ATP-binding Modes and Functionally Important Interdomain Bonds of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase Revealed by Mutation of Glycine 438, Glutamate 439, and Arginine 678*§

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ATP binds to sarcoplasmic reticulum Ca\(^{2+}\)-ATPase both in a phosphorylating (catalytic) mode and in a nonphosphorylating (modulatory) mode. The latter leading to acceleration of phosphoenzyme turnover (Ca\(_2\)E\(_1\)P → E\(_2\)P and E\(_2\)P → E\(_3\)P reactions) and Ca\(^{2+}\) binding (E\(_3\) → Ca\(_2\)E\(_3\)). In some of the Ca\(^{2+}\)-ATPase crystal structures, Arg\(^{678}\) and Glu\(^{439}\) seem to be involved in the binding of nucleotide or an associated Mg\(^{2+}\) ion. We have replaced Arg\(^{678}\), Glu\(^{439}\), and Glu\(^{438}\) with alanine to examine their importance for the enzyme cycle and the modulatory effects of ATP and MgATP. The results point to the key role of Arg\(^{678}\) in nucleotide binding and to the importance of interdomain bonds Glu\(^{439}\)–Ser\(^{186}\) and Arg\(^{678}\)–Asp\(^{203}\) in stabilizing the E\(_3\)P and E\(_2\)P intermediates, respectively. Mutation of Arg\(^{678}\) had conspicuous effects on ATP/MgATP binding to the E\(_1\) form and ADP binding to Ca\(_2\)E\(_{1}\)P, as well as ATP/MgATP binding in modulatory modes to E\(_2\)P and E\(_3\)P, whereas the effects on ATP/MgATP acceleration of the Ca\(_2\)E\(_3\)P → E\(_3\)P transition were small, suggesting that the nucleotide that accelerates Ca\(_2\)E\(_3\)P → E\(_3\)P binds differently from that modulating the E\(_2\)P → E\(_2\) and E\(_2\) → Ca\(_2\)E\(_3\)1 reactions. Mutation of Glu\(^{439}\) hardly affected nucleotide binding to E\(_1\), Ca\(_2\)E\(_3\)P, and E\(_2\), but it led to disruption of the modulatory effect of ATP on E\(_2\)P → E\(_3\) and acceleration of the latter reaction, indicating that ATP normally modulates E\(_2\)P → E\(_2\) by interfering with the interaction between Glu\(^{439}\) and Ser\(^{186}\). Gly\(^{438}\) seems to be important for this interaction as well as for nucleotide binding, probably because of its role in formation of the helix containing Glu\(^{439}\) and Thr\(^{441}\).

The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (1) is a membrane-bound energy transducer ("nanomotor") that couples ATP hydrolysis with Ca\(^{2+}\) translocation against a concentration gradient by means of a reaction cycle (Scheme 1) in which the ATPase enzyme is transiently phosphorylated at a conserved aspartic acid residue and undergoes major conformational transitions between Ca\(_2\)E\(_{1}\)/Ca\(_2\)E\(_{1}\)P and E\(_2\)/E\(_3\)P forms (2, 3). In recent years, several high resolution crystal structures of the Ca\(^{2+}\)-ATPase, each thought to represent a particular intermediate state in the pump cycle, have been determined (4–11). The Ca\(^{2+}\)-ATPase consists of a membrane-spanning domain of 10 mostly helical segments and a large cytoplasmic headpiece, comprising three distinct domains, named N (nucleotide binding), P (phosphorylation), and A (actuator) (4). In the Ca\(_2\)E\(_{1}\) and Ca\(_2\)E\(_{1}\)P conformations, the catalytic ATP-binding site is made up by residues in the N- and P-domains, and during the Ca\(_2\)E\(_{1}\)P → E\(_2\)P transition the departing ADP molecule is replaced by the TGES loop of the A-domain, which subsequently assists in catalysis of E\(_2\)P dephosphorylation (8, 9, 12). In addition to being the phosphorylating substrate in the E\(_1\) state, ATP exerts modulatory effects on various steps of the Ca\(^{2+}\)-ATPase cycle (boxed ATP in Scheme 1). Hence, the Ca\(_2\)E\(_{1}\)P → E\(_2\)P, E\(_3\)P → E\(_2\), and E\(_2\) → Ca\(_2\)E\(_{1}\) transitions are all accelerated by the binding of ATP or MgATP in a nonphosphorylating mode (13–23). The apparent affinity for the nucleotide is generally lower when it binds in the nonphosphorylating modulatory mode as compared with the phosphorylating mode, but it varies depending on the step being modulated. A subject of much controversy is the question whether the phosphorylating and modulatory ATP molecules are at the same locus, exhibiting variable affinity during the transport cycle depending on conformational state, or whether a separate low affinity allosteric site exists on the same Ca\(^{2+}\)-ATPase polypeptide chain (18, 22, 24–28).

In previous site-directed mutagenesis studies aimed at locating the amino acid side chains involved in ATP binding, we have examined the functional roles of several conserved residues of the N- and P-domains (29–34) and found that the N-domain residues Thr\(^{441}\), Glu\(^{442}\), Phe\(^{487}\), Arg\(^{489}\), Lys\(^{515}\), Arg\(^{560}\), and Leu\(^{562}\), as well as the P-domain residues Asp\(^{551}\) (the phosphorylated aspartic acid), Lys\(^{532}\), Thr\(^{353}\), Gly\(^{526}\), Lys\(^{528}\), and Asp\(^{703}\), are important for the binding of ATP together with Mg\(^{2+}\) at the catalytic site. These results are consistent with the more recently published crystal structures of the Ca\(^{2+}\)-ATPase with bound nucleotide. On the basis of the crystal structures in con-
In this study, we have addressed the functional roles of P-domain residue Arg$^{678}$ and N-domain residues Gly$^{438}$ and Glu$^{439}$.

