Critical Roles of Interdomain Interactions for Modulatory ATP Binding to Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase* 

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ATP has dual roles in the reaction cycle of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase. Upon binding to the Ca\textsubscript{2}E1 state, ATP phosphorylates the enzyme, and by binding to other conformational states in a non-phosphorylating modulatory mode ATP stimulates the dephosphorylation and other partial reaction steps of the cycle, thereby ensuring a high rate of Ca\textsuperscript{2+} transport under physiological conditions. The present study elucidates the mechanism underlying the modulatory effect of ATP and the importance therein of interdomain bonds.

Using the energy liberated by ATP hydrolysis the sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase pumps Ca\textsuperscript{2+} ions into the endoplasmic reticulum, a critical aspect of a variety of important physiological processes in animal cells such as contraction and relaxation of muscle and secretion of hormones, enzymes, and neurotransmitters. The Ca\textsuperscript{2+}-ATPase is an integral membrane protein made up of 10 transmembrane helixes (M1–M10) connected to a large cytoplasmic headpiece comprised of three distinct and loosely connected domains, N (“nucleotide binding”), P (“phosphorylation”), and A (“actuator”). Ca\textsuperscript{2+} transport is achieved by means of a reaction cycle (Scheme 1) involving the formation and decay of an aspartyl-phosphorylated intermediate coupled to protein conformational changes, whereby the Ca\textsuperscript{2+} binding sites sequentially alter affinity for Ca\textsuperscript{2+} and exposure to the cytosol and endoplasmic reticulum lumen, thus enabling the translocation of Ca\textsuperscript{2+} to the lumen against a large Ca\textsuperscript{2+} gradient. The determination by x-ray crystallography of atomic structures of the Ca\textsuperscript{2+}-ATPase in several different physiologically relevant conformations has, in combination with a wealth of functional studies, provided unique insight into the nanomachinery of the Ca\textsuperscript{2+}-ATPase (reviewed in Refs. 1 and 2). Thus, during the reaction cycle, the A- and N-domains undergo large displacements relative to the P-domain. Phosphorylation from ATP bound mainly to the N- and P-domains in the E1 conformation is triggered by the binding of the two Ca\textsuperscript{2+} ions in the membrane domain forming the Ca\textsubscript{2}E1 state. Crystal structures indicate that Ca\textsuperscript{2+} binding to M4 directs a movement of M1–M2 required for bending of the P-domain to form a more compact structure of the ATP site that allows the phosphoryl transfer to the Asp\textsuperscript{351} carboxylate group (3, 4). The dephosphorylation of E2P occurs as a consequence of the movement of the conserved phosphatase motif of the A-domain, 181TGES, into close contact with the Asp\textsuperscript{351} aspartyl phosphate, allowing the side chain of the glutamate Glu\textsuperscript{183} to catalyze a nucleophilic attack of the aspartyl phosphate in a S\textsubscript{N}2 reaction, forming a pentacoordinated phosphoryl transition state (5, 6). The dephosphorylation proceeds from

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the E2P ground state\(^2\) through the E2-P phosphoryl transition state and into the E2-P, product state, and these enzyme intermediate states ("E2P-like states") are mimicked by the complexes of the Ca\(^{2+}\)-ATPase with the respective metal fluorides BeF, AlF\(^3\), and MgF\(^6\) (6–9).

ATP is an important mediator of interdomain interactions, with all three cytoplasmic domains contributing to nucleotide binding at various stages of the reaction cycle (9–17). Aside from being the phosphorylating substrate in the Ca\(^{2+}\)E1 state, ATP also fulfills a role as a non-phosphorylating modulator of the pump cycle, accelerating certain partial reaction steps without being hydrolyzed (boxed ATP in Scheme 1) (16–29). Some of the residues that contribute to ATP binding in the phosphorylating mode in E1 are also involved in ATP binding in the modulatory mode, in particular N-domain residues. When the A-domain approaches the P-domain in E2 and E2P-like states, the N-domain is partially displaced, leading to a less compact structure of the ATP site and affinities for modulatory ATP 10–100-fold lower than the affinity of E1 for phosphorylating ATP (17). The apparent affinity for ATP binding to E2P is further lowered in the presence of Mg\(^{2+}\), because only free ATP and not MgATP can bind to this state, which already contains Mg\(^{2+}\)-deprived enzyme with the respective phosphate analogs in the presence of Mg\(^{2+}\).

Scheme 1. Ca\(^{2+}\)-ATPase reaction cycle. Major conformational changes and substrate binding and dissociation steps are shown. Boxed ATP indicates steps for which the rate is enhanced by additional binding of ATP or MgATP in a non-phosphorylating mode, i.e. without being hydrolyzed ("modulatory ATP"). Encircled AlF, VO\(^4\)\(^-\) (orthovanadate), and BeF are indicated below the reaction intermediates, for which stable analogs can be formed by incubation of Ca\(^{2+}\)-deprived enzyme with the respective phosphate analogs in the presence of Mg\(^{2+}\).

2 Throughout this text the terms "E2P ground state" and "E2-P phosphoryl transition state" are used to designate the enzyme intermediates with the bound phosphoryl group in the ground state and transition state, respectively, as occurring during dephosphorylation (5, 6). According to classic transition state theory, the catalytic rate of an enzyme increases with the transition state and then decreases again from the E2-P, product state to the E2 ground state, suggesting that ATP accelerates E2P dephosphorylation by tightening the interactions around the catalytic site in the E2-P phosphoryl transition state, thereby stabilizing this intermediate and lowering the activation energy of the reaction.

