Multiple and Distinct Effects of Mutations of Tyr$^{122}$, Glu$^{123}$, Arg$^{324}$, and Arg$^{334}$ Involved in Interactions between the Top Part of Second and Fourth Transmembrane Helices in Sarcoplasmic Reticulum Ca$^{2+}$-ATPase

We explored, by mutational substitutions and kinetic analysis, possible roles of the four residues involved in the hydrogen-bonding or ionic interactions found in the Ca$^{2+}$-bound structure of sarcoplasmic reticulum Ca$^{2+}$-ATPase, Tyr$^{122}$-Arg$^{324}$, and Glu$^{123}$-Arg$^{334}$ at the top part of second transmembrane helix (M2) connected to the A domain and fourth transmembrane helix (M4) in the P domain. The observed substitution effects indicated that Glu$^{123}$, Arg$^{324}$, and Tyr$^{122}$ contributed to the rapid transition between the Ca$^{2+}$-unbound and bound states of the unphosphorylated enzyme. Results further showed the more profound inhibitory effects of the substitutions in the M4/P domain (Arg$^{324}$ and Arg$^{334}$) upon the isomeric transition of phosphorylated intermediate (EP) (loss of ADP sensitivity) and those in M2/A domain (Tyr$^{122}$ and Glu$^{123}$) upon the subsequent processing and hydrolysis of EP. The observed distinct effects suggest that the interactions seen in the Ca$^{2+}$-bound structure are not functionally important but indicate that Arg$^{334}$ with its positive charge and Tyr$^{122}$ with its aromatic ring are critically important for the above distinct steps. On the basis of the available structural information, the results strongly suggest that Arg$^{334}$ moves downward and forms new interactions with M2 (likely Asn$^{111}$); it thus contributes to the inclination of the M4/P domain toward the M2/A domain, which is crucial for the appropriate gathering between the P domain and the largely rotated A domain to cause the loss of ADP sensitivity. On the other hand, Tyr$^{122}$ most likely functions in the subsequent Ca$^{2+}$-releasing step to produce hydrophobic interactions at the A/P domain interface formed upon their gathering and thus to produce the Ca$^{2+}$-released form of EP. During the Ca$^{2+}$-transport cycle, the four residues seem to change interaction partners and thus contribute to the coordinated movements of the cytoplasmic and transmembrane domains.

Sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA1a) is a representative member of P-type ion-transporting ATPases; it catalyzes Ca$^{2+}$ transport coupled with ATP hydrolysis (Fig. 1; Refs. 1 and 2, and for recent reviews, see Refs. 3 and 4). In the catalytic cycle, the enzyme is activated by the binding of two Ca$^{2+}$ ions (E2 to E1Ca$_{2-}$, steps 1 and 2) and then autophosphorylated at Asp$^{351}$ by MgATP to form ADP-sensitive phosphoenzyme (E1P, step 3). Upon formation of this EP, the bound Ca$^{2+}$ ions are occluded in the transport sites. The subsequent isomeric transition to the ADP-insensitive form (E2P) will result in a reduction in affinity and a change in orientation of the Ca$^{2+}$-binding sites and, thus, the Ca$^{2+}$ release into lumen (steps 4–5). Finally, 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, in the presence of Mg$^{2+}$ and the absence of Ca$^{2+}$ by reversal of its hydrolysis.

The enzyme has three cytoplasmic domains (N, P, and A) which are widely separated in the Ca$^{2+}$-bound form (E1Ca$_{2-}$) but are associated in the Ca$^{2+}$-unbound and thapsigargin-bound form E2(TG) (Refs. 5 and 6; Fig. 2). In E2(TG), the A domain has largely rotated, and the P domain has significantly inclined together with the transmembrane helices M4 and M5 toward the A domain to associate with A domain. We showed previously in the proteolysis experiments (7, 8) that the large rotation of A domain and its gathering with P and N domains most likely occur during the E1P to E2P transition and Ca$^{2+}$-release (steps 4–5) to form the most compactly organized single headpiece in the Ca$^{2+}$-released form of E2P; we further suggested that the stabilization energy provided by the intimate contacts between three cytoplasmic domains in E2P will provide energy for moving transmembrane helices and release the bound Ca$^{2+}$ into lumen. To gain further insight into the energy coupling between cytoplasmic and transmembrane domains, it is crucial to find out the structural elements essential for the changes in the cytoplasmic domain organization and the coordinated movements of transmembrane helices and reveal the actual roles of each of the elements in the Ca$^{2+}$-transport cycle. In this regard, we have recently identified the Glu$^{40}$-Ser$^{48}$ loop as a critical element for the rotation of A domain and coordinated unique motions of M1 during the E1P to E2P transition (9); we also identified the Lys$^{189}$-Lys$^{205}$ outermost loop of A

$^*$ This work was supported by grant-in-aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
$\dagger$ To whom correspondence should be addressed: Dept. of Biochemistry, Asahikawa Medical College, Midorigaoka-bijishi, Asahikawa 078-8510, Japan. Tel.: 81-166-68-2350; Fax: 81-166-68-2398; E-mail: hisuzuki@asahikawa-med.ac.jp.
changes previously (13). Expression level of wild-type SERCA1a was determined in the presence of 5 mM EGTA without added CaCl₂, and 50 mM MOPS/Tris (pH 7.0). The Ca²⁺-ATPase activity was quantitated by digital autoradiography as described (16). The amount of EP formed with the expressed SERCA1a was obtained by subtracting the background radioactivity with the control microsomes. This background was less than 5% of the radioactivity of EP formed with the expressed wild-type SERCA1A. The amount of EPing of SERCA1a protein was calculated from the amount of EP thus obtained and the amount of expressed SERCA1a.

