Conformational Fluctuations of the Ca$^{2+}$-ATPase in the Native Membrane Environment

**EFFECTS OF pH, TEMPERATURE, CATALYTIC SUBSTRATES, AND THAPSIGARGIN**

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Digestion with proteinase K or trypsin yields complementary information on conformational transitions of the Ca$^{2+}$-ATPase (SERCA) in the native membrane environment. Distinct digestion patterns are obtained with proteinase K, revealing interconversion of E1 and E2 or E1-P and E2-P states. The pH dependence of digestion patterns shows that, in the presence of Mg$^{2+}$, conversion of E2 to E1 pattern occurs (even when Ca$^{2+}$ is absent) as H$^+$ dissociates from acidic residues. Mutational analysis demonstrates that the Glu$^{309}$ and Glu$^{771}$ acidic residues (empty Ca$^{2+}$-binding sites I and II) are required for stabilization of E2. Glu$^{309}$ ionization is most important to yield E1. However, a further transition produced by Ca$^{2+}$ binding to E1 (i.e. E1-2Ca$^{2+}$) is still needed for catalytic activation. Following ATP utilization, H$^+$/Ca$^{2+}$ exchange is involved in the transition from the E1-P-2Ca$^{2+}$ to the E2-P pattern, whereby alkaline pH will limit this conformational transition. Complementary experiments on digestion with trypsin exhibit high temperature dependence, indicating that, in the E1 and E2 ground states, the ATPase conformation undergoes strong fluctuations related to internal protein dynamics. The fluctuations are tightly constrained by ATP binding and phosphoenzyme formation, and this constraint must be overcome by thermal activation and substrate-free energy to allow enzyme turnover. In fact, a substantial portion of ATP free energy is utilized for conformational work related to the E1-P-2Ca$^{2+}$ to E2-P transition, thereby disrupting high affinity binding and allowing luminal diffusion of Ca$^{2+}$. The E2 state and luminal path closure follow removal of conformational constraint by phosphate.

The Ca$^{2+}$-ATPase of sarcoplasmic reticulum membranes (SERCA) includes multiple isoforms and splice variants with variable tissue distribution. In this study, we used the SERCA1a isoform of skeletal muscle, a well characterized enzyme (1, 2) that utilizes the free energy of ATP for Ca$^{2+}$ transport against a concentration gradient. The functional unit is a protein monomer consisting of 994 amino acid residues. The sequence is folded into a cluster of 10 segments forming a transmembrane region, and three relatively large domains ("N", "P", and "A") protruding from the cytosolic surface of the membrane (3, 4). The ATPase cycle begins with high affinity binding of two Ca$^{2+}$ derived from the cytosolic medium ("outside"), followed by ATP utilization to form a phosphorylated enzyme intermediate. Isomerization of the phosphoenzyme intermediate is then coupled to active transport of the bound Ca$^{2+}$ across the membrane ("inside"). Hydrolytic cleavage of the phosphoenzyme is the final step that allows enzyme turnover.

The cooperative character of Ca$^{2+}$ binding as well as the relatively large distance between the catalytic site in the headpiece and the Ca$^{2+}$-binding sites of the ATPase within the transmembrane region imply that conformational rearrangements of the ATPase protein are involved in the mechanism of catalytic activation and energy transduction. Within the general context of cation transport, these rearrangements were envisioned as interconversions of the E1 and E2 conformations of the phosphorylated intermediate (5). High resolution structures were then obtained by crystallographic studies and attributed to different catalytic intermediates (6). On the other hand, the occurrence of conformational transitions in the native membrane environment is revealed by changes in the patterns of proteolysis (7). We report here a series of experiments on limited proteolysis with proteinase K or trypsin, yielding complementary information on the conformational effects of pH, temperature, catalytic ligands, and the specific inhibitor thapsigargin (TG). The experimentation was extended to measurements of ATP hydrolysis and Ca$^{2+}$ transport, thereby providing an understanding of how functional events and conformational transitions are related in the native membrane environment, and how free energy is utilized for conformational work linked to active transport.

**MATERIALS AND METHODS**

SR vesicles were obtained from rabbit skeletal muscle (8). Recombinant Ca$^{2+}$-ATPase was obtained from COS-1 cells.
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Infected with adenovirus vectors carrying rabbit WT (3) or mutant cDNA (9).

