The Molecular Basis for Cyclopiazonic Acid Inhibition of the Sarcoplasmic Reticulum Calcium Pump*

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The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase is essential for calcium reuptake in the muscle contraction-relaxation cycle. Here we present structures of a calcium-free state with bound cyclopiazonic acid (CPA) and magnesium fluoride at 2.65 \(\AA\) resolution and a calcium-free state with bound CPA and ADP at 3.4 \(\AA\) resolution. In both structures, CPA occupies the calcium access channel delimited by transmembrane segments M1–M4. Inhibition of Ca\(^{2+}\)-ATPase is stabilized by a polar pocket that surrounds the tetrameric acid of CPA and a hydrophobic platform that cradles the inhibitor. The calcium pump residues involved include Gln\(^{56}\), Leu\(^{61}\), Val\(^{62}\), and Asn\(^{101}\). We conclude that CPA inhibits the calcium pump by blocking the calcium access channel and immobilizing a subset of transmembrane helices. In the E2(CPA) structure, ADP is bound in a distinct orientation within the nucleotide binding pocket. The adenine ring is sandwiched between Arg\(^{480}\) of the nucleotide-binding domain and Arg\(^{678}\) of the phosphorylation domain. This mode of binding conforms to an adenine recognition motif commonly found in ATP-dependent proteins.

The sarcoplasmic reticulum (SR)\(^{2}\) Ca\(^{2+}\)-ATPase is the best characterized member of the P-type ATPases. The members of this family convert ATP into a high energy aspartylphosphate, which is subsequently used in the concentrative transport of cations across the membrane. The Ca\(^{2+}\)-ATPase moves calcium out of the cytosplam in a process that is central to the use of this cation as an intracellular signaling molecule. During the muscle contraction-relaxation cycle, the recovery phase relies on transport of calcium into the SR by the action of Ca\(^{2+}\)-ATPase. In the last 7 years, we have experienced remarkable insights into the Ca\(^{2+}\)-ATPase structure and mechanism of transport (1–7). The Ca\(^{2+}\)-ATPase is an integral membrane protein of 994 amino acids that consists of three cytoplasmic domains containing the ATP binding site and ten transmembrane helices (M1–M10) containing the two calcium binding sites. A phosphorylation domain (P domain) is coupled to the calcium sites through transmembrane helices M4 and M5 and contains the invariant Asp\(^{351}\) residue. A nucleotide binding domain (N domain) is connected to the P domain and contains the nucleotide binding pocket. An actuator domain (A domain) is connected to the M1–M3 helices and communicates the conformational rearrangements that regulate the binding and release of calcium. At present, we have a sophisticated understanding of calcium transport (8–10).

The reaction cycle of Ca\(^{2+}\)-ATPase is characterized by calcium-bound E1 and calcium-free E2 forms. The phosphorylated calcium-free form (E2-P) is a low energy state following a conformational change from a high energy phosphorylated form (E1-P). The energy captured from ATP is released in the concentrative transport of two calcium ions into the SR. Subsequent hydrolysis of the phosphorylated enzyme results in the formation of a protonated ground state. This E2 ground state is ready to countertransport protons, bind calcium and ATP, and begin the cycle anew. In the transition from the E2-P to the E2 state, modest domain movements serve to open a cavity around Asp\(^{351}\), allowing the hydrolysis and release of inorganic phosphate. The potential drawbacks associated with these “static” images from crystallographic studies include structural changes caused by the inhibitors and nucleotide analogues used to stabilize the different conformational states of the enzyme. For instance, an electron cryomicroscopy structure originally visualized an exit pathway from the calcium transport sites to the lumen of the SR (11, 12), and this channel was postulated to close in the presence of an inhibitor (13, 14). However, a luminal exit channel has not been observed in the available crystal structures (4, 6). This has recently been addressed in a biochemical study (15), which demonstrated that luminal access to the calcium binding sites is dependent on the inhibitors and/or phosphate analogues used to stabilize the complex.

