Roles of Interaction between Actuator and Nucleotide Binding Domains of Sarco(endo)plasmic Reticulum Ca\(^{2+}\)-ATPase as Revealed by Single and Swap Mutational Analyses of Serine 186 and Glutamate 439*1

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Roles of hydrogen bonding interaction between Ser\(^{186}\) of the actuator (A) domain and Glu\(^{439}\) of nucleotide binding (N) domain seen in the structures of ADP-insensitive phosphorylated intermediate (E2P) of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase were explored by their double alanine substitution S186A/E439A, swap substitution S186E/E439S, and each of these single substitutions. All the mutants except the swap mutant S186E/E439S showed markedly reduced Ca\(^{2+}\)-ATPase activity, and S186E/E439S restored completely the wild-type activity. In all the mutants except S186E/E439S, the isomerization of ADP-sensitive phosphorylated intermediate (E1P) to E2P was markedly retarded, and the E2P hydrolysis was largely accelerated, whereas S186E/E439S restored almost the wild-type rates. Results showed that the Ser\(^{186}\)-Glu\(^{439}\) hydrogen bond stabilizes the E2P ground state structure. The modulatory ATP binding at sub mM range largely accelerated the E2P isomerization in all the alanine mutants and E439S. In S186E, this acceleration as well as the acceleration of the ATPase activity was almost completely abolished, whereas the swap mutation S186E/E439S restored the modulatory ATP acceleration with a much higher ATP affinity than the wild type. Results indicated that Ser\(^{186}\) and Glu\(^{439}\) are closely located to the modulatory ATP binding site for the E2P isomerization, and that their hydrogen bond fixes their side chain configurations thereby adjusts properly the modulatory ATP affinity to respond to the cellular ATP level.

Sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA1a)\(^2\) is a representative member of P-type ion-transporting ATPases and catalyzes Ca\(^{2+}\) transport coupled with ATP hydrolysis (Fig. 1) (1–9). In the catalytic cycle, the enzyme is activated by binding of two Ca\(^{2+}\) ions at the transport sites (E2 to E1Ca\(_2\), steps 1–2) and then autophosphorylated at Asp\(^{351}\) with MgATP to form ADP-sensitive phosphoenzyme (E1P, step 3), which can react with ADP to regenerate ATP. Upon formation of this E1P, the bound Ca\(^{2+}\) ions are occluded in the transport sites (E1PCa\(_2\)). The subsequent isomeric transition to ADP-insensitive form (E2P) results in a change in the orientation of the Ca\(^{2+}\) binding sites and reduction of their affinity, and thus Ca\(^{2+}\) release into lumen (steps 4 and 5). Finally, the hydrolysis takes place and returns the enzyme into an unphosphorylated and Ca\(^{2+}\)-unbound form (E2, step 6). E2P can also be formed from P\(_1\) in the presence of Mg\(^{2+}\) and the absence of Ca\(^{2+}\) by reversal of its hydrolysis.

The cytoplasmic three domains N, A, and P largely move and change their organization states during the Ca\(^{2+}\) transport cycle (10–22). These changes are linked with the rearrangements in the transmembrane helices. In the E2P isomerization (loss of ADP sensitivity) and Ca\(^{2+}\) release, the A domain largely rotates (by ~110° parallel to membrane plane), intrudes into the space between the N and P domains, and the P domain largely inclines toward the A domain. Thus in E2P, these domains produce the most compactly organized state (see Fig. 2) for the change E1Ca\(_2\)-AlF\(_4\)-ADP → E2-MgF\(_2\) as the model for the overall process E1PCa\(_2\)-ADP\(^{3+}\) → E2P.

