first setup is a standard equipment for steady-state measurements. The light source is a green HeNe laser LHGR 0200M from LASER 2000 GmbH, Wessling, Germany. Output power is 2 mW and the wavelength is $\lambda = 543$ nm. The cuvette in the measuring chamber is thermostated and the solutions in the cuvette are mixed by a magnetic stirrer. The laser light beam is dispersed to illuminate most of the cuvette volume. The light emitted by the styryl dyes in the solution passes a narrow-band filter with a transmission wavelength of $\lambda = 589$ nm ($T > 70\%$) and a half width of $\lambda = 10.6$ nm, before it is detected by a photomultiplier (R 2066 from Hamamatsu Photonic K. K., Hamamatsu Japan). High voltage for the photomultiplier is supplied by a Keithley 247 High Voltage Supply and is adjusted between -780 V and -850 V. The photo current is fed into a Keithley 427 current amplifier from Keithley Instruments, Cleveland Ohio USA. The output voltage is subsequently digitized with an AdLink PCI-9112 data-acquisition board from Imtec, Backnang Germany, and recorded on a PC at sampling rates between 1 Hz and 10 Hz. The high intensity of the excitation laser produces a relatively high yield of emitted fluorescent light in this setup and allows the measurement of even weak signals with a relatively good S/N.

The second setup was designed for time-resolved kinetics measurements with an advanced resolution in the millisecond and sub-millisecond time range. A schematic representation is shown in Figure 13. To excite the styryl dye, the same type of HeNe laser with a wavelength of 543 nm is used in this fluorimeter. The laser is mounted in horizontal position above the cuvette and the laser beam is deflected by a mirror, then passing in a vertical direction through a dichroic mirror and a lens into the cuvette. A specific feature of this fluorimeter is the measuring chamber with its ellipsoidal mirror. The cuvette is positioned at the upper focus of the mirror on a thermostated plate. The second focus of the ellipsoidal mirror is in the lower part of the measuring chamber, where a photo-multiplier is placed. That allows the collection of a much higher amount of emitted light, collecting the light in the half-sphere around the cuvette. A type R928 photomultiplier was purchased from Hamamatsu Photonic K. K. in Japan. High voltage is supplied by a Fluke 405B power supply from Fluke MFG. Co. Inc., Everett, Washington, USA, with an output voltage between -770 V and -950 V. The photomultiplier signal is converted by an I/V-converter (Electronic workshop at the University of Konstanz) and fed into an A/D-converter board in a standard PC with a sampling frequency of up to 100 kHz.
To start a time-resolved measurement the caged compound in the homogeneous solution is uncaged by a high-energy UV laser flash. The UV laser is an excimer laser, type EMG 100 from LAMBDA PHYSIK, Göttingen Germany. The laser tube is filled with a mixture of 20 mbar xenon, 220 mbar helium/fluorine 5% and 2260 mbar helium (purity 4.6). To trigger the laser flash with the thyratron, a voltage of 30 kV was set at the power supply. All chosen gas mixtures in the laser produced a UV flash of 14 ns duration with a wavelength of 351 nm and an average energy of 100 mJ. The power released in one flash was about 6 MW, which is sufficient to split off the covalently bound protection group from the caged compounds. The horizontally oriented UV flash passes a cylinder lens and is deflected by the dichroic mirror to a vertical direction. Passing the second lens the beam is widened to produce an almost homogeneous illumination of the total cuvette volume. The excitation and activation laser light illuminate together the cuvette from above, and both beams are blocked by the thermostated cuvette socket in the measurement chamber. To reduce the effect of interfering light on the photomultiplier a narrow-band interference filter from AMKO GmbH, Tornesch Germany, with a transition wavelength of 589 nm (T > 70%) and a bandwidth of 10 nm ± 2 nm are placed in front of the photomultiplier entrance window. An additional UV cut-off filter with 420 nm is added to minimise the artefact of the high-energy UV flash.

The A/D board, which converts the analogue voltage signal into digital data, which was used during early measurements, was a 16-bit A/D converter type DSP 32C from Loughborough Sound Images Ltd., Loughborough UK. That board used a C++ program (oszi5.exe by (Bühler, 1994)) running under DOS/Windows 3.1. Each data sample consists of 500 values.
and the recording time depends on the sampling rate. These data were normalised and stored in a linear time scale by a program named norm3.exe, which is programmed in Fortran.

A new A/D-board applied in more recent measurements was the AdLink PCI-9112 data-acquisition board from Imtec, Backnang Germany. This board works with sample rates up to 100 kHz and a resolution of 12 bit. The data are stored in a buffer and the recording length with high sampling rates is much higher than in the old equipment. Instead of a few milliseconds of collected data, it is now possible to save the signal data over a couple of seconds with the highest available time resolution. A new method to normalise the stored data is applied by the Fortran program redlog.exe. The huge amount of sampled data are reduced and normalised in a specific way. The time scale is transformed to a logarithm scale, to obtain also a high time resolution in the millisecond and sub-millisecond region, and to produce data with constant increment on a logarithm time scale. To improve the S/N ratio, data averaging according to the boxcar method is included. Thereby, a defined number of adjacent values in a recorded signal were summed up and the average was set as the new value. By doing this with the whole data sample, the noise was reduced. Both methods reduced also the file size and improved the representation of the data.

The computers with the PCI-9112 data-acquisition board use a working environment with special algorithms for each measurement setup which were created to record the signals of the photomultipliers. With the data acquisition program DASYLab from Geitmann Messtechnik, Menden Germany, it was possible to create these algorithms for each application. Features like the sampling rate for steady-state or enzyme kinetics measurements can be set in this environment, as well as the prearrangement steps, data display and trigger signals.

Two working modules and a flowchart for experiments with the laser fluorimeter with low sampling rates are shown in Figure 14. It consists of two lanes, one is the data acquisition lane with the A/D-converter, display and data storage. The other lane contains two additional timers to display the elapsed time.
The software environment for the high time resolution laser fluorimeter is much more comprehensive and comprises of two flowcharts. The first one (not shown) guided the user through the prearrangement and the second one (shown in Figure 15) starts the measurement with an output trigger pulse and records the photomultiplier data with high sampling rates.

Figure 14: DASYLab flowchart of the data-sampling program for the laser fluorimeter with low sampling rates.

Figure 15: Trigger, data acquisition and display of the fluorescence signal (DASYLab flowchart). The high time resolution laser fluorimeter works with sampling rates up to 100 kHz.
2.12 Software

2.12.1 WinMaxC

To compare results of all kinetic measurements of the SR Ca-ATPase it was necessary to determine the concentration of free divalent cations in the buffer solution. The equilibrium concentration of free ions depends on environmental parameters such as temperature and pH, as well as the concentration of chelating agents and ion-complexing compounds like ATP (in the case of Mg$^{2+}$). In this case the Windows program WinMaxC Version 1.70 from http://www.stanford.edu/~cpatton/maxc.html was used. The model file for the determination of the parameters was the bers.tcm file, which contains the individual complexation properties of each compound used in the electrolytes. Parameters like temperature, pH and ion strength were also included in the calculations. The concentration of different chelators, mono and divalent ions, Pi and ATP led to exact concentrations of the free ions or the necessary amount of salt added to obtain the desired free concentrations. So each result of the measurements could be assigned to the distinct amount of free ions in the buffer and different buffer solution setups could be compared to each other.

2.12.2 Drifter

Drifter is a graphical-mathematical software programmed by Dirk Reimann (Reimann, 2000). The results of the steady-state experiments in the laser fluorimeter were recordings over a long time period of 15 min and up to 30 min. During this period some fluorescence traces showed frequently under otherwise equilibrium conditions no stable fluorescence level. Some of them had increasing or decreasing fluorescence drifts without any interference from the experiment. Such drifts can be corrected by Drifter. To be able to compare the fluorescence signals from various experiments it is necessary to normalize the data in a way that the arbitrary voltage signal of the photomultiplier output is related to a defined reference.

The signals could be normalised by a zero and 100 % level and the volume error, which was produced by each addition of substrates, could also be corrected. To reduce the data of the recorded signal Drifter could create the average over a selected range of values, which represents one fluorescence level between two additions to the cuvette.

The data were stored as Drifter (*.drf) or Excel files (*.csv).

2.12.3 Fig.P

Fig.P is a software for data analysis of the Fig.P Software Corporation, Durham NC, USA, published by BIOSOFT, Cambridge UK. The version used was No. 2.98. This software imports data and displays the data in various manners. Mathematical tools allow the performance of a statistical analysis and of fits of the recorded data with mathematical models.
such as the Hill function and exponential kinetics of the time-resolved ion binding of the protein. Most of the data were analysed with this software.

2.12.4 UN-SCAN-IT gel
This program is an automated digitizing system, which digitises and analyses for example SDS-PAGE gels. It was provided in version 5.1 by Silk Scientific Inc. Orem, Utah, USA. A digitised picture of a gel could be analysed. The intensity of the protein bands are transformed to numerical values. It allows the determination of the running distance of each band, and by calibrating with a molecular weight marker, the determination of the molecular mass of the protein in each band.

2.12.5 PyMol
To present 3D X-ray structure models of the enzymes, the software PyMol from DeLano Scientific LLC, Palo Alto California USA was used. The applied version is the freely available 0.99 version, downloaded from http://pymol.sourceforge.net. Protein-structure data for the PyMol viewer were obtained from the RCBS Protein Data Bank (PDB) (http://www.rcbs.org/pdb/home/home.do). Snapshots of the 3D model of the SR Ca-ATPase for illustration were obtained by this tool.

2.12.6 BioDraw Ultra
BioDraw Ultra is a software package with tools for biological and chemical drawings. It is provided by CambridgeSoft, Cambridge, MA, USA. The used version was 11.0. Chemical structures and biological pathways and schemes were created with this software package.

2.13 Measurement of the SR Ca-ATPase concentration of the purified enzyme preparations
In all enzyme preparations of the SR Ca-ATPase the enzyme concentration was unknown. To determine the amount of protein in solution the Markwell method was used (Markwell, et al., 1978). The Markwell test is a colorimetric method derived from the methods of (Wu, 1922) and (Lowry, et al., 1951) especially for membrane proteins and lipoproteins.

An alkaline copper reagent with SDS was used to incubate the protein solution for 15 minutes at room temperature. Then the samples were mixed forceful with a 1 : 1 dilution of Folin-Ciocalteu Phenol Reagent and incubated for another 45 minutes at room temperature. After incubation the absorption at 660 nm was measured in a photo spectrometer. As weight standard a bovine serum albumin (BSA) solution with known concentrations (w / v) was used. Each concentration of the SR Ca-ATPase, as well as the BSA test series, was determined as the average of three samples. The regression line fitted through the BSA standard was used to determine the SR Ca-ATPase concentration.
2.14 Determination of enzyme activity by the PK/LDH-test

Each SR Ca-ATPase protein preparation varied not only in its protein concentration but also in its specific enzyme activity. Therefore, each batch was tested for the enzyme activity by measuring the turnover of ATP to ADP with the coupled pyruvate-kinase / lactate-dehydrogenase test (PK/LDH-test) described in (Schwartz, et al., 1971). In this photospectrometric test the cuvette was filled with a solution of ATP, pothoenoipruvate (PEP) and the protein mixture of PK and LDH. The reduced nicotinamide adenine dinucleotide (NADH) was added in a concentration to obtain an absorbance of about 1.5 at a wavelength of \( \lambda = 340 \) nm. Following the scheme in Figure 16, the SR Ca-ATPase activity starts the reaction sequence and the oxidation of NADH lead to a decreasing absorbance level. In this reaction scheme the rate-limiting process is the phosphorylation of the SR Ca-ATPase by ATP hydrolysis. The PK and LDH steps have much higher turnover for the PEP phosphotransfer and the oxidation of the NADH than ATP consumption by the added P-type ATPases.

Due to the 1 : 1 stoichiometry in the reaction sequence the decrease of the absorption of NADH at the wavelength \( \lambda = 340 \) nm is the indicator for the enzyme activity. The decrease per minute is used to determine the absolute enzyme activity of the SR Ca-ATPase by Equation 4. Known parameters like the volume (V) of the test solution, the length (d) of the light pathway through the solution, the absorption coefficient (\( \varepsilon \)) of NADH and the mass (m) of the used SR Ca-ATPase are sufficient to calculate the enzyme activity (\( a \)) in \( \mu \text{mol Pi per min and mg of protein} \).

\[
a = \frac{\left( \frac{\Delta A_{\lambda=340 \text{ nm}}}{\Delta t} \right)}{d \cdot \varepsilon \cdot m} \cdot V
\]

\( Figure 16: \) Reaction scheme of the coupled PK/LDH test for the determination of enzyme activity.
2.15 Mathematical models of the binding kinetics

With all the equipment and the analysis tools it was possible to produce and record signals, which are associated to the function of the SR Ca-ATPase. These signals are modelled by the relaxation and rearrangement kinetics of the enzyme, and, therefore, these signals represent properties of the ion binding and release steps in the SR Ca-ATPase.

By a synchronised start of the enzyme activity the recorded fluorescence signals describe in the simplest case a one-step transition into a new steady-state of the enzyme which is monitored by an increasing or decreasing fluorescence change that runs into a constant final level. The transition kinetics from a state 1 to a state 2 can be described by the following function:

\[ F = F_{t=0} + \Delta F_{kin} \cdot \left(1 - e^{\frac{-t}{\tau_{kin}}}\right) \]

It represents a function with a starting state at \( F_{t=0} \). \( \tau_{kin} \) is the characteristic time constant of the transition process. The second characteristic parameter of the transition is \( \Delta F_{kin} \), which describes the maximum change in the fluorescence amplitude. This mathematical model fits well to simple transition signals in the SR Ca-ATPase experiments, and the parameters \( \tau_{kin} \) and \( \Delta F_{kin} \) can be determined by a numerical fitting procedure, provided by the analytical software Fig.P.

In cases when the performed partial reaction consists of one reaction step, a fit with one exponential function is sufficient to describe the time course of the fluorescence signal. In the case of two sequential reaction steps the theory of chemical kinetics predicts a behaviour that can be described by the sum of two exponentials as shown in Equation 6.

\[ F = \Delta F_{kin,1} \cdot \left(1 - e^{\frac{-t}{\tau_{kin,1}}}\right) + \Delta F_{kin,2} \cdot \left(1 - e^{\frac{-t}{\tau_{kin,2}}}\right) + F_{t=0} \]

If the time constants significantly differ, the first process with a lower time constant dominates the first phase of the time course, whereas the slower process emphasises the long-lasting trend. This function is adequate to describe several partial reactions of the SR Ca-ATPase kinetics. If there are more than two processes in a recorded signal, the model would have to be supplemented by additional exponential terms.
3 Results

3.1 Kinetics studies of the vesicular SR Ca-ATPase

With the SR Ca-ATPase preparation in vesicular SR membranes as described in chapter 2.7, a series of experiments were performed, to investigate the ion binding kinetics of the enzyme. Starting with the coupled PK/LDH enzyme activity test and steady-state control experiments, the series comprise ion equilibrium titration measurements and the kinetics analysis of the ion binding.

To allow the comparison of the experiments containing different SR Ca-ATPase charges, enzyme was chosen from preparations with nearly similar protein activity and concentration. In Table 4 the preparations used are shown with their typical characteristics. These preparations were the starting material for SDS preparations, to get the open microsomal membrane fragments with inserted SR Ca-ATPase. The following tests were performed to obtain results for a direct comparison of the SR Ca-ATPase in vesicular membranes and the open membrane fragments.

Table 4: Protein concentrations and enzyme activities of the vesicular SR Ca-ATPase preparations.

<table>
<thead>
<tr>
<th>Date of preparation</th>
<th>Protein concentration mg/ml</th>
<th>Enzyme activity in ( \mu \text{mol P/mg protein min} )</th>
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<td>2.7</td>
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</tr>
</tbody>
</table>

3.1.1 Standard substrate binding tests in steady-state experiments

Figure 17 shows a typical steady-state experiment. These tests were control experiments to demonstrate ion-binding dependent measurable fluorescence changes and the activity of the SR Ca-ATPase. Used buffer compositions vary and were dependent on the requirements of the intended experiments. The electrolyte contained 25 mM or 50 mM of one of the buffering substances HEPES, Tricine, or MOPS as well as mono- and divalent ions such as Na\(^+\), K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\). In some experiments choline chloride (ChCl) was used. The ion radius of the
monovalent choline is much bigger than that of the other monovalent ions used, and it doesn't interact with the enzyme or its binding sites. Each sample contained one of the styryl dyes 2BITC, 2HITC or F52 in final concentrations between 200 nM and 800 nM. All steady-state experiments were performed either in the LS 50B fluorospectrometer or, more often, in the laser fluorimeter. The laser fluorimeter has the benefit of a much higher yield of fluorescence light than the fluorospectrometer. Sampling rates lay between 1 Hz and 10 Hz. The cuvette was an optical glass fluorescence cuvette with a maximum volume of about 4 ml. Just 2 ml of the test solution were needed to achieve a sufficient filling for all the measurements.

A general example of such a standard experiment is shown in Figure 17. It illustrates the behaviour of a vesicular SR Ca-ATPase with a concentration of 9 µg/ml in a 50 mM Tricine buffer at pH 7.2. The numbers indicate the equilibrium states. (1) is the zero level of the buffer. (2) shows the addition and the slow balancing of the 200 nM 2HITC dye. The large increase in fluorescence between (2) and (3) is caused by the arrangement of the hydrophobic dye molecules in the phospholipid bilayer around the SR Ca-ATPase. With the addition (4) of the Ca\(^{2+}\) chelator BAPTA (200 µM), Ca\(^{2+}\) ions in the solution were bound to the chelator and a new equilibrium of bound and free ions was adjusted. The release of positive charged ions from the enzyme increased the fluorescence of the dye. Upon adding 300 µM CaCl\(_2\) in step (5), the binding sites of the SR Ca-ATPase were saturated with Ca\(^{2+}\) ions evidenced by a lower fluorescence level as in step (3). The difference between level (3) and (5) shows the partial Ca\(^{2+}\) saturation of the SR Ca-ATPase in the stock solution. The addition of 1 mM

![Figure 17: Raw data of a steady-state measurement. The levels show the fluorescence after addition of (1) buffer solution, (2) styryl dye, (3) vesicular SR Ca-ATPase, (4) BAPTA, (5) CaCl\(_2\), (6) Na\(_2\)ATP and (7) highly concentrated CaCl\(_2\).](image-url)
Na$_2$ATP is shown in step (6). Here various effects may occur, depending on pH-value, the buffer compound and other ingredients in the cuvette. In this example the fluorescence level decreased, representing the additional bindings of positive charged ions in the enzymes. Upon the addition of 32 mM CaCl$_2$ in (7) a fully saturating concentration was achieved and the fluorescence attained a level significant lower than the starting level of the SR Ca-ATPase.

The native results were normalised and corrected by the Drifter software, to reduce the drift of the fluorescence in some equilibrium states. This treatment allows the comparison of different measurements and enzyme charges (Figure 18). During this normalisation the fluorescence level in step (3) was set to 1.0 to allow the description of the relative changes in respect to that starting point in the Post-Albers cycle.

*Figure 18:* Corrected and normalised data of Figure 17. The fluorescence levels marked from (1) to (7) correspond to the substrate addition steps in Figure 17.
3.1.2 Ca\textsuperscript{2+} equilibrium titration

Ca\textsuperscript{2+} titration experiments were performed to determine the half saturating Ca\textsuperscript{2+} concentration, the Hill coefficient, zero Ca\textsuperscript{2+} and saturated Ca\textsuperscript{2+} fluorescence levels. The buffer was composed of 25 mM MOPS, 50 mM KCl and the pH was adjusted to 7.2. All experiments contained 1 mM MgCl\textsubscript{2} which is necessary for the enzyme phosphorylation by ATP. The styryl dye 2HITC was used as indicator in a concentration of 200 nM. 18 mg/ml of the SR Ca-ATPase in vesicular membranes were applied. To allow the free Ca\textsuperscript{2+} exchange across the membranes, 12.5 µM of the calcium ionophore A23187 were added to the solution. Steps (1) to (4) as mentioned above (Figure 18) were made in all these titrations to check the function of the Ca-ATPase. By the addition of the chelator BAPTA an almost Ca\textsuperscript{2+}-free SR Ca-ATPase was obtained. This represents the starting level in the titration experiments. By gradual increase of the Ca\textsuperscript{2+} concentration new fluorescence levels were detected and plotted against the corresponding ion concentration. The free Ca\textsuperscript{2+} concentration depended on the concentration of chelator, ATP and all other ions in the solution, and was calculated by the WinMaxC Software.