**Miscellaneous**—Protein concentrations were determined by the method of Lowry et al. (17), with bovine serum albumin as a standard. Data were analyzed by nonlinear regression using the program Origin (Microcal Software, Inc., Northampton, MA). Three-dimensional models of the enzyme were reproduced by the program VMD (18).

**RESULTS**

**Effects of Substitutions on Ca²⁺-ATPase Activity**—The specific Ca²⁺-ATPase activities of the expressed mutant and wild-type SERCA1a were determined at 25 °C. All the substitutions resulted in the reduction of the activity (4.30 ± 0.27 μmol/min/mg SERCA1a protein (n = 4) observed with the wild type). The substitution of Tyr122 with phenylalanine moderately reduced the activity to 51% of the activity of the wild type, and the substitution with alanine almost completely diminished it (to 1%). The alanine substitutions of Glu123 and Arg324 slightly or moderately reduced the activity to 75 and 80%, respectively, and the introduction of the opposite charge to Glu123 and Arg324 caused a much stronger inhibition to 30 and 41%, respectively. The substitutions of Arg334 (especially with glutamate) resulted in strong or almost complete inhibition (to 6% of the activity of wild type). The result with R334A (reduction to 45%) is consistent with the previously observed inhibition of the activity with this mutant (actually the reduction in the previous study (to 22%) was even larger; Ref. 19).

**Ca²⁺ Concentration Dependence of EP Formation from ATP and the E2 to E1Ca₂ Transition**—In Fig. 3, the amount of EP formed from ATP was determined at steady state (at 15 s) after the addition of ATP to the enzyme preincubated with various concentrations of Ca²⁺. All the mutants formed EP, and the amount formed with a saturating Ca²⁺ was comparable with that of wild type. Furthermore, the dissociation constants for Ca²⁺ and Hill coefficient obtained by least squares fit with these mutants (0.08–0.19 and 1.7–2.2 μM, respectively) were nearly the same as those obtained with the wild type (0.13 and 1.7 μM, respectively). The results indicate that the Tyr122, Arg324 and Glu123-Arg334 interactions found in E1Ca₂ and these four residues do not contribute significantly to the steady-state equilibrium between E2 and E1Ca₂. In the kinetics of the EP formation at the saturating Ca²⁺ (100 μM), we further found that the first-order rate in all the mutants (2.0–2.9 s⁻¹) is almost the same as that of the wild type (2.5 s⁻¹) (E1Ca₂ to E1PCa₂ in Table 1). Thus, the substitutions have essentially no effect upon EP formation from E1Ca₂ in step 3.

We then examined the rate of the E2 to E1Ca₂ transition in steps 1 and 2. For this experiment, the mutants and wild type were preincubated in the absence of Ca²⁺ at pH 6, where the equilibrium between E1 and E2 is most shifted to E2 (20), and then phosphorylated by the simultaneous addition of saturating
ing Ca\(^{2+}\) and ATP (Fig. 4). The time course of EP formation was well described by the first-order kinetics (although the maximum level of EP formed with R334A was somewhat lower than that at pH 7). The rates obtained are summarized in Table I. When ATP was added to the enzyme preincubated with Ca\(^{2+}\)/H\(\text{11001}\) otherwise as above, the EP formation was much faster and almost completed within 1 s for all the mutants and wild type; therefore, the rates obtained above actually reflect the rate-limiting \(E_2\) to \(E_1\) transition.

The substitutions of either of the residues of the Glu 123-Arg334 interaction pair reduced the rate of the \(E_2\) to \(E_1\) transition with the larger magnitudes in the Arg 334 mutants (Fig. 4A). In each of Glu 123 and Arg\(^{334}\), the introduction of an opposite charge had a stronger effect than the alanine substitution. The results indicate that the side chains of these residues, especially of Arg\(^{334}\), are important for the rapid \(E_2\) to \(E_1\) transition.

The substitutions in M4/P domain (Arg 324 and Arg\(^{334}\)) and those in M2/A domain (Tyr 122 and Glu 123) both have serious effects, but on the distinct steps. We first determined the fraction of ADP-insensitive EP (\(E_2\)) accumulated at 15 s (nearly the steady state) after the addition of ATP to the enzyme preincubated with Ca\(^{2+}\)/H\(\text{11001}\) (as described in the legend to Fig. 5). In the presence of K\(^{+}\), which accelerates the decay of \(E_2\) and thus causes its low accumulation in the wild type (18% of the total amount of EP under the present conditions; Ref. 21), the fraction of accumulated \(E_2\) was again higher in the Tyr122 and Glu123 mutants and was also at the higher level in the wild type (58% (Y122F), 50% (Y122A), 31% (E123A), 77% (E123K), and 43% (wild type)). In contrast, the fraction of accumulated \(E_2\) in the Arg324 mutants was still low (25% (R324A) and 14% (R324A)), or very low in the Arg\(^{334}\) mutants (5% in R334A and R334E).

**Fig. 2.** Interactions between M2/A domain and M4/P domain. The coordinates for the structures \(E_1\) and \(E_2\) (\(E_2\) stabilized with thapsigargin) were obtained from the Protein Data Bank (accession codes 1EUL and 1IWO, respectively; Refs. 5 and 6). Red arrows in \(E_1\) indicate the direction of movement of the cytoplasmic domains from \(E_1\) to \(E_2\). The Glu\(^{123}\)-Arg\(^{334}\) ionic interaction and the Tyr\(^{122}\)-Arg\(^{334}\) hydrogen bond are present in \(E_1\) but lost in \(E_2\), as indicated.