We have found that the interactions between the A and P domains at the Val\(^{200}\)-loop (Asp\(^{196}\)-Asp\(^{203}\)) with the residues of the P domain (Arg\(^{678}\)/Glu\(^{680}\)/Arg\(^{656}\)/Asp\(^{660}\) (23) and at the Tyr\(^{122}\) hydrophobic cluster (24–26) (see Fig. 2) play critical roles for Ca\(^{2+}\) deocclusion/release in E2PCa\(_2\) → E2P + 2Ca\(^{2+}\) after the loss of ADP sensitivity (E1PCa\(_2\) to E2PCa\(_2\) isomerization). The proper length of the A/M1 linker is critical for inducing the inclining motion of the A and P domains for the Ca\(^{2+}\) deocclusion and release from E2PCa\(_2\) (27, 28). The importance of the interdomain interaction between Arg\(^{678}\) (P) and Asp\(^{203}\) (A) in stabilizing the E2P and E2 intermediates and its influence on modulatory ATP activation were pointed out by the mutation R678A (29). Regarding the N domain, the importance of Glu\(^{439}\) in the E2P isomerization and E2P hydrolysis was previously noted by its alanine substitution, and possible importance of its interaction with Ser\(^{186}\) on the A domain has been suggested since Glu\(^{439}\) forms a hydrogen bond with Ser\(^{186}\) in the E2P analog structures (29) (see Fig. 2). The Darier disease-causing mutations of Ser\(^{186}\) of SERCA2b, S186P and S186F also alter the kinetics of the EP processing and its impor-
tance as the residue in the immediate vicinity of TGES\textsuperscript{184} has been pointed out (30, 31). Notably also, Glu\textsuperscript{439} is situated near the adenine binding pocket and its importance in the ATP binding and ATP-induced structural change have been shown (32, 33). In the structure \textit{E2(TG)AMPPCP (E2-ATP),} Glu\textsuperscript{439} interacts with the modulatory ATP binding via Mg\textsuperscript{2+}, and is involved in the acceleration of the Ca\textsuperscript{2+}-ATPase cycle (16).

Considering these critical findings on each of Glu\textsuperscript{439} and Ser\textsuperscript{186}, it is crucial to reveal the role of the Ser\textsuperscript{186}-Glu\textsuperscript{439} hydrogen-bonding interaction between the A and N domains in the EP processing and its ATP modulation (i.e. regulatory ATP-induced acceleration). We therefore made a series of mutants on both Ser\textsuperscript{186} and Glu\textsuperscript{439} including the swap substitution mutant, S186A, E439A, S186A/E439A, S186E, E439S, S186E/ E439S, and explored their kinetic properties. Results showed that the Ser\textsuperscript{186}-Glu\textsuperscript{439} hydrogen bond is critical for the stabilization of the E2P ground state structure, and possibly functioning as to make the E2P resident time long enough for Ca\textsuperscript{2+} release (E2P\textsuperscript{Ca2+} \rightarrow E2P + 2Ca\textsuperscript{2+}) thus to avoid its hydrolysis without Ca\textsuperscript{2+} release. Results also revealed that the side-chain configurations of Ser\textsuperscript{186} and Glu\textsuperscript{439} are fixed by their hydrogen bond, thereby conferring the proper modulatory ATP binding to occur at the cellular ATP level to accelerate the rate-limiting EP isomerization.

**EXPERIMENTAL PROCEDURES**

\textit{Mutagenesis and Expression}—The Stratagene QuikChange\textsuperscript{TM} site-directed mutagenesis method (Stratagene, La Jolla, CA) was utilized for the substitution in the rabbit SERCA1a cDNA. The Apal-KpnI or KpnI-SalI restriction fragments with the desired mutation were excised from the plasmid and ligated back into the corresponding region in the full-length SERCA1a cDNA in the pMT2 expression vector (34). The pMT2 DNA was transfected into COS-1 cells by the liposome-mediated transfection method. Microsomes were prepared from the cells as described (35). “Control microsomes” were prepared from COS-1 cells transfected with the pMT2 vector containing no SERCA1a cDNA. The amount of expressed SERCA1a was quantified by immunosorbent assay (36). Expression levels of wild-type SERCA1a and the mutants were 2–3% of total microsomal proteins.

\textit{Ca\textsuperscript{2+}-ATPase Activity}—The rate of ATP hydrolysis was determined at 25 °C with 20 μg/ml microsomal protein in various concentrations of ATP, 1 μM A23187, 0.1 M KCl, 7 mM MgCl\textsubscript{2}, 0.05 mM CaCl\textsubscript{2}, or 5 mM EGTA, and 50 mM MOPS/Tris (pH 7.0), otherwise as noted in the legends to figures. The Ca\textsuperscript{2+}-ATPase activity of the expressed SERCA1a of the microsomes was obtained by subtracting the Ca\textsuperscript{2+}-ATPase activity of the control microsome. 