![Graph](image)

*Figure 19: Ca\textsuperscript{2+} titration of the SR Ca-ATPase in vesicular membranes. Black line represent the E\textsubscript{1} state, the red line shows the results with added ATP and the enzyme in the P-E\textsubscript{2} state.*

The results of the Ca\textsuperscript{2+} titration were normalised with respect to the starting fluorescence level, and the obtained concentrations dependence of the fluorescence was fitted by an allosteric Hill kinetics function, as shown in Equation 7, to determine the Ca\textsuperscript{2+} binding affinity. F\textsubscript{max} and F\textsubscript{min} describe the fluorescence levels. The concentration c\textsubscript{50} is the half saturation of the Ca\textsuperscript{2+} binding and P\textsubscript{Hill} the characteristic Hill coefficient. In Figure 19 the curves in both states of the enzyme are shown. Both the E\textsubscript{1} state and the P-E\textsubscript{2} state of the SR Ca-ATPase reveal a fluorescence decrease, ΔF\textsubscript{max}, of approximately 40 % between the
Ca\(^{2+}\)-free and the Ca\(^{2+}\)-saturated level obtained at 10 µM of free Ca\(^{2+}\). For the E\(_1\) state ΔF showed a 44.9 % and the P-E\(_2\) state a 38.5 % loss of fluorescence. The derived half saturating constants \(c_{50}\) were 290 nM ±17 nM Ca\(^{2+}\) in the E\(_1\) state and 385 nM ±17 nM Ca\(^{2+}\) in the P-E\(_2\) state. The value of the Hill coefficient \(P_{\text{Hill}}\), which is relevant to characterise the binding affinity, shows significant different results for both states. In the E\(_1\) state the Hill coefficient was 1.4 ±0.1 and in the P-E\(_2\) state it was 3.3 ±0.4.

\[
\frac{F}{F_0} = F_{\text{max}} - \left( F_{\text{max}} - F_{\text{min}} \right) \frac{1}{1 + \left( \frac{c}{c_{50}} \right)^{-P_{\text{Hill}}}}
\]

At data points above the concentration of 100 µM free Ca\(^{2+}\) ions, the fluorescence consistently decreased very fast to zero level by adding exceeding Ca\(^{2+}\). That data points were not a result of the physiological function of the SR Ca-ATPase. This artificial decrease was not shown in Figure 19 and not included in the Hill fits.

### 3.1.3 H\(^+\) concentration jump experiments in the E\(_1\) conformation

The proton binding kinetics of the SR Ca-ATPase was tested with the caged H\(^+\), MNPS.Na. In the transport cycle (Post-Albers cycle) of the SR Ca-ATPase the protons act as transported counter ions to Ca\(^{2+}\). With these experiments the exchange kinetics in the binding sites of the enzyme was determined. A test sample contained so-called pseudo buffer with monovalent cations like sodium, potassium or choline chloride but no buffering substance. This is a crucial requirement to produce pH jumps when protons were released from cg H\(^+\). Typical concentrations of these cations were 50 mM and 100 mM. For the measurements of the SR Ca-ATPase kinetics the styryl dyes 2HITC, 2BITC or F52 and caged proton were added to the mixture. Since no ATP and Mg\(^{2+}\) ions were present the enzyme was in its E\(_1\) conformation. 300 µl of this testing solution were filled into a round quartz glass cuvette. The cuvette was placed in the laser fluorimeter setup and thermally equilibrated at 20 °C. Thereafter, the test solution was continuously illuminated by the He/Ne-laser with a wavelength of 543 nm for the excitation of the styryl dye. The measurement started with a pre-trigger signal initiated by the user. All fluorescence data were acquired by the A/D converter-board in a PC system with a sampling rate of 100 kHz. The pre-trigger data were used for the normalisation of the fluorescence levels in the following analysis. About 1.5 s after the pre-trigger pulse an automated second trigger pulse activated the high-energy UV-flash which led to the release of the protons from MNPS.Na. The free H\(^+\) ions increased the proton concentration of the sample solution whereby decreasing the pH. The following fluorescence data represent the exchange and movement of ions in the enzyme binding sites.
One measurement consists of 10 seconds of acquired data which were stored in a file by the DASY-Lab Software.

Figure 20 shows the data of an experiment on a linear time axis. The fluorescence level at \( t < 0 \) was 1. As shown, the first decreasing part of the signal is represented by a nearby vertical line. Using this data representation no further analysis was able with adequate results of the fast decrease. Therefore, all stored data of these experiments were normalised and reduced by the redlog02.exe software for further analysis. The reduction of the data occurred in a logarithmic manner for better visualisation of the signals. This method emphasises the millisecond time range for a better illustration of the signals in the diagram.

In Figure 21 the same experimented data are shown as in Figure 20, but in logarithmic representation. With the logarithmic time axis the fast decreasing part of the signal is clearly visible. For further analysis the signals were fitted by an exponential kinetics function as described in Equation 8.

\[
F = \Delta F_{kin,1} \cdot \left( 1 - e^{-\frac{t}{\tau_{kin,1}}} \right) + \cdots + \Delta F_{kin,i} \cdot \left( 1 - e^{-\frac{t}{\tau_{kin,i}}} \right) + F_{t=0}
\]
The kinetics function represents a sum of several exponential functions. Each function contains its characteristic parameters $\Delta F_{\text{kin},x}$ and $\tau_{\text{kin},x}$ which describes the fluorescence level changes respectively the time constants of that function. The indices $x = 1 .. i$ represent the different exponentials in the sum and the term $F_{t=0}$ shows the fluorescence starting level of the signal, which was 1 due to the normalisation.

For the fitting of the cg $H^+$ signal in Figure 21 a function with three exponentials was necessary to achieve an adequate fit. The green line in this figure describes the trend of the fluorescence signal after the $H^+$ concentration jumps. Due to the logarithmic time scale the emphasised fast decrease of the signal is shown within a satisfying resolution which allows an easy analysis of the characteristic parameters.

**Figure 21:** The same experiment as shown in Figure 20. The normalisation and reduction of the fluorescence data was done by the redlog02.exe software. The green line represents the fit of the signal data with three exponentials.
Figure 22 shows two results achieved at a buffer pH of about 7.0 in the E₁ state of the SR Ca-ATPase. The used electrolyte contained 100 mM KCl. The styryl dye concentration was 400 nM and 18 µg/ml SR Ca-ATPase was applied. 50 µM BAPTA and additional CaCl₂ were given into the electrolyte to achieve a free Ca²⁺ ion concentration of 20 µM. The CaCl₂ amount was calculated with the WinMaxC software. In the experiment with cg H⁺ 300 µM of the MNPS.Na were added to the solution. The black curve represents the signal of the measurement with SR Ca-ATPase and cg H⁺, whereas the red signal shows the result without cg H⁺. On the x axis the elapsed time since the automated trigger pulse is plotted with a logarithmic scale to allow the detailed overview on numerous magnitudes. Normalised amplitudes of the fluorescence signal are represented by the scale on the y axis. To determine the kinetics of this partial reaction a sum of exponential functions was used according to Equation 8 (page 38). The signals in the absence of cg H⁺ were fitted by one and the experiments with cg H⁺ were fitted by three exponential functions.

The numerical analysis of each sample of data was executed by the Software FigP. Fits are shown as green lines in Figure 22. This method provides values for the variables ΔF_{kin,x} and τ_{kin,x} which characterise the UV-flash induced artefact and the proton binding kinetics of the SR Ca-ATPase. Results of the H⁺ jump experiments with the SR-Ca-ATPase (black signal in Figure 22) are shown in Table 5.
The signal showed only one exponential characteristic when the experiment was done without cg H⁺ (red signal in Figure 22). A comparison of the results indicated the differences between both signals. The last four seconds of each measurement show an almost stable fluorescence level which can be described as the ending level \( F_{t \to \infty} \). In the experiment without any changes of the proton concentration (in the absence of cg H⁺) the fluorescence level reached an averaged end level \( F_{t \to \infty} = 99.3 \pm 0.1 \% \) during the last four seconds of the measurement. The fluorescence average of the last four seconds of the experiment in the presence of cg H⁺ results in an end value of \( F_{t \to \infty} = 93.7 \pm 0.1 \% \). Without cg H⁺ the fluorescence change was about 0.7 % of the starting level. In contrast, the experiments with cg H⁺ showed a much higher fluorescence change of about 6.3 % and of course the tri-exponential fit. This indicated that there was a charge movement or ion binding in the SR Ca-ATPase caused by the H⁺ concentration jump.

Table 6: Results for the SR Ca-ATPase experiment without cg H⁺.

<table>
<thead>
<tr>
<th>SR Ca-ATPase without cg H⁺</th>
<th>x = 1</th>
<th>x = 2</th>
<th>x = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time constant (( \tau_{\text{kin,} x} ))</td>
<td>2.44 ms ± 0.05 ms</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amplitude (( \Delta F_{\text{kin,} x} ))</td>
<td>-7.7 ± 0.1 %</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Starting amplitude (( F_{t=0} ))</td>
<td>107.1 ± 0.1 %</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

To study and determine quantitatively how the proton concentration affects the enzyme kinetics of the SR Ca-ATPase a couple of experiments were performed. In this series the starting pH was varied to achieve values after the H⁺ jump between pH 5.5 and pH 8.0 in steps of a tenth pH unit. Each sample was analysed to extract the characteristic time constants and the fluorescence amplitudes for the kinetics of the ATPase. Different samples within a range of 0.1 pH units were combined, and the average values were used for further analysis.
Figure 23 (A) shows the three time constants determined for the used enzyme. Although the pH was varied by almost 3.5 units, which corresponds to a factor of 3000 in the proton concentration, the time constants did not change significantly. The overall average of $\tau_{\text{kin,1}}$ (black circles) is 13.5 ms $\pm$ 3.3 ms. For $\tau_{\text{kin,2}}$ (red circles) this average value is about 167.3 ms $\pm$ 58.7 ms and for $\tau_{\text{kin,3}}$ (green circles) it is 1.93 s $\pm$ 0.52 s.

Each H$^+$ concentration jump produces a disturbance of the proton-binding equilibrium and induces a relaxation into a new equilibrium. This process resulted in a binding and/or a release of positive charge in the SR Ca-ATPase, which caused a change of the fluorescence signal. The amplitudes, $\Delta F_{\text{kin,x}}$, relating to the time constants $\tau_{\text{kin,x}}$ are shown in Figure 23 (B). $\Delta F_{\text{kin,1}}$ (black circle) and $\Delta F_{\text{kin,2}}$ (red circle) possess negative values in the whole pH range, which represents the decreasing fluorescence amplitude. A slight pH dependence for $\Delta F_{\text{kin,1}}$ and $\Delta F_{\text{kin,2}}$ can be observed by increasing amplitudes from lower to higher proton concentrations. The amplitudes of $\Delta F_{\text{kin,1}}$ are in the order of $-11.7 \pm 1.2 \%$, whereas the $\Delta F_{\text{kin,2}}$ amplitudes are around $-2.2 \pm 0.6 \%$. The slopes of each regression line is 1.3 $\pm$ 1.8 for $\Delta F_{\text{kin,1}}$ and 1.3 $\pm$ 0.9 for $\Delta F_{\text{kin,2}}$. Both regression lines have almost identical slightly slopes but in respect to the standard error of all results the slope is not significant. In contrast, $\Delta F_{\text{kin,3}}$ (green circle) showed a completely different pH dependence. The fluorescence amplitudes start with positive values at high proton concentrations which indicates an increasing fluorescence level and it decreases with rising pH. A regression line through all measurement results for $\Delta F_{\text{kin,3}}$ has a considerably slope of $-4.4 \pm 0.9$. At pH 7.37 the regression line through the data points crosses the zero level which corresponds to an absence of the third exponential function in the mathematical model. With higher pH values the fluorescence amplitude has a negative value, indicating a decreasing fluorescence signal.
3.2 Characterisation of the SR Ca-ATPase in open microsomal membrane fragments

The well-established SR Ca-ATPase preparation obtained by the method of Heilmann et al., 1977 consists of a vesicular membrane preparation in which the SR Ca-ATPases are oriented with the cytosolic domain facing outside. The purity of the enzyme is excellent and the protein shows very good enzyme activity. Such preparations were used in all experiments presented above. But the vesicular structure of the SR membranes entails a significant
problem for the desired extensive studies of the luminal binding sites of the SR Ca-ATPase. Therefore, these vesicular preparations have to be processed by an additional treatment, which produces open microsomal membrane fragments which contain the SR Ca-ATPases with preferably unaffected activity. Such an advanced preparation method is described in chapter 2.10, page 21, in all details. To compare the properties of vesicular SR Ca-ATPase preparations and SR Ca-ATPase preparations of open membrane fragments a series of tests was performed.

### 3.2.1 SDS-PAGE test of the SR Ca-ATPase

The first test of the SDS treated and untreated SR Ca-ATPase vesicles was a SDS polyacryl amide gel electrophoresis. Both samples were prepared with the sample buffer as described in the standard procedure for the SDS-PAGE (Laemmli, 1970). The same amount of these preparations and the molecular weight marker was applied in each slot of a 10 % SDS polyacryl amide gel and the electrophoresis was started with a current of about 40 mA.

An image of that gel is shown in Figure 24. Lane M contains molecular mass markers with the indicated mass in kDa. Lane A represents the untreated vesicular SR membranes with Ca-ATPases and lane B the SDS treated open membrane fragments with SR Ca-ATPases.

![Figure 24: SDS-PAGE of the untreated, vesicular (lane A) and SDS treated flat membrane (lane B) SR Ca-ATPase preparations. BMA ProSieve Marker set 15-225 kDa was applied in lane M.](Image)

No significant differences can be observed between lane A and B. The protein mass pattern shows the same allocation for the untreated and treated SR Ca-ATPase. A majority of the
proteins could be found in the region of the 100 kDa marker. From the amino acid sequence of the SR Ca-ATPase a molecular mass of 110 kDa was calculated, corresponding to these major bands in the SDS-PAGE. The other minor bands in the gel are membrane channel proteins or denatured and cleaved fractions of the SR Ca-ATPases.

3.2.2 Electron-microscopic images to approve the open membrane fragments

For the visualisation of the physical differences between the SDS treated and untreated SR Ca-ATPase preparations electron-microscopic images were taken to the EM service unit of the Biological Department at the University of Konstanz. As method for investigations on vesicular lipid structures in the EM, a negative staining preparation of the SR lipid vesicles and fragments was chosen (Degushi, et al., 1977). Samples of the SR Ca-ATPase in vesicular and open fragment SR membranes were stained by a 1 % ammonium heptamolybdate solution on a copper grid slide. After the vaporisation of the staining solution the negative-stained samples were placed in the transmission electron microscope (TEM) type ZEISS TEM 912 OMEGA. Sample images were taken at a magnification of 100,000 x.

Figure 25 shows an EM image of the vesicular SR Ca-ATPase. Globular structures of the SR lipid vesicles can be observed in this picture. All of these vesicles have a perfect spherical shape and they are in the same size range with a diameter between 150 and 200 nm.

Figure 25: TEM image of the vesicular SR Ca-ATPase preparation. Taken with a ZEISS TEM 912 OMEGA, University of Konstanz, Germany.
The SDS treated SR Ca-ATPase vesicles are shown in Figure 26. There are, in contrast, irregular structures observable and the diameter of the lipid fragments varies between 50 and 200 nm. With the ammonium heptamolybdate staining the boundaries of the lipid patches appear much more differentiated than in the vesicular preparation in Figure 25. The inner area of the fragments shows a constant staining without any evidence for the existence of a spherical structure. A few speckles in the fragment's area indicate the overlapping or an overlay of more than one flat lipid fragment structure, which occurs during the vaporisation of the staining solutions. These pictures show, that the incubation with SDS and the following dialysis process produce an open lipid membrane structure in which the SR Ca-ATPases are integrated. Both sides of the enzyme are thus directly accessible in the aqueous phase without any barrier.

Figure 26: TEM image of the SDS treated SR Ca-ATPase preparation. Irregular formed flat lipid fragments are observable with diameters between 50 and 200 nm.

3.2.3 Changes in protein concentration and enzyme activity after SDS treatment

During the incubation of the SR Ca-ATPase with SDS the protein concentration was changed in two ways. First of all, the stock concentration was diluted to obtain a protein : SDS ratio of 2.2 mg/ml : 1.9 mM. The dilution of the incubated SR Ca-ATPase was checked by determination of the protein concentration using the method of Markwell (Markwell et al., 1978). The second way, which changed the protein concentration during the preparation steps, was a dilution of the incubated protein solution by the dialysis buffer. Due to the osmotic pressure across the tube membrane, the buffer diffused into the dialysis tubes and diluted the protein solution. Dialysis over three days led to a much higher dilution, than the dialysis with
duration of just one hour. The averaged protein concentrations after the 3 days dialysis were about 0.76 mg/ml ± 0.33 mg/ml (31 preparations). In contrast, the 1h dialysis resulted in an averaged SR Ca-ATPase concentration of 1.13 mg/ml ± 0.06 mg/ml (18 preparations). Later, a few of the 3 days preparations were discarded, due to missing enzyme activities.

The enzyme activity was determined by the coupled pyruvate kinase/lactate hydrogenase test as described in chapter 2.14 and (Schwartz, et al., 1971). During each test the activity was measured with and without the Ca$^{2+}$ ionophore A23187 and the SR Ca-ATPase inhibitor TG. The activity test with A23187 showed the difference between SR Ca-ATPase in vesicular and microsomal membrane fragment structures. In SR vesicles the ATPases increased their activity by abolishing the Ca$^{2+}$ ion gradient. The addition of TG showed the residual activity produced by other ATPases and depicted the level of contamination.

The tests provided information on inactivation or denaturation of the SR Ca-ATPase caused by the SDS treatment. Therefore, the activities were determined from both, the vesicular and the open fragment preparations and the results of the particular preparation were averaged. For the SR Ca-ATPase in the vesicular preparation it was $5.74 \pm 0.73 \mu\text{mol Pi/mg protein min}$ in the absence of the ionophore A23187 and $15.45 \pm 1.80 \mu\text{mol Pi/mg protein min}$ in the presence. The latter was the maximum reachable enzyme activity for the SR Ca-ATPase since the ionophore prevented the accumulation of Ca$^{2+}$ and depletion of H$^+$ in the lumen of the vesicles, which caused a fractional inhibition of the ion pump. The enzyme activity of the SDS treated Ca-ATPase was $5.64 \pm 0.59 \mu\text{mol Pi/mg protein min}$ in the absence of A23187. But in contrast to vesicular SR Ca-ATPases the addition of the ionophore resulted in just a not significantly reduced activity of only $5.30 \pm 0.56 \mu\text{mol Pi/mg protein min}$. This may have been caused by the influence of the ionophore integrated in the SR membrane or the solvent ethanol which reduced the enzyme activities.

In Figure 27 the averaged values of the enzyme activity of the three different preparation methods are shown. Data set A represents the activities of the vesicular SR Ca-ATPase. The shown error bars represent the standard error of the mean and were determined from the averaged results of seven different preparations. SR Ca-ATPase in open microsomal membrane fragments with a 3 days dialysis is shown as data set B. The activity values are the average of 23 enzyme preparations. The third part, set C, shows the SR Ca-ATPase in open membrane fragments with a 1 h dialysis preparation. These activities are averaged values of 18 different preparations. The blank columns represent the SR Ca-ATPase activity under normal conditions. The hatched columns are the enzyme activities measured in the presence of the Ca$^{2+}$ ionophore A23187, and the solid columns show the residual ATPase activities after addition of TG. All of these results are shown in Table 6.
Table 7: The averaged enzyme activities of the SR Ca-ATPase in membrane vesicles and open membrane fragments (3 days dialysis and 1 h dialysis).