Despite these complications, the inhibitors and substrate analogs are necessary to stabilize calcium-free conformations for crystallization. To prevent calcium-free forms from denaturing, Ca\(^{2+}\)-ATPase has been crystallized with the inhibitors thapsigargin (TG) (2, 4, 6) and 2,5-di-tert-butyl-1,4-dihydrobenzene (BHQ) (5). In addition, the enzyme has been further stabilized by phosphate analogs, such as aluminum fluoride (6).
and magnesium fluoride (4). Cyclopiazonic acid (CPA) is another specific inhibitor of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases, yet the structure of this inhibitory complex is unknown. Comparatively, CPA and TG have dissociation constants in the nanomolar range, although CPA has a 1000-fold lower affinity (\(K_d = 120\) nM) than TG (\(K_d = 0.1\) nM) (16–19).

CPA is a toxic indole tetracyclic acid produced by certain fungi (Penicillium or Aspergillus) (20) that are found as contaminants in foods (21). Inhibitor binding occurs for the calcium-free conformations of the enzyme (17), and the inhibitory mechanism may be similar to that used by TG. Mutagenesis studies suggest that the CPA and TG binding sites on Ca\(^{2+}\)-ATPase are distinct with some possible overlap (18, 22). CPA inhibits all sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases but does not affect other cation pumps, such as the Na\(^{+}\)/K\(^{+}\)-ATPase, the gastric H\(^{+}\)/K\(^{+}\)-ATPase pump, and the plasma membrane calcium pump (16).

We have determined the structure of Ca\(^{2+}\)-ATPase in the presence of CPA. Herein, we describe crystal structures of calcium-free conformations of Ca\(^{2+}\)-ATPase solved to 2.65 Å resolution with bound CPA and magnesium fluoride and solved to 3.4 Å resolution with bound CPA and ADP. These structures provide a molecular description of CPA inhibition. In addition, a distinct mode of adenine recognition is identified in the E2 calcium-free conformation with bound ADP.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Crystallization**—Ca\(^{2+}\)-ATPase was prepared from sarcoplasmic reticulum vesicles isolated from rabbit hind leg muscle (23) and purified by Reactive-Red affinity chromatography after solubilization in octaethyleneglycol mono-n-dodecylether (C\(_{12}\)E\(_{8}\)) as described previously (24). All Ca\(^{2+}\)-ATPase preparations used for crystallization trials contained 10 mM ADP as a result of the Reactive-Red affinity chromatography elution buffer. Crystals were grown by hanging drop vapor diffusion at 11 °C using 4 mM EGTA, 0.2 mM Na\(_2\)EDTA, 0.02% NaN\(_3\), and 20 mM MES, pH 6.1. Crystals grew between 3 and 8 days to a size of about 350 × 100 × 100 μm for E2(CPA) and E2P(CPA) and 150 × 150 × 50 μm for E2(TG). Crystals were mounted directly from mother liquor in nylon loops and flash-frozen in liquid nitrogen.

**X-ray Diffraction Data Collection**—Numerous data sets were collected at 100 K on beam lines 8.2.2, 8.3.1, and 12.3.1 at the Advanced Light Source (Berkeley, CA). Data were processed and merged using the HKL package (HKL Research, Inc., Charlottesville, VA). For E2(CPA), the crystals possessed P2\(_1\) symmetry, and the diffraction intensities from the three best crystals were merged. For E2P(CPA), the crystals possessed C2 symmetry, and data from the single best crystal were used. For E2(TG), the crystals possessed P4\(_1\) symmetry, and data from the single best crystal were used. The E2(CPA) and E2P(CPA) crystals contain one Ca\(^{2+}\)-ATPase molecule per asymmetric unit, and the E2(TG) crystals contain two molecules per asymmetric unit. The solvent content for the E2(CPA) and E2P(CPA) crystals were 71.0% (Matthews’ \(V_m\) coefficient, 4.24 Å\(^3\) Da\(^{-1}\)) and 67.8% (Matthews’ \(V_m\) coefficient, 3.82 Å\(^3\) Da\(^{-1}\)), respectively. Data collection statistics are shown in Table 1.

