Roles of Tyr\textsuperscript{122}-hydrophobic Cluster and K\textsuperscript{+} Binding in Ca\textsuperscript{2+}-releasing Process of ADP-insensitive Phosphoenzyme of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase\textsuperscript{(*)}

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Tyr\textsuperscript{122}-hydrophobic cluster (Y122-HC) is an interaction network formed by the top part of the second transmembrane helix and the cytoplasmic actuator and phosphorylation domains of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase. We have previously found that Y122-HC plays critical roles in the processing of ADP-insensitive phosphoenzyme (E2P) after its formation by the isomerization from ADP-sensitive phosphoenzyme (E1PCa\textsubscript{2}) (Wang, G., Yamasaki, K., Daiho, T., and Suzuki, H. (2005) J. Biol. Chem. 280, 26508–26516). Here, we further explored kinetic properties of the alanine-substitution mutants of Y122-HC to examine roles of Y122-HC for Ca\textsuperscript{2+} release process in E2P. In the steady state, the amount of E2P decreased so that of E1PCa\textsubscript{2} increased with increasing luminal Ca\textsuperscript{2+} concentration in the mutants with $K_{0.5}$ 110–320 μM at pH 7.3. These luminal Ca\textsuperscript{2+} affinities in E2P agreed with those estimated from the forward and lumenal Ca\textsuperscript{2+} when K\textsuperscript{+} was omitted from the medium of the wild type, the propensities further indicated that the rates of luminal Ca\textsuperscript{2+} access and binding to the transport sites of E2P were substantially slowed by the mutations. Therefore, the proper formation of Y122-HC and resulting compactly organized structure are critical for both decreasing Ca\textsuperscript{2+} affinity and opening the luminal gate, thus for Ca\textsuperscript{2+} release from E2PCa\textsubscript{2}. Interestingly, when K\textsuperscript{+} was omitted from the medium of the wild type, the properties of the wild type became similar to those of Y122-HC mutants. K\textsuperscript{+} binding likely functions via producing the compactly organized structure, in this sense, similarly to Y122-HC.

Sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA1a)\textsuperscript{2} of the P-type ion-transporting ATPase family catalyzes Ca\textsuperscript{2+} transport coupled with ATP hydrolysis from the cytoplasm to lumen against a concentration gradient of ~10,000-fold (1–8). In the initial steps (steps 1 and 2 in Scheme 1), the enzyme is activated by binding of two cytoplasmic Ca\textsuperscript{2+} ions at the transport sites with a submicromolar high affinity (E2 to E1Ca\textsubscript{2}). The activated enzyme is then auto-phosphorylated at Asp\textsuperscript{351} by ATP and forms a phosphoenzyme intermediate (EP) (step 3), thereby the bound Ca\textsuperscript{2+} ions are occluded in the transport sites. This EP is rapidly dephosphorylated by ADP in the reverse reaction reproducing ATP, therefore “ADP-sensitive EP” (E1P). In the next step (step 4), E1PCa\textsubscript{2} is isomerized to the ADP-insensitive form, E2PCa\textsubscript{2}. Upon this change at the catalytic site, the Ca\textsuperscript{2+} sites are deoccluded and opened to the luminal side, and the Ca\textsuperscript{2+} affinity is largely reduced, releasing the bound Ca\textsuperscript{2+} ions into the lumen (step 5). The Ca\textsuperscript{2+} release process is thought to be very rapid with the wild-type Ca\textsuperscript{2+}-ATPase, and the accumulation of E2PCa\textsubscript{2} intermediate had actually never been found until we recently identified and trapped successfully this intermediate by a mutation study (9). In the final step, the Asp\textsuperscript{351}-acylphosphate of E2P is hydrolyzed to reproduce the dephosphorylated and inactive E2 form (step 6). The transport cycle is totally reversible, e.g. E2P can be formed from E2 by P\textsubscript{i} in the absence of Ca\textsuperscript{2+}, and the subsequent luminal Ca\textsuperscript{2+} binding to E2P produces E1PCa\textsubscript{2}.

Three-dimensional structures in several intermediate states and their analogs have been solved (10–18). The Ca\textsuperscript{2+}-ATPase has three cytoplasmic domains, P (phosphorylation), N (nucleotide binding), and A (actuator or anchor), and ten transmembrane helices (M1–M10). The two Ca\textsuperscript{2+} binding sites consist of residues on M4, M5, M6, and M8 (10). The P domain possesses the phosphorylation site (Asp\textsuperscript{351}) and is directly linked to the long helices M4 and M5. The ATP binding site is on the N domain connected to the P domain. The A domain is linked to M1, M2, and M3 via the A/M1-, A/M2-, and A/M3-linkers. The cytoplasmic three domains largely move and change their organization states during the Ca\textsuperscript{2+}-transport cycle (19–21), and these changes are linked with the rearrangements in the transmembrane helices for the Ca\textsuperscript{2+} transport. As a most remarkable change, in the EP isomerization (loss of ADP sensitivity) and Ca\textsuperscript{2+} release, the A domain largely rotates and the P domain largely inclines toward the A domain, and these domains produce their tight association (see Fig. 1 for the change E1Ca\textsubscript{2}·AlF\textsubscript{4}·ADP → E2·MgF\textsubscript{2} as the model for the overall process E1·PCa\textsubscript{2}·ADP → E2·P\textsubscript{i}, including the EP isomerization and Ca\textsuperscript{2+} release). These structural changes therefore involve distinct events in distinct regions, yet they

\* This work was supported by a grant-in-aid for scientific research (C) (to K. Y.) and (B) (to H. S.) from the Ministry of Education, Culture, Sports, Science and Technology of 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.