Phosphorylation of SERCA1a in microsomes with \textit{[γ\textsuperscript{32}P]ATP or \textit{32}P} and dephosphorylation of \textit{32}P-labeled SERCA1a were performed as described in the legends to figures. The reactions were quenched with ice-cold trichloroacetic acid
containing \( P_i \). Rapid kinetics measurements were performed with a handi¬made rapid mixing apparatus (37). The precipitated proteins were separated by 5% SDS-PAGE at pH 6.0 according to Weber and Osborn (38). The radioactivity associated with the separated Ca\(^{2+}\)-ATPase was quantitated by digital autoradiography (39). The amount of \( \text{Ca}^{2+} \) accumulated was determined with 0.1 mM \[^{32}\text{P} \]ATP as described under “Experimental Procedures.” The activities are divided by the amount of EP formed at steady state (see supplemental Fig. S1), and the turnover rates thus obtained are shown as percentage of that of the wild type (7.29 \pm 0.42 s\(^{-1}\) (n = 5)). The values of the mutants presented are the mean \pm S.D. (n = 3–5).

**FIGURE 3.** \( \text{Ca}^{2+} \)-ATPase activities of expressed SERCA1a. The \( \text{Ca}^{2+} \)-ATPase activity of microsomes expressing the wild-type or mutant SERCA1a was determined with 0.1 mM \[^{32}\text{P} \]ATP as described under “Experimental Procedures.” The activities are divided by the amount of EP formed at steady state (see supplemental Fig. S1), and the turnover rates thus obtained are shown as percentage of that of the wild type (7.29 \pm 0.42 s\(^{-1}\) (n = 5)). The values of the mutants presented are the mean \pm S.D. (n = 3–5).

**TABLE 1**

Kinetic parameters determined for partial reaction steps

| Affinity for \( \text{Ca}^{2+} \) | \( E_2 \) to \( E_1\text{Ca}_2 \) | Loss of ADP sensitivity | Decay of \( E_2\ PATP \) | Hydrolysis of \( E_2\text{PPi} \) | \( K_{\text{Ca}} \) in formation of \( E_2\text{BeF}_3^- \) and \( E_2\text{AlF}_4^- \) from the \( E_2 \) state | \( \text{BeF}_3^- \) analog | \( \text{AlF}_4^- \) analog |
|---|---|---|---|---|---|---|---|
| \( K_{n,2} \) | \( n_i \) | \( x \) | (\%) | \( x \) | (\%) | \( x \) | (\%) | \( x \) | (\%) | \( x \) | (\%) | \( x \) | (\%) | \( x \) | (\%) | \( x \) | (\%) | \( x \) | (\%) | \( x \) | (\%) | \( x \) | (\%) | \( \mu \mu \) | (\%) | \( \mu \mu \) | (\%) |
| WT | 0.149 | 2.26 | 0.184 | (100) | 0.270 | (100) | 0.0250 | (100) | 0.144 | (100) | 3.2 | (100) | 6.7 | (100) |
| S186A | 0.181 | 1.76 | 0.194 | (106) | 0.160 | (98) | 0.0034 | (14) | 0.590 | (410) | 5.2 | (162) | 8.3 | (124) |
| S186E | 0.127 | 1.84 | 0.254 | (139) | 0.184 | (68) | 0.0159 | (64) | 0.119 | (83) | 2.2 | (68) | 4.3 | (64) |
| E439A | 0.172 | 2.00 | 0.174 | (95) | 0.136 | (79) | 0.0067 | (27) | 0.452 | (314) | 6.7 | (217) | 6.2 | (92) |
| E439S | 0.142 | 1.86 | 0.250 | (136) | 0.136 | (79) | 0.0121 | (49) | 0.306 | (213) | 8.7 | (273) | 13.4 | (200) |
| S186A/E439A | 0.154 | 1.78 | 0.116 | (63) | 0.136 | (79) | 0.0052 | (21) | 0.491 | (341) | 5.4 | (169) | 6.6 | (99) |
| S186E/E439S | 0.146 | 1.64 | 0.363 | (198) | 0.185 | (68) | 0.0221 | (89) | 0.261 | (181) | 3.5 | (110) | 5.9 | (88) |

* The rate most likely reflects the rate-limiting \( E_1\text{PCa}_2 \) to \( E_2 \) transition in step 4 of the presence of 0.1 mM \( \text{K}^+ \).