**Structure Determination and Refinement**—The structure of E2(CPA) was solved by molecular replacement using Phaser (25) and the E2(TG) Ca\(^{2+}\)-ATPase structure (Protein Data Bank accession code 1WPG) (4) as the search model. The initial model was subjected to rigid body refinement using refmac5 in the CCP4 program suite (26). Thereafter, the structure was refined with alternate cycles of restrained refinement with TLS (parameterization of the translation, libration, and screw rotation displacements of pseudorigid bodies) in reffmac5 (27) and manual rebuilding using XtalView (28) after inspection of both 2Fo – Fo and Fo – Fc maps. Extensive model building was done in the following regions: helices M1/M2 and their associated cytoplasmic loops and a loop in the nucleotide binding domain (Ala\(^{501}\)–Gly\(^{509}\)). The R\(_{cryst}\) and R\(_{free}\) values were monitored closely throughout the refinement. The chemical structure of CPA was obtained from the PubChem Compound index (CID 65261), which is a slightly different isomer from that originally reported (20). The molecular structure of CPA was generated with the program CORINA (29) and added to the model after several rounds of refinement. CPA was located as outstanding electron density between the transmembrane segments M1 and M4 in both the 2Fo – Fc and 2Fc – Fo maps (density extending to the 6σ level). At 2.65 Å resolution, the overall conformation and orientation of CPA were well defined based on refinement statistics; however, we could not differentiate between the tau-tomers mentioned above. The Ca\(^{2+}\)-ATPase molecule was well defined in the electron density map except for the M3/M4 luminal loop (Ile\(^{276}\)–Ile\(^{389}\)), for which there was no observed density. The final model at a resolution of 2.65 Å (R\(_{cryst} = 0.246\), R\(_{free} = 0.287\)) consisted of Ca\(^{2+}\)-ATPase, Mg\(^{2+}\), Na\(^{+}\), Mg\(^{2+}\), CPA, and 75 water molecules. All residues were in the most favored and allowed regions of the Ramachandran plot (87.4/ 12.2/0.3/0) as evaluated by PROCHECK (30).

The model of E2(CPA) was built in a similar manner as described above. The structure of E2(CPA) was solved by molecular replacement using the E2(TG + BHQ) Ca\(^{2+}\)-ATPase...
Structure of SERCA Bound to Cyclopiazonic Acid

structure (Protein Data Bank accession code 2AGV) (5) as the search model. Restricted refinement in reflmac5 used bulk solvent correction and incorporated a TLS refinement only in the later cycles. The final model at a resolution of 3.4 Å ($R_{cryst} = 0.290$, $R_{free} = 0.328$) includes Ca$^{2+}$-ATPase, Mg$^{2+}$, ADP, and CPA. ADP was located as outstanding electron density sandwiched between Arg$^{389}$ and Arg$^{578}$ in both the $F_o - F_c$ and $2F_o - F_c$ maps (density extending to the $7\sigma$ level). All residues are in the most favored and allowed regions of the Ramachandran plot (82.5/17.2/0.2/0) as evaluated by PROCHECK (30).

For the E2(TG) crystals, an isomorphous difference map was calculated for the sole purpose of identifying bound ligands (using Protein Data Bank accession code 2AGV) (5).

Activity and Fluorescence Measurements—All biochemical measurements were performed with detergent-solubilized, affinity-purified Ca$^{2+}$-ATPase to allow comparison with the crystal structures. The ATPase activity of purified Ca$^{2+}$-ATPase was measured by a coupled enzyme assay (34). Briefly, 2 µg of Ca$^{2+}$-ATPase were incubated with or without inhibitor in assay buffer (50 mM imidazole, pH 7.0, 100 mM KCl, 5 mM MgCl$_2$, 0.5 mM EGTA, 0.5 mM phosphoenolpyruvate, 0.18 mM NADH, 0.6 mg/ml C$_{12}$E$_6$, 9.6 units/ml pyruvate kinase, 9.6 units/ml lactate dehydrogenase) containing varying concentrations of ATP from 1.6 µM to 4 mM. The reaction was started by the addition of calcium to a final free calcium concentration of 10 µM ($p\mathrm{Ca} = 5$). The absorbance of NADH was monitored at 340 nm at an assay temperature of 25 °C. Data were plotted as ATPase-specific activity (µmol of ATP hydrolyzed min$^{-1}$ mg$^{-1}$ of Ca$^{2+}$-ATPase) versus ATP concentration (mM). The $K_{ATP}$ and $V_{max}$ were calculated based on fitting the data to the four-parameter logistic curve using Sigma Plot software (SPSS Inc., Chicago, IL). Errors shown for the control are the S.E. for four independent measurements. The ATPase activities in the presence of either 150 nM CPA or 17 nM thapsigargin, concentrations similar to those used in crystallization, were the average of two independent experiments.