Arg$^{678}$ and Glu$^{439}$ have both been suggested to be involved in the binding of nucleotide. The first indication associating Arg$^{678}$ with nucleotide binding came from studies showing that chemical cross-linking of the side chains of Arg$^{678}$ and Lys$^{992}$ by glutaraldehyde is prevented by ATP or ADP (36, 37). Furthermore, in some of the Ca$^{2+}$-ATPase crystal structures with bound AMPPCP$^3$ (nonhydrolyzable ATP analog) or ADP, the side chain of Arg$^{678}$ is sufficiently close to the nucleotide for bond formation with the ribose part (Fig. 1, upper and lower left panels, and Table 1) or even with the adenine ring (Fig. 1, lower right panel, and Table 1). The involvement of Arg$^{678}$ in nucleotide binding and the binding mode may vary depending on conformational state. A role for Glu$^{439}$ in nucleotide binding has been inferred from studies showing that mutation of Glu$^{439}$ interferes with the protection by AMPPCP against proteolytic cleavage (35). On the basis of nucleotide-dependent Fe$^{2+}$-catalyzed oxidative cleavage of the peptide chain near Glu$^{439}$ (38, 39) or its homolog in Na$^+$,K$^+$-ATPase, Asp$^{442}$ (40, 41), these residues have been suggested to be part of the catalytic site and to be in contact with the nucleotide via the Mg$^{2+}$ ion at site 2. Although an intervening water molecule seems to be needed to mediate interaction between this Mg$^{2+}$ ion and Glu$^{439}$ in the Ca$_2$E$_1$ crystal structures (the distance between Mg$^{2+}$ and Glu$^{439}$ being 4.9–5.3 Å; see Table 1 and Fig. 1, upper left panel), the crystal structure of the E$_2$ form with bound AMPPCP suggests a closer interaction between the Mg$^{2+}$ at site 2 and Glu$^{439}$ (cf. Table 1 and Fig. 1, lower left panel), which might indicate that Glu$^{439}$ is of importance for the binding of MgATP in the modulatory mode to the E$_2$ form (10). In Ref. 10 it was also speculated that the Mg$^{2+}$ ion bound at site 2 by Glu$^{439}$ might play an important role following phosphorylation, first by facilitating ADP dissociation, through attracting the Mg$^{2+}$ and thereby the phosphoryl groups of ADP away from their binding in a catalytic configuration in Ca$_2$E$_1$P and acting as a flexible helping arm, and second through binding the modulatory ATP promoting the Ca$_2$E$_1$P $\rightarrow$ E$_2$P transition (cf. Scheme 1). To clarify the roles of Arg$^{678}$ and Glu$^{439}$, we have determined the functional properties of Ca$^{2+}$-ATPase mutants in which Arg$^{678}$ or Glu$^{439}$ is replaced by alanine. The

The abbreviations used are: AMPPCP, adenosine 5'-triphosphate; CPA, cyclopiazonic acid; EPPS, N-2-hydroxyethylpiperezine-N'-3-propanesulfonic acid; MES, 2-[N-morpholino]ethanesulfonic acid; MOPS, 3-[N-morpholino]propanesulfonic acid; Tg, thapsigargin; TNP-8N3-ATP, 2',3'-O-(2,4,6-trinitrophenyl)-8-azidoadenosine 5'-triphosphate; PDB, Protein Data Bank.
function of the adjacent glycine residue Gly438, which appears too far away for direct interaction with the nucleotide or its associated Mg$^{2+}$ (cf. Table 1 and Fig. 1), but is well conserved, was also examined by replacement with alanine. All the partial reactions of the Ca$^{2+}$-ATPase reaction cycle indicated in Scheme 1 and their ATP dependences were analyzed in the mutants to address the effects of the mutations on the conformational changes of the enzyme as well as the interaction with ATP in catalytic as well as modulatory modes.

**EXPERIMENTAL PROCEDURES**

Site-directed mutagenesis of cDNA encoding the rabbit fast twitch muscle Ca$^{2+}$-ATPase (SERCA1a isoform) inserted into the pMT2 vector (42) was carried out using the QuikChange site-directed mutagenesis kit, and the mutant cDNA was sequenced throughout. To express wild type or mutant cDNA, COS-1 cells were transfected using the calcium phosphate precipitation method (43). Microsomal vesicles containing either expressed wild type or mutant Ca$^{2+}$-ATPase were isolated by differential centrifugation (44). The concentration of expressed Ca$^{2+}$-ATPase was determined by an enzyme-linked immunosorbent assay (45). The ATPase activity was determined by following the liberation of P$_i$ (46). Measurements of phosphorylation and dephosphorylation at 0°C were carried out by manual mixing (46, 47). Transient state kinetics at 25°C was analyzed using the Bio-Logic quench-flow module QFM-5 (Bio-Logic Science Instruments, Claix, France) with mixing protocols as described previously (48). The determination of the phosphorylation level by acid quenching followed by acid SDS-PAGE and quantification of the radioactivity associated with the Ca$^{2+}$-ATPase band as well as the photolabeling with [γ-32P]TNP-8N3-ATP, the inhibition of photolabeling by ATP/MgATP, and the quantification of the label bound specifically to the Ca$^{2+}$-ATPase were carried out using the previously established procedures (29–34, 49).

The experiments were generally conducted at least twice, and average values are shown in the figures. The data were analyzed by nonlinear regression using the SigmaPlot program (SPSS, Inc.) or by computation using the SimZyme program (48). Monoexponential functions were fitted to the phosphorylation and dephosphorylation time courses, except when the phosphorylation time course exhibited an overshoot. In the latter case the SimZyme program was used as described (48). A hyperbolic function plus a constant or linear component was fitted to the [γ-32P]TNP-8N3-ATP labeling data, and the “true” dissociation constant, $K_D$, for ATP/MgATP binding was calculated from the measured $K_{0.5}$ values using the validated equation for competitive inhibition (29). For analysis of the modulatory effect of nucleotide (“N”) on the rates of the partial reaction steps, the nucleotide concentration dependence of the rate constant was fitted according to Equation 1.

$$k_{obs} = k_0 + (k_{max} - k_0)[N]/(K_{0.5} + [N])$$  \hspace{1cm} (Eq. 1)

Here, $k_{obs}$ is the rate constant observed at the indicated nucleotide concentration; $k_0$ is the rate constant in the absence of nucleotide (“basic rate”), and $k_{max}$ is the extrapolated value of the rate constant corresponding to infinite nucleotide concentration. The enhancement factor $k_{max}/k_0$ describes the extent of the modulatory effect, and the $K_{0.5}$ value describes the affinity for the modulatory nucleotide.

**RESULTS**

**Expression and Ca$^{2+}$ Affinity**—The expression levels of the three mutants, G438A, E439A, and R678A, were similar to that obtained with wild type Ca$^{2+}$-ATPase, as evaluated by immunoreactivity in a specific enzyme-linked immunosorbent assay. Like the wild type, all the mutants were able to form a phosphenzyme in the presence of [γ-32P]ATP and Ca$^{2+}$, and the mutants showed an apparent Ca$^{2+}$ affinity for activation...
FIGURE 2. Rapid kinetics of phosphorylation of Ca$_2$E$_1$ with [γ-32P]ATP. Using a QFM-5 quench-flow module at 25 °C, microsomes suspended in 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl$_2$, and 100 mM CaCl$_2$ were mixed with an equal amount of the same medium containing [γ-32P]ATP to produce a final concentration of 5 μM, followed by acid quenching at the indicated time intervals. The lines show the best fits to the data obtained using the SimZyme program (for wild type, cf. Ref. 48) or the SigmaPlot program with a monoexponential rise to maximum function, giving the indicated rate constants for Ca$_2$E$_1$ → Ca$_2$E$_2$. For further explanation of the fitting procedure, see supplemental Fig. S7.