\* The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

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\* The abbreviations used are: SERCA1a, adult fast-twitch skeletal muscle sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase; EP, phosphoenzyme; E1P, ADP-sensitive phosphoenzyme; E2P, ADP-insensitive phosphoenzyme; MOPS, 3-(N-morpholino)propanesulfonic acid; TG, thapsigargin; Y122-HC, Tyr\textsuperscript{122}-hydrophobic cluster.
Tyr122-hydrophobic Cluster of SERCA1a for Ca2+ Release

FIGURE 1. Structure of SERCA1a and formation of Tyr122-hydrophobic cluster. The coordinates for the structures E1Ca2-AlF4-ADP (the analog for the transition state of the phosphoryl transfer E1-PCa2-ADP, left panel) and E2Mg2P (E2P, analog (21), right panel) of Ca2+-ATPase were obtained from the Protein Data Bank (PDB accession codes 1T5T and 1WPG, respectively (12, 13)). In these Y122-HC mutants, the high affinity binding of ADP to E2P, produced by P2, without Ca2+. Thus the observation is consistent with the involvement of Y122-HC in the Ca2+ release process from E2PCa2.

In the present study, to further clarify roles of Y122-HC in the Ca2+ deocclusion/release processes and thus in the long range communication between the cytoplasmic and transmembrane regions, we explored kinetic features of the alanine-substitution mutants of Y122-HC. The results revealed that the mutations cause a marked increase in the apparent affinity for E2P for luminal Ca2+ and also a substantial retardation of the luminal Ca2+ access to E2P. Therefore, the formation of Y122-HC is critical for decreasing the affinity for Ca2+, for luminal gating (opening of the release pathway), and thus for Ca2+ release into lumen. Importantly, the assembling manner of the seven residues in Y122-HC in the recently revealed crystal structure E2BeF3 (17, 18) somewhat differs from that in E2AlF4 and E2Mg2P. Therefore, we discussed the significance of this difference in terms of the possible sequential gathering of the seven residues into Y122-HC on the basis of the observed difference in the extents of their mutational effects. In addition, we found with the wild type that its kinetic behavior became similar to that of Y122-HC mutants when K+ was omitted from the medium of the wild type. Results revealed for the first time the critical role of K+ binding in the wild type for Ca2+ deocclusion/release from E2PCa2.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression—Mutations were created by the QuikChange™ site-directed mutagenesis kit (Stratagene) and plasmid pGEM7-Zf (+) or pGEM3-Zf (+) (Promega, Madison, WI) containing Apal-KpnI or KpnI-Sall fragments of rabbit SERCA1a cDNA as a template. The Apal-KpnI or KpnI-Sall fragments were then excised from the products and used to replace the corresponding region in the full-length SERCA1a cDNA by the elongation of the A/M1-linker with two or more amino acid insertions (9). In the elongation mutants, Y122-HC is not formed properly yet in E2PCa2 trapped, but it is properly formed in the Ca2+-released form of E2P produced by P2 without Ca2+. Thus the observation is consistent with the involvement of Y122-HC in the Ca2+ release process from E2PCa2.

are coordinated; namely 1) the loss of ADP sensitivity at the cytoplasmic region, 2) the decrease in the Ca2+ affinity at the transmembrane region, and 3) the opening of the Ca2+ releasing pathway (lumenal gating).

Recently, we found that mutations in a specific hydrophobic interaction network, “Tyr122-hydrophobic cluster” (Y122-HC), at the A-P domain interface disrupt markedly the processing of ADP-insensitive EP formed from ATP with Ca2+ and also the hydrolysis of E2P formed from P2 without Ca2+, thus causing nearly complete inhibition of the Ca2+-ATPase activity (22, 23). In these Y122-HC mutants, the high affinity binding of cytoplasmic Ca2+, the resulting E1-PCa2, and the loss of the ADP sensitivity were all found to occur normally as in the wild type (22, 23). Y122-HC is formed by gathering of the seven residues of the three regions upon their motions; i.e. the largely rotated A domain (Ile179, Leu180, and Ile232), the inclined P domain (Val705 and Val726), and the top part of the largely inclined M2 (or the A/M2-linker) (Leu19 and Tyr122). Thus Y122-HC produces the compactly organized structure of E2P. Our previous analyses indicate that, in the Y122-HC mutants, there is a kinetic limit after the loss of ADP sensitivity and before the hydrolysis of the Ca2+-free E2P, therefore the Ca2+ release from E2PCa2 is likely retarded (22, 23). Almost the same kinetic results were found with the mutations in another A-P domain interaction network at the Val200 loop of the A domain (24). Notably, E2PCa2, the ADP-insensitive EP with two Ca2+ ions occluded at the transport sites was recently identified and trapped successfully by the elongation of the A/M1-linker with two or more amino acid insertions (9). In the elongation mutants, Y122-HC is not formed properly yet in E2PCa2 trapped, but it is properly formed in the Ca2+-released form of E2P produced by P2 without Ca2+. Thus the observation is consistent with the involvement of Y122-HC in the Ca2+ release process from E2PCa2.

