Crystal Structure of D351A and P312A Mutant Forms of the Manmalian Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase Reveals Key Events in Phosphorylation and Ca\(^{2+}\) Release*§

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In recent years crystal structures of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA1a), stabilized in various conformations with nucleotide and phosphate analogs, have been obtained. However, structural analysis of mutant forms would also be valuable to address key mechanistic aspects. We have worked out a procedure for affinity purification of SERCA1a heterologously expressed in yeast cells, producing sufficient amounts for crystallization and biophysical studies. We present here the crystal structures of two mutant forms, D351A and P312A, to address the issue whether the profound functional changes seen for these mutants are caused by major structural changes. We find that the structure of P312A with ADP and AlF\(_4\)- bound (3.5-Å resolution) and D351A with AMPPCP or ATP bound (3.4- and 3.7-Å resolution, respectively) deviate only slightly from the complexes formed with that of wild-type ATPase. ATP affinity of the D351A mutant was very high, whereas the affinity for cytosolic Ca\(^{2+}\) was similar to that of the wild type. We conclude from an analysis of data that the extraordinary affinity of the D351A mutant for ATP is caused by the electrostatic effects of charge removal and not by a conformational change. P312A exhibits a profound slowing of the Ca\(^{2+}\)-translocating Ca\(^{2+}\)E1P → E2P transition, which seems to be due to a stabilization of Ca\(^{2+}\)E2P rather than a destabilization of E2P. This can be accounted for by the strain that the Pro residue induces in the straight M4 helix of the wild type, which is removed upon the replacement of Pro\(^{312}\) with alanine in P312A.

The sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) plays a crucial role in muscle relaxation by re-accumulating Ca\(^{2+}\) into the sarcoplasmic reticulum lumen at the end of the contractile event (1, 2). The fast twitch muscle isoform (SERCA1a) can easily be prepared in large yield from rabbit skeletal muscle, which has facilitated many biochemical and biophysical studies, thus leading to a detailed description of its functional and structural properties (3–7). SERCA belongs to the family of P-type ATPases characterized by the formation during the catalytic cycle of an energy-rich covalent aspartyl-phosphorylated intermediate. The cycle of phosphorylation and dephosphorylation is coupled with Ca\(^{2+}\)/H\(^{+}\) exchange through conformational changes between the so-called “E1” and “E2” forms in phosphorylated and dephosphorylated states. Ca\(^{2+}\) binding from the cytoplasmic side in the E1 form is required for phosphorylation of the enzyme from ATP, whereas the dephosphorylation occurs subsequently to the luminal release of Ca\(^{2+}\) from E2P and leads to proton countertransport (Scheme 1). Key residues involved in the binding of Ca\(^{2+}\) and ATP (8–11) and in the phosphorylation reaction (12, 13) were initially identified by site-directed mutagenesis, and essential information about residues involved in conformational...
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Scheme 1. Ca²⁺-ATPase reaction cycle. Simplified scheme illustrating the partial reaction of the Ca²⁺-ATPase reaction cycle.

changes and energy transduction has also been gained from the detailed studies of SERCA mutants (see e.g. Refs. 8 and 13–17). A very significant step forward in the understanding of the structure-function relationships of SERCA was taken by the crystallization and three-dimensional structure clarification at atomic resolution of catalytic intermediates, stabilized in several cases by structural analogues of ATP and phosphoryl groups (see e.g. Refs. 18–25). The cytosolic part of the protein consists of a transduction (“actuator”) domain A, a phosphorylation domain P containing the phosphorylated aspartic acid residue Asp-351, and a nucleotide-binding domain N. The membrane-embedded region to which these cytosolic domains are linked is organized in 10 transmembrane spans, M1–M10, which accommodate the Ca²⁺ binding sites, nested in internal loops or kinks of M4 and M6 (23, 26). The formation of a compact Ca²⁺-occluding Ca₂E₁P state ensures coupling between phosphoryl transfer and vectorial Ca²⁺ transport (21, 25). The phosphorylation/Ca²⁺ occlusion step is followed by a major conformational rearrangement involving both the cytosolic and the transmembrane regions, resulting in the Ca₂E₁P → E₂P transition (cf. Scheme 1), in which the A domain rotates (24, 25) and the membrane domains separate, allowing release of Ca²⁺ toward the lumen (27).

Many structural details correlate well with the results of functional studies of mutants with changes to specific residues, thus providing a sound basis for the use of site-directed mutagenesis to obtain further insight into how the long range communications between the cytoplasmic and membranous parts of the protein are brought about. The analysis of the functional consequences of mutations has reached a level where it encompasses rapid kinetic quench-flow studies of the partial reaction steps (28) and most recently also biophysical measurements of conformational transitions by fluorescence changes, made feasible by large scale expression in yeast (15, 29). A number of mutations appear to accelerate or block partial reactions in the functional cycle and displace E₁→E₂ and/or E₁P→E₂P equilibria. However, in the absence of direct structural information the fundamental question arises whether the effect of a given mutation is caused by the displacement of the equilibrium between already existing conformations in the wild-type enzyme, or whether it is the result of induction of local or global structural changes not seen in the wild type. To deal with this question there is a need for an analysis of the structures of the mutant proteins.

So far, the success in three-dimensional crystallization of Ca²⁺-ATPase, as well as other eukaryotic membrane proteins like cytochrome oxidase (30), bovine rhodopsin (31), and aquaporins (32), has to a large extent been dependent on their purification in relatively large amounts from natural sources, enabling optimization of the conditions for obtaining well diffracting protein crystals (33). This fortunate situation does not exist for Ca²⁺-ATPase mutants or for the majority of membrane proteins from mammalian sources, which, however, can be prepared by recombinant expression systems. The first reports on successful crystal structure determination of eukaryotic membrane proteins, purified from an overexpression host (yeast), came in 2005 (SERCA1a (34), the shaker K⁺ channel protein (35)). With a new procedure for affinity purification of SERCA1a heterologously expressed in yeast cells (36), we were able to produce sufficient amounts, in the milligrams range, of SERCA1a for crystallization and biophysical studies (34). Extending this technique to Ca²⁺-ATPase mutants would open the possibility of linking, in a more definitive way than before, the structural data obtained by x-ray diffraction with those arising from the functional analysis of Ca³⁺-ATPase mutants, to address key functional aspects of the Ca²⁺ transport mechanism.

To explore these possibilities, we have focused on two mutants, D351A and P312A, which have been selected for crystallization due to their important functional characteristics as previously determined in the mammalian COS cell expression system (12, 16, 37). Both of these mutations prevent the ATPase from hydrolyzing ATP and transporting Ca²⁺. In mutant D351A the negatively charged side chain of the conserved aspartate, constituting the phosphorylation site in the P-domain, has been removed, resulting in an inability of the mutant enzyme to undergo phosphorylation (12). It was demonstrated that ATP and MgATP bind with much higher apparent affinity to D351A as compared with the wild-type enzyme (37), leading to the hypothesis that in the wild type a significant electrostatic repulsion exists between the γ-phosphate of ATP and the negative charge of Asp³⁵¹, which was considered to be overcome by highly favorable interactions of ATP with other residues. This hypothesis would also explain the observation of an enhanced nucleotide protection against proteolysis in the D351A mutant (38). However, an alternative possibility could be that the high affinity state is the result of a particular structure of the mutant, induced by the mutation per se, and as such irrelevant to the function of the wild-type enzyme. The other mutant, P312A, is characterized by an extremely slow transition from the ADP-sensitive Ca₂E₁P phosphoenzyme intermediate to the ADP-insensitive E₂P state, the step that releases the Ca²⁺ ions toward the lumen (16). Pro³¹² is located at the border of the unwound part of transmembrane segment M4, next to the 308PEGL motif containing the Ca²⁺ binding residue Glu³⁰⁹, and the question here is also whether the block of Ca₂E₁P → E₂P reflects an abnormal structure of the mutant or different kinetic properties of the Ca₂E₀P → E₂P transition.

