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Conformational Changes in Sarcoplasmic Reticulum Ca^{2+}-ATPase Mutants: Effect of Mutations either at Ca^{2+}-Binding Site II or at Tryptophan 552 in the Cytosolic Domain†

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ABSTRACT: By analyzing, after expression in yeast and purification, the intrinsic fluorescence properties of point mutants of rabbit Ca^{2+}-ATPase (SERCA1a) with alterations to amino acid residues in Ca^{2+}-binding site I (E^{771}), site II (E^{309}), in both sites (D^{800}), or in the nucleotide-binding domain (W^{552}), we were able to follow the conformational changes associated with various steps in the ATPase catalytic cycle. Whereas Ca^{2+} binding to purified wild-type (WT) ATPase in the absence of ATP leads to the rise in Trp fluorescence expected for the so-called E2 → $E1Ca_2$ transition, the Ca^{2+}-induced fluorescence rise is dramatically reduced for the $E^{309}Q$ mutant. As this purified $E^{309}Q$ mutant retains the ability to bind Ca^{2+} at site I (but not at site II), we tentatively conclude that the protein reorganization induced by Ca^{2+} binding at site II makes the major contribution to the overall Trp fluorescence changes observed upon Ca^{2+} binding to both sites. Judging from the fluorescence response of $W^{552}F$, similar to that of WT, these changes appear to be primarily due to membranous tryptophans, not to W^{552}. The same holds for the fluorescence rise observed upon phosphorylation from P_i (the so-called E2 → E2P transition). As for WT ATPase, Mg^{2+} binding in the absence of Ca^{2+} affects the fluorescence of the $E^{309}Q$ mutant, suggesting that this Mg^{2+}-dependent fluorescence rise does not reflect binding of Mg^{2+} to Ca^{2+} sites; instead, Mg^{2+} probably binds close to the catalytic site, or perhaps near transmembrane span M3, at a location recently revealed by Fe^{2+}-catalyzed oxidative cleavage. Mutation of W^{552} hardly affects ATP-induced fluorescence changes in the absence of Ca^{2+}, which are therefore mostly due to membranous Trp residues, demonstrating long-range communication between the nucleotide-binding domain and the membranous domain.

Ca^{2+} is a ubiquitous second messenger involved in a wide range of cellular processes, e.g., fertilization, muscle contraction, and exocytosis (*1*). In resting cells, the intracellular level of free Ca^{2+} is kept at low, submicromolar concentrations, and signaling can be achieved through transient release of Ca^{2+} from internal compartments such as the sarco/endoplasmic reticulum. Such compartments are subsequently reloaded thanks to the action of ATP-dependent Ca^{2+} pumps, the sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs),[1] which belong to the family of P-type cation-pumping ATPases that actively transport cations (like Ca^{2+}, Na^+, K^+, and H^+, or even heavy metals) by a mechanism involving the formation of a phosphorylated intermediate (for recent reviews, see refs *2* and *3*). A remarkable member of this family, the Ca^{2+}-ATPase (SERCA1a) present in adult skeletal muscle sarcoplasmic reticulum (SR), allows muscle relaxation. It contains 994 amino acid residues, has a molecular mass of 110 kDa (*4*), and transports into the reticulum lumen two Ca^{2+} ions per ATP hydrolyzed. Transport is achieved through a reversible cycle, during which Ca^{2+}-ATPase conformation is thought to alternate between various states of low or high affinity for Ca^{2+} (so-called E2 or E1 states, respectively), and Scheme 1 illustrates a popular description of this transport cycle, in which the two transported Ca^{2+} ions are supposed to bind sequentially to the "E1" conformation of the ATPase. Recently, X-ray diffraction analysis of three-dimensional crystals of this protein corresponding to some of the intermediates of this cycle, combined with biochemical analysis of mutants, has provided a structural basis for the understanding of the transport mechanism (*5*−*10*).

The protein has 10 transmembrane spans (M1−10), as well as a bulky cytosolic region consisting of three major domains that are connected to the membrane-embedded part by a stalk. Two of these domains, the nucleotide-binding domain (N) and the phosphorylation domain (P), are formed by the amino

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[1] Abbreviations: SR, sarcoplasmic reticulum; SERCA, the sarco-(endo)plasmic reticulum Ca^{2+}-transporting adenosinetriphosphatase; WT, wild type; Ni-NTA, Ni^{2+}-nitrilotriacetic acid; DDM, *n*-dodecyl β-D-maltoside; EYPC, egg yolk L-α-phosphatidylcholine; EYPA, egg yolk phosphatidic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; $K_{0.5}$, ligand concentration giving half-maximum effect; V_m, maximal value of the ATPase activity.

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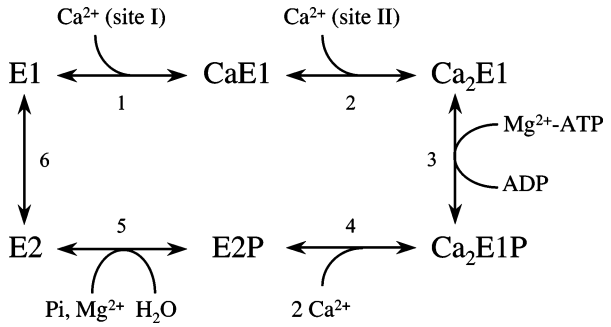
Scheme 1: Ca^{2+}-ATPase Reaction Cycle for Ca^{2+} Transport



acid residues located between the membrane-spanning segments M4 and M5. The third domain (termed A) is formed from both the N-terminal tail and the amino acids located between segments M2 and M3. The membrane domain contains the two Ca^{2+}-binding sites located side by side, with α-helices M4−M6 and M8 containing the residues that coordinate the two Ca^{2+}. Binding of Ca^{2+} to the ATPase is a crucial event in the catalytic ATPase cycle, as it leads to the formation of an ATPase species reactive toward phosphorylation from ATP and results in D^{351} phosphorylation (steps 1, 2, and 3 in Scheme 1). This binding is accompanied by large conformational changes in the protein cytosolic region as well as in its membrane region (*6*). As illustrated in Scheme 1, binding of Ca^{2+} to the ATPase is generally described as being sequential (*11*−*13*): transmembrane helices M5, M6, and M8 contribute to Ca^{2+} binding at "site I" through side chains of residues N^{768}, E^{771}, T^{799}, D^{800}, and E^{908}, while M4 and M6 helices contribute to binding of a second Ca^{2+} ion at "site II" through side chains of residues N^{796}, D^{800}, and E^{309} (thus, D^{800} contributes to both binding sites) and main-chain carbonyl oxygen atoms of V^{304}, A^{305}, and I^{307} (*5*, *13*, *14*). After mutation of a residue involved in Ca^{2+} binding at site II (e.g., E^{309}), Ca^{2+}-ATPase only retains binding of one of the two Ca^{2+} ions, a priori at site I (*15*, *16*), and is no longer phosphorylatable from ATP, emphasizing the critical role of Ca^{2+} binding at site II for the protein conformation (*14*).

