Cyclopiazonic Acid Is Complexed to a Divalent Metal Ion When Bound to the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase*\(^{5}\)

Mette Laursen\(^{1,4,5}\), Maike Bublitz\(^{1,4,5}\), Karine Moncq\(^{4}\), Claus Olesen\(^{1,6}\), Jesper Vuust Møller\(^{1,3}\), Howard S. Young\(^{1,3}\), Poul Nissen\(^{5,6}\), and J. Preben Morth\(^{5,6}\)

From the \(^{1}\)Centre for Membrane Pumps in Cells and Disease–PUMPKIN, Danish National Research Foundation, and the \(^{2}\)Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10C, DK-8000 Aarhus C, Denmark, the \(^{3}\)Department of Biochemistry and National Institute for Nanotechnology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

We have determined the structure of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) in an E2-P-like form stabilized as a complex with Mg\(^{2+}\), an ATP analog, adenosine 5’-(β,γ-methylene)triphosphate (AMPPCP), and cyclopiazonic acid (CPA). The structure determined at 2.5 Å resolution leads to a significantly revised model of CPA binding when compared with earlier reports. It shows that a divalent metal ion is required for CPA binding through coordination of the tetratomic acid moiety at a characteristic kink of the M1 helix found in all P-type ATPase structures, which is expected to be part of the cation access pathway. Our model is consistent with the biochemical data on CPA function and provides new measures in structure-based drug design targeting Ca\(^{2+}\)-ATPases, e.g. from pathogens. We also present an extended structural basis of ATP modulation pinpointing key residues at or near the ATP binding site. A structural comparison to the Na\(^{+}\),K\(^{+}\)-ATPase reveals that the Phe\(^{93}\) side chain occupies the equivalent binding pocket of the CPA site in SERCA, suggesting an important role of this residue in stabilization of the potassium-occluded E2 state of Na\(^{+}\),K\(^{+}\)-ATPase.

The Ca\(^{2+}\)-ATPase from sarco(endo)plasmic reticulum of rabbit skeletal muscle (SERCA,\(^{5}\) isoform 1a) is a thoroughly studied member of the P-type ATPase family (1). SERCA possesses 10 transmembrane helices (M1 through M10) with both the N terminus and the C terminus facing the cytoplasmic side and three cytoplasmic domains, inserted in loops between M2 and M3 (A-domain) and between M4 and M5 (P- and N-domain) (2). The enzyme mediates the uptake of Ca\(^{2+}\) ions into the lumen of the sarcoplasmic reticulum (SR) after their release into the cytoplasm through calcium release channels during muscle contraction (3). SERCA, plasma membrane Ca\(^{2+}\)-ATPase, and a third, Golgi-located secretory pathway Ca\(^{2+}\)-ATPase are important factors in calcium and manganese homeostasis, transport, signaling, and regulation (4, 5).

Crystal structures of all major states in the reaction cycle of SERCA have been determined. These include the Ca\(^{2+}\)E\(^{1}\)P\(^{+}\)ATP state (6, 7) with high affinity Ca\(^{2+}\) binding sites accessible from the cytoplasmic side of the SR membrane, the calcium-occluded Ca\(^{2+}\)E\(^{1}\)ADP\(^{2-}\)AlF\(^{4-}\) transition state (6), the open E2P state with luminal facing ion binding sites that have low affinity for Ca\(^{2+}\) and high affinity for protons (8) and the proton-occluded H\(^{2-}\)E\(^{2}\)[ATP] state with a bound modulatory ATP (9). This considerable amount of structural information has turned the Ca\(^{2+}\)-ATPase into a valuable model system for studies on structural rearrangements that take place during the catalytic cycle of P-type ATPases. SERCA is considered a promising drug target in medical research, with a particular focus on prostate cancer and infectious diseases. Several compounds have already been shown to bind and inhibit SERCA by stabilizing the enzyme in a particular conformational state. Thapsigargin (TG), cyclosporinic acid (CPA), and 2,5-di-(tert-butyl)hydroquinone (BHQ) stabilize an E2-like state, and 1,3-dibromo-2,4,6-tri (methylisothiouronium)benzene stabilizes an E1-P-like conformation (10–13). CPA is a toxic indole tetraacetic acid first isolated from Penicillium cyclopium (14) and later found to be produced by Aspergillus versicolor and Aspergillus flavus. Like TG, CPA specifically binds to and inhibits SERCA with nanomolar affinity (15). Indeed, CPA is widely used in biochemical and physiological studies on Ca\(^{2+}\) signaling and muscle function, where it causes Ca\(^{2+}\) store depletion due to specific inhibition of Ca\(^{2+}\) reuptake by SERCA. CPA and TG were...
The SERCA-CPA Complex