In this communication we have addressed these questions by determining the crystal structure of the above mutants with
bound nucleotide. The large scale expression in the yeast system of the D351A mutant has also been utilized to investigate by fluorescence measurements whether the change induced by the mutations has any consequences for the long range communication between the Ca\(^{2+}\)-binding sites and the phosphorylation site. As will be shown this approach, in conjunction with data obtained by previously established methodology, has allowed us to obtain an improved understanding of the mechanistic roles of Asp351 and Pro312 in ATPase function.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemical products were purchased from Sigma-Aldrich unless specified otherwise. Restriction and modification enzymes were from New England Biolabs (Ozyme, Saint Quentin en Yvelines, France). High activity bovine thrombin was from Calbiochem (VWR International, Fontenay sous Bois, France), the streptavidin-Sepharose\(^{TM}\) High Performance was purchased from GE Healthcare Biosciences AB (Orsay, France). All products for GE Healthcare and bacteria cultures were purchased from Difco (BD Biosciences, Le Pont de Claix, France). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was from Avanti Polar Lipids (Alabaster, AL), n-dodecyl-β-D-maltoside (DDM) was from Anatrace (Maumee, OH) and octaethylene glycol mono-n-dodecyl ether (C\(_{12}\)E\(_{8}\)) was purchased from Nikkol Chemical (Tokyo, Japan).

**Plasmid Constructions**—SERCA1a mutants (SERCA1amut) in pMT2 vector (D351A) or p91023 (P312A) were the ones previously used for expression in COS cells (16, 37). The yeast expression plasmid pYeDP60-SERCA1a-BAD was as previously described (36). Each plasmid was digested by EcoRI (cut on the plasmid upstream the cDNA of SERCA1a) and BssHII (internal site at +2038 in SERCA1a). Fragments were purified using the QIAquick gel extraction kit (Qiagen). The 2.1-kb EcoRI-BssHII fragment of SERCA1amut cDNA containing the mutation was subcloned in the opened EcoRI-BssHII pYeDP60-SERCA1a-BAD, replacing the corresponding WT SERCA1a cDNA. To keep the same environment between the EcoRI site and the ATG, which is important for expression in yeast, another step of subcloning was performed: pYeDP60-SERCA1a-BAD and new pYeDP60 plasmids containing SERCA1amut-BAD cDNA were digested by KpnI (internal site at +640 in SERCA1a and at +3304 in pYeDP60). After purification, the 2.76-kb KpnI fragment containing the mutation was ligated to KpnI-cleaved pYeDP60 (using an NEB Quick ligation kit, Ozyme, Saint Quentin en Yvelines, France) to obtain the pYeDP60-SERCA1amut-BAD containing the D351A or P312A mutation. The constructions were checked by sequencing. These plasmids were transformed and amplified in *Escherichia coli* JM109. Plasmids DNA were purified using a QIAPrep 8 Turbo Miniprep kit (Qiagen).

**Yeast Transformation**—The *Saccharomyces cerevisiae* yeast strain W303.1b/Gal4 (a, leu2, his3, trp1::TRP1-GAL10-GAL4, ura3, ade2–1, can', civ') was the same as previously described (39). Transformation was performed according to the lithium acetate/single-stranded carrier DNA/PEG method (40).

**Yeast Culture, Mutant Expression, and Preparation of Membrane Fractions**—Growth conditions and criteria for expression of the mutants were the same as previously published for native SERCA1a expressed in yeast (36). Yeast cells were broken with glass beads. For membrane preparation by differential centrifugation (36, 39), the P3 pellet, corresponding to the light membrane fraction (where the level of SERCA1a activity is highest), was finally suspended in Hepes-sucrose buffer (20 mM Hepes-Tris (pH 7.5), 0.3 mM sucrose, 0.1 mM CaCl\(_2\)) at a final volume corresponding to 0.5 ml/g of the initial yeast pellet). From 1 liter of culture, 40 g of yeast was obtained and from the P3 pellet, 250 mg of membrane proteins was obtained with a SERCA1a (wild type or mutants) content of ~1% as estimated by Western blot analysis using an anti-SERCA1a antibody (39). SR-SERCA1a from rabbit muscle, used as a standard for protein estimation, was prepared as previously described (41).

**Purification of the Mutants by Streptavidin-Agarose Chromatography**—The light membrane fraction ("P3" fraction), suspended in the Hepes-sucrose buffer, was first solubilized by DDM (6 mg/ml) at a protein concentration of 2 mg/ml in a buffer containing 50 mM Tris-HCl (pH 7), 0.1 mM NaCl, 20% glycerol, 1 mM CaCl\(_2\), 1 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. After stirring for 2.5 h at room temperature, non-solubilized material was pelleted by centrifugation at 120,000 × g for 30 min at 4°C. All subsequent steps (unless otherwise specified) were then performed in a cold room. The supernatant after the centrifugation step was mixed with streptavidin-Sepharose\(^{TM}\) High Performance resin at a ratio of 40:1 (v/v), using typically 1 ml of resin per 0.4 mg of SERCA1a (corresponding to 40 ml of solubilized proteins with an average solubilization yield of 50%), and stirred gently overnight at 4°C. The suspension was then loaded onto a number of 11 × 100 mm columns (BioSepra) and washed, first with a "high salt" buffer (50 mM Tris-HCl (pH 7), 1 mM NaCl, 20% glycerol, 1 mM CaCl\(_2\), 0.05% DDM, buffer:resin, 12:1 (v/v)), and then with a "low salt" KCl buffer (50 mM Tris-HCl (pH 7), 50 mM KCl, 20% glycerol, 2.5 mM CaCl\(_2\), 0.05% DDM, buffer:resin, 12:1 (v/v)). All the resin in the columns was resuspended in the "low salt" KCl buffer (buffer:resin, 1:1 (v/v)) and introduced into a larger column (40 × 200 mm, BioSepra, MA) and thrombin was added (20 units of thrombin/ml of resin). The column was sealed and placed on a wheel, and the mixture was stirred gently at room temperature for 30 min, followed by a second addition of thrombin and stirring of the mixture for another 30 min. To inactivate thrombin, 2.5 mM phenylmethylsulfonyl fluoride was then added, and the proteolytically cleaved SERCA1a proteins eluted from the column by three successive additions of the KCl buffer (buffer:resin, 1:1 (v/v)). The eluted fractions containing the Ca\(^{2+}\)-ATPase (~2 mg of protein with a Ca\(^{2+}\)-ATPase content of ~40%, as evidenced by gel electrophoresis) were pooled, and the glycerol concentration increased to 40% before freezing the samples in nitrogen and storage at −80°C.

**HPLC Gel Filtration**—For crystallization experiments, DDM was exchanged with C\(_{12}\)E\(_{8}\) by size-exclusion chromatography, an additional step that also led to improvement of the purity of the Ca\(^{2+}\)-ATPase preparation. For this, the pool of SERCA1a mutant proteins (typically 30 ml) was first concentrated on Centricon Ultracel-YM 30 (Amicon, Millipore, Bedford, MA) until the volume was smaller than 500 μl. The concentrate was applied at 0.5 ml/min to a silica gel-filtration column (0.78 cm × 30 cm Tosohaas TSK-gel G3000SW XL column, Sigma-
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Aldrich), mounted on a Gold HPLC System (Beckman Coulter, Roissy CDG, France), and equilibrated with 100 mM MOPS (pH 6.8), 80 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 15% sucrose, and 0.5 mg/ml C$_{12}$E$_8$. The eluted fractions containing the Ca$^{2+}$-ATPase peak were pooled (1.5 ml) and concentrated on Centricon-30 to a final volume of ~50 μl, resulting in a final protein concentration of 8–10 mg/ml and a concentration of C$_{12}$E$_8$ estimated to be ~10–12 mg/ml on the basis of previous C$_{12}$E$_8$ binding data (42).