E1-PCa2 (E1Ca2AlF4-ADP) E2P2 (E2Mg2P2)

SCHEME 1
protein, 0.1 mM \([\gamma-32P]ATP, 3 \mu M A23187, 0.1 \mu M KCl, 7 \mu M MgCl_2\), various concentrations of CaCl_2 up to 3 mM, 0.01 mM EGTA, and 50 mM MOPS/Tris (pH 7.3).

Formation and Hydrolysis of EP—Phosphorylation of SERCA1a in microsomes with \([\gamma-32P]ATP or 32P_2\), and dephosphorylation of \(32P\)-labeled SERCA1a were performed under conditions described in the figure legends. The reactions were quenched with ice-cold trichloroacetic acid containing P_i. Rapid kinetics measurements of phosphorylation and dephosphorylation were performed with a handmade rapid mixing apparatus (27), otherwise the method was as above. The precipitated proteins were separated at pH 6.0 by 5% SDS-PAGE, according to Weber and Osborn (28). The radioactivity associated with the separated Ca^{2+}-ATPase was quantitated by digital autoradiography as described previously (29). The amount of EP formed with the expressed SERCA1a was obtained by subtracting the background radioactivity with the control microsomes. This background was <1% of the radioactivity of EP formed with the expressed wild-type SERCA1a.

Miscellaneous—Protein concentrations were determined by the method of Lowry et al. (30) with bovine serum albumin as the standard. Free Ca^{2+} concentrations were calculated by the Calcon program. Data were analyzed by nonlinear regression using the program Origin (MicroCal Software, Inc., Northampton, MA). Three-dimensional models of the enzyme were reproduced by using the program VMD (31).

RESULTS

Ca^{2+}-induced Change in Accumulation of ADP-insensitive EP in the Presence of 0.1 mM K^+ at Steady State—We first determined the steady state Ca^{2+}-ATPase activity in the presence of increasing Ca^{2+} and ionophore A23187 with the alanine-substitution mutants of the seven residues of Y122-HC and the wild type. The Ca^{2+}-ATPase activity was nearly completely inhibited in all the mutants in agreement with our previous observation (22, 23), and the complete inhibition was found at all the Ca^{2+} concentrations examined (see supplemental Fig. S1 for the representative mutant Y122A). Thus the possible luminal Ca^{2+} effect was not revealed by this type of measurements. Therefore in Fig. 2, to assess the affinity of the luminal-oriented Ca^{2+} transport site of E2P (known as the low affinity sites with the mm over \(\sim 10 \mu M K_d\) value), the amounts of ADP-insensitive EP were determined with the representative mutant Y122A at steady state at various Ca^{2+} concentrations and pH values in the presence of A23187 and KCl. The total amounts of EP (ADP-sensitive EP plus ADP-insensitive EP) were nearly the same under all the sets of conditions.

In the mutant Y122A, the fraction of the ADP-insensitive EP was very high at the low Ca^{2+} concentrations at all pHs (Fig. 2). This agrees with the property of this mutant (22, 23) that the hydrolysis of E2P is nearly completely inhibited thus causing its accumulation. The fraction of ADP-insensitive EP in the mutant markedly decreased, and it was converted to the ADP-insensitive EP with increasing Ca^{2+} concentration at several tens of micromolar to the sub-millimolar range. The apparent Ca^{2+} affinity in this Ca^{2+}-induced change increased with increasing pH, and the Hill coefficients were found to be 2 in all pH values (see the legend to Fig. 2). In the wild type, the fraction of ADP-insensitive EP was low at pH 7.3 and 7.8 being \(<10\%\) or less, and was a significant level, 35% at pH 6.8 (supplemental Fig. S2). These levels were not changed at 1 \(\mu M\) to 3 \(\mu M\) Ca^{2+}. Consistently, the luminal Ca^{2+} affinity of E2P of the wild type is known to be in the millimolar to 10 mm range (see Ref. 32–34). The results suggested that the luminal Ca^{2+} affinity of transport sites of E2P in the mutant may be significantly higher than that in the wild type.