Changes in Organization of A and P Domains in SERCA1a

---

2204 Changes in Organization of A and P Domains in SERCA1a
Fig. 3. Ca\(^{2+}\) dependence of EP formation from ATP. Microsomes expressing the wild-type or mutant SERCA1a were preincubated with various concentrations of Ca\(^{2+}\) as indicated at 25 °C for 15 min in 50 μl of a mixture containing 2 μg of microsomal protein, 1 μM A23187, 0.1 mM KCl, 7 mM MgCl\(_2\), 50 mM MOPS/Tris (pH 7.0) and various concentrations of CaCl\(_2\) with 0.4 mM EGTA, and then cooled and phosphorylated at 0 °C for 15 s by the addition of a small volume of [γ\(^{-}\)\(^{32}\)P]ATP to give 10 μM. The amount of EP formed was determined as described under “Experimental Procedures.” In A, wild-type (○) and mutants E123A (●), E123K (△), R324A (□), and R324E (△) are indicated. In B, wild-type (○) and mutants Y122F (●), Y122A (△), R324A (□), and R324E (△) are indicated. The data were best-fitted with the Hill equation (EP = EP\(_{max}\)/1 + (K\(_{app}\)/[Ca\(^{2+}\)]\(^n\))), and the EP\(_{max}\) obtained in the fitting in each mutant was normalized to 100%. Broken lines show the least squares fit for the wild type. The dissociation constants for Ca\(^{2+}\) (K\(_{app}\)) and Hill coefficient (n) thus obtained are 0.13 μM and 1.7, respectively, for the wild type, and those for the mutants are almost the same (0.08–0.19 and 1.7–2.2 μM, respectively). The EP\(_{max}\) obtained was comparable in the mutants and wild type, as, in fact, the amounts of EP (the mean ± S.D. (n = 3–4)) determined with the saturating 100 μM Ca\(^{2+}\), were 4.62 ± 0.06 (wild type), 3.93 ± 0.16 (E123A), 3.40 ± 0.09 (E123K), 4.49 ± 0.18 (R334A), 3.89 ± 0.33 (R334E), 3.78 ± 0.07 (Y122F), 3.95 ± 0.26 (Y122A), 5.41 ± 0.05 (R324A), and 5.06 ± 0.13 (R324E).

The results indicate that the E1P to E2P transition in step 4 is inhibited in the Arg\(^{224}\) and Glu\(^{334}\) mutants (especially the Arg\(^{224}\) mutants) but not in the Tyr\(^{222}\) and Glu\(^{123}\) mutants.

We then determined the time course of E2P formation from E1Ca\(_2\) with ATP in the absence (Fig. 5A) and presence (Fig. 5B) of K\(^{+}\) with the Tyr\(^{222}\) and Glu\(^{123}\) mutants, which were shown above to accumulate the large amount of E2P. Under both sets of conditions, the total amount of EP reached its maximum level very rapidly (within ~1 s) and remained unchanged during the experiments (60 s); thus, the time course actually reflects the E2P accumulation from E1P in step 4. In the absence of K\(^{+}\) (Fig. 5A), a large amount of E2P accumulated in the wild type as well as in the mutants, and thus the time courses can be clearly compared. The E2P accumulation apparently proceeded with first-order kinetics (the rates obtained are summarized in Table I). The rate in the mutants was similar (Y122A and Y122F) or even faster (E123A and E123K, by ~3-fold), as compared with that of the wild type. In each of the mutants, which accumulate a fair amount of E2P also in the presence of K\(^{+}\) (Y122F, Y122A, E123K), the apparent rate obtained in the presence of K\(^{+}\) was similar to that obtained in its absence (Fig. 5B and Table I). The substitutions of Tyr\(^{222}\) and Glu\(^{334}\) thus did not inhibit the rapid E1P to E2P transition. The results suggest that the substitutions caused the E2P accumulation because of possible inhibition of the subsequent E2P decay.

For the Arg\(^{224}\) and Arg\(^{334}\) mutants, which do not accumulate E2P but accumulate mostly E1P, the decay of EP formed from ATP was determined in the presence of K\(^{+}\) instead of the E2P formation (Fig. 6A). This EP decay most likely reflects the rate-limiting E1P to E2P transition (as also known for the wild type; Ref. 22). In Fig. 6, the EP decay was determined by first phosphorylating with [γ\(^{-}\)\(^{32}\)P]ATP in the presence of K\(^{+}\) and Ca\(^{2+}\) for 15 s and then terminating phosphorylation by adding excess EGTA to prevent further phosphorylation and thus allow for decay of \(^{32}\)P-labeled EP. The EP decay was markedly slowed by the substitutions, especially of Arg\(^{334}\). The time courses were fitted well with a single exponential, and the rates obtained are summarized in Table I. The rate was reduced in R324A to ~40% of that in the wild type, further reduced to ~20% in R324E and R334A, and almost completely inhibited in R334E (6%). In each of the time points in the EP decay, almost all of the total amount of EP was E1P (data not shown). The results indicate that these two residues on the M4/P domain are important for the rapid E1P to E2P transition in step 4, and that Arg\(^{334}\) is especially crucial.