**Protein Crystallization**—For crystallization, the concentrated, delipidated, and C$_{12}$E$_8$-solubilized Ca$^{2+}$-ATPase was supplemented with phospholipids (DOPC), to reach a C$_{12}$E$_8$:DOPC ratio of 3:1 (w/w). This was done by adding the sample to a centrifuge tube that had been deposited with a thin layer of N$_2$-dried DOPC, vortexing the tube, and storing it overnight at 4 °C. The next day the sample often exhibited a slight haziness, arising from non-solubilized lipids, in which case it was clarified by addition of a slight amount of C$_{12}$E$_8$ stock solution, until having a clear solution. In addition the sample was subjected to centrifugation at 120,000 × g for 20 min to remove aggregated (inactive) protein, arising during the preparation. Agents were then added to induce the formation of ATPase crystals in the presence of 10 mM Ca$^{2+}$ together with either 1 mM ATP, AMP-PCP, or ADP together with 1 mM AlF$_3$ (as a structural analogue of phosphate). An initial 96 conditions of PEG/salt screening (33) were performed using a liquid-handling Mosquito robot (TTP Labtech, Cambridge UK), which distributed 100-μl drops of protein and crystallization buffer for equilibration with 100 μl of well solution. After a few hours (up to 2 days) at 19 °C, crystals started to appear. The best crystals were obtained at 10% sucrose, 12% PEG 2000 monomethyl ether, 0.2 M NaOAc, and 3% tert-butanol or 6% 2-methyl-2,4-pentanediol for P312A; and 7% PEG 6000, 0.2 M Na$_2$C$_3$H$_6$O$_4$, and 6% 2-methyl-2,4-pentanediol for D351A. These conditions were subjected to further grid screen optimization with 1- 1-μl hanging drops equilibrating against 450 μl of well solution at 19 °C. After 1 week suitable crystals were cryoprotected (2 μl of 40% PEG 4000 or 5 μl of 50% sucrose added to the drop) and picked up in nylon-fiber loops (Hampton Research, Riverside, CA) mounted on 18-mm metal pins. Crystals were flash-cooled and stored in liquid nitrogen. Data were collected at the Swiss Light Source (for P312A mutant) or at the Berliner Elektronen Speicher Synchrotron (for the D351A mutant) beamlines. The crystals diffracted anisotropically, to 3.4 Å for the D351A crystals and to 3.5 Å for the P312A crystals. Crystallographic data were processed and scaled by using the XDS package (43). An unbiased difference Fourier map using experimental $F_{\text{obs}}$(SERCA1a P312A mutant) − $F_{\text{obs}}$(SERCA1a D351A mutant) difference coefficients was calculated to reveal the structural differences between SERCA1a D351A and P312A mutants.

**Ca$^{2+}$-ATPase Reconstitution into Proteoliposomes**—After the streptavidin chromatography step and concentration of the affinity-purified SERCA1a on Centricon 30 to a Ca$^{2+}$-ATPase concentration of 0.1 mg/ml in a medium containing 0.5 mg of DDM/ml, 20% glycerol, 2.5 mM Ca$^{2+}$, 50 mM Tris (pH 7.0), and 50 mM KCl, the protein was mixed gently with DOPC (protein: DOPC, 1:3 (w/w)) for 30 min at 4 °C. Biobeads SM-2 absorbent (Bio-Rad) were prepared as described in a previous study (44). Detergent was removed by adding Biobeads at a Biobeads:DDM ratio of (200:1, w/w) and incubating with gentle stirring at 4 °C for 3 h. Biobeads were then removed, and the reconstituted protein was stored at 4 °C.

**ATP Affinity Measurement**—ATP equilibrium binding to mutant D351A reconstituted into proteoliposomes was measured by Millipore filtration. 10 μg of D351A was incubated with various amounts of [γ$^{32}$P]ATP (0.5 nm to 5 μM) in 2 ml of buffer (50 mM MOPS, pH 7.2, 100 mM KCl, 1 mM MgCl$_2$) in the presence of either 100 μM CaCl$_2$ or 2 mM EGTA, for 1 min at 20 °C. Then the sample was filtered on two superposed Millipore GS 0.22-μm filters under mild vacuum. The radioactivity of the filters was measured by liquid scintillation counting. The $^{32}$P radioactivity of the lower filter (corresponding to the wet volume) was subtracted from the $^{32}$P radioactivity of the upper filter to obtain the amount of ATP bound to the reconstituted protein.

**Fluorescence Measurements**—Fluorescence measurements were performed with a Spex fluorolog instrument at 20 °C. Intrinsic fluorescence of SERCA1a or mutants was measured with excitation and emission wavelengths set at 290 and 330 nm, with bandwidths of 2 and 20 nm, respectively (Fig. 3), or 295 and 320 nm, with bandwidths of 5 and 10 nm, respectively (Figs. 4 and 5). Changes in tryptophan fluorescence were monitored at 20 °C after 20-fold dilution of concentrated WT SERCA1a or mutants (to final concentrations of 0.1 mM Ca$^{2+}$ and 7.5 μg of protein/ml for WT SERCA1a or D351A, and 5 μg of protein/ml for P312A) into a medium containing 40 mM MOPS-Tris, pH 7.0, 80 mM KCl, 40% glycerol, 1 mg/ml DDM, and supplemented with Ca$^{2+}$, EGTA, or Mg-EDTA (according to previous studies (45, 46)) as indicated in the legends of Figs. 3–5. SR vesicles from rabbit muscle (at 8 μg/ml) served as a control. In ATP binding measurements, 5 mM MgCl$_2$ and EGTA were also added to the buffer, and various additions were performed, as indicated in the legends of Figs. 4 and 5.

**Phosphorylation Studies on P312A Expressed in COS Cells**—The P312A mutant was also expressed in COS-1 cells (12), and measurements of phosphorylation from [γ$^{32}$P]ATP or $^{32}$P, (in the absence of a Ca$^{2+}$ gradient), and of dephosphorylation, were carried out essentially as previously described (14, 16). The Ca$^{2+}$ gradient-dependent phosphorylation from $^{32}$P, (47, 48) was studied following passive Ca$^{2+}$ loading of the microsomal vesicles by incubation overnight with CaCl$_2$ added in concentrations ranging from 10 μM to 40 mM. The Ca$^{2+}$-loaded vesicles were diluted into a phosphorylation medium containing $^{32}$P, and EGTA to remove Ca$^{2+}$ from the medium inside of the vesicles. Further experimental details are given in the figure legends.

For quantification of enzyme phosphorylation, the acid-precipitated protein was washed by centrifugation and subjected to SDS-PAGE in a 7% polyacrylamide gel at pH 6.0, and the radioactivity associated with the separated Ca$^{2+}$-ATPase band was determined by imaging using a Packard Cyclone™ Storage Phosphor System. The experiments were generally conducted at least twice, and the data were analyzed by nonlinear regression using the SigmaPlot program (SPSS, Inc.).
**RESULTS**

**Protein Expression and Purification**—The strategy used to obtain the D351A and P312A proteins suitable for crystallization and biophysical measurements was to express in yeast these mutated forms of rabbit SERCA1a, fused to a biotinylation acceptor domain (BAD) by a linker containing a thrombin cleavage site (36). This construct allowed purification of the mutant by avidin chromatography after in vivo biotinylation in yeast, using thrombin to release the mutant protein from the affinity column.