Time Courses of Forward and Ca^{2+}-induced Reverse Conversions between E1P-Ca_2 and E2P—In Fig. 3 with Y122A, the ADP-insensitive EP, and the ADP-sensitive EP was first accumulated at steady state at 10 \(\mu M\) Ca^{2+} and 1 \(\mu M\) Ca^{2+}, respectively, at pH 7.3. Then the Ca^{2+} concentration jump was made from 10 \(\mu M\) to 1 \(\mu M\) or from 1 \(\mu M\) to 80 \(\mu M\), and the change in the fraction of the ADP-insensitive EP was followed. Because the hydrolysis of E2P was nearly completely blocked in Y122A (with the rate \(< 0.01 \text{s}^{-1}\) (22, 23), the time courses represent the forward and reverse isomerization between E1P-Ca_2 and E2P. When Ca^{2+} was increased from 10 \(\mu M\) to 1 \(\mu M\), the fraction of ADP-insensitive EP rapidly decreased from 80% to 10% (i.e. it was converted to the ADP-sensitive EP) with a rate 0.4 \text{s}^{-1}. On the other hand, when the Ca^{2+} concentration was decreased from 1 \(\mu M\) to 80 \(\mu M\), i.e. virtually Ca^{2+} was removed, the ADP-sensitive EP was converted to the ADP-insensitive EP with a rate 0.022 \text{s}^{-1}.

Effect of Ca^{2+} Ionophore A23187 in Accumulation of ADP-insensitive EP—To ascertain that the Ca^{2+}-dependent changes in the fraction of ADP-insensitive EP (Figs. 2 and 3) are caused by luminal Ca^{2+}, we examined also in the absence of A23187


FIGURE 3. Time course of the change in the fraction of ADP-insensitive EP upon Ca\(^{2+}\)-concentration jump. Microsomes expressing the mutant Y122A (20 \(\mu\)g/ml) were phosphorylated with \([\gamma-\text{P}]\text{ATP}\) at pH 7.3 for 5 min in the presence of 10 \(\mu\)M (○) or 1 mM CaCl\(_2\) (●) without EGTA in the phosphorylation solution otherwise as described in Fig. 2. Then an equal volume of the solution containing 2 mM CaCl\(_2\) or 2 mM EGTA (otherwise as in the phosphorylation solution) was added to give final free Ca\(^{2+}\) concentrations 1 mM and 80 nM, respectively. At the indicated times after this Ca\(^{2+}\) jump, the total amount of EP and the amount of ADP-insensitive EP was determined as in Fig. 2. Solid lines show the least squares fit to a single exponential. The total amount of EP was not changed during the period of observation in both cases (data not shown). The amount of ADP-insensitive EP is shown as a percentage of the total amount of EP.

FIGURE 4. Effect of calcium ionophore on the Ca\(^{2+}\) dependence of ADP-insensitive EP accumulation in the steady state. Microsomes expressing the wild type and Y122A but here in the absence of K\(^{+}\) were phosphorylated with \([\gamma-\text{P}]\text{ATP}\) at pH 7.3 for 5 min in the medium containing 50 mM MOPS/Tris (pH 7.3), 0.1M KCl, 7 mM MgCl\(_2\), 2 mM EGTA, and various concentrations of CaCl\(_2\) (20/11001/11545). Then an equal volume of a solution containing 2 mM CaCl\(_2\) or 2 mM EGTA (otherwise as described in Fig. 2) for unknown reasons. The results showed that all these Y122-HC mutants possess the lumenally oriented transport sites. The fitting parameters, including the apparent Ca\(^{2+}\) affinity and the Hill coefficient, thus obtained are listed in Table 1.

FIGURE 5. Ca\(^{2+}\) dependence of accumulation of ADP-insensitive EP in mutants for Tyr\(^{122}\)-hydrophobic cluster. Microsomes expressing each of the seven Y122-HC mutants (indicated in the figure) were phosphorylated with \([\gamma-\text{P}]\text{ATP}\) at various concentrations of CaCl\(_2\) and pH 7.3, otherwise as described in Fig. 2. Solid lines show the least squares fit to the Hill equation. The fitting parameters, including the apparent Ca\(^{2+}\) affinity and the Hill coefficient, thus obtained are listed in Table 1.

The maximal level of the ADP-insensitive EP of L180A at low Ca\(^{2+}\) concentrations (44% of total amount of EP) was significantly lower than those of the other mutants. It may be due to the significantly faster EP hydrolysis rate in L180A as compared with the rates in the others, as shown in supplementary Fig. S4.
the wild type is markedly slowed, and therefore the ADP-insensitive \( E_P \) significantly accumulates. The fraction of ADP-insensitive \( E_P \) in the wild type in the absence of \( K^+ \) decreased with increasing \( Ca^{2+} \) concentration as in Y122A with the Hill coefficient \( \sim 2 \). The apparent affinity for luminal \( Ca^{2+} \) increased with increasing pH in the wild type as in Y122A. The pH-dependent changes are consistent with the fact that the residues for \( Ca^{2+} \) ligation at the transport sites are also involved in the proton binding (and its counter transport); the observed \( Ca^{2+} \)-induced changes reflect the \( Ca^{2+} \) binding to the luminal oriented transport sites of \( E_{2P} \). At each pH, the affinity of the wild type was similar to or slightly lower than that of Y122A. Thus in the absence of \( 0.1 \) \( M \) \( K^+ \), the property of the wild type became similar to that of Y122A. In Y122A, elimination of \( K^+ \) exhibited no significant effect on the apparent affinity for luminal \( Ca^{2+} \) (cf. Fig. 2).