Nevertheless, the high-resolution structures of the Ca^{2+}-ATPase obtained so far (*5*−*10*) only provide snapshots of the Ca^{2+}-ATPase conformation in a few particular states. Many questions remain unaddressed concerning the exact mechanism for energy transduction between the ATP hydrolysis site and the ion transport sites, two regions 50 Å apart. Which are the detailed events occurring during the transitions between the various ATPase forms revealed by X-ray crystallography or, more generally, in the various intermediate steps of the ATPase cycle? For instance, what is the exact sequence of events resulting in the conformational changes that accompany Ca^{2+} binding? What is the contribution of each of the two Ca^{2+} ions to these conformational changes (steps 1 and 2 in Scheme 1)? Which parts of the protein are experiencing significant changes during the various transitions in Scheme 1?

Almost 30 years ago, intrinsic fluorescence was found to be a powerful tool for examining in a global way the various conformations of the ATPase, both under equilibrium conditions (*17*) and under transient conditions (*18*, *19*). However, due to the lack of purified recombinant Ca^{2+}-ATPase suitable for fluorescence studies, it has up to now been difficult to make more specific the information derived from the overall fluorescence of the many (13) Trp residues of the rabbit SR Ca^{2+}-ATPase.

We recently made a first step in this direction by showing that purification of recombinant Ca^{2+}-ATPase does make it possible to monitor with Trp fluorescence the conformational changes associated with various transitions in the catalytic cycle of ATPase expressed in the yeast *Saccharomyces cerevisiae*. In that work, Trp fluorescence was used for understanding more accurately the functional consequences resulting from simultaneous mutation ($D^{813}A/D^{818}A$) of two aspartates in the cytosolic L6−7 loop (*20*), and the results were consistent with those obtained independently after a single mutation at D^{813} (*21*). In the present work, we make a further step ahead and use Trp fluorescence spectroscopy (in addition to classical phosphorylation assays) to characterize conformational changes of a purified and reconstituted ATPase mutated at one of the two Ca^{2+}-binding sites (site II), the $E^{309}Q$ mutant. We also describe results obtained with another ATPase mutant in which 1 of the 13 Trp residues has been replaced by a phenylalanine: specifically, the $W^{552}F$ mutant was studied here to evaluate the contribution to the overall fluorescence changes of a tryptophan residue (W^{552}) located far away from the membranous domain but close to the ATP-binding region. We provide evidence that binding of Ca^{2+} at site II (CaE1 → Ca_2E1 transition), not at site I, is probably responsible for the rise in tryptophan fluorescence observed upon binding of the two Ca^{2+} ions, a rise experienced by tryptophan residues located in the membrane region. Our data also suggest that the fluorescence enhancement observed upon Mg^{2+} binding to the ATPase (e.g., at pH 7) is due to Mg^{2+} binding in the cytosolic domain, not at the Ca^{2+}-binding sites. Lastly, as ATP-induced fluorescence changes in the absence of Ca^{2+} are unaltered in the $W^{552}F$ mutant, our data give further evidence for long-range transmission of signals in Ca^{2+}-ATPase between the nucleotide-binding domain and the membranous domain.

MATERIALS AND METHODS

Solutions and Chemicals. Yeast culture products were from Difco Laboratories. *n*-Dodecyl β-D-maltoside (DDM) was obtained from Anatrace Inc. (Anagrade). Low molecular weight markers were from Amersham Biosciences. Other products were purchased from Sigma.

Expression and Purification of C-Terminal His-Tagged WT and Mutant Ca^{2+}-ATPases. His-tagged WT and mutant ($E^{309}Q$, $E^{771}Q$, $D^{800}N$, or $W^{552}F$) SERCA1a Ca^{2+}-ATPases were expressed in yeast using a previously described procedure (*22*), and expressed Ca^{2+}-ATPases were purified (using Ni-NTA affinity chromatography) and reconstituted as in refs *20* and *22*. Briefly, after SERCA1a expression, yeast cells were broken with glass beads, and light membranes containing the expressed ATPase were separated by differential centrifugations. These membranes were solubilized using DDM at a 3/1 (w/w) detergent/protein ratio and incubated with Ni-NTA beads. After Ca^{2+}-ATPase recovery from the Ni-NTA beads in a column, lipid vesicles (100−300 nm diameter) made of EYPC and EYPA at a 10/1 ratio (w/w) were added at a final concentration of 1 mg of lipid/mL (detergent concentration was 0.5 mg/mL). DDM was then eliminated using Bio-Beads at a 200/1 (w/w) Bio-Beads/

detergent ratio over 3 h at 4 °C under gentle stirring. The final reconstituted sample contained 100−200 μg/mL expressed ATPase; the medium also contained 1 mM Ca^{2+} to optimize stability.

SR Membrane Preparation. As control, sarcoplasmic reticulum microsomal vesicles were isolated from rabbit skeletal muscle as previously described (*23*, *24*), except that initial homogenization was performed in the presence of 1 μg/mL amylase and rabbits had been subjected to a 48 h starvation diet before preparation.

Functional Studies. ATPase hydrolytic activity was assessed as described previously using an ATP-regenerating coupled enzyme assay (*20*, *25*) and a HP8452A spectrophotometer equipped with a cuvette holder kept at 20 °C. The sample in the cuvette was continuously stirred. The reaction was performed with purified and reconstituted ATPase at about 5 μg of protein/mL in 2 mL of a medium containing 50 mM MOPS/Tris, pH 7.0, 100 mM KCl, and 5 mM $MgCl_2$ (buffer A), supplemented with various concentrations of Ca^{2+} or Ca^{2+}/EGTA buffer to yield the desired Ca^{2+}-free concentrations ($K_{d\,Ca-EGTA}$ = 0.5 μM at pH 7.0 and 5 mM $MgCl_2$). The reaction was started by the addition of 1 mM Na_2ATP.

Fluorescence measurements were performed with a Spex Fluorolog instrument. The temperature was kept at 20 °C, and samples were continuously stirred. Intrinsic fluorescence was measured with excitation and emission wavelengths of 290 and 330 nm and bandwidths of 2 and 20 nm, respectively, with about 5 μg/mL purified and reconstituted WT or mutant ATPase (or native SR vesicles, for control) in 150 mM MOPS/Tris at pH 7.0 (in the absence of KCl, to maximize fluorescence signals). To monitor intrinsic fluorescence changes upon removal or addition of Ca^{2+}, this buffer was supplemented with EGTA and Ca^{2+}; to monitor the changes occurring upon addition of inorganic phosphate, it was supplemented from the start with 5 mM $MgCl_2$, 0.5 mM EGTA, and 20% Me_2SO; to monitor those occurring upon addition of ATP, it was supplemented with 2 mM EGTA (in the presence of 55 μM total Ca^{2+}), and in some cases with $MgCl_2$, at a concentration of 5 mM.