* Not determined because \( E_2P \) was not accumulated.
accumulation largely increased in the wild type, S186E, and S186E/E439S. By contrast, the E2P accumulation in S186A, E439A, E439S, and S186A/E439A was very low even in the absence of K⁺, therefore the E1PCa₂ to E2P isomerization was likely retarded and/or the E2P hydrolysis was accelerated in these mutants.

In Fig. 5, the time course of E2P accumulation upon the addition of ATP to E1Ca₂ was determined in the absence of K⁺. The total amount of E2P reached its maximum level very rapidly (within ~1 s) and remained unchanged during the period of observation and therefore the time course actually reflects the accumulation of E2P from E1PCa₂. The E2P accumulation proceeded with first-order kinetics, and the rates obtained are listed in Table 1. The rates in the mutants S186E and S186E/E439S were only slightly slowed. In S186A, E439A, S186A/E439A, and E439S, E2P was not accumulated.

For the analysis of the E1PCa₂ to E2P isomerization in the presence of K⁺, E1PCa₂ was first accumulated with ATP and its decay time course was determined in Fig. 6. This is because, as well known with the wild type (44), the E1PCa₂ decay reflects the rate-limiting E1PCa₂ to E2P isomerization followed by the rapid E2P hydrolysis. In fact, almost all of E2P present at each time point was E1PCa₂ in the mutants as well as in the wild type (data not shown, but see Fig. 4A). The decay time courses were well fitted with a single exponential, and the decay rates obtained are listed in Table 1. In S186A, E439A, and S186A/E439A, the decay rate was markedly reduced to 14–27% of the wild type. The rate was also reduced significantly in E439S and to some extent in S186E. In the swap mutant S186E/E439S, the wild-type rate was almost restored.

Hydrolysis of E2P Formed from Pᵢ —The E2P hydrolysis was examined by first phosphorylating the enzyme with 32Pᵢ in the absence of Ca²⁺ and K⁺ and presence of 35% (v/v) Me₂SO, which extremely favors E2P formation (45), and then by dilut-
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![Graphs](FIGURE_7.png)  ![Graphs](FIGURE_8.png)

FIGURE 7. Hydrolysis of E2P formed from P_i without Ca^{2+}. Microsomes expressing the wild type or mutant were phosphorylated with 0.1 mM [32P]ATP at 25 °C for 10 min in 5 μl of a mixture containing 2 μg of microsomal protein, 20 μM E2, 1 mM EGTA, 7 mM MgCl_2, 50 mM MOPS/Tris (pH 7.0), and 35% (v/v) MeSO. The mixture was then cooled and diluted at 0 °C by 100 μl of a mixture containing 1.05 mM non-radioactive P_i, 105 mM KCl, 15.8 mM EGTA, and 50 mM MOPS/Tris (pH 7.0). At different times after the dilution, the E2P hydrolysis was quenched by acid. The amounts of E2P formed with [32P]P_i, at zero time are normalized to 100%. Solid lines show the least squares fit to a single exponential, and the rates obtained are given in Table 1.

FIGURE 8. MgATP dependence of Ca^{2+}-ATPase activity. The Ca^{2+}-ATPase activity of microsomes expressing the wild type or mutant was determined at various concentrations of [γ-^{32}P]ATP otherwise as in Fig. 3. Almost all of ATP (more than 97% of total ATP) is in MgATP. The activities of the mutants are presented as a percentage of that of the wild type at 5 mM MgATP.

