ADP-fluoroaluminate intermediate analog of the Ca\(^{2+}\)-ATPase

Ca\(^{2+}\) OCCLUSION AND GATING FUNCTION OF Glu309 IN THE ADP-FLUOROALUMINATE ANALOG OF THE Ca\(^{2+}\) ATPase PHOSPHOENZYME INTERMEDIATE

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**SUMMARY**

In the absence of ATP the sarcoplasmic reticulum ATPase (SERCA) binds two Ca\(^{2+}\) with high affinity. The two bound Ca\(^{2+}\) rapidly undergo reverse dissociation upon addition of EGTA, but can be distinguished by isotopic exchange indicating fast exchange at a superficial site (site II), and retardation of exchange at a deeper site (site I) by occupancy of site II. Site II mutations that allow high affinity binding to site I, but only low affinity binding to site II, show that retardation of isotopic exchange requires higher Ca\(^{2+}\) concentrations with the N796A mutant, and is not observed with the E309Q mutant even at mM Ca\(^{2+}\). Fluoroaluminate forms a complex at the catalytic site yielding stable analogs of the phosphoenzyme intermediate, with properties similar to E2-P or E1-P\(\text{Ca}_2\). Mutational analysis indicates that D351, K352, T353, D703, N706, D707, T625 and K684 participate in stabilization of fluoroaluminate and Mg\(^{2+}\) at the phosphorylation site. In the presence of fluoroaluminate and Ca\(^{2+}\), ADP (or AMPPCP) favors formation of a stable ADP-E1-P\(\text{Ca}_2\) analog. This produces strong occlusion of Ca\(^{2+}\) bound to both sites (I and II), whereby dissociation occurs very slowly even following addition of EGTA. Occlusion by fluoroaluminate and ADP is not observed with the E309Q mutant, suggesting a gating function of E309 at the mouth of a binding cavity with a single path of entry. This phenomenon corresponds to the earliest step of the catalytic cycle following utilization of ATP. Experiments on limited proteolysis reveal that a long range conformational change, involving displacement of headpiece domains and transmembrane helices, plays a mechanistic role.
ADP-fluoroaluminate intermediate analog of the Ca\textsuperscript{2+}-ATPase

INTRODUCTION

The Sarco-Endoplasmic Reticulum Ca\textsuperscript{2+} ATPase (SERCA) is a ubiquitous Ca\textsuperscript{2+} pump, required for intracellular Ca\textsuperscript{2+} storing and Ca\textsuperscript{2+} signaling mechanisms. The SERCA1 isoform is a 994 amino acid protein that includes ten transmembrane segments and a cytosolic headpiece comprising three (N, P and A) distinct domains (1, 2). The reaction sequence (scheme I) of the catalytic and transport cycle (3) includes high affinity binding of 2 Ca\textsuperscript{2+}, ATP binding, formation of a phosphorylated intermediate, release of ADP, isomerization of the phosphorylated intermediate from a state of high affinity (E1-P\textsubscript{Ca2}) to a state of low affinity for Ca\textsuperscript{2+} (E2-P\textsubscript{Ca2}), vectorial dissociation of bound Ca\textsuperscript{2+} and, finally, hydrolytic cleavage of phosphate.

![Scheme I](image)

The initial binding of two Ca\textsuperscript{2+} (in exchange for H\textsuperscript{+}) occurs sequentially (4, 5, 6) and involves two neighboring sites (I and II) within the ATPase membrane region where stabilization is provided by amino acid residues deriving from the M4, M5, M6 and M8 transmembrane helices (2, 7). On the other hand binding and catalytic utilization of ATP occurs within the cytosolic headpiece of the enzyme with direct or indirect intervention of several residues of the N, P and A domains. In the absence of ATP, the two bound Ca\textsuperscript{2+} undergo rapid reverse...
dissociation when the Ca\(^{2+}\) concentration in the medium is lowered by the addition of EGTA (6). However, upon addition of ATP, occlusion (i.e., lack of reverse dissociation) of the two bound Ca\(^{2+}\) is observed upon enzyme phosphorylation (8, 9).

It was previously reported (10) that incubation of ATPase with fluoroaluminate yields an ATPase complex with characteristics attributed to E2-P or E1-P\(\text{Ca}_2\) enzyme intermediate. We have therefore studied the reverse dissociation and exchange of bound Ca\(^{2+}\) in the absence of ATP, and the Ca\(^{2+}\) dissociation following formation of a phosphoenzyme analog with fluoroaluminate. In addition, we performed a mutational analysis of the Ca\(^{2+}\) binding site and of the catalytic domain, to clarify the role of various residues in stabilization of fluoroaluminate and in the mechanism of Ca\(^{2+}\) occlusion.

**MATERIALS AND METHODS**

Native sarcoplasmic reticulum (SR) vesicles were obtained by homogenization and differential centrifugation of rabbit white muscle (11). Recombinant ATPase protein was obtained with the microsomal fraction of COS-1 cells infected with adenovirus vectors carrying WT or mutated (chicken or rabbit) SERCA1 cDNA. The methods for construction of vectors, cultures and preparation of microsomes were previously described in detail (12). The total microsomal protein was determined using bicinehonic acid with the biuret reaction (Pierce, Rockford, IL). In all experiments with recombinant ATPase, the total protein concentrations in reaction mixtures, or the experimental results, were adjusted to reflect comparable SERCA concentrations as determined by Western blotting. Contaminant Ca\(^{2+}\) in various reaction mixtures was estimated by titration with EGTA in the presence of metallochromic indicators in a double wavelength spectrophotometer. Ca\(^{2+}\) dependent hydrolytic activity and Ca\(^{2+}\) transport were measured as previously described (13).
Ca\(^{2+}\) binding at equilibrium was measured by incubating SR vesicles (40 µg/ml) or COS-1 cell microsomes (60 µg/ml) in reaction mixtures containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 0.2 mM EGTA, \(^{45}\text{Ca}\)-CaCl\(_2\) to yield various concentrations of free Ca\(^{2+}\) (14), and 5 µM A23187 calcium ionophore. Thapsigargin (TG) was added to half of the samples (1 µM) to provide controls exhibiting no specific Ca\(^{2+}\) binding. Following 15 minutes incubation at 25°C, 1 ml samples were filtered by suction on 0.45 µm Millipore filters, and the filters were collected, blotted and processed for scintillation counting.

The time course of \(^{45}\text{Ca}\)-Ca\(^{2+}\) dissociation from the ATPase, was determined by first incubating SR vesicles (40 µg/ml) or COS-1 cell microsomes (100 µg/ml) in a medium containing 50 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl\(_2\), 5 µM A23187 calcium ionophore, and 20 µM \(^{45}\text{Ca}\)-Ca\(^{2+}\) (including contaminant Ca\(^{2+}\)). TG was added to half of the samples (1 µM) to provide controls exhibiting no specific Ca\(^{2+}\) binding. Following 10 minutes incubation at 25 °C, 1 ml samples were placed on a 0.65 µm Millipore filter and the medium removed by suction. The vesicles were then perfused with the same medium, but containing 10 mM EGTA for dissociation of bound \(^{45}\text{Ca}\)-Ca\(^{2+}\), or various concentrations of \(^{40}\text{Ca}\)-Ca\(^{2+}\) for exchange with bound \(^{45}\text{Ca}\)Ca\(^{2+}\). The experiments were performed in a BioLogic rapid filtration apparatus, and increasing perfusion times were applied to individual samples. The filters were then collected, blotted and processed for scintillation counting. Controls with TG were obtained for each perfusion time.