The binding of a fluorescent nucleotide analogue, TNP-ATP, to purified Ca$^{2+}$-ATPase was measured using an established method (35). Samples were prepared in fluorescence buffer (20 mM MOPS, pH 7.0, 100 mM KCl, 1 mM EDTA, 1 mM EGTA) that promoted the E2 conformation of Ca$^{2+}$-ATPase. The fluorescence emission intensity due to formation of the complex between Ca$^{2+}$-ATPase and TNP-ATP. The fluorescence emission intensity due to formation of the complex between Ca$^{2+}$-ATPase and 10 µM TNP-ATP was set to 100%. In the absence of Ca$^{2+}$-ATPase, TNP-ATP had a fluorescence emission intensity that was proportional to the nucleotide concentration up to a concentration of 10 µM (data not shown). Higher TNP-nucleotide concentrations were avoided, because inner filter effects caused this relationship to deviate from linearity.

RESULTS

Our current understanding of Ca$^{2+}$-ATPase includes several structures in the absence of calcium and in the presence of phosphate analogues and/or inhibitors (2, 4–6, 36). The maximum resolution of these structures is 2.3 Å; however, a variety of other Ca$^{2+}$-ATPase intermediates have been solved in the range of 2.4–3.3 Å resolution. The crystal structures presented herein are the first for Ca$^{2+}$-ATPase bound to the specific inhibitor CPA.

Overall Structure of E2P—In the absence of calcium and in the presence of magnesium fluoride (E2-MgF$_2$), the Ca$^{2+}$-ATPase adopts a transition state that represents the dephosphorylated enzyme immediately following hydrolysis with inorganic phosphate still bound. This structure represents an E2 PO$_4$ state of the calcium pump; however, this conformation is commonly designated E2P, and we will use this convention herein. The structure of E2P at 2.65 Å resolution reveals a well defined Ca$^{2+}$-ATPase molecule and bound ligands (Mg$^{2+}$, MgF$^{2-}$, and CPA) in the electron density map (Fig. 1 and Table 1). Despite crystallization in the presence of 10 mM ADP, no density was observed for this ligand. A comparison of our E2-PO$_4$ structure with previously determined E2P(TG) structures (4, 6) revealed an overall conservation of the molecular structure. The root mean square deviations after alignment of all peptide backbone atoms were 0.6 Å (versus 1WPQ) (4) and 1.2 Å (versus 1XP5) (6). Our E2P(CPA) structure resembles previous structures, and our goal is not to reiterate its detailed description. Rather, we will focus on the observed structural differences.

Our structure of E2P(CPA) differs from the previous structures in the relative positions of M1–M3 and in the disordered M3/M4 luminal loop. These differences are explained by the use of inhibitors to stabilize this intermediate, CPA, in our structure and TG in previous structures (4, 6). Despite changes in transmembrane helices M1–M3 and the associated loops (Fig. 2), the position of the A domain remains unchanged. It is unlikely that the position of the A domain is altered by crystal contacts, because our C2 crystal lattice is distinct from the previously determined structures (4, 6). Nonetheless, the conformational coupling between the A domain and M1–M3 accommodates significant plasticity. Helix M2 undergoes a bending displacement that leaves the luminal end of the helix in place but moves the cytoplasmic end of the helix by ~10 Å (12° change in inclination). The associated loop adopts a different conformation extending to Ala$^{12}$, where the rest of the loop and the A domain occupy their expected positions. The short
cytoplasmic helix of M1 undergoes a 26° rotation along the membrane surface in the direction of the movement of M2. The associated loop adopts a distinct conformation that begins at Glu^45, whereas the remainder of the loop and the A domain are unchanged. The conformational changes in M1 and M2 are explained by the binding of CPA to a site above Glu^459 that locks these helices against M4. CPA fills the calcium access channel in the E2 calcium-free form of the enzyme and stabilizes M1 and M2 in positions that are incompatible with calcium binding.