The structural basis for this variation of ATP affinity along the reaction coordinate of dephosphorylation has not been clear, and in the present work we focus on the important roles of two interdomain interactions: the ionic bond between Asp\(^{203}\) (A-domain) and Arg\(^{678}\) (P-domain) and the hydrogen bond between Ser\(^{186}\) (A-domain) and Glu\(^{439}\) (N-domain). Common to these two interactions is that they seem to exist only in the E2P class of substrates of the pump cycle (i.e. in the E2P ground state and the E2-P phosphoryl transition state). Hence, from crystal structures it appears that the Asp\(^{203}\)-Arg\(^{678}\) and Ser\(^{186}\)-Glu\(^{439}\) bonds break when the enzyme liberates P, and enters the E2 state, and the residues remain apart throughout the rest of the reaction cycle as a consequence of the A-domain movements (cf. Fig. 1 and Table 1). Ser\(^{186}\), Asp\(^{203}\), Glu\(^{439}\) and Arg\(^{678}\) have been implicated as critical for Ca\(^{2+}\)-ATPase function (16, 28, 30–33), and both interaction sites are positioned close to the bound nucleotide in the crystal structure of the Ca\(^{2+}\)-ATPase stabilized in the E2-AlF-AMP-PCP state (9), with the Asp\(^{203}\)-Arg\(^{678}\) interaction near the \(\gamma\)-phosphate of AMP-PCP and the Ser\(^{186}\)-Glu\(^{439}\) interaction near the adenine moiety (Fig. 1). We now demonstrate the importance of the two cytoplasmic domain interactions for the progression of E2P dephosphorylation as well as the ATP modulation of the dephosphorylation reaction, and by analyzing the ATP affinity profiles of the intermediates in mutants we provide insight in the mechanism underlying the modulatory effect.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis of cDNA encoding the rabbit fast twitch muscle Ca\(^{2+}\)-ATPase (SERCA1a isoform) inserted into the pMT2 vector (34) was carried out using the QuikChange site-directed mutagenesis kit (Agilent Technologies), and the mutant cDNA was sequenced throughout. The cDNA encoding mutants E439A and R678A was the same as used previously (17, 28). To express wild type and mutant cDNA, COS-1 cells were transfected using the calcium phosphate precipitation method (35). Microsomal vesicles containing either wild type or mutant Ca\(^{2+}\)-ATPase were isolated by differential centrifugation (36). The concentration of expressed Ca\(^{2+}\)-ATPase was determined by an enzyme-linked immunosorbent assay (37) and by determination of the maximum capacity for phosphorylation with ATP or P\(_i\) ("active site concentration") (38).

Formation of the complexes of wild type or mutant Ca\(^{2+}\)-ATPase in the E2 state with AlF or BeF prior to photolabeling was achieved by pre-equilibration of the enzyme for 30 min at
FIGURE 1. Comparison of the structural arrangements near the Asp^{203}-Arg^{678} and Ser^{186}-Glu^{439} interaction sites in Ca\(^{2+}\)-ATPase in various crystal structures. The Protein Data Bank accession codes corresponding to the structures shown are 3B9R (E\(^2\)P-AMP-PCP state (9)), 1T5S (Ca\(^2+\)E\(^1\)-AMP-PCP state (10)), 3AR8 (E\(^2\)AIF-TNP-AMP state (43)), and 3AR9 (E\(^2\)BeF-TNP-AMP state (43)). Amino acid side chains are shown for residues discussed in the text. The Asp^{203}-Arg^{678} and Ser^{186}-Glu^{439} interactions as well as the Glu^{183}-H\(_2\)O-AlF\(^-\)-Asp^{351} bonding network are indicated by green dotted lines. Aluminum atoms are shown in gray, nitrogen in blue, oxygen in red, phosphorous in orange, and fluoride in cyan. Carbon atoms are shown in gray for the side chains and yellow for the nucleotides.

TABLE 1
Distances of the Asp^{203}-Arg^{678} and Ser^{186}-Glu^{439} interactions in selected wild type Ca\(^{2+}\)-ATPase crystal structures

| Crystal structure                                    | Reaction state analog | Asp\(^{203}\) carboxyl-Arg\(^{678}\) guanidinium (Å) | Glu\(^{439}\) carboxyl-Ser\(^{186}\) hydroxyl (Å) |
|----------------------------------------------------|-----------------------|----------------------------------------------------|-------------------------------------------------|
| E\(^2\)BeF (PDB code 3B9R; Ref. 9)                 | E\(^2\)P ground state | 2.6                                                | 2.8                                             |
| E\(^2\)BeF-thapsigargin (PDB code 2ZBF; Ref. 45)   | E\(^2\)P ground state | 3.3                                                | 2.5                                             |
| E\(^2\)BeF-thapsigargin-TNPAMP (PDB code 3AR8; Ref. 43) | E\(^2\)P-ATP ground state | 3.0                                                | 2.5                                             |
| E\(^2\)AIF (PDB code 1XP5; Ref. 6)                 | E\(^2\)P transition state | 3.0                                                | 2.6                                             |
| E\(^2\)AIF (PDB code 2ZBG; Ref. 45)                | E\(^2\)P transition state | 2.5                                                | 3.7                                             |
| E\(^2\)AIF-AMP-PCP (PDB code 3B9R; Ref. 9)         | E\(^2\)P-ATP transition state | 3.1                                                | 2.6                                             |
| E\(^2\)AIF-TNPAMP (PDB code 3AR8; Ref. 43)         | E\(^2\)P-ATP transition state | 2.5                                                | 3.6                                             |
| E\(^2\)MgF (PDB code 1WPG; Ref. 8)                 | E\(^2\)P, product state | 2.5                                                | 2.8                                             |
| E\(^2\)hthapsigargin (PDB code 2EAR; Ref. 46)      | E\(^2\)P ground state | 10.4                                               | 12.2                                            |
| E\(^2\)hthapsigargin-AMP-PCP (PDB code 2C8K; Ref. 12) | E\(^2\)P-ATP ground state | 11.2                                               | 9.4                                             |
| E\(^2\)hthapsigargin-ATP (PDB code 3AR4; Ref. 43)   | E\(^2\)P-ATP ground state | 10.9                                               | 11.3                                            |
| Ca\(^2+\)E\(^1\) (PDB code 1SU4; Ref. 47)          | Ca\(^2+\)E\(^1\) ground state | 46.7                                               | 32.5                                            |
| Ca\(^2+\)E\(^1\)-AMP-PCP (PDB code 3AR2; Ref. 43)   | Ca\(^2+\)E\(^1\)-AMP ground state | 40.2                                               | 16.8                                            |
| Ca\(^2+\)E\(^1\)-AlF-ADP (PDB code 1TST; Ref. 10)   | Ca\(^2+\)E\(^1\)-ADP transition state | 39.1                                               | 16.6                                            |
| Ca\(^2+\)E\(^1\)-AMPNN (PDB code 3BA6; Ref. 9)      | Ca\(^2+\)E\(^1\)-ADP product state | 39.1                                               | 15.8                                            |
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25 °C in 25 mM MOPS/TMAH (pH 7.0), 80 mM KCl, 2 mM EGTA, 2 mM NaF, 0.2 mM MgCl₂, and either 0.5 mM AlCl₃ or 0.1 mM BeSO₄. The complex with orthovanadate was formed by incubation of the enzyme for 1 h at 25 °C in 25 mM MOPS/TMAH (pH 7.0), 80 mM KCl, 2 mM EGTA, 5 mM MgCl₂, and the indicated concentration of orthovanadate.