Mutant D351A was expressed in yeast at about the same level as WT SERCA1a-BAD, whereas P312A was expressed at a somewhat lower, but nevertheless comparable level (Fig. 1). From 4 liters of yeast culture, we were able to purify the two mutants in the milligrams range at a concentration of 8–10 mg/ml as required by the crystallization protocol.

**[γ-32P]ATP Binding Measurements on D351A**—As described in the introduction, previous studies of D351A using the mammalian expression system had demonstrated an extraordinarily high affinity of this mutant for ATP (37). Using the yeast-expressed, purified, and reconstituted mutant D351A, it was feasible to measure directly [γ-32P]ATP equilibrium binding over a wide range of ATP concentrations from ~0.1 nM to 1 μM by filtration (Fig. 2). An extremely tight [γ-32P]ATP binding to D351A was indeed obtained in the presence of Ca2+, resulting in a $K_{0.5}$ value of 11 nM (at pH 7.2). In the absence of Ca2+ (i.e. after addition of EGTA), the binding was weaker (with a $K_{0.5}$ of 117 nM), in accordance with the previous observation using an indirect method for the same mutant (37). But still this value represents a much higher affinity than observed for WT SERCA1a ($K_d = 3–5 \mu M$ at pH 7.0–7.2 (see e.g. Refs. 18 and 49)).

**Fluorescence Measurements on Mutants**—By tryptophan fluorescence measurements, it was also possible to estimate the affinity of the yeast-expressed SERCA1a mutants for Ca2+ and ATP. When excited in the UV regions the protein with its 13 tryptophan residues responds with a fluorescence signal, which is affected by changes in the conformation of the native protein induced by addition of ligands such as Ca2+ or EGTA, and Mg2+ or MgATP. Previous measurements of fluorescence with wild-type yeast-expressed SERCA1a were performed after reconstitution of the purified (detergent-solubilized) SERCA1a into proteoliposomes (29, 36). This method has been used in preference to detergent-solubilized SERCA1a, which, as is well known (e.g. Refs. 50 and 51), inactivates very rapidly in the presence of calcium chelators. Moreover, the detergent concentration is known to affect (and reduce) the amplitude of the fluorescence signals (e.g. Refs. 52 and 53). Yet, reconstitution generally leads to a certain loss of material, and possibly to partial inactivation of the SERCA1a (in our hands ATPase activity was somewhat decreased after reconstitution of the purified proteins, data not shown).

As an alternative to reconstitution, we found here that it was possible to perform the fluorescence experiments directly on detergent-solubilized Ca2+-ATPase by the use of the same high concentration of glycerol (40% v/v) in which we stored our preparations in the deep-freezer after affinity chromatography. Under these conditions, SERCA1a was not only very efficiently protected from inactivation, but simultaneously the ligand-dependent fluorescence signals remained stable and of a decent size, both when DDM (Fig. 3) and C12E8 (data not shown) were used. These conditions with 40% glycerol, where SERCA1a remained stable, were therefore used to investigate the response of wild-type or mutant SERCA1a to ligand binding.

**Ca2+ Binding Affinity Measurements**—Fig. 3 shows the fluorescence changes observed when Ca2+ chelators were added at concentrations designed (as in Ref. 46) to result in a change of the free Ca2+ concentration from 100 μM to 1 μM and 0.04 μM, before a final shot of Ca2+ re-established the free Ca2+ concentration at its initial value of 100 μM. The similar relative amplitudes in all four cases excludes that a major proportion of the
yeast-expressed SERCAs could have adopted a non-native structure (except perhaps for P312A).

The fluorescence changes occurring upon decreasing the free concentration of Ca^{2+} from 100 μM to 1 μM free Ca^{2+}, relative to the maximal changes occurring upon reduction to still lower free concentrations of Ca^{2+}, were similar for all four SERCA1a proteins, indicating that the D351A mutation did not result in any significant difference in Ca^{2+} affinity, as compared with the wild-type yeast-expressed SERCA1a (and this was probably the case also, although less clear-cut, for the P312A mutation). The apparent $K_f$ for Ca^{2+} deduced from these data and from similar experiments performed at other free Ca^{2+} concentrations (data not shown) is ∼1.5–2 μM. This value is only slightly higher (due to DDM) than the absence of detergent under otherwise identical conditions, which is in accordance with previous studies of SR Ca^{2+}-ATPase (e.g. Ref. 54). Note, however, that detergent seems to accelerate very much movements of Ca^{2+} between the bulk medium and SERCA1a Ca^{2+} fixation site II (55), which might blur any effect of the mutations in native membranes.

Effects of Mg^{2+} and ATP Binding—Fluorescence changes are also observed upon binding of Mg^{2+} and MgATP to Ca^{2+}-free SR SERCA1a (56, 57). This effect is shown in Figs. 4 (A and B) for native SR SERCA1a and for yeast-expressed and purified WT SERCA1a, respectively. Here, a first addition of EGTA resulted in Ca^{2+} chelation and formation of a Ca^{2+}-free conformation with low fluorescence. The following addition of 5 mM Mg^{2+} caused an increase of the fluorescence signal. Finally, various concentrations of MgATP were added (0.3, 3, 30, and 300 μM), revealing the anticipated increase of fluorescence caused by ATP binding to the protein. At the highest concentrations of ATP, e.g. 300 μM, a drop in intensity was observed instead of a rise, because of the inner-filter effect caused by the UV absorbance by the nucleotide. The magnitude of the inner filter effect, which might mask a real increase in fluorescence due to ATP binding to the protein, is best evaluated by repeating the ATP additions in increments of 300 μM, beyond the levels required to saturate the ATP binding site on the protein, to allow true fluorescence changes to be deduced from these measurements at the lower concentrations of ATP (see Ref. 58).

For SR and WT SERCA1a, the addition of 0.3 μM MgATP in the absence of Ca^{2+} only gave rise to a slight increase in SERCA1a fluorescence, whereas 50% of the maximal fluorescence increase was observed at ∼3 μM MgATP for both enzymes (see traces A and B in Fig. 4), a value in the expected range (e.g. Ref. 57). A similar value was found for the P312A mutant, although the fluorescence changes were lower and noisier, and it seems that P312A and WT SERCA1a have similar ATP affinities (data not shown). For mutant D351A (see Fig. 4C), the addition of Mg^{2+} raised Trp fluorescence in a manner similar to that found with wild-type enzyme. On the other hand, with this mutant 0.3 μM MgATP added in the absence of Ca^{2+} was sufficient to increase the fluorescence signal by >50% of the maximal change observed upon saturation with ATP (see Fig. 4C). At these low ATP concentrations, inner filter effects are not significant. Thus, the estimated ATP_{0.5} value was ∼0.2 μM for D351A. This confirmed the high ATP affinity of the D351A mutant illustrated in Fig. 2.
Fig. 5A shows that addition of 3 μM MgATP to the D351A mutant in the presence of Ca²⁺ induced a rise in Trp fluorescence similar to that seen in the absence of Ca²⁺. Such an experiment is not feasible with the WT SERCA1a, which would become phosphorylated and start turning over utilizing the ATP in the presence of Ca²⁺. The fluorescence responses observed upon addition of lower concentrations of ATP demonstrated that the affinity of D351A for ATP in the presence of Ca²⁺ is higher than in the absence of Ca²⁺ with a $K_d$ of the order of 0.01 μM (data not shown), consistent with the $[^{32}P]$ATP binding measurements illustrated in Fig. 2. This experiment allows us to check that affinity for nucleotide seems to be not affected by the conditions of measurement, i.e. in the presence of DDM and glycerol. In Fig. 5B the same type of experiment as in Fig. 5A was repeated in the presence of 10 mM Ca²⁺ and 50 μM AMPPCP (i.e. the concentrations and type of nucleotide used in the crystallization conditions) leading to the same Trp fluorescence responses as described above (see controls in supplemental Fig. S1).