Ca\(^{2+}\) uptake by SR vesicles was measured at 25 or 35 °C in a reaction mixture containing 50 mM MOPS, pH 7, 80 mM KCl, 3 mM MgCl\(_2\), 50 μg of microsomal protein/ml, and 50 μM CaCl\(_2\), including 45Ca-labeled isotopic tracer. ATP (3 mM) was added to start the reaction, and at various times, the protein contained in the 1-ml reaction mixture was loaded onto a 0.45-μm Millipore filter by vacuum suction and washed with 15 ml of 2 mM LaCl\(_3\) and 10 mM MOPS, pH 7.0. The filter was then processed for determination of radioactivity by scintillation counting. A zero-time sample was taken before addition of ATP.

ATPase activity was measured at 25 °C, in a reaction mixture containing 30 μg of SR protein/ml, 50 mM MOPS, pH 7 (or 50 mM HEPES, pH 8), 50 mM KCl, 3 mM MgCl\(_2\), 1 μg of A23187 ionophore, and 2 mM EGTA, in the presence or in the absence of 2 mM CaCl\(_2\). The reaction was started by addition of 2 mM ATP, and samples were taken at serial times for P\(_i\) determination by colorimetry (10).

Enzyme phosphorylation with P\(_i\) was estimated following equilibration (5 min at 25 °C) of SR vesicles (0.12 mg/0.2 ml) with 2.0 m\(^{32}\)P\(_i\) in a medium containing 100 mM MES, pH 6.0 (or 100 mM HEPES, pH 8), and 3 mM EGTA, in the presence or the absence of 10 mM MgCl\(_2\) and/or 0.1 mM free Ca\(^{2+}\). The reaction was quenched with 0.2 ml of 2.0 M perchloric acid. The quenched reaction mixture (0.3 ml) was filtered through 0.45-μm Millipore filters, and the protein collected on the filters was washed three times with 5 ml of cold 0.125 M perchloric acid, once with 5 ml of cold water, and finally dissolved with dimethylformamide and processed for scintillation counting. Control experiments were performed by quenching samples with perchloric acid before addition of radioactive substrate.

Limited proteolytic digestion was performed in reaction mixtures containing 50 mM MOPS, pH 7.0 (or MES, pH 6.0, or HEPES, pH 8), 50 mM NaCl, 0.05 mg of SR microsomal protein/ml (or 0.4 mg of COS-1 cell microsomes containing recombinant SERCA/ml) and 0.05 mg of proteinase K or trypsin. CaCl\(_2\), MgCl\(_2\), EGTA, and AMPPCP were added as indicated in the figures. In some experiments, 2 mM KF and 0.1 mM AlCl\(_3\) were added, and the reaction mixture was incubated for 30 min to obtain the fluoromaluminate complex, in the absence or in the presence of nucleotide. Following incubation at 25 °C for various time intervals, the reaction was quenched with trichloroacetic acid (2.5%), and the protein was solubilized with a medium containing lithium dodecyl sulfate (1%), MOPS (0.312 M), pH 6.8, sucrose (3.75%), β-mercaptoethanol (1.25 mM), and bromphenol blue (0.025%). The samples were then subjected to electrophoretic analysis on 12% gels, and the protein bands stained with Coomassie Blue.

Mapping of the fragments with respect to their proximity to the amino or carboxyl termini was confirmed by use of complementary monoclonal antibodies. A 29–30-kDa band, due to cleavage at Ser\(^{550}\)–Thr\(^{357}\) and Ile\(^{611}\), is observed as well (12). A faint 54-kDa band corresponding to the segment between Glu\(^{243}\) and Val\(^{343}/\text{Val}^{247}\) is also visible. The pattern of digestion appears to be the same at pH 6–8, although proteolysis occurs more rapidly at alkaline pH. This pattern, obtained in the presence of Ca\(^{2+}\), is attributed to the E1 state (including various sequential conformations yielding high Ca\(^{2+}\) affinity, cooperative binding of 2 Ca\(^{2+}\), and catalytic activation; see “Discussion”).