Although the movements of M1 and M2 are due to CPA binding, this does not explain the position of M3 or the disordered M3/M4 loop. In our structure, M3 is shifted upward by approximately one-half of a turn of the helix, and the M3/M4 loop is disordered between residues Asn^275 and Arg^290 (Fig. 2). In contrast, M4 is well defined, and its position is unchanged in comparison with previous structures (4, 6). Electron cryomicroscopy of helical crystals revealed that TG binding causes a small downward movement of M3 and a restructuring of the M3/M4 loop (13, 14). We believe that the position of M3 observed in previous structures (4, 6) and in the helical crystals (13) is a characteristic of TG inhibition. The M3 helix has been shown to be a major component of the TG binding site (2). In addition, the M3/M4 loop participates in crystal contacts in the previous structures (4, 6) and in the helical crystals (13), and its structure may be due to TG binding and intermolecular contacts. This loop does not participate in a crystal contact in our E2P(CPA) structure.

**Overall Structure of E2**—Following the release of calcium and inorganic phosphate, the cytoplasmic domains undergo concerted movements that rotate the conserved TGES^184^-loop away from Asp^151^ and generally open up the phosphorylation site in the E2 conformation (4, 6, 14). The structure of E2 at 3.4 Å resolution reveals a well defined Ca^2+^-ATPase molecule and bound ligands (CPA, ADP, and Mg^{2+}) in the electron density map (Fig. 1 and Table 1). A comparison of our E2(CPA) structure with the previously determined E2(TG) structures (2, 5) revealed an overall conservation of the molecular structure. The root mean square deviations after alignment of all peptide backbone atoms were 0.8 Å (versus 1IWO) (2) and 0.7 Å (versus 2AGV) (5).

In the presence of CPA, our structure of the E2 conformation differs in the positions of transmembrane helices M1–M3. As before, M2 undergoes a bending displacement that leaves the luminal end of the helix in place but moves the cytoplasmic end of the helix. The loops connecting M1 and M2 to the A domain adopt distinct conformations, whereas the A domain occupies its expected position. Again, this may be partly explained by intermolecular contacts in our P2 crystal lattice. Additional conformational changes are observed for the M1/M2 luminal loop and part of the M3/M4 luminal loop (Fig. 1D). The M1/M2 luminal loop adopts a different conformation that is bent away...
from the rest of the molecule and toward the membrane surface, and the luminal loop at the base of M4 is bent toward M1. Although the movements of M1 and M2 are a consequence of CPA binding, the positions of the M1/M2 and M3/M4 luminal loops do not appear to be influenced by CPA binding. As before, neither loop participates in a crystal contact in our E2(CPA) structure, and this is in contrast to previous structures (2, 5, 36).

Mechanism of Inhibition—CPA is an amphipathic molecule that was thought to bind in the general vicinity of Phe\textsuperscript{256} along the cytoplasmic end of M3, involving a distinct set of residues compared with TG (18, 37). Mutation of Phe\textsuperscript{256} had dramatic effects on the binding affinity for TG with very little effect on CPA binding; however, other amino acid mutations in this region of M3 appeared to alter CPA binding (18). Although a detailed analysis of CPA binding to Ca\textsuperscript{2+}/H\textsubscript{11001}-ATPase was lacking, it was thought that TG and CPA share a common mechanism of inhibition and occupy overlapping binding sites.

The inhibitor binding site is unambiguously identified as the calcium access channel consisting of \(400 \text{ Å}^2\) of surface area (Fig. 1A). CPA is a relatively planar molecule with a charged tetramic acid at one end and a hydrophobic ring structure at the other end. The amino acid residues that form the binding site include Leu\textsuperscript{61} and Val\textsuperscript{62} on M1 (3.5 and 3.3 Å away, respectively), Asn\textsuperscript{101} and Ala\textsuperscript{102} on M2 (2.7 and 3.4 Å away, respectively), Leu\textsuperscript{311} and Pro\textsuperscript{312} on M4 (3.6 and 2.8 Å away, respectively), and Gly\textsuperscript{257} on M3 (3.8 Å away) (Fig. 3). CPA sits at the level of Phe\textsuperscript{256}, surrounded by transmembrane helices M1–M4, and is situated 4.1 Å above the putative gating residue, Glu\textsuperscript{309}. A defining residue of the TG binding site, Phe\textsuperscript{256}, is located 4.1 Å away from the binding site, confirming the minimal effect of mutations at this position on CPA inhibition (22). Previous mutagenesis studies have suggested an important role for Gly\textsuperscript{257} in CPA binding (22), and this is confirmed by our crystal structures. Mutations in this region, converting residues 254–257 to the homologous sequence in the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, produce a 250-fold reduction in sensitivity to CPA. This effect is almost entirely due to the substitution of an isoleucine for
It is clear from our structures that the insertion of an isoleucine at position 257 would cause a steric clash with the indole ring of CPA.