The same sets of experiments were done with the Tyr\(^{222}\) and Glu\(^{123}\) mutants (Fig. 6B), but in this case, to examine the possible inhibition of processing of E2P after the E1P to E2P transition. The time courses were apparently fitted very well with a single exponential, although a substantial fraction of the total amount of EP was ADP-insensitive in these mutants at the start of the decay reaction (see Fig. 5). The EP decay was almost completely blocked in Y122A and substantially slowed in E123A and E123K (Table I). In Y122F, the rate was similar to that in the wild type.

Hydrolysis of E2P Formed from P\(_{i}\) —To examine hydrolysis of E2P without Ca\(^{2+}\) in the presence of K\(^{+}\) (step 6), the enzyme was first phosphorylated with \(^{32}\)P, in the absence of Ca\(^{2+}\) and K\(^{+}\) and in the presence of 35% (v/v) Me\(_2\)SO, which favors E2P formation (23); then, the phosphorylated samples were diluted at 0 °C with a large volume of solution containing K\(^{+}\) and non-radioactive P\(_{i}\) without Ca\(^{2+}\) (Fig. 7, A and B). Thus, the conditions were made in all other respects identical to those used for the E2P formation from ATP (Fig. 5) and the decay of EP formed from ATP in the presence of Ca\(^{2+}\) (Fig. 6). Hydrolysis of \(^{32}\)P-labeled E2P proceeded with first-order kinetics, and the rates obtained were summarized in Table I. In the Arg\(^{224}\) and Arg\(^{334}\) mutants, the rate of E2P hydrolysis was similar to or even faster (by ~3-fold) than that of wild type. On the other hand, in the Tyr\(^{222}\) and Glu\(^{123}\) mutants, the E2P hydrolysis was markedly slowed (except E123A, which showed the enhanced rate) and almost completely inhibited in Y122A.

Inhibition of Processing of E2P Formed from ATP in Tyr\(^{222}\) and Glu\(^{123}\) Mutants—In the above experiments performed at 0 °C with the mutant Y122A (Figs. 6B and 7B), the decay of EP formed from ATP with Ca\(^{2+}\) and the E2P hydrolysis without Ca\(^{2+}\) were both not detected in the experimental time scale and thus could not be compared with each other. Therefore, we performed the same set of experiments at a higher temperature (25 °C; Fig. 7C) and actually could observe both the EP decay and hydrolysis, which were, again, very strongly inhibited and extremely slow, as compared with those in the wild type (which
were definitely slower than the hydrolysis of E2P without Ca\(^{2+}\).

The rate constants for the partial reaction steps were obtained in the experiments shown in Fig. 4 (formation of EP from E2, i.e. the rate-limiting E2 to E1Ca\(_2\) transition in steps 1-2), Fig. 5 (formation of E2P from ATP, i.e. E1PCa\(_2\), E2PCa\(_2\) in step 4), Fig. 6 (decay of EP formed from ATP), and Fig. 7 (hydrolysis of E2P formed from P, in step 6). The rate constants for the E1PCa\(_2\) formation from ATP (E1Ca\(_2\), E1PCa\(_2\) in step 3) were also determined (see text). In parenthesis, the values obtained with the wild-type are normalized to 100%.

### Table I

| Reaction Step | WT | Y122F | Y122A | E123A | E123K | R324A | R324E | R334A | R334E | Y122A | Y122F |
|---------------|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| E2 to E1Ca\(_2\) | 0.172 (100) | 0.108 (63) | 0.054 (31) | 0.096 (56) | 0.029 (17) | 0.350 (221) | 0.345 (201) | 0.051 (30) | 0.005 (3) | 0.054 (31) | 0.108 (63) |
| E1Ca\(_2\) to E1PCa\(_2\) | 2.46 (100) | 2.47 (101) | 2.85 (116) | 2.13 (87) | 2.64 (107) | 2.22 (90) | 2.5 (102) | 2.92 (119) | 1.97 (80) | 2.85 (116) | 2.47 (101) |
| E1PCa\(_2\) to E2PCa\(_2\) | 0.155 (100) | 0.106 (113) | 0.172 (75) | 0.513 (331) | 0.223 (349) | — | — | — | 0.0239 (41) | 0.0114 (20) | 0.0107 (18) |
| Decay of EP formed from ATP | 0.0578 (100) | 0.0482 (84) | 0.0410 (157) | 0.0257 (39) | 0.2559 (45) | — | — | — | 0.14 (46) | 0.06 (10) | 0.168 (256) |
| Hydrolysis of E2P to E2 | 0.66 (100) | 0.21 (32) | 0.21 (32) | 1.04 (157) | 0.06 (10) | — | — | — | 0.46 (69) | 0.61 (92) | 1.69 (256) |

\(a\) Not determined because E2P accumulation was low.

\(b\) Almost all of EP accumulated was E1P (see text in “Results”).

\(c\) The rate most likely reflects the rate-limiting E1PCa\(_2\) to E2PCa\(_2\) transition (loss of ADP sensitivity) in step 4.

---

**Fig. 4.** Phosphorylation upon simultaneous addition of ATP and Ca\(^{2+}\). Microsomes expressing the wild-type or mutant SERCA1a (2 
\(\mu\)g of microsomal protein) were preincubated in the absence of Ca\(^{2+}\) at 25 °C for 15 min in 50 
\(\mu\)l of a buffer containing 0.8 mM EGTA, 1 
\(\mu\)M A23187, 0.1 M KCl, 7 mM MgCl\(_2\), and 50 mM MES/Tris (pH 6.0) and then cooled to 0 °C. At zero time, 50 
\(\mu\)l of the buffer containing 20 
\(\mu\)M [\(\gamma\)-\(^{32}\)P]ATP and 200 
\(\mu\)M CaCl\(_2\) in place of EGTA were added at 0 °C to the microsome suspension. At various times after this addition, the amount of EP formed was determined. In A, wild type (○) and mutants E123A (○), E123K (△), R334A (□), and R334E (△) are indicated. In B, wild type (○) and mutants Y122F (○), Y122A (△), R324A (□), and R324E (△) are indicated. Solid lines show the least squares fit to a single exponential. The rate constants thus obtained are given in Table I.