FIGURE 3. Binding of ATP determined by inhibition of TNP-8N$_3$-ATP photolabeling. Photolabeling was performed in the presence (closed symbols) or absence (open symbols) of Mg$^{2+}$ and without thapsigargin (TG) or with thapsigargin. The photolabeling medium contained 25 mM EPPS/tetramethylammonium hydroxide (pH 8.5), 20% (v/v) glycerol, a [γ-32P]TNP-8N$_3$-ATP concentration of 3 × $K_{0.5}$ (mutants G438A and E439A and wild type) or 0.2 × $K_{0.5}$ (mutant R678A, because of its low affinity), and the indicated concentrations of ATP with either 1 mM MgCl$_2$ and 0.5 mM EGTA (presence of Mg$^{2+}$) or 2 mM EDTA (absence of Mg$^{2+}$). The lines represent the best fits to the data of a hyperbolic function as described under “Experimental Procedures.” Table 2 lists the $K_{0.5}$ values derived from the data. For comparison, the panels include the best fits to the data for the expressed wild type obtained in the presence and absence of Mg$^{2+}$ (short dashed and long dashed lines, respectively) (29, 30).

TABLE 2

|                  | TNP-8N$_3$-ATP$^a$ | ATP$^a$     |
|------------------|--------------------|-------------|
|                  | Mg$^{2+}$ | EDTA       | Mg$^{2+}$ | EDTA       |
| WT               | 0.79      | 0.19       | 0.51      | 21         |
| WT + TG          | 1.2       | 0.15       | 130       | 20         |
| G438A            | 0.30      | 0.14       | 2.1       | 99         |
| G438A + TG       | 0.84      | 0.14       | 290       | 68         |
| E439A            | 0.28      | 0.083      | 1.2       | 9.5        |
| E439A + TG       | 1.3       | 0.29       | 140       | 50         |
| R678A            | 40        | 12         | 11        | 950        |
| R678A + TG       | 19        | No labeling| 310       | Not feasible|

$^a$ $K_{0.5}$ values are from the data in supplemental Fig. S2.

$^b$ $K_{0.5}$ values were calculated from the measured $K_{0.5}$ values (Fig. 3) under the assumption of competitive inhibition as described previously (29).

similar to that of the wild type or slightly higher (supplemental Fig. S1).

Phosphorylation Rate and Affinity for MgATP and ATP—The physiological substrate for phosphorylation of the enzyme in the Ca$_2$E$_1$ form is MgATP (cf. Scheme 1). Fig. 2 shows the time course of phosphorylation with 5 μM [γ-32P]MgATP of enzyme pre-equilibrated with Ca$^{2+}$. The measured phosphorylation rate depends on the rate constant for transfer of the γ-phosphoryl group of the bound MgATP to Asp$^{351}$ as well as the saturation of the catalytic site with MgATP. Because the MgATP concentration used is subsaturating in the wild type, any change of MgATP affinity induced by the mutations should be revealed in this experiment. Compared with the wild type, the phosphorylation rate was significantly reduced for G438A (to 80%) and R678A (to 50%), whereas it was wild type-like in E439A. The nucleotide binding properties of the mutants were examined in more detail by using a previously described and validated assay in which the ATPase is photolabeled with [γ-32P]TNP8N$_3$-ATP (29–34). The [γ-32P]TNP8N$_3$-ATP concentration dependence of the photolabeling and the competitive inhibition with ATP/MgATP were studied. This assay was carried out with the enzyme being either in a Ca$^{2+}$-free E$_2$ state at pH 8.5 (50), the absence of Ca$^{2+}$ ensuring that the ATP is not being utilized, or in a thapsigargin-bound E$_2$ state, with and without Mg$^{2+}$ present. Besides allowing studies of nucleotide binding to an E$_2$ form, the thapsigargin-bound state is pertinent as the E$_2$ crystal structures have either thapsigargin or another inhibitor (CPA in Ref. 11) bound. Fig. 3 depicts the ATP and MgATP concentration dependences of inhibition of photolabeling, and Table 2 summarizes the resulting $K_{0.5}$ values for ATP/MgATP binding as well as the $K_{0.5}$ values obtained from the TNP-8N$_3$-ATP/TNP-8N$_3$-MgATP concentration dependence of photolabeling (see the corresponding data in supplemental Fig. S2). As documented previously (29, 30), Mg$^{2+}$ and thapsigargin have only moderate effects on the affinity of the wild type for the TNP-8N$_3$-ATP photolabel. The affinity of the wild type for MgATP is, on the other hand, about 40-fold higher than the affinity for metal-free ATP, and the presence of thapsigargin reduces the affinity for MgATP about 250-fold. We did not find any effect of thapsigargin on the affinity for ATP in the absence of Mg$^{2+}$, thus suggesting that ATP binds differently from MgATP as described previously (32). As is shown in Fig. 3 and Table 2, mutant G438A showed reduced binding affinity for ATP as well as MgATP, relative to the wild type (4–5-fold in the absence of thapsigargin and 2–3-fold in its presence). There
was no significant effect of mutation G438A on the affinity for the TNP-8N₃-ATP photolabel under any of the conditions tested (Table 2). R678A, on the other hand, had significant effects on the binding of the photolabel as well as the binding of ATP, in the presence or absence of Mg²⁺ or thapsigargin. A very large reduction of the nucleotide affinity (20–50-fold) was seen with R678A in the absence of thapsigargin, whereas the effect was more modest in its presence (2.4-fold reduced affinity for MgATP, the affinity for metal-free ATP not being measurable because of poor photolabeling; see Fig. 3 and Table 2).

The effects of E439A on nucleotide binding were generally much smaller than the effects of the other two mutations. Only a 2-fold reduction of MgATP affinity, relative to wild type, was seen with E439A in the absence of thapsigargin, and in its presence there was no significant difference from wild type. In the absence of thapsigargin, E439A increased the affinity for the photolabel or metal-free ATP about 2-fold. In the presence of thapsigargin, the affinity for metal-free ATP was reduced 2.5-fold. The difference between the three mutants with respect to the magnitude of the mutational effect on MgATP affinity in the E₁ form (absence of thapsigargin) matches well the difference between the mutational effects on the phosphorylation rate described above (R678A > G438A > E439A = wild type), indicating that the major reason for the reduced phosphorylation rate in R678A and G438A is the reduced MgATP affinity.