Reaction with fluoroaluminate to test ATPase inhibition was obtained by incubating SR vesicles (0.06 mg/ml) in media containing 40 mM MOPS, pH 7.0, 80 mM KCl, 2 mM MgCl\(_2\), 2 µM A23187 Ca\(^{2+}\) ionophore, 1 mM EGTA, 2 mM KF and various concentrations (0 – 50 µM) of AlCl\(_3\), in the presence or in the absence of 0.95 mM CaCl\(_2\). The incubation was carried out for
30 minutes at 25 °C, and the samples were then used directly for ATPase measurements by adding 0.95 mM Ca\(^{2+}\) (when Ca\(^{2+}\) was not present), and ATP (1 mM).

Reaction with fluoroaluminate to determine Ca\(^{2+}\) binding or dissociation of bound Ca\(^{2+}\) from the ATPase was obtained by incubating SR vesicles with \(^{45}\)Ca-Ca\(^{2+}\) as explained above, but with the addition of 2 mM KF and 5 or 50 µM AlCl\(_3\), in the absence or in the presence of various concentration of ADP or AMPPCP. Following 30 minutes incubation at 25 °C, 1 ml samples were placed on 0.65 µm Millipore filter and the medium removed by suction. The filters were then processed for determination of bound calcium. Alternatively, the vesicles were perfused with the same medium as for pre-incubation, but containing 10 mM EGTA for dissociation of bound \(^{45}\)Ca-Ca\(^{2+}\). The experiments were performed in a BioLogic rapid filtration apparatus, and increasing perfusion times were applied to individual samples. The filters were then collected, blotted and processed for scintillation counting. Controls with TG were obtained for each perfusion time.

For the experiments on limited proteolytic digestion, microsomes containing recombinant (chicken or rabbit) ATPase were preincubated for 40 minutes at 25 °C in media containing 50 mM MOPS, pH 7.0, 50 mM NaCl, 1.2 mg microsomal protein/ml and 2.0 mM EGTA, in the presence or in the absence of 2.1 mM CaCl\(_2\), 5 mM MgCl\(_2\), 2 mM NaF, 5 or 50 µM AlCl\(_3\), and 0.1 mM ADP. After 60 minutes, limited proteolytic digestion was started by the addition of either 0.04 mg proteinase K or 0.012 mg trypsin/ml. The reaction was quenched after 50 minutes of digestion by the addition of trichloroacetic acid to reach a 2.5% final concentration. The quenched protein was then solubilized by adding sodium dodecylsulfate (1%), Tris (0.312 M), pH 6.8, sucrose (3.75%), beta-mercaptoethanol (1.25 mM, and bromophenol blue (0.025%). The samples were then subjected to electrophoretic analysis (15) on 12 % gels followed by staining.
with Coomassie Blue. Western Blots were obtained with monoclonal antibody mAb CaF3-5C3 for chicken SERCA-1 or MA911 for rabbit SERCA1, followed by goat antimouse IgG-horseradish peroxidase-conjugated secondary antibodies and visualization with an enhanced chemiluminescence-linked detection system (Amersham Biosciences).

**RESULTS**

**Ca\(^{2+}\) binding and dissociation in the absence of ATP**

Equilibrium binding of Ca\(^{2+}\) to the native ATPase of SR vesicles has been previously observed within the micromolar concentration range (4). However, measurements of Ca\(^{2+}\) binding to recombinant ATPase are more difficult due to low concentrations of the enzyme in COS-1 cell microsomes, and restrictive conditions required for subsequent kinetic experiments (i.e. filtration and low Ca\(^{2+}\)/EGTA concentrations, as opposed to column chromatography and high Ca\(^{2+}\)/EGTA buffer). Under our present conditions, we obtained equilibrium binding isotherms that yield a maximal stoichiometry of two Ca\(^{2+}\) per ATPase, and are best fitted with a cooperative equation assuming Keq values of 7 x 10\(^5\) and 2 x 10\(^6\) M\(^{-1}\) for two interacting sites (16). Identical values, adjusted for the ATPase molar concentration, are obtained with native and recombinant WT enzyme.

Dissociation of bound Ca\(^{2+}\) was studied by pre-incubating microsomes with 20 µM (\(^{45}\)Ca)-Ca\(^{2+}\) for 15 minutes, which results in saturation the high affinity binding sites (2 Ca\(^{2+}\)/ATPase). We placed pre-incubated SR vesicles on filters for removal of the medium by suction, and subjected them to perfusion with 10 mM EGTA and no added calcium (2 x 10\(^{-10}\) M free Ca\(^{2+}\)) for various time intervals in the rapid filtration apparatus. We found that, following addition of EGTA, most of the bound calcium dissociates with a 21-26 sec\(^{-1}\) rate constant (Fig 1), while approximately 10% of the total signal dissociates slightly slower (see also 6). It is apparent
that under these conditions the bound Ca\textsuperscript{2+} dissociates rapidly and the two sites cannot be distinguished.

Contrary to dissociation in the presence of EGTA, isotopic exchange of bound (\textsuperscript{45}Ca)-Ca\textsuperscript{2+} following addition of (\textsuperscript{40}Ca)-Ca\textsuperscript{2+} \cite{6} yields two distinct kinetic components. We studied the (\textsuperscript{40}Ca)-Ca\textsuperscript{2+} concentration dependence of isotopic exchange with regard to rate constants as well as the size of the two kinetic components. We found that in the presence of (\textsuperscript{40}Ca)-Ca\textsuperscript{2+} concentrations in the µM range the (\textsuperscript{45}Ca)-Ca\textsuperscript{2+} dissociation curve exhibits a component approximately as fast as that observed in the presence of EGTA (kd = 16-18 sec\textsuperscript{-1}), corresponding to 75-80% of the total signal), and a definitely slower component (kd = 1 sec\textsuperscript{-1}), corresponding to 20-25% of the total signal. On the other hand, when the (\textsuperscript{40}Ca)-Ca\textsuperscript{2+} concentration is raised up to the mM range, the size of the slow component of (\textsuperscript{45}Ca)-Ca\textsuperscript{2+} increases to a maximum of 50% of the total signal, while its rate constant is reduced to 0.01-0.02 sec\textsuperscript{-1} (Fig. 1 and Table 1). No further change in the size of the two components is obtained by increasing the (\textsuperscript{40}Ca)-Ca\textsuperscript{2+} concentration above 1 mM. Considering that the stoichiometry of (\textsuperscript{45}Ca)-Ca\textsuperscript{2+} bound is 2 per ATPase, it is clear that in the presence of mM Ca\textsuperscript{2+} the initial dissociation (and exchange) of one Ca\textsuperscript{2+} occurs unimpeded, while dissociation of the remaining Ca\textsuperscript{2+} is strongly retarded. This indicates that (\textsuperscript{40}Ca)-Ca\textsuperscript{2+} occupancy of one site produces retardation of exchange on the other site.