The enzyme-inhibitor complexes were formed immediately prior to the initiation of the photolabeling experiments and were kept on ice throughout (<1 h). The high stability of the E2-ALF, E2-BeF, and E2-orthovanadate complexes during the course of the photolabeling experiments has been documented previously (17).

The synthesis of the [γ-³²P]TNP-8N₃-ATP photolabel, its application as a specific photolabel of the Ca²⁺-ATPase, the competitive inhibition by ATP of [γ-³²P]TNP-8N₃-ATP photolabeling, and the quantification of ³²P-labeled bands by electronic autoradiography following SDS-PAGE were carried out using the previously established procedures (13, 39). The present application of the experimental setup for the [γ-³²P]TNP-8N₃-ATP photolabeling of the Ca²⁺-ATPase is described in detail in Ref. 17. The medium used for photolabeling of the complexes of the enzyme with ALF, BeF, or orthovanadate contained 25 mM EPPS/TMAH (pH 8.5), 2 mM EDTA, 17.4% (v/v) glycerol, and [γ-³²P]TNP-8N₃-ATP without or with ATP at the concentrations indicated in the figures. The concentration of [γ-³²P]TNP-8N₃-ATP used in competition experiments with ATP was 3 times the K₀.₅ where K₀.₅ is the [γ-³²P]TNP-8N₃-ATP concentration giving half-maximum labeling.

Measurements of phosphorylation from [γ-³²P]ATP or ³²P were carried out by acid quenching followed by acid SDS-polyacrylamide gel electrophoresis and quantitation of the radioactivity associated with the Ca²⁺-ATPase band using the previously established procedures (16, 28, 40). For studies of the ATP dependence of dephosphorylation of E₂P, phosphorylation with 0.5 mM [³²P]ATP was carried out for 10 min at 25 °C in 100 mM MES/Tris (pH 6.0), 10 mM MgCl₂, 2 mM EGTA, and 30% (v/v) dimethyl sulfoxide. The phosphorylated sample was chilled in ice water, and dephosphorylation was followed at 0 °C by a 19-fold dilution into ice-cold medium containing 50 mM MOPS/Tris (pH 7.0), 2 mM EDTA, 10 mM DTA, 5 mM H₃PO₄, and various concentrations of ATP.

The data were analyzed by nonlinear regression using the SigmaPlot program (SPSS, Inc.). The analysis of the TNP-8N₃-ATP photolabeling data were based on the hyperbolic function, \( Y = Y_{\text{max}} \cdot \left[ \text{TNP-8N₃-ATP} \right] / \left( K_{0.₅} + \left[ \text{TNP-8N₃-ATP} \right] \right) \), in which \( Y \) is the amount of photolabeled Ca²⁺-ATPase, \( Y_{\text{max}} \) is the maximum amount of photolabeled Ca²⁺-ATPase, \( K_{0.₅} \) is the concentration of TNP-8N₃-ATP giving half-maximum labeling, and \( m \left[ \text{TNP-8N₃-ATP} \right] \) is a linear background component, which has been subtracted from the data shown (13). The analysis of the data obtained from ATP inhibition of TNP-8N₃-ATP photolabeling was based on the Hill equation modified to describe inhibition, \( Y = Y_{\text{max}} \cdot (1 - [\text{ATP}])/\left( K_{0.₅}^\prime \cdot ([\text{ATP}] + K_{0.₅}^\prime \prime) \right) \), in which \( Y \) and \( Y_{\text{max}} \) are defined as above, \( K_{0.₅} \) is the concentration of ATP giving half-maximum effect, and \( n \) is the Hill coefficient (varying between 0.74 and 1.02 for the present data). The “true” dissociation constant, \( K_{0.₅} \), for ATP binding was calculated from the measured \( K_{0.₅} \) values using the validated equation for competitive inhibition (13). For analysis of the modulatory effect of ATP on the rate of E₂P dephosphorylation, the ATP concentration dependence of the rate constant was analyzed according to the hyperbolic function, \( k_{\text{obs}} = k_0 + (k_{\text{max}} - k_0) \left[ \text{ATP} \right] / \left( K_{0.₅} + \left[ \text{ATP} \right] \right) \), in which \( k_{\text{obs}} \) is the rate constant observed at the indicated ATP concentration, \( k_0 \) is the rate constant in the absence of ATP ("basal rate"), and \( k_{\text{max}} \) is the extrapolated value of the rate constant corresponding to infinite ATP concentration (16). The experiments were conducted at least twice on independent microsomal preparations, and average values are shown on the graphs, with error bars when larger than the size of the symbols.