originally proposed to bind to similar sites on SERCA (16), but recent crystal structures have shown a distinct site of interaction (17, 18). Despite these structural insights, a previously demonstrated magnesium dependence of CPA binding (19) remained unexplained, and opposing CPA binding modes were observed (see below).

Tetramic acids are synthesized naturally, and more than 150 natural derivatives have been isolated from bacterial and fungal species (reviewed in Ref. 20). Tetramic acids possessing a 3-acyl group have the ability to chelate divalent metal ions. For instance, tenuazonic acid from the fungus Phoma sorghina has been shown to form complexes with Ca\(^{2+}\) and Mg\(^{2+}\) (21), as well as heavier metals such as Cu(II), Ni(II), and Fe(III) (22).

Previously published crystallographic structures of the SERCA-CPA complex (PDB ID 2O9J and 2EAS) demonstrated that CPA binds within the proposed calcium access channel of SERCA. However, the structures did not reveal a role for magnesium, and the orientation of CPA within this binding site differed in the two studies (17, 18). To address these ambiguities, we have determined the crystal structure of SERCA in complex with Mg\(^{2+}\), AMPPCP (an ATP analog), and Mn\(^{2+}\)-CPA. The structure reveals novel insight into CPA binding, which we find to be mediated by a divalent cation, as demonstrated by means of the anomalous scattering properties of Mn\(^{2+}\). Further and improved refinement using previously deposited data (PDB ID 2O9J and 2OAO), in light of our new findings, also revealed a strong plausibility for a magnesium ion bound at this site. Furthermore, we find a new configuration of the bound AMPPCP nucleotide, addressing the modulatory role of ATP binding to the E2-P\(_i\) occluded conformation of SERCA.

EXPERIMENTAL PROCEDURES

Protein Preparation and Crystallization—SERCA1a was prepared from SR vesicles isolated from rabbit fast-twitch skeletal muscle (SERCA1a) and purified by extraction with deoxycholate, according to established protocols (23). To produce E2-stabilized protein, the purified membranes were solubilized in 20 mg/ml octaethyleneglycol mono-\(n\)-dodecylether (C\(_\text{12}\)E\(_\text{8}\)) in 85 mM MOPS-KOH (pH 6.8), 67 mM KCl, 17% glycerol (v/v), 1.5 mM EGTA, 2.8 mM MgCl\(_2\), 1.0 mM NaF, 1.0 mM AMPPCP, and 0.2 mM CPA. The solubilization was followed by ultracentrifugation, and the supernatant, with a protein concentration of ~12 mg/ml, was used directly for crystallization experiments by the vapor diffusion method in hanging drops. Protein solution (80% supernatant, 20% 5 mM dithiothreitol) and crystallization buffer (14% polyethylene glycol 6000, 6% 2-methyl-2,4-pentanediol, 70 mM sodium acetate (pH 6.8), 10 mM MnCl\(_2\)) were mixed in a ratio 1:1 and supplemented with 3 mM Zwittergent 3-12 as an additive. Large, single triangular-shaped crystals grew over 2 weeks at 19°C. Crystals were cryoprotected by soaking in 20—30% ethylene glycol. The crystals were mounted in litholoops (molecular dimensions) and flash-cooled in liquid nitrogen.