**Effects of site directed mutations**

Consistent with the cooperative mechanism, single mutations in site I interfere with Ca\textsuperscript{2+} binding on both site I and II, even when the residue undergoing mutation does not participate directly in Ca\textsuperscript{2+} binding at site II \cite{17}. On the other hand, high affinity binding (Keq = 10\textsuperscript{6} M\textsuperscript{-1}) of one Ca\textsuperscript{2+} on site I is still retained following single mutations on site II (i.e., E309Q or N796A),
while site II looses high affinity binding (16). Consequently, no binding to site II is observed at µM Ca^{2+} concentrations.

We found that, following addition of EGTA, Ca^{2+} dissociation from these mutants (i.e., from site I) occurs with a single rate constant of 2 - 4 sec^{-1} (Fig 2A and B). This is significantly slower than observed with WT enzyme, and may reflect additional stabilization of Ca^{2+} in site I by oxygen functions of site II. For instance, the Asp800 side chain that contributes one oxygen function to site I and one oxygen function to site II in the WT enzyme, is likely to contribute both its oxygen functions to site I in the site II mutants.

We measured calcium isotope exchange in the two mutants N796A and E309Q. As explained above, N796 and E309 residues contribute side chain oxygen atoms for Ca^{2+} complexation on site II. Therefore, while retaining high affinity Ca^{2+} binding to site I, these mutants loose high affinity Ca^{2+} binding to site II. On the other hand, low affinity binding is still retained by the altered site II, as suggested by inhibition of enzyme phosphorylation with Pi (18). We then tested if addition of (^{40}\text{Ca})-Ca^{2+} would result in isotopic exchange with (^{45}\text{Ca})-Ca^{2+} bound to site I. We found that the two mutants exhibit a different behavior, as dissociation (i.e., isotopic exchange) of bound (^{45}\text{Ca})-Ca^{2+} is increasingly retarded by (^{40}\text{Ca})-Ca^{2+} within the 0.1-1.0 mM range in the N796A mutant (Fig 2A), while the E309Q mutant exhibits dissociation as rapid as that observed in the presence of EGTA even following addition of 1 mM (^{40}\text{Ca})-Ca^{2+} (Fig 2B). This indicates that E309 makes a greater contribution than N796 to the affinity of site II for Ca^{2+}. Therefore, the E309 side chain plays a very important role in retardation of Ca^{2+} dissociation from site I and in gating the calcium binding cavity.

Formation of fluoroaluminate-ATPase complex
Intrinsic fluorescence and Ca\(^{2+}\) binding measurements were previously reported (10) indicating that incubation of ATPase with fluoroaluminate yields a fluoroaluminate-ATPase complex with characteristics attributed to the E2-P or E1-P\(\text{Ca}_2\) enzyme intermediate. In our experiments, we tested the formation of stable intermediate analogs by incubating the ATPase with various concentrations of fluoroaluminate in the absence or in the presence of Ca\(^{2+}\), and then measured the residual Ca\(^{2+}\) dependent ATPase activity. It is shown in Fig 3 that ATPase inactivation occurs in either case, but higher concentrations of fluoroaluminate are required when the incubation is carried out in the presence of Ca\(^{2+}\). This demonstrates that a stable complex is formed in either case, most likely corresponding to E2-P in the absence of Ca\(^{2+}\), and to E1-P\(\text{Ca}_2\) in the presence of Ca\(^{2+}\). Formation of the latter analog requires higher fluoroaluminate concentrations.

We also explored whether formation of fluoroaluminate complex would produce the ATPase conformational changes as expected of phosphoenzyme intermediate formation. For this purpose we conducted experiments using partial digestion with proteinase K or trypsin, which may reveal protection of specific sites on the peptide loops intervening between the A domain and the M3 transmembrane segment. This protection is observed following reaction of the ATPase with phosphate analogs, suggesting large conformational changes that include various degrees of A domain rotation and gathering of the cytosolic domains (19). Accordingly we found that, although the pattern of digestion by proteinase K is different in the absence or in the presence of Ca\(^{2+}\), protection is obtained following reaction with 50 µM fluoroaluminate in either case (Fig 4). In fact even Mg\(^{2+}\) makes little difference, indicating that while Mg\(^{2+}\) is required for electrophilic assistance in the catalytic mechanism, its contribution to steric stabilization of fluoroaluminate is not apparent in our experiments with WT enzyme. On the other hand,
following mutation of residues (i.e., D601, K352, N706 and K684) that contribute to fluoroaluminate stabilization in the WT enzyme (see below and Table 2), the Mg$^{2+}$ contribution to stabilization of the complex becomes apparent.

A conformational effect of Ca$^{2+}$ on the phosphoenzyme analog is demonstrated by the experiment shown in Fig 5. In fact, protection from trypsin (as opposed to proteinase K) is obtained when the reaction with fluoroaluminate is performed in the absence of Ca$^{2+}$, but not in the presence of Ca$^{2+}$ (Fig 5A and B). These results are consistent with conformational changes due to the fluoroaluminate reaction, and a significantly different A domain rotation in the E2-P and E1-PCa$_2$ analogs allowing protection of the R198 trypsin site only in the former analog. Furthermore, it is shown in Fig 5C that, in the presence of Ca$^{2+}$, low fluoroaluminate concentrations (5 µM) afford only partial protection from proteinase K. In this case, addition of ADP produces total protection, as acquisition of the ADP-E1-PCa$_2$ conformation is highly favored. It is noteworthy that formation of the E2-P analog is not influenced by ADP, as complete protection is already observed with 5 µM fluoroaluminate.

We found that protection is not observed at all when the fluoroaluminate reaction is carried out with the D351N or the D351A mutant (Table 2). Considering that D351 is the residue involved directly in the phosphorylation reaction, the lack of reactivity of D351 mutants demonstrates unambiguously that the fluoroaluminate complex obtained with the WT enzyme is in fact a phosphoenzyme analog. It is remarkable that the same D351 mutations favor protection by nucleotide binding, while interfering with protection by fluoroaluminate (see discussion).

We then performed a mutational analysis of several residues whose involvement in substrate binding and/or utilization was indicated by previous studies with SERCA (2, 13) or with the highly analogous phosphoserine phosphatase (PSPase) enzyme (20). All of these
mutations produce catalytic inhibition when either ATP or acetylphosphate are used as the substrate (Table 2). A special case is the R560A mutant in which a greater inhibition of ATP utilization is observed, evidently due to a prevalent role of R560 in ATP (13), as compared with acetylphosphate binding. It is noteworthy that derivatization of K515 (N domain) with fluoroisothiocyanate produces total inhibition of ATP utilization, while allowing partial utilization of acetylphosphate (not shown).