The observed effect of \( K^+ \) on the wild type is probably due to its binding in the cytoplasmic region. In crystallographic as well as mutational studies (12, 37), the \( K^+ \) binding site of the \( Ca^{2+} \)-ATPase was identified to be in the cytoplasmic region but not in the luminal or transmembrane regions (see Fig. 11). Actually, we found experimentally that, when \( K^+ \) at \( 0.1 \) \( M \) was added without any \( K^+ \) ionophore to the \( Ca^{2+} \)-ATPase in SR vesicles phosphorylated in the absence of \( K^+ \), the \( Ca^{2+} \)-dependence of the ADP-insensitive \( E_P \) fraction observed as in Fig. 6A became immediately (within 10 s after the \( K^+ \) addition) that in the presence of \( 0.1 \) \( M \) \( K^+ \) as in supplemental Fig. S2 (data not shown).

**Kinetics of Lumenal \( Ca^{2+} \)-induced \( E_{2P} \) to \( E_{1PCa^2} \) Reverse Transition Followed by Its ADP-induced Rapid Decay to \( E_{1Ca^2} \) in the Presence of \( 0.1 \) \( M \) \( K^+ \)—Then with the representative mutant Y122A, we explored kinetically the luminal \( Ca^{2+} \) accessibility to the luminal oriented transport sites of \( E_{2P} \) formed from \( P \) with \( Ca^{2+} \) and the resulting luminal \( Ca^{2+} \)-induced \( E_{2P} \) to \( E_{1PCa^2} \) reverse transition. In Fig. 7, we included ADP and thereby followed the \( Ca^{2+} \)- and ADP-induced decay of \( E_{2P} \) to \( E_{1Ca^2} \) via \( E_{1PCa^2} \) in the reverse reaction. The \( E_{2P} \) hydrolysis in the absence of \( Ca^{2+} \) was extremely slow (as previously demonstrated with the Y122-HC mutants (22, 23)), and the \( E_{2P} \) decay was dramatically accelerated by the addition of \( Ca^{2+} \) and ADP (Fig. 7A). For example, the rate in the presence of \( 1 \) \( mM \) \( Ca^{2+} \) was 200-times faster than that of the forward \( E_{2P} \) hydrolysis in the absence of \( Ca^{2+} \). ADP alone without \( Ca^{2+} \) or \( Ca^{2+} \) alone without ADP did not accelerate the \( E_P \) decay (data not shown). As shown in Fig. 3, the increase of \( Ca^{2+} \) to \( 1 \) \( mM \) converted the ADP-insensitive \( E_P \) (\( E_{2P} \)) to the ADP-sensitive one (\( E_{1PCa^2} \)), and \( E_{1PCa^2} \) thus formed was not decomposed in the absence of ADP. Therefore the \( Ca^{2+} \)- and ADP-induced decay of \( E_{2P} \) in Fig. 7A obviously occurred in the reverse reaction by the luminal \( Ca^{2+} \) binding: \( E_{2P} + 2Ca^{2+} \rightarrow E_{1PCa^2} \), then \( E_{1PCa^2} + ADP \rightarrow E_{1Ca^2} + ATP \) (Scheme 1). This view agrees with the previous demonstration with SR \( Ca^{2+} \)-ATPase (34). The rate of the \( E_P \) decay in the presence of \( Ca^{2+} \) and ADP increased almost linearly with increasing \( Ca^{2+} \) concentrations and was not saturated even at 3 \( mM \) (Fig. 7B).4 Here, note that

4 Its slope was approximately 0.2 \( s^{-1} \) \( mM^{-1} \) at 0–2 \( mM \) \( Ca^{2+} \). In Fig. 3, the rate of the forward \( E_{1PCa^2} \) to \( E_{2P} \) conversion was estimated to be 0.02 \( s^{-1} \). Therefore the calculation with these values, 0.02 \( s^{-1} \) divided by

**TABLE 1**

Parameters obtained for \( Ca^{2+} \) dependence of accumulation of ADP-insensitive \( E_P \) in Y122-HC mutants

As shown in Fig. 5, the luminal \( Ca^{2+} \)-induced change in the steady-state accumulation of ADP-insensitive \( E_P \) of the seven Y122-HC mutants in the presence of 0.1 \( M \) \( K^+ \) were fitted to the Hill equation. The parameters thus obtained by the least squares fit are listed here. \( K_{0.5} \) is the \( Ca^{2+} \) concentration giving the half-maximum change in the fraction of ADP-insensitive \( E_P \) among the total amount of \( E_P \), therefore the apparent affinity for luminal \( Ca^{2+} \). The highest fraction of the ADP-insensitive \( E_P \) at the low \( Ca^{2+} \) concentration range (0–10 \( \mu M \)) and its lowest fraction at the high \( Ca^{2+} \) concentration range (over ~100 \( \mu M \)) are also listed as the obtained parameters in the fitting (see Fig. 5). The value \( n_H \) is the Hill coefficient.

| Mutant    | \( \% \) of total amount of \( E_P \) | \( K_{0.5} \) | \( n_H \) |
|-----------|-----------------------------------|--------------|----------|
| High \( Ca^{2+} \) | Low \( Ca^{2+} \) |                          |
| L119A     | 11 78 86 12 92 8 90 78 27 80 | 0.12 1.6 0.25 1.6 0.11 1.6 0.32 2.0 0.27 1.6 | 2.1 1.9 2.3 1.6 2.0 |