RESULTS

Design and Expression of the Mutant Ca²⁺-ATPases—To address the importance of the Asp²⁰³Arg⁶⁷⁸ (A-P domains) and Ser¹⁸⁶-Glu¹⁴⁹ (A-N domains) interaction sites in the E₂P states, we analyzed four mutations replacing these residues individually with the residue corresponding to the interaction partner (mutants D203R, R678D, S186E, E439S) as well as two double mutations, D203R/R678D and S186E/E439S, in which the side chains of the interaction partners were swapped. In addition, the point mutations R678Q, S186A, S186P, and Q202A as well as the double mutation Q202A/D203A were included in the study, the latter two motivated by previous reports that replacement of Gln²⁰² with alanine causes reduced Ca²⁺ transport (32) and ATPase activity (33). The wild type and mutant Ca²⁺-ATPase constructs were expressed in COS-1 cells to similar high levels, allowing the study of the kinetics of the partial reactions of the pump cycle as well as direct measurements of nucleotide binding.

Rate and ATP Dependence of E₂P Dephosphorylation—The rate of dephosphorylation of E₂P phosphoenzyme was measured by first phosphorylating Ca²⁺-deprived enzyme by [³²P]P in the backward direction of the normal reaction cycle (cf Scheme 1) and then chasing the E₂P phosphoenzyme with EDTA to remove free Mg²⁺, thereby terminating phosphorylation. Fig. 2 shows the time courses observed in the absence of ATP in the dephosphorylation medium, with the extracted rate constants (the basal rate "k₀") listed in Table 2. Fig. 3 shows the E₂P dephosphorylation rate constants obtained for wild type and mutants at varying concentrations of ATP in the dephosphorylation buffer. The apparent affinities for modulatory ATP (K₀.₅) extracted from the analysis are listed in Table 2 together with the ratio between the dephosphorylation rates at 1 and 0 mM ATP (kₒ/k₀).

Mutations to the Asp²⁰³Arg⁶⁷⁸ interaction site inhibited E₂P dephosphorylation, most markedly for D203R, which displayed a 5-fold lower basal dephosphorylation rate compared with the wild type. Importantly, the swap mutant D203R/R678D displayed a basal dephosphorylation rate ~2-fold higher than that of the D203R point mutant, corresponding to a partial restoration of the dephosphorylation rate, which may result from ionic interaction between the A- and P-domains through the swapped side chains at positions 203 and 678.

For the wild type, the E₂P dephosphorylation rate was ~3-fold higher in the presence of 1 mM ATP, relative to the
The basal dephosphorylation rate constant ($k_0$), the ATP concentration giving half-maximum activation ($k_{sat,ATP}$), and the dephosphorylation rate constant at 1 mM ATP ($k_1$) were derived from the data shown in Figs. 2 and 3, see “Experimental Procedures.” The accumulated $2P$ phosphoenzyme was then chased by dilution into ATP-free dephosphorylation medium containing excess EDTA to remove Mg$^{2+}$, followed by acid quenching after various time intervals as indicated on the abscissa. The dephosphorylation rate constants, obtained by fitting of an exponential decay function to the data, are listed in Table 2 ("basal rate", $k_0$). The basal dephosphorylation rate, 9-fold in the case of E439S and as much as 18-fold in the case of S186P, relative to the wild type. S186E was an exception, showing little effect on the basal dephosphorylation rate. The mutants with single substitutions of Arg$^{679}$ displayed little or no stimulatory effect of ATP on the dephosphorylation rate in the 0–1 mM ATP concentration range (reflected by the $k_1/k_0$ ATP enhancement factors being close to 1, cf. Fig. 3 and Table 2). This indicates that Arg$^{679}$ is critical for modulatory ATP binding, or, at least, for making the binding productive in terms of activation of dephosphorylation. Remarkably, in the swap mutant D203R/R678D ATP modulation of $2P$ dephosphorylation was much less affected as compared with the respective mutants with single substitutions D203R and R678D (Fig. 3 and Table 2). Hence, for D203R/R678D, the dephosphorylation rate increased by a factor of 2.2 over the 0–1 mM ATP concentration range with a $k_{sat,ATP}$ of 181 $\mu$M, thus clearly demonstrating a gain of function relative to the D203R and R678D point mutants, which might result from the interaction between the swapped side chains.

Also Q202A and Q202A/D203A displayed markedly reduced basal $2P$ dephosphorylation rates. However, only the double mutant involving Asp$^{203}$ showed a significantly reduced modulatory effect of ATP, whereas Q202A displayed a slightly higher affinity for modulatory ATP than the wild type and wild type-like $k_1/k_0$ enhancement factor (Fig. 3 and Table 2).

Mutations to individual residues Ser$^{186}$ and Glu$^{439}$ generally resulted in substantial increases of the basal $2P$ dephosphorylation rate, 9-fold in the case of E439S and as much as 18-fold for S186P, relative to the wild type. S186E was an exception, showing little effect on the basal dephosphorylation rate. The swap mutation S186E/E439S restored the basal dephosphorylation rate to a level only ~2-fold faster than that of the wild type (Fig. 2 and Table 2). However, for S186E/E439S as well as the mutants with single substitutions of Ser$^{186}$ and Glu$^{439}$ no or little stimulation of the dephosphorylation by ATP was seen.
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in the 0–1 mM concentration range (Fig. 3 and Table 2). Hence, although the Ser^{186}-Glu^{349} swap mutation rescues the basal dephosphorylation rate of the E439S mutant to a wild type-like level, it does not rescue the stimulation by ATP of dephosphorylation.