Data Collection and Refinement—Diffraction data were collected at 100 K on the end stations X06SA at the Swiss Light Source (SLS) in Villigen, Switzerland and at I1911-3 at MAX-lab in Lund, Sweden. The diffraction data were processed and scaled with XDS (24). Phases were obtained by molecular replacement using the program PHASER (25) and a search model of the Ca\(^{2+}\)-ATPase in the E2-Mg\(^{2+}\)–(CPA) form (PDB ID 2O9J). Model building was performed using Coot (26), and model refinement was performed with phenix.refine (27) for all models. For anomalous map calculation and reflection file handling, programs from the CCP4 package were used (28). All structural figures in this study were prepared with PyMOL (DeLano Scientific, Palo Alto, CA).

RESULTS

Indications for the Presence of a Divalent Cation—Early studies on fungal toxins had demonstrated that the tetramic acids tend to occur naturally as metal-chelate complexes (29). This was confirmed in a recent biochemical experiment demonstrating CPA binding to SERCA in an Mg\(^{2+}\)-dependent manner (19). There is a striking discrepancy from previous reports on how CPA is oriented in its binding pocket within the putative calcium entry channel in SERCA. A structure by Toyoshima and co-workers (18) shows the tetramic acid moiety of CPA in a buried position, pointing toward transmembrane segment M3 of SERCA (PDB entry 2EAS), whereas the Young group (17) has presented a structure with the tetramic acid group pointing outwards toward transmembrane segment M2 of SERCA (PDB entry 2O9J). The two structural models represent inhibitor orientations deviating by roughly 180°. Being interested in identifying the correct binding mode of CPA to SERCA and intrigued by the absence of a divalent cation and proper planarity of the tetramic acid moiety in both structures, we first resorted to further refinement using the structure factor amplitudes deposited by the Young group (17). Data from the Toyoshima group (18) were not available. We found the 2O9J structure to be substantiated by unbiased omit maps, but we also noted a strong positive peak in the Fo — Fc map located at the position of a water molecule (numbered 2074 in PDB ID 2O9J) coordinated by O1 and O2 of the tetramic acid moiety of CPA, and furthermore, noted that it displayed a substantially lower B-factor when compared with the oxygens of the tetramic acid (~55 versus ~90 Å\(^2\)). Given the recently demonstrated magnesium dependence of CPA inhibition (19) and the metal-chelating properties of tetramic acids, we suspected that a divalent cation might occupy this position. This assumption was supported by the fact that exchanging the water molecule with Mg\(^{2+}\) and re-refining the model led to improved B-factor consistency while also eliminating the positive peak of the difference map. However, to resolve this issue with independent crystallographic data, we identified crystallization conditions for the E2-Mg\(^{2+}\)–AMPPCP-CPA-Mn\(^{2+}\) complex in the presence of manganese, with the aim of replacing the suspected Mg\(^{2+}\) at the CPA site by Mn\(^{2+}\) displaying anomalous scattering properties.

Crystallization of SERCA-CPA in the Presence of Mn\(^{2+}\)—The crystals were obtained by screening against an in-house screen (30) with sparse matrix optimization in 24-well vapor diffusion hanging drop format followed by a detergent optimization procedure developed for the Na\(^+\),K\(^+-\)ATPase crystals (31). SERCA stabilized in the E2-Mg\(^{2+}\)–AMPPCP-CPA-Mn\(^{2+}\) form crystallized in a new crystal form exhibiting P\(_2\)\(_2\)\(_2\) space group symmetry with two...
The SERCA-CPA Complex

The structure was readily determined by molecular replacement using the structure of the Young group (17) (PDB ID 2O9J, now 3FPB) as a search model with CPA omitted.