Various mutations within the P domain interfere with nucleotide or fluoroaluminate protection from proteinase K (Table 2). This is the case of K352A, T353A, T625A, K684A, D703A, N706 and D707. It is noteworthy that the T353, D707, and T625 mutations have approximately the same degree of interference with fluoroaluminate protection in the absence or in the presence of Ca²⁺ and/or Mg²⁺. On the other hand, interference by the K352, D703, N706, and K684 mutations is relieved to some extent by the presence of Mg²⁺. Furthermore, in the K684A, N706A and D703A mutants the effect of Mg²⁺ is much more prominent when Ca²⁺ is also present, suggesting a different contribution of fluoroaluminate stabilization by these residues in the E1-PCa₂ as compared to the E2-P conformation. Mutation of G626 has very little effect on protection by fluoroaluminate, although it interferes with protection by nucleotide and produces catalytic inhibition.

A case of special interest is the N706A mutation which, in analogy to the D351 mutation, favors protection by nucleotide (see discussion) while interfering with protection by fluoroaluminate only in the presence of Ca²⁺. This interference is totally relieved by Mg²⁺ (Table 2). The N706 mutation produces complete inhibition of catalytic activity (see discussion).

Mutations of R560, E439, R489 (N domain), R174 and E183 (A domain) do not interfere significantly with fluoroaluminate protection from proteinase K, indicating that these residues do
not contribute to stabilization of fluoraluminate. On the other hand, interference of nucleotide protection is produced by the R560 and E439 (but not R489) mutations, suggesting that these residues are involved in nucleotide binding (13).

**Ca^{2+} binding and dissociation in the fluoroaluminate-ATPase complex**

In order to study Ca^{2+} dissociation from the fluoroaluminate complex, we first obtained a preliminary assessment of Ca^{2+} binding when the ATPase is incubated for 30 minutes in the presence of 20 µM Ca^{2+} with fluoroaluminate at concentrations yielding enzyme inactivation (Fig 3). We found that when the ATPase is incubated in the presence of 20 µM (^{45}Ca)Ca^{2+} in the absence or in the presence of fluoroaluminate, the maximal level of high affinity Ca^{2+} binding (2 Ca^{2+}/ATPase) is reduced to 85% if 5 µM fluoroaluminate is present, and to 60% if 50 µM fluoroaluminate is present (Table 3). This reduction is evidently related to formation of the E2-P analog which does not retain high affinity binding, and accounts for 15 and 40% following incubation with 5 or 50 µM fluoroaluminate, respectively (Table 3).

When we studied the time course of Ca^{2+} dissociation from the fluoroaluminate-ATPase upon addition of EGTA, we found a fast and a slow kinetic component, occurring with 20-30 sec^{-1} and 0.3-0.4 sec^{-1} rate constants (Fig. 6). It is apparent that the fast dissociation is quite similar to that observed in the absence of fluoroaluminate (E1-Ca_{2}), while the slow component is likely due to formation of fluoroaluminate-ATPase complex (i.e. E1-PCa_{2}) and consequent occlusion of bound Ca^{2+}. The sizes of the fast and slow components differ depending on the fluoroaluminate concentration: 58 and 42% of the observed signal in the ATPase incubated with 5 µM fluoroaluminate; 25 and 75% in the ATPase incubated with 50 µM fluoroaluminate. Considering that Ca^{2+} binding is reduced to 85 or 60% by 5 or 50 µM fluoroaluminate respectively, we conclude that incubation with 5 µM fluoroaluminate yields 15% E2-P (no Ca^{2+}
bound), 52% E1-Ca\(^{2+}\) (rapid dissociation), and 33% E1-PCa\(_2\) (slow dissociation). On the other hand, incubation with 50 µM fluoroaluminate yields 40% E2-P (no bound Ca\(^{2+}\)), 13% E1-Ca\(^{2+}\) (rapid dissociation), and 47% E1-PCa\(_2\) (slow dissociation). These levels are summarized in Table 3.

Since ADP favors formation of the E1-PCa\(_2\) analog (Fig 5C), we tested whether dissociation of bound Ca\(^{2+}\) would be also affected. We found that addition of ADP to the incubation mixture with a fluoroaluminate concentration as low as 5 µM allowed 100% Ca\(^{2+}\) binding. In addition, it produced total occlusion of bound Ca\(^{2+}\) with no significant dissociation throughout a two second perfusion with EGTA (Fig. 7A). Interestingly, the effective ADP concentration was in the µM concentration range. A similar high affinity (Fig 7B) for the fluoroaluminate complex was exhibited by AMPPCP, an ATP analog that is not utilized for enzyme phosphorylation. It is noteworthy that in the absence of fluoroaluminate, ADP and AMPPCP produced no retardation of Ca\(^{2+}\) dissociation even at mM concentrations.

Finally, we tested whether occlusion of bound Ca\(^{2+}\) was produced by fluoroaluminate and ADP in the E309Q and N796A mutants. It is shown in Fig 8 that dissociation of bound Ca\(^{2+}\) occurs rapidly following perfusion of the E309Q mutant with EGTA, demonstrating that the gating role of E309 plays an important role for Ca\(^{2+}\) occlusion in the ADP-E1-PCa\(_2\) analog. Interestingly, the N796A mutant does not display tight occlusion as the WT enzyme does (Fig 7). Rather, a slow dissociation of Ca\(^{2+}\) is noted (Fig 8) upon addition of EGTA. It is then apparent that even though E309 plays a direct role in gating, engagement of all site II residues is required to obtain tight occlusion of bound Ca\(^{2+}\) in the ADP-E1-PCa\(_2\) analog. The levels of Ca\(^{2+}\) bound, the rates of dissociation, and the distribution of enzyme states under these conditions are summarized in Table 3.
DISCUSSION

The Ca\(^{2+}\) binding mechanism

The stoichiometry (2 Ca\(^{2+}\) per ATPase) and the cooperative character of high affinity Ca\(^{2+}\) binding to SERCA was established by early equilibrium measurements in the absence of ATP (4). Mutational (7) and crystallographic studies (2) then showed that the two bound Ca\(^{2+}\) reside within the transmembrane region of the ATPase (2), separated by a 5.7 Å distance, and surrounded by the M4, M5, M6 and M8 helices (Fig. 9). The side chains of E771 (M5), N768 (M5), T799 (M6), D800 (M6) and E908 (M8), as well as two water molecules, contribute oxygen atoms for stabilization of one Ca\(^{2+}\) (site I). Stabilization of the other Ca\(^{2+}\) (site II) is obtained with side chain oxygen atoms of E309 (M4), N796 (M6) and D800 (M6), and main chain carbonyl oxygen atoms of V304, A305 and I307. D800 contributes its two acidic side chain oxygen atoms to coordinate both calcium ions (site I and II). Participation of all these residues is rendered possible by unwinding of the M4 and M6 helices. Our present experiments were designed to characterize the mechanism of reverse dissociation of the bound Ca\(^{2+}\) and its dependence on local effects, as well as its occlusion through long range effects of phosphorylation site occupancy in a stable analog of the phosphoenzyme intermediate.