"Back door" ATPase phosphorylation from $[^{32}P]P_i$ was measured by suspending 1 μg of purified and reconstituted ATPase in 100 μL of a final reaction mixture containing 100 mM MOPS/Tris, pH 7.0 (20 °C), 20 mM $MgCl_2$, 20% Me_2SO, and various concentrations of free Ca^{2+}; the reaction was started by the addition of 100 μM $[^{32}P]P_i$ (2 mCi/μmol) and continued for 30 s. Samples were then acid-quenched with trichloroacetic acid (TCA) and H_3PO_4 at final concentrations of 1 M and 67 mM, respectively. Samples (final volumes were typically 800 μL) were chilled on ice for 20 min and centrifuged at 28000*g* for 25 min at 4 °C. Pellets were washed with 800 μL of 75 mM TCA and 5 mM H_3PO_4 and centrifuged again, and this second pellet was finally resuspended by vortexing for 1 min in 50 μL of buffer containing 150 mM Tris-HCl, pH 6.8, 2% SDS, 10 mM EDTA, 16% glycerol, 0.8 M β-mercaptoethanol, and 0.04% bromophenol blue. Finally, 20 μL aliquots were subjected to SDS−PAGE according to the method of Sarkadi (*26*). Radioactivity was revealed with a STORM 860 PhosphorImager, from Amersham Biosciences, and quantified by comparison with known amounts of $[^{32}P]P_i$.

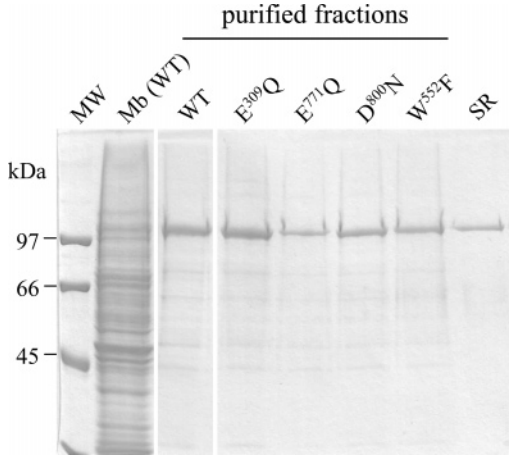


FIGURE 1: SDS−PAGE analysis of Ca^{2+}-ATPase mutants expressed in yeast and purified by Ni-NTA affinity chromatography. Purification of various Ca^{2+}-ATPase mutants and their reconstitution into EYPC/EYPA liposomes were performed as described in the Materials and Methods section. Samples (corresponding to about 2 μg of total protein for the most concentrated purified fractions) were loaded onto various lanes of an 8% acrylamide gel, submitted to SDS−PAGE according to Laemmli (*52*), and visualized by Coomassie blue staining. Key: MW, standard proteins, with the indicated molecular masses; Mb(WT), yeast light membranes, expressing about 2% WT Ca^{2+}-ATPase; WT, Ni-NTA purified and reconstituted WT Ca^{2+}-ATPase; $E^{309}Q$, $E^{771}Q$, $D^{800}N$, and $W^{552}F$, Ni-NTA purified and reconstituted $E^{309}Q$, $E^{771}Q$, $D^{800}N$, and $W^{552}F$ Ca^{2+}-ATPase mutants, respectively; SR, rabbit SR vesicles (1 μg of protein in this case).

RESULTS

Ni-NTA Affinity Purification of Mutants Expressed in Yeast. Wild-type Ca^{2+}-ATPase (WT) and Ca^{2+}-ATPases mutated either at residues within the Ca^{2+}-binding sites ($E^{309}Q$ for site II, $E^{771}Q$ for site I, and $D^{800}N$ for a residue common to both sites) or at a Trp residue in the cytosolic nucleotide-binding "N"-domain ($W^{552}F$) were overexpressed in yeast and purified in a single step by a protocol involving affinity purification on a Ni-NTA column (*20*). Purified ATPase was then reconstituted into a lipid mixture consisting of EYPC and EYPA (with a 10-fold excess of EYPC). As can be seen from the SDS−PAGE gel displayed in Figure 1, heterologously expressed Ca^{2+}-ATPase ran as the major band in the purified fractions, with an apparent molecular mass identical to that of native SR enzyme (last lane). WT and mutant ATPases were obtained at a purity approaching 50−60%, as estimated by densitometry and by comparison with native SR from rabbit sarcoplasmic reticulum (about 75% purity for native SR). The total amount of SERCA1a collected after purification varied from one mutant to another. For instance, the yield was much lower for $E^{771}Q$ than for $E^{309}Q$ (compare lanes $E^{309}Q$ and $E^{771}Q$ in Figure 1); this can be attributed to the fact that, for $E^{771}Q$, only 10% of the expressed ATPase was solubilized by DDM under our conditions, compared to 60−70% solubilization for $E^{309}Q$. This poor extractability of the $E^{771}Q$ mutant expressed in yeast has already been mentioned elsewhere (*27*).

Control Functional Assays: After Purification, the $E^{309}Q$ Mutant Remains Able To Bind Ca^{2+} at Site I. Purified $W^{552}F$ Behaves Normally. We first measured the influence of the free Ca^{2+} concentration on the catalytic activity of purified ATPases. As shown in panel A of Figure 2, ATP hydrolysis by WT ATPase was stimulated by micromolar Ca^{2+} con-

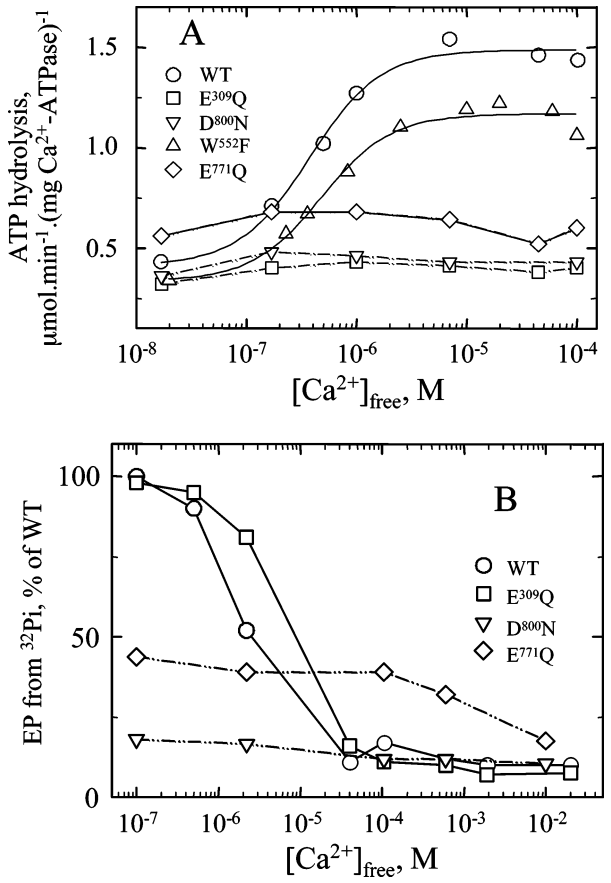


FIGURE 2: After purification, the $E^{309}Q$ mutant remains able to bind Ca^{2+} at site I; purified $W^{552}F$ behaves normally. (A) ATPase activity. Following the protocol in ref *20*, the rate of ATP hydrolysis by purified and reconstituted WT (○), $E^{309}Q$ (□), $E^{771}Q$ (◇), $W^{552}F$ (△), or $D^{800}N$ (▽) ATPase was measured in buffer A [50 mM MOPS/Tris, pH 7 (20 °C), 100 mM KCl, 5 mM $MgCl_2$] supplemented with 50 μM Ca^{2+}, added together with the 20-fold diluted purified and reconstituted samples. The amount of SERCA protein present in each case was deduced from SDS−PAGE experiments similar to the one in Figure 1. (B) Inhibition by Ca^{2+} of phosphoenzyme formation from $[^{32}P]P_i$. Phosphoenzyme was formed by incubating purified and reconstituted WT, $E^{309}Q$, $E^{771}Q$, or $D^{800}N$ ATPase with $[^{32}P]P_i$ at pH 7, 20% Me_2SO, and 20 °C, as described under Materials and Methods. The same amount of WT or mutant ATPase was used in all experiments (as checked, again, by Coomassie blue staining of the ATPase bands after SDS−PAGE). The amount of phosphorylated intermediate obtained at equilibrium for the WT ATPase in the presence of 10^{-7} M $[Ca^{2+}]_{free}$ was taken as 100%.