<table>
<thead>
<tr>
<th></th>
<th>Enzyme activity µmol P/mg protein min</th>
<th>Enzyme activity with ionophore A23187 µmol P/mg protein min</th>
<th>Enzyme activity with inhibitor TG µmol P/mg protein min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicular SR Ca-ATPase</td>
<td>5.74 ± 0.73</td>
<td>15.45 ± 1.80</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>Open membrane SR Ca-ATPase 3 days dialysis</td>
<td>2.85 ± 0.38</td>
<td>2.43 ± 0.35</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Open membrane SR Ca-ATPase 1 h dialysis</td>
<td>5.64 ± 0.59</td>
<td>5.30 ± 0.56</td>
<td>0.31 ± 0.11</td>
</tr>
</tbody>
</table>

For the investigation of partial and reversible inactivation of the enzymes, a SDS treated SR Ca-ATPase produced by the 1 h dialysis preparation was studied. The enzyme activities were determined to 5.51 µmol Pi/mg protein min (without A23187) and 5.00 µmol Pi/mg protein min (with A23187), which proved that the open membrane fragments were obtained (Figure 59, black line, page 98). A fraction of this enzyme preparation was transferred into a dialysis tube and dialysed in buffer with a decreased ionic strength for one
hour at + 4 °C to minimize the residual SDS concentration and reduce the polar properties of the electrolyte. The aim was to reobtain closed vesicles with integrated SR Ca-ATPases and to demonstrate the preservation of enzyme functions. A following pyruvate kinase/lactate hydrogenase test (Figure 59, red line, page 98) showed enzyme activities of 4.13 µmol Pi/mg protein min (without A23187) and 6.93 µmol Pi/mg protein min (with A23187). These results reflect two findings. First, the open membrane fragments could be reconstituted to vesicular structures, indicated by the increasing enzyme activity (+ 67.9 %) after the ionophore A23187 addition. And second, a partial reversible inactivation of the SR Ca-ATPase as consequence of the open membrane preparation step was shown, which was indicated by the reactivation and the increased enzyme activity of the regained vesicles in the presence of A23187 (+ 25.8 %). Both enhancements of the enzyme activities were observed despite the denaturation of the protein, which was provoked by the additional dialysis step and the remaining SDS concentration and led to generally lower enzyme activities. This indicates that some SR Ca-ATPases at the edge of the open membrane fragments are inactivated and could be reactivated, if the membranes rebuild closed vesicles in which these SR Ca-ATPases are fully surrounded by phospholipids again.

3.2.4 Comparison of steady-state experiments

To determine ion- and substrate-binding characteristics as quality control, both preparations, the vesicular and the open microsomal fragment SR Ca-ATPase, were tested by the laser fluorimeter in steady-state experiments. A comparison of both measurements with the vesicular SR Ca-ATPase (A) and the open microsomal SR Ca-ATPase (B) is shown in Figure 28. The values are normalised to the fluorescence level (3) of the protein solution at pH 7.2 before any addition of substrates (normalised fluorescence 1.0).
The time course of the fluorescence signal displays in both experiments a similar shape. It started with the preparation of the non-fluorescent buffer solution (step 1) and the addition of the styryl dye 2HITC (step 2). The next fluorescence level of the SR Ca-ATPase was set to 1.0 (100 %) during the normalisation of all fluorescence data (step 3). The subsequent step 4 (BAPTA addition) showed the Ca$^{2+}$ release from the binding sites and, therefore, a function of the SR Ca-ATPase. This fluorescence level represented the highest reachable level during the experiment. In step 5 CaCl$_2$ was given to the solution in a saturating concentration. The concentration of free Ca$^{2+}$ ions was calculated by WinMaxC software. In this figure all Ca$^{2+}$ saturated levels show a slightly lower fluorescence level than the SR Ca-ATPase level which was normalised to 1.0. One reason is the ion-saturated Ca$^{2+}$-bound E$_1$ state of the SR Ca-ATPase at the beginning of the experiment. The addition of ATP in step 6 showed generally different results depending on the electrolyte conditions and the ATP composition. An electrolyte with pH 7.2 and a Na$_2$ATP concentration of 500 µM induced a decreasing fluorescence level. A difference between the vesicular and the open membrane SR Ca-ATPase can be observed in Figure 28 A and B. The slight decrease of fluorescence is much smaller in the titration with the new SR Ca-ATPase preparation but it is still observable. Experiments with other ATP compositions, like pH-adjusted ATP or MgATP instead of Na$_2$ATP showed an increasing fluorescence at both enzyme preparations. The last addition of CaCl$_2$ (step 7) showed the influence of high Ca$^{2+}$ concentrations and functioned as control for the steps before. A decrease of the fluorescence to a level of 30 % to 40 % was the normal result and indicated the functioning of the protein.
For a quantitative analysis the relative fluorescence changes were determined. The relative fluorescence levels of both preparation methods are listed in Table 8.

**Table 8: The fluorescence levels of the SR Ca-ATPase in vesicular and open membrane preparations. Results of the steady-state titration in Figure 28.**

<table>
<thead>
<tr>
<th></th>
<th>Vesicular preparation % of SR Ca-ATPase level</th>
<th>Open membrane preparation % of SR Ca-ATPase level</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Buffer</td>
<td>-100 %</td>
<td>-100 %</td>
</tr>
<tr>
<td>(2) Dye</td>
<td>-80 %</td>
<td>-64 %</td>
</tr>
<tr>
<td>(3) SR-Ca-ATPase</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(4) BAPTA</td>
<td>+44 %</td>
<td>+28 %</td>
</tr>
<tr>
<td>(5) CaCl₂</td>
<td>-28 %</td>
<td>-6 %</td>
</tr>
<tr>
<td>(6) Na₂ATP</td>
<td>-39 %</td>
<td>-6 %</td>
</tr>
<tr>
<td>(7) Saturating CaCl₂</td>
<td>-68 %</td>
<td>-49 %</td>
</tr>
</tbody>
</table>

A distinct difference between both preparations is that the observation of the relative fluorescence changes of the microsomal fragment preparation are significantly smaller than those of the vesicular preparation. This observation is valid also for the absolute photomultiplier signal of all fluorescence levels (not shown). The open fragment preparation has an overall fluorescence by a factor of about $\frac{3}{4}$.

This fact of a reduced fluorescence yield with respect to the vesicular preparation was observed in all open microsomal membrane fragment preparations of the SR Ca-ATPases. But the step by step comparison of the vesicular and the open membrane fragments SR Ca-ATPase showed the same trend for each single addition.

**3.2.5 Tryptic digestion in E₁ and P-E₂ states**

To provide evidence that the enzyme transition from E₁ state to the P-E₂ state is catalysed by ATP also in the open microsomal SR Ca-ATPase a partial tryptic digestion followed by a SDS-PAGE was performed. The three different enzyme preparations were tested and compared. The first preparation consisted of an unmodified vesicular SR Ca-ATPase. The enzyme activities obtained for the three preparations of Figure 30 were shown in Table 9.
Table 9: The Enzyme activity of three SR Ca-ATPase preparations used in the tryptic digestion test.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Enzyme activity with ionophore A23187</th>
<th>Enzyme activity with inhibitor TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol P&lt;sub&gt;i&lt;/sub&gt;/mg protein min</td>
<td>μmol P&lt;sub&gt;i&lt;/sub&gt;/mg protein min</td>
<td>μmol P&lt;sub&gt;i&lt;/sub&gt;/mg protein min</td>
</tr>
<tr>
<td>Vesicular SR Ca-ATPase</td>
<td>6.61</td>
<td>19.59</td>
</tr>
<tr>
<td>Open membrane SR Ca-ATPase 1 h dialysis</td>
<td>5.26</td>
<td>4.95</td>
</tr>
<tr>
<td>Open membrane SR Ca-ATPase 3 days dialysis</td>
<td>0.29</td>
<td>0.24</td>
</tr>
</tbody>
</table>

The best conditions for the tryptic digestion were found at a concentration of 0.4 mg/ml protein with 2% trypsin in a buffer containing 25 mM MOPS, 50 mM KCl at pH 8.2. Each sample had a volume of 100 µl, with or without 1 µl of a 500 mM MgATP solution depending on the desired P-E<sub>2</sub> or E<sub>1</sub> state. Control samples were prepared without trypsin and ATP.

All samples were kept for 10 min at 37 °C in an incubator. To stop the digestion process after that time, a respective number of new tubes filled with 64 mg urea were prepared. The incubated solutions were transferred into these tubes and mixed properly before placed on ice. 10 µl of each solution were mixed with a 5 µl aliquot of the electrophoresis loading buffer and then filled into a slot of the gel in the SDS-PAGE chamber. As a reference a mixture of marker proteins with molar masses of 5-225 kDa were loaded into another slot on the gel, too. After the electrophoresis, the gel was treated by the normal developing steps of fixing, Coomassie-blue staining and destaining.

The gels were scanned and analysed with UN-SCAN-IT. In Figure 29 the undigested SR Ca-ATPases are shown. All three enzyme lanes (A, B, and C) show a major band with high intensity. The molecular weight of the enzymes forming these bands was determined to 112 kDa. This fact and the strong bands imply that the bands contain the entirely undigested SR Ca-ATPase. Minor bands between 35 kDa and 65 kDa seems to be artefacts of other SR proteins.
The gel in Figure 30 shows the tryptic digested SR Ca-ATPases in both the initial state E₁ and the P-E₂ state obtained by an ATP-induced conformation transition. The idea is that trypsin has access to different cleavage sites when the Ca-ATPase assumes different conformations during the pump cycle. And the cleavages at the accessible trypsin-specific amino acid sequences produce different digestion pattern which demonstrate the structural rearrangements of the SR Ca-ATPase on the gels. Lane A, C and E show the basic E₁ state of the three SR Ca-ATPase preparations. The according P-E₂ state of each protein preparation is shown in lane B, D, and F.
The inactive SR Ca-ATPase from the 3 days dialysis preparation in lane E and F reveal no significant differences between the E₁ and P-E₂ conformation. This result was expected, because in an inactive enzyme no conformational changes happen when adding ATP. More meaningful are the lanes of the active vesicular SR Ca-ATPase (A and B) and the open microsomal fragment SR Ca-ATPase (C and D). In both enzyme preparations similar differences were detected between the E₁ state and the P-E₂ state. In all four lanes a series of bands is present in the range between 47 kDa and 66 kDa. Differences between both states are perceptible by the intensity of two of these bands. The 52 kDa bands and the 57 kDa bands possess a much higher intensity in the P-E₂ state than in the E₁ state. Another evidence for the conformational change are the bands at 28 kDa and 33 kDa in the lanes B and D, which correspond to the phosphorylated P-E₂ state.

For a more detailed analysis, the band intensities along each lane were determined and converted into a spectrum for the further evaluation. The best way to detect discrepancies of two spectra, representing E₁ and P-E₂ states, is to generate a difference spectrum. The results of B minus A and D minus C are shown in Figure 31. (A) represents the intensity difference of the vesicular SR Ca-ATPase and (B) of the microsomal fragment enzyme. The intensity data of M, the molecular weight marker, is inserted to the bottom of the diagram for a better orientation. Their intensity values are shown in arbitrary units. To detect significant discrepancies in the difference-spectra, the zero line is shown (green line). The overall
average and the standard deviation of both difference-spectra were calculated, and the standard deviation of this average is a good marker for the significance of the divergence in the cleavage pattern between E₁ and P-E₂ state. The dashed green lines represent the band of the standard deviation.

Outstanding significance of band differences can be observed at about 52 kDa and 57 kDa, what could be assumed from the gel images, too. A less significant, but still distinguished difference of the E₁ and P-E₂ state can be detected by the bands at 22 kDa and 33 kDa and in case of the microsomal fragment preparation also at the band of 28 kDa.

Figure 31: Difference spectra from the SDS gels of the P-E₂ and the E₁ state of the (A) vesicular SR Ca-ATPase and (B) open microsomal SR Ca-ATPase. The solid green line represents the zero line and the two dashed green lines are the ± standard deviation marker of an overall spectra average. The red spectrum represents the molecular weight marker intensity, which is inserted and shifted down in arbitrary intensity units scale.
The principally comparable traces of the intensity differences between both preparations, the vesicular SR Ca-ATPase and the microsomal fragment enzyme, indicate that the SDS treatment that leads to the open microsomal membrane fragments preparation does not inhibit the transition into a corresponding P-E₂ state.

3.3 Ion-binding kinetics of the SR Ca-ATPase in open membrane fragments

The new introduced preparation method for the SR Ca-ATPase allows free access to both sides (luminal and cytosolic) of the ATPase. With the flat open membrane fragments we are now able to observe the SR Ca-ATPase ion binding properties without additional Ca^{2+}-ionophore A23187 in the E₁ and also the P-E₂ state of the enzyme. Standard titration experiments with Mg^{2+}, Ca^{2+} and H⁺ ions were performed using these preparations. In these fluorescence experiments the electrochromic styryl dye 2HITC at a concentration of 200 nM was used as indicator to reveal the ion binding in the SR Ca-ATPase ion binding sites. Kinetics of the SR Ca-ATPase was determined at high time-resolutions in the millisecond range by using the caged compounds NPE-ATP and MNPS.Na. By the addition of Mg^{2+} ions and ATP to the enzyme, the SR Ca-ATPases could follow the Post-Albers cycle scheme and the conformational changes from E₁ state to the P-E₂ state allows the opening of the access channel on the luminal side of the membrane to the ion binding sites. In this state the Ca^{2+}/H⁺ exchange could be observed and the kinetics of that process could be determined.

3.3.1 Ca^{2+} equilibrium titration with open membrane SR Ca-ATPase

In steady-state experiments the new SR Ca-ATPase preparation with open SR membrane fragments was tested. With the Ca^{2+} titration the binding constant of this divalent ion was determined for the E₁ and the P-E₂ state of the enzyme. The electrolyte contained 25 mM MOPS, 50 mM KCl and 1 mM MgCl₂ at a pH of 7.1. For the experiment the buffer was completed with 200 nM 2HITC, 18 µg/ml of the SR Ca-ATPase in open membrane fragments and 12.5 µM of the Ca^{2+} ionophore A23187. The ionophore was given into this solution only for a better comparison with the experiments of the SR Ca-ATPase in vesicular membranes. In case of the P-E₂ state measurements, the solution contained 200 µM of Na₂ATP and 280 µM additional KOH to stabilise the pH value. 400 µM BAPTA Ca^{2+} chelator was added in the experiment to obtain a virtually Ca^{2+} free state of the ATPase. Starting with these conditions the Ca^{2+} titration began by adding CaCl₂ step by step. Each CaCl₂ step was calculated by the WinMaxC software for the determination of free Ca^{2+} ions in respect to the other agents in the electrolyte. The concentration range of the calcium titrations comprised 1 nM up to 30 mM.
In Figure 32 both titrations of the SR Ca-ATPase in open membrane fragments are shown. The fits of the concentration dependence represent allosteric Hill kinetics as described in Equation 7 on page 37. The half saturating concentration $c_{50}$ of the Ca$^{2+}$ ions was determined to be 133 nM ± 8 nM in the $E_1$ state of the enzyme and to 411 nM ± 53 nM in the P-E$_2$ state. The loss of fluorescence signal $\Delta F$ was 15.3 % and 8.8 %, respectively, and the corresponding Hill coefficients $P_{Hill}$ were 1.5 ± 0.1 and 1.5 ± 0.3 at each state of the enzyme. The Hill fit ends at a Ca$^{2+}$ concentration of 10 µM whereas the titration continued. At higher calcium concentrations from 10 µM to 30 mM (not shown) the fluorescence signal rapidly decreases to a level near the protein-free fluorescence signal.

Significant differences between the $E_1$ and the P-E$_2$ state of the SR Ca-ATPase in open membrane fragments can be observed with respect to the half saturating concentration and the fluorescence change during this calcium titration. The concentration of free Ca$^{2+}$ for the half saturation in P-E$_2$ state is about three times higher than in $E_1$ state. In contrast the fluorescence change between the Ca$^{2+}$ free and the Ca$^{2+}$ saturated enzyme is in state P-E$_2$ about half of the fluorescence change of that in the $E_1$ state. The Hill coefficient $P_{Hill}$ shows no significant difference and describes a characteristic of the ion binding in the enzyme binding sites. For the further results a couple of these experiments were performed at different pH values to determine the pH dependence of the characteristically parameters.
3.3.2 pH dependence of the Ca\(^{2+}\) titration in E\(_1\) and P-E\(_2\) states and the comparison to the SR Ca-ATPase in vesicles

Ca\(^{2+}\) and its counter ion H\(^+\) are competitors for the binding sites of the SR Ca-ATPase in the Post-Albers transport cycle. The influence of the proton concentration on the Ca\(^{2+}\) binding in the enzyme was determined by a series of Ca\(^{2+}\) titration experiments. Enzyme preparations of open microsomal fragments containing the SR Ca-ATPase were used in the E\(_1\) and the P-E\(_2\) state. A vesicular SR Ca-ATPase preparation was also tested in both states as a reference for the new experiments.

The electrolyte was composed of 25 mM MOPS, 50 mM KCl, 1 mM MgCl\(_2\) and 200 mM of choline chloride (ChCl). To adjust the pH to the desired values KOH was added to the solution. For a Ca\(^{2+}\) free electrolyte the chelator BAPTA was added in a concentration of 400 \(\mu\)M. The necessary CaCl\(_2\) concentrations for the titration steps were calculated with the WinMaxC Software in consideration of the electrolyte composition. The Ca\(^{2+}\) ionophore A23187 was used in the experiments with the vesicular SR Ca-ATPase. It was added in a concentration of 12.5 \(\mu\)M. For all experiments with the enzyme in the P-E\(_2\) state the addition of 200 \(\mu\)M ATP was necessary. A couple of experiments was performed with the laser fluorimeter for steady-state experiments. Each titration experiment was analysed and fitted by an allosteric Hill kinetics function according to Equation 7 on page 37 and as described in chapter 3.1.2.

For the representation of the pH dependence of the Ca\(^{2+}\) binding in the SR Ca-ATPase the pH was varied between 6.2 and 7.4. The experiments were performed at least twice at each pH value and the averages of the data were plotted against their corresponding pH values. Figure 33 shows the pH dependence of the half-saturating constant \(c_{50}\) of the SR Ca-ATPase. The first eye-catching result is the corresponding pH dependence between the SR Ca-ATPase preparation in the open microsomal membrane fragments and the preparation in closed lipid vesicles containing the Ca\(^{2+}\) ionophore A23187. Both show the same pH dependence of the half-saturating concentrations in these experiments. The enzymes in the E\(_1\) initial state (Figure 33 A) show an increased half-saturating concentration \(c_{50}\) for Ca\(^{2+}\) at low pH values which corresponds with the highest proton concentration in these measurements. With the increasing pH the half saturating concentration decreased to the minimum at pH 7.4 at the upper range of the experiment. Table 9 shows the maxima and minima results of these experiments.
Table 10: Maxima and minima of the half saturating concentration $c_{50}$ are shown in this table for the $E_1$ state of the SR Ca-ATPase in buffer solutions with pH 6.2 and pH 7.4.

<table>
<thead>
<tr>
<th>SR Ca-ATPase in open membranes</th>
<th>Maximum half saturating concentration $c_{50}$ of Ca$^{2+}$ ions at pH 6.2</th>
<th>Minimum half saturating concentration $c_{50}$ of Ca$^{2+}$ ions at pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.15 ± 1.15 µM</td>
<td>0.33 ± 0.07 µM</td>
</tr>
<tr>
<td>SR Ca-ATPase in membrane vesicles</td>
<td>1.01 ± 0.21 µM</td>
<td>0.21 ± 0.01 µM</td>
</tr>
</tbody>
</table>

Figure 33: pH dependence of half saturating constant $c_{50}$. In the (A) $E_1$ and the (B) $P$-$E_2$ state of the SR Ca-ATPase. The black circles represent the enzymes in open microsomal membrane fragments and the red circles show the values of SR Ca-ATPases in vesicular membranes. Each result is the average of at least two experiments.
In the P-E₂ state the SR Ca-ATPase shows a contrariwise pH dependence trend (Figure 33 B). A minimum of the half saturating concentration is observable at pH 6.4. Starting at this point with decreasing and increasing pH the concentration \( c_{50} \) increased constantly. The results of both preparations, the open membranes and the vesicular membranes, correspond to each other, indicating that there is no significant difference between both enzyme preparations.

Table 11 shows the values of the minima and maxima of the half saturating concentrations \( c_{50} \) determined in the experiments for pH dependence of Ca\(^2+\) ion binding. The results demonstrate the consistence of the SR Ca-ATPase preparations and their characteristics. All values concur within the standard deviation of their averages.

Table 11: Maxima and minima of the half saturating concentration \( c_{50} \) of Ca\(^2+\) ions are shown in this table for the P-E₂ state of the SR Ca-ATPase in buffer solutions with pH 6.2, pH 6.4 and pH 7.4.