---

**Fig. 5.** Time course of E2P accumulation from ATP with the Tyr122 and Glu123 mutants. Microsomes expressing the wild type or the Tyr122 or Glu123 mutants were phosphorylated with [\(\gamma\)-\(^{32}\)P]ATP in the presence of 100 
\(\mu\)M Ca\(^{2+}\) at 0 °C for various periods, which are indicated on the abscissa in 50 
\(\mu\)l of a mixture containing 1 
\(\mu\)g of microsomal protein in the presence of 0.1 M choline-Cl without added KCl (○) or 0.1 M KCl (△); otherwise, they are as described in the legend to Fig. 3. The reaction was quenched with trichloroacetic acid at the indicated time, and the total amount of EP was determined. For determination of E2P, an equal volume (50 
\(\mu\)l) of a mixture containing 10 
\(\mu\)M ADP, 7 mM MgCl\(_2\), 10 mM EGTA, 50 mM MOPS/Tris (pH 7.0), and 0.1 M choline-Cl without added KCl (○) or 0.1 M KCl (△) was added to the above phosphorylation mixture at the indicated time. At 1 s after this addition, the reaction was quenched with trichloroacetic acid. E1P disappeared entirely within 1 s after the addition of ADP. The amount of E2P is shown as a percentage of the total amount of EP. Solid lines show the least squares fit to a single exponential, and the apparent rates to reach the steady-state E2P level thus obtained are given in Table I. Wild type (○) and mutants Y122F (○), Y122A (△), E123A (□), and E123K (△) are indicated.
We also observed that the formation of the ADP-insensitive EP from ATP in Y122A was completed within 1 s after the addition of ATP at 25 °C (data not shown). The results indicate that the step after the loss of ADP-sensitivity (in step 4) but before the E2P hydrolysis without Ca\(^{2+}\) (in step 6) is also strongly inhibited in Y122A. It is noteworthy that the effects of the alanine-substitution of Tyr\(^{122}\) are very similar to those of substitutions of Val\(^{200}\) on the Lys\(^{189}\)-Lys\(^{205}\) loop on the A domain (10), in which both the rate of processing of E2P after the loss of ADP-sensitivity and the rate of E2P hydrolysis are dramatically reduced (but not the rate of loss of ADP-sensitivity).

The kinetic results with the Glu\(^{123}\) mutants showed that the decay of EP formed from ATP is slower than the E2P hydrolysis (step 6) and also substantially slower than the accumulation of E2P from E1P (step 4) in these mutants (Figs. 5–7 and Table I). Especially with the mutant E123K, it was clearly shown in the presence of K\(^+\) that the large amount of E2P accumulation occurred with the much faster rate than that of decay of EP. The results are consistent with the view that the step after the loss of ADP-sensitivity but before the E2P hydrolysis is inhibited in these mutants.

**DISCUSSION**

In the present study, we explored the possible roles of the residues involved in the ionic and hydrogen-bonding interactions present in the crystal structure E1Ca\(_2\) between the top part of M4 in P domain (Arg\(^{324}\) and Arg\(^{334}\) on M4/P domain) and that of M2 (or the loop) connected to A domain (Tyr\(^{122}\) and Glu\(^{123}\) on M2/A domain; Fig. 2). We found that Arg\(^{324}\) (especially) and Glu\(^{123}\) are important for the rapid E2 to E1Ca\(_2\) transition, and that Tyr\(^{122}\) also contributes to some extent. Furthermore, we found the more profound inhibitory effects of the substitutions in each of the two regions on the successive but distinct catalytic steps after the formation of E1P, i.e. the substitutions in M4/P domain (particularly of Arg\(^{324}\)) seriously affect the loss of ADP-sensitivity, and those in the M2/A domain (particularly of Tyr\(^{122}\)) seriously affect the subsequent processing and hydrolysis of E2P. The results likely reflect the distinct structural roles of the residues in the specific steps as discussed in the following, but the interactions seen in the E1Ca\(_2\) structure are not functionally important, as may be deduced from the pattern of functional changes induced by mutations.

Arg\(^{324}\) and Arg\(^{334}\) on M4/P Domain for the E1P to E2P Transition—Because the substitutions of Arg\(^{324}\) and Arg\(^{334}\) inhibited strongly the E1P to E2P transition and, in sharp contrast, those of Tyr\(^{122}\) and Glu\(^{123}\) did not affect them seriously (or rather, they increased the E2P accumulation; Figs. 5–6, Table I, and “Results”), the Tyr\(^{122}\) and Arg\(^{324}\) and Glu\(^{123}\) Arg\(^{334}\) interactions present in E1Ca\(_2\) are likely lost during or before the E1P to E2P transition. In fact, in E2V, which is analogous to E2P (5, 7), the residues in each of the interaction pairs are separated by the relative downward movement of M4/P domain by ~10 residues (Fig. 8A).