If the digestion is performed at pH 6 in the absence of Ca\(^ {2+}\), additional 95 and 14 kDa bands are noted (Fig. 1), corresponding to complementary fragments between Lys\(^{120}\) and the carboxyl terminus, and a 28-kDa band corresponding to the carboxyl terminus (Fig. 1). Mapping of the fragments with respect to their proximity to the amino or carboxyl termini was confirmed by use of complementary monoclonal antibodies. A 29–30 kDa band, due to cleavage at Ser\(^{550}\)–Thr\(^{357}\) and Ile\(^{611}\), is observed as well (12). A faint 54-kDa band corresponding to the segment between Glu\(^{243}\) and Val\(^{343}/\text{Val}^{247}\) is also visible. The pattern of digestion appears to be the same at pH 6–8, although proteolysis occurs more rapidly at alkaline pH. This pattern, obtained in the presence of Ca\(^{2+}\), is attributed to the E1 state (including various sequential conformations yielding high Ca\(^{2+}\) affinity, cooperative binding of 2 Ca\(^{2+}\), and catalytic activation; see “Discussion”).

If the digestion is performed at pH 6 in the absence of Ca\(^{2+}\), the E2 digestion pattern changes to that observed in the presence of Ca\(^{2+}\), corresponding to the proteolytic fragment intervening between Glu\(^{243}\) and the carboxyl terminus, and a 28-kDa band corresponding to the complementary fragment between Thr\(^{242}\) and the amino terminus (Fig. 1). Mapping of the fragments with respect to their proximity to the amino or carboxyl termini was confirmed by use of complementary monoclonal antibodies. A 29–30 kDa band, due to cleavage at Ser\(^{550}\)–Thr\(^{357}\) and Ile\(^{611}\), is observed as well (12). A faint 54-kDa band corresponding to the segment between Glu\(^{243}\) and Val\(^{343}/\text{Val}^{247}\) is also visible. The pattern of digestion appears to be the same at pH 6–8, although proteolysis occurs more rapidly at alkaline pH. This pattern, obtained in the presence of Ca\(^{2+}\), is attributed to the E1 state (including various sequential conformations yielding high Ca\(^{2+}\) affinity, cooperative binding of 2 Ca\(^{2+}\), and catalytic activation; see “Discussion”).
that H produced by raising the pH. Overall, these experiments indicate that E1 digestion pattern even in the absence of Ca$^{2+}$, whereas H$^-$ ionization to the E1 conformation. On the other hand, it is also shown that in the absence of Ca$^{2+}$, digestion of E309Q yields the E1 pattern at any pH, regardless of whether Mg$^{2+}$ is present or not (Fig. 2). This indicates that in the WT protein ionization of the Glu$^{309}$ carboxyl group has an important role for transition to the E1 conformation. On the other hand, it is also shown in Fig. 2 that the E771Q mutant acquires in great part the E1 digestion pattern as the pH is raised from 6.0 to 7.0, either in the presence or in the absence of Mg$^{2+}$. This suggests that in the WT protein, the Glu$^{771}$ carboxyl group is involved in conformational stabilization of the E2 state. Lack of such stabilization then allows disruption of the E2 conformation when ionization of the Glu$^{309}$ (and/or other residues) carbonyl chain is produced by raising the pH. Overall, these experiments indicate that H$^-$ binding to acidic residues at the Ca$^{2+}$ sites favors the E2 digestion pattern, whereas H$^+$ dissociation favors the E1 digestion pattern even in the absence of Ca$^{2+}$. The effect of Mg$^{2+}$ observed with WT protein is likely due to a requirement for additional perturbation when both Glu$^{309}$ and Glu$^{771}$ contribute to stabilization of E2 (see “Discussion”).

Because the physiologic role of shifting E2 to E1 is usually observed in the presence of Ca$^{2+}$, resulting in catalytic activation, we checked whether acquisition of the E1 digestion pattern at pH 8 in the absence of Ca$^{2+}$, but in the presence of Mg$^{2+}$, would be accompanied by ATPase activation. We found no ATP utilization under these conditions (not shown), indicating that Ca$^{2+}$ binding to E1 is still required to obtain catalytically active E1:2Ca$^{2+}$. At any rate, this group of experiments suggests that in the WT protein and in the absence of Ca$^{2+}$, E2 is stabilized by a cooperative mechanism, including the Glu$^{309}$ and Glu$^{771}$ side chains. H$^+$ dissociation from their acidic chains favors a conformation yielding the E1 digestion pattern.