The CPA binding pocket is formed by two subcavities (Fig. 3, A and B). A polar pocket interacts with the tetramic acid portion of CPA (primarily formed by Gln$$^{56}$$ and Asn$$^{101}$$), and a hydrophobic platform cradles the indole ring portion of CPA (primarily formed by Leu$$^{61}$$ and Val$$^{62}$$). The indole nucleus of CPA bridges the M1 and M4 helices, and this interaction seems to be vital to its inhibitory action. This is consistent with a previous study comparing different tetramic acids, where the deletion of the indole ring completely abolished Ca$$^{2+}$$-ATPase inhibition (38). Another determining interaction with CPA occurs through Asn$$^{101}$$, which appears to form a hydrogen bond with a hydroxyl of the tetramic acid. Notably, significant displacement of M2 begins at residue Asn$$^{101}$$, suggesting a causative effect. Additional hydrogen bonds to Gln$$^{56}$$ in this region are mediated by a water molecule. Together, the close interactions between CPA, Asn$$^{101}$$, and Pro$$^{312}$$ serve to draw M2 toward M4. As a result, the movement of M1 may be a natural consequence of the movement of M2, which could be subsequently stabilized by van der Waals interactions with Leu$$^{61}$$ and Val$$^{62}$$ and water-mediated hydrogen bonding with Gln$$^{56}$$. This latter residue is well conserved among the P-type ATPases (except in the plasma membrane calcium pump), whereas Leu$$^{61}$$, Val$$^{62}$$, and Asn$$^{101}$$ are unique to the SR Ca$$^{2+}$$-ATPases (Fig. 3C). Interestingly, Leu$$^{61}$$ and Val$$^{62}$$ are replaced by Leu-Ile in a secretory pathway Ca$$^{2+}$$-ATPase, SPCA1, which is weakly sensitive to CPA (39). By comparison, other P-type ATPases are insensitive.

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Formation of the TG and CPA binding sites depends on the Ca\(^{2+}\)-ATPase conformation, where binding occurs to the calcium-free conformations. In the case of TG, inhibition is achieved by locking M3, M5, and M7 against one another in an E2-like arrangement that is essentially irreversible due to the high affinity binding (19). For CPA, inhibition is achieved by blocking the calcium access channel and locking M1 and M2 against M4, and inhibition is reversible in the presence of elevated ATP and calcium. CPA and BHQ (5) occupy a common binding site, although the size of the CPA binding site and the structural changes associated with binding are much more extensive. We conclude that the mechanisms of inhibition used by TG and CPA are distinct; TG stabilizes a Ca\(^{2+}\)-ATPase conformation that is incompatible with calcium binding, whereas CPA blocks the calcium access channel and immobilizes a subset of transmembrane helices.