Reaction of the Phosphoenzyme with ADP—The phosphoenzyme intermediate formed immediately by phosphorylation with ATP is the Ca₂E₁P form, which subsequently is converted into E₂P in association with the translocation of Ca²⁺ (Scheme 1). Ca₂E₁P is ADP-sensitive, i.e. able to donate the phosphoryl group back to ADP forming ATP, whereas E₁P is ADP-insensitive. The reaction of the phosphoenzyme with ADP was examined by addition of 1, 0.1, or 0.02 mM ADP (Fig. 4). It is seen that 1 mM ADP was able to rapidly dephosphorylate more than 90% of the phosphoenzyme in the wild type and all three mutants, thus indicating that Ca₂E₁P is the major phosphoenzyme intermediate accumulated. However, at the lower ADP concentrations the R678A mutant behaved distinctly different from the wild type and the other mutants by showing a significantly reduced reactivity with ADP. Hence, in R678A 92% of the phosphoenzyme remained 2 s after addition of 0.02 mM ADP, which should be compared with 20% for the wild type, 14% for G438A, and 11% for E439A (Fig. 4). This shows that the affinity of the Ca₂E₁P form for ADP is reduced in R678A, thus matching the reduction of ATP/MgATP affinity in this mutant described above. On the other hand, the affinity of Ca₂E₁P for ADP does not appear to be reduced by mutations G438A and E439A.

The Forward Processing of the Ca₂E₁P Phosphoenzyme and Its Modulation by MgATP and ATP—The dephosphorylation of the Ca₂E₁P phosphoenzyme was also examined in the absence of ADP, using a chase with EGTA to terminate phosphorylation (removing Ca²⁺ but not Mg²⁺). Under these conditions Ca₂E₁P is converted into E₂P, which subsequently undergoes hydrolysis, liberating Pᵢ (cf. Scheme 1). Various concentrations of non-radioactive MgATP were added with the chase (0, 1, and 5 mM MgATP shown in supplemental Fig. S3). For each MgATP concentration, a monoeponential decay function was fitted to the time course of phosphoenzyme decay, and the dependence of the rate constant on the MgATP concentration is displayed in Fig. 5. The data could be fitted satisfactorily by the hyperbolic function described under “Experimental Procedures” (see Equation 1), and the extracted parameters are displayed in Table 3 (Ca₂E₁P → E₂P (+ Mg²⁺)).
mode, and as seen in Table 3 the analysis in Fig. 5 showed that MgATP, acting with a $K_{0.5}$ of 1.44 mM, caused a 5-fold increase of the rate of Ca$_2^+$ turnover in the wild type. In E439A, MgATP induced a 14-fold increase of the dephosphorylation rate with a $K_{0.5}$ of 1.45 mM, thus indicating that the modulatory effect of MgATP on the Ca$_2^+$ turnover is fully intact, and even enhanced, in E439A. In G438A and R678A, MgATP enhanced the rate of Ca$_2^+$ turnover 5- and 9-fold, with $K_{0.5}$ values of 1.51 and 3.55 mM, respectively, i.e. a wild type-like apparent affinity for MgATP in G438A and a 2.5-fold reduced affinity in R678A (Table 3).

To examine whether the modulatory effect of metal-free ATP is different from that of MgATP, experiments were conducted in which the phosphoenzyme was chased with an excess of EDTA (removing Ca$_2^+$ as well as Mg$_2^+$) together with various concentrations of metal-free ATP (see the data for 0, 0.2, and 3 mM ATP in supplemental Fig. S5). In this case, the phosphoenzyme decay was not strictly monoeponential, because a small but significant fraction (<30%) decayed much more slowly than the major part. The slow phase may represent phosphoenzyme in which the Mg$_2^+$ bound at site 1 (needed for rapid dephosphorylation of E$_2^P$) dissociated before the dephosphorylation had taken place. It is also possible that because the Mg$_2^+$ concentration present during the phosphorylation was reduced to 1 mM in these experiments, to allow the EDTA to remove Mg$_2^+$ efficiently, a significant fraction of the enzyme was phosphorylated by CaATP instead of MgATP. This would result in a biphasic phosphoenzyme decay, because the turnover of phosphoenzyme formed from CaATP (presumably having Mg$_2^+$ bound at site 1 occupied by Ca$^{2+}$) is much slower than that of phosphoenzyme formed from MgATP (51). The two phases were well resolved, thus allowing extraction of the rate constants, and Fig. 6 shows the ATP concentration dependence of the rate constant corresponding to the fast phase (the majority of the decay). Hence, this rate constant is supposed to reflect the rate-limiting part of the Ca$_2^+$-dependent E$_2^P$ → E$_2$ transition sequence, i.e. the Ca$_2^+$-dependent E$_2^P$ → E$_2$ transition, of enzyme that has Mg$_2^+$ bound at site 1 and is being modulated by the added metal-free ATP. It is seen in Fig. 6 and Table 3 that metal-free ATP enhanced the Ca$_2^+$-dependent E$_2^P$ → E$_2$ transition with an apparent affinity that was quite similar in the wild type and E439A ($K_{0.5}$ 0.35 and 0.31 mM, respectively) and less than 2-fold reduced in G438 and R678A ($K_{0.5}$ 0.58 and 0.53 mM, respectively). The apparent affinities for metal-free ATP were 3–7-fold higher than for MgATP. Again the basic dephosphorylation rate without added nucleotide ($k_0$) was found 4–6-fold reduced in the mutants relative to wild type, and the enhancement induced by ATP was most pronounced in E439A, the enhancement factor being as high as 11-fold for E439A (compare with 4-fold for the wild type). The $k_0$ and the $k_{max}$ values were generally higher than those determined in the presence of free Mg$_2^+$, 2–3-fold for the wild type and R678A and somewhat more for G438A, whereas E439 showed only a slight difference. This could be a consequence of removing the Mg$^{2+}$ ion at site 2, which is supposed to be more loosely bound than the Mg$^{2+}$ at site 1.