In the absence of Ca^{2+}, the marked increased rate of E439S toward the wild-type one. The inhibition of the Ca^{2+}-ATPase activity was significantly inhibited in all the alanine mutants, S186A, E439A, S186A/E439A, and also in E439S at a lower extent. The result of E439A is consistent with the previous observation by Clausen et al. (29). The rate was slightly decreased in S186E, in contrast to the marked increase in S186A and the Darier disease mutant S186F of SERCA2b (30). The swap mutant S186E/E439S exhibited an intermediate rate between those of E439S and S186E, and thus brought the markedly increased rate of E439S toward the wild-type one.

Note that the overall Ca^{2+}-ATPase activity was significantly inhibited in all the alanine mutants, S186E, and E439S, despite the markedly accelerated E2P hydrolysis (or unretarded hydrolysis in S186E). Therefore, the inhibition of the Ca^{2+}-ATPase activity in the mutants is ascribed to the retardation of the rate-limiting E1P-iCa to E2P isomerization.

**BeF_3 and AlF_4 Affinities in Formation of E2-BeF_3 and E2-AlF_4**—The E2 state Ca^{2+}-ATPase in the absence of Ca^{2+} forms the complexes E2-BeF_3 and E2-AlF_4, which are analogs of the E2P ground state and of the transition state of the E2P hydrolysis, respectively (22). In supplemental Fig. S3 and Table 1, the effects of the mutations on the affinities for BeF_3 and AlF_4 were determined by changing the beryllium and aluminum concentrations in the presence of excess 2 mM fluoride and by determining the inhibition of P formation from ATP. In the E2-BeF_3 formation, the mutations S186A, E439A, S186A/E439A, and E439S significantly decreased the BeF_3 affinity, S186E increased slightly, and the swap mutation S186E/E439S restored the wild-type affinity. In the E2-AlF_4 formation, the mutation effects were much less pronounced or not exhibited. The results agree with the mutation effects on the E2P hydrolysis (Fig. 7) that the alanine mutations and E439S markedly enhance the hydrolysis rate, S186E reduces slightly, and the swap mutation S186E/E439A restores the wild-type rate. The results indicated that the hydrogen-bonding interaction between Ser^{186} and Glu^{439} functions to stabilize the E2P ground state.

**Modulatory MgATP-induced Acceleration of Ca^{2+}-ATPase Activity**—As known for a long time (46), the Ca^{2+}-ATPase activity of the wild type is markedly increased by MgATP at sub-mM–μM range. This modulatory MgATP effect was examined at various MgATP concentrations in Fig. 8. The mutants S186A, E439A, and S186A/E439A exhibited the marked increase with increasing MgATP concentration despite their significantly reduced activity at the low ATP concentrations (see Fig. 3). The result on E439A is consistent with the previous observation by Clausen et al. (29). The activity was increased only slightly in E439S with MgATP and not increased at all in S186E. In the swap mutant S186E/E439S, the activity was increased with MgATP, and thus the MgATP modulatory effect as well as the activity was almost restored. In Figs. 9–11, the modulation by MgATP and free ATP was further explored in each of the steps; the E1P-iCa to E2P isomerization and the E2P hydrolysis.

**Modulatory MgATP- and ATP-induced Acceleration of E1P-iCa to E2P Isomerization**—E1P-iCa was first formed with 10 μM MgATP in the presence of K^+ under conditions in which E1P-iCa accumulates dominantly (see Fig. 4A). Then, the MgATP modulation of the E1P-iCa decay (the rate-limiting E1P-iCa to E2P isomerization) was examined by the subsequent addition of various concentrations of MgATP together with an excess EGTA to remove free Ca^{2+}. The decay time courses were fitted well with a single exponential (data not shown). In Fig. 9, the rates thus determined are plotted versus the MgATP concentration. The values V_0 (the rate at 10 μM MgATP), V_max (the maximum rate at a saturating MgATP concentration), and K_0.5 (giving the half-maximum rate) are obtained by fitting the
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FIGURE 9. MgATP dependence of the decay rate of E1PCa, formed from ATP. E1PCa was first formed in 50 μl of a microsomes suspension in 10 μM γ-32PATP, 10 μM Ca^2+ (0.98 mM CaCl_2 with 1 mM EGTA), and 0.1 mM KCl otherwise as in Fig. 4. Phosphorylation was terminated by 100 μl of a buffer containing 8 mM EGTA, 1 mM A23187, 0.1 mM KCl, 50 mM MOPS/Tris (pH 7.0), and various concentrations of ATP and MgCl_2 (producing MgATP (more than 97% of the total ATP) with 6.2 mM free Mg^{2+}). At different times after this MgATP addition, the decay reaction of E1PCa at 0 °C was quenched by acid. The rate of the single exponential decay of E1PCa, obtained was plotted versus the MgATP concentration. Solid lines show the least squares fit to the Hill equation, and the parameters V_max (the rate at the lowest 10 μM MgATP), V_max (the maximum rate), and K_M (MgATP concentration giving the half-maximal change) are given in Table 2.