It is clear that, as a consequence of Ca\(^{2+}\) binding, the side chains of residues involved in binding undergo a change in orientation, most prominently that of E309 (Fig 9). These changes are permitted by responsive displacement and reorganization of pertinent transmembrane helices, such as M4, M5 and M6, and the resulting coordination geometry is different for the two sites. The denomination I and II assumes that the two sites are occupied sequentially, as predicted by the cooperative mechanism. In fact, single mutations of site I residues prevent binding on both
site I and II. On the other hand, single mutations of site II residues interfere with binding on site II, but not on site I (12).

**Ca^{2+}** dissociation and exchange

When the Ca^{2+} concentration in the medium is lowered below 10^{-8} M by addition of EGTA, reverse dissociation of bound Ca^{2+} occurs rapidly, and a distinct behavior of the two bound Ca^{2+} cannot be demonstrated. However, a clear distinction is obtained by the exchange experiments. It is shown in Fig 1 that when (^{40}Ca)-Ca^{2+} is added to enzyme saturated with (^{45}Ca)-Ca^{2+}, the time course of (^{45}Ca)-Ca^{2+} dissociation becomes biphasic, with a first component exhibiting a rate constant (19-25 sec^{-1}) similar to that observed following addition of EGTA, and a second slower component whose contribution increases as the concentration of added (^{40}Ca)-Ca^{2+} is raised. It should be noted that exchange depends both on the rate of dissociation of bound (^{45}Ca)-Ca^{2+} from site I and then II, and the rate of (^{40}Ca)-Ca^{2+} binding to the empty site within a corresponding time interval. As dissociation of bound (^{45}Ca)-Ca^{2+} is a first order phenomenon, and binding of (^{40}Ca)-Ca^{2+} is second order, the latter is influenced by raising the (^{40}Ca)-Ca^{2+} concentration up to the mM level. For this reason, both the percentage of enzyme undergoing exchange, and the dissociation rate constant of the slow component of (^{45}Ca)Ca^{2+} dissociation are influenced by the (^{40}Ca)-Ca^{2+} concentration. An important point is that in the presence of (^{40}Ca)-Ca^{2+} concentrations (1 to 10 mM) producing maximal effect, the exchange is limited to half of the total Ca^{2+} bound, and does not increase further. This demonstrates unambiguously that occupancy of site II by (^{40}Ca)-Ca^{2+} blocks dissociation of (^{45}Ca)-Ca^{2+} from site I and, therefore, the two Ca^{2+} exit through a single path, as previously suggested by Inesi (23), and Petithory and Jencks (24).
An important finding is that retardation of exchange of Ca\(^{2+}\) on site I is still obtained with the N796A mutant when 0.1-1.0 mM (\(^{40}\text{Ca})\text{-Ca}^{2+}\) is added, but is not observed with the E309Q mutant even in the presence of 1 mM (\(^{40}\text{Ca})\text{-Ca}^{2+}\) (Fig 2). This indicates that the binding affinity of site II is lower in the E309 than in the N796 mutant, and the E309 side chain has a very important role in Ca\(^{2+}\) binding and capping the binding cavity. Structural studies (21) have demonstrated clearly that the E309 side chain undergoes a pronounced change in orientation upon Ca\(^{2+}\) binding, thereby sealing site II (Fig 9).

Fluroaluminate analog of the phosphorylated enzyme intermediate

An early and important step of the catalytic cycle is the rapid occlusion of bound Ca\(^{2+}\) that occurs in parallel with phosphoenzyme formation by utilization of nucleotide substrate (8, 9). Ca\(^{2+}\) occlusion is also produced by reaction of ATPase monomers with Cr-ATP (25). In the experiments reported here we have used fluoroaluminate to obtain a stable analog of the phosphorylated enzyme intermediate (10), and to define its effects on the ATPase conformational state, as well as its ability to bind and occlude Ca\(^{2+}\). No effects were observed with D351 mutants, demonstrating that interaction of fluoroaluminate with this residue is required and the fluoroaluminate-ATPase complex is in fact an analog of the phosphoenzyme intermediate. It is of interest that mutation of D351 appears to relieve a repulsive effect of its acidic side chain on the nucleotide gamma-phosphate (13 and Table 2) while it interferes strongly with fluoroaluminate stabilization. It is then clear that stabilization occurs only in concomitance with the covalent phosphoryl transfer reaction, or the transition state thereof. It is interesting that mutation of N706 has an effect similar to the D351 mutation, suggesting that in the WT enzyme N706 may contribute to the relative positioning of gamma-phosphate and the D351 side chain.
Experiments on limited proteolytic digestion are a useful device to study conformational changes associated with formation of phosphorylated enzyme intermediates, since protection of proteolytic sites within the ATPase loop connecting the A domain and the M3 transmembrane segment are indicative of cytosolic domains rotation and gathering (19). Using this technique, we found that resistance to proteinase K was obtained by preincubating the ATPase with high concentrations (50 µM) of fluoroaluminate in the absence or in the presence of Ca^{2+} (Fig 4A). However, much lower protection to trypsin (T2 site) was observed when the fluoroaluminate reaction was conducted in the presence of Ca^{2+}, as compared with the absence of Ca^{2+} (Fig 4B). This difference is attributed to a specific conformational difference due to rotation of the A domain, resulting in protection of the T2 site (R198) in E2-P but not in E1-PCa_{2} (19). This demonstrates that conformational analogs of E2-P or E1-PCa_{2} are prevalently obtained when Ca^{2+} is absent or present during the incubation with fluoroaluminate. In the presence of low (5 µM) concentrations of fluoroaluminate (Fig 6C), total protection from proteinase K is observed in the absence of Ca^{2+}, but only partial protection in the presence of Ca^{2+} due to low production of E-P'Ca_{2} analog. On the other hand, full protection is obtained by the addition of ADP, demonstrating unambiguously that under these conditions formation of the ADP-E-P'Ca_{2} analog is favored and its conformation is highly resistant to digestion.

Mutational analysis of the fluoroaluminate protection of SERCA from proteinase K can be interpreted with reference to the high resolution structure of the fluoroaluminate intermediate of the analogous PSPase (20). For this purpose, the SERCA residues corresponding to the PSPase residues are shown in parenthesis in Fig 10. Comparison of our mutational analysis (Table 2) with the PSPase intermediate structure (Fig 10) shows that the aluminum atom is stabilized by the three fluorine atoms, and by oxygen atoms from the D351 side chain and a
water molecule. In turn, the fluorine atoms are stabilized by hydrogen bonding with backbone amino groups of G626, K352 and T353, as well as with K684, N706 and T625 side chains. Furthermore, SERCA T353 can still provide side chain oxygen for hydrogen bonding with water, even though T353 corresponds to D13 of the PSPase. It should be pointed out that although the residue corresponding to D707 is not included in the interactions shown in the structure of the PSPase intermediate (Fig 10), mutation of this residue interferes strongly with binding of ATP and fluoroaluminate in the SERCA enzyme (Table 2). D707 resides in close proximity of D351, and its mutation produces complete catalytic inactivation of SERCA (13). Mutation of D601 also interferes with nucleotide and fluoroaluminate protection (Table II). In fact this residue is likely to provide stabilization to K352 which, in turn, is involved in stabilization of one of the fluorine atoms.