The Dephosphorylation of the E$_2$P Phosphoenzyme and Its Modulation by ATP—All the mutants could be phosphorylated in the reverse direction (“backdoor”) by $^{32P}$, under favorable conditions (absence of Ca$^{2+}$, acid pH, presence of dimethyl sulfoxide, and absence of K$^+$). To examine the modulatory

### Table 3: Apparent affinities and kinetic parameters for the modulatory effects of ATP/MgATP on the partial reaction steps

| ATP/MgATP Effect | Ca$_2^+$E$_2^P$ → E$_2P^{2-}$ (+Mg$^{2+}$) | Ca$_2^+$E$_2^P$ → E$_2P$ (−Mg$^{2+}$) | E$_2$P → E$_2$ (−Mg$^{2+}$) | E$_2$ → Ca$_2^+$E$_2$P$^{2-}$ (+Mg$^{2+}$) |
|-----------------|----------------------------------|----------------------------------|----------------------------|----------------------------------|
| $K_{0.5}$ (mM)  | $k_0$ (s$^{-1}$) | $k_{max}$ (s$^{-1}$) | $K_{0.5}$ (mM) | $k_0$ (s$^{-1}$) | $k_{max}$ (s$^{-1}$) | $K_{0.5}$ (mM) | $k_0$ (s$^{-1}$) | $k_{max}$ (s$^{-1}$) | $K_{0.5}$ (mM) | $k_0$ (s$^{-1}$) | $k_{max}$ (s$^{-1}$) |
| WT              | 1.44 | 0.12 | 0.56 | 0.35 | 0.30 | 1.14 | 19 | 1.2 | 3.5 | 45 | 1.3 | 25 |
| G438A           | 1.51 | 0.02 | 0.10 | 0.54 | 0.07 | 0.66 | 35 | 6.3 | 12.6 | 37 | 2.1 | 10 |
| E439A           | 1.45 | 0.03 | 0.44 | 0.31 | 0.05 | 0.56 | ND | 13.4 | ND | 25 | 1.2 | 10 |
| R678A           | 3.55 | 0.02 | 0.19 | 0.53 | 0.06 | 0.31 | ND | 0.63 | ND | ND | 4.4 | ND |

$a$ Data are from Fig. 5 (MgATP effect).

$b$ Data are from Fig. 6 (ATP effect).

$c$ Data are from Fig. 7 (ATP effect).

$d$ Data are from Fig. 9 (MgATP effect).

$e$ ND indicates not determined because the affinity is too low, see data in figures.
**Gly**<sup>438</sup>, **Glu**<sup>439</sup>, and **Arg**<sup>678</sup> of SR Ca<sup>2+</sup>-ATPase

The effect of metal-free ATP on the $E_2P \rightarrow E_3$ step, the dephosphorylation of the $E_2P$ phosphoenzyme intermediate formed backdoor from $^{32}$P, was examined following dilution of the phosphorylated sample in a chase medium containing excess of EDTA together with various concentrations of ATP (see supplemental Fig. S6). The ATP dependence of the rate constant for $E_2P \rightarrow E_3$ derived from these data is shown in Fig. 7. When possible, the data were analyzed according to Equation 1 (see “Experimental Procedures”), and the results of the analysis are indicated in Fig. 7 and Table 3. The basic rate observed in the absence of ATP ($k_0$) was found markedly higher in G438A (5-fold) and E439A (11-fold), whereas it was 2-fold reduced in R678A, compared with the wild type rate. ATP, acting with a $K_{0.5}$ of 19 $\mu$M, enhanced the rate of $E_2P$ dephosphorylation 3-fold in the wild type, which is in accordance with results obtained with sarcoplastic reticulum Ca<sup>2+</sup>-ATPase isolated from rabbit skeletal muscle (16, 22). It is of note that the apparent affinity of $E_2P$ of the wild type enzyme for metal-free ATP is more than 10-fold higher than the apparent affinity for modulation of Ca<sub>2</sub>_E<sub>1</sub>P $\rightarrow E_3P$ discussed above (cf. Table 3). It has been demonstrated previously that only metal-free ATP, and not MgATP, binds to $E_2P$ with reasonable affinity (22, 52). The latter observation was confirmed in the present study, as we found the rate of dephosphorylation of $E_2P$ insensitive to ATP added in millimolar concentration in the presence of excess Mg<sup>2+</sup> (data not shown). Fig. 7 and Table 3 indicate that the modulatory effect of metal-free ATP on the dephosphorylation of $E_2P$ was conserved in mutant G438A, albeit with a 2-fold reduction of the apparent affinity relative to wild type. By contrast, E439A showed no enhancement of the dephosphorylation rate upon addition of ATP, neither in the absence (Fig. 7) nor in the presence (not shown) of Mg<sup>2+</sup>. In fact, in this mutant the dephosphorylation rate was slightly reduced by ATP in the millimolar concentration range. In R678A, there was a slight, hardly significant enhancement of the dephosphorylation rate in the millimolar ATP concentration range, but there was no effect of ATP in the concentration range where the dephosphorylation of the wild type and G438A was accelerated (Fig. 7). Hence, E439A as well as R678A interfered profoundly with the ATP modulation of $E_2P$ dephosphorylation. A concern was whether the lack of ATP modulation of the dephosphorylation of $E_2P$ in E439A could in some way be a consequence of the high basic rate of dephosphorylation in this mutant (one possibility simply being that the time resolution of the manual mixing technique used for this type of experiment is too low to allow detection of the ATP-induced acceleration of a reaction whose rate is already very high). Therefore, we also examined the ATP modulation of $E_2P$ dephosphorylation in another mutant, P248A, for which we previously had demonstrated a very rapid dephosphorylation of $E_2P$ in the absence of ATP (53). The data obtained with this mutant are also shown in supplemental Fig. S6 and are summarized in Fig. 7, from which it appears that even though the basic rate of dephosphorylation was about as high for P248A as for E439A, ATP induced an easily measurable 3-fold acceleration of the dephosphorylation in P248A with a $K_{0.5}$ of 14 $\mu$M, i.e. very similar to that of the wild type. Supplemental Fig. S6 and Fig. 7 also show results obtained with three other mutants, T441A, F487S, and R560L, which we previously had found defective with respect to ATP binding at the catalytic site in the $E_1$ conformation (29, 32). F487S and R560L displayed conspicuous reductions of the apparent affinity for ATP modulation of $E_2P$ dephosphorylation (>50- and 30-fold, respectively). T441A showed a less pronounced, but significant, reduction of the apparent affinity, similar to that described above for G438A.