FIGURE 10. ATP dependence of the decay rate of E1PCa, formed from ATP. E1PCa decay was followed after addition of various concentrations of ATP and 30 mM EDTA without MgCl_2 (in place of 8 mM EGTA) otherwise as in Fig. 9. The single exponential decay rate of E1PCa obtained was plotted versus the metal-free ATP concentration, and the parameters V_max, V_max and K_M estimated as in Fig. 9 are given in Table 2.

curves to the Hill equation and shown in Table 2. In the wild-type, MgATP enhanced the EP isomerization rate by 18-fold (V_max/V_0) with K_M of 1.35 mM. In the alanine mutants S186A, E439A, and S186A/E439A, the magnitudes of MgATP-induced acceleration were much larger (54-, 50-, and 56-fold, respectively) with slightly higher MgATP affinities than the wild type. In E439S and the swap mutant S186E/E439S, the magnitudes of MgATP-induced acceleration were much larger (54-, 50-, and 56-fold, respectively) with slightly higher MgATP affinities than the wild type. The results of E439A are in agreement with the previous study by Clausen et al. (29).

In the wild type, the affinity of metal-free ATP on the EP isomerization was also explored, in this case, by adding various concentrations of ATP together with an excess EDTA to remove free Ca^{2+} and Mg^{2+} (Fig. 10). The E1PCa decay time courses of the wild type and mutants were apparently fitted to a single exponential kinetics but not strictly (data not shown). This complicated kinetics may be because Mg^{2+} bound at the catalytic site was likely removed by the added EDTA in some E1PCa fraction as previously also noted (29). Nevertheless, for simplicity, the single exponential rates estimated were plotted in Fig. 10. The parameters V_max, V_0 (at 10 μM ATP), V_max in the ATP dependence curve are listed in Table 2.

In the wild type, the affinity of metal-free ATP for the EP isomerization was significantly higher than that of MgATP (29); thus Mg^{2+} in MgATP brings its modulatory binding to correspond to cellular level of ATP (mostly MgATP complex). In the alanine mutants S186A, E439A, and S186A/E439A (most profoundly in S186A), the increase in free ATP concentration from 0.1 to 40 mM exhibited the marked acceleration of the E1PCa decay by 38-, 15-, and 17-fold, respectively, which are even much more than that of the wild type (8-fold). The free ATP affinities in the alanine mutants were similar to and not higher than the wild type. The results of E439A are in agreement with the previous study by Clausen et al. (29).

In E439S, the extent of the acceleration was 5.1-fold and slightly smaller than that of the wild type with somewhat increased ATP affinity. In S186E, the ATP-induced acceleration of EP isomerization was very small (2.7-fold), and actually MgATP concentrations, e.g. at 10 mM became much lower than the wild type. Note that this markedly reduced rate in S186E was increased by the swap mutation S186E/E439S to the significant level close to the wild type.

The modulatory effect of metal-free ATP on the EP isomerization was also explored, in this case, by adding various concentrations of ATP with a mixture containing 16.6 μM of microsomal protein, 0.1 mM ^32P, 20 μM A23187, 1 mM EGTA, 7 mM MgCl_2, 50 mM MOPS/Tris (pH 7.0), 15.3 mM EDTA, and various concentration of ATP. At different times after the addition, the E2P hydrolysis was quenched by acid. The rate of the single exponential E2P hydrolysis was plotted versus the ATP concentration, and the parameters V_max (the rate at the lowest 10 μM ATP), V_max (the rate at the highest ATP concentration, 1 mM), and K_M (ATP concentration giving the half-maximal change) are given in Table 2. It should be noted that Mg^{2+} bound at the catalytic site of E2P is occluded (53), and therefore the E2P hydrolysis takes place even after removal of free Mg^{2+}.
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**Table 2**