With respect to the structural role of Arg\(^{324}\), the strong or almost complete inhibition of the loss of ADP sensitivity by its alanine- and glutamate-substitutions indicates that Arg\(^{324}\), with its positive charge, is crucial for the E1P to E2P transition (step 4), and further, it suggests that such roles may involve interactions with a certain polar or negatively charged residue(s). In E2V, Arg\(^{324}\) in the P domain actually protrudes onto M2 and is in close contact with Asn\(^{111}\) (Fig. 8A), of which alanine-substitution was previously shown to suppress almost completely the activity without inhibiting phosphorylation with ATP (24). (In E2(TG), Arg\(^{324}\) is very close to Asn\(^{114}\), of which substitution was also shown to reduce strongly the activity (24).) Therefore, Arg\(^{324}\) likely moves on M2 from Glu\(^{123}\) to Asn\(^{111}\). For such movement of Arg\(^{324}\), it is of interest that there are several negatively charged (Glu\(^{123}\), Glu\(^{121}\), Glu\(^{117}\), and Glu\(^{113}\)) and polar (Asn\(^{114}\) and Asn\(^{111}\)) residues situated on M2 between Glu\(^{123}\) and Asn\(^{111}\) (Fig. 8A). It is tempting to speculate that those residues on M2 (and possibly the neighboring Glu\(^{44}\), Glu\(^{45}\), and Glu\(^{115}\)) together effectively guide Arg\(^{324}\) to the final interaction site during the large rotation of the A domain in the E1P to E2P transition (hence the limited substitutions of each of the single glutamates on M2 may possibly be compensated for by other glutamates, which, in fact, we found (24)). In any case, the downward movement of Arg\(^{324}\) and its interaction with the new partner (Asn\(^{111}\)) would cause the inclination and large shift of M4/P domain toward the M2/A domain. In fact, P domain and M4/M5, which are directly linked with P domain, are significantly inclined (or bent) forward in E2V (as well as in E2(TG)). The possible interactions of Arg\(^{334}\) with the negatively charged residues may also be important during the E2 to E1Ca\(_2\) transition (moving back from Asn\(^{114}\) (E2(TG)) to Glu\(^{123}\) (E1Ca\(_2\))), because the rate of this transition was also markedly reduced by the Arg\(^{334}\) substitutions (Fig. 4 and Table I).

With respect to Arg\(^{324}\) on M4, the observed inhibition of the
Changes in Organization of A and P Domains in SERCA1a

Hydrolysis of E2P—The results on Tyr122 indicate that the hydrophobic aromatic ring of Tyr122 is critically important for decay and hydrolysis of E2P after the loss of ADP-sensitivity in step 4 (Figs. 5–7). Such a structural role may involve some hydrophobic interactions. In E2V, Tyr122 protrudes into and is situated at the center of the hydrophobic cluster composed of the residues on the P domain (Val105 and Val129), the A domain (Leu119 and Tyr122), and the M2/A domain (Leu139 and Tyr122) (Fig. 8A). The gathering of these residues is obviously realized as a consequence of the large rotation of the A domain and the inclination of the P domain. Importantly, such motions and docking of the A and P domains most likely occur during the E1P to E2P transition in step 4 and are essential for the subsequent processing and hydrolysis of E2P. For the rapid E2P hydrolysis (step 6), the 4′-OH group of Tyr122 also likely contributes to some extent (by the possible hydrogen bond within the A domain (Lys158)), because the rate was appreciably reduced by the removal of the OH group in the mutant Y122F.

With the Glu123 mutants, the kinetic results suggest that the native structure at Glu123 juxtaposed to Tyr122 on M2/A domain is also important for the processing of E2P before its hydrolysis. It is also of interest that the strong block of the hydrophobic aromatic ring of Tyr122/(without Ca2+) was found in E123K, as opposed to the enhanced rate in E123A. Because Glu123 is more exposed to solvent in E2V than in E2(TG) but closer in E2(TG) than in E2V to the Glu10 backbone on the A domain, the conformational changes during the transition from E2P to E2 may possibly involve desolvation at this residue and formation of the interaction within A domain, which is not favored with the introduced positive charge in E123K.

Integrated Picture of Conformational Changes in the E1P to E2P Transition and Subsequent Processing of E2P—The above findings indicate that two distinct structural groups operate in distinct steps during the E1P to E2P transition and subsequent E2P processing (to contribute altogether for the final release of Ca2+). We have also recently found (9, 10) that the Glu42/Ser48 loop is critical for the E1P to E2P transition in step 4 and that the Lys158-Lys160 loop (Val160 loop in Fig. 8) on A domain is essential in the subsequent processing of E2P for the intimate contact of A and P domains. In other previous studies (25–32), several residues were found to be crucial for the E1P to E2P transition. By gathering all these findings, we can speculate and gain an integrated picture of the conformational changes required for the processes to release Ca2+. Although these processes in the wild-type enzyme may occur kinetically as a single step (E1PCa2→E2P+2Ca2+), and the existence of the E2PCa2 intermediate is a controversial issue in the literature on the wild type, the reaction scheme with E2PCa2 (Fig. 1) can be conveniently used in a discussion about our findings upon the distinct functions of the identified residues. It should
be noted that accumulation of $E_2PCa_2$ is a hypothesis and not a fact (as it is impossible to measure $Ca^{2+}/H_1$ binding and dissociation in the expressed enzyme that comprises only $2$–$3\%$ of total proteins in the microsomes).