Nucleotide Affinity in Analog States of the E2P Dephosphorylation Reaction Sequence—To understand the mechanism underlying the altered ATP sensitivity of the E2P dephosphorylation rate seen for some of the mutants we proceeded to study the ATP affinity of individual states of the dephosphorylation reaction sequence, the E2P ground state represented by E2-BeF (7, 9), and the E2-P phosphoryl transition state represented by E2-AlF (6, 7). Like the wild type enzyme, the mutants formed stable complexes of the E2 state with the metal fluorides, as proved by measuring the phosphorylation from $[^{32}P]ATP$ in the presence of Ca$^{2+}$ following pre-equilibration with AlF or BeF in the absence of Ca$^{2+}$. Because of the mutually exclusive binding of these metal fluoride phosphoryl analogs and the γ-phosphate of ATP at the phosphorylation site, and the low rate of dissociation of the metal fluorides, the $[^{32}P]$ incorporation determined under these conditions was inhibited. Hence, as demonstrated below, we were able to determine the affinity of these complexes for the modulatory ATP by use of our previously validated TNP-8N$_3$-ATP photolabeling assay (13, 17).

Fig. 4 shows the $[^{32}P]TNP-8N$_3-ATP concentration dependence of $[^{32}P]TNP-8N$_3-ATP photolabeling of the wild type and mutants in E2-AlF (circles) and E2-BeF (triangles) states, with the extracted $K_{0.5}$ values for the $[^{32}P]TNP-8N$_3-ATP binding listed in Table 3. The binding site for TNP-8N$_3$-ATP overlaps sufficiently with the binding site for modulatory ATP in the E2 states to allow competition binding assays in which the affinity for modulatory ATP is obtained from the ATP concentration dependence of inhibition of TNP-8N$_3$-ATP photolabeling (17). Results of such competition experiments are shown in Fig. 5, with the calculated ATP affinity constants ($K_{0.5}$) listed in Table 3. Two mutants, R678D and R678Q, showed poor labeling with TNP-8N$_3$-ATP in either metal fluoride complexed state, thus preventing the determination of their affinity for the photolabel or for ATP. For the remaining mutants, the levels of photolabeling at saturating concentrations of TNP-8N$_3$-ATP were as high as those obtained with the wild type in the E2-AlF state as well as the E2-BeF state, thus allowing determination of the $K_{0.5}$ for TNP-8N$_3$-ATP and the $K_D$ for ATP. Importantly, this was also the case for the swap mutant D203R/R678D. Furthermore, whereas the point mutation D203R markedly lowered TNP-8N$_3$-ATP affinity of both E2-AlF and E2-BeF (4- and 9-fold, respectively), as well as ATP affinity (12- and 30-fold, respectively), the affinity obtained with D203R/R678D was wild type-like for both TNP-8N$_3$-ATP and ATP in the case of E2-AlF and only slightly reduced for E2-BeF (2.1-fold for TNP-8N$_3$-ATP and 1.4-fold for ATP, relative to wild type), thus once again implying that the Asp$^{203}$-Arg$^{678}$ side chain swap is much less disruptive than each of the individual D203R and R678D mutations (Figs. 4 and 5, and Table 3).

The affinity of the wild type for TNP-8N$_3$-ATP and ATP is 4- and 3-fold higher, respectively, in the E2-AlF state than in E2-BeF (Figs. 4 and 5, and Table 3, BeF/AlF affinity constant ratio 4 and 3, respectively, for the wild type). In the wild type, ATP binding therefore appears to stabilize the E2-P phosphoryl transition state (mimicked by E2-AlF) relative to the E2P ground state (mimicked by E2-BeF), thus accounting for the stimulatory effect of ATP on dephosphorylation. Mutants with alterations to the Ser$^{186}$-Glu$^{349}$ interaction site differed markedly from the wild type enzyme in this respect. Hence, for S186A and S186P there was only a marginal difference between the ATP affinities of E2-AlF and E2-BeF states, and for mutants S186E and E439S, as well as S186E/E439S, the ATP affinity was actually higher in E2-BeF than in E2-AlF (Table 3, BeF/AlF...
**TABLE 3**

| Affinity for TNP-8N₃-ATP and ATP of E₂-AlF and E₂-BeF states |
|-------------------------------------------------------------|
|                 | K_{D(AlF)} (TNP-8N₃-ATP) | E₂-P | E₂-P | BeF/AlF | K_{D(ATP)} |
| Wild type       | [AlF] (n = 11)            | 100 ± 3 | 100 ± 3 | 3.85 | (AlF) (n = 8) | 100 ± 4 | 100 ± 4 | 3.02 |
|                 | [BeF] (n = 12)            |        |        |       | (BeF)         |        |        |
| D203R           | [0.5 nM]                  | 105 ± 29 | 89 ± 24 | 8.55 | [1.39 μM]     | 1190 ± 212 (n = 2) | 3024 ± 788 (n = 2) | 7.67 |
| R678D           |                          | No labeling | No labeling |       |              | Not feasible | Not feasible |       |
| D203R/R678D     | [73 ± 6 (n = 3)]          | 211 ± 13 (n = 2) | 11.19 |       |              | 98 ± 5 (n = 2) | 138 ± 11 (n = 2) | 4.26 |
| Q202A           | [70 ± 6 (n = 2)]          | 176 ± 7 (n = 2) | 9.67 |       |              | 89 ± 8 (n = 2) | 61 ± 5 (n = 3) | 2.08 |
| Q202A/D203A     | [281 ± 20 (n = 2)]        | 208 ± 12 (n = 2) | 2.84 |       |              | 678 ± 75 (n = 2) | 148 ± 13 (n = 2) | 0.66 |
| S186E           | [97 ± 6 (n = 2)]          | 46 ± 2 (n = 2) | 1.81 |       |              | 128 ± 7 (n = 2) | 49 ± 3 (n = 3) | 1.14 |
| S186E           | [405 ± 24 (n = 2)]        | 460 ± 32 (n = 2) | 4.34 |       |              | 280 ± 26 (n = 2) | 14.5 ± 1.5 (n = 3) | 0.16 |
| S186P           | [104 ± 6 (n = 2)]         | 36 ± 3 (n = 2) | 1.31 |       |              | 329 ± 20 (n = 2) | 82 ± 4 (n = 2) | 0.76 |
| E439A*          | [177 ± 11 (n = 3)]        | 22 ± 3 (n = 2) | 0.56 |       |              | 631 ± 74 (n = 3) | 32 ± 5 (n = 3) | 0.19 |
| E439S           | [192 ± 16 (n = 2)]        | 18 ± 2 (n = 2) | 0.36 |       |              | 268 ± 20 (n = 3) | 7.3 ± 0.3 (n = 2) | 0.08 |
| S186E/E439S     | [117 ± 7 (n = 2)]         | 17 ± 2 (n = 2) | 0.57 |       |              | 69 ± 5 (n = 2) | 4.3 ± 0.2 (n = 2) | 0.19 |