<table>
<thead>
<tr>
<th></th>
<th>Half saturating concentration ( c_{50} ) of Ca(^2+) ions at pH 6.2</th>
<th>Half saturating concentration ( c_{50} ) of Ca(^2+) ions at pH 6.4</th>
<th>Half saturating concentration ( c_{50} ) of Ca(^2+) ions at pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Ca-ATPase in open membranes</td>
<td>1.08 ± 0.38 µM</td>
<td>0.58 ± 0.24 µM</td>
<td>2.37 ± 0.61 µM</td>
</tr>
<tr>
<td>SR Ca-ATPase in membrane vesicles</td>
<td>1.06 ± 0.13 µM</td>
<td>0.75 ± 0.12 µM</td>
<td>1.89 ± 0.64 µM</td>
</tr>
</tbody>
</table>

A second characteristic parameter of the Hill kinetics function is the Hill coefficient \( P_{\text{Hill}} \) which describes the distinctive slope of the transition from the unsaturated enzyme to the saturated enzyme. From all experiments the Hill coefficients were presented in Figure 34. The black data points display the pH dependence of the Hill coefficients of SR Ca-ATPases in the new open microsomal membrane preparation and the red data points were Hill coefficients of the enzymes in vesicular SR membranes. In the E₁ state of the Post-Albers cycle the Hill coefficients (Figure 34 A) show no agreement in the lower pH range (pH 6.2 – 6.8). Above pH 6.4 at least the trends of the pH dependence of the Hill coefficients were similar, with decreasing coefficients at higher pH. Above pH 7.2 the coefficients were close for both preparations in open and vesicular membranes. A subset of results is shown in Table 12.

In contrast, the pH dependence of the Hill coefficients in the P-E₂ state of the SR Ca-ATPases (Figure 34 B) shows no significant difference between both preparations. Almost constant Hill coefficients were observed over the whole pH range of the experiments, which indicates an independence from the electrolyte pH. The overall average of all Hill coefficients calculated for the open membrane preparations was 0.8 ± 0.1 and for the vesicular membrane preparations it was 0.8 ± 0.1. These data indicate the similarity of both preparations.
Table 12: pH dependence of the Hill coefficients $P_{\text{Hill}}$ for the $\text{Ca}^{2+}$ titration experiments with the Ca-ATPase in its $E_1$ state. The buffer solutions were equilibrated to pH 6.2, pH 6.4 and pH 7.4. In the $P-E_2$ state pH independent Hill coefficients were observed, which are not shown in this table.

<table>
<thead>
<tr>
<th></th>
<th>Hill coefficient $P_{\text{Hill}}$ of $\text{Ca}^{2+}$ ions at pH 6.2</th>
<th>Hill coefficient $P_{\text{Hill}}$ of $\text{Ca}^{2+}$ ions at pH 6.4</th>
<th>Hill coefficient $P_{\text{Hill}}$ of $\text{Ca}^{2+}$ ions at pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Ca-ATPase in open membranes</td>
<td>4.2 ± 0.6</td>
<td>2.8 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>SR Ca-ATPase in membrane vesicles</td>
<td>1.2 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

Figure 34: The pH dependence of Hill coefficients $P_{\text{Hill}}$ in the $E_1$ (A) and the $P-E_2$ (B) state conformation of the Ca-ATPase. The black circles represent the enzymes in open membrane fragments and the red circles show the values for SR Ca-ATPase in vesicular membranes. Each result is the average of at least two experiments.
A third parameter of the Hill function for the Ca$^{2+}$ ion binding in SR Ca-ATPase was the fluorescence level change $\Delta F$ between the Ca$^{2+}$ unsaturated and the saturated state of the enzyme. That parameter was determined from the Hill fits as difference of the variables $F_{\text{max}}$ and $F_{\text{min}}$, which represent the starting and the end fluorescence level of the Ca$^{2+}$ titration. The pH dependence of these results is shown in Figure 35. Part A presents the results of both enzyme preparations in the E$_1$ state and part B displays values for the enzymes in P-E$_2$ state.

Figure 35: The pH dependence of fluorescence amplitudes $\Delta F$ in the E$_1$ (A) and P-E$_2$ (B) state of the SR Ca-ATPase. The black circles represent the enzyme in open membrane fragments and the red circles show the values of SR Ca-ATPases in vesicular membranes. Each result is the average of at least two experiments.

A pH dependency of $\Delta F$ was identified between $\Delta F_{\text{pH 6.2}} = 23.0 \pm 1.3\%$ and $\Delta F_{\text{pH 7.2}} = 31.9 \pm 0.9\%$. In contrast the E$_1$ state of the SR Ca-ATPase in open membrane fragments shows in Figure 35 (A, black) a decreasing fluorescence amplitude within this pH range. It
starts at $\Delta F_{\text{pH} \ 6.2} = 24.5 \pm 1.1 \%$ and reaches $\Delta F_{\text{pH} \ 7.2} = 19.8 \pm 0.1 \%$. The disagreement between the SR Ca-ATPase in vesicular and open membrane structures may be explained by the different membrane potential. A variation of the $\text{H}^+$ concentration occurred with the pH changes. In the vesicular membrane preparations this led to a change of the membrane potential. The SR Ca-ATPases and the styryl dye molecules, integrated in the membrane, show results influenced by the changed membrane potential. In contrast enzyme preparations in open membrane fragments are exposed to the same buffer conditions with identic ionic strength on the luminal and cytoplasmic side of the SR Ca-ATPase.

The P-E$_2$ state in Figure 35 (B) shows not clearly such an increasing respectively decreasing trend for SR Ca-ATPase in vesicular and open membrane fragments. With larger standard deviations at each measurement in the P-E$_2$ state, it is difficult to proclaim the same pH dependence as in the E$_1$ state. Nevertheless, regarding the results at pH 6.2 and pH 7.4 in Figure 35 (B) the tendency of an increasing fluorescence amplitude $\Delta F$ for SR Ca-ATPases in vesicular membranes and a decreasing tendency for these in open membrane fragments could be implied, comparable to the trends in the E$_1$ state of the SR Ca-ATPase. This indicates that the enzymes in both preparations allow different accessibilities for the ions. It can be assumed that in the case of open membrane fragments, the ions have a free access to the SR Ca-ATPase from both sides.

### 3.3.3 $\text{H}^+$ titration with open membrane preparation of the SR Ca-ATPase

The binding and release of protons, as transported counter ions to the Ca$^{2+}$ ions, is an essential part of transport cycle of the SR Ca-ATPase. Therefore, the binding characteristics of H$^+$ ions were determined by equilibrium titrations. These experiments were performed at the laser fluorimeter for steady-state titrations. Starting at low pH values the H$^+$ concentration was decreased by adding NaOH to the measurement solutions. The electrolyte contained 10 mM NaCl and 200 µM MgCl$_2$ without any buffering agent, as described for the H$^+$ concentration jump experiments. The styryl dye 2HITC was used in a concentration of 400 nM with 9 µg/ml of the SDS treated SR Ca-ATPase. To bind the free Ca$^{2+}$ ions 10 µM BAPTA were applied. With the WinMaxC software the amount of CaCl$_2$ was calculated to reach a free Ca$^{2+}$ concentration of 5 µM depending on the composition of the electrolyte. For the measurements in the P-E$_2$ state 200 µM of Na$_2$ATP were added into this cuvette. The titrations started at a pH of about 6.5 with the additions of a 10 mM NaOH solution. Step by step the NaOH was added and the pH was increased. Respectively, the H$^+$ concentration decreased at each step up to a pH value of about 7.8.

The results of the titration were fitted by an increasing allosteric Hill function as shown in Equation 9 with the characteristically parameter $F_{\text{min}}$, $F_{\text{max}}$, pH$_{50}$ and $P_{\text{Hill}}$. 

- 63 -
Equation 9:

\[
\frac{F}{F_0} = F_{\text{min}} + \frac{(F_{\text{max}} - F_{\text{min}})}{\left(1 + \left(\frac{10^{-pH}}{10^{-pH_{50}}}\right)^{-PH_{Hill}}\right)}
\]

Figure 36 represents both titrations with their fits. The half saturating pH\textsubscript{50} was determined to pH 6.7 ± 0.1 for the E\textsubscript{1} state and pH 7.0 ± 0.1 in the P-E\textsubscript{2} state of the SR Ca-ATPase which shows a significant difference between both states. The Hill coefficient for both states of the ATPase was almost the same in values of 3.0 ± 0.4 in the E\textsubscript{1} state and 3.1 ± 0.5 in the P-E\textsubscript{2} state. With the change in the proton concentration the fluorescence level changed from the ion-saturated state to a higher fluorescence level with fewer ions. In the E\textsubscript{1} state of the SR Ca-ATPase the fluorescence change Δ\textit{F} was about 21.9 % and in the P-E\textsubscript{2} state it was about 18.4 %. The main difference between both states is represented by the half saturating pH\textsubscript{50}. All the other constants of the Hill fit in E\textsubscript{1} and P-E\textsubscript{2} are in the similar range.

Figure 36: \textit{H}\textsuperscript{+} titration of the SDS treated SR Ca-ATPase in open membrane fragments. The titration was performed in both enzyme conformations E\textsubscript{1} (black) and P-E\textsubscript{2} (red) of the SR Ca-ATPase.

To calculate the proton concentration the definition of the pH value was used. This definition indicates that the pH can be calculated as a logarithmic function of the known concentration of proton donor and vice versa. The function is shown in Equation 10 in its form for the calculation with the proton concentration parameter.
Equation 10:

\[ pH = -\log_{10} (A) = -\log_{10} (c_H) \]

\[ \rightarrow c_H = 10^{-pH} \]

For the determination of the H\(^+\) half saturation of the SR Ca-ATPase the pH\(_{50}\) of the E\(_1\) and P-E\(_2\) state was taken. The calculation resulted in a H\(^+\) concentration of 191 nM ± 9 nM for the E\(_1\) state and 89 nM ± 5 nM in the P-E\(_2\) state. This shows that the binding affinity for protons is about two times higher in the P-E\(_2\) state than in the E\(_1\) state of the SR Ca-ATPase.

### 3.3.4 H\(^+\) jump experiments with SR Ca-ATPase in open microsomal membranes in E\(_1\) state

The proton binding kinetics of the SR Ca-ATPase in open microsomal fragments was determined with proton concentration jump experiments. Using \(\text{cg H}^+\), MNPS.Na, homogeneous pH jumps could be performed in the prepared sample solution triggered by the UV-flash induced photo-cleavage of that compound. During these H\(^+\) concentration jump experiments the samples were placed in the laser fluorimeter for advanced kinetics experiments and the styryl dye was used as a probe for the ion movements in the protein. For the excitation of the styryl dyes F52 a HeNe-Laser with a wavelength of 543 nm illuminated the whole sample volume for a maximum of fluorescence yield.

![Proton concentration jump experiment](image)

*Figure 37: Proton concentration jump experiment with the SR Ca-ATPase in open microsomal membrane fragments with the enzyme in its E\(_1\) conformation. The black signal was achieved with 300 \(\mu\)M \(\text{cg H}^+\). The red signal is the control experiment without \(\text{cg H}^+\). The normalisation and the time axis are in a linear manner. The green lines represent the mathematical fit of the original signals. Each signal is the average of two experiments.*
A test solution contained an electrolyte without any pH buffering agents. The ion strength was adjusted by 50 mM KCl. For the detection of the electrogenic ion movements in the SR Ca-ATPase the styryl dye F52 was added to the solution in a concentration of 800 nM. The concentration of the open membrane preparation of the SR Ca-ATPase was 36 µg/ml. For the experiment in the E1 state the residual Ca$^{2+}$ ion concentration was used without any additional CaCl$_2$ or Ca$^{2+}$ chelator BAPTA. The cg H$^+$ was applied in a concentration of 300 µM determined to produce the best H$^+$ concentration jump results. Starting with these conditions, 300 µl of the sample solution were filled into the cuvette and placed in the measuring chamber of the fluorimeter. During the equilibration of the temperature at 20 °C the sample was covered by a light protection cap to prevent the dye from bleaching and the photolysis of the cg H$^+$. That non-illuminated signal was defined as level zero. The cap was removed shortly before the measurements started. With the automated measuring procedure the high-energy UV-flash was triggered and the recorded fluorescence data were stored for the further analysis in a file on the PC computer system with a sampling rate of 100 kHz. The analysis started with the normalisation of the fluorescence data to allow the comparison of all experiments. For this normalisation the range between the detected level zero and the averaged fluorescence level before the UV-flash was set as 1.0 of normalised fluorescence. All fluorescence changes during the proton concentration jumps were shown in the diagrams with respect to the starting level. The normalisation was done by the redlog02.exe software which normalises the fluorescence signals and reduces the data by the boxcar method in a logarithmic manner for a better visualisation of the millisecond range (as described in chapter 2.11.3, page 28). In a first experiment the reaction on proton concentration jump was determined by measuring the fluorescence signal after UV-flash with and without the cg H$^+$ in the sample solution. Figure 37 shows the linearly normalised experiments. The time range was reduced to 300 ms to visualise the rapid decrease of the fluorescence signal. Information about the trend following the first 300 ms were lost. Both experiments were shown in Figure 38 with the logarithmic normalisation and the full time range of 10 s.
Dissertation Andreas Fibich

The black signal represents the ion movement in the SR Ca-ATPase in open membrane fragments following the rapidly increasing proton concentration at a pH of 6.8. As a control a similar solution was used but without the cg H⁺ (red signal). Both signals are the averages of two experiments. During the first 10 ms the fluorescence level quickly decreased in both experiments. This indicated that the first fast decrease in fluorescence level belongs to the UV-flash artefact. Without cg H⁺ the signal could be fitted by a bi-exponential function according to Equation 6 on page 32. The kinetics parameters τ_{kin,x} and ΔF_{kin,x} only correspond to the fast decreasing part of the fluorescence signal (Table 13). The result of the first time constant τ_{kin,1} was 4.13 ms ± 0.10 ms with a high fluorescence loss of -7.1 ± 0.1 % and the second exponential function was composed of a larger time constant of 41.8 ms ± 5.4 ms with minor decreasing fluorescence of -1.3 ± 0.1 %. At times longer than 100 ms the signal shows no significant reactions of the SR Ca-ATPase. At the end of that experiment the fluorescence level for the steady-state was determined to 98.5 ± 0.1 %. That fluorescence change of 1.5 % represents the bleaching of the styryl dye caused by the 6 MW UV-flash.

Figure 38: The same data as shown in Figure 37 with logarithmic normalisation and time axis. More characteristic details are observable in the black signal from the millisecond range up to 10 seconds. Each green line represents the suitable fit of the signals.
Table 13: Results of the control experiments without cg H⁺. The characteristic time constants and fluorescence amplitudes of the bi-exponential fit for the SR Ca-ATPase signals in open microsomal fragments are shown.

<table>
<thead>
<tr>
<th>Control experiment SR Ca-ATPase without cg H⁺</th>
<th>x = 1</th>
<th>x = 2</th>
<th>x = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time constant ( \tau_{\text{kin},x} )</td>
<td>4.13 ms ± 0.10 ms</td>
<td>41.8 ms ± 5.4 ms</td>
<td>-</td>
</tr>
<tr>
<td>Amplitude ( \Delta F_{\text{kin},x} )</td>
<td>-7.1 ± 0.1 %</td>
<td>-1.3 ± 0.1 %</td>
<td>-</td>
</tr>
<tr>
<td>Starting fluorescence level ( F_{t=0} )</td>
<td>106.9 ± 0.1 %</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The experiment with cg H⁺ (Figure 38, black curve), can be described by an equation with three exponential functions as presented in Equation 8 (page 38) in chapter 3.1.3. The first one is assigned to the UV-flash artefact and contains a fast decrease in the range of a few milliseconds as seen before in the control experiment (control without cg H⁺ \( \tau_{\text{kin},1} = 4.13 \text{ ms ± 0.10 ms} \) and with cg H⁺ \( \tau_{\text{kin},1} = 5.10 \text{ ms ± 0.06 ms} \)). After that decay a slightly increasing part of the signal followed between 30 ms and 350 ms. This second, increasing part of the signal appears only in the presence of cg H⁺, but not in the cg H⁺-free control experiment described above. A third part of the signal shows also a slightly decreasing exponential function with a time constant in the order of one second. The most outstanding difference between both measurements was the steady-state fluorescence level which was reached after the proton concentration jumps. For the cg H⁺ experiment that level was 93.3 ± 0.1 %. These results in the fluorescence change of 6.7 % were in contrast to the experiment without cg H⁺ with 1.5 %. A higher fluorescence change of 5.2 % indicates the binding of positive charges (protons) in the enzyme. The observed kinetics functions show the characteristics of the conformation relaxation of the SR Ca-ATPase. Time constant \( \tau_{\text{kin},2} \) is the first characteristic related to the protein function. The data for all function parameters are shown in Table 14. A detailed analysis of the kinetic fluorescence changes showed that \( \Delta F_{\text{kin},2} \) and \( \Delta F_{\text{kin},3} \) possess the significantly similar absolute value of about 0.7 % and 0.9 %, with an increasing trend for \( \Delta F_{\text{kin},2} \) and a decreasing trend for \( \Delta F_{\text{kin},3} \).
Table 14: A kinetics experiment with cg $H^+$ MNPS.Na. Values of the characteristic time constants and fluorescence amplitudes of the tri-exponential fit for the SR Ca-ATPase signals in open microsomal fragments are shown.

<table>
<thead>
<tr>
<th>SR Ca-ATPase with cg $H^+$</th>
<th>$x = 1$</th>
<th>$x = 2$</th>
<th>$x = 3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time constant $\tau_{\text{kin},x}$</td>
<td>5.11 ms ± 0.06 ms</td>
<td>221.8 ms ± 97.9 ms</td>
<td>1.37 s ± 0.56 s</td>
</tr>
<tr>
<td>Amplitude $\Delta F_{\text{kin},x}$</td>
<td>-9.3 ± 0.1 %</td>
<td>-0.7 ± 0.3 %</td>
<td>-0.9 ± 0.2 %</td>
</tr>
<tr>
<td>Starting fluorescence level $F_{t=0}$</td>
<td>102.8 ± 0.1 %</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Excluding the signal of the UV-flash artefact, which is described by the first exponential function, the characteristic kinetics of the SR Ca-ATPase is represented by the exponential functions starting with $\tau_{\text{kin},2}$ and $\tau_{\text{kin},3}$.

3.3.5 pH dependence of the proton binding kinetics in the E$_1$ state

A series of proton concentration jump experiments was performed for the determination of the pH dependence of the proton binding kinetics. The SR Ca-ATPase in open microsomal membrane fragments was tested in the E$_1$ state as well as the P-E$_2$ state of the enzyme. To reach the P-E$_2$ state in the Post-Albers cycle the sample solution with the SR Ca-ATPase contained ATP, MgCl$_2$ and CaCl$_2$. The electrolyte in these experiments contained 50 mM KCl, 1 mM MgCl$_2$, but no pH buffering agent. 800 nM of the electrochromic styryl dye F52 was used for the observation of the ion movements in the enzyme and a concentration of 36 $\mu$g/ml of the SR Ca-ATPase was adjusted in the solution. The CaCl$_2$ concentration was 20 $\mu$M and the sample solutions for the P-E$_2$ measurements contained 400 $\mu$M Na$_2$ATP. To generate the proton concentration jump 300 $\mu$M of the cg $H^+$ MNPS.Na were added into the sample solution. The kinetics measurements with high time-resolution were performed in the UV-laser fluorimeter which was described in chapter 2.11.3. Temperature of all samples was adjusted to 20 °C by the thermostated measuring chamber. A trigger signal released the 351 nm UV flash, started data acquisition, and the achieved fluorescence signal data were normalised and reduced by the redlog02.exe software. Following the normalisation and data reduction the results were analysed with the FigP Software. The enzyme kinetics was determined by numerically fits (Marquardt, 1963) of the signals with a couple of exponential functions.

To determine the pH dependence of the proton binding kinetics in the E$_1$ state of the SR Ca-ATPase in open membranes, H$^+$ concentration jump experiments were performed between pH 6.6 and pH 8.3. The measurements were performed several times at the same pH value for...
an improved analysis of the signals. All corresponding results of the same pH step were
normalised, the data were reduced and the signals were averaged to improve the signal-to-
noise ratio. Each analysed signal was an average of at least 2 measurements up to 8
measurements in this series of experiments. These signals were fitted and analysed by
typically a four exponential function as described in Equation 11, whereas the signal at pH 7.4
could just be fitted with a function without the term (b) of Equation 11 (highlighted in green).
The achieved values of this signal could be assigned to the time constants $\tau_{\text{kin,1}}$, $\tau_{\text{kin,2}}$ and $\tau_{\text{kin,4}}$
and fluorescence amplitudes $\Delta F_{\text{kin,1}}$, $\Delta F_{\text{kin,2}}$, and $\Delta F_{\text{kin,4}}$. A similar result was observed at
pH 8.3 where the signal contained just three exponential functions excluding the term (a) in
Equation 11 (highlighted in red). In that case the determined values were assigned to their
respective time constants $\tau_{\text{kin,x}}$ and fluorescence amplitudes $\Delta F_{\text{kin,x}}$ with $x = 1, 3$ and 4.