A Novel Mode of ADP Binding—Based on previous structures (3, 4, 7), the nucleotide binding site is formed by packing of the adenine ring against Phe\(^{487}\), capping of the adenine binding site by Lys\(^{678}\) and Arg\(^{560}\), and positioning of the phosphate groups by Thr\(^{353}\), Arg\(^{489}\), Arg\(^{560}\), Thr\(^{625}\), Gly\(^{562}\), Lys\(^{680}\), and Asn\(^{706}\). A magnesium ion sits in the canonical site and is coordinated by Asp\(^{705}\), Asp\(^{351}\), and Thr\(^{353}\). A comparison of the ADP and magnesium bound in our E2(CPA) structure with the previously determined structures of Ca\(^{2+}\)-ATPase revealed a very similar mode of interaction for many of the residues described above. However, in our structure, the adenine ring of ADP sits in a distinct site between the N and P domains, sandwiched between Arg\(^{489}\) and Arg\(^{678}\) (Figs. 1 and 4). The adenine ring of ADP is positioned between the N and P domains with the \(\beta\)-phosphate and magnesium ion arranged around Asp\(^{351}\). The adenine ring stacks between the side chains of Arg\(^{489}\) and Arg\(^{678}\), and the binding site is capped on either end by Glu\(^{680}\) and Lys\(^{678}\) (Fig. 4). This site is transiently formed during the reaction cycle as the N and P domains come together, and the adenine ring sits \(\sim 10\) Å from its expected position against Phe\(^{487}\). Previous labeling studies have implicated Lys\(^{679}\) and Arg\(^{678}\) in nucleotide binding (40–47), and in our structure these residues participate in adenine recognition. The \(\beta\)-phosphate and magnesium ion are positioned 7.5 and 4.3 Å from the side chain of Asp\(^{351}\), respectively. The magnesium ion sits in a large polar cavity that is formed by the \(\beta\)-phosphate of ADP, Asp\(^{351}\), Thr\(^{353}\), and Asp\(^{705}\). Last, the \(\beta\)-phosphate of ADP is positioned 7.5 Å from Asp\(^{351}\). Surprisingly, the \(\beta\)-phosphate of ADP is closer to Asp\(^{351}\) than the \(\gamma\)-phosphate in a recent complex with AMPPCP, a nucleotide analogue (9 Å away (36)). In our structure, positioning of ADP proximal to the phosphorylation site is enabled by the ribose interaction with Gly\(^{562}\) and the \(\beta\)-phosphate interaction with Thr\(^{353}\).

The striking difference observed in our E2(CPA) model is that Glu\(^{680}\), Arg\(^{489}\), Lys\(^{679}\), and Arg\(^{678}\) form a distinct adenine binding site, where Arg\(^{678}\) may participate in a cation-\(\pi\) interaction. The cation-\(\pi\) interaction of positively charged residues is a common mechanism for the molecular recognition of adenine (48, 49). Arg\(^{678}\) appears to fit this role, because it is highly conserved among members of the P-type ATPases, whereas Arg\(^{489}\) is not (Fig. 4).

Ca\(^{2+}\)-ATPase (SPCA1) is substituted with a serine, another amino acid commonly found in adenine recognition motifs (48). Approximately one-third of ATP-dependent proteins contain an adenine recognition motif (48, 50) that is similar to the binding site in our E2(CPA) model. The two arginines found
in Ca\(^{2+}\)-ATPase closely resemble the adenine recognition motif in a 70-kDa heat shock protein (Protein Data Bank code 1BUP) (51).

Nucleotide Binding in the Presence of Inhibitors—To compare the effects of TG on ADP binding, we calculated an electron density map for a P4\(_1\) crystal form of the E2(TG) intermediate grown in the presence of an identical concentration of ADP (Table 1). Our E2(TG) crystals are identical to those previously published, yet the nucleotide was not observed in the map (using Protein Data Bank codes 1IWO and 2AGV as search models) (2, 5). In contrast to this finding, recent structures of the E2(TG) intermediate in the presence of AMPPCP revealed nucleotide binding in the canonical site defined by Phe\(^{587}\) (36, 52). Whether CPA affects nucleotide binding remains uncertain, since the nucleotide binding sites are nearly identical in the E2(CPA) and E2(TG) structures.

We next wished to examine the effect of inhibitors on nucleotide binding utilizing biochemical methods. First, we measured the ATP dependence of Ca\(^{2+}\)-ATPase activity in the absence and presence of a range of inhibitor concentrations. Over a range of concentrations that included our crystallization conditions (17 nM TG and 150 nM CPA), the nucleotide affinity was measured to be \(~0.1\) \(\mu\)M (Fig. 5, A and B). Thus, both inhibitors perturbed the nucleotide binding affinity. Second, we used a fluorescent ATP analogue, TNP-ATP, to measure nucleotide binding to Ca\(^{2+}\)-ATPase in the absence and presence of inhibitors. The fluorescence emission intensity of TNP-ATP increases upon binding to Ca\(^{2+}\)-ATPase, and inhibitor binding to Ca\(^{2+}\)-ATPase alters the fluorescence enhancement. Whereas the TNP-ATP analogue may not bind to Ca\(^{2+}\)-ATPase in the same manner as ATP, the fluorescent analogue is a reliable indicator of conformational changes in the vicinity of the nucleotide binding site. Our fluorescence measurements show that TG reduces the signal from TNP-ATP by 27.8 \pm 2.6\% \((n = 9)\), whereas CPA reduces the signal by 14.1 \pm 3.7\% \((n = 9)\) (Fig. 5C). Therefore, both inhibitors perturb nucleotide binding.