In the $E1P$ to $E2P$ transition (step 4), the A domain largely rotates and then docks with the P domain, which has inclined forward to the A domain, likely by forming the interactions involving the TGES loop on the A domain (34), the residues surrounding Asp$^{311}$ on the P domain (Asp$^{661}$-Pro$^{663}$, Asp$^{627}$, Asp$^{705}$, Asn$^{706}$, Lys$^{302}$-Asn$^{302}$, or at least some of them), and Arg$^{550}$ on the N domain at the three-domain interface at the central part of the molecule (Fig. 8B, the semitransparent blue cylinder on E2V, an E2P analogue).

The Arg$^{334}$ movement on the M2/A domain and the formation of a hydrophobic cluster with Tyr$^{122}$ during the $E1P$ to $E2P$ transition, the subsequent processing of E2P (Ref. 7; Protein Data Bank accession code 1FQU; Ref. 5) is analogous to E2P (Ref. 7; Protein Data Bank accession code 1FQU; Ref. 5). Red arrows in E1Ca$_2$ indicate the movement of A and P domains to E2V. Several glutamates and asparagines are situated on M2 between Glu$^{123}$ and Glu$^{326}$ and also on the loop connecting the A domain and M1, together forming the negatively charged, groove-like space in E1Ca$_2$, (Glu$^{123}$ just above Tyr$^{122}$ is omitted for simplicity.) The hydrophobic cluster with Tyr$^{122}$ is formed in E2V by the gathering of the residues on the A domain (Ile$^{179}$ and Ile$^{229}$), P domain (Val$^{705}$ and Val$^{726}$), and M2/A domain (Leu$^{179}$ and Tyr$^{122}$), as indicated by the large rotation of the A domain and the inclination of the P domain.

The residues essential for the loss of ADP sensitivity (step 4) are represented in dark blue, with Asp$^{351}$ (phosphorylation site), Arg$^{550}$, and Asn$^{706}$ (32) on the E2V structure in its side view and top view (from the top of the semitransparent cylinder). The residues on the cytoplasmic domains gathered in the central part of the molecule are depicted with the semitransparent blue cylinder, upon which Asp$^{351}$, the $Ca^{2+}$-transport sites, and Gly$^{770}$ on M5 (the pivoting point where M5 and M4 tilt; Ref. 6) are situated. The other residues and regions crucial for the loss of ADP sensitivity, the conserved Gly$^{233}$ (25), the Gly$^{233}$-Pro$^{248}$ loop (33, 35–37), the Glu$^{40}$-Ser$^{48}$ loop (9), and Arg$^{734}$ (this study), are located on the outer side. The interactions between Arg$^{334}$ and the M2/A domain and between the Gly$^{233}$-Pro$^{248}$ loop and the P6 helix are shown with dotted red circles in E2V, side view (left). The residues surrounding Val$^{500}$ on the Val$^{200}$ loop (10) and Tyr$^{122}$ (this study) are most likely essential for the intimate A-P domain contact in the subsequent processing of E2P and are located at the opposite ends of the A-P domain interface, as shown with the dotted blue circles in E2V, top view (right). The forward shift of the whole P domain is due to its inclination toward the A domain (from E1Ca$_2$ to E2V, revealed by manually fitting these structures with M5-M10, which do not move in these structures) and is represented by the movement of Asp$^{351}$ with the dotted orange arrow. The likely rotation of the P domain (by ~25°), another component of the motion of the P domain (from E1Ca$_2$ to E2V), is depicted by the solid orange arrow on the circle. Dotted red arrow indicates the rotation of the A domain.
likely crucial for the large rotation of the A domain and the coordinated unique motions of M1 (upward movement and bending at the membrane surface forming M1') to cause the appropriate domain docking (and possibly contributing to the movement of M3 by van der Waals interactions of M1; Ref. 9). Arg334 on the M4/P domain moves downward to Asn111 on M2 during the E1P to E2P transition and hence contributes to the inclination of the P domain with the top part of M4/M5 and, thus, anchors the inclined P domain on the A domain, as discussed above. The Glu233-Pro248 loop connecting the A domain and M3 also functions to anchor the inclined P domain by the interaction with helix P6 (33). For the appropriate domain docking and anchoring, the positioning of three regions is necessary: the TGES184 loop for docking at the central part, and the other two (Arg334 and the Gly233-Pro248 loop) for docking at the lower and outer parts but separated at each side of the P domain for anchoring in the inclined state (Fig. 8B, dotted red circles). The Glu46-Ser48 loop connected to M1' at the membrane surface may also stabilize such a state.

The movement of the top part of M2 is large as the directly connected A domain rotates; therefore, the interaction of Arg334 upon the M4/P domain with the M2/A domain is likely crucial for the large forward movement of the top part of M4/M5 during the E1P to E2P transition. The previously observed blocking of the E1P to E2P transition by mutations on M4, M5, and M8 (26, 30, 31) is consistent with the view that the transmembrane helices are significantly rearranged with the motions of A and P domains during this transition in step 4, although these movements are likely not enough to open the Ca2+ pathway to lumen.

It should be noted that E1PcA2 has the P and N domains, with their most closed configuration as realized in the E1Ca2+ATP complex and the loops connecting the A domain with M2 and M3 being repositioned close to the P domain (thus, they are ready for the subsequent A domain rotation; Ref. 8). During the E1P to E2P transition, therefore, the P and N domains should be opened to some extent, and, hence, the A domain can rotate in and associate with the P and N domains (thus to interfere with the access of β-phosphate of ADP to the acylphosphate; Ref. 8).