centrations to a maximal velocity (V_m) of about 1.5 μmol of ATP hydrolyzed min^{-1} (mg of Ca^{2+}-ATPase)$^{-1}$, and the K_m for Ca^{2+} was about 0.4 μM; the measured V_m is smaller than the one found for SR ATPase activity under similar conditions (not shown), while the measured K_m is consistent with earlier studies performed with SR-derived nonpurified WT ATPase or with microsomes derived from COS-1 cells transfected with WT SERCA1a cDNA under similar experimental conditions (*28*). As previously discussed (*20*, *22*), the modest V_m and the normal K_m can be accounted for by the presence of fully inactive ATPase in our partially purified preparation, and the fraction of fully inactive protein does not prevent us from exploiting the fraction of active protein. Under the same conditions, the V_m for the purified $W^{552}F$ mutant was 80% of that for WT, and the K_m for Ca^{2+} was again normal, showing that W^{552} is not essential for the SERCA1a catalytic cycle. In contrast, ATPase activity of the purified high-affinity Ca^{2+}-binding site mutants $E^{309}Q$, $D^{800}N$, and $E^{771}Q$ was no longer activated by Ca^{2+}; the low Ca^{2+}-independent ATPase activity observed was in the same range as that observed for WT and $W^{552}F$ ATPases at the lowest Ca^{2+} concentrations, suggesting that it corresponds to ATP hydrolysis by contaminating proteins present in these partially purified fractions. These results with purified mutants are in agreement with previous results obtained with nonpurified mutants (*16*) and confirm the need for two Ca^{2+} ions at the high-affinity binding sites for initial phosphorylation from ATP and, therefore, for overall ATP hydrolysis.

Solubilized Ca^{2+}-ATPase is known to be susceptible to irreversible inactivation by detergents in the absence of Ca^{2+} (*29*). To evaluate the possibility that our purified mutants could have suffered from the purification step that involves solubilization by detergent and to confirm that the purified and reconstituted $E^{309}Q$ mutant remains able to bind Ca^{2+} at an intact site I, as before purification, we measured the Ca^{2+} dependence of phosphorylation from P_i of these mutants. Upon binding to site I, Ca^{2+} is known to be able to prevent Ca^{2+}-ATPase phosphorylation from P_i (*14*, *30*). For WT ATPase as well as for the $E^{309}Q$ mutant, panel B in Figure 2 shows phosphorylation from P_i at various free Ca^{2+} concentrations (i.e., the dependence on Ca^{2+} of the formation of the so-called E2P state, from E2, here in the presence of Me_2SO at pH 7). In the absence of Ca^{2+}, the same amount of phosphorylated intermediate was formed for WT and $E^{309}Q$. The Ca^{2+} concentration required for half-inhibition of phosphoenzyme formation was about 10 μM for the $E^{309}Q$ mutant, not very different from that for the WT under the same conditions, 2 μM, and consistent with values obtained in previous studies with the nonpurified same mutant (*13*). This sensitivity to Ca^{2+} of the purified $E^{309}Q$ mutant demonstrates that its ability to bind Ca^{2+} at site I (E2 → CaE1) has been fully retained, despite the purification step, at least for the fraction that has not experienced full inactivation.

Note, in contrast, that, for the $E^{771}Q$ purified mutant, phosphoenzyme formation in the absence of Ca^{2+} was significantly reduced, while for $D^{800}N$, it was hardly detectable (Figure 2B). When phosphorylation from P_i was measured at pH 6, both $E^{771}Q$ and $D^{800}N$ had a reduced phosphorylation level (data not shown). Yet, nonpurified forms of $D^{800}N$ and $E^{771}Q$ have been previously shown to remain able to form a phosphoenzyme from P_i in the absence of Ca^{2+} (*30*); thus, $D^{800}N$ and to a lesser extent $E^{771}Q$ mutated Ca^{2+}-ATPases probably do experience denaturation during purification. From these results, we can infer that binding of one of the two Ca^{2+} ions at the transport sites (as occurs in $E^{309}Q$) is required and sufficient to prevent rapid inactivation in a solubilized state.

Trp Fluorescence Changes upon Binding of Ca^{2+}: Binding of Ca^{2+} to Purified $E^{309}Q$ (i.e., Binding to Site I Only) Does ***Not*** *Induce Significant Changes in Trp Fluorescence, While $W^{552}F$ Responds to Ca^{2+} Like WT.* Ca^{2+} binding is a crucial event in the Ca^{2+}-ATPase cycle, resulting in clear changes in Trp fluorescence (*17*−*19*, *31*−*33*). However, the detailed mechanism for the binding of the two Ca^{2+} ions and the associated conformational changes remain elusive. Here, as our purified $E^{309}Q$ mutant has retained after purification the ability to bind Ca^{2+} at one (and only one) of the two Ca^{2+}-