To investigate whether Mn$^{2+}$ was bound, data were collected close to the manganese absorption edge at an energy of 7.74 keV (wavelength 1.6 Å). Several strong peaks were observed in the anomalous difference Fourier map at equivalent positions in both molecules of the asymmetric unit. A major peak at the CPA binding pocket corresponding to the suspected Mg$^{2+}$ site at the tetramic acid, another peak between the α- and β-phosphates of the AMPPCP molecule in the nucleotide binding site between the N-, A-, and P domains (Fig. 1, A and B, and supplemental Fig. 1), and a peak in the P-domain K$^+$ site indicating a bound K$^+$ (supplemental Fig. 2) were found. Remaining peaks appeared at the positions of well ordered sulfur atoms.

The Architecture of the CPA Binding Pocket—In agreement with the previously presented models, we find that the CPA binding pocket is located at a groove formed between transmembrane segments M1, M2, M3, and M4 of SERCA (Fig. 1, A and C). The quality of the electron density maps allowed us to unambiguously determine an orientation of the CPA molecule in accordance with the model of the Young group (17). The hydrophobic indole group of CPA thus sits in a wide hydrophobic groove between M3 and M4, whereas the tetramic acid moiety is involved in a network of polar interactions with residues on M1 and M2. The Mn$^{2+}$ ion has replaced the probable Mg$^{2+}$ located at this site in the previous structures (Figs. 1C and 2A). Gln$^{56}$-Oe1 is the only side chain directly linked to the Mn$^{2+}$ ion, whereas backbone carbonyls from Gln$^{56}$, Asp$^{95}$, and Asn$^{101}$ coordinate two water molecules within the CPA binding pocket in accordance with the model of the Young group (17).

molecules in the asymmetric unit. The crystals display favorable diffraction properties with low mosaic spread (as low as 0.07°), and a 2.5 Å resolution data set was collected (supplemental Table 1).

The structure of the Young group (17) (PDB ID 2O9J, now 3FPB) as a search model with CPA omitted.

To investigate whether Mn$^{2+}$ was bound, data were collected close to the manganese absorption edge at an energy of 7.74 keV (wavelength 1.6 Å). Several strong peaks were observed in the anomalous difference Fourier map at equivalent positions in both molecules of the asymmetric unit. A major peak at the CPA binding pocket corresponding to the suspected Mg$^{2+}$ site at the tetramic acid, another peak between the α- and β-phosphates of the AMPPCP molecule in the nucleotide binding site between the N-, A-, and P domains (Fig. 1, A and B, and supplemental Fig. 1), and a peak in the P-domain K$^+$ site indicating a bound K$^+$ (supplemental Fig. 2) were found. Remaining peaks appeared at the positions of well ordered sulfur atoms.

The Architecture of the CPA Binding Pocket—In agreement with the previously presented models, we find that the CPA binding pocket is located at a groove formed between transmembrane segments M1, M2, M3, and M4 of SERCA (Fig. 1, A and C). The quality of the electron density maps allowed us to unambiguously determine an orientation of the CPA molecule in accordance with the model of the Young group (17). The hydrophobic indole group of CPA thus sits in a wide hydrophobic groove between M3 and M4, whereas the tetramic acid moiety is involved in a network of polar interactions with residues on M1 and M2. The Mn$^{2+}$ ion has replaced the probable Mg$^{2+}$ located at this site in the previous structures (Figs. 1C and 2A). Gln$^{56}$-Oe1 is the only side chain directly linked to the Mn$^{2+}$ ion, whereas backbone carbonyls from Gln$^{56}$, Asp$^{95}$, and Asn$^{101}$ coordinate two water molecules within the CPA binding pocket in accordance with the model of the Young group (17).

molecules in the asymmetric unit. The crystals display favorable diffraction properties with low mosaic spread (as low as 0.07°), and a 2.5 Å resolution data set was collected (supplemental Table 1).
The SERCA-CPA Complex