After the loss of ADP sensitivity, the final process of gathering the A and P domains is accomplished (in the subsequent step 5) by the formation of interactions at two distinct regions (Fig. 8B, dotted blue circles), i.e., the ionic interactions of residues surrounding Val200 (Arg198 and Asn201, Glu202, and Asp203 on the Val200 loop) with those on the P domain (Arg62, Glu60, and Arg680 on the basis of E2V; Ref. 10) and the hydrophobic interactions of Tyr222 with P domain in the hydrophobic cluster (present study). These interactions will likely further distort the P domain (further inclination and/or possible rotation; see the following paragraph) and rearrange the transmembrane helices to release Ca2+. The intimate A-P domain contact also likely crucial for the large rotation of the A domain and the twisting of transmembrane helices. In any case, the exquisitely positioning of the two interaction regions (Val200 loop and Tyr222) at each side of the A-P domain interface may be most suitable for appropriately causing the further forward inclination of the P domain, the tilting of M4/M5 and the possible rotation of P domain, and the twisting of transmembrane helices for Ca2+ release.

Acknowledgments—We thank Dr. David H. MacLennan, University of Toronto, for his generous gift of SERCA1a cDNA and Dr. Randal J. Kaufman, Genetics Institute, Cambridge, MA, for his generous gift of the expression vector pMT2. We also thank Dr. Chikashi Toyoshima, University of Tokyo, for helpful discussions.

REFERENCES

1. Hasselbach, W., and Makineno, M. (1961) Biochem. Z. 333, 518–528
2. Khash, S., and Lipmann, F. (1962) J. Biol. Chem. 149, 389–400
3. Møller, J. V., Juul, B., and le Maire, M. (1996) Biochim. Biophys. Acta 1286, 35–51
4. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) J. Biol. Chem. 272, 28815–28818
5. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Nature 405, 647–655
6. Toyoshima, C., and Nomura, H. (2002) Nature 418, 605–611
7. Danko, S., Daito, T., Yamashita, K., Kamidochi, M., Suzuki, H., and Toyoshima, C. (2001) FEBS Lett. 505, 125–133
8. Danko, S., Yamashita, K., Daito, T., Suzuki, H., and Toyoshima, C. (2001) J. Biol. Chem. 276, 9624–9629
9. Kaufman, R. J., Davies, M. V., Pathak, V. K., and Hershey, J. W. B. (1989) Mol. Cell. Biol. 9, 946–958
10. Maruyama, Y., and MacLennan, D. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3314–3318
11. Daito, T., Yamashita, K., Suzuki, H., Saito, T., and Kanazawa, T. (1999) J. Biol. Chem. 274, 23910–23915
12. Kanazawa, T., Saito, M., and Tomonura, Y. (1970) J. Biochem. (Tokyo) 67, 693–711
13. Weber, K., and Oshorn, M. (1969) J. Biol. Chem. 244, 4406–4412
14. Daito, T., Suzuki, H., Yamashita, K., Saito, T., and Kanazawa, T. (1999) FEBS Lett. 444, 54–58
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
16. Humphrey, W., Dalke, A., and Schulten, K. (1996) J. Mol. Graphics 14, 33–38
17. Zhang, Z., Bumbilla, C., Lewis, D., Summers, S., Klein, G. M., and Inesi, G. (1999) J. Biol. Chem. 274, 16290–16298
18. Pick, U., and Karlish, S. D. J. (1992) J. Biol. Chem. 257, 6120–6126
19. Shigekawa, M., and Dougherty, J. P. (1978) J. Biol. Chem. 273, 1451–1457
20. Inesi, G., Kurumizaka, M., and Lewis, D. (1988) Methods Enzymol. 157, 154–190
21. de Mina, L., Martinis, O. B., and Alves, E. W. (1980) Biochemistry 19, 4252–4261
22. Clarke, D. M., Maruyama, K., Lee, T. W., Leberer, E., Inesi, G., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 11246–11251
23. Andersen, J. P., Vilsen, B., Leberer, E., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 21018–21023
24. Vilsen, B., Andersen, J. P., Clarke, D. M., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 21024–21030
25. Clarke, D. M., Lee, T. W., and MacLennan, D. H. (1990) J. Biol. Chem. 265, 14088–14092
26. Clarke, D. M., Lee, T. W., and MacLennan, D. H. (1990) J. Biol. Chem. 265, 22223–22227
27. Vilsen, B., Andersen, J. P., and MacLennan, D. H. (1991) J. Biol. Chem. 266, 16157–16164
28. Vilsen, B., Andersen, J. P., and MacLennan, D. H. (1991) J. Biol. Chem. 266, 18839–18845
29. Rice, W. J., and MacLennan, D. H. (1996) J. Biol. Chem. 271, 31412–31419
30. Ma, H., Inesi, G., and Toyoshima, C. (2003) J. Biol. Chem. 278, 28938–28943
31. Møller, J. V., Lenoir, G., Marchand, C., Montigny, C., le Maire, M., Toyoshima, C., Juul, B. S., and Champsali, P. (2002) J. Biol. Chem. 277, 38647–38659
32. Patchornik, G., Goldshleger, R., and Karlish, S. J. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11954–11959
33. Inamuro, Y., and Kawai, M. (1989) J. Biochem. (Tokyo) 105, 775–781
34. le Maire, M., Lund, S., Veil, A., Champsali, P., and Møller, J. V. (1990) J. Biol. Chem. 265, 1111–1123
35. Juul, B., and Møller, J. V. (2000) Na/K-ATPase and Related ATPases (Taniguchi, K., and Kaya, S., eds) pp. 233–236, Elsevier, Amsterdam, The Netherlands