Equation 11:

$$F = \Delta F_{\text{kin,1}} \cdot \left(1 - e^{\frac{-t}{\tau_{\text{kin,1}}}}\right) + \Delta F_{\text{kin,2}} \cdot \left(1 - e^{\frac{-t}{\tau_{\text{kin,2}}}}\right)^{(a)} + \Delta F_{\text{kin,3}} \cdot \left(1 - e^{\frac{-t}{\tau_{\text{kin,3}}}}\right)^{(b)} + \Delta F_{\text{kin,4}} \cdot \left(1 - e^{\frac{-t}{\tau_{\text{kin,4}}}}\right) + F_{t=0}$$

With these results the pH dependence of proton binding of the SR Ca-ATPase in the $E_1$
state was determined. Figure 39 shows the time constants of the binding process depending on the
pH in the buffer solution. The time constant $\tau_{\text{kin,1}}$ (black) shows an almost pH independent
trend. The average of these values in the whole pH range was 0.80 ms ± 0.07 ms. Regression
lines of the following time constants $\tau_{\text{kin,2}}$ (red) and $\tau_{\text{kin,3}}$ (green) show an decreasing trend
with the increasing pH values. Difficulties in the numerical determination of the characteristic
parameter at higher pH, led to missing results at these pH values. The highest time constant of
$\tau_{\text{kin,2}}$ was found at pH 6.6 with 9.35 ms ± 0.51 ms and the lowest one at pH 7.8 with 1.61 ms
± 0.14 ms. At pH 8.3 the fit of the signal contained just three exponential functions. The
comparison of the time and fluorescence values allowed the classification of these values as
$\tau_{\text{kin,1}}$, $\tau_{\text{kin,3}}$ and $\tau_{\text{kin,4}}$, and their respective fluorescence amplitudes. For the third time constant
$\tau_{\text{kin,3}}$ a high value was found at pH 6.6 whereas a second maximum was determined at pH 7.6.
A highest characteristic value with 166.5 ms ± 63.5 ms was found at pH 6.6. At pH 7.6 an
erratic value was observed with 189.4 ms ± 55.9 ms which differ significantly from the
regression line calculated for all data points. At pH 7.4 the fit function again consists of only
three exponential terms. A comparison of the determined values led to the classification of these values to be the time constants $\tau_{\text{kin,1}}$, $\tau_{\text{kin,2}}$ and $\tau_{\text{kin,4}}$, and their fluorescence amplitudes.
A pH dependence of the decreasing time constant $\tau_{\text{kin,3}}$ could be observed in these experiments. With the decreasing proton concentration at higher pH the ion binding process
seems to be expedited. Where at pH 6.6 a time constant $\tau_{\text{kin},3}$ of $166.5 \text{ ms} \pm 63.5 \text{ ms}$ was observed, the time constant at pH 8.3 was only $12.2 \text{ ms} \pm 0.9 \text{ ms}$. That indicates a faster $\text{Ca}^{2+}/\text{H}^+$ exchange in the absence of protons.

![Graph showing pH dependence of characteristic time constants](image)

*Figure 39: The pH dependence of the characteristic time constants $\tau_{\text{kin}, x}$ determined with the SR Ca-ATPase preparation in open membrane fragments in the $E_1$ state of the enzyme. The lines represent the regression lines through the data points.*

In contrast, the regression line of the fourth time constant $\tau_{\text{kin},4}$ (cyan) shows an increasing trend with increasing pH values. The minimum of the time constant $\tau_{\text{kin},4}$ was observed at pH 7.0 with $0.77 \text{ s} \pm 0.23 \text{ s}$. With the increasing pH $\tau_{\text{kin},4}$ reaches $3.98 \text{ s} \pm 0.88 \text{ s}$ at pH 8.3. An outlier was observed at pH 7.6 with $4.26 \text{ s} \pm 1.31 \text{ s}$. But this outlier is within the range of the standard error of $\tau_{\text{kin},4}$ at pH 8.3 and consequently not significant, whereas the whole increasing trend of the time constant is a significant characteristic. The underlying process became slower with increasing pH.

The corresponding fluorescence amplitude changes $\Delta F_{\text{kin},1}$ (black), $\Delta F_{\text{kin},2}$ (red), $\Delta F_{\text{kin},3}$ (green) and $\Delta F_{\text{kin},4}$ (cyan) of the analysed kinetics functions in the $E_1$ state are shown in Figure 40. $\Delta F_{\text{kin},1}$ and $\Delta F_{\text{kin},2}$ have negative values all over the tested pH range. This indicates the decreasing fluorescence level of the kinetics signal, which describes an ion binding process of the SR Ca-ATPase. The regression lines of the amplitude changes $\Delta F_{\text{kin},1}$ and $\Delta F_{\text{kin},2}$ show a positive slope between pH 6.6 and pH 8.3 that represent the decreasing amplitude changes at higher pH values. In contrast, the results of the fourth amplitude $\Delta F_{\text{kin},4}$ show just minor but also negative values with a negative slope of the regression line at increasing pH. An eye-catching result of these experiments represents the fluorescence amplitude changes of the third exponential function $\Delta F_{\text{kin},3}$. This is the only exponential
function which reports an increasing fluorescence level below pH 7.4 and, therefore, an
electrogenic ion release at lower pH. The positive values of $\Delta F_{\text{kin,3}}$ decreased with the
increasing pH and have their zero point at pH 7.4. With the further increasing pH up to pH 8.3
the amplitude change $\Delta F_{\text{kin,3}}$ shows negative values. As described for the time constants
the determination of the corresponding amplitude changes was not possible at all pH conditions.
Crossing the zero line of $\Delta F_{\text{kin,3}}$ at pH 7.4 and the indeterminable term of $\Delta F_{\text{kin,2}}$ at pH 8.3
were the gaps in the pH-dependence experiments of the SR Ca-ATPase in E$_1$ state. A
common result for all fluorescence amplitudes is the decrease of the amplitude changes
following the proton concentration jump at higher pH values.

![Image](image_url)

**Figure 40:** The pH dependence of the characteristic fluorescence amplitude changes $\Delta F_{\text{kin,x}}$ determined with the SR Ca-ATPase preparation in open membrane fragments in the E$_1$ state of the enzyme. The lines represent the regression lines through the data points.

### 3.3.6 pH dependence of the proton binding kinetics in the P-E$_2$ state

With the same conditions of the electrolyte and setup of the experiments as described in
chapter 3.1.3, page 37, the pH dependence of the proton-binding kinetics of the SR Ca-
ATPase in the P-E$_2$ state was determined. The experiments in the P-E$_2$ state of the SR Ca-
ATPase in open membrane fragments were performed in the range between pH 6.6 and
pH 8.5. For the transfer of the proteins from the E$_1$ to the P-E$_2$ state 500 µM MgATP were
added to the sample solution. The pH of the electrolyte was adjusted by the addition of KOH
and each pH step was measured at least two times to average the achieved signals. In Figure
41 three averaged signals and their fits with four exponential functions is presented. The fit
function was the four exponential kinetics equation as presented in Equation 11, page 70.
In both measurements shown in Figure 41 the first rapid decreases of the fluorescence signal belong to the UV laser flash artefact and minor structural rearrangements of the SR Ca-ATPase. After 25 ms a slightly increase was observed at lower pH (black signal in Figure 41) followed by a slight decay to the end fluorescence level at $t \to \infty$. The increasing and decreasing parts of the signals show a pH dependence as could be observed in the comparison of both signals at pH 6.9 and pH 7.9.

![Figure 41: Two exemplary proton concentration jump experiments (black and red signals) and a control experiment (green) with the SR Ca-ATPase in open membrane fragments in the P-E2 conformation of the enzyme and their fits (green lines). The black signal was obtained at pH 6.9 and the red signal at pH 7.9. Both signals are the average of two measurements. The green signal represents the control experiment without $c_g H^+$ (average of three measurements).](image)

For the determination of the pH dependence of proton binding kinetics all experiments from this series were analysed, averaged and the characteristic time constants $\tau_{\text{kin},x}$ and the fluorescence change values $\Delta F_{\text{kin},x}$ were plotted in a graph. All results were averages of at least two experiments. Figure 42 displays the time constants $\tau_{\text{kin},x}$ obtained from the measurements. $\tau_{\text{kin},1}$ (black) and $\tau_{\text{kin},2}$ (red) show constant values all over the pH range between pH 6.6 and pH 8.5. For the parameter $\tau_{\text{kin},1}$ an average of 1.25 ms $\pm$ 0.16 ms was determined and the time constant $\tau_{\text{kin},2}$ showed an average of 9.42 ms $\pm$ 0.80 ms. The standard deviation of all results indicated their consistency. So these time constants represent the part of the UV laser flash artefact.
Dissertation Andreas Fibich

For the $\tau_{\text{kin},3}$ (green) and $\tau_{\text{kin},4}$ (cyan) time constants only a small trend in the pH dependence was observed. These time constants possess higher standard deviations, and their values were not as consistent as for the other two time constants. For $\tau_{\text{kin},3}$ an apparent pH effect was observed in the range around pH 7.1 where a transition from the increasing fluorescence signal to a decreasing signal took place. This results in a less accurate determination of the time constant $\tau_{\text{kin},3}$ and its fluorescence change $\Delta F_{\text{kin},3}$. The average of the $\tau_{\text{kin},3}$ time constant in the whole pH range was 208.3 ms ± 82.5 ms. For $\tau_{\text{kin},4}$ the average was determined to 3.32 s ± 0.30 s.

Figure 43 shows the fluorescence amplitude changes $\Delta F_{\text{kin},x}$. All increasing $\Delta F_{\text{kin},x}$ values own a positive sign. The negative fluorescence values represent the decreasing parts of the exponential kinetics functions. As for the time constants, each averaged fluorescence change, $\Delta F_{\text{kin},1}$ (black) and $\Delta F_{\text{kin},2}$ (red), had a good accuracy with small standard deviation. The regression lines show for both parameters an increasing characteristic in the range from pH 6.6 to pH 8.5. The increase of the negative amplitudes represents the smaller fluorescence changes at higher pH. All fluorescence amplitudes were decreasing at higher pH. The maximum amplitude of $\Delta F_{\text{kin},1}$ was determined -9.1 ± 0.5 % at pH 6.5 and the minimum amplitude was -4.6 ± 0.1 % at pH 8.3. The second amplitude $\Delta F_{\text{kin},2}$ of the exponential function had a maximum at pH 6.9 with -4.7 ± 0.2 % and a minimum at pH 8.5 with -2.8 ± 0.2 %.
Dissertation Andreas Fibich

The fluorescence decrease $\Delta F_{\text{kin},4}$ (cyan) of the fits shows an almost constant value in the pH range between pH 6.6 and pH 8.5. The average of $\Delta F_{\text{kin},4}$ was $-0.8 \pm 0.1$ % of the normalised fluorescence level. A pH dependence was observed for the fluorescence amplitude of the third exponential term in the kinetics function, $\Delta F_{\text{kin},3}$. The eye-catching transition from an increasing to a decreasing signal with rising pH. At about pH 7.1 this term disappeared which was represented in Figure 43 by the zero point of $\Delta F_{\text{kin},3}$. Following this sign exchange the fluorescence amplitude further decreased and ended at the level of $-0.8 \pm 0.1$ % at pH 8.5.

3.3.7 TG and H$^+$ concentration jump experiments in P-E$_2$ state of the open membrane SR Ca-ATPase

Thapsigargin is an inhibitor of the SR Ca-ATPase, which binds in a cavity (M3, M5 and M7 helices) of the luminal access channel in the P-E$_2$ state and stabilises this conformation of the protein. The H$^+$ concentration jump experiments were used to determine the effects of the inhibitor to the proton binding kinetics of the SR Ca-ATPase. In this sample solution no pH buffering agent was present to permit the pH changes. The electrolyte was composed of 50 mM KCl, 800 nM of the styryl dye F52 and 10 $\mu$M CaCl$_2$. This solution contained 36 $\mu$g/ml of the SR Ca-ATPase in open microsomal SR membranes, and 500 $\mu$M Mg-ATP were added to reach the P-E$_2$ state. With 300 $\mu$M of the cg H$^+$ and 1 $\mu$M TG the sample solution was completed.

Figure 43: pH dependence of the fluorescence amplitude changes $\Delta F_{\text{kin},x}$ corresponding to the time constants shown in Figure 42. SR Ca-ATPase preparation in open membrane fragments in the P-E$_2$ state. The lines represent the parabolic regression through the data points.
The kinetics experiments were performed in the laser fluorimeter for high time-resolution measurements. The experiments were performed at pH 6.4, 7.2, 7.4 and 7.9 to analyse the pH dependence of the kinetics of the proton binding in the presence of TG.

For the sake of clarity only the (perfect) fits to the data at all pH values are shown in Figure 44. For a better visualisation the time axis is shown in a logarithmic scale. Each fit represents the average of several individual experiments, as noted by the numbers in brackets in Figure 44. The representation of the data by a sum of four exponentials is analogous to the experiments without the inhibitor TG. A first rapidly decreasing part of the fluorescence signal is characterised by two exponential functions. This part is followed by the third exponential with increasing or decreasing amplitude, dependent on pH, and a fourth decreasing exponential. The time constants $\tau_{\text{kin},x}$ and the fluorescence amplitude changes $\Delta F_{\text{kin},x}$ were determined by these fits to the data.

Figure 44: Fits of the averaged fluorescence results from proton concentration jump experiments at various pH. In brackets the number of individual measurements is shown. The SR Ca-ATPase was in the P-E$_2$ state and inhibited by 1 µM TG.
The results are presented in Figure 45 and Figure 46 which show the pH dependence of the time constants and the fluorescence changes. Data points and regression lines for all time constants show no significant pH dependence, and the comparison of the overall averages of the time constants $\tau_{\text{kin},x}$ with that of the previously performed experiments without TG shows a significant congruence, see also Table 15.

**Figure 45:** pH dependence of the time constants $\tau_{\text{kin},x}$ determined with the SR Ca-ATPase in open microsomal membrane fragments in the P-E$_2$ state with 1 µM TG. The lines represent the regression lines through the data points.

**Table 15:** Comparison of the time constants $\tau_{\text{kin},x}$ determined for the SR Ca-ATPase in open membrane fragments in P-E$_2$ and P-E$_2$-TG inhibited states.

<table>
<thead>
<tr>
<th></th>
<th>$\tau_{\text{kin}, 1}$ ms</th>
<th>$\tau_{\text{kin}, 2}$ ms</th>
<th>$\tau_{\text{kin}, 3}$ ms</th>
<th>$\tau_{\text{kin}, 4}$ s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without TG</td>
<td>1.25 ± 0.16</td>
<td>9.42 ± 0.80</td>
<td>208.3 ± 82.5</td>
<td>3.23 ± 0.30</td>
</tr>
<tr>
<td>With 1µM TG</td>
<td>0.98 ± 0.16</td>
<td>9.93 ± 2.37</td>
<td>227.9 ± 46.9</td>
<td>3.30 ± 0.76</td>
</tr>
</tbody>
</table>
The fluorescence amplitudes $\Delta F_{\text{kin},x}$ are negative except for the fluorescence amplitude $\Delta F_{\text{kin},3}$ (green) at pH 6.4, where $\Delta F_{\text{kin},3}$ indicates an increasing fluorescence amplitude. At pH $> 7.1$ the fluorescence amplitude change of $\Delta F_{\text{kin},3}$ describes a decreasing exponential function for the fluorescence signal. The amplitude $\Delta F_{\text{kin},4}$ (cyan) shows no significant pH dependence. The average of the $\Delta F_{\text{kin},4}$ data was determined to be $-1.0 \pm 0.1\%$ over the pH range of the experiments. The fluorescence amplitude changes $\Delta F_{\text{kin},1}$ (black) and $\Delta F_{\text{kin},2}$ (red) decreased with the increasing pH. The largest fluorescence change for $\Delta F_{\text{kin},1}$ was observed at pH 6.4 with $-6.3 \pm 0.1\%$ and the lowest change was at pH 7.8 with $-5.0 \pm 0.1\%$ except for the outlier at pH 7.2. $\Delta F_{\text{kin},2}$ shows a similar trend with $-4.0 \pm 0.1\%$ at pH 6.4 and $-1.4 \pm 0.1\%$ at pH 7.8. In summary, for all four $\Delta F_{\text{kin},x}$ a behaviour was found comparable to that in the experiments without TG (Figure 43, page 75).

3.3.8 Activation energy of the rate-limiting reaction steps for proton binding in P-E$_2$ state of the open membrane SR Ca-ATPase

The activation energy $E_A$ of the rate-limiting reaction steps for proton binding was determined for the SR Ca-ATPase in the P-E$_2$ state in open microsomal membrane fragments. For these investigations the Arrhenius equation was used to determine the activation energies of the
participating reaction steps related to luminal H\(^{+}\) binding. A series of experiments was started to get the necessary kinetics information at temperatures of 10 °C, 20 °C and 30 °C. With these data the evaluation of ion binding and the rearrangement of the enzyme structure of the observed processes was possible.

The electrolyte for these proton-concentration jump experiments contained 50 mM of KCl and 1 mM of MgCl\(_2\). KOH was used to adjust the pH to pH 6.6. For the detection of ion movements in the SR Ca-ATPase the styryl dye F52 was applied with a concentration of 800 nM. 36 µg/ml of the enzyme were added to the electrolyte, and the sample solution contained 20 µM of Ca\(^{2+}\) ions. To reach the P-E\(_2\) state of the SR Ca-ATPase 400 µM of Na\(_2\)ATP were given to the electrolyte. For the H\(^{+}\) concentration jumps the cg H\(^{+}\) was applied with a concentration of 300 µM. All measurements were performed in the UV-laser fluorimeter for a determination of enzyme kinetics at a high time resolution. 300 µl of the prepared sample solution were filled into the cuvette which was placed on the thermostat plate in the detector chamber. The achieved fluorescence signals were normalised and the data were reduced by the redlog02.exe software.

In this series four experiments were performed at each condition. All signals of the same temperature were averaged for a better signal-to-noise ratio. The signals were fitted by a sum of the four or five exponential functions as described in Equation 12.

\[
F = \Delta F_{\text{kin,1}} \cdot \left( 1 - e^{-t/\tau_{\text{kin,1}}} \right) + \Delta F_{\text{kin,2}} \cdot \left( 1 - e^{-t/\tau_{\text{kin,2}}} \right) \\
+ \Delta F_{\text{kin,add}} \cdot \left( 1 - e^{-t/\tau_{\text{kin,add}}} \right) + \Delta F_{\text{kin,3}} \cdot \left( 1 - e^{-t/\tau_{\text{kin,3}}} \right) \\
+ \Delta F_{\text{kin,4}} \cdot \left( 1 - e^{-t/\tau_{\text{kin,4}}} \right) + F_{t=0}
\]

Figure 47 shows the fits of the averaged signals at the different temperatures 10 °C (black curve), 20 °C (red curve) and 30 °C (green curve). A striking characteristic of these results is the increase in speed of the enzyme kinetics at higher temperatures. The arrows in Figure 47 mark the transient maximum of the functions belonging to the SR Ca-ATPase kinetics. It was observed that these maxima appeared earlier during the measurements. This effect was caused by the accelerated protein kinetics at increased temperatures. In the experiments at 10 °C the four exponential functions did not fit the signal sufficiently, and a fifth exponential term was added. That additional exponential term was characterised by the parameters Δ F\(_{\text{add}}\) and \(\tau_{\text{add}}\).
and appeared as an decreasing function in the transition between the first two decreasing exponentials (kin,1 and kin,2) and the increasing exponential (kin,3).

The respective kinetics parameters of the fits shown in Figure 47 are listed in Table 16. Data were obtained from the average of four experiments. The positive and negative values of the fluorescence change amplitudes $\Delta F_{\text{kin,x}}$ describe the increasing, respectively, the decreasing terms in the sum of exponential functions. A comparison of the parameters at normal conditions ($T = 20 \, ^\circ \text{C}$, pH 6.6) show an agreement with the results of experiments presented in the chapters above.