There are two possible explanations for the binding of ADP in our E2(CPA) crystal structure. The simplest explanation is that CPA has perturbed the nucleotide binding site, causing ADP to bind in a nonphysiological manner. At present, we cannot exclude this possibility. Nonetheless, an alternate explanation may be that ADP binding has revealed an adenine recognition motif that participates in the calcium pump transport cycle.

DISCUSSION

Our understanding of the transition from E2P to E2 in the calcium transport cycle revealed modest rearrangements of the cytoplasmic domains with consequences in the positions of a subset of the transmembrane helices. The N and P domains of Ca\(^{2+}\)-ATPase tilt away from the A domain, and the A domain rotates away from the phosphorylation site. As a result of the A domain movement, the positions of M1, M2, and the luminal end of the M4 are slightly altered. The major conformational change that marks the transition to the E2 ground state is an opening of the cytoplasmic domains around the phosphorylation site. Luminal access to the cation binding sites is closed, and the enzyme is prepared for ATP binding and cytosolic calcium access. Both the E2P and E2 structures have a cytosolic calcium access channel that is surrounded by M1–M4 and leads to Glu\(^{308}\) and the calcium binding sites.

What new information is provided by our E2P and E2 structures? The structures differ in the positions of M1–M3 as a consequence of inhibitor binding, yet there is no change in the positions of the A domain. This suggests an inherent plasticity in the transmembrane helices and connecting loops that is tolerated by the A domain interaction. Perhaps this is a consequence of the large interfaces formed between the N, P, and A domains in the E2P and E2 conformations (14). The largest
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cytosolic domain interface occurs in the E2P intermediate, which is transiently stabilized by the interaction of the TGES$^{184}$ loop. After the release of phosphate and the formation of the E2 state, the A domain movement has a minimal effect on the calcium access channel and binding sites. This is because small changes in the disposition of the A domain are tolerated by M1–M3. Access to the calcium binding sites is gated by Glu$^{309}$ and helix dynamics are a likely component in binding of the first calcium ion. This binding causes a conformational change that creates cooperativity for binding of the second calcium ion and sets up the enzyme for activation by ATP. The structure of the enzyme with a single bound calcium ion is unknown, but binding of the second calcium ion causes significant changes in the membrane domain (1). The movements of M1–M3 pull on the loops connecting the A domain, and this force is sufficient to swing the A domain away from the N and P domains. This is the activation step that allows the N and P domains to associate for aspartyl-phosphate formation from ATP.

CPA is a specific inhibitor of the sarco(endo)plasmic reticulum Ca$^{2+}$-ATPases, and it is known to be a human health concern in food contamination. Inhibition of Ca$^{2+}$-ATPases results in cell death through the activation of stress-response and apoptotic pathways within the endoplasmic reticulum and mitochondria (53). Our structures reveal that CPA inhibits Ca$^{2+}$-ATPase by blocking the calcium access channel and rigidifying a subset of transmembrane helices in a nonnative configuration that is incompatible with calcium binding. A network of hydrogen bonds lock M2 against M4 via the tetrameric acid moiety of CPA, whereas nonpolar interactions lock M1 against M4. These interactions are completely distinct from those made by TG. Phe$^{256}$ is an important residue in TG binding, yet this residue makes only a minor contribution to the hydrophobic pocket occupied by CPA. Despite having different binding pockets, TG and CPA both rigidify a nonnative arrangement of the transmembrane helices. However, unlike TG, CPA plugs the calcium access channel.

Finally, the ADP binding site in our E2(CPA) model appears to conform to an adenine recognition motif. The position of the adenine ring differs from the canonical nucleotide binding mode revealed in prior crystal structures (1, 3, 4, 7, 36). At present, it remains uncertain if the mode of ADP binding observed herein is a consequence of CPA inhibition or a genuine nucleotide binding mode that modulates the calcium transport cycle.

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