Parameters determined for the MgATP/ATP-induced acceleration of partial reaction steps

The modulatory MgATP- and metal-free ATP-induced acceleration of the decay rate of EP\textsubscript{ATP} formed from ATP and Ca\textsuperscript{2+} (i.e. the rate-limiting EP\textsubscript{1PCa\textsubscript{2}} to EP\textsubscript{2PCa\textsubscript{2}} isomerization) and that of the hydrolysis of EP\textsubscript{P} formed from P\textsubscript{i} in the absence of Ca\textsuperscript{2+} were determined as described in Figs. 9–11. The kinetic parameters in the nucleotide dependence curves were obtained by the fitting to the Hill equation in Figs. 9 and 10, or roughly by the eye inspection in Fig. 11; $K_{\text{m}}$ (the MgATP or ATP concentration giving the half-maximum acceleration), $V_{0}$ (the rate at 10 mM MgATP or ATP in Figs. 9 and 10, and no ATP in Fig. 11), and $V_{\text{max}}$ (the maximum rate in Figs. 9 and 10, or the rate at the highest 1 mM ATP in Fig. 11).

| Parameters for the MgATP/ATP-induced acceleration of partial reaction steps | MgATP dependence of EP\textsubscript{ATP} decay | ATP dependence of EP\textsubscript{ATP} decay | ATP dependence of EP\textsubscript{P} hydrolysis |
|---|---|---|---|
| | $K_{0.5}$ | $V_{0}$ | $V_{\text{max}}$ | $K_{0.5}$ | $V_{0}$ | $V_{\text{max}}$ | $K_{0.5}$ | $V_{0}$ | $V_{\text{max}}$ |
| | $\text{mM}$ | $s^{-1}$ | $s^{-1}$ | $\text{mM}$ | $s^{-1}$ | $s^{-1}$ | $\text{mM}$ | $s^{-1}$ | $s^{-1}$ |
| Wild type | 1.351 | 0.033 | 0.591 | 0.224 | 0.088 | 0.714 | 60 | 0.016 | 0.066 |
| S186A | 0.738 | 0.009 | 0.489 | 0.612 | 0.033 | 1.248 | 70 | 0.011 | 0.178 |
| S186E | | | | | | | 30 | 0.009 | 0.118 |
| E439A | 0.774 | 0.017 | 0.657 | 0.195 | 0.035 | 0.513 | 10 | 0.011 | 0.010 |
| E439S | 0.170 | 0.030 | 0.494 | 0.065 | 0.082 | 0.418 | 70 | 0.011 | 0.010 |
| S186A/E439A | 0.913 | 0.014 | 0.786 | 0.360 | 0.039 | 0.647 | 12 | 0.141 | 0.164 |
| S186E/E439S | 0.281 | 0.043 | 0.357 | 0.032 | 0.010 | 0.295 | 12 | 0.033 | 0.029 |

$^{a}$ Not determined.

$^{b}$ The value at the highest (10 mM) ATP.

$^{c}$ The value at the highest (40 mM) ATP.

at the highest 40 mM ATP, the isomerization rate was only 17% of the wild-type rate. However, in the swap mutation S186E/E439S, the marked ATP-induced acceleration (by 30-fold) was restored with the ATP affinity much higher than the wild type.