Crystallization—To increase the purity of the samples, the mutants purified by avidin chromatography were submitted to an additional step of size-exclusion chromatography. We also used this step as an opportunity for detergent exchange (from DDM to C₁₂E₈, present at 0.5 mg/ml in the HPLC buffer), because C₁₂E₈ is better suited than DDM for the preparation of Ca²⁺-ATPase crystals (33). The HPLC fractions containing the D351A or P312A proteins were pooled and concentrated to ~10 mg of protein/ml, followed by relipidation with DOPC. After incubation overnight at 4 °C, the detergent/phospholipid ratio was finally adjusted, and the sample was ultracentrifuged to eliminate aggregated material. With the help of a nanoliter dispensing robot, many conditions for crystallization could be tested and optimal conditions could be identified using a total of only 500 μg of pure protein per mutant.
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Crystals of D351A were obtained in the presence of 10 mM Ca\(^{2+}\) and 1 mM AMPPCP as for native SR SERCA1a (21) or expressed WT SERCA1a (34). In addition a successful crystallization of D351A was obtained with ATP replacing the non-hydrolyzable analogue, which was feasible with this mutant, because of the inability to hydrolyze ATP. For P312A, which is characterized as a mutant for which the ADP-sensitive phosphoenzyme (Ca\(_2\)E1P) piles up at steady state (16), the crystallization condition contained AlF\(_4\) and ADP together with 10 mM Ca\(^{2+}\) (21). The crystals displayed the same morphology as those of the native SERCA1a under comparable conditions. A full data set was collected at 3.4 Å for D351A in complex with AMPPCP and at 3.7 Å for D351A in complex with ATP, and the crystals of P312A with ADP plus AlF\(_4\) gave a data set at 3.5 Å (summarized in Table 1). There is a high level of isomorphism between WT SERCA1a and the two mutants, P312A and D351A, as can be seen from Figs. 6B and 7A, respectively. However, for a close comparison with that of the wild-type enzyme (34) we were confronted with the obstacle that the mutants crystallized in a different space group, P2\(_1\) instead of C2\(_1\). The P2\(_1\) crystal form is nearly identical to the C2\(_1\) form, but a slight change of the crystal packing has lead to deterioration of the C-centering, yielding now two copies in the asymmetric unit. This prevented us from computing difference Fourier maps between the mutants and the wild-type enzyme. On the other hand, we were able to compute a difference map between the mutants, \(F_{\text{obs}}(\text{P312A:ADP:AlF}_4) - F_{\text{obs}}(\text{D351A:AMPPCP})\), which confirmed both the absence of the aspartate side chain in the D351A mutant (Fig. 7B), the absence of the proline side chain in the P312A mutant (Fig. 6C), and slight differences in the binding of the nucleotides. However, apart from this no other significant differences between the two mutants were observed. Finally, the difference map of D351A with bound ATP and AMPPCP, \(F_{\text{obs}}(\text{D351A:ATP}) - F_{\text{obs}}(\text{D351A:AMPPCP})\) showed that, while both nucleotides are bound in the same overall bent conformation, the position of the \(\beta\)-phosphate appears to be slightly different (Fig. 7C). Taken together the structural information obtained from the crystals of D351A and P312A allow the conclusion that the mutations per se only result in subtle conformational changes, at least in the respective Ca\(_2\)E1P/AMPPCP and Ca\(_2\)E1P forms.

Phosphorylation Studies on P312A—The normal structure of P312A in the Ca\(_2\)E1P form raises the question whether the block of the Ca\(_2\)E1P \(\rightarrow\) E2P transition previously described (16) is due to an inability to form an E2P intermediate in the ground state. As illustrated in Fig. 8, we therefore re-examined the Ca\(_2\)E1P \(\rightarrow\) E2P transition and the functional properties of E2P in the P312A mutant in more detail than previously, using the COS-1 cell-expressed enzyme. At 0°C the rate constant of phosphorylation following phosphorylation with [\(\gamma\)-\(^32\)P]ATP was reduced 60- to 70-fold in P312A, relative to that of the wild type, and the phosphoenzyme remained ADP-sensitive (i.e. Ca\(_2\)E1P) throughout the experiment (Fig. 8A). At 25°C the turnover of the phosphoenzyme formed from ATP...
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TABLE 1
Crystalllographic data obtained for D351A and P312A crystals

| Data                  | D351A-AMPPCP | D351A-ATP | P312A-ADP-AlF₄ |
|-----------------------|--------------|-----------|-----------------|
| Space group           | P2₁          | P2₁       | P2₁             |
| Cell dimension (Å, °) | a = 152.9    | a = 152.7 | a = 150.6       |
|                       | b = 75.8     | b = 76.0  | b = 75.6        |
|                       | c = 162.6    | c = 163.1 | c = 161.8       |
| Beam angle            | β = 108.9    | β = 108.8 | β = 108.6       |
| Resolutions (Å)       | 30-3.4       | 30-3.5    | 30-3.5          |
| Completeness (%)      | 98.2 (83.0)  | 96.3 (87.2) | 99.7 (99.8) |
| Unique reflections    | 48,648       | 37,013    | 44,047          |
| Redundancy            | 3.4 (3.4)    | 3.0 (2.1) | 4.2 (4.2)       |
| Rmerge (%)            | 10.5 (66.7)  | 20.3 (57.4) | 25.4 (88.3) |
| I/σ(I)                | 11.89 (2.55) | 6.7 (2.4) | 7.0 (2.5)       |
| Wilson B-factor       | 87.5         | 66.3      | 82.1            |
| Model Resolution (Å)  | 30-3.4       | No model refined | 30-3.5 |
| R/|σ| (%)  | 26.5/29 (46.9/42.9) | No model refined | 20.8/25.8 (31.5/33.8) |
| Ramachandran (%)      | 77.9/19.9/20.0/3.0 | No model refined | 78.5/19.3/19.0/3.0 |
| r.m.s.d. bonds (Å)    | 0.009        | No model refined | 0.008 |
| r.m.s.d. angles (°)   | 1.54         | No model refined | 0.98 |
| Average B-value (Å²)  | 102          | No model refined | 97.38 |

* Values in parentheses refer to the highest resolution shell, except when otherwise stated.
* Rmerge = Σ|I(h) − 〈I(h)〉|/ΣI(h), where I(h) is the i th measurement.
* Determined by TRUNCATE of the CCP4 program package.
* Rfree is the R-factor calculated for a randomly picked subset with ~1000 reflections excluded from the refinement.
* Fractions of residues in the most favorable/allowed/generous/disallowed regions of the Ramachandran plot according to PROCHECK.