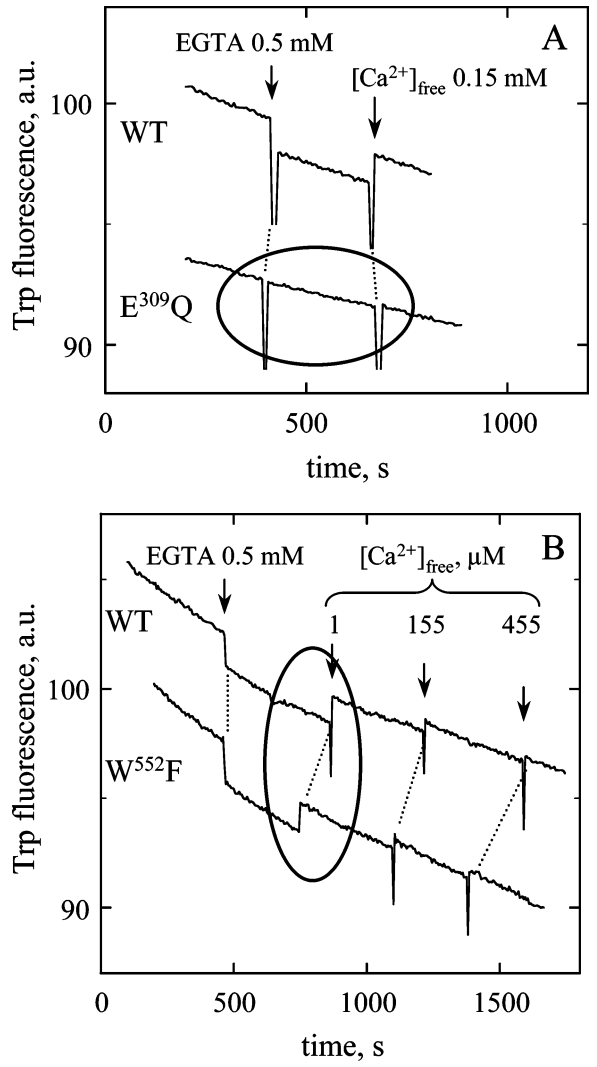


FIGURE 3: Binding of Ca^{2+} to purified $E^{309}Q$ (i.e., binding to site I only) does not induce significant changes in Trp fluorescence; $W^{552}F$ behaves normally. (A, B) Tryptophan fluorescence was monitored after 20-fold dilution (to about 5−10 μg/mL) of purified and reconstituted WT, $E^{309}Q$, or $W^{552}F$ Ca^{2+}-ATPase into 150 mM MOPS/Tris, pH 7 (20 °C). The final total Ca^{2+} was 55 μM, due to the presence of contaminating Ca^{2+} and Ca^{2+} added together with the enzyme. First, 0.5 mM EGTA was added to reach a free Ca^{2+} concentration of about 0.05 μM (as $K_{d\,Ca-EGTA} = 0.4$ μM at pH 7.0 in the absence of $MgCl_2$). Ca^{2+} was then added to restore the fluorescence level of the Ca^{2+}-bound form: for the experiments in panel A, performed with WT and $E^{309}Q$, 0.6 mM total Ca^{2+} was then added, leading to a free Ca^{2+} concentration of 0.155 mM; for the experiments in panel B, with WT and $W^{552}F$, sequential additions of 300 μM total Ca^{2+} were allowed to reach $[Ca^{2+}]_{free}$ concentrations of 1, 155, and 455 μM, respectively. The fluorescence traces have been slightly shifted with respect to each other for clarity, and the very small changes (0.1%) due to dilution have been corrected. Additions were identical for both traces in each panel, as indicated by the dotted lines.

binding sites, we searched for intrinsic fluorescence changes upon Ca^{2+} binding to this mutant (or upon Ca^{2+} dissociation from it).

For preliminary control, Figure 3A shows that dissociation of Ca^{2+} from WT ATPase previously incubated in the presence of 55 μM free Ca^{2+} triggered a fluorescence drop of about 1.5%, while subsequent addition of Ca^{2+} (to reach a free Ca^{2+} concentration of 155 μM) restored the fluorescence level of the initial Ca^{2+}-saturated form. The amplitude observed for these fluorescence changes is relatively small compared to that for native ATPase in the same conditions (5−6%), but its dependence on free Ca^{2+} is normal (not shown here, but see ref *20*); as discussed above for ATPase activity measurements and in ref *20*, this can be accounted for by the presence of contaminating proteins in our partially purified preparation as well as by the presence of fully denatured ATPases. Again, these unresponsive proteins do not prevent us from observing the properties of the nondenatured ATPases.

When EGTA was now added to the $E^{309}Q$ mutant (again preincubated with 55 μM Ca^{2+}), fluorescence remained essentially unaltered, and this was also true when Ca^{2+} was subsequently re-added (bottom trace in Figure 3A). Yet, we know from Figure 2B that, at 55 μM free Ca^{2+}, phosphorylation of $E^{309}Q$ from P_i (at the same pH) is fully prevented, so that Ca^{2+} site I in $E^{309}Q$ must have been fully occupied at the beginning of the experiment in Figure 3A. Thus, it appears that dissociation of Ca^{2+} from site I in $E^{309}Q$ does not affect Trp fluorescence.

It is known that SERCA1a contains 13 Trp residues, 12 of which are located close to the membrane while the last 1 (W^{552}) is part of the cytosolic nucleotide-binding N-domain (*5*). To establish whether W^{552} contributes to the Ca^{2+}-dependent fluorescence changes, we compared the fluorescence properties of the $W^{552}F$ mutant to those of WT ATPase (Figure 3, panel B). The results show that, in the presence of 1 μM free Ca^{2+}, the Ca^{2+}-binding sites were almost, but not fully, saturated both for WT and for $W^{552}F$. From this and other similar fluorescence records, the K_d for Ca^{2+} was estimated to be about 0.4 μM for both WT and $W^{552}F$, close to that for native sarcoplasmic reticulum Ca^{2+}-ATPase under identical experimental conditions (data not shown); the maximal amplitude of the observed changes in Trp fluorescence was also similar for WT and $W^{552}F$. Thus, W^{552} hardly contributes to the overall Ca^{2+}-dependent fluorescence changes, which are therefore primarily due to changes in intramembranous or interfacial Trp residues.

Trp Fluorescence Changes upon Phosphorylation from P_i (E2 → E2P): Phosphorylation from P_i Induces a Fluorescence Rise for both $E^{309}Q$ and $W^{552}F$ Mutants (as for WT). We also analyzed the intrinsic fluorescence changes induced by phosphorylation from P_i (*34*) under conditions similar to those of the ^{32}P experiments previously illustrated in Figure 2B, namely, in the presence of Me_2SO (at pH 7) which favors phosphorylation from P_i (to the "E2P" form) (*13*, *24*, *35*). As a control, panel A of Figure 4 first shows that addition of 0.2 mM P_i to Ca^{2+}-free purified WT ATPase (upper trace) resulted in the expected enhancement of intrinsic fluorescence (the P_i-dependent rise was about 50% of the initial drop induced by the addition of EGTA) and that further addition of Ca^{2+} restored the fluorescence level of the Ca^{2+}-saturated form, in agreement with fluorescence recordings obtained with rabbit sarcoplasmic reticulum Ca^{2+}-ATPase (data not shown and ref *34*). Then, the $E^{309}Q$ purified mutant was subjected to the same additions (Figure 4A, middle trace). As mentioned above, addition of EGTA to Ca^{2+}-saturated $E^{309}Q$ produced essentially no effect on fluorescence intensity; however, subsequent addition of P_i did lead to fluorescence enhancement, indicating that formation of the E2P phosphoenzyme in $E^{309}Q$ was accompanied by the usual changes in Trp fluorescence. But subsequent addition of Ca^{2+}