Figure 47: $H^+$ concentration jump experiments performed with the SR Ca-ATPase in open membranes ($P-E_2$ state) at different temperatures. Signal data were normalised, reduced and fitted by a sum of exponential functions. Because of the overlap of the noisy fluorescence traces only the fits are shown for clarity.
Table 16: Kinetics parameters of the fitted signals are shown in this table. The results were obtained in the pH jump experiments with the SR Ca-ATPase in open microsomal membranes. The enzyme was in its P-E₂ conformation.

<table>
<thead>
<tr>
<th></th>
<th>T = 10 °C</th>
<th>T = 20 °C</th>
<th>T = 30 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>τₖin,1</td>
<td>1.36 ms ± 0.20 ms</td>
<td>1.37 ms ± 0.29 ms</td>
<td>0.98 ms ± 0.16 ms</td>
</tr>
<tr>
<td>ΔFₖin,1</td>
<td>-5.5 ± 0.7 %</td>
<td>-8.0 ± 1.8 %</td>
<td>-9.2 ± 1.0 %</td>
</tr>
<tr>
<td>τₖin,2</td>
<td>11.6 ms ± 3.0 ms</td>
<td>12.3 ms ± 3.4 ms</td>
<td>9.97 ms ± 1.99 ms</td>
</tr>
<tr>
<td>ΔFₖin,2</td>
<td>-5.2 ± 0.8 %</td>
<td>-7.3 ± 0.9 %</td>
<td>-7.3 ± 1.0 %</td>
</tr>
<tr>
<td>τₖin,add</td>
<td>71.0 ms ± 18.0 ms</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ΔFₖin,add</td>
<td>-2.8 ± 0.5 %</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>τₖin,3</td>
<td>244.7 ms ± 30.2 ms</td>
<td>123.2 ms ± 38.0 ms</td>
<td>19.0 ms ± 4.8 ms</td>
</tr>
<tr>
<td>ΔFₖin,3</td>
<td>2.2 ± 0.4 %</td>
<td>2.3 ± 1.3 %</td>
<td>3.0 ± 1.0 %</td>
</tr>
<tr>
<td>τₖin,4</td>
<td>5.18 s ± 1.18 s</td>
<td>1.37 s ± 0.22 s</td>
<td>0.56 s ± 0.03 s</td>
</tr>
<tr>
<td>ΔFₖin,4</td>
<td>-0.9 ± 0.2 %</td>
<td>-1.4 ± 0.4 %</td>
<td>-1.1 ± 0.2 %</td>
</tr>
</tbody>
</table>

For the determination of the activation energies for SR Ca-ATPase related reaction steps the results of the experiments were plotted in an Arrhenius plot (ln(1/τ) versus 1/T) shown in Figure 48. The temperature was converted according to the function described in Equation 13, and the time constants τₖin,x representing the kinetics parameter of these reaction steps were converted into rate constants k of the Arrhenius equation (Equation 14) as shown below.

Equation 13:

\[
\frac{1}{T} \cdot 1000 = \frac{1000}{(273.15 K + \theta)}
\]

\[
k = \left(\frac{1}{\tau_{kin,x}}\right)
\]

Equation 14:

\[
k = A \cdot \exp\left(-\frac{E_A}{RT}\right)
\]

transformed, using the natural logarithm:

\[
\ln k = -\frac{E_A}{R} \cdot \left(\frac{1}{T}\right) + \ln A
\]
Figure 48 shows the Arrhenius plot of four determined rate constants received from the time constants of the proton concentration jump experiments with the SR Ca-ATPase in open microsomal membrane fragments. The linear regression lines with their slopes \( m \) in the Arrhenius plot were used to determine the activation energy \( E_A \) of the rate-limiting reaction steps of the SR Ca-ATPase using Equation 15. The temperature dependence observed for the first two time constants \( \tau_{\text{kin,1}} \) and \( \tau_{\text{kin,2}} \) is represented by the black and red data points. Regression lines are shown for both results. The slope \( m \) (Table 17) of these lines is very small or almost zero and indicates that these processes are more or less temperature independent. In contrast the slopes of the third and the fourth reaction steps showed significant temperature dependences. From the slopes determined by a regression line through the data points the activation energies \( E_A \) of each reaction step of the SR Ca-ATPase was calculated.

\[
E_A = -R \frac{\delta \ln k}{\delta(1/T)} = -R \cdot m
\]

Compared the transformed Arrhenius Equation in Equation 14 to a standard linear function

\[
y = m \cdot x + b
\]
The activation energies are shown in Table 17. For the third, protein-related process, an activation energy $E_A$ of 94.2 kJ/mol was determined, and the fourth process showed an $E_A$ of 77.3 kJ/mol. The values of the first and second observed process were just $E_A = 12.4$ kJ/mol, respectively, $E_A = 1.4$ kJ/mol.

### Table 17: Slopes m of the regression lines and the calculated activation energies. They are determined in experiments with the SR Ca-ATPase in its P-E$_2$ conformation in the open membrane preparation.

<table>
<thead>
<tr>
<th>Exponential function</th>
<th>Slope m of regression lines</th>
<th>Activation energy kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\tau_{kin,1} / \Delta F_{kin,1}$ (black)</td>
<td>-1.5 $\pm$ 0.8</td>
<td>12.4</td>
</tr>
<tr>
<td>$\tau_{kin,2} / \Delta F_{kin,2}$ (red)</td>
<td>-0.2 $\pm$ 0.7</td>
<td>1.4</td>
</tr>
<tr>
<td>$\tau_{kin,3} / \Delta F_{kin,3}$ (green)</td>
<td>-11.3 $\pm$ 2.9</td>
<td>94.2</td>
</tr>
<tr>
<td>$\tau_{kin,4} / \Delta F_{kin,4}$ (cyan)</td>
<td>-9.3 $\pm$ 1.0</td>
<td>77.3</td>
</tr>
</tbody>
</table>

Comparing the four values of the activation energies with typical specifications in textbooks, indicates that the high results obtained from protein reaction steps 3 and 4 point to a structural rearrangements of the protein, which have to be involved in a rate-limiting way. This rearrangement may correlate to the opening of the access channel to the ion binding sites. The much smaller activation energies of the reaction steps 1 and 2 suggest to be protonation effects on the surface of the enzyme, due to the increased proton concentration after the H$^+$ jump.

### 3.3.9 ATP jump experiments with the open microsomal membrane preparation

The kinetics of the transition from the $E_1$ state to the P-E$_2$ state of the SR Ca-ATPase in open membrane fragments was determined with the ATP-jump experiments. By photo-releasing the ATP from its inert precursor within a few milliseconds an ATP jump induced a triggered enzyme activity. The observed fluorescence signals of the synchronised ion pump action indicate the electrogenic exchange of ions in the binding sites of the SR Ca-ATPase. For these measurements the electrolyte contained 25 mM Tricine as buffering agent and 50 mM KCl. pH was adjusted by the addition of KOH. As fluorescence probe for electrogenic reactions, the styryl dye F52 was used in a concentration of 800 nM. The SR Ca-ATPase in its open membrane preparation was applied with 36 µg of the enzyme in 1 ml of the buffer solution. Because of its photolability the cg ATP was added to the cuvette directly prior the start of the measurement. The cg ATP solution contained NPE-ATP, MgCl$_2$ and the enzyme apyrase in a very low concentration (0.020 units/ml) to remove the traces of released ATP. The final
cg ATP concentration in the cuvette was 100 µM. After the thermal equilibration at 20 °C the experiment was started with the UV-flash at a wavelength of 351 nm, an energy of 6 MW and a duration of 14 ns. The UV flash cleaves the bond between the ATP and the NPE protection group and the released ATP activates the SR Ca-ATPase. When calcium ions are bound, the SR Ca-ATPase progresses through the Post-Albers cycle to its P-E\(_2\) state in which the calcium ions are released from and protons bound to ion-binding sites in the membrane domain of the enzyme.

![Graph showing fluorescence signal](image)

**Figure 49:** The fluorescence signal shows the result of an ATP jump experiment at physiological pH 7.1 with the SR Ca-ATPase in open membrane fragments. The time scale is in linear and the signal is normalised to the pre-trigger level (data not shown). The flash artefact caused by the high energy UV-laser is visible as the rapidly decreasing, almost vertical line at the experiment’s starting point at 0 seconds.

The data of the fluorescence signals were collected as described in the Methods section (Chapter 2.11.3, page 25). Figure 49 shows a typical measurement of an ATP concentration jump experiment on a linear time scale. Pre-trigger data are not shown in this figure but were used to normalize the fluorescence. The initial level was set to 1.00. The large number of data points collected (with 100 kHz) was reduced within the normalisation program rdl13.exe by averaging adjoining data by a boxcar method with variable length. This mathematical method also increased the S/N ratio. Starting at 0 s an UV-flash induced artefact was recorded. After uncaging the cg ATP the time-dependent fluorescence signal shows the electrogenic ion movements between the aqueous phase and the enzyme binding sites by the increasing and decreasing fluorescence level as can be seen more clearly in Figure 50 when logarithmic time scale has been chosen.
The UV-flash artefact was mostly reduced electronically. There is just a minor remnant visible during the first milliseconds of the fluorescence signal. To analyse the ATP concentration jump experiments a sum of exponential functions was used to fit the fluorescence signals. Covering the whole signal trace in these experiments a sum of four or five exponential functions was necessary as shown in Equation 12 (see page 79).

The time course of the fluorescence signal representing the ATP concentration jump experiments with the SR Ca-ATPase could be fitted with Equation 12 by the FigP software. In this numerical method (Marquardt, 1963) the parameters of the exponential functions were determined. A plot of a fluorescence signal and the corresponding fit is shown in Figure 51. The first region (1) encloses two of the decreasing exponential functions in the mathematical model. In case of a five exponential fit, this region is expanded by a third decreasing function (to which, if needed, the characteristic parameters $\tau_{\text{kin,add}}$, pH and $\Delta F_{\text{kin,add}}$, pH are assigned). The fluorescence signal in region (1) is assigned to UV-flash induced artefacts. The second region (2) contains one increasing exponential function in these cg ATP experiments. The increasing fluorescence level indicates an electrogenic net release of Ca$^{2+}$ ions. Following this increasing part the third region (3) shows a slight and long lasting (in the order of a second) decreasing part, which corresponds to the fourth, respectively, fifth exponential function of the mathematical model. This trend was observed in all ATP jump experiments and the mathematical model was used to determine the time constants and fluorescence change amplitudes.

Figure 50: The same experiment as in Figure 49 is shown. The data were normalised with the new redlog02.exe software and plotted over a logarithmic time scale for a better visualisation.
Table 18: The time constants and amplitudes of the cg ATP experiment (Figure 51) with SR Ca-ATPase in open membrane fragments. The pH of the electrolyte was 7.1 and it contained 36 µg/ml of the enzyme. 100 µM of cg ATP were applied to produce the ATP concentration jump. The temperature was equilibrated to 20 °C.

<table>
<thead>
<tr>
<th>Open membrane SR Ca-ATPase with cg ATP</th>
<th>x = 1 (region 1)</th>
<th>x = 2 (region 1)</th>
<th>x = 3 (region 2)</th>
<th>x = 4 (region 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time constant $\tau_{\text{kin},x}$</td>
<td>0.99 ms ± 0.05 ms</td>
<td>7.31 ms ± 0.97 ms</td>
<td>113.0 ms ± 39.7 ms</td>
<td>0.60 s ± 0.39 s</td>
</tr>
<tr>
<td>Amplitude $\Delta F_{\text{kin},x}$</td>
<td>-5.4 ± 0.2 %</td>
<td>-2.8 ± 0.2 %</td>
<td>1.5 ± 0.4 %</td>
<td>0.6 ± 0.4 %</td>
</tr>
<tr>
<td>Starting level $F_{t=0}$</td>
<td>108.2 ± 0.1 %</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3.3.10 ATP-jump experiments and pH dependence of SR Ca-ATPase

A series of these cg ATP experiments was performed to determine the characteristic time constant and fluorescence changes of the SR Ca-ATPase in the open microsomal membrane preparation. In this series of experiments the pH of the electrolyte was adjusted to various values. Starting at pH 6.6, covering the physiological range up to the alkaline pH at pH 8.5 the properties of the SR Ca-ATPase kinetics were determined. The electrolyte contained 25 mM Tricine, 50 mM KCl and the pH was adjusted by the addition of a KOH solution. As
fluorescence probe the styryl dye F52 was applied in a concentration of 800 nM. 36 µg/ml of the SR Ca-ATPase were used in this test solution.

At each pH several experiments were performed. The fluorescence signal of each measurement was normalised and the data were reduced with the redlog02.exe program. After this procedure all measurements at one specific pH were averaged for an improved signal-to-noise ratio. This leads to a more accurate fluorescence signal with a better chance to obtain a significant fit. These fits were a sum of exponential functions as described in Equation 8, page 38. In Figure 52 the best fits of the averaged experiments of all pH values are plotted on a logarithmic time axis to visualise the processes in the millisecond range. The number of averaged experiments at each pH is included in brackets in the diagram. In the pH range between pH 6.6 and pH 7.9 the fits were a sum of four exponential functions. At the higher pH values of pH 8.2 and pH 8.5 it was necessary to use five exponential functions to obtain an adequate fit. As in the case of the pH jump experiments the additional function was needed in the first region with the decreasing fluorescence intensity, which then consists of three exponentials.

The first decreasing part (compare Figure 51, region (1)) of all signals was assigned to the decay of the artefact UV flash peak and minor rearrangements of the enzyme structure. This part could be fitted with the sum of two, respectively, three exponential functions. In Figure 53 the determined time constants are shown on a logarithmic axis against buffer pH. The determination of the first exponential time constant $\tau_{\text{kin},1}$ shows an average of 1.49 ms ± 0.19 ms and was pH independent. The following decreasing part was analysed with
the time constant $\tau_{\text{kin},2}$ and that average of all determined values over the whole pH range was 8.11 ms ± 2.19 ms. This time constant is not significantly pH dependent, too. The optional time constant, $\tau_{\text{add}}$ is not shown in Figure 53. It has values of $\tau_{\text{add}} = 27.2$ ms ± 11.9 ms at pH 8.2 and $\tau_{\text{add}} = 44.9$ ms ± 16.5 ms at pH 8.5. The reason for the obviously huge standard error of the time constant $\tau_{\text{add}}$ was the scattering of the data, but the hardly determinable, additional time constant is necessary for an accurate fit of the total fluorescence signal.

At the transition from the first to the second region (Figure 51, (1) and (2)) data and fits reach a minimum fluorescence level. This level corresponds approximately to the level of the pre-trigger signal. Upon the minimum a strong increasing fluorescence signal follows which is described by the next exponential term of the sum. The time constants of increasing fluorescence signal is pH independent and the averaged $\tau_{\text{kin},3}$ is 78.2 ms ± 9.0 ms over the whole pH range. Individual values are shown in Figure 53 by green points. Each value is within a narrow band around the regression line.

In region (3) of Figure 51 the fit continues with the fourth (respectively, fifth) exponential function of Equation 12 (page 79). The time constants are shown in Figure 53 as cyan points. In the lower and physiological pH regions between pH 6.6 and pH 7.5 $\tau_{\text{kin},4}$ showed an average of 0.61 s ± 0.01 s. At buffer pH > 7.5 the time constant between the experiments varied significantly, which can be observed in inaccurate averages. The values of $\tau_{\text{kin},4}$ increased up to 3.20 s at pH 8.5.
The second fitted parameter of each exponential function is the change of the fluorescence amplitude which is described by the value \( \Delta F_{\text{kin},x} \) (with \( x = 1...4 \) and 'add' for the additional term). This normalised amplitude represents the maximum change of each exponential function, which corresponds to the amount positive charge released from or bound in the binding sites following enzyme phosphorylation and the P-E1 – P-E2 transition. Figure 54 displays the normalised amplitudes as function of the buffer pH in the colour code of the time constants \( \tau_{\text{kin},1}...\tau_{\text{kin},4} \) as used in Figure 53. The initial decreasing exponential functions of the flash artefact have amplitudes with a negative sign whereas the increasing functions are shown with amplitudes larger than zero. The amplitudes of the fifth additional exponential (at pH 8.2 and pH 8.5) are not shown in Figure 54.

![Figure 54: Normalised amplitudes \( \Delta F_{\text{kin},1} \) (black), \( \Delta F_{\text{kin},2} \) (red), \( \Delta F_{\text{kin},3} \) (green) and \( \Delta F_{\text{kin},4} \) (cyan) of the exponential fits with Equation 12 (page 79) are plotted against the buffer pH. Corresponding to Figure 53 the additional amplitude in the first decreasing signal part, which appears solely in the upper pH range, is not shown.](image)

In the first region (1) in Figure 51 both amplitudes \( \Delta F_{\text{kin},1} \) and \( \Delta F_{\text{kin},2} \), seem to be pH independent and may be represented by an average of \( \Delta F_{\text{kin},1} = -5.1 \pm 0.2 \% \) and \( \Delta F_{\text{kin},2} = -2.4 \pm 0.4 \% \). The amplitude \( \Delta F_{\text{kin,add}} \) of the additional exponential function was determined to -5.2 \% at pH 8.2 and -4.9 \% at pH 8.5.

In contrast, the increasing fluorescence signal represented by the amplitude \( \Delta F_{\text{kin},3} \) shows a significant pH dependence. With increasing pH the fluorescence amplitude increases. Starting at pH 6.6 with an amplitude of +0.6 \% it reaches the highest amplitude at pH 8.5 with +6.8 \%, see Table 19.
The fluorescence amplitude $\Delta F_{\text{kin,4}}$, describing the fourth exponential function in the fits, shows again small and pH independent values. With the ATP-jump experiments in the pH range between pH 6.6 and pH 8.5 the average of all amplitudes $\Delta F_{\text{kin,4}}$ was determined to 0.7 ± 0.2 %. These amplitudes, with increasing fluorescence, possess a positive sign and correlated to the slow time constant $\tau_{\text{kin,4}}$. Therefore, this indicates a minor electrogenic charge movement in the ion binding sites.

### 3.4 Tests with new synthesised styryl dyes

Since more than ten years ion movements in the SR Ca-ATPase have been detected very successfully by electrochromic styryl dyes. In the beginning so-called nXITC styryl dyes have been established for the SR Ca-ATPase tests (Birmes, 1995), (Butscher, et al., 1999). The styryl dyes 2BITC and 2HITC were the most effective probes in experiments with the SR Ca-ATPase. Both dyes were provided by the group of Prof. Dr. H.-D. Martin from the University of Düsseldorf (Birmes, 1995). Recently, a set of new electrochromic styryl dyes have been synthesised by Karl Janko in our group at the University of Konstanz. They possess the same chromophore as the nXITC dyes, but differ in the attached head group (Figure 8, page 15). In the new dyes the isothiocyanate group is exchanged against an alkyl or a carboxyl group. The length of headgroup varies between the different dyes. Table 3, page 14 lists the styryl dyes which were tested with the SR Ca-ATPase to identify the dye with the highest changes of the fluorescent yield and the best protein/dye ratio. Tests were performed in standard steady-state experiments as well as in kinetic measurements with caged compounds.

### 3.4.1 Standard fluorescence test with the new styryl dyes

Standard experiments were used to determine the fluorescence level changes of the dyes with respect to ion-saturated and ion-free states of the SR Ca-ATPase to identify the dye with the

---

Table 19: The pH dependence of the increasing amplitudes is shown.

<table>
<thead>
<tr>
<th>pH</th>
<th>Number of individual experiments</th>
<th>$\Delta F_{\text{kin,3}}$</th>
<th>Standard error for the numerically fitted function</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.6</td>
<td>4</td>
<td>+0.6 %</td>
<td>0.2 %</td>
</tr>
<tr>
<td>7.1</td>
<td>6</td>
<td>+1.5 %</td>
<td>0.4 %</td>
</tr>
<tr>
<td>7.5</td>
<td>4</td>
<td>+4.1 %</td>
<td>1.2 %</td>
</tr>
<tr>
<td>7.9</td>
<td>8</td>
<td>+3.8 %</td>
<td>0.3 %</td>
</tr>
<tr>
<td>8.2</td>
<td>4</td>
<td>+6.4 %</td>
<td>4.0 %</td>
</tr>
<tr>
<td>8.5</td>
<td>4</td>
<td>+6.8 %</td>
<td>4.3 %</td>
</tr>
</tbody>
</table>
largest effect caused by Ca$^{2+}$ binding. For each dye steady-state experiments were performed with an electrolyte containing 25 mM MOPS, 50 mM KCl and 500 µM MgCl$_2$. Buffer pH was adjusted to 7.0 and the temperature was equilibrated at 20 °C. Subsequently, 800 nM styrly dye and 36 µg/ml protein were added to start the measurements. Addition of 50 µM BAPTA induces an almost Ca$^{2+}$ free E$_1$ state of the SR Ca-ATPase. The resulting levels represent the highest fluorescence in these experiments. Adding CaCl$_2$, to maintain a concentration of 20 µM of free Ca$^{2+}$ in the electrolyte leads to the Ca$^{2+}$ saturated state (Figure 20, page 38), E$_1$Ca$_2$, which is the second relevant fluorescence level. The difference of both fluorescence levels is an indicator of the suitability of the dyes. The larger the fluorescence change is, the more useful is the dye to resolve charge movements in the SR Ca-ATPase.