To examine whether the E2P state of the P312A mutant also exposed the Ca²⁺ binding sites to the lumen in a normal way, we studied the well known (see e.g. Refs. 47 and 48) Ca²⁺ gradient-dependent formation of E2P from P, (Fig. 9). This is an assay that up to now had never been performed with expressed ATPase. The microsomal vesicles containing expressed WT SERCA1a or mutant were loaded with Ca²⁺ at a high concentration of 20 mM and then diluted into a medium containing 32P, and EGTA (to chelate Ca²⁺ in the medium outside the vesicles and thereby allow phosphorylation from P). The high luminal Ca²⁺ concentration is known to allow the reaction of the wild type with P, even under conditions that in the absence of a Ca²⁺ gradient promote dephosphorylation (neutral pH, presence of K⁺ without organic solvent) (48), the effect of the luminal Ca²⁺ being attributed to a shift toward the right of the equilibrium E2P + 2Ca²⁺(lumen) ↔ Ca⁺E₂P ↔ Ca⁺E₁P (Scheme 1, steps 3–5). Phosphorylation with 32P dependent on the high luminal Ca²⁺ concentration was likewise observed with the P312A mutant, indicating that the mutation did not interfere with the luminal exposure of the Ca²⁺ sites in E2P (Fig. 9A). The phosphoenzyme formed with the wild type as well as the mutant under these conditions disappeared rapidly upon addition of ADP, as expected because of the equilibrium Ca⁺E₂P ↔ Ca⁺E₁P, leading to the formation of ADP-sensitive Ca⁺E₁P, whereas the E2P formed in the absence of a high luminal Ca²⁺ concentration was likewise markedly (~10-fold) reduced in P312A relative to wild type, although it is also noteworthy that the temperature dependence is steeper in the mutant than in the wild type. On the other hand, the dephosphorylation rate was wild type-like following backward phosphorylation of E2 with P, i.e. the rate of E2P → E2 + P, is normal, in the P312A mutant (Fig. 8D), cf. Scheme 1. This, together with the close to normal (~2-fold reduced) apparent affinity for P, in the backward phosphorylation with P, (Fig. 8C), excludes any significant destabilization of the Ca²⁺-free E2P form of the mutant. Hence, in P312A as opposed to certain other SERCA mutants with mutation in the cytoplasmic domains (8), accumulation of Ca⁺E₁P is not caused by alteration of the properties of the Ca²⁺-free E2P form.
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FIGURE 7. Crystal structures of yeast-expressed D351A mutants of SERCA1a in the presence of Ca\textsuperscript{2+} and AMPPCP (or ATP). A, overall view of the crystal structure of D351A-AMPPCP mutant showed in yellow, aligned with the WT structure of Ca\textsubscript{2}E1-AMPPCP (PDB code: 1T55), shown in green. B, enlarged view of the phosphorylation site where the structure of WT Ca\textsubscript{2}E1-AMPPCP is aligned with the structure of the D351A mutant. In the WT structure, the residues Asp\textsuperscript{351}, Phe\textsuperscript{487}, Asp\textsuperscript{703}, and the Ca\textsuperscript{2+}-AMPPCP substrate analogue are displayed; in the mutant structure, Ala\textsuperscript{351}, Phe\textsuperscript{487}, Asp\textsuperscript{703}, and Ca\textsuperscript{2+}-AMPPCP are displayed. F\textsubscript{obs}(P312A-ADP-AlF\textsubscript{4}) - F\textsubscript{obs}(D351A-AMPPCP) difference Fourier map reveals a clear positive peak (displayed at 7\textsigma, red mesh), which confirms the absence of the aspartate side chain in D351A mutant. (Differences between ADP-AlF\textsubscript{4} and AMPPCP are also apparent in the difference map albeit at lower (3.0 \textsigma) contour level, not shown.) C, the computed structure of D351A with AMPPCP shown here was similar to that of D351A with ATP-bound except for a positive and a negative peak (displayed at 4\textsigma and -4\textsigma, purple and orange mesh, respectively) in the F\textsubscript{obs}(D351A-ATP) - F\textsubscript{obs}(D351A-AMPPCP) difference Fourier map, which we interpret as a slight shift of the position of \beta-phosphate in ATP, otherwise there were no clear differences between the D351A-AMPPCP and the D351A-ATP structures.

was exclusively ADP-insensitive (Fig. 9A) (47). By loading the vesicles with various Ca\textsuperscript{2+} concentrations, it was also feasible to determine an apparent affinity for luminal Ca\textsuperscript{2+}. Importantly, we found that the P312A mutant displayed a significant increase of the luminal Ca\textsuperscript{2+} affinity relative to the wild type (Fig. 9B), which may be accounted for by a displacement of the equilibrium E2P \leftrightarrow Ca\textsubscript{2}E1P \leftrightarrow Ca\textsubscript{2}E1P in favor of one or both of the latter intermediates. What is excluded is that Ca\textsubscript{2}E2P can be considered to be destabilized with respect to Ca\textsubscript{2}E1P, i.e. that formation of Ca\textsubscript{2}E1P from the Ca\textsubscript{2}E2P species is impossible in the mutant.

DISCUSSION

Recent methodological and technological progress has led to advances in the clarification of the structure of membrane proteins by x-ray crystallography (33), especially for a number of prokaryotic membrane transporters. Compared with this, structural determination at atomic resolution of eukaryotic membrane proteins is still lagging behind, but the preparation of diffracting crystals of SERCA1a with structural analogues of the physiological ligands, representing many different functional states, has led to significant progress in understanding the pump function of this P-type ATPase (59, 60), and this progress has been made possible by the ready availability of the protein in large amounts at an already sufficient purity level from an animal source (skeletal muscle). However, when dealing with Ca\textsuperscript{2+}-ATPase mutants we are in the same situation as with most eukaryotic membrane proteins that are only available in small amounts and require extensive purification for crystallization, including solubilization by detergents with no loss of functional properties. The yeast-expression method developed in our laboratory for this purpose relies on the expression of a fusion protein consisting of SERCA1a and the BAD domain, which has allowed us to obtain fair amounts of purified and active protein amenable to both functional investigation and crystallization (34, 36). Our results provide for the first time an answer to a fundamental question in relation to Ca\textsuperscript{2+}-ATPase mutants: whether the functional changes resulting from mutations reflect major structural deviations from the wild type. At least for the two mutations under study here, D351A and P312A, despite the profound changes in the functional properties, we can now conclude that they are only associated with rather subtle structural changes, which makes it imperative to discuss the structural basis of the functional disturbance from this point of view.

The D351A Mutant—For this mutant we have confirmed a previous observation, namely that it exhibits an extraordinarily high affinity for ATP. We were able to deduce this both from classic [\gamma\textsuperscript{32}P]ATP binding measurements, performed by filtration of D351A reconstituted into proteoliposomes (Fig. 2), and from Trp fluorescence measurements performed using purified protein still solubilized in DDM (Fig. 4). From a methodological point of view it is of interest that we found that under appropriate conditions (40% glycerol added to Ca\textsuperscript{2+}-ATPase solubilized by 1 mg/ml DDM) information could be obtained directly from the detergent-solubilized state, obviating the need to go through the complete reconstitution procedure used previously (15, 36). In addition to confirming the reliability of the previous measurement of an ATP affinity in the nanomolar
range in D351A (37), we also show that the D351A mutation does not alter the affinity for Ca\(^{2+}\) (Fig. 3), thus demonstrating that the removal of this negative charge at the catalytic site does not influence the long range interaction with the Ca\(^{2+}\) binding sites and the equilibrium between the E1 and E2 forms. In this respect there seems to be a difference from the corresponding Na\(^{+}\),K\(^{+}\)-ATPase mutation, which appears to displace the E1–E2 conformational equilibrium in favor of E2 (61).