**FIGURE 6.** \( Ca^{2+} \) dependence of accumulation of ADP-insensitive \( E_P \) in the absence of \( K^+ \). Microsomes expressing the wild type (A) or Y122A (B) SERCA1a were phosphorylated with \( [\gamma-32P]ATP \) at various \( Ca^{2+} \) concentrations and pH (6.8 (○), 7.3 (●), and 7.8 (△)) in the presence of 0.1 \( M \) LiCl in place of KCl otherwise under exactly the same conditions as those described in Fig. 2. The amount of ADP-insensitive \( E_P \) was determined by addition of the ADP solution that contains 0.1 \( M \) LiCl in place of KCl otherwise as in Fig. 2. The total amount of \( E_P \) was nearly constant under all the conditions (data not shown). The amount of ADP-insensitive \( E_P \) is shown as a percentage of the total amount of \( E_P \). Solid lines show the least squares fit to the Hill equation. Apparent \( Ca^{2+} \) affinities and Hill coefficients thus obtained with the wild type (panel A) were 930 \( \mu M \) and 1.8 (pH 6.8), 400 \( \mu M \) and 1.5 (pH 7.3), and 310 \( \mu M \) and 1.9 (pH 7.8), and those obtained with Y122A (panel B) were 1000 \( \mu M \) and 1.9 (pH 6.8), 220 \( \mu M \) and 2.2 (pH 7.3), and 130 \( \mu M \) and 1.7 (pH 7.8).
The Ca\(^{2+}\) - and ADP-dependent acceleration of the reverse E2P decay was assayed also with all the other Y122-HC mutants (supplemental Fig. S3). The rates of the reverse E2P decay increased almost linearly with increasing Ca\(^{2+}\) concentrations even at 3 mM, except those of I232A and V705A over \(-1\) mM Ca\(^{2+}\). Nevertheless, the slope of the Ca\(^{2+}\) dependence below 1 mM Ca\(^{2+}\) was estimated to be \(-0.2\) s\(^{-1}\)mM\(^{-1}\) in all the mutants as in Y122A. Therefore, the rate of the luminal Ca\(^{2+}\) access and binding to the transport sites is similar in all the mutants of Y122- HC.

**Kinetics of Lumenal Ca\(^{2+}\) Access to E2P of Wild Type and Y122A with and without K\(^{+}\)**—Then in Fig. 8, with the wild type and the representative mutant Y122A in the presence and absence of 0.1 mM K\(^{+}\), we analyzed the Ca\(^{2+}\) - and ADP-dependent acceleration of the reverse decay of E2P formed from P\(_i\) without Ca\(^{2+}\). As the well characterized property of the wild type, the forward hydrolysis of E2P without bound Ca\(^{2+}\) is very slow in the absence of K\(^{+}\), but markedly accelerated and thus very rapid in the presence of 0.1 mM K\(^{+}\) (35, 36) (see the rates without Ca\(^{2+}\) in Fig. 8A). Nevertheless, even with the wild type in the presence of K\(^{+}\), we observed an apparently single exponential decay of E2P after the addition of Ca\(^{2+}\) and ADP at all the Ca\(^{2+}\) concentrations examined (time courses are not shown for simplicity). This is consistent with the kinetics described in the textbook by Fersht (39) that, in the parallel reactions in which a compound undergoes two or more single-step reactions simultaneously, its disappearance rate is described by a single exponential decay. In our case, the two reactions are the forward E2P hydrolysis and the Ca\(^{2+}\)-/ADP-induced reverse E2P decay. The single decay rates thus obtained are plotted in Fig. 8A.

In the wild type in the presence of 0.1 mM K\(^{+}\), the Ca\(^{2+}\) dependence of the E2P decay rate was complicated because of the rapid E2P hydrolysis without Ca\(^{2+}\) (\(-0.4\) s\(^{-1}\)), no change in the rate at 0–0.6 mM Ca\(^{2+}\), and the gradual increase above 0.6 mM. On the other hand, in the wild type in the absence of K\(^{+}\), in which the E2P hydrolysis without bound Ca\(^{2+}\) is markedly slowed, the nearly linear increase in the rate of Ca\(^{2+}\)-/ADP-induced reverse E2P decay was observed at least up to \(-3\) mM Ca\(^{2+}\) as in the Y122- HC mutants in the presence of K\(^{+}\). The slope of the wild type without K\(^{+}\) was actually close to that of Y122A with K\(^{+}\). Therefore, the rate of luminal Ca\(^{2+}\) access and binding to the transport sites of E2P of the wild type in the absence of K\(^{+}\) is similar to that of Y122A. With the wild type in the presence of K\(^{+}\), evaluation of the luminal Ca\(^{2+}\) access rate by this approach was not possible because of the complicated Ca\(^{2+}\)-dependence curve. In Y122A, little effect was seen when K\(^{+}\) was omitted at 0–1 mM Ca\(^{2+}\), although the slope became gradually less steep at the higher Ca\(^{2+}\) concentration in the absence of K\(^{+}\).