The Nucleotide Binding Site—In both structures (PDB ID 3FGO and 3FPB), the nucleotide is not fully occupied, confirming an antagonistic effect of CPA against ATP binding, which has already been observed in earlier biochemical studies on CPA-SERCA interaction (10, 34). These are the first structures with AMPPCP present in the H⁺-E2-P(CPA) state, with the liberated phosphate group mimicked by MgF⁴⁻. We find this configuration of the bound AMPPCP to be different from the modulatory AMPPCP in the H⁺-E2(TG) state, which is the dephosphorylated state, where the α- and β-phosphates are bridged via a magnesium ion to Glu⁶³⁹ (9). The AMPPCP is, however, in an equivalent conformation as in H⁺-E2-AlF⁴⁻T(G) (8), but with our improved resolution of 2.5 Å, we are able to make a more accurate description of the nucleotide binding site. In our structure, the γ-phosphate of the AMPPCP is in direct hydrogen-bonding distance to Lys²⁰⁵-N(H). Mutational studies on Lys²⁰⁵ confirm a modulatory effect of ATP on SERCA (Fig. 1B) (35). Additional residues in close contact to the γ-phosphate are Arg⁶⁷⁸ and Asn⁶²⁸ in the P-domain. Arg⁶⁷⁸ is coordinating a water molecule (Fig. 1B, W4) that is bifurcated by the γ-phosphate, whereas Asn⁶²⁸ and Asp²⁰³ make hydrogen bonds to Arg⁶⁷⁸, presumably stabilizing its conformation Arg⁶⁷⁸ (Fig. 1B). Arg⁶⁷⁸ is important for modulation of the ATP in the E2 to E1 transition (9). Mutation of Arg⁶⁷⁸ has been shown not to affect the low affinity ATP site in this particular functional transition state and is indeed found not to interact with the AMPPCP (36). Although Arg⁶⁷⁸ has been proposed to be involved with the modulatory effect of ATP in the E2-P, occluded state and E2 state (37), Asp²⁰³ has been proposed to have influence on the transport rate and ATP hydrolysis (23, 38). Thus, our structural data confirm Asp²⁰³ as an important stabilizing residue of Arg⁶⁷⁸. It is interesting that the stabilization of Arg⁶⁷⁸ is also mediated by a direct hydrogen bond from Asn⁶²⁸. This stabilizing triplet is also evident in the occluded H⁺-E2-AlF⁴⁻T(G) stabilized structure (8), but no water molecules were visible in the electron density. The interaction to the γ-phosphate is mediated through a hydrogen-bonding network including only water (Fig. 1B) but no direct hydrogen bonds. A direct hydrogen bond is formed between Glu⁴³⁹ and Arg⁶⁷⁸. The residues Lys⁴⁹² and Phe⁴⁷⁸ have both been shown to have a catalytic and a modulatory role (supplemental Fig. 1) (39), and they are both within van der Waals distance (<3.5 Å) of the ATP analog. The modulatory effect observed on Ile¹⁸⁸ (35) is not apparent. However, Ile¹⁸⁸ is within van der Waals distance of the AMPPCP. This structure allows for the first accurate structural characterization of the bound ATP and especially of the water-mediated hydrogen-bonding network around the γ-phosphate in the modulatory state prior to dephosphorylation (E2P → E2).