**Rate of the Ca<sup>2+</sup> Binding Transition and Its Modulation by MgATP—**Figs. 8 and 9 show the results of rapid mixing experiments carried out to determine the rate of the Ca<sup>2+</sup> binding transition of the phosphoenzyme (i.e. the $E_2 \rightarrow E_1$ conformational change and accompanying Ca<sup>2+</sup> binding leading to Ca<sub>2</sub>_E<sub>1</sub>) and the modulatory effect of MgATP on this transition. The enzyme was pre-equilibrated in the absence of Ca<sup>2+</sup> (presence of EGTA) at acid pH (pH 6.0) to accumulate $E_2$, and the
Ca\textsuperscript{2+}-depleted enzyme was mixed with Ca\textsuperscript{2+} without nucleotide and incubated for the time intervals indicated in the figure. The amount of phosphorylatable Ca\textsubscript{2}E\textsubscript{1}, accumulated during the Ca\textsuperscript{2+} incubation step was determined for each time interval by a further 34-ms incubation with [\gamma\textsuperscript{32P}]MgATP prior to acid quenching. To follow the transition to Ca\textsubscript{2}E\textsubscript{1} in the presence of MgATP, [\gamma\textsuperscript{32P}]MgATP was added together with Ca\textsuperscript{2+} to the Ca\textsuperscript{2+}-depleted enzyme, and the phosphorylation level was determined by acid quenching at the time intervals indicated in the figure. These two protocols have been previously validated and the resulting rate constants shown to reflect the Ca\textsuperscript{2+} binding transition (48, 54). A requirement is that the reaction of MgATP with Ca\textsubscript{2}E\textsubscript{1} is relatively rapid compared with the Ca\textsuperscript{2+} binding transition, and this was the case even with R678A (see supplemental Fig. S7). The results of determining the rate of the Ca\textsuperscript{2+} binding transition in the absence of MgATP and in the presence of 10 or 50 \textmu M MgATP are displayed in Fig. 8, and Fig. 9 shows the analysis of the MgATP dependence according to Equation 1 under "Experimental Procedures"; the results are summarized in Table 3. The basic rate of the Ca\textsuperscript{2+} binding transition observed in the absence of MgATP (k\textsubscript{0}) was 1.6- and 3.4-fold higher in G438A and R678A, respectively, as compared with wild type, whereas it was wild type-like in E439A. In the wild type, MgATP, acting with a K\textsubscript{(0.5)} of 45 \textmu M, enhanced the rate of the Ca\textsuperscript{2+} binding transition 19-fold, which is in accordance with results obtained with sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase isolated from rabbit skeletal muscle using a similar method as described here or measurements of the fluorescence change associated with the Ca\textsuperscript{2+} binding transition (15, 17, 23, 54, 55). In G438A, the K\textsubscript{(0.5)} value was 37 \textmu M, i.e. similar to that of the wild type, and in E439A it was 25 \textmu M, corresponding to a less than 2-fold increase of affinity for the modulatory MgATP. The enhancement factors were somewhat lower than that of the wild type (5- and 9-fold for G438A and E439A, respectively). R678A showed only a very weak MgATP dependence of the Ca\textsuperscript{2+} binding transition, consistent with a strongly reduced affinity of the E\textsubscript{2} form for MgATP. The effect of metal-free ATP was not examined, as the method applied here requires Mg\textsuperscript{2+} to be present to catalyze phosphorylation.

Nucleotide Dependence of the Steady-state Rate of ATP Hydrolysis—The ATP/MgATP modulation of the various partial reaction steps is reflected in the nucleotide dependence of the ATPase activity, which is rather complex in the wild type Ca\textsuperscript{2+}-ATPase, consisting of at least three activation phases (20, 21, 24). The K\textsubscript{(0.5)} values corresponding to these phases depend on the affinities of the various intermediate states for the nucleotide as well as the rate constants of the partial reaction steps. The ATPase activation profiles shown in Fig. 10 were determined at 37 °C (highest possible temperature, to obtain an activity significantly higher than background even at the lowest nucleotide concentrations) and in the presence of 2 \textmu M free Mg\textsuperscript{2+}, thereby allowing ~4% of the nucleotide to be metal-free ATP, which can modulate Ca\textsubscript{2}E\textsubscript{1} to E\textsubscript{2}P and E\textsubscript{3}P to E\textsubscript{4} as described above, and possibly also E\textsubscript{2} → Ca\textsubscript{2}E\textsubscript{4}, whereas the major part of the nucleotide is MgATP, which is the substrate of the phosphorylation reaction and modulates only the Ca\textsubscript{2}E\textsubscript{1} to E\textsubscript{2}P and E\textsubscript{3}P → Ca\textsubscript{2}E\textsubscript{4} transitions. For the wild type, the activation below 100 \textmu M nucleotide likely reflects the role of MgATP.
as substrate of the phosphorylation reaction in combination with the modulation by MgATP of the Ca\(^{2+}\) binding transition, \textit{i.e.} the MgATP activation of the reaction sequence $E_1 \rightarrow CaE_1 \rightarrow CaE_2$. Two more activation phases may be distinguished for the wild type, one between 0.1 and 1 mM nucleotide and one above 1 mM. The latter two activation phases reflect the ATP/MgATP-modulating Ca\(^{2+}\) binding transition, whereas the modulatory effect of metal-free ATP on $E_1P$ (e.g. $E_1\text{AlF}_4^-\cdot$ADP), the Arg\(^{678}\)–Asp\(^{203}\) pair being ~40 Å apart in $E_1\text{AlF}_4^-\cdot$ADP (6, 8), \textit{cf.} supplemental Fig. S8. It is clear from supplemental Fig. S8 that if the interactions of Glu\(^{439}\) and Arg\(^{678}\) in the $E_2\text{MgF}_4^-$ structure also exist in genuine $E_2P$ and possibly are formed during the transition from $CaE_2P$ to $E_2P$ (certainly feasible for the Glu\(^{439}\) and Ser\(^{186}\) pair as the residues are close to the pivot point at Glu\(^{468}\) and Thr\(^{171}\), then they could be very important in guiding or stabilizing the rearrangements of the $A\cdot$, $N\cdot$, and $P\cdot$-domains, thus explaining the slowing of the $CaE_2P \rightarrow E_2P$ transition in mutants E439A and R678A.

Of the three mutants studied here, R678A showed the most conspicuous effects on ATP/MgATP binding in the phosphorylating as well as the modulatory modes. Thus, in mutant R678A the affinity of the $E_1$ form for MgATP was 20-fold reduced relative to wild type (Fig. 3 and Table 2). The phosphorolytic rate (Fig. 2) and the affinity of $CaE_2P$ for ADP (Fig. 4) were likewise reduced significantly in R678A, and a modulatory effect of ATP/MgATP on the $E_2P \rightarrow E_2$ and $E_2 \rightarrow CaE_2$ transitions was hardly discernible in R678A (Figs. 7 and 9 and Table 3). The most obvious conclusion seems to be that Arg\(^{678}\) must be directly involved in the ligation of the phosphorylating nucleotide in $CaE_1$ and the leaving ADP in $CaE_2$, as well as in the ligation of the modulatory nucleotide binding to $E_2P$ and $E_2$. By contrast, the role played by Arg\(^{678}\) in the ligation of the MgATP/ATP-modulating $CaE_2P \rightarrow E_2P$ seems less crucial, as the R678A mutant showed only 2.5/2-fold reduced apparent affinity for MgATP/ATP activation of the latter transition (Figs. 5 and 6 and Table 3).