* Data for mutant E439A have been published previously (17) and are included for comparison.

K_{D(AlF)} ratio < 1), thus explaining the lack of ATP stimulation of E₂P dephosphorylation seen for these mutants (Fig. 3). The for the Ser^{186} and Glu^{439} single substitutions, both an ATP affinity increase in E₂-BeF and an ATP affinity decrease in E₂-AlF, relative to wild type, contributed to make the affinity constant ratio differ markedly from that of the wild type. For the swap mutant the situation was different, because a marked 25-fold increase of the ATP affinity in E₂-BeF constituted the major reason for the higher ATP affinity in E₂-BeF compared with E₂-AlF. In fact, the ATP affinity of E₂-AlF was slightly higher for S186E/E439S than for the wild type and, notably, 4-fold higher than for the point mutants S186E and E439S, thus showing gain of a wild type-like function in the swap mutant with respect to ATP binding to E₂-AlF, i.e. the E₂-P phosphoryl transition state with bound ATP was more stable in the swap mutant than in the point mutants. However, due to the very high affinity of the E₂-BeF state for ATP the ratio between the K_{D(AlF)} values of E₂-BeF and E₂-AlF in S186E/E439S was still only a fraction of that of the wild type (Table 3). For ATP binding to E₂-BeF, no gain of a wild type-like function was obtained with S186E/E439S relative to the point mutants, because the ATP affinity was increased even more in S186E/E439S (25-fold) than in S186E (7-fold) and E439S (14-fold), thus showing that the ground state with bound ATP was more stable in the swap mutant than in the point mutants.

The ATP affinity was unaffected by the Q202A mutation in the E₂-AlF state and only ~2-fold increased relative to wild type in the E₂-BeF state, which is in accordance with the wild type-like ATP modulation of dephosphorylation in this mutant (Fig. 3). The double mutation Q202A/D203A lowered ATP affinity as much as 7-fold in E₂-AlF but only 1.5-fold in E₂-BeF, thus explaining the reduced sensitivity of the dephosphorylation rate to ATP modulation in this mutant.

To confirm the gain of wild type-like function observed for the D203R/R678D swap mutant, in comparison with the corresponding point mutants, we also carried out TNP-8N₃-ATP photolabeling experiments with wild type and mutants D203R, R678D, and D203R/R678D stabilized in the E₂-P phosphoryl transition state-like conformation by use of another phosphoryl transition state analog, orthovanadate (17, 41, 42). As seen in Fig. 6A, the D203R and R678D point mutations reduced the affinity for orthovanadate 16- and 17-fold, respectively, whereas the D203R/R678D swap mutant displayed 43-fold lower affinity for orthovanadate than the wild type enzyme. Fig. 6B shows the TNP-8N₃-ATP concentration dependence of photolabeling of wild type and mutants inhibited by a saturating concentration (0.5 mM) of orthovanadate. Similar to the situation with R678D in E₂-AlF and E₂-BeF states, the E₂-orthovanadate state of this mutant was poorly labeled with TNP-8N₃-ATP (not shown). In contrast, the E₂-orthovanadate state of the D203R/R678D swap mutant was labeled to the same extent as the wild type, and indeed with an affinity for TNP-8N₃-ATP almost identical to that of the wild type (Fig. 6B). The gain-of-function of the D203R/R678D swap mutant with respect to TNP-8N₃-ATP binding is further supported by the fact that point mutation D203R lowered the affinity for the photolabel ~8-fold relative to the wild type in the E₂-orthovanadate state. A similar picture emerged from the titrations of the competitive inhibition by ATP of TNP-8N₃-ATP photolabeling shown in Fig. 6C. Hence, mutant D203R displayed 48-fold reduced ATP affinity relative to wild type in the E₂-orthovanadate state, contrasting the wild type-like ATP affinity of the D203R/R678D swap mutant.

**DISCUSSION**

During the dephosphorylation of the E₂P state of the Ca^{2+} -ATPase, the conserved Glu^{103} of the 181TGES motif of the A-domain positions the water molecule responsible for aspartyl phosphate hydrolysis, and the optimal geometry for the nucleophilic attack of the aspartyl phosphate by water is reached in the pentacoordinated E₂P phosphoryl transition state (5, 6). ATP seems to accelerate E₂P dephosphorylation by tightening the interactions around the catalytic site in the E₂P phosphoryl transition state, thereby lowering the activation energy of the reaction (17). This modulatory effect of ATP is a consequence of acceleration of the forward dephosphorylation reaction and
is not due to prevention by ATP of reversal of the dephosphorylation, because it is observable in the absence of a significant concentration of Pi, and both by transient kinetic studies and at steady state (28).