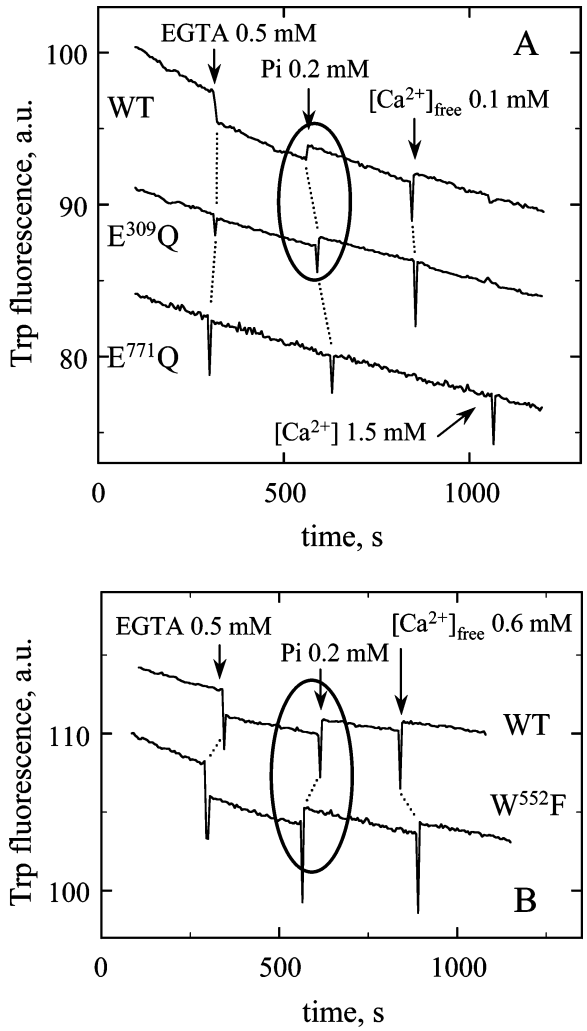


FIGURE 4: Phosphorylation from P_i induces a fluorescence rise for both $E^{309}Q$ and $W^{552}F$ mutants (as for WT). (A, B) Tryptophan fluorescence was monitored after 20-fold dilution of purified and reconstituted WT, $E^{309}Q$, or $E^{771}Q$ Ca^{2+}-ATPase (panel A), or $W^{552}F$ (panel B), into 150 mM MOPS/Tris, pH 7 (20 °C), 5 mM $MgCl_2$, and 20% Me_2SO. Total Ca^{2+} was 55 μM, due to the presence of contaminating Ca^{2+} and the Ca^{2+} added together with the enzyme. 0.5 mM EGTA was first added to clamp free Ca^{2+} to a very low concentration; 0.2 mM P_i was then added, and lastly, excess Ca^{2+} was re-added to restore a high free Ca^{2+} concentration (as indicated on the various traces). The various traces have been slightly shifted with respect to each other, for clarity, and the very small changes (0.1%) due to dilution have been corrected. Additions that were identical for the different traces are indicated by dotted lines.

to $E^{309}Q$ failed to further raise its fluorescence; instead, it induced a slight and slow drop of fluorescence intensity (hardly visible in Figure 4A but reproducible in several experiments), corresponding probably to slow return to the fluorescence level expected for the nonphosphorylated E1Ca form, a level which, for the $E^{309}Q$ mutant, is lower than the fluorescence level observed for the E2P form. Taken together, these data show that phosphorylation from P_i of Ca^{2+}-free $E^{309}Q$ is accompanied by "normal" Trp fluorescence changes, although Ca^{2+} binding at site I does not raise the fluorescence of this mutant.

Note that when we examined the purified and reconstituted $E^{771}Q$ or $D^{800}N$ mutants, their Trp fluorescence did *not* respond to P_i addition (only the results with the former are shown in Figure 4A, bottom trace), as expected from the poor residual ability of these mutants to be phosphorylated from $[^{32}P]P_i$, even in the absence of Ca^{2+} (see Figure 2B), and confirming that ATPase mutants that are completely unable to bind Ca^{2+} experience irreversible denaturation during the purification procedure.

In the experiment illustrated in Figure 4B, we made use of the purified $W^{552}F$ mutant to evaluate the contribution of W^{552} to the fluorescence changes observed after phosphorylation from P_i. As can be seen, changes in fluorescence intensity upon dissociation of Ca^{2+} and upon phosphorylation from P_i were similar for $W^{552}F$ (bottom trace in Figure 4B) and WT ATPase (upper trace in Figure 4B); the affinity for P_i also proved similar for WT and $W^{552}F$ ATPase, about 50 μM, close to that for the native enzyme under identical experimental conditions (not shown). These results show that W^{552}, located in the nucleotide-binding domain (N), does not contribute significantly to the rise in intrinsic fluorescence observed upon phosphorylation from P_i. The major contribution of membranous or interfacial Trp residues to the P_i-induced rise in fluorescence also emphasizes the long-range communication existing between the cytosolic phosphorylation site, where P_i binds, and the membranous domain, where the Trp residues other than W^{552} reside.

The Effect of Mg^{2+} on Ca^{2+}-Free ATPase Fluorescence Is Retained in $E^{309}Q$. The Effect of Mg·ATP on Ca^{2+}-Free ATPase Fluorescence Is Retained in $W^{552}F$. It has also been known for years that Trp residues in Ca^{2+}-ATPase can be used to probe the interaction of Mg^{2+} ions or ATP with Ca^{2+}-deprived ATPase (*32*, *36*), but again with little insight concerning the exact mechanism for this; for instance, the effect of Mg^{2+} could be due either to competition with Ca^{2+} for binding to one of the high-affinity Ca^{2+} sites (which would drive the enzyme to a dead-end complex) or, alternatively, to Mg^{2+} binding close to the phosphorylatable aspartate D^{351} or the substrate-binding site (*33*). Here we monitored the effects, in the absence of Ca^{2+}, of Mg^{2+} or ATP binding to the same ATPase mutants as above (Figure 5).

As shown in the upper trace of Figure 5A for control, addition of 5 mM $MgCl_2$ to Ca^{2+}-deprived WT enzyme at pH 7 allowed the reversal of more than 50% of the fluorescence drop induced by the previous chelation of Ca^{2+} by EGTA, as previously described for the native enzyme using the same experimental settings (*32*), and subsequent addition of 50 μM ATP (Mg·ATP in the cuvette) further raised Trp fluorescence (*36*) to a saturating level, while higher ATP concentrations only resulted in a small inner filter effect; the dissociation constant for the Mg·ATP-induced rise was estimated to be about 5 μM, as for binding of ATP to intact SR under these conditions (data not shown). When the $E^{309}Q$ mutant was used, no change in fluorescence was detected upon addition of EGTA to Ca^{2+}-saturated $E^{309}Q$, as previously described (Figures 3A and 4A), but remarkably, subsequent addition of Mg^{2+} induced a fluorescence rise similar to the one observed for WT ATPase. As Ca^{2+} binding to this mutant does *not* result in any fluorescence change, it is therefore likely that the effect of Mg^{2+} on fluorescence is due to Mg^{2+} binding to a site that is *not* a Ca^{2+}-binding site.