**Effects of AMPPCP and ATP**—It was previously reported that nucleotide binding protects the ATPase from digestion by protease K (7, 14). The inactive analog AMPPCP must be used instead of ATP when Ca$^{2+}$ is present, to avoid rapid substrate consumption by the activated enzyme. We show here that in the presence of Ca$^{2+}$ the protective effect of AMPPCP is the same at pH 6–8, whereas digestion retains the E1 pattern (Fig. 3). Half-maximal protection is obtained with a 0.1 mM concentration of AMPPCP, as determined by gel densitometry.

In the absence of Ca$^{2+}$, ATP has a low protective effect at pH 6, whereas the digestion pattern remains that of E2. However, as the pH is raised to pH 8, as expected (Fig. 1), the digestion pattern is transformed to that of E1. In this case, a more prominent protection by ATP (as well as AMPPCP) is observed (Fig. 3). This indicates that in the E1 state obtained in the presence of Mg$^{2+}$ and alkaline pH, the level of ATP protection is similar to that observed in the presence of Ca$^{2+}$. Nevertheless, as stated above, addition of Ca$^{2+}$ is still required to obtain catalytic activation.

**Phosphoenzyme Intermediate and the E1-P to E-2P Transition**—Following ATP binding in the presence of Ca$^{2+}$, formation of phosphorylated enzyme intermediate is the next...
step in the ATP catalytic cycle. It was previously shown that formation of a stable fluoroaluminate transition state analog of E1-P-2Ca^{2+} protects the ATPase from digestion with proteinase K (7, 15). When we studied the proteinase K digestion pattern of the phosphoenzyme analog in the presence and in the absence of Ca^{2+} (E-AlF_{4}2Ca^{2+} versus E-AlF_{4}), we found that E-AlF_{4}2Ca^{2+} was digested with the E1 pattern at acid as well as alkaline pH (Fig. 4). On the other hand, the E-AlF_{4} (as well as the MgF_{4}) analog of E2-P exhibited the E2 digestion pattern at pH 6, while exhibiting the E1 digestion pattern at pH 8 (even though Ca^{2+} was absent). This experiment indicates that the E1-P to E2-P transition requires H^{+} binding, and E2-P is stabilized by H^{+} binding in analogy to E2. For this reason, digestion with proteinase K exhibits the E1-P pattern at alkaline pH, even in the absence of Ca^{2+} (Fig. 4). Therefore, H^{+}/Ca^{2+} exchange is a determining factor in the E1-P-2Ca^{2+} to E2-P transition, and Ca^{2+} dissociation depends on this transition.

We also studied the effect of pH on the ability of the enzyme to acquire protection from proteinase K digestion following exposure to P_{i} in the absence of Ca^{2+}. In this case, the E2-P ground state of the catalytic cycle is obtained by reverse phosphorylation. It is shown in Fig. 5A that formation of E2-P by equilibration of the ATPase with P_{i} (i.e. reversal of the ATPase cycle) produces strong protection, and the remaining digestion exhibits the E2 pattern. Protection by P_{i} appears similar to that produced by TG (Fig. 5A). However, whereas experiments with TG yield the E2 pattern and a low rate of digestion under all conditions (including alkaline pH, absence of Mg^{2+}, and presence of Ca^{2+}), experiments with P_{i} yield protection and E2 pattern of digestion only under conditions permitting its covalent reaction with the enzyme (i.e. acid pH, presence of Mg^{2+}, and absence of Ca^{2+}). It is shown in Fig. 5B that the same conditions required for ATPase protection from proteinase K (i.e. acid pH in the absence of Ca^{2+}) are also required for formation of phosphorylated intermediate by utilization of P_{i} (16). The P_{i} concentration yielding half-maximal activity was found to be ~0.5 mM, both for protection and phosphorylation. It is then apparent that detection of the E1 or E2 digestion pattern is a convenient expedient to monitor both the E1 to E2 and E1-P to E2-P through the ATPase cycle.