The very small effect of E183Q mutation on fluoroaluminate protection from proteinase K (Table 2) indicates that E183 is not directly involved in fluoroaluminate stabilization. It is apparent that the catalytic inactivation produced by E183 mutation is related to the role of this residue in coordination of water (Fig 10) for the final hydrolytic reaction (26).

It should be pointed out that structural studies indicate (Fig 10) that Mg$^{2+}$ is coordinated with side chain oxygen atoms of D703 and D351, the backbone oxygen of T353, and a fluorine atom. However, our proteinase K protection experiments with WT enzyme (Table 2) do not reveal an important role for Mg$^{2+}$ in stabilization of fluoroaluminate, in spite of the known Mg$^{2+}$ requirement for electrophilic assistance in catalysis. On the other hand, an effect of Mg$^{2+}$ is observed in the protection experiments with the K352, D706, K684 and D601 mutants (Table 2). This suggests that Mg$^{2+}$ coordination can compensate for interference by mutations of residues that are normally involved (directly or indirectly) in stabilization of fluoroaluminate, and its
importance in conformational stabilization becomes evident in experiments with selected mutants.

Fluoroaluminate phosphoenzyme analog and Ca\textsuperscript{2+} occlusion

The importance of Ca\textsuperscript{2+} binding is related to its requirement for enzyme activation, which is transmitted to the extramembranous domains through a long range intramolecular linkage triggered by Ca\textsuperscript{2+} occupancy of site II (16). This conformational state is identified as E1Ca\textsubscript{2}. The structural changes produced by the final complex are quite extensive, as direct interaction of Ca\textsuperscript{2+} with M4, M5 and M6 affects other transmembrane helices and, in turn, results in separation of the three headpiece domains (2, 21). Opening of the headpiece domains in the Ca\textsuperscript{2+} activated enzyme (E1Ca\textsubscript{2}) then allows binding of ATP, whereby closure and crosslinking of the headpiece is produced by interactions of the adenosine moiety with the N domain and of the gamma-phosphate with the P domain (13, 19, 27). Phosphoryl transfer and formation of phosphoenzyme intermediates with high and low affinity for Ca\textsuperscript{2+} occur as outlined in scheme I.

Equilibrium measurements of Ca\textsuperscript{2+} binding in the presence of fluoroaluminate are consistent with distribution of the enzyme into a fraction sustaining no high affinity Ca\textsuperscript{2+} binding (i.e., E2-P analog), and a fraction sustaining high affinity Ca\textsuperscript{2+} binding. Kinetic measurements then demonstrate that the latter fraction contains E1Ca\textsubscript{2} and E1-PCa\textsubscript{2}, which undergo fast or slow dissociation upon addition of EGTA (Fig 5 and Table 3). It is interesting that ADP or AMP-PCP produce strong stabilization of the E1-PCa\textsubscript{2} analog and great enhancement of Ca\textsuperscript{2+} occlusion (Fig 6). This effect is likely due to approximation of P and N domains through crosslinking by the nucleotide (13). The associated A domain rotation (19.) is then transmitted to transmembrane helices that affect the Ca\textsuperscript{2+} binding sites.
Considering that the combination of fluoroaluminate (i.e., analog of phosphate) and ADP produce strong occlusion, we wondered why we did not observe occlusion with AMP-PCP or AMP-PNP. In fact, both in our experiments with fluoroaluminate and in those by Vilsen and Andersen with Cr-ATP (28), Ca$^{2+}$ occlusion occurred slowly and in parallel with engagement of D351 by the ATP terminal phosphate or its analog (AlF$_3$). An explanation may be found in the tendency of the nucleotide substrate to bind with the phosphate chain either in a folded (29) or an extended (13) configuration. The folded configuration keeps the two terminal phosphates and bound Mg$^{2+}$ near T441, and does not crosslink effectively the N and P domains. On the other hand the extended configuration, which is required for the conformational change producing Ca$^{2+}$ occlusion, is subjected to unfavorable statistics due to repulsion of the terminal phosphate by D351 (Table 2) and is only stabilized by the covalent phosphorylation reaction. It is possible that the conformational change producing occlusion may also occur under special conditions that favor the extended configuration even in the absence of covalent interaction (30).

A point of great interest is that while both Ca$^{2+}$ bound to the E1-PCa$_2$ analog are strongly occluded by fluoroaluminate and ADP in the WT enzyme (Fig 7), rapid dissociation is observed with the E309Q mutant. Therefore, E309 has a determinant role not only in retardation of Ca$^{2+}$ dissociation from site I through occupancy of site II (i.e., Ca$^{2+}$ exchange), but also in the occlusion of both Ca$^{2+}$ sites which is produced by the long range conformational change triggered by formation of the ADP$\cdot$E1-PCa$_2$ intermediate. It is also interesting that occlusion by fluoroaluminate and ADP is not produced efficiently even in the N796A mutant, and significant Ca$^{2+}$ dissociation from this mutant occurs upon addition of EGTA. It was previously shown that occurrence of a similar long range linkage (i.e., catalytic activation by Ca$^{2+}$ binding) requires engagement of all site II residues by Ca$^{2+}$, including N796 (16, 31). It is then apparent that, in the
reverse direction, a full effect of the long range signal also requires engagement of Glu309 as well as other site II residues.

It should be understood that as opposed to retardation of site I \(^{45}\text{Ca})-\text{Ca}^{2+}\) exchange in \(E_1\text{Ca}_2\) (Fig 1), which is due to local engagement of the E309 side chain by \(^{40}\text{Ca})-\text{Ca}^{2+}\) bound to site II, occlusion of both \(\text{Ca}^{2+}\) (Figs 6 and 7) is observed in the dissociation experiments upon addition of EGTA to the \(E_1\text{-P}\text{Ca}_2\) analog. In this case, occlusion is produced by locking the E309 side chain in the closed position through an extensive and long range conformational change which is triggered at the catalytic site and includes headpiece domains and transmembrane helices. Detailed structural analysis (30) shows that the binding cavity of \(E_1\text{Ca}_2\) is capped by Glu309, but a water filled space still allows movements of the Glu309 side chain. The consequent \(\text{Ca}^{2+}\) dissociation from site II then leads to exchange with medium \(\text{Ca}^{2+}\) (Fig 1). On the other hand, following the long range conformational change, this aqueous space is filled by M1 (30), which now locks the Glu309 side chain (compare Figs 7 and 8) through a suitable switch of local hydrogen bonds and van der Waals interactions. The functional relevance of M1 has been also shown by mutational analysis (32). Gating of the occluded state must be then considered a specific function of Glu309 (33), in addition to its prominent role in determining the high affinity of site II for \(\text{Ca}^{2+}\). A general discussion of the gating role of single amino acids in ion transport was recently published by Gadsby (34).