**ATP Modulation of E2P Hydrolysis**—The modulation of the E2P hydrolysis was examined only with metal-free ATP, because metal-free ATP but not MgATP is able to bind to E2P for the modulation with a reasonable affinity (47, 48). E2P was first formed by $^{32}$P\textsubscript{i} in the absence of Ca\textsuperscript{2+} and K\textsuperscript{+} and presence of Me\textsubscript{2}SO as in Fig. 7, then the phosphorylated sample was largely diluted with a buffer containing excess EDTA and various concentrations of ATP, and the E2P hydrolysis was followed in the absence of K\textsuperscript{+} (in the presence of K\textsuperscript{+}, the hydrolysis especially at high ATP was too fast to be followed). In Fig. 11, the single exponential rates of E2P hydrolysis were plotted versus the ATP concentration. In the absence of K\textsuperscript{+} without ATP, the hydrolysis rate ($V_{0}$ in Table 2) was significantly faster in the mutants S186A, E439A, S186A/E439A, and E439S than in the wild type, as was found in the presence of K\textsuperscript{+} without ATP (Fig. 7 and Table 1). The $V_{0}$ of S186E was similar to that of the wild type, and the swap mutant S186E/E439S exhibited the intermediate $V_{0}$ value between S186E and E439S; thus, restored almost the wild-type rate from the markedly enhanced one in E439S.

With increasing ATP to 1 mM, the hydrolysis rate in the wild type increased by 4.1-fold. In S186E and in S186E/E439S, the rate was not increased or rather decreased slightly by 1 mM ATP; therefore, the swap mutation did not restore the ATP modulation. No ATP acceleration was found with E439S, although its rate was markedly elevated as compared with the wild type. In E439A, S186A, and S186A/E439A, the markedly elevated rates without ATP were slightly increased with increasing ATP by 1.2–1.5-fold.

**Discussion**

**Roles of Ser\textsuperscript{186} - Glu\textsuperscript{439} Interactions in E1PCa\textsubscript{2} to E2P Isomerization and E2P Hydrolysis in the Absence of Modulatory ATP**—In the absence of modulatory ATP, we observed here that the substitutions of Ser\textsuperscript{186} and Glu\textsuperscript{439}, especially by alanine(s) S186A, E439A, and S186A/E439A result in the markedly retarded E1PCa\textsubscript{2} to E2P isomerization and markedly accelerated E2P hydrolysis. Such changes were also found in E439S and S186E with a somewhat less extent (in S186E the change was only the retardation of the EPI isomerization). Most importantly, these changes as well as the inhibition of the overall Ca\textsuperscript{2+}-ATPase activity were almost relieved by the swap mutation S186E/E439S, and thus the wild-type properties were restored. The results demonstrated that the Ser\textsuperscript{186} - Glu\textsuperscript{439} hydrogen bond between the A and N domains functions to stabilize the E2P structure, consistent with the prediction made with the mutation E439A (29). The loss of the Ser\textsuperscript{186} - Glu\textsuperscript{439} interdomain interaction destabilized the E2P structure. In theory, the transition state structure is close to the product state (e.g. see the textbook by Fersht, Ref. 49); therefore, the transition state of the E1PCa\textsubscript{2} to E2P isomerization is probably also destabilized by the loss of the interaction causing the retardation of EPI isomerization.

Note in Table 1 that the disruption of the Ser\textsuperscript{186} - Glu\textsuperscript{439} hydrogen bond by the mutations caused the reduction of the Be\textsuperscript{3+} affinity for the E2-BeF\textsubscript{2} formation concomitantly with the marked acceleration of the E2P hydrolysis, and that the swap mutation S186E/E439S restored the wild type properties. The results obviously show that the E2P ground state is stabilized by the Ser\textsuperscript{186} - Glu\textsuperscript{439} hydrogen bond. This interaction therefore functions to retard the catalytic structural events, which involve rearrangement of the catalytic site for the attack of the TGES\textsuperscript{184} - coordinated specific water on the Asp\textsuperscript{351} acylphosphate. The E2P stabilization may be important to avoid the too (un-physiologically) rapid E2P hydrolysis, and thereby making the resident time of E2P long enough for Ca\textsuperscript{2+} release (E2PCa\textsubscript{2} $\rightarrow$ E2P + 2Ca\textsuperscript{2+}) and preventing a possible E2PCa\textsubscript{2} hydrolysis without Ca\textsuperscript{2+} release for the energy coupling.

Regarding the positioning of the TGES\textsuperscript{184} loop, the A domain largely rotates for the E1PCa\textsubscript{2} to E2P isomerization and its outermost TGES\textsuperscript{184} comes above the Asp\textsuperscript{351} region of the P domain; therefore blocking the access of the ADP $\beta$-phosphate...
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Acknowledgments

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