Temperature Dependence of Proteolytic Digestion and Effects of Catalytic Substrates—It is noteworthy that protection by catalytic substrate (Fig. 3) does not involve a primary effect on the digestion pattern but rather a delay of the time course of digestion occurring with the same pattern (E1 or E2, respectively). This suggests that protection may be due to constraint of conformational fluctuations related to internal protein dynamics (17) and required for suitable interaction of the ATPase with the proteolytic enzyme. This is best demonstrated by comparing the ATPase digestion with trypsin at various temperatures.

Trypsin produces an initial cut on the surface of the ATPase N domain, acting on a site (Arg^{198}) freely accessible from the aqueous medium. Consequently, a 57- and 46-kDa complementary fragment are produced immediately, with no apparent temperature dependence under our conditions. On the other hand, further digestion is much slower, as the pertinent site (Arg^{198}) is enclosed within the ATPase structure. In fact, it is shown in Fig. 6 that further digestion, yielding the 35- and 23-kDa fragments from the 57-kDa fragment and even smaller fragments derived from the 46-kDa fragment, is temperature-
dependent and is much faster at 35 than at 25 °C. Protection by ATP is also much more evident at 35 °C (Fig. 7). It is interesting that the effect of ATP appears to be more pronounced on the 23-kDa than on the 35-kDa fragment, suggesting greater exposure of the latter in the ATP-bound conformation of the enzyme.

In the experiments shown in Figs. 6 and 7, the effect of temperature does not involve direct trypsin activation, because the first cut is produced very fast at all temperatures studied. It is clear that the effect of temperature is to promote conformational fluctuations and unfolding of the ATPase, whereby trypsin access to further proteolytic sites is facilitated. Protection by ATP binding is then produced by domain cross-linking and stabilization of conformational fluctuations, thereby limiting productive interaction of the ATPase with trypsin. We also found that the ADP/E1−P•2Ca\(^{2+}\) fluoroaluminate analog exhibits very strong protection of the ATPase from digestion with trypsin. The protection is most evident at 35 °C (Fig. 8, left panel). If ADP is omitted, protection by the E1−P•2Ca\(^{2+}\) analog is significantly weaker (Fig. 8, right panel). The experiments with trypsin digestion demonstrate that substrate (nucleotide) binding and the subsequent formation of phosphoenzyme intermediate produce strong stabilization of conformational fluctuations in the ATPase protein.

**Functional Effects of Alkaline pH**—Addition of ATP to SR vesicles is followed by rapid Ca\(^{2+}\) transport, coupled to utilization of ATP. In the absence of oxalate trapping, maximal levels of Ca\(^{2+}\) uptake are reached within 2 min, and these levels are lower at alkaline than at neutral or acid pH (Fig. 9). The levels of uptake are not limited by passive leak because if TG is added after reaching maximal level, at any pH, no reduction of these levels is observed for several minutes even though the ATPase activity is totally inhibited (not shown). In fact, the maximal levels of uptake are limited by the high Ca\(^{2+}\) concentration formed in the lumen of the vesicles and the E2-P•2Ca\(^{2+}\) dissociation constant. Assuming a luminal volume of 10 μl per mg of SR protein (18), the luminal Ca\(^{2+}\) concentrations reached at pH
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6–8, can be calculated to be 9.8, 8.1, and 3.7 mM, respectively. However, if we consider the significant quantity of Ca\(^{2+}\) binding to internal sites such as calsequestrin, the free luminal Ca\(^{2+}\) concentration at alkaline pH is likely to be within the 0.1 mM range. The lower levels of Ca\(^{2+}\) uptake observed at alkaline pH indicate that lack of protons is a limiting factor for Ca\(^{2+}\)/H\(^+\) exchange and net Ca\(^{2+}\) dissociation from the phosphoenzyme into the lumen of the vesicles. This is consistent with Figs. 1 and 5 showing that the E1 and E1-P states (high Ca\(^{2+}\) affinity) are favored by alkaline pH, whereas the E2 and E2-P states (low Ca\(^{2+}\) affinity) are favored by acid pH.