We present here the results of investigating the importance of the dephosphorylation reaction and its modulation by ATP, because it is observable in the absence of a significant concentration of Pi, and both by transient kinetic studies and at steady state (28).
ATP. Dissection of the mutational effects on ATP stimulation of E2P dephosphorylation, resolved by studying the nucleotide affinity of analogs of the intermediate states in the dephosphorylation reaction sequence, revealed a clear difference between the functions of the two interaction sites. The point mutations to Asp203 and Arg678 (A-P domains interaction), but importantly not the D203R/R678D swap mutation, were detrimental to ATP binding both in the E2P ground state (mimicked by E2-BeF) and in the E2-P phosphorylation transition state (mimicked by E2-AlF) with consequent disruption of the stimulatory effect of ATP on dephosphorylation. The point mutations to Ser186 and Glu439 (A-N domains interaction), on the other hand, increased the ATP affinity of the E2P ground state and lowered the ATP affinity of the E2-P phosphorylation transition state. Unlike the D203R/R678D swap mutation the S186E/E439S swap mutation was unable to rescue the abolishment of the modulatory effect of ATP, because S186E/E439S increased the affinity of the E2P ground state for ATP even more than the corresponding point mutations.

An important distinction that must be made when interpreting these data is whether the mutational effects occur as a consequence of destabilization of the respective domain contacts, or the individual amino acid side chains play direct roles in interaction with nucleotide.

Importance of Asp203 and Arg678 and Their Ion Bond Interaction in E2P States—A key finding in our study is the remarkable gain of wild type-like function of the D203R/R678D swap mutant with respect to nucleotide affinity in the E2P-like states, relative to the respective point mutants, D203R and R678D. Thus, D203R displayed 12- and 48-fold reduced ATP affinity relative to the wild type in E2-AlF and E2-orthovanadate states (E2-P phosphorylation transition state analogs), respectively, and 30-fold reduced ATP affinity in the E2-BeF state (E2P ground state analog), and R678D was unable to become photolabeled with TNP-8N3-ATP in either of the three E2P-like states, suggesting severely reduced nucleotide affinity. In sharp contrast hereto the D203R/R678D swap mutant displayed wild type-like affinities for TNP-8N3-ATP and ATP in both E2-AlF and E2-orthovanadate states and only slightly reduced affinity for the two nucleotides in the E2-BeF state (Figs. 4–6 and Table 3). The results of the nucleotide binding measurements with D203R, R678D, and D203R/R678D rationalize the finding that the two point mutations abolished the stimulatory effect of ATP on E2P dephosphorylation, whereas the swap mutant retained significant ATP modulation with reasonable apparent ATP affinity for activation (Fig. 3 and Table 2). Hence, the nucleotide binding measurements indicate that the effects of the point mutations on ATP modulation are caused by reduced binding affinity for ATP, and not merely by interference with the transmission to the catalytic site of the conformational changes elicited by ATP binding. In the crystal structure of the E2-AlF-AMP-PCP state (Fig. 1, upper left panel) the side chain of Arg678 seems poised for ion bond interaction with the γ-phosphate of the nucleotide, the guanidinium group of Arg678 pointing toward the γ-phosphate at a distance of 4.2 Å, and Asp203 contributes to position Arg678. Hence, at least part of the effects of the Arg678 and Asp203 point mutations on the affinity for ATP could arise from disruption of such binding interaction. However, because of the gain of function seen with the swap mutant, the Asp203Arg678 ionic interaction appears more important for the ATP affinity than the exact positioning of the arginine side chain. The Asp203Arg678 interaction might contribute to stabilization of the ATP binding pocket as a whole, including tightening of other ATP-protein interactions than that between the γ-phosphate and Arg678, which is supported also by the finding that TNP-8N3-ATP binding is profoundly affected in the Asp203 and Arg678 point mutants, but not in the swap mutant, although TNP-nucleotides are positioned in the binding pocket rather differently from nucleotides without the TNP moiety, as seen in various crystal structures (43) (see Fig. 1, and further discussion below).

Role of Glu202 in E2P Dephosphorylation—The Q202A mutant displayed the lowest basal rate of E2P dephosphorylation of all the mutants studied in the present work. The stimulation by ATP of the dephosphorylation reaction, however, proceeded with wild type-like K0.5(ATP) and rate enhancement factor (k1/k0) for Q202A (Table 2). Accordingly, Q202A also behaved in a wild type-like manner with respect to the affinities for TNP-8N3-ATP and ATP in E2-BeF and E2-AlF states (Table 3), suggesting that the very slow dephosphorylation rate of Q202A is unrelated to the actions of modulatory ATP. In the various published crystal structures of E2P-like states (e.g. Refs. 6, 8, and 9), the Gln202 side chain amide is within hydrogen bonding distance (~3 Å) of the backbone carbonyl of Gly626, which is a critical residue at the catalytic site (15, 44). Because of the A-domain rearrangements during the Ca2+-transport cycle, Gln202 and Gly626 are much further apart in E2 (10 Å) than in the E2P-like states and more than 30 Å apart in Ca2E1, excluding the possibility of Gln202-Gly626 interaction in the latter states. The close contact of Gln202 with the Gly626 backbone carbonyl in the E2P-like states appears to explain the marked effect by the Q202A mutation on the E2P dephosphorylation rate.