In Fig. 8B, by using the rates of the E2P decay in the presence of added Ca\(^{2+}\) and ADP (determined in Fig. 8A) and the rates of
the fact that nearly all the phosphorylation sites are phosphorylated at steady state (in either E1P or E2P form) under the conditions used for the steady-state and kinetic analyses at all the Ca\(^{2+}\) concentrations in this study. Namely, the E2 to E1Ca\(_2\) transition and the E1PCa\(_2\) formation from E1Ca\(_2\) with ATP are rapid enough to be ignored from the simulation. Therefore, the fraction of ADP-insensitive EP (E2P) in the steady state will be determined by the rate of its formation in the forward E1PCa\(_2\) to E2P transition, \(v_1\), and by the rate of its decay, \(v_2\), that includes both the forward hydrolysis of Ca\(^{2+}\)-unbound E2P to E2 and the Ca\(^{2+}\)-induced reverse transition to E1PCa\(_2\) with the subsequent ADP-induced decay. This means that the simulation can be made even with the wild type in the presence of K\(^+\) (as \(v_2\) can include the forward E2P hydrolysis). In the steady-state conditions, the decay rate \(v_2\) and formation rate \(v_1\) should be equal, therefore the fraction of ADP-insensitive EP (F\(_{E2P}\)) in the total amount of EP will be estimated by an equation: F\(_{E2P}\) = \(v_1 / (v_1 + v_2)\). Here, the E2P decay rate \(v_2\) was obtained in Fig. 8A at each Ca\(^{2+}\) concentration. The E1PCa\(_2\) to E2P transition rate \(v_2\) was estimated from the Ca\(^{2+}\)-unbound E2P to E2 jump experiments from high (1 mM) to low (80 nM) virtually Ca\(^{2+}\)-removal for Y122A with 0.1 mM K\(^+\) or Li\(^+\) (without K\(^+\)) and for the wild type with 0.1 mM Li\(^+\) (without K\(^+\)). For the wild type with 0.1 mM K\(^+\), the forward decay rate of E1PCa\(_2\) formed from ATP was used as \(v_1\), because the E1PCa\(_2\) to E2P transition (the loss of ADP sensitivity) is rate-limiting for the E1PCa\(_2\) decay via E2P and its hydrolysis.

The Ca\(^{2+}\)-dependent curves thus obtained by the simulation for the steady-state level of ADP-insensitive EP for Y122A with and without K\(^+\) and the wild type without K\(^+\) agreed very well with the respective ones determined at the steady state (cf. Figs. 2 (with K\(^+\)) and 6 (without K\(^+\)) at pH 7.3). The affinities for luminal Ca\(^{2+}\) estimated from the simulated curves are in fact almost the same as those actually determined at steady state (Table 2). The agreements assure the validity of the simulation and further allow us to estimate the luminal Ca\(^{2+}\) affinity of E2P of the wild type in the presence of K\(^+\) (open circles and inset in Fig. 8B), the fraction of ADP-insensitive EP was very low, and the extent of its change was extremely small as expected from the

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**Table 2**

Affinities of E2P for luminal Ca\(^{2+}\) estimated by kinetic analyses and those determined at steady-state analyses

| K\(^+\) (0.1 mM) | Estimated by kinetic analyses | Determined by steady-state analyses |
|-----------------|-----------------------------|-----------------------------------|
|                 | \(K_{0.5}\) | \(n_H\) | \(K_{0.5}\) | \(n_H\) |
| WT (+)          | 1.48 | 2.2 | \(\text{Not determined}\) |
| WT (+)          | 0.45 | 1.7 | 0.40 | 1.5 |
| WT (+)          | 0.15 | 1.9 | 0.16 | 1.9 |
| WT (+)          | 0.22 | 1.7 | 0.22 | 2.2 |

\(\text{Not determined because the accumulation of ADP-insensitive EP was very low at all the Ca}^{2+}\text{concentrations examined, and therefore possible change was not revealed.}\)
steady-state measurements (cf. supplemental Fig. S2 (pH 7.3)). The apparent affinity of wild type for lumenal Ca\textsuperscript{2+} in the presence of K\textsuperscript{+} was thus estimated by the small change to be 1.5 mM (see Table 2). This affinity was ~3.5-times lower than that of wild type without K\textsuperscript{+} and 10-times lower than that of Y122A with and without K\textsuperscript{+}. Thus, by omitting K\textsuperscript{+}, the lumenal Ca\textsuperscript{2+} affinity of E2P in the wild type became higher and similar to that in Y122A.