**DISCUSSION**

Original diffraction studies (19, 20) provided detailed representations of the ATPase crystal structure in the presence of high Ca\(^{2+}\) or in the absence of Ca\(^{2+}\) and presence of TG. The two resulting structures are quite different, including an open (E1–2Ca\(^{2+}\)) or closed (E2-TG) headpiece configuration, where the N, P, and A domains are separate or clustered, and their displacement is coupled to rearrangement of transmembrane segments to increase or decrease the binding affinity of the two Ca\(^{2+}\) sites. Additional crystal structures were obtained in the presence of ligands (i.e. fluoroaluminate or MgF\(_4\)) producing stable transition state analogs of phosphoenzyme in the presence (i.e. analog of E1-P/2Ca\(^{2+}\)) or in the absence (i.e. analog of E2-P) of Ca\(^{2+}\) (21–23). These structures provide very informative atomic models representing diverse conformations of mechanistic relevance, acquired by the ATPase protein under crystallization conditions, although ground states in the absence of analogs are expected to present some differences. On the other hand, with the experiments reported here, we studied the occurrence of conformational fluctuations and transitions in the native membrane environment, as they are revealed by the patterns and time course of ATPase digestion by proteinase K or trypsin. We characterized the dependence of these transitions on pH, temperature, catalytic ligands, and a specific inhibitor, and thereby their involvement in the mechanism of ATP utilization for Ca\(^{2+}\) transport.

A specific feature of the experiments with proteinase K is the diversity of digestion patterns obtained in the presence or in the absence of Ca\(^{2+}\). This difference is related to the appearance of additional fragments (95 and 14 kDa), because of a more favorable exposure of the Leu\(^{119}\)/Lys\(^{120}\) site in the E2 configuration (Fig. 10). This site is located within the M2 helix that extends from the lumen of the SR vesicles to the A domain, and is inaccessible to proteinase K in the E1–2Ca\(^{2+}\) state. On the other hand, in the E2-TG structure, the Leu\(^{119}\)/Lys\(^{120}\) is located within a short helical segment that is evidently accessible to proteinase K, because of unwinding of the M2 helix around Asn\(^{111}\) and Ala\(^{115}\). This conformation change allows a longer path for M2 and rotation of the A domain to form the compact headpiece observed in the E2-TG crystal structure. The limited proteolysis observed in our experiments reveals then a functionally relevant transition from an E2 to an E1 (or “vice versa”) pattern. It should be understood that in fact the E1 pattern is a common feature of sequential states related to H\(^+\) dissociation, cooperative binding of 2Ca\(^{2+}\), and catalytic activation (see below). Although only the final state (E1–2Ca\(^{2+}\)) has been characterized by crystallography (20), the occurrence of sequential conformational adjustments was inferred by experiments on Ca\(^{2+}\) binding and catalytic activation (24).

We now find that, in the native membrane environment, transition between the two patterns of digestion is not strictly dependent on the presence of Ca\(^{2+}\). In fact, the E1 pattern can be obtained by raising the pH in the presence of Mg\(^{2+}\). This suggests that, at the physiological pH, the enzyme resides mostly in an E1 state even in the absence of Ca\(^{2+}\) (25). However, Ca\(^{2+}\) binding to E1 is still required to obtain catalytically active E1–2Ca\(^{2+}\). In fact, even though ATP can bind in the absence of Ca\(^{2+}\) (Fig. 3), its terminal phosphate acquires a productive position (interacting with Asp\(^{391}\) and Mg\(^{2+}\)) only in the presence of Ca\(^{2+}\) (26–29) (see also supplemental Fig. 1).