Importance of Ser186 and Glu439 and Their Hydrogen Bond Interaction in E2P States—With the exception of S186E, the point mutations to Ser186 and Glu439 substantially increased the basal E2P dephosphorylation rate determined in the absence of ATP, and the swap mutation S186E/E439S almost restored the rate to a wild type-like level. These findings are in accordance with those of Liu et al. (30), who from analysis of the E2-BeF crystal structures (9, 45) pointed to a role for the hydrogen bond between Ser186 and Glu439 in stabilization of the interaction between domains A, N, and P in the E2P ground state. The pKa of the introduced glutamate in S186E was estimated to be extremely high, consistent with protonation and hydrogen bond formation between this glutamate and Glu439, thereby explaining the minimal effect of the mutation S186E (30). Structural modeling suggested that in the swap mutant, serine and glutamate may still be able to interact by hydrogen bonding in the E2-BeF state, thus explaining the gain of function seen for S186E/E439S with respect to attaining a wild type-like basal E2P dephosphorylation rate. The present study, furthermore, encompasses mutant S186P, which was found to display the highest basal E2P dephosphorylation rate of the Ser186 mutants, likely a consequence of the influence of the
unique properties of proline on the backbone conformation of the 181-TGES loop.

Also in accordance with Liu et al. (30) we found that S186A, S186E, and E439S, as well as the S186E/E439S swap mutation, abolished the stimulatory effect of ATP on E2P dephosphorylation (Fig. 3 and Table 2). The results of our direct measurements of nucleotide affinity (Fig. 5 and Table 3) now provide an explanation of this loss of ATP modulation. The mutants with alterations to Ser186 or Glu439 displayed a markedly reduced ratio between the $K_{D(\text{ATP})}$ values of E2-BeF and E2-AIF, relative to the ratio of 3 seen for the wild type (Table 3). In several of these mutants, the ATP affinity was actually higher in E2-BeF than in E2-AIF. Such a destabilization of the ATP-bound form of the E2-P phosphoryl transition state relative to that of the E2P ground state is indeed expected to lead to the observed abolishment of the enhancing effect of ATP on the dephosphorylation rate. The swap mutation S186E/E439S was not different from the individual point mutants with respect to the lack of ATP modulation (Fig. 3), which was mirrored in its reduced ratio between the $K_{D(\text{ATP})}$ values of E2-BeF and E2-AIF (Table 3). This should, however, not mislead one to conclude that there was no gain of function for the swap mutant relative to the point mutants in relation to the binding of modulatory ATP in the E2-P phosphoryl transition state. Although the individual Ser186 and Glu439 point mutants displayed reduced affinity of E2-AIF for ATP (higher $K_{D(\text{ATP})}$ value), relative to the wild type, the swap mutant showed a slightly enhanced affinity of E2-AIF for ATP relative to the wild type and indeed a 4-fold higher affinity relative to the S186E and E439S point mutants, thus clearly indicating that the hydrogen bond between Ser186 and Glu439 stabilizes the E2-P phosphoryl transition state in the form with bound ATP. For the E2P ground state analog, E2-BeF, the situation is quite different. Here we found an enhanced ATP affinity for the individual Ser186 and Glu439 point mutants (lower $K_{D(\text{ATP})}$ value), relative to that of the wild type, and the simultaneous presence of S186E and E439S in the swap mutant did not lead to any compensation; on the contrary, the ATP affinity was even more enhanced in the swap mutant, thus leading to the reduced ratio between the $K_{D(\text{ATP})}$ values of E2-BeF and E2-AIF, relative to that of the wild type, even though the $K_{D(\text{ATP})}$ actually was reduced also in E2-AIF of the swap mutant (Table 3).

Hence, the S186E and E439S mutations work independently in an additive way to generate a more stable nucleotide-bond E2-BeF state. These findings indicate that the ATP binding mode differs considerably between E2-AIF and E2-BeF, which is in line with structural data for TNP-AMP bound forms (43). Hence, in E2-BeF crystalized with TNP-AMP the TNP moiety of the nucleotide is slotted into a groove formed between the A- and N-domains, with the Ser186 and Glu439 side chains within interaction distance (3–4 Å) of TNP, whereas in the corresponding structure of E2-AIF, TNP-AMP is displaced relative to its position in the E2-BeF state, with the TNP moiety now 5–7 Å distant from the Ser186 and Glu439 side chains (Fig. 1, right panels). No structures of the E2-BeF state have so far been solved with bound ATP, AMP-PCP, or any other nucleotide devoid of the TNP moiety, but a comparison of the TNP-AMP bound structures with the structure of E2-AIF with bound AMP-PCP (Fig. 1, upper left panel) shows that the TNP moiety of TNP-AMP is found roughly at the location where the adenine of AMP-PCP binds, near Phe487. It is, thus, conceivable that the marked increase of ATP affinity in the E2P ground state of the Ser186 and Glu439 mutants is related to altered interaction of Ser186 and Glu439 directly with the adenine part of ATP. In the wild type E2P ground state, the binding of ATP might cause destabilization by disrupting the hydrogen bond between Ser186 and Glu439. Such a destabilization will not occur if the Ser186-Glu439 bond has already been disrupted by mutation. Furthermore, both single and swap mutations might lead to favorable interaction of the substituents with the adenine ring, thereby enhancing the ATP affinity.

**Conclusion**—The Asp203-Arg678 (A-P domains) and the Ser186-Glu439 (A-N domains) interactions are both critical for the binding of the modulatory ATP that enhances dephosphorylation of Ca$^{2+}$-ATPase. The Asp203-Arg678 ionic bond stabilizes the nucleotide binding pocket in the E2-P phosphoryl transition state as well as in the E2P ground state, and the stabilizing effect on the E2-P phosphoryl transition state contributes to the accelerating effect of ATP on the dephosphorylation. The Ser186-Glu439 hydrogen bond likewise stabilizes the nucleotide binding pocket in the E2-P phosphoryl transition state, whereas in the E2P ground state, ATP binding and the Ser186-Glu439 hydrogen bond are mutually exclusive. Both of these effects of the Ser186-Glu439 bond contribute to the mechanism underlying the accelerating effect of ATP on the dephosphorylation.

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