Figure 55 represents the relative fluorescence changes between the Ca$^{2+}$-saturated and the Ca$^{2+}$-free states of the SR Ca-ATPase for all six dyes tested. The most convenient styrly dye is the F52, which shows the maximal fluorescence changes of 29 %.

![Graph showing relative fluorescence changes between Ca$^{2+}$-saturated and Ca$^{2+}$-free states for different dyes.]

Figure 55: Test of the new styrly dyes for the best suitability. The fluorescence change was determined by standard experiments. The difference of the fluorescence levels between the Ca$^{2+}$-free E$_1$ and the E$_1$Ca$_2$ state of the SR Ca-ATPase was used to find the best dye.
3.4.2 Determination of the dye / protein ratio

The dye with the maximum fluorescence change, F52, was chosen to perform experiments to determine the best dye / protein ratio for kinetical measurements with the enzyme.

The electrolyte of these experiments contained 25 mM Tricine and 50 mM KCl at pH 7.0. For the kinetical measurements with caged ATP the presence of Ca$^{2+}$ and Mg$^{2+}$ ions is necessary. They were added to the buffer at a concentration of 10 µM free Ca$^{2+}$, 1 mM MgCl$_2$ and 100 µM cg ATP. In a first series of experiments the F52 concentration was varied between 50 nM and 800 nM. Figure 56 (A) shows the fits to the fluorescence traces obtained with the indicated F52 concentrations. The fitted traces were the average of four individual measurements under identical conditions. The data were taken with the laser fluorimeter and normalised by the redlog02.exe software.

The fluorescence increase in the time interval between 10 ms and 100 ms represents ion release from the binding sites of the SR Ca-ATPase. The fluorescence level obtained thereafter (between 100 ms and 1 s) represents an equilibrium state of the ion pumps. It was observed, that with the increasing dye concentration up to 800 nM the fluorescence level could not be elevated much more. The doubling of the F52 concentration from 400 nM to 800 nM caused just a slightly higher fluorescence level in the equilibrium state of the SR Ca-ATPase. An exceeding dye concentration above 800 nM had no benefit to the fluorescence signals, which led to the conclusion that higher dye concentrations were not necessary.
Figure 56 shows experiments with various enzyme concentrations. At a constant dye concentration of 800 nM, SR Ca-ATPase concentration of 18 µg/ml, 36 µg/ml and 72 µg/ml were used in the standard experiments. The variation of the protein concentration appears to have a smaller effect on the fluorescence signal than variations of the dye concentration (Figure 56 A). But the fluorescence signal at higher protein concentrations is much more stable and reproducible than that at 18 µg/ml. The additional analysis of the voltage output of the photomultiplier detector as measure of the absolute fluorescence level were used to select...
the best dye/protein ratio (Figure 57). The plotted voltages were obtained as difference between illuminated and dark solutions, as described above.

The photomultiplier output voltage and, therefore, the total fluorescence, had the highest yield results in the experiment with 36 µg/ml SR Ca-ATPase and 800 nM F52. The buffer contained 25 mM Tricine and 50 mM KCl at pH 7.0 and 10 µM free Ca\(^{2+}\), 1 mM MgCl\(_2\) and 100 µM cg ATP. Assuming a molecular weight of 110 kDa for the SR Ca-ATPase, leads to a dye/protein ratio of about 2.4 dye molecules per SR Ca-ATPase under these experimental conditions. The other dye/protein ratios show a lower fluorescence yield (Figure 57).

![Figure 57](image_url)

*Figure 57: Dependence of the photomultiplier (PM) output voltage on protein concentration. Each result is the average of four measurements.*
4 Discussion

The SR Ca-ATPase is one of the favourite enzymes for the investigation of function-structure relations of the P-type ATPases. The preparation of the protein in its native SR membrane or reconstituted in DOPC vesicles are still state-of-the-art technique for the analysis of the enzyme kinetics (Inesi, et al., 1983 (b)), (Villalobo, 1990). But vesicular membrane structures are handicapped by great restrictions in the determination of detailed characteristics of the enzyme kinetics. The permeability of monovalent, and much more divalent cations through the SR membrane is dramatically restricted (Meissner, et al., 1976), and access of ions to the luminal binding sites in the P-E\textsubscript{2} conformation can hardly be controlled. A new preparation method was introduced by (Fibich, et al., 2008) to circumvent this disadvantage. This method produces flat, non-vesicular SR membrane fragments in which the SR Ca-ATPase proteins are still integrated. A unique benefit of this preparation is the barrier-free access for the ions to both, the cytoplasmic and luminal access channels of the ion pumps. The cations in the electrolyte are able to enter and bind to the ion sites without affecting the kinetics of protein-related reaction steps. Therefore, the detected electrogenic behaviour is controlled by the rate-limiting characteristic parameters of the reaction kinetics which are related to the changes of the enzyme conformation and the relaxation steps during the transport cycle. The new preparation allows, therefore, detailed and reliable investigations of the binding kinetic characteristics for the transported cations in the P-E\textsubscript{2} state of the enzyme.

It allows in particular the study of the cation binding kinetics at the luminal sites of the SR Ca-ATPase. Rapid concentration jumps of the transported protons or the substrate ATP are possible with physiological inactive precursors, so-called caged compounds (Kaplan, et al., 1978), (Barth, et al., 2002), (Fibich, et al., 2007). The electrochromic styryl dyes detect charge movements in the membrane domain of proteins and are able to detect even rapid ion movements (Grinvald, et al., 1982), (Apell, et al., 1985), (Ephart, et al., 1993), (Stürmer, et al., 1989), (Birmes, 1995), (Butscher, et al., 1999). The experiments presented here show new detailed results on the ion binding kinetics of the SR Ca-ATPase in its E\textsubscript{1} and P-E\textsubscript{2} state. Fast proton binding processes have been observed and their characteristic parameters in both states of the enzyme have been analysed. The activation energies $E_A$ of these processes were determined. Binding affinities for Ca\textsuperscript{2+} and H\textsuperscript{+} ions are the important data to understand and refine the transport mechanism, the so called Post-Albers cycle, of the SR Ca-ATPase (Post, et al., 1972), (Albers, 1967). The usage of caged proton and caged ATP allows the analysis on discrete steps in this transport cycle, and the determination of various kinetic parameters of the ion binding processes of the SR Ca-ATPase and conformational changes of the enzyme structure.
4.1 Comparison of vesicular and open SR membrane preparations containing the SR Ca-ATPase

When the new preparation was introduced the first questions to be answered were: are both preparations, in the vesicular membranes and the open membrane fragments, comparable to each other, and are there any details or parameters in which they differ? The first steady-state experiments showed for the preparation with open membrane fragments no significant difference in the protein function (Chapter 3.2.4, page 49). Each substrate addition showed a similar response (Figure 58). Differences were observed solely in the amplitude of the respective fluorescence levels, which were slightly reduced in the experiments with open membrane fragments. The fluorescence levels were normalised in the vesicular and open membrane fragment experiments with respect to the level obtained when the membrane preparations were equilibrated in the F52-containing electrolyte (Figure 58, fluorescence level 3). The subsequent substrate-induced fluorescence responses were related to that level.

A couple of reasons are responsible for the minor changes of amplitudes observed with the open membrane preparation. These findings are similar to the results in (Pedersen, et al., 2002), (Fibich, et al., 2008). One explanation is the alteration of the dielectric constant \( \varepsilon \) of the membrane. During the SDS treatment phospholipids were removed from the membrane to obtain flat almost inflexible membrane rafts, which couldn’t form vesicles again. Thereby the enzyme : lipid ratio increased and alter the dielectric constant and therefore the spatial range of electric fields in the membrane. This results in a minor effect of the free and enzyme-bound...
ion charges to the membrane integrated styryl dye molecules. The increased protein density leads also to another effect, which reduces the fluorescence amplitude. Dye molecules have to insert in the membrane for their correct function, but the reduced amount of lipid molecules and the high amount of proteins prevent partly the correct placement of the dye molecules in the membrane. A small shift in the Ångstrom range can cause a reduced ion charge - dye interaction. An obviously reason for the amplitude reduction in open membrane preparation, finally is the insertion of dye molecules in both leaflets of the lipid bilayer. In the vesicular membrane structures the styryl dye molecules solely insert in the outer cytoplasmic leaflet of the membrane. The polar head group does not allow a flip-flop of the molecules to the inner luminal leaflet. Whereas in the open membrane preparation the dye molecules can insert in both, the cytoplasmic and the luminal, leaflets. The molecules in the luminal leaflet do not contribute to the changes in fluorescence levels, due to the minor spatial range of the ion charges and the increased distance between the luminal leaflet and the ion binding sites. With respect to these findings it was expected that the amplitude of the open membrane preparation is slightly reduced, but it can conclude that the function of the styryl dye still allow the observation of ion movements in the membrane domain of the SR Ca-ATPase.

Evidence for the correct enzyme function after the SDS treatment were found in the enzyme activity tests and confirm the comparability of vesicular and open membrane preparations. The first result was that both enzyme preparations show enzyme activities, which could be assigned to the SR Ca-ATPase. In the coupled pyruvate kinase/lactate dehydrogenase test the validation occurred by the addition of the SR Ca-ATPase specific inhibitor TG. In both preparations the activity was reduced to almost zero after the inhibition. One expected and of course desired difference between both preparations was the identical enzyme activity in the open membrane preparation without and with Ca\(^{2+}\) ionophore A23187. The absence of an activity increase is expected since in the open fragments both Ca\(^{2+}\) and H\(^{+}\) ions have free access to the luminal binding sites. An accumulation of Ca\(^{2+}\) in the vesicle lumen and subsequently a fractional inhibition of the SR Ca-ATPase activity could not occur with open membrane fragments. When the maximum activity of both preparations is compared it shows that the remaining enzyme activity is 34.4 % after the SDS treatment compared to the activity obtained from the original vesicular preparation. This reduction is caused by an inactivation of remaining proteins after the SDS preparation. As described in (Fibich, et al., 2008) one obviously reason for the decreased enzyme activity is denaturation of the SR Ca-ATPase by the SDS treatment. The reduced lipid density allows the SDS molecules a better access to the enzymes and therefore a higher denaturation impact. A second explanation for the reduction of enzyme activity was discussed to be the alteration of enzyme : lipid ratio and the lipid composition of the membranes. To obtain the open membrane fragments these alterations are necessary, but this accompanied with decreased membrane elasticity and membrane bending,
which influence the enzyme activity. A third reason, a high residual SDS concentration after the dialysis was eliminated, because enzyme activities were stable during all the storage periods, throughout all preparations of the 1 h and the 3 days dialysis. This showed that the SDS concentration was rapidly decreased in the dialysis step and do not further contribute to the denaturation of the SR Ca-ATPase.

Furthermore an additional finding was observed. As described in chapter 3.2.3 on page 46 it could be shown that the decreased enzyme activity is also caused by a reversible inactivation. A SDS treated Ca-ATPase preparation with open membrane fragments, approved in the pyruvate kinase / lactate dehydrogenase test, was dialysed in a second step with a low ionic strength dialysis buffer, which led to a reconstitution of membrane vesicles, confirmed in the test with Ca$^{2+}$ ionophore A23187. Figure 59 showed this experiment with recovered enzyme activity and vesicle formation. These results allow the conclusion to that by a reduction of SDS and decreased ionic strength the vesicular structure of the SR membrane can be regained, as indicated by the increase of the enzyme activity (+ 67.9 %) in the presence of A23187. The enhancement was also reflected in the fluorescence experiments with F52 in which the SDS- and ionic strength-reduced preparations produced larger fluorescence amplitudes. To describe these effects we have to take a look at the density alteration and the formation of the lipid rafts as shown before. The residual SDS molecules are bound at the edge of the open membrane fragments to stabilise the lipid bilayer against the polar buffer solution. With the

![Figure 59: Enzyme activity test (coupled PK/LDH-Test) with two different enzyme preparations. The black line represents the SR Ca-ATPase activity with the open membrane fragments after 1 h dialysis and the red line the enzyme activity after a reconstitution of the membrane vesicles after a second dialysis step in a buffer with lower ionic strength. The consumption of ATP by the SR Ca-ATPase caused the decreasing absorbance signal. The regions 1, 2 and 3 represent the different substrate effects. (1) normal enzyme activity, (2) enzyme activity with ionophore A23187 and (3) inhibited enzyme activity with TG (control).]
continuing reduction of the SDS molecules the rafts began to form larger membrane fragments, until they bend and form new vesicles. As a result the SDS influence to the membrane integrated SR Ca-ATPases didn’t play a major role in the enzyme activity test and an altered lipid composition allows a higher flexibility of the lipid bilayer and supports the increase of the enzyme activity.

The first introduced method used to obtain open microsomal membrane fragments containing SR Ca-ATPases included a 3 days dialysis to remove the SDS (Fibich, et al., 2008). An advantage of the alternative 1 h dialysis method for the SR Ca-ATPase is not only the faster availability but also the increased enzyme activity and the reproducibility with this procedure. During the three days of dialysis the enzyme activity significantly decreased and a few preparations had to be discarded, because of the total loss of enzyme activity. Compared to the SR Ca-ATPase in untreated membrane vesicles the remaining enzyme activity after three days was 15.7 % in the average and 45.9 % of the activity obtained after the 1 h dialysis. The excellent reproducibility of the always active enzyme preparations obtained by the 1 h method led to shorter dialysis times and was a great progress to achieve SR Ca-ATPase preparations with comparable properties for the further experiments. The SDS concentration was rapidly decreased in the 1 h dialysis, that residual SDS molecules have no further influence to the enzyme activity during storage and experiments with the SR Ca-ATPase.

The further analysis of these open membrane fragments by the visualisation with the TEM as shown in chapter 3.2.2 (page 45) approves the predicted flat membrane structures and confirms the results and conclusions of the experiments described above.

Evidence for the fully functioning SR Ca-ATPase in open membrane fragments has been provided by the SDS-Page analysis. Partial tryptic digestion may be performed under buffer conditions that stabilise either the E₁ or the P-E₂ state of the enzyme. When partial tryptic digestion is passed, different band pattern on the SDS-PAGE gel are observed (see Figure 30, page 54). It has been shown that the conformation changes between the E₁ to the P-E₂ state resulted in two characteristic dominant bands of the SR Ca-ATPase at 50-55 kDa (see Figure 31, page 55). The different band pattern is explained by the altered access of the trypsin to the SR Ca-ATPase structure in both enzyme conformations. That these band pattern correlate with the P-E₂ state has been shown in addition by the partial tryptic digestion of the inactive enzyme. In this preparation the ATP-induced transition to the P-E₂ state is inhibited, and, therefore, the band pattern in the SDS-PAGE gel showed no differences between the E₁ state and the anticipated P-E₂ state of the enzyme (see Figure 30 (E) and (F), page 54).

In contrast to the inactivated enzyme, when ATP was added to transfer the SDS treated SR Ca-ATPase into its P-E₂ state, the band pattern of the open membrane fragments displays the same characteristic scheme in the SDS-PAGE gels as in the case of the vesicular SR Ca-
ATPase. This finding provides a solid basis for meaningful investigations of the binding and transport characteristics of the open membrane preparation in both states, the E₁ state, to compare the properties obtained in experiments performed with vesicular Ca-ATPase preparations, and the P-E₂ state, which was not directly accessible so far. The first time, the new preparation method allows an unrestricted entry of the transported ions to the luminal ion access channels of the enzyme.

These findings confirm the assumption, that ion pumps contained in SR membrane vesicles and in open membrane fragments are comparable. The protein structure and function of both preparations is identical and not affected by the SDS treatment. That result permits further analysis of the ion binding and release.

4.2 Proton binding in the P-E₂ state of the SR Ca-ATPase

Protons play a major role in the transport mechanism of the SR Ca-ATPase (Inesi, et al., 1983 (a)). As counter ions to the transported Ca^{2+} ions they are necessary for the stabilisation of the enzyme cavities and the binding sites (Møller, et al., 2005). The exchange of Ca^{2+} and H^{+} at the binding sites, and vice versa, is one of the crucial steps during the Post-Albers cycle and correlates with the structure relaxations of the enzyme. The experimental approach of the determination of the binding kinetics was possible by the usage of the cg H^{+} MNPS.Na (Fibich, et al., 2007). This cg H^{+} allows the generation of a fast and homogeneous H^{+} concentration jump in the sample electrolyte. The pH jump perturbed the initial equilibrium of the transport system, consisting of the SR Ca-ATPase and the ions, and the new environmental conditions induced a transition of the enzymes into a new equilibrium. With the electrochromic styryl dye the rearrangement of the enzyme structure and its related ion binding and release was observed (Peinelt, et al., 2004) (Fibich, et al., 2007). Protein-transported cations in the aqueous electrolyte are available sufficiently and the rate-limiting steps are defined by the relaxation rate constants of the respective reaction steps of the SR Ca-ATPase. The observed fluorescence signal could be assigned to the protein function and the ion movements in the membrane domain of the SR Ca-ATPase. Another evidence is, that the kinetics of the SR Ca-ATPase exhibited time constants in the range of milliseconds or sub-milliseconds and, therefore, were slow compared to the rate constants of the ion diffusion processes in the electrolyte and the access channels.

4.2.1 The Ca^{2+} H^{+} exchange in the P-E₂ conformation

Former studies analysed the Ca^{2+} and H^{+} binding to the P-E₂ conformation of the SR Ca-ATPase in SR vesicles (Peinelt, 2002). One major handicap was the vesicular SR membrane, in which the enzymes are inserted. This structure prevents a fast access of the ions to the...
Dissertation Andreas Fibich

enzyme binding sites in the P-E_2 conformation of the enzymes. Therefore, the ionophore A23187 was used to allow the ions passing through the membrane and obtain an equilibrium between the inner and outer electrolyte. This process allowed equilibrium titration experiments with Ca^{2+} and H^+ ions. The interpreted results lead to a description of a branched reaction scheme in the P-E_2 conformation of the SR Ca-ATPase, which includes in one hand a mixed occupation of the binding sites in the so-called P-E_2 Ca H state at low pH and in the other hand an ion free P-E_2 state at high pH (Scheme 1). As shown in (Toyoshima, et al., 2004 (c)) the P-E_2 conformation of the SR Ca-ATPase could be populated only transiently, due to the instability of the membrane domain under ion free conditions.

Scheme 1:

$$\cdots \rightarrow P - E_2 Ca_2 \leftrightarrow P - E_2 Ca \leftrightarrow P - E_2 \uparrow \downarrow$$

$$P - E_2 Ca H \leftrightarrow P - E_2 H \leftrightarrow P - E_2 H_2 \rightarrow \cdots$$

These processes were further analysed. The experiments were performed with exceed Ca^{2+} ions and ATP to keep the protein running continuously through the Post-Albers cycle. In (Peinelt, et al., 2004) it was shown that under these conditions most of the proteins settle down in a steady-state before the rate-limiting steps, which were investigated by the performed concentration jump experiments. Former results confirmed that a major fraction of the SR Ca-ATPase is maintained in the P-E_2 Ca state under these conditions. The H^+ concentration jumps trigger the protein to run through its subsequent conformation steps, which represent the Ca^{2+} release and the H^+ binding and provide new results for the SR Ca-ATPase function.

The pH jumps drive the protein into a new steady-state. This transition is linked with protein relaxation and/or conformational changes, which were measured as fluorescence amplitudes $\Delta F_{kin,x}$ and time constants $\tau_{kin,x}$. An analysis of the first time constant $\tau_{kin,1}$ was quite difficult, because it is the fastest reaction step and it is overlain by the UV-flash artefact. So the determined time of $\tau_{kin,1} = 1.25$ ms is a sum of reaction step and the flash artefact (see Figure 42, page 74). In contrast to the improper time constant, the fluorescence amplitude $\Delta F_{kin,1}$ allow further analysis. It shows an electrogenic reaction assigned to the enzyme binding sites with an initial fluorescence decrease of about 8 % at physiological pH (see Figure 43, page 75). The activation energy for this process was determined to be 12.4 kJ/mol (Table 17, page 83), with respect to the artefact and the derived inaccuracy of the time constant. However, these results are in agreement with the concept that protons diffuse after their release in the bulk phase through the access channel of the SR Ca-ATPase and bind in the ion-binding sites of the protein membrane-domain. The measured activation energy allows the
conclusion, that this diffusion process is accompanied by a minor protein relaxation. In summary, these results indicate that the first and very fast step after the proton concentration jump represent an increased occupation of the SR Ca-ATPase binding sites with H⁺. Corresponding to the reaction scheme this process describes the P-E₂ Ca → P-E₂ Ca H branch or the P-E₂ → P-E₂ H branch depending on buffer pH and Ca²⁺ concentration.