The results obtained with the E309Q and E771Q mutants (Fig. 2) indicate that, even in the absence of Ca\(^{2+}\), control of the E2 to E1 transition resides within the empty Ca\(^{2+}\) sites (Fig. 2). Various residues that are likely to be involved in the stabilization of the empty Ca\(^{2+}\) sites in the E2 configuration are shown in Fig. 11. Glu\(^{771}\) is a critical residue as its mutation abolishes completely cooperative Ca\(^{2+}\) binding at both sites (30). Glu\(^{309}\) is also a critical residue, because its mutation interferes with Ca\(^{2+}\) binding at the second site, as well as long range catalytic
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The Mg$^{2+}$ requirement for the E2 to E1 transition of the WT ATPase as the pH is raised (Fig. 2, top) indicates that, even following H$^+$ dissociation from Glu$^{309}$ and Glu$^{771}$, the E2 conformation may still be retained in virtue of residual weak interactions, which are then disrupted by Mg$^{2+}$ binding. In fact, under similar conditions, Mg$^{2+}$ binding occurs in the P domain, coordinated by Asp$^{203}$ and Asp$^{707}$. Its presence is likely to interfere with stability of the A domain within the gathered headpiece, thereby allowing displacement of M2 and exposure of Leu$^{119}$/Lys$^{120}$ site to protease K. On the other hand, in addition to its primary location at the catalytic site, Mg$^{2+}$ may also bind to the empty Ca$^{2+}$ sites. It was reported that, in the absence of Ca$^{2+}$, interaction of Mg$^{2+}$ with SR-ATPase induces a fluorescence change because of pH-sensitive binding to the Ca$^{2+}$ sites and formation of an E-Mg dead-end complex (34). Furthermore, fluorescence measurements suggest that Mg$^{2+}$, in the millimolar concentration range, is able to bind to the empty Ca$^{2+}$ sites, and this binding is competitively inhibited by Ca$^{2+}$ and H$^+$ (35). At any rate, note that under all circumstances Ca$^{2+}$ binding is still required to yield the catalytically active E1-2Ca$^{2+}$ complex (Scheme 1).

It is of interest that the E1 and E2 patterns of digestion by protease K are also observed in the fluoroaluminate transition state analogs of E1-2Ca$^{2+}$ and E2-P (Fig. 4), as well as in the physiologic E2-P state obtained by ATPase phosphorylation with P$_i$, in the absence of Ca$^{2+}$ (Fig. 5). This is helpful in demonstrating the effect of H$^+$ and of H$^+$/Ca$^{2+}$ exchange in the E1-2Ca$^{2+}$ to E2-P conformational transition. It is clear that, following ATP utilization, alkaline pH favors the E1-P over the E2-P state (Scheme 1 and supplemental Fig. 1) not only from the functional (36) but also from the structural (Fig. 4) standpoint.

Another important aspect of our experiments is the evidence for prominent conformational fluctuations of the ATPase E1 and E2 ground states in the native membrane environment, as demonstrated (Fig. 6) by the thermal energy requirement for exposure of the Arg$^{198}$ site (Fig. 10) to trypsin. Consider that stabilization by multiple weak interactions depends on anisotropy, and thermally induced motion reduces electrostatic forces because of averaging effects by dipole rotations. In fact, spectroscopic studies have shown conformational heterogeneity of the ATPase protein (37), as well as prominent effects of ligands on the internal dynamics of the enzyme protein (17).

Strong restraint of fluctuations is then observed upon acquisition of intermediate catalytic states. This is the case, for example, of the phosphoenzyme obtained with P$_i$ (i.e., E2-P) that displays a high resistance to digestion (Fig. 5), whereas the E2 digestion pattern is maintained. Stabilization upon formation of the ADP:E1-2Ca$^{2+}$ intermediate analog occurs as well.

activation (31). It is likely that a specific network of hydrogen bonding, involving Glu$^{771}$, Glu$^{309}$, Asn$^{796}$ and other residues (32), is critical to the stability of the E2 state (Fig. 11). Measurements of charge movements upon Ca$^{2+}$ binding indicate that these residues release their protons within the 6–7 pH range (33). Glu$^{309}$ appears most important because its (single) mutation to Gln (Fig. 2) yields the E1 digestion pattern at any pH. Its ionization is evidently important for transition to the E1 conformation. Glu$^{771}$ is also important because its (single) mutation to Gln allows transition to E1, even in the absence of Mg$^{2+}$. In this case, however, a pH rise is required, most likely to induce H$^+$ dissociation from Glu$^{309}$ and/or possibly other residues.