Conclusions

Our findings are consistent with a sequential binding mechanism, beginning with \(\text{Ca}^{2+}\) entry through an aqueous cavity lined with polar residues (K47, E55, E58, D59, E109, K246, K254 and Q250) on the cytosolic side of the ATPase near the membrane interface (21). Progression through the cavity leads to \(\text{Ca}^{2+}\) stacking in site I and II. Formation of an enzyme
intermediate (ADPE1-P-Ca_2) analog with fluoroaluminate produces tight occlusion of both bound Ca^{2+}. This phenomenon corresponds to the mechanism of Ca^{2+} occlusion that occurs early in the catalytic cycle, in parallel with phosphoenzyme formation by utilization of ATP. Engagement of E309 at the gateway of the binding cavity serves as an important gating device for occlusion of bound Ca^{2+}. Mutational analysis indicates that the amino acid residues involved in stabilization of fluoroaluminate at the SERCA phosphorylation site are apparently identical to the corresponding residues in the high resolution structure of the fluoroaluminate intermediate analog of PSPase (20).

**LEGENDS TO FIGURES**

**Fig. 1- Time course of Ca^{2+} dissociation and exchange in native ATPase.** Following equilibration with 20 micromolar (^{45}Ca)-Ca^{2+} (see METHODS), SR vesicles were placed on a rapid filtration apparatus and perfused for various time intervals with either 10 mM EGTA (○) or different concentrations of (^{40}Ca)-Ca^{2+}: 1 x 10^{-6} M (●), 5x10^{-6} M (□), 2x10^{-5} M (■), 1 x 10^{-4} M (∆) and 1x10^{-3} M (▲). The data points were fitted with exponential functions. The standard deviation per each point varied between 0.2 and 0.3 mole/mole.

**Fig. 2-Time course of Ca^{2+} dissociation or isotopic exchange using the N796A (A) or E309Q (B) ATPase mutants.** Following equilibration with 20 micromolar (^{45}Ca)-Ca^{2+} (see METHODS), microsomes obtained from COS-1 cells expressing either mutant were placed on a rapid filtration apparatus and perfused for various time intervals with 10 mM EGTA (○) or 1x10^{-4} M mM (∆), or 0.5x10^{-3} M (●), or 1x10^{-3} M (▲) (^{40}Ca)Ca^{2+}. 

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Fig. 3- Inactivation of Ca^{2+} ATPase by reaction with fluoroaluminate. SR vesicles (0.06 mg/ml) were incubated as described in METHODS with various concentrations (0 – 50 µM) of fluoroaluminate, in the presence (○) or in the absence (□) of 0.95 mM CaCl_{2} (1 mM EGTA present in both cases). The incubation was carried out for 30 minutes at 25 °C, and the samples were then used directly for ATPase measurements by adding 0.95 mM Ca^{2+} (when Ca^{2+} was not present) and ATP (1 mM). The dotted lines were drawn empirically.

Fig. 4- Limited digestion of ATPase with proteinase K and protective effect of fluoroaluminate in the absence or in the presence of Ca^{2+} and/or Mg^{2+}. Incubation with fluoroaluminate, in the presence of Ca^{2+} and/or ADP, was carried out as described in METHODS. The concentrations of AlF_{3}, Ca^{2+} and Mg^{2+} were 50 µM, 0.1 mM and 5 mM respectively.

Fig. 5- Limited digestion of ATPase with proteinase K or trypsin, and protective effect of fluoroaluminate in the absence or in the presence of Ca^{2+} and/or ADP. Incubation with fluoroaluminate, in the presence of Ca^{2+} and/or ADP, was carried out as described in METHODS. A: Full protection from proteinase K is produced by preincubation with 50 µM fluoroaluminate both in the presence or in the absence of Ca^{2+}. B: Full protection from trypsin (A1 fragment) is produce by preincubation with 50 µM fluoroaluminate in the absence of Ca^{2+}, but only partial protection in the presence of Ca^{2+}. C: Full protection from proteinase K is produced by preincubation with fluoroaluminate at a concentration as low as 5 µM, in the absence of Ca^{2+}; ADP makes no difference. In the presence of Ca^{2+} however, 5 µM...
fluoroaluminate protects only partially in the absence of Ca$^{2+}$, but full protection is obtained by the addition of ADP.

**Fig. 6- Occlusion of bound Ca$^{2+}$ by reaction of native ATPase with fluoroaluminate.** SR vesicles were equilibrated with 20 µM ($^{45}$Ca)-Ca$^{2+}$ as for the experiment of Fig 2, but with the addition of 2 mM KF and 0 (○), 5 (□) or 50 (◊) µM fluoroaluminate. Following 30 minutes incubation at 25 °C, 1 ml samples were placed on 0.65 um Millipore filter and the medium removed by suction. The vesicles were then perfused with the same medium, but containing 10 mM EGTA for dissociation of bound ($^{45}$Ca)-Ca$^{2+}$. The standard deviation varied between 0.1 and 04 mole/mole. Fitting was obtained with exponential functions.

**Fig. 7-Enhancement of fluoroaluminate dependent Ca$^{2+}$ occlusion by ADP (A) or AMPPCP (B) in native ATPase.** SR vesicles were equilibrated with 20 µM ($^{45}$Ca)-Ca$^{2+}$ as explained above, but with the addition of 2 mM KF and 5 or 50 µM fluoroaluminate, in the absence or in the presence of various concentration of ADP or AMPPCP. Following 30 minutes incubation at 25 °C, 1 ml samples were placed on 0.65 µm Millipore filter and the medium removed by suction. The vesicles were then perfused with the same medium, but containing 10 mM EGTA for dissociation of bound ($^{45}$Ca)-Ca$^{2+}$. The experiments were performed in a BioLogic rapid filtration apparatus, and increasing perfusion times were applied to individual samples. The filters were then collected, blotted and processed for scintillation counting. Controls with TG were obtained for each perfusion time. The dotted lines were drawn empirically. The standard deviation varied between 2 and 6%.
Fig 8- Dissociation of bound Ca\textsuperscript{2+} from the N796A and E309Q mutants reacted with fluoroaluminate and ADP. Microsomes containing recombinant E309Q or N796 mutants were equilibrated with 20 µM (\textsuperscript{45}Ca)-Ca\textsuperscript{2+} as explained above, but with the addition of 2 mM KF, 5 µM AlF\textsubscript{3}, and 1 mM ADP. Following 30 minutes incubation at 25 °C, 1 ml samples were placed on 0.65 µm Millipore filter and the medium removed by suction. The vesicles were then perfused with the same medium, but containing 10 mM EGTA for dissociation of bound (\textsuperscript{45}Ca)-Ca\textsuperscript{2+}. The experiments were performed in a BioLogic rapid filtration apparatus, and increasing perfusion times were applied to individual samples. The filters were then collected, blotted and processed for scintillation counting. Controls with TG were obtained for each perfusion time. The data points were fitted with single exponential functions.