So far, the identity of the Trp residues implicated in the response of ATPase fluorescence to addition of Mg·ATP in the absence of Ca^{2+} also remains uncertain. Conceivably, the location of W^{552} near the nucleotide-binding pocket might allow this residue to contribute to these changes. Yet, as can

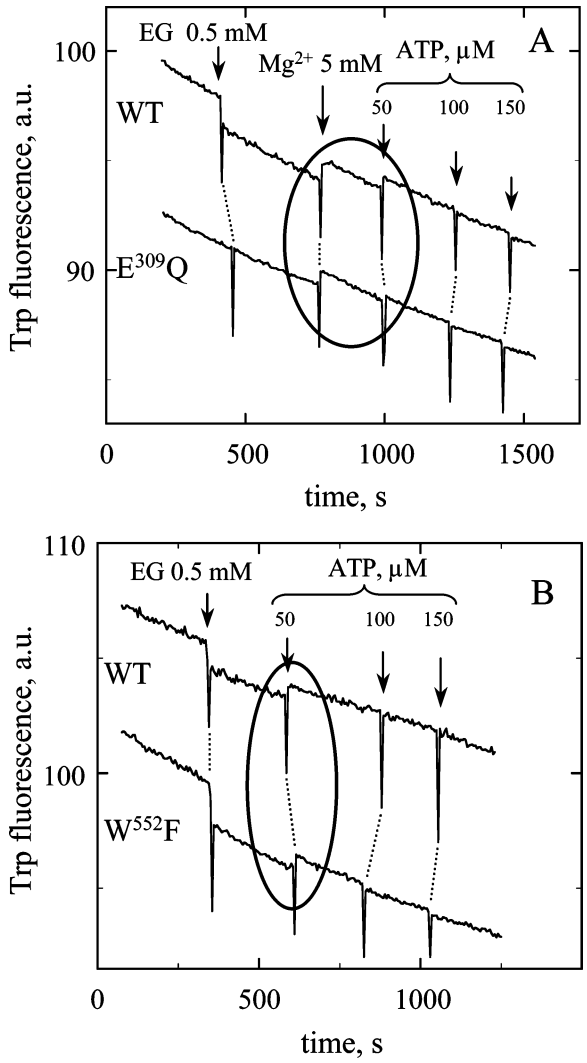


FIGURE 5: Effects of Mg^{2+} and ATP binding on the fluorescence of Ca^{2+}-free ATPase. (A, B) Tryptophan fluorescence was monitored after 20-fold dilution of purified and reconstituted ATPase (to about 5−10 μg/mL) into either 150 mM MOPS/Tris, pH 7 at 20 °C (panel A, for WT or $E^{309}Q$), or the same medium supplemented with 5 mM $MgCl_2$ (panel B, for WT or $W^{552}F$). Total Ca^{2+} was 55 μM, due to the presence of contaminating Ca^{2+} and the Ca^{2+} added together with the enzyme. 0.5 mM EGTA was first added to reach a low concentration of free Ca^{2+} (0.05 μM for panel A and only slightly higher for panel B) and then 5 mM $MgCl_2$ (for panel A only). Lastly, various amounts of ATP were sequentially added to reach final concentrations of 50, 100, and 150 μM. The traces have been slightly shifted with respect to each other for clarity, and the very small changes (0.1%) due to dilution have been corrected. Identical additions are indicated by the dotted lines.

be seen from Figure 5B, swapping the W^{552} residue for a phenylalanine did not produce any significant effect on the ATPase response to ATP, showing that W^{552} does *not* make a dominant contribution to the fluorescence changes upon binding of Mg·ATP in the absence of Ca^{2+}. Hence, it must be concluded that nucleotide binding in the cytosolic portion of SERCA1a triggers reorganization of the distant membrane domain.

DISCUSSION

Monitoring the changes in fluorescence of intrinsic Trp residues has previously allowed various groups to reveal conformational changes of native rabbit SERCA1a (*17*−*19*, *31*−*33*, *36*, *37*), but with one or two exceptions (*20*, *38*) this has so far been impossible in recombinant and mutated ATPase due to the poor enrichment of Ca^{2+}-ATPase in the corresponding final microsomes. In this paper, purification of a few mutants of SERCA1a ATPase allowed us to perform initial studies of the Trp fluorescence of these mutants and to gain information about the events revealed by the fluorescence changes associated with various steps in the ATPase cycle (namely, Ca^{2+}, P_i, ATP, or Mg^{2+} binding). We particularly focused here on the experiments made possible by the purification of two mutants in nondenatured forms: the $E^{309}Q$ mutant, known to bind only one of the two Ca^{2+} ions, at Ca^{2+} site I, and the $W^{552}F$ mutant, in which, to start with, 1 of the 13 ATPase Trp residues (namely, the one located in the cytosolic domain of the ATPase) has been exchanged for the nonfluorescent phenylalanine, thereby allowing us to attribute conformational changes either to the membrane part of the enzyme or to the cytosolic region.

Changes Associated with Ca^{2+} Binding to ATPase. Our control phosphorylation measurements make clear that, under the conditions used in our experiments, the purified and reconstituted $E^{309}Q$ mutant remains phosphorylatable from P_i *and* able to bind one of the two Ca^{2+} ions with high affinity (at site I), as judged by the sensitivity to Ca^{2+} of P_i-dependent phosphorylation of this mutant, at pH 7 (Figure 2B) or at pH 6 (data not shown). Inhibition by Ca^{2+} in those phosphorylation experiments revealed an apparent affinity of the $E^{309}Q$ mutant for Ca^{2+} of about 10 μM at pH 7 (see Figure 2B), as expected from earlier studies with the nonpurified mutant (*13*). Yet, the results in Figure 3A show that binding or dissociation of that single Ca^{2+} ion does *not* result in major Trp fluorescence changes. Assuming that the $E^{309}Q$ mutant otherwise behaves as normal Ca^{2+}-ATPase, this implies that the events induced by Ca^{2+} binding at site II make the major contribution to the overall Trp fluorescence changes observed upon binding of Ca^{2+} to both sites in WT Ca^{2+}-ATPase.

This conclusion, derived from experiments with mutated ATPase, is consistent with the previous suggestion derived from kinetic studies of Ca^{2+} dissociation from rabbit SR Ca^{2+}-ATPase (*39*), in which the Ca^{2+} ion that plays the largest role in the Trp fluorescence changes was found to be the first ion to leave upon dissociation [i.e., presumably (*6*, *40*), the ion bound at site II]. An independent study at equilibrium of Sr^{2+}-induced Trp fluorescence changes in FITC-labeled SR vesicles had previously indicated that the Sr^{2+} concentrations required to enhance the Trp fluorescence were larger than those required to reduce FITC fluorescence, again suggesting that Trp residues were sensitive to late steps in the Sr^{2+} (and presumably Ca^{2+}, too).

As labeling of WT ATPase with FITC has already been found possible (*20*), we are currently trying to label with FITC our purified and reconstituted $E^{309}Q$ mutant (which nevertheless seems to be more fragile than WT under our traditional labeling conditions). If Ca^{2+}-dependent changes in *FITC* fluorescence can indeed be observed upon binding of Ca^{2+} to site I of this labeled $E^{309}Q$ mutant, this will definitely exclude the possibility that the lack of response to Ca^{2+} of the *Trp* fluorescence of $E^{309}Q$ could be due to adoption by this mutant of conformations unrelated to those (E2, E1, or CaE1) in the classical Scheme 1 (ongoing experiments to be published later). Irrespective of the result, the fact that Ca^{2+}-dependent changes in *Trp* fluorescence

were similar for WT ATPase and the $W^{552}F$ mutant suggests that W^{552} does not play a major role in these Ca^{2+}-dependent changes.