The importance of Arg\(^{678}\) in the binding of MgATP in $CaE_1$ and ADP in $CaE_2P$ is consistent with the $E_1$ crystal structures, where the side chain of Arg\(^{678}\) is within hydrogen bonding distance to the ribose O-3’ atom of AMPPCP or ADP and within van der Waals interaction distance to ribose O-2’ (Fig. 1, upper left panel, and Table 1). To understand the crucial role of Arg\(^{678}\) in MgATP modulation of the $E_2 \rightarrow CaE_1$ transition, the most relevant crystal structures to use for reference are $E_2\text{AMPPCP}$ and $E_2\text{ADP}$ (Fig. 1, lower panels). In addition to the nucleotide, the $E_2\text{AMPPCP}$ structure contains Mg\(^{2+}\) bound at site 2, as well as thapsigargin for stabilization of the membrane part (10).
There is no Mg\(^{2+}\) at site 1, in agreement with the view that the latter Mg\(^{2+}\) leaves with the phosphate (Scheme 1). In the \(E_2\)-AMPPCP structure, the side chain of Arg\(^{678}\) is within van der Waals interaction distance of the ribose O-3’ atom of AMPPCP (cf. Table 1). Consistent with such interaction, mutation R678A was found to reduce the affinity of the thapsigargin-bound enzyme for MgATP 2.4-fold (Table 2). The almost complete disruption of the modulatory effect of MgATP on the \(E_2 \rightarrow Ca_2E_1\) transition by mutation R678A (Fig. 9) indicates, however, that Arg\(^{678}\) is even more important for MgATP binding in the functioning enzyme in the \(E_2\) state than it is for MgATP binding to the thapsigargin-inhibited enzyme. Likewise, the difference between the MgATP affinity constants of the \(E_2\) conformation of the thapsigargin-inhibited wild type enzyme (\(K_{m,\text{avg}} 130 \mu M\); cf. Table 2) and the functioning wild type enzyme (45 \(\mu M\), cf. Table 3) suggests some perturbing influence of thapsigargin. It is possible that in the \(E_2\) state of the functioning Ca\(^{2+}\)-ATPase, Arg\(^{678}\) is able to approach the ribose of MgATP somewhat closer than in the thapsigargin-bound state. Moreover, an alternative relevant possibility for the binding of the modulatory MgATP to the \(E_2\) form has appeared from the recently published crystal structure of \(E_2\)-ADP (11). In this structure the adenine ring of ADP is sandwiched between Arg\(^{678}\) and Arg\(^{489}\), the guanidino group of Arg\(^{678}\) being involved in cation-π interaction (Fig. 1, lower right panel); a Mg\(^{2+}\) ion binds near the canonical site 1, and the β-phosphate of ADP takes the place of the enzyme-bound phosphoryl group as Mg\(^{2+}\) ligand, and the membrane domain is stabilized by the inhibitor CPA. A similar central role of Arg\(^{678}\) in the binding of MgATP in the \(E_2\) form of the native enzyme would clearly be in good accordance with the conspicuous effect of the R678A mutation on the modulatory effect of MgATP seen in Fig. 9. Both of the above mentioned modes of interaction of the MgATP with Arg\(^{678}\) would interfere with the ion pairing between Arg\(^{678}\) and Asp\(^{203}\) that attaches the A-domain to the P-domain in the \(E_2\)-MgF\(^{2+}\) structure (cf. Fig. 1). Because the basic rate of the \(E_2 \rightarrow Ca_2E_1\) transition was found 3.4-fold higher in R678A as compared with the wild type (cf. Table 3), it seems that \(E_2\) is stabilized by the ion pairing. Hence, it is likely that the competition between MgATP and Asp\(^{203}\) for bond formation with Arg\(^{678}\) destabilizes \(E_2\) and promotes the \(E_2 \rightarrow Ca_2E_1\) transition by helping release the A-domain.

By contrast, the basic rate of the \(E_2P \rightarrow E_3\) step was found 2-fold reduced in R678A. Hence, in this case the mutation exerts an effect opposite that of the nucleotide, and it is therefore not likely that interference of the nucleotide with the ion pairing between Arg\(^{678}\) and Asp\(^{203}\) plays any significant role in the strong effect of mutation R678A on the modulation of \(E_2P \rightarrow E_3\) by ATP. In \(E_2\)-MgF\(^{2+}\), the closest analog of \(E_2P\) with bound nucleotide for which a structure is available, the ADP is bound such that the side chain of Arg\(^{678}\) is too far away to interact with the ribose or adenine ring. The β-phosphate of the ADP is, however, only 5.0 Å from the side chain of Arg\(^{678}\) (Fig. 1, upper right panel, and Table 1), and it is therefore reasonable to speculate that in \(E_2P\) Arg\(^{678}\) interacts with the γ-phosphate of ATP. Importantly ADP, unlike ATP, does not accelerate the dephosphorylation of \(E_2P\) (13). Hence, the interaction of Arg\(^{678}\) with the γ-phosphate of ATP may be required for the modulatory effect on \(E_2P \rightarrow E_2\). This hypothesis could also explain that the modulatory ATP binding to \(E_2P\) needs to be Mg\(^{2+}\)-free, because a Mg\(^{2+}\) ion near the γ-phosphate might prevent the interaction with Arg\(^{678}\). Because the α- and β-phosphates of the ADP bound in the \(E_2\)-MgF\(^{2+}\) structure seem to interact with residues in the A-domain, ATP bound at the same position, and with the γ-phosphate near Arg\(^{678}\), might actually stabilize the attachment of the A-domain to the P-domain even more than the ion pairing between Arg\(^{678}\) and Asp\(^{203}\), and this might lead to the acceleration by ATP of \(E_2P \rightarrow E_3\) through stabilization of the transition state of the reaction.