The inability of D351A to utilize ATP in the presence of Ca\(^{2+}\) allowed for the first time crystallization of the intermediate with Ca\(^{2+}\) and ATP simultaneously bound. Moreover, D351A measurements of ATP-induced Trp fluorescence responses were feasible even in the presence of Ca\(^{2+}\), which activates phosphorylation in the wild type. Hence, with this mutant it was possible to compare the fluorescence response induced by MgATP at a moderate (100 μM) Ca\(^{2+}\) concentration with that observed upon addition of AMPPCP under crystallization conditions at 10 μM Ca\(^{2+}\) (i.e. presumably as a Ca-AMPPCP complex, see Ref. 62) (Fig. 5). This provides an interesting confirmation of the physiological relevance of the x-ray data obtained with crystals formed in the presence of the very high Ca\(^{2+}\) concentration of 10 mM. Furthermore, we found that the effect of Mg\(^{2+}\) on the conformation of the SERCA1a pump remained observable with the D351A mutant (Fig. 4C). This long studied effect of Mg\(^{2+}\) (56, 63), was previously suggested not to depend on the on the hypothesized binding of Mg\(^{2+}\) to the first Ca\(^{2+}\)
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binding site (29). The present data indicate that it does not depend, either, on the putative binding of Mg\(^{2+}\) to the Asp\(^{351}\) -negative charge.

With the D351A mutant, the crystals obtained diffracted to 3.4-Å resolution in the presence of AMPPCP and Ca\(^{2+}\), and to 3.7-Å resolution in the presence of ATP and Ca\(^{2+}\). The difference Fourier maps show that the triphosphate moiety of both nucleotides are bound in nearly the same bent conformation as in wild-type Ca\(^{2+}\)-ATPase (Fig. 7C). The elevated affinity of the D351A mutant for ATP (37) and the similar effect of the corresponding Na\(^{+},K^{+}\)-ATPase (61) mutation have been interpreted in terms of a decrease in binding energy of the wild type, which is caused by the approach of the negatively charged γ-phosphate and the Asp\(^{351}\) carboxylate group leading to formation of the aspartyl-phosphoanhydride. Fig. 10 illustrates the electrostatic environment of the phosphorylation site, as based on the Ca\(_{21}\)-AMPPCP structure (21). The negatively charged residues of this site are Asp\(^{351}\), Asp\(^{703}\), and Asp\(^{707}\), which are counterbalanced by Lys\(^{684}\), also essential to phosphorylation (13), and by a bound divalent cation (Ca\(^{2+}\) in the crystal structure (62), Mg\(^{2+}\) in the physiological situation) coordinated by Asp\(^{351}\), Asp\(^{703}\), Thr\(^{353}\), and the γ-phosphate of the trinucleotide. Lys\(^{684}\) and the divalent cation, by interaction with Asp\(^{351}\), will provide charge delocalization of the carboxylic group of Asp\(^{351}\), permitting the approach of the γ-phosphate to form the transition state of the phosphorylated Ca\(_{21}\)E1P intermediate with MgATP as the substrate. Furthermore, Lys\(^{684}\) and Mg\(^{2+}\), by their interposition between the negatively charged groups, will exert a partial screening effect of the electrostatically negative field, arising from Asp\(^{703}\) and Asp\(^{707}\). Replacement of Asp\(^{351}\) by alanine or asparagine (37) will tip the balance in favor of positively charged groups at the phosphorylation site and thereby enhance the affinity for nucleotide binding. The similarity between the structures of the wild-type Ca\(^{2+}\)-ATPase and the D351A mutant, with no difference between the main chains around Asp\(^{351}\) (Fig. 7), supports the hypothesis of a purely electrostatic effect with no major conformational changes in D351A.

On the basis of the distances among the relevant charged groups, the magnitude of the effect can be roughly quantified from electrostatic theory. Such calculations call for an estimate of the change in electrostatic potential (Δψ), caused by removal of the electrostatic charge from the carboxylate group of Asp\(^{351}\). According to Coulomb’s law, the presence of an electrostatic point charge causes a change in the surrounding electrostatic potential, Δψ ∼ e k / D r, where e is the electronic unit charge (1.6 × 10^-19 C), k is Coulomb’s constant (9 × 10^9 N m^2 C^-2), D is the dielectric constant at the binding site, r is the distance (in meters), C is coulombs, and N is newtons. As indicated in Fig. 10, the distance between the charges of the carboxylate group and the γ-phosphate can be estimated to be 4 Å, which by insertion of a suitable value for the dielectric constant inside the protein (D = 20) leads to a Δψ value of 0.18 V induced by removal of the charge from the Asp\(^{351}\) carboxylate group. By application of the Nernst equation this corresponds to a 1250-fold increase in binding affinity at 20 °C, as calculated from the expression, ln K\(_{eq}\) = ln K\(_{eq,int}\) + zFΔψ/R T, where K\(_{eq}\) represents the (intrinsic) equilibrium constant of the charged state, z is ion charge, F is Faraday, R is gas constant, and T is absolute temperature. An increase in affinity of this order of magnitude will suffice to account for the marked increase in nucleotide binding affinity as indicated by a K\(_{d}\) value for the dissociation constant ATP of 0.11 μM for the Ca\(^{2+}\)-bound D351A mutant. Similarly, the effect of the mutation on the nucleotide binding affinity in the Ca\(^{2+}\)-depleted ATPase can be estimated from the E2-TG-AMPPCP structure (PDB code 2C88), where TG is thapsigargin (18). Here the γ-phosphate is located at the periphery of the P-domain, with the result that the distance from Asp\(^{351}\) is increased to 9 Å. Assuming this conformation will be retained after removal of one electronic charge at the phosphorylation site, the estimated increase in binding affinity will be 24-fold, we then compared it with an experimentally determined increase of ~45-fold (from a K\(_{d}\) of 0.11 μM for the D351A mutant in the present work, to 5 μM in native Ca\(^{2+}\)-ATPase (18)). Thus, the data are consonant with binding of monovalently (rather than divalently) charged MgATP (which appears to be a reasonable proposition at pH 7.2 and D = 20), and the electrostatic interactions become more prominent when the Ca\(^{2+}\)-ATPase resulting from binding of Ca\(^{2+}\) is converted to the Ca\(_{21}\)E1 state. It should be emphasized that the above calculations are dependent on the near identity of the mutant and wild-type structure, such that any movements of other charged groups (including the coordinated Mg\(^{2+}\)) can be neglected. The good agreement between the calculation and the measurement of binding affinity underscores
FIGURE 11. The structural consequence of the P312A mutation. Upper part: comparison of the M4 helix between the native E1P (1T5S) (magenta sticks) and the E1P mutant P312A in Ca\textsubscript{2}E1\textsubscript{1}/ADP/AlF\textsubscript{4}/AlF\textsubscript{4} form (yellow sticks) structure. On the right, the two structures are superimposed on residues 605–745. Lower part: comparison of P312A (E1P) and native SERCA1a in E2·BeF\textsubscript{3} form (E2P). Yellow: P312A in Ca\textsubscript{2}E1·ADP·AlF\textsubscript{4} form (E1P). Green: native SERCA in E2·BeF\textsubscript{3} form (E2P).
the conclusion drawn from the comparison of the crystal structures of mutant and wild type.