Fig. 9- The coordination geometry of the two Ca\textsuperscript{2+} sites. Site I includes six side chain oxygen atoms and two water molecules. Site II includes four side chain and three main chain oxygen atoms. The side chains are shown with magenta color for E1\textsuperscript{1}Ca\textsubscript{2}, and yellow color for E2-TG (2, 21). Note the wide swing of the E309 side chain upon Ca\textsuperscript{2+} binding. The figures were derived from PDB Accession number 1EUL for E1\textsuperscript{1}Ca\textsubscript{2} and 1IWO for E2-TG. Water and main chain oxygen atoms are left out to show clearly the movements of side chains upon Ca\textsuperscript{2+} binding or dissociation. A complete representation is given by Toyoshima and Inesi (22).

Fig 10- Structure of the fluoroaluminate complex in the transition state analog of the PSPase enzyme. The structure was obtained by Wang et al. (20), and the figure derived from PDB Accession number 1L7N. The corresponding SERCA residues subjected to mutational analysis in our experiments are shown in parenthesis.
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Table 1. Percentage of bound Ca\(^{2+}\) undergoing slow isotopic exchange, and rate constants of fast and slow exchange components (see legend to Fig 1).
### Table 2. Effects of various mutations on ATPase and Ca²⁺ transport activity and nucleotide (1 mM AMP-PCP) or aluminum fluoride (50 µM) protection from digestion with proteinase K. Activity was measured with ATP (1 mM) or acetylphosphate (5 mM) as the substrate, in the presence of Mg²⁺ (5 mM) and Ca²⁺ (0.1 mM). Nucleotide and aluminum fluoride protection was measured in the absence or in the presence of Ca²⁺ and/or Mg²⁺ as indicated. Protection was evaluated from the type of disappearance of the ATPase band on electrophoretic gels (see Figs 4 and 5). The values pertinent to mutants are given in fractional values of controls obtained with WT protein under the same conditions, and are averages of 3–4 experiments. The standard deviations varied between 5 and 18%.

| Mutants | Domain | Activ. (ATP) | Activ. (AcP) | Nucleotide Protection (Ca, Mg) | AlF₃ protection (Mg) | AlF₃ protection (Ca) | AlF₃ protection (Ca, Mg) |
|---------|--------|--------------|--------------|--------------------------------|---------------------|---------------------|------------------------|
| WT      | -      | 1.00         | 1.00         | 1.00                           | 1.0                 | 1.0                 | 1.0                    |
| R560A   | N      | 0.3          | 0.5          | 0                              | 0.8                 | 1.0                 | 0.8                    |
| E439A   | N      | 0            | 0.1          | 0                              | 0.8                 | 0.9                 | 0.8                    |
| R489A   | N      | 0            | 0.1          | 1.2                            | 0.9                 | 0.9                 | 0.9                    |
| R489E   | N      | 0            | 0            | 1.2                            | 0.9                 | 0.9                 | 0.9                    |
| D601N   | N      | 0            | 0.2          | 0.3                            | 0.1                 | 0.9                 | 0.1                    |
| D351N   | P      | 0            | 0            | 1.2                            | 0                   | 0                   | 0                      |
| D351A   | P      | 0            | 0            | 5.0                            | 0                   | 0                   | 0                      |
| K352A   | P      | 0            | 0            | 0                              | 0.8                 | 0                   | 0.5                    |
| T353A   | P      | 0.4          | 0.2          | 0.3                            | 0.5                 | 0.5                 | 0.5                    |
| D703A   | P      | 0.1          | 0.2          | 0.3                            | 0.5                 | 0.5                 | 0.5                    |
| N706A   | P      | 0            | 0            | 4.0                            | 1.0                 | 1.0                 | 1.0                    |
| D707A   | P      | 0            | 0            | 0.3                            | 0                   | 0                   | 0                      |
| K684A   | P      | 0            | 0            | 0                              | 0.2                 | 0.3                 | 0                      |
| T625A   | P      | 0            | 0            | 1.1                            | 0                   | 0.1                 | 0                      |
| G626A   | P      | 0.1          | 0            | 0.2                            | 1.0                 | 1.0                 | 1.0                    |
| R174A   | A      | 0.6          | 0.6          | 1.3                            | 1.0                 | 1.0                 | 1.0                    |
| R174E   | A      | 0.2          | 0.3          | 3.0                            | 1.0                 | 1.0                 | 1.0                    |
| E183Q   | A      | 0            | 0            | 1.2                            | 1.0                 | 1.0                 | 0.7                    |
### Table 3. Maximal level of Ca$^{2+}$ bound, rates of Ca$^{2+}$ dissociation and distribution of enzyme states in the absence or in the presence of fluoroaluminate and ADP. See Figures 6 and 7.

| Fluoro-aluminate | Ca$^{2+}$ bound | Dissociation | E1•Ca$_2$ | E1•AlF$_3$•Ca$_2$ | E2•AlF$_3$ |
|------------------|-----------------|--------------|------------|------------------|------------|
|                  |                 | fast         | slow       |                  |            |
| 0                | 100%            | 90%          | 10%        | 100%             | -          |
| 5 µM             | 85%             | 61%          | 39%        | 52%              | 33%        | 15%        |
| 50 µM            | 60%             | 21%          | 79%        | 13%              | 47%        | 40%        |
| 5 µM and 5 µM ADP| 100%            | -            | 100%       | -                | 100%       | -          |

ADP-fluoroaluminate intermediate analog of the Ca$^{2+}$-ATPase
Figure 1
Figure 2

A

B

Bound $^{45}$Ca - Ca$^{2+}$ (mole/mole ATPase)

Time (sec)

N796A

E309Q
Figure 3

[Graph showing ATPase activity (%) against Fluoraluminate (µM)]
Figure 4
Figure 5

A

Ca\textsuperscript{2+}-ATPase
95KDa
83KDa

Ca\textsuperscript{2+} 0.1 µM — — + +
AlF\textsubscript{4} 50 µM — + — +

B

Ca\textsuperscript{2+}-ATPase

A
A1
A2

Ca\textsuperscript{2+} — — + +
AlF\textsubscript{4} — + — +

C

Ca\textsuperscript{2+}-ATPase
95KDa
83KDa

Ca\textsuperscript{2+} 0.1 µM — — — — + + + +
AlF\textsubscript{4} 5 µM — + — + — + — +
ADP 0.1 µM — — + — — + + +
Figure 6
Figure 7
Ca\textsuperscript{2+} occlusion and gating function of Glu309 in the ADP-fluoroaluminate analog of the Ca\textsuperscript{2+} ATPase phosphoenzyme intermediate
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