of CPA, the Gln⁵⁶ side chain of SERCA, and three water molecules. The top water molecule (Fig. 1C, W1) is coordinated by the carbonyl oxygen from Asn¹⁰¹ and Gln⁵⁶-Oe. The bottom water molecule (W2) is bifurcated between the carbonyl oxygen of Gln⁵⁶ and Oδ2 from Asp⁵⁹. The tetracatic acid is likely to be in the non-protonated state (pKₐ ~ 3) (32), with the negative charge distribution polarized toward the Mn²⁺ ion. The 9-carbonyl and the tertiary 8-amino group of CPA are both within weak hydrogen-bonding distance (3.6 Å) to Asn¹⁰¹-Nδ. The hydrogen on Asn¹⁰¹-NδH may act as hydrogen donor to the conjugated π-acceptor system above the tetracatic ring. This type of π-HX interaction (where HX is any hydrogen bond donor) is often observed in protein structures (33), with the indole ring of a tryptophan acting as the acceptor. It is unlikely that a hydrogen bond between Asp²⁵⁴-Oe and the indole nitrogen (position N-2) of CPA exists given their approximate distance of 5.4 Å (Fig. 2A). For a thorough description of the hydrophobic binding pocket for the indole group of CPA, please refer to Moncoq et al. (17).

DISCUSSION

The Nucleotide Binding Site—In both structures (PDB ID 3FGO and 3FPB), the nucleotide is not fully occupied, confirming an antagonistic effect of CPA against ATP binding, which has already been observed in earlier biochemical studies on CPA-SERCA interaction (10, 34). These are the first structures with AMPPCP present in the H⁺-E2-P(CPA) state, with the liberated phosphate group mimicked by MgF⁴⁻. We find this configuration of the bound AMPPCP to be different from the modulatory AMPPCP in the H⁺-E2(TG) state, which is the dephosphorylated state, where the α- and β-phosphates are bridged via a magnesium ion to Glu⁶³⁹ (9). The AMPPCP is, however, in an equivalent conformation as in H⁺-E2-AlF⁴⁻T(G) (8), but with our improved resolution of 2.5 Å, we are able to make a more accurate description of the nucleotide binding site. In our structure, the γ-phosphate of the AMPPCP is in direct hydrogen-bonding distance to Lys²⁰⁵-N(H). Mutational studies on Lys²⁰⁵ confirm a modulatory effect of ATP on SERCA (Fig. 1B) (35). Additional residues in close contact to the γ-phosphate are Arg⁶⁷⁸ and Asn⁶²⁸ in the P-domain. Arg⁶⁷⁸ is coordinating a water molecule (Fig. 1B, W4) that is bifurcated by the γ-phosphate, whereas Asn⁶²⁸ and Asp²⁰³ make hydrogen bonds to Arg⁶⁷⁸, presumably stabilizing its conformation Arg⁶⁷⁸ (Fig. 1B). Arg⁶⁷⁸ is important for modulation of the ATP in the E2 to E1 transition (9). Mutation of Arg⁶⁷⁸ has been shown not to affect the low affinity ATP site in this particular functional transition state and is indeed found not to interact with the AMPPCP (36). Although Arg⁶⁷⁸ has been proposed to be involved with the modulatory effect of ATP in the E2-P, occluded state and E2 state (37), Asp²⁰³ has been proposed to have influence on the transport rate and ATP hydrolysis (23, 38). Thus, our structural data confirm Asp²⁰³ as an important stabilizing residue of Arg⁶⁷⁸. It is interesting that the stabilization of Arg⁶⁷⁸ is also mediated by a direct hydrogen bond from Asn⁶²⁸. This stabilizing triplet is also evident in the occluded H⁺-E2-AlF⁴⁻T(G) stabilized structure (8), but no water molecules were visible in the electron density. The interaction to the γ-phosphate is mediated through a hydrogen-bonding network including only water (Fig. 1B) but no direct hydrogen bonds. A direct hydrogen bond is formed between Glu⁴³⁹ and Arg⁶⁷⁸. The residues Lys⁴⁹² and Phe⁴⁷⁸ have both been shown to have a catalytic and a modulatory role (supplemental Fig. 1) (39), and they are both within van der Waals distance (<3.5 Å) of the ATP analog. The modulatory effect observed on Ile¹⁸⁸ (35) is not apparent. However, Ile¹⁸⁸ is within van der Waals distance of the AMPPCP. This structure allows for the first accurate structural characterization of the bound ATP and especially of the water-mediated hydrogen-bonding network around the γ-phosphate in the modulatory state prior to dephosphorylation (E2P → E2).
The Role of the Divalent Cation—We find that a divalent metal (Mg\(^{2+}\) or Mn\(^{2+}\)) is important for CPA interaction with SERCA, forming a SERCA-CPA-M\(^{2+}\) complex. The Mg\(^{2+}\)/Mn\(^{2+}\) ion bound with CPA also interacts with residues of SERCA lining the putative ion access channel between M1 and M2 at the cytoplasmic membrane interface. This may hint at a transient Ca\(^{2+}\) site important for selectivity in the entry channel. An earlier finding that Ca\(^{2+}\) can compete with CPA and prevent inhibition, if added to SERCA before CPA (34), makes it tempting to speculate that the CPA-Mn\(^{2+}\) site indeed mimics a transient Ca\(^{2+}\) site along the entry pathway. It has been proposed based on transport experiments that CPA is able to uncouple ATP hydrolysis in SERCA in a Ca\(^{2+}\)-dependent manner (40). These studies concluded that only one Ca\(^{2+}\) ion is bound in the presence of CPA. However, our present data suggest that under appropriate conditions, a Ca\(^{2+}\) ion could be stabilized in the entry channel with CPA, corresponding to the single Ca\(^{2+}\) ion detected (40). Metal chelation by the tetrameric acid seems to be important for transport across membranes in biological tissues (20), and presumably, CPA may form a stable complex with a range of other divalent cations. The residues that are structurally important for CPA binding are conserved (supplemental Table 2). Homology models of two Ca\(^{2+}\)-ATPases from *Plasmodium falciparum* (causative of malaria), pfATP4 and pfATP6, and of a putative Ca\(^{2+}\)-ATPase from *Mycobacterium tuberculosis* (MTB) Locus tag: Rv1997 were generated with 3FGO as template, and all indicate that the residues of mode of CPA binding is possible. In particular, the residues Gln\(^{56}\) and Asn\(^{101}\) that we find to be important for binding of the tetrameric acid moiety are conserved. In support of such arguments, CPA inhibition has been observed for pfATP4 (41) and the Ca\(^{2+}\)-ATPase LCA1 from tomato (*Solanum lycopersicum*) (42).