![FIGURE 11. Atomic model (stereo pair) of the empty Ca$^{2+}$-binding sites in the E2(TG + DBHQ) structure (Protein Data Bank code 2agv). The explicit presence of hydrogen atoms was deduced from continuum electrostatic calculations (32). Our experiments demonstrate that in fact the E2 state is stabilized by the presence of these protons, and H$^+$ dissociation favors transition to E1 even in the absence of Ca$^{2+}$ (and in the absence of TG and DBHQ stabilization). The broken lines show hydrogen bonds. Hydrogen bonds between main chain atoms are shown in green.](image-url)

![SCHEME 1. Reaction scheme of the Ca$^{2+}$-ATPase catalytic and transport cycle based on the original Post-Albers model. The scheme includes exchange of 2H$^+$ for 2Ca$^{2+}$, as well as the dead-end complex formed by stabilization with the inhibitor TG. Our experiments demonstrate a pH dependence of the E2 to E1 transition, whereby H$^+$ dissociation from acidic residues at the binding sites favors transition of 2H$^+$/E2 to E1 even in the absence of Ca$^{2+}$. Furthermore, the experiments demonstrate that the E1 and E2 ground states undergo strong conformational fluctuations. These fluctuations are tightly constrained by interaction with substrate, whereby thermal activation and substrate-free energy are required for the E1→P-2Ca$^{2+}$ transition. Therefore, conformational work is a key for transduction of ATP free energy to active transport. The structures of some of the sequential states (or analogs thereof) were solved by crystallographic studies and are now available in the literature (for review see Ref. 6, and supplemental Fig. 1).](image-url)
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(Fig. 4), whereas the E1 pattern of digestion with proteinase K is retained. Thermal activation and substrate-free energy are then required to overcome conformational stabilization and promote catalytic turnover. It is well known that phosphoenzyme can be obtained by utilization of ATP at low temperature (2–3 °C), but thermal activation energy is required for progress to hydrolytic cleavage (38). On the other hand, regarding utilization of substrate-free energy, consider that a 3 orders of magnitude reduction of the binding affinity constant, as produced with the E1→P-2Ca\(^{2+}\) to E2-P-2Ca\(^{2+}\) transition, entails an input of 8–9 kcal per cycle (−2RT ln(10\(^3\) M\(^{-1}\)/10\(^6\) M\(^{-1}\))). It is then apparent that a large portion of the energy derived from ATP is actually utilized for conformational work to promote this transition. In fact, under conditions limiting the E1→P-2Ca\(^{2+}\) to E2-P-2Ca\(^{2+}\) transition (slippage of the pump), hydrolytic cleavage of E1→P-2Ca\(^{2+}\) yields extra heat rather than work (39). It is noteworthy that the conformational work related to the E1→P-2Ca\(^{2+}\) to E2-P-2Ca\(^{2+}\) transition results in disruption of the high affinity Ca\(^{2+}\) sites and opening of the exit path on the luminal side (see Scheme 1). Considering a 10\(^{-3}\) m \(K_{eq}\) luminal dissociation of Ca\(^{2+}\) from E2-P-2Ca\(^{2+}\) will proceed spontaneously as long as the luminal Ca\(^{2+}\) concentration is below mM. Furthermore the \(K_{eq}\) for hydrolytic cleavage of E2-P to E2-P is nearly 1, and the \(K_{eq}\) value for P\(_i\) dissociation from E2-P is \(~1 \times 10^{-3}\) M. Therefore, these reactions require negligible energy input to proceed forward. Therefore, the return to the E2 ground state and closure of the luminal Ca\(^{2+}\) path occur as soon as the long range constraint (Fig. 5) produced by the presence of phosphate at the catalytic site is relieved (Scheme 1). A full account of the \(K_{eq}\) values for the ATPase sequential reactions, and the resulting free energy changes (including second order reactions), is given in Ref. 40.

Additional evidence for the functional relevance of dynamic fluctuations and conformational transitions, as revealed by patterns of proteolysis, is provided by the effects of the specific inhibitor TG. In fact, ATPase digestion by protein kinase K in the presence of Ca\(^{2+}\) occurs with the E2 pattern if TG is present (i.e. just as if Ca\(^{2+}\) were not present). It is clear that the mechanism of inhibition is based on interference with conformational transitions, whereby the enzyme is locked in an inactive dead-end complex (Scheme 1), which is in some respects different from the physiological E2 state (Fig. 5), but nevertheless prevents dynamic fluctuations and conformational transitions that are required for catalytic turnover.

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