As mentioned in the Result section the second process, described by \( \tau_{\text{kin,2}} \) and \( \Delta F_{\text{kin,2}} \), seems to be an artefact of the experimental setup. The activation energy 1.4 kJ/mol (Table 17, page 83) is too low to be related to an enzyme relaxation or a conformation change. Another evidence, which intensifies the assumption of a setup artefact, is the almost pH independent time constant and fluorescence amplitude. So these results may not be assigned to an enzyme reaction and have to be accepted as an experimental artefact.

The analysis of the third components \( \tau_{\text{kin,3}} \) and \( \Delta F_{\text{kin,3}} \) in the mathematical model show a higher correlation to the enzyme reaction steps. With increasing pH in the electrolyte the third time constant \( \tau_{\text{kin,3}} \) disappeared before reaching pH 7.1 and then reappeared at values above pH 7.2 with a decreasing fluorescence signal. The mathematical model shows a difficult determination with larger standard deviation for the time constant \( \tau_{\text{kin,3}} \) at this transition. Much more meaningful than the time constant is the fluorescence amplitude \( \Delta F_{\text{kin,3}} \), which is shown in Figure 43, page 75. The decreasing amplitude at increasing pH crosses the zero line at pH 7.1 and then settles down to an average of \(-0.9 \pm 0.2\ %\) between the physiological pH of 7.1 up to pH 8.5 at the endpoint of the plot. That behaviour indicates that at low pH a net charge release from the enzyme binding sites occurred and with increasing pH this release changes to a net charge binding. These findings correlate with the reaction scheme for the P-E₂ conformation of the SR Ca-ATPase. The SR Ca-ATPases were distributed between the P-E₂ Ca H, the P-E₂ H and the P-E₂ H₂ state of Scheme 1, at which the Ca²⁺ concentration of 20 µM shifts the equilibrium to the Ca²⁺ bound state under normal pH conditions. The pH jumps of about 0.1 units lead to an increased H⁺ concentration, which allows a Ca²⁺ H⁺ exchange in the protein binding site. The equilibrium is shifted slightly to the right in the reaction scheme P-E₂ Ca H ↔ P-E₂ H ↔ P-E₂ H₂ and the exchange of the divalent against the monovalent ion in a minor fraction of proteins results in a small fluorescence increase in this reaction step. For the third process an activation energy of 94.2 kJ/mol was determined (Table 17, page 83). This result and the measured pH-independent time constant \( \tau_{\text{kin,3}} = 208.3 \text{ ms} \pm 82.5 \text{ ms} \) are indicators for further determination of the reaction step. Both are evidence for a process of conformational rearrangements of the protein structure, due to the ion exchange and H⁺ binding in the ion binding sites. As shown in (Fibich, et al., 2007) a comparable activation energy and time constant was found in the E₁ state of the SR Ca-ATPase. These earlier experiments allowed the conclusion that an H⁺ binding in the E₁ state is followed by a minor conformational relaxation and the reaction sequence has to be enlarged with an
additional step. Transferred to the P-E₂ state of the SR Ca-ATPase the reaction scheme is now described by Scheme 2. The new step represents a rate-limiting structural rearrangement of the protein, which facilitates the binding of the second proton.

\[
\begin{align*}
P - E₂ Ca H & \leftrightarrow P - E₂ H \leftrightarrow P - E₂^* H \leftrightarrow P - E₂^* H₂
\end{align*}
\]

A look at the third component of the SR Ca-ATPase reaction after H⁺ jump experiments at higher pH showed that the equilibrium of the initial steady-state may be distributed between the P-E₂ Ca and the P-E₂ H states, depending on pH. At alkaliescent pH the P-E₂ conformation is a transient state of the protein. With excess Ca²⁺ ions in the buffer the most highly populated state will be the P-E₂ Ca conformation. Additional protons after an H⁺ concentration jump lead to monovalent cation binding in the protein and, therefore, to the observed small fluorescence decrease. The corresponding transition for the H⁺ binding is the P-E₂ Ca → P-E₂ Ca H step, which results in the initial state described in Scheme 2 above.

The last (fourth) component of the mathematical model for P-E₂ reaction scheme has a slow time constant of \( \tau_{\text{kin},4} = 3.32 \text{ s} \pm 0.30 \text{ s} \) and the fluorescence amplitude was \( \Delta F_{\text{kin},4} = -0.8 \pm 0.1 \% \) as showed in Chapter 3.3.6, page 72 and in the described Figure 42, page 74 and Figure 43, page 75. This time constant and fluorescence amplitude is almost pH-independent. The decreasing fluorescence amplitude indicates a minor additional cation binding in the protein which can be assigned to the H⁺ binding after the release from its inactive precursor. These minor changes in the fluorescence signal represent just a small increase of occupation of the ion access channel and the protein binding sites.

Further analysis of this reaction step showed high activation energy of 77.3 kJ/mol (Table 17, page 83). With this fact and with respect to the pH-independence and the extremely slow time constant \( \tau_{\text{kin},4} \) this reaction step could be assigned to another conformational relaxation of the SR Ca-ATPase. One possibility is the conformation transition from the E₂ state to the E₁ state of the SR Ca-ATPase. The transition \( E₂(H₂) \rightarrow E₁H₂ \) was already identified as a slow reaction step, which is associated with high activation energy (Fibich, et al., 2007).

In respect to the buffer conditions and the results from the measurements, a second explanation may be preferred. At low proton and Ca²⁺ concentrations no cations are bound in the binding sites of the SR Ca-ATPase. To stabilise the protein structure a minor conformational change lead to a new P-E₂(*) state, which is in agreement with an additional branch in the Post-Albers-cycle. Structural investigations (Toyoshima, et al., 2004 (a)), (Møller, 2010) described such behaviour of the SR Ca-ATPase and the result in this work and
(Fibich, et al., 2011) showed the kinetics properties for this expanded Post-Albers-cycle (Scheme 3) in the E_2 state of the protein.

Scheme 3:

\[
\begin{align*}
P - E_2^{(c)} \\
\uparrow \\
\ldots &\rightarrow P - E_2 Ca_2 \leftrightarrow P - E_2 Ca \leftrightarrow P - E_2 \\
\downarrow &
\downarrow \\
P - E_2 Ca H &\leftrightarrow P - E_2 H \leftrightarrow P - E_2 H_2 \rightarrow \ldots
\end{align*}
\]

4.2.2 Characterisation of the measured processes by their activation energies

The determination of the activation energies for each exponential function of the fit to the data has been used to determine the character of the underlying molecular processes. Generally, it can be stated that diffusion-controlled processes show low activation energies in the range of the thermal energy (< 9 kJ/mol). Binding to or release of the ions (or substrate molecules) from binding sites, processes that include coordination with complex moiety in the protein require a higher activation energy which is typically in the order of 15-25 kJ/mol. Rearrangements and relaxations of the enzyme structures need much more energy which has to be placed in the order of 30-100 kJ/mol. These well separated ranges of activation energies allow at least a preliminary classification of the processes which have been observed in the kinetics experiments with caged compounds (Table 14, page 69).

The process responsible for the second exponential function with the time constant \( \tau_{\text{kin},2} \) exhibited an activation energy of 1.4 kJ/mol which is even below the level expected for a simple diffusion process. Since a decrease in the fluorescence signal could be observed also in control experiments without \( \text{cg H}^+ \), it stands to reason to assign this component of an artefactual fluorescence signal not to a transport-related process in the SR Ca-ATPase. An adequate explanation is an UV-flash induced fluorescence transition. The high UV intensity may induce a transient modification of the chromophore of the styryl dye that leads to delayed, phosphorescence-like emission with time constants in the order of a few milliseconds.

In contrast, the processes represented by the exponential functions with the time constants \( \tau_{\text{kin},3} \) and \( \tau_{\text{kin},4} \) have significantly higher activation energies. The measurements provided activation energies of 94.2 kJ/mol (\( \tau_{\text{kin},3} \)) and 77.3 kJ/mol (\( \tau_{\text{kin},4} \)). These high energies underlying kinetic reactions are related to protein relaxations and rearrangements. With this
argument the observed third and fourth process was defined as rearrangement processes of the SR Ca-ATPase following the H⁺ concentration jump.

An exception described the activation energy of the first exponential process in the kinetics of the SR Ca-ATPase. The energy was determined to 12.4 kJ/mol. This value is neither clearly in the range of system constants or diffusion-controlled processes nor in the range of a substrate-binding reaction.

Therefore, further analysis were performed. In experiments without cg H⁺ also two fast processes could be observed. But the determined time constant \( \tau_{\text{kin,1}} \) was in the absence of cg H⁺ much shorter than in the experiments containing the cg H⁺. In the experiments with the SR Ca-ATPase and cg H⁺ the average of the values for \( \tau_{\text{kin,1}} \) was determined to 1.25 ms ± 0.16 ms, whereas the caged compound free samples showed time constants in the sub-millisecond range. This fact indicates that there are two fast processes which overlap each other and don’t allow a differentiated determination of one or the other process. So the activation energy determined for the first time constants describes a combination of the system dependent fast process and the protein correlated slower process.

For a detailed visualisation of the fast processes cg H⁺ experiments were compared with experiments without cg H⁺ at the same conditions of the electrolyte. A couple of identical measurements either with or without cg H⁺ were averaged for further analysis and normalised to the fluorescence level before the pH jump. Then the cg H⁺ free signal was subtracted from the cg H⁺ signal. For an easier comparison of the fluorescence level before the pH jump was set to 1.00 for all signals. These analysis were performed in both the E₁ and the P-E₂ state of the SR Ca-ATPase.

Further analysis of the difference signals gives evidence of the described fast binding process, observed in the P-E₂ experiments by the determination of the activation energy. A fast process with a time constant of 1 ms at 20 °C and an activation energy of 12.4 kJ/mol was observed. But the time constant of the SR Ca-ATPase specific reaction could not be separated from the limiting time constant of the UV-flash artefact which was inevitably present in all measurements, with and without cg H⁺. The analysis of the difference signal elucidates the presence of a fast specific fluorescence decrease that is concealed by the unspecific fluorescence artefact. The difference signal shows a fluorescence level of 98.5 ± 0.4 % at 1 ms corresponding to an initial fluorescence loss of 1.5 % during the sub-millisecond time span between the UV flash at \( t = 0 \) and 1 ms. A comparable decrease of the fluorescence level at 11.4 ms after the UV flash is obtained for the SR Ca-ATPase in the E₁ state. In the E₁ conformation an additional decreasing fluorescence amplitude with a slower kinetics (\( \tau \approx 10 \text{ ms} \)) than in the P-E₂ state became visible.
A fluorescence decrease indicates, according to the mechanism of the styryl dyes, an uptake of positive charge into the membrane domain of the SR Ca-ATPase, which has to be assigned to proton binding in either conformation, E₁ and P-E₂, in the case of pH jump experiments. The comparison of the results from both enzyme conformations reveals a sub-millisecond process. An obvious explanation for this fast process in the P-E₂ conformation is that the pH jump induces a right shift in the binding equilibrium of the reaction, like shown here in Scheme 4.

Scheme 4:

\[ P - E_2 + H_\text{luminal}^+ \leftrightarrow P - E_2 + H_\text{inside}^+ \leftrightarrow P - E_2 + H_\text{bound}^+ \]

The permeation of protons through the access channel from the surface of the protein membrane domain to the ion-binding sites is a fast diffusion process that explains a time constant in the sub-millisecond range. The subsequent second step describes the binding of the proton to a carboxylate group at the side chain of an amino acid in the binding site. This reaction would be expected to have an activation energy in agreement with the observed 12.4 kJ/mol. In the E₁ conformation a relaxation process of the SR Ca-ATPase was observed with a time constant \( \tau_{\text{kin,1}} \) of 9.02 ms ± 2.02 ms in addition to the initial, unresolved fluorescence decrease. This finding indicates that a rearrangement in the enzyme must be necessary for a small fraction of the proton binding. As was shown in a recent study, (Fibich, et al., 2007), in the E₁ conformation an equilibrium between several states exists, and the transition between some of these is slow since conformational relaxations are required before (additional) protons may bind. Those reaction steps may hold for the observed slower process. In the E₁ conformation activation energies were determined in the experiments with the SR Ca-ATPase in vesicular SR membranes (Fibich, et al., 2007) The most rapid process with a time constant \( \tau_{\text{kin,1}} \) of 8.3 ms ± 0.4 ms had an activation energy of 40.3 kJ/mol ± 5.7 kJ/mol. \( \tau_{\text{kin,1}} \) is in reasonable agreement with 9.02 ms ± 2.02 ms obtained in the experiments with the open membrane fragment preparation. In contrast, in the P-E₂ state proton binding after the pH jump is faster than 1 ms and independent from protein relaxation. With all these data it can be concluded, that this is an E₁ state specific relaxation process, which does not appear in the P-E₂ state of the SR Ca-ATPase. In the P-E₂ state the proton binding kinetics in the sub-millisecond range is not controlled by an energy consuming, huge rearrangement of the protein structure. Ion binding is a minor relaxation process in the protein structure with a high rate constant.

In summary all the results give new insights in the proton binding kinetics of the SR Ca-ATPase in the E₂ state of the Post-Albers-cycle, which allowed to define or refine new transition steps during the cation binding and release.
5 List of Abbreviations

<table>
<thead>
<tr>
<th>ABBREVIATION</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>2BITC</td>
<td>C_{22}H_{38}N_{3}BrS 1- [4-(isothiocyanato)-n-butyl]-4-[p-(N,N-diethylamino)-styryl]-pyridiniumbromide, electrochromic styryl dye</td>
</tr>
<tr>
<td>2HITC</td>
<td>C_{24}H_{32}N_{3}BrS 1- [6-(isothiocyanato)-n-butyl]-4-[p-(N,N-diethylamino)-styryl]-pyridiniumbromide, electrochromic styryl dye</td>
</tr>
<tr>
<td>A23187</td>
<td>Calmycin, Ca^{2+} ionophore</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate, C_{10}H_{16}N_{5}O_{10}P_{2}</td>
</tr>
<tr>
<td>Apyrase</td>
<td>A water-soluble ATPase, which hydrolyse ATP and ADP to AMP and inorganic phosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine diphosphate, C_{10}H_{16}N_{5}O_{13}P_{3}</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane- N,N,N’,N’-tetraacetic acid, C_{22}H_{24}N_{2}O_{10} Ca^{2+} ion specific chelator</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cg ATP</td>
<td>Caged ATP, see below: NPE-ATP, photo-activated ATP</td>
</tr>
<tr>
<td>cg Ca^{2+}</td>
<td>Caged Calcium, see below: DM-nitrophen, photo-activated Ca^{2+} ions</td>
</tr>
<tr>
<td>cg H^+</td>
<td>Caged Proton, see below: MNPS.Na, photo-activated protons</td>
</tr>
<tr>
<td>ChCl</td>
<td>Cholinchloride C_{3}H_{14}ClNO</td>
</tr>
<tr>
<td>d/p</td>
<td>Dye / protein ratio, molecules of styryl dye per protein molecules, For the calculation with the SR Ca-ATPase a molecular weight of 110 kDa was assumed.</td>
</tr>
<tr>
<td>DM-nitrophen</td>
<td>1-(2-Nitro-4,5-dimethoxyphenyl)-1,2-diaminoethane-N,N,N’,N’-tetraacetic acid, tetrasodium salt</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide, (CH_{3})_{2}SO</td>
</tr>
<tr>
<td>DOPC</td>
<td>Dioleyl phosphatidyl choline</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscope</td>
</tr>
<tr>
<td>ABBREVIATION</td>
<td>DESCRIPTION</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>EtOH</td>
<td>Ethanol, C₂H₆O</td>
</tr>
<tr>
<td>F42, (F42-H₃)</td>
<td>C₂₁H₂₉N₂Br new synthesised electrochromic styryl dye</td>
</tr>
<tr>
<td>F52, (F52-H₃)</td>
<td>C₂₂H₃₁N₂Br new synthesised electrochromic styryl dye</td>
</tr>
<tr>
<td>F52-O₂H</td>
<td>C₂₂H₂₉O₂N₂Br new synthesised electrochromic styryl dye</td>
</tr>
<tr>
<td>F62, (F62-H₃)</td>
<td>C₂₃H₃₃N₂Br new synthesised electrochromic styryl dye</td>
</tr>
<tr>
<td>F62-O₂H</td>
<td>C₂₃H₃₁O₂N₂Br new synthesised electrochromic styryl dye</td>
</tr>
<tr>
<td>F82, (F82-H₃)</td>
<td>C₂₅H₃₇N₂Br new synthesised electrochromic styryl dye</td>
</tr>
<tr>
<td>h</td>
<td>Planck constant, H = 6.626·10⁻³⁴ Js</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazinethansulfonic acid, C₈H₁₈N₂O₄S</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital, excited state of a molecule</td>
</tr>
<tr>
<td>Lac</td>
<td>Lactate, C₃H₆O₃</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LUMO</td>
<td>Lowest unoccupied molecular orbital, lowest electron-free orbital of a molecule</td>
</tr>
<tr>
<td>MNPS.Na</td>
<td>Methoxynitrophenolsulfate sodium salt</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid, C₇H₁₅NO₄S</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide, C₂₁H₂₇N₇O₁₄P₂</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide, C₂₃H₂₈N₇O₁₄P₂</td>
</tr>
<tr>
<td>NPE-ATP</td>
<td>Adenosine 5’triphosphate, P³⁻(1-(2-nitrophenyl)ethyl) ester disodium salt</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid, C₅H₆O₉P</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl choline</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidyl ethanolamine</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate, C₃H₅O₆P</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>PM</td>
<td>Photomultiplier</td>
</tr>
<tr>
<td>pp</td>
<td>Percentage points</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>Pyr</td>
<td>Pyruvate, C₃H₄O₃</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium lauryl sulfate, NaC₁₂H₂₅SO₄</td>
</tr>
<tr>
<td>SDS Ca-ATPase</td>
<td>Sarcoplasmic Calcium-ATPase after the treatment with SDS. The</td>
</tr>
<tr>
<td>ABBREVIATION</td>
<td>DESCRIPTION</td>
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<tr>
<td>microsomal membranes vesicles were opened and flat structures. Luminal a cytosolic sides of the SR Ca-ATPase are accessible without any restrictions.</td>
<td></td>
</tr>
<tr>
<td><strong>SDS-PAGE</strong></td>
<td>SDS polacryl amine gel electrophoresis</td>
</tr>
<tr>
<td><strong>SR</strong></td>
<td>Sarcoplasmic Reticulum</td>
</tr>
<tr>
<td><strong>SR Ca-ATPase</strong></td>
<td>Sarcoplasmic Calcium-ATPase in microsomal membranes obtained from the Sarcoplasmic Reticulum of the rabbit psoas muscle.</td>
</tr>
<tr>
<td><strong>TEM</strong></td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td><strong>TG</strong></td>
<td>Thapsigargin, C\textsubscript{34}H\textsubscript{50}O\textsubscript{12}, an SR Ca-ATPase-specific inhibitor</td>
</tr>
</tbody>
</table>
6 References


Thanks!

I would like to thank especially Prof. Dr. Hans-Jürgen Apell, his family and Claudia Hoffmann for their sustained support, Milena Roudna and Nadège Devaux for the excellent technical assistance and Karl Janko for the preparation of the cg H⁺ and the fluorescence dyes.

And of course I would like to thank all the colleagues in the groups of Prof. Dr. Kay Diederichs and Prof. Dr. Wolfram Welte at the University of Konstanz for their support and collaboration.

Last but not least I would like to thank my family.

“The searching eye finds often more than it desires to see.”
Curd von Stauffen, in Nathan the Wise, Gotthold Ephraim Lessing

“Der Blick des Forschers fand nicht selten mehr, als er zu finden wünschte.”
Curd von Stauffen, in Nathan der Weise, Gotthold Ephraim Lessing