Fragment-based Drug Design—CPA and BHQ bind to the same region in the entry channel. Despite the overall differences in the helical arrangement of M3 and M4, the cyclic ring systems of both BHQ and the indole moiety in CPA are accommodated at the same binding pocket (Figs. 1D and 2B). The binding pocket seems to accommodate three distinct interaction sites, with selectivity for specific chemical groups. First, the tetrameric acid binds near the access channel entry site between M1 and M2, where Gln\(^{56}\) (M1) participates directly in metal coordination and Asp\(^{59}\) (M1) and Asn\(^{101}\) (M2) coordinate water molecules that interact with the metal (Fig. 2A). Second, a hydrophobic pocket provides selectivity for chemical groups with conjugated π-systems represented by the indole moiety of CPA and the hydroquinone of BHQ (Fig. 2B). Third, an extension of the same hydrophobic binding pocket between M3 and M5 can accommodate aliphatic moieties, as was demonstrated when the butanoyl group on O-8 of TG was replaced with an N-tert-butoxycarbonyl-12-aminododecanoyl (Boc12-ADT) group. The TG-Boc12-ADT derivative had similar inhibitory properties as TG itself, and the crystal structure of SERCA with TG-Boc12-ADT revealed the presence of the Boc12-ADT group extending from the TG binding side between helices M3, M5, and M7 into the binding pocket occupied by BHQ and CPA (43) (Fig. 1D). These observations invite new drug development strategies for derivatizing the indole group of CPA with the addition of aliphatic groups extending toward the TG binding pocket (Fig. 2B) as one possible strategy to gain specificity for specific Ca\(^{2+}\)-ATPase targets.