The P312A Mutant—The other mutant studied, P312A, was crystallized in a transition state of phosphorylation as Ca$_2$E1P (which structurally resembles Ca$_2$E1P). The crystallization conditions were similar to those used for crystallization of the native enzyme in the same state (21), and the crystals diffracted to 3.5-Å resolution. Like the D351A mutant, the P312A structure shows no significant deviation from the wild-type structure, even in the 308PEGLP segment where only the absence of the Pro$^{312}$ side chain is noted (Figs. 6 and 11, upper part). In the wild type, this part of the M4 helix is unwound at Pro$^{308}$ to accommodate one of the bound Ca$^{2+}$ ions, and there is no reason to believe that there is any difference in this regard for the P312A mutant. In agreement with this view we observed that the Ca$^{2+}$ affinity of the cytoplasmically facing high affinity sites is probably not altered very significantly by the P312A mutation (Fig. 3). The reason why the ability of the P312A mutant to hydrolyze ATP and translocate Ca$^{2+}$ is severely curtailed was previously shown to be due to a slowing of the Ca$_2$E1P → E2P transition (16), and this conclusion is confirmed by the more extensive functional measurements in the present study (Fig. 8, A and B). It is of note that mutation in the same region of the closely related Na$_3$K$^+$-ATPase (where Leu rather than Pro is present in the homologous position to Pro$^{312}$) likewise causes a block of the Na$_3$E2P → E2P transition (64). Fig. 11 (lower part) compares the conformation of M4 of the P312A mutant and of the wild type in the E2P ground state (resulting from the Ca2E1P → E2P transition), based on the E2-BeF3 crystal structures as reported recently by us (27) and by Toyoshima’s group (65). Although the two new E2P structures differ in important respects, especially concerning the presence of a wide open luminal channel for release of Ca$^{2+}$ in our structure (27), they do align well with respect to the kink that is formed by the unwound part of M4 (Fig. 11). The change of orientation of this unwound part of M4 in all E2 conformations, which in the E2P ground state contributes to the formation of the luminal exposure of the Ca$^{2+}$ sites in E2P, is likely caused by a push, after phosphorylation, of the P-domain on the M4 helix. This leads to its downward displacement (~5 Å) in the membrane (25, 27). What then could explain the slowing of the Ca$_2$E1P → E2P transition in the mutant? Apart from a possible destabilization of transition states along this overall transition, for yet unknown reasons, several hypotheses were considered. Initially, before performing the phosphorylation experiments illustrated in Fig. 9, we had tentatively considered the possibility that the absence of Pro$^{312}$ in the P312A mutant might prevent this helix from adopting the best conformation that apparently is required (27) for opening the phosphoenzyme Ca$^{2+}$ site toward the lumen. But such destabilization of the Ca$_2$E2P form was subsequently excluded by our measurements of the Ca$^{2+}$-dependent phosphorylation in P312A, which showed that luminal Ca$^{2+}$ had normal access to its sites on E2P (Fig. 9). Therefore Ala$^{312}$ does not inhibit the formation of either Ca$_2$E2P or the open form (ground state) of E2P. We have therefore considered another explanation for the slowing of the Ca$_2$E1P → E2P transition in the P312A mutant. We propose that the replacement of Pro$^{312}$ by alanine is relieving an inbuilt constrained region$^7$ corresponding to the Ca$^{2+}$ binding site I. This could explain the slowing of the Ca$_2$E1P → E2P transition in the mutant. Stabilization of a close to normal Ca$_2$E1P conformation by the P312A mutation is consistent with the similar crystal structures of the mutant and the wild type and would also be consistent with the right shift of the equilibrium E2P + 2Ca$^{2+}$lumen ↔ Ca$_2$E2P ↔ Ca$_2$E1P observed in the presence of a Ca$^{2+}$ gradient (Fig. 9B). Additionally, our data reveal that the temperature dependence between 4 °C and 20 °C for the E1P → E2P transition is steeper for the mutant than for WT, meaning that higher activation energy is needed for the transition between E1P and E2P for the mutant.

Finally, how can the “proline effect” be compared with other known structure of membrane proteins, transporters or channels? Interestingly, in the molybdate and sulfate ABC transporters, residues with significant conservation include a Pro-XXX-Pro sequence as in M4 of SERCA1a. These prolines are present in what has been pointed out as one of the gate regions in the molybdate transporter structure (66). In the voltage-dependent potassium channel Kv1.2 (35, 67), there is a Pro-X-Pro sequence in S6 inner helices, which allow these inner helices to curve so that they can form the correct interaction with the rest of the structure, an essential feature for the coupling of the voltage sensor movements to pore opening and closing. In several other membrane transporters the proline appear to be critical residues for transport (see e.g. Refs. 68 and 69). Conformational dynamics simulations indicate that proline can create a weak point in a helix that is thought to facilitate movements required for the function of some membrane proteins (see e.g. Ref. 70). On the other hand, Pro residues are sometimes found to be replaced by other residues in critical kink regions of membrane proteins, e.g. by Gly in the KcsA channel, and by Leu in Na$_3$K-ATPase in the position homologous to Pro$^{312}$ in Ca$^{2+}$-ATPase. For such data Yohannan et al. (71) have advanced evidence in favor of the view that other residues, as a result of evolutionary changes, can replace Pro in critical kink regions, when the optimized packing of the surrounding structure puts the non-Pro residue under strain to the same degree as by a Pro residue in a helical or other folded structure. The advantage of having a leucine present in the Na$^+$/K$^+$-ATPase at the position corresponding to Pro$^{312}$ of the Ca$^{2+}$-ATPase may be related to the difference between the affinities of the “high affinity” sites for Na$^+$ and Ca$^{2+}$ in the respective proteins, which amounts to ~1000-fold. Hence, because the Na$^+$-bound E1P form of the Na$^+$/K$^+$-ATPase is less stable than the Ca$^{2+}$-bound E1P form

$^7$ It is worth noting that the transmembrane segment M4 in both E1P (1TSS) and E2P (3R9B) contains a canonical type I turn in the 308PEGLP$^{312}$ region separating the M4 segment into two α-helices, and type I turns are well fitted for places in a sequence where conformational readjustments must occur (J.-M. Neumann, CEA, Saclay, personnel observation). Indeed, the phi, psi angles in 1TSS are ~−100, +6 for Gly$^{310}$ (which is i+2 of Pro$^{308}$, the beginning of this turn) and ~−59, −22 for Glu$^{328}$ (which is i+1), consistent with a canonical type I turn. Having two prolines as PXXP constrains the choice of a hydrogen bond between i (Pro$^{308}$ and i+3 (Leu$^{311}$) through this turn type I. Introducing an alanine in 312 instead of a proline gives a second opportunity to Pro$^{308}$ to make an i+4 hydrogen bond with the NH of Ala$^{312}$, which is impossible with a proline in 312. That this possibility can occur has been tested using simple modeling protocols (J.-M. Neumann and M. le Maire, CEA, Saclay, unpublished observation).
of the Ca$^{2+}$-ATPase, there may not be the same requirement for a proline in the Na$^+$,K$^+$-ATPase as in the Ca$^{2+}$-ATPase, to allow the transition from E1P to E2P to occur at a reasonable rate.

Concluding Comments—The methodology worked out here for yeast expression and purification of Ca$^{2+}$-ATPase mutants in relatively large yield holds promise for correlating functional and structural properties of Ca$^{2+}$-ATPase investigated by biophysical techniques such as x-ray crystallography, intrinsic fluorescence, etc. As many Ca$^{2+}$-ATPase mutants with different functional deficiencies have previously been characterized enzymatically, mainly by phosphorylation and transport assays, the structural background for these changes should become clearer. The present results are in accordance with the simplifying concept that the mutations either affect the affinity for a ligand binding to a particular conformational state, due to local changes in the binding site (the electrostatics in the case of D351A), or displace the equilibrium between basic conformations that already exist in the wild-type enzyme (as seen for P312A). The third, highly relevant possibility, that aberrant properties of mutants are caused by the induction of new conformational states not existing in the wild type is not supported by the extent to which this is also the case for other mutants of central importance. Of course, for such investigations to be fruitful there are important difficulties that have to be overcome for each mutant, such as the proper expression and stability of the purified mutated proteins in detergent. Beyond this scope, our methods for yeast expression and purification hopefully also will find a place in the study of the structure of other membrane proteins.

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