Considering the conspicuous effects of mutation R678A on the modulation of the \(E_2P \rightarrow E_2\) and \(E_2 \rightarrow Ca_2E_1\) by ATP/MgATP, it is remarkable that the affinity for ATP/MgATP modulation of the \(Ca_2E_1P \rightarrow E_2P\) transition was little affected by mutation R678A. Importantly, a similar situation exists with Phe\(^{487}\) and Arg\(^{560}\), which are key residues in the binding of MgATP at the catalytic site (6, 7, 29, 32), and like Arg\(^{678}\) were found critical to ATP modulation of \(E_2P \rightarrow E_2\) in the present study (Fig. 7), whereas they could be substituted without significant effect on the MgATP modulation of \(Ca_2E_1P \rightarrow E_2P\) (32). Thus, the picture that emerges is one in which the nucleotide that modulates the \(E_2P \rightarrow E_2\) and \(E_2 \rightarrow Ca_2E_1\) steps is ligated by some of the same residues (including Arg\(^{678}\)) that form the catalytic site in \(Ca_2E_1P\) and \(Ca_2E_1\). Whereas the ATP/MgATP that accelerates the \(Ca_2E_1P \rightarrow E_2P\) transition binds differently, perhaps at a completely different locus, which would be in agreement with a previous proposal based on the finding that ATP does not compete with ADP for binding to \(Ca_2E_1P\) (28). The higher apparent affinity of ATP compared with MgATP for modulation of \(Ca_2E_1P \rightarrow E_2P\) (Table 3) would be consistent with a critical role of electrostatic forces in the interaction involved here (note that the situation is opposite at the catalytic site, MgATP binding with higher affinity than ATP; cf. Table 2). The need for a nucleotide site for enhancement of the \(Ca_2E_1P \rightarrow E_2P\) transition differing from the nucleotide site involved in the modulation of the rate of \(E_2 \rightarrow Ca_2E_1\) may arise from the fact that the rearrangements of the contacts between the A-, N-, and P-domains occurring in relation to the \(Ca_2E_1P \rightarrow E_2P\) transition are opposite those associated with \(E_2 \rightarrow Ca_2E_1\). The binding of MgATP to \(E_2\) at the site containing Phe\(^{487}\), Arg\(^{560}\), and Arg\(^{678}\) as key residues promotes the \(E_2 \rightarrow Ca_2E_1\) transition by tying the N- and P-domains together and loosening the contact with the A-domain, thereby turning this modulatory site into the catalytic site by a sort of “induced fit” mechanism. Because the opposite rearrangements of the cytoplasmic domains take place during the \(Ca_2E_1P \rightarrow E_2P\) transition, interactions of the N- and P-domains with the A-domain being established and the TGES loop of domain A being inserted into the catalytic site, it may seem logical to find that the nucleotide interaction needed to promote \(Ca_2E_1P \rightarrow E_2P\) is very different from that needed to promote \(E_2 \rightarrow Ca_2E_1\).

The closeness of Glu\(^{439}\) to the Mg\(^{2+}\) ion at site 2 in the \(E_2\)-ALF\(^{-1}\)-ADP and \(E_2\)-AMPPCP crystal structures (Fig. 1 and Table 1) has led to the suggestion that this glutamate is important in the binding of MgATP in catalytic as well as modulatory modes, and perhaps in aiding the release of the ADP leaving from \(Ca_2E_1P\) (10, 35, 38–41). We found small and opposite
effects of the E439A mutation on the affinities for MgATP (2-fold reduction) and metal-free ATP (2-fold increase) in the absence of thapsigargin (Fig. 3 and Table 2), which could indeed reflect a weak electrostatic interaction between Glu439 and a Mg\(^{2+}\) ion associated with ATP in \(E_1\). It appears from Fig. 2 that such interaction, if it exists, is too weak to influence the rate of phosphorylation. There was likewise no significant effect of E439A on the apparent affinity of Ca\(_2\)E\(_P\) for ADP (Fig. 4), thus contradicting the suggestion that Glu439 interacts in a functionally important way with the leaving ADP molecule (10). Furthermore, Glu439 was not found to be critical for ATP/MgATP ligation at the site involved in the activation of the Ca\(_2\)E\(_P\) → E\(_2\)\(_P\) transition, as the activation was retained with normal apparent affinity in E439A and a markedly increased enhancement factor relative to wild type (Figs. 5 and 6 and Table 3). The apparent affinity with which MgATP modulates the E\(_2\) → Ca\(_2\)E\(_1\) transition was slightly increased (less than 2-fold) in E439A, relative to wild type (Table 3), and the affinity of the thapsigargin-bound E\(_2\) form of E439A for MgATP was wild type-like (Table 2). Hence, it seems clear that despite the close-ness of Glu439 to the Mg\(^{2+}\) ion at site 2 in the E\(_2\)-AMPPCP crystal structure, Glu439 contributes very little, if at all, to the binding of MgATP in the E\(_2\) form of the native enzyme.

A highly interesting finding with E439A was the complete disruption of the modulatory effect of metal-free ATP on the dephosphorylation of E\(_2\)\(_P\). E439A furthermore showed a conspicuous 11-fold increase of the basic rate of E\(_2\)\(_P\) dephosphorylation in the absence of ATP, i.e. constitutive activation (Fig. 7 and Table 3). Our studies of mutant P248A, which like E439A showed a marked enhancement of the basic rate of E\(_2\)\(_P\) dephosphorylation, but nevertheless retained a wild type-like modulation by ATP (Fig. 7), demonstrate that the disruption of ATP modulation in E439A is not a consequence of the increased basic rate of E\(_2\)\(_P\) dephosphorylation. In E439A, the destabilization of E\(_2\)\(_P\) may be caused by the absence of the hydrogen bond between Gly\(^{438}\) and Ser\(^{186}\) mentioned above (cf. Fig. 1, upper right panel, and supplemental Fig. S8). This explanation would be consistent with a previous finding that the S186F mutation leads to a block of the basic rate of E\(_2\)\(_P\) dephosphorylation (Table 3). Furthermore, G438A showed moderate reductions of the affinity for ATP/MgATP in the binding assays (Table 2) and a slight 2-fold reduction of the apparent affinity for the ATP modulating E\(_2\)\(_P\) → E\(_1\) (Fig. 7 and Table 3), an effect very similar to that of mutation T441A (Fig. 7). Thr\(^{441}\) was previously shown to be an important residue for ATP/MgATP binding to E\(_1\) (32), which seems to be a consequence of its interaction with Arg\(^{560}\), holding the latter residue in position to bind the β-phosphate of the nucleotide (6, 7). The crystal structures show that Glu439 and Thr\(^{441}\) are located in the first part of an α-helix that is preceded by a loop, whose structure depends on the presence of Gly\(^{38}\). The bend between the α-helix and the loop at this glycine is very acute (cf. Fig. 1, upper right corner of upper left panel), and a Ramachandran plot analysis reveals dihedral angles that would be energetically very unfavorable for residues with side chains. It is therefore rather unlikely that these angles would be maintained in mutant G438A. The consequence of the mutation might well be that the loop expands to make the bend less acute, thereby partially unwinding the N-terminal part of the helix containing Glu439 and Thr\(^{441}\). This would clearly disturb the interactions of the Glu439 and Thr\(^{441}\) side chains to some extent, thus providing a neat explanation of the resemblance of the functional effects of the G438A mutation to some of those seen for the E439A and T441A mutations.

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