Comparison with Na\(^{+}\),K\(^{+}\)-ATPase Indicates a Structural Importance of Phe\(^{93}\)—The recently determined crystal structure of the potassium-bound Na\(^{+}\),K\(^{+}\)-ATPase was stabilized by MgF\(^{2-}\), thus in an E2-P, like state presented here for SERCA. A structural alignment of the Na\(^{+}\),K\(^{+}\)-ATPase and SERCA was performed on transmembrane helices 1, 2, and 4 that define the CPA binding site. The structural alignment offers a clear explanation as to why CPA is unable to inhibit Na\(^{+}\),K\(^{+}\)-ATPase and the closely related H\(^{+}\),K\(^{+}\)-ATPase (10). Residues in the kink region of the M1 helix protrude into the equivalent site of the CPA binding pocket, preventing CPA and the related BHQ from binding due to sterical hindrance. The F95Y motif of the M1 is specific for the Na\(^{+}\),K\(^{+}\)-ATPase, and it was well defined from the experimental electron density map of the Na\(^{+}\),K\(^{+}\)-ATPase structure (31). The presence of the aromatic Phe\(^{93}\) side chain at the M1 kink binding pocket replacing the hydroquinone of BHQ or the indole moiety of CPA indicates that the pocket has a preference for compounds containing a conjugated π-system. We speculate that this pocket is of functional importance in the Na\(^{+}\),K\(^{+}\)-ATPase, possibly in an autoregulatory mechanism where Phe\(^{93}\) prevents other ions and water from entering the buried ion binding sites. The corresponding residue in rat has been investigated by mutagenesis (Phe\(^{95}\)) and was shown to be essential because COS cells expressing the mutants F95A and F95R were not viable, whereas the F95Y and F95L mutants showed decreased sodium affinity (44). The role of the two glycines leading up to Phe\(^{93}\) have also been investigated by mutagenesis, indicating that both residues are important for Na\(^{+}\) and K\(^{+}\) binding, and thus, their mutation prevents proper reverse transition into the E2P state (45). We now offer a unifying model for these mutagenesis data guided by the finding that a pocket with similar chemical characteristics exists in both SERCA and Na\(^{+}\),K\(^{+}\)-ATPase. This observation points to Phe93 of Na\(^{+}\),K\(^{+}\)-ATPase as an intramolecular determinant of E2 stabilization where the aromatic phenyl group blocks the cytoplasmic access channel by a mechanism reminiscent of CPA or BHQ inhibition of SERCA. This further suggests that the Mn\(^{2+}\) ion trapped in the CPA complex with SERCA may have pinpointed a pre-entry site for Ca\(^{2+}\) ions centered on Gln\(^{96}\) at the water-membrane interface.

Acknowledgments—We express gratitude for beam line support by Thomas Ursby (MAX-lab) and Clemens Schulze-Briese, Anuschka Fauluhn, and Rouven Bingel-Erlenmeyer (Swiss Light Source) for making our experiments possible and to Anne-Marie Lund Winther and Anna Marie Nielsen for valuable discussions and technical support.

REFERENCES

4. Van Baalen, K., Dode, L., Vanoevelen, J., Callewaert, G., De Smedt, H.,...
The SERCA-CPA Complex


Cyclopiazonic Acid Is Complexed to a Divalent Metal Ion When Bound to the Sarcoplasmic Reticulum Ca$^{2+}$-ATPase
Mette Laursen, Maike Bublitz, Karine Moncoq, Claus Olesen, Jesper Vuust Møller, Howard S. Young, Poul Nissen and J. Preben Morth

J. Biol. Chem. 2009, 284:13513-13518. doi: 10.1074/jbc.C900031200 originally published online March 16, 2009

Access the most updated version of this article at doi: 10.1074/jbc.C900031200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2009/03/17/C900031200.DC1

This article cites 45 references, 15 of which can be accessed free at http://www.jbc.org/content/284/20/13513.full.html#ref-list-1