Effect of Ca^{2+} on ATPase Stability. We were able to exploit the results with the $E^{309}Q$ mutant because this mutant (like the $W^{552}F$ mutant) survived purification (at least in part) without being denatured. From the various functional assays performed in this study (e.g., Figure 2B), it appears that this was not the case for our mutants of site I ($E^{771}Q$ or $D^{800}N$), despite the fact that all of them were functional *before* the purification step, as judged from their ability to be phosphorylated from [^{32}P]P_i (refs *14*, *28*, and *30* and unpublished data). Irreversible inactivation of SERCA1a in the presence of detergent and absence of Ca^{2+} is known to occur rapidly (e.g., ref *29*); in that respect, the fact that $E^{771}Q$ and $D^{800}N$ are not functional after purification is not so surprising, as these mutants are no longer able to bind any Ca^{2+} ion with high affinity. Conversely, it can be concluded that binding of the first Ca^{2+} ion (to site I) is *sufficient* to protect the ATPase polypeptide from irreversible denaturation in the presence of detergent.

A simple mechanism for the protective effect of Ca^{2+} could be that Ca^{2+} ions bridge the various helices bearing the residues to which Ca^{2+} binds, so that the bundle of transmembrane helices in solubilized ATPase, despite its loosening due to lipid replacement by a monolayer of detergent, is prevented from experiencing large amplitude movements that would lead to irreversible denaturation. Along this view, it is understandable that binding of only one Ca^{2+} ion to this transmembrane bundle already affords significant protection. It is, however, not impossible that binding of the first Ca^{2+} ion changes the ATPase conformation in the cytosolic domain, too, and makes it more resistant to denaturation. Note that binding of (only one) Ca^{2+} ion to $E^{309}Q$ is also sufficient, after initial cleavage of ATPase at E^{242}, to protect the C-terminal p83C peptide formed from further proteolysis (*42*). In contrast, binding of the mere first Ca^{2+} ion to a mutant of site II, $N^{796}A$, is *not* sufficient to confer to AMPPCP (an ATP analogue) a protective role during ATPase cleavage by proteinase K (*43*).

Mg^{2+} Binding to the ATPase. Mg^{2+} binding to sarcoplasmic reticulum Ca^{2+}-ATPase is a prerequisite for ATPase phosphorylation, either from P_i in the absence of Ca^{2+} or from ATP in its presence (Scheme 1 and refs *36* and *44*−*46*). In the absence of Ca^{2+}, Mg^{2+} addition to ATPase induces a change in ATPase fluorescence, a change which is strongly dependent on pH, being minimal at acidic pH (*32*). The reason for this change, however, is not clear. One of the previously discussed possibilities is that, at neutral or alkaline pH, Mg^{2+} might compete with Ca^{2+} for binding to one of the two high-affinity Ca^{2+} sites on ATPase (*32*, *33*). The results obtained in the present study with the $E^{309}Q$ mutant seem to completely exclude this possibility, as binding of Mg^{2+} to the Ca^{2+}-deprived $E^{309}Q$ mutant *raises* fluorescence, whereas binding of Ca^{2+} to site I does *not* (Figure 5A); the Mg^{2+} ion responsible for the fluorescence rise is unlikely to bind to the high-affinity Ca^{2+}-binding site I, and it would be surprising that the mutated site II, which has lost the ability to bind Ca^{2+}, could have retained the ability to bind Mg^{2+}.

Where then could binding of this Mg^{2+} ion take place? A first hypothesis could be that Mg^{2+} binds near the catalytic site, at the site whose occupancy is known to be required for phosphorylation. However, at pH 6, when P_i phosphorylation is at its maximum, Mg^{2+} hardly raises tryptophan fluorescence, as recalled above (*32*); in this hypothesis, we would therefore have to find an explanation for the fact that, at pH 6, the binding of Mg^{2+} to the catalytic site (that makes the ATPase nicely reactive to P_i) is *not* reflected in the Trp fluorescence level, whereas it is at pH 7, where phosphorylation is less favored. An alternative and more speculative hypothesis could then be that Mg^{2+} binds to a separate site, distinct from either the catalytic site or the Ca^{2+}-binding site(s). At present, two possibilities could be thought of: Mg^{2+} might bind at the second Mg^{2+}-binding site known to exist under certain conditions in the cytosolic ATPase domain, close to ADP, as exemplified in the ATP-containing fluoride complexes of the ATPase (*7*−*9*); otherwise, Mg^{2+} could bind at still another position, for instance, near Glu^{255}, where Fe^{2+} can bind (*47*). Future work will have to clarify these issues. In either case, the pH-dependent occupancy by Mg^{2+} of this putative new binding site could be related to the previously described pH dependence of the so-called E2 to E1 transition of the ATPase, driving the enzyme in a conformation suitable for subsequent ATP and Ca^{2+} binding (*33*, *44*, *48*) and again revealing long-distance effects of the binding of a ligand for rearrangement of the enzyme structure or dynamics.

Response of ATPase Trp Residues to P_i and ATP, Especially for $W^{552}F$. Previous studies using a membrane-bound ionophore able to quench the fluorescence of Ca^{2+}-ATPase tryptophan residues located at a distance compatible with energy transfer have proposed that W^{552} does *not* contribute to the overall rise in fluorescence induced by phosphorylation from P_i (and that, instead, the fluorescence emitted by W^{552} *drops* upon such phosphorylation, although this is generally obscured by the dominating rise in fluorescence of other Trp residues) (*49*, *50*). The present results with the $W^{552}F$ mutant (Figure 4B) fully support this previous suggestion and prove that the membranous Trp residues are responsible for the phosphorylation-dependent rise in fluorescence.

On the other hand, on the basis of similar measurements it has also been suggested that W^{552} contributes significantly to the fluorescence rise observed after addition of ATP to Ca^{2+}-deprived enzyme preincubated with Mg^{2+} (*49*). That second suggestion is contradicted by the present measurements with the $W^{552}F$ mutant (Figure 5B): we found that replacing W^{552} with a phenylalanine did not affect the fluorescence rise recorded upon addition of ATP in the absence of Ca^{2+} and presence of Mg^{2+}, excluding a prominent role of W^{552} in these variations. The present result shows that mainly tryptophans located near the membrane interface are responsible for these fluorescence changes and that ATP binding is followed by a reorganization of the membrane domain [a fact that is not completely unexpected, in view of the accelerating effect of ATP on Ca^{2+} binding (*44*, *45*, *51*)].

Future Mutations of Trp Residues. Our results suggest that W^{552} is not critical for any of the ligand-induced changes that we have been monitoring here. Concerning the Trp residues borne by the membrane helices and comparing the available structures of Ca^{2+}-ATPase in various conformations, it appears that M1−M6 helices undergo the most drastic rearrangements when going from one intermediate

to the other in the catalytic cycle, whereas C-terminal M7−M10 helices show barely any movement (*5*−*10*). Thus, one can speculate that the tryptophan residues responsible for fluorescence changes upon binding of Ca^{2+}, P_i, or ATP are localized on M1−M6 helices. Examining the response of these Trp residues will therefore be an obvious goal for future directed mutagenesis of the Trp residues. Indeed, deletion of a large number of Trp residues in the Ca^{2+}-ATPase sequence (ideally, leaving only one Trp residue per mutant), combined with purification and reconstitution of appropriate amounts of such mutants, can be expected to be both necessary and useful for a more precise description of the motion of membrane helices during the various steps in the catalytic cycle of Ca^{2+}-ATPase. The present paper opens the way toward this goal.

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