**Kinetics of Lumenal Ca\textsuperscript{2+}-induced E2P to E1PCa\textsubscript{2} Reverse Transition of E2P of Wild Type in the Presence of 0.1 mM K\textsuperscript{+} Was Revealed by the Absence of ADP—Unfortunately, in the above experimental design and approach of Fig. 8A, we were not able to estimate the lumenal Ca\textsuperscript{2+} access rate in E2P of the wild type in the presence of K\textsuperscript{+} because of the observed complexity of the Ca\textsuperscript{2+}-dependent curve. In Fig. 9, we therefore employed a modified and thus different approach to examine the lumenal Ca\textsuperscript{2+}-induced reverse conversion from E2P to E1PCa\textsubscript{2}. Namely, E2P was formed with P\textsubscript{i} and then a medium containing various concentrations of Ca\textsuperscript{2+} but without ADP (in contrast to its presence in Fig. 8) was added to E2P, and the subsequent E\textsubscript{P} decay was followed (Fig. 9A). In the absence of Ca\textsuperscript{2+}, E2P was all hydrolyzed rapidly to E2 in a single exponential function. The E2P hydrolysis was inhibited gradually with increasing Ca\textsuperscript{2+} concentrations (over 0.1 mM), and the decay time course became biphasic as typically seen with 1 mM Ca\textsuperscript{2+} (22, 23). Thus, the proper formation of E1PCa\textsubscript{2} from E2P was all dependent curve as described above in Fig. 8.

In this kinetics, we eliminated the contribution of forward E2P hydrolysis on the overall E2P decay kinetics, and thereby revealed the rate of reverse E2P transition to E1PCa\textsubscript{2} induced by the lumenal Ca\textsuperscript{2+} binding of the wild type in the presence of K\textsuperscript{+}. For comparison in Fig. 9C, the rates of the lumenal Ca\textsuperscript{2+}-induced reverse E2P decay estimated for the wild type without K\textsuperscript{+} and Y122A with K\textsuperscript{+} in Fig. 8A were replotted. Note again that, in these cases, the hydrolysis of Ca\textsuperscript{2+}-unbound E2P was very slow and retarded; therefore, the observed Ca\textsuperscript{2+}/ADP-induced decay rates in their linear regions up to 3 mM Ca\textsuperscript{2+} reflect mostly the rates of the luminal Ca\textsuperscript{2+} access and binding to E2P in the reverse E2P decay. Note also that the experimental design in Fig. 9A employed for the wild type with K\textsuperscript{+} was not applicable to the wild type without K\textsuperscript{+} and Y122A, because the E2P hydrolysis is very slow and almost completely retarded in these cases, and therefore the E2P decay upon the Ca\textsuperscript{2+} addition cannot be described as the biphasic decay. Conversely, the experimental design employed in Fig. 8A to estimate the rates of the luminal Ca\textsuperscript{2+} access was not applicable to the wild type in the presence of K\textsuperscript{+} because of the complexity of the Ca\textsuperscript{2+}-dependent curve as described above in Fig. 8A.

Thus in Fig. 9C, employing the inevitably different but most suitable experimental designs depending on the different kinetic properties, we were able to compare the rates of the E2P to E1PCa\textsubscript{2} reverse transition induced by the luminal Ca\textsuperscript{2+} binding to the transport sites of E2P at the limited Ca\textsuperscript{2+} concentration range up to 3 mM. In the wild type in the presence of K\textsuperscript{+}, the rate was Ca\textsuperscript{2+}-dependent and not saturated even at 3 mM, thus reflecting at least the Ca\textsuperscript{2+}-dependent and rate-limiting process; *i.e*. the lumenal Ca\textsuperscript{2+}-induced change from E2P to E1PCa\textsubscript{2}. This reverse transition rate in the wild type in the presence of K\textsuperscript{+} was significantly faster than those in the wild type in the absence of K\textsuperscript{+} and in Y122A (as well as in the other Y122-HC mutants (supplemental Fig. S3)) especially at the high Ca\textsuperscript{2+} concentration over 1 mM.

Here it is also interesting to note that the affinity of E2P for the luminal Ca\textsuperscript{2+} in the wild type without K\textsuperscript{+} and the Y122-HC mutants is significantly higher than in the wild type with K\textsuperscript{+} (see Fig. 8B). If the rate of luminal Ca\textsuperscript{2+} access and binding to E2P is solely slowed in the wild type without K\textsuperscript{+} and Y122-HC mutants, a decrease in the affinity is rather the consequence, which is in contrast to the observed increase. Therefore the rates of the Ca\textsuperscript{2+} release from E2PCa\textsubscript{2} in the wild type without K\textsuperscript{+} and in the Y122-HC mutants are also presumably retarded significantly as compared with that in the wild type in the presence of K\textsuperscript{+}. Namely, the mutations of Y122-HC and the lack of K\textsuperscript{+} binding affect the energy levels of Ca\textsuperscript{2+}-free and -bound E2P states, as well as that of the transition state for luminal gating (opening), and favor the Ca\textsuperscript{2+}-bound state E2PCa\textsubscript{2} and the closed luminal gate.

**DISCUSSION**

Roles of Y122-HC in Ca\textsuperscript{2+} Release from E2PCa\textsubscript{2} and in E2P Hydrolysis—In this study, we found that the mutations of any of the seven residues in Y122-HC increase the luminal Ca\textsuperscript{2+} affinity and retard the luminal Ca\textsuperscript{2+} access to the transport sites in E2P. These mutations also retard markedly the hydrolysis of the Ca\textsuperscript{2+}-released form of E2P (22, 23). Thus, the proper formation of Y122-HC from the seven residues is critical for both Ca\textsuperscript{2+} release


**FIGURE 9.** Kinetics of luminal Ca\(^{2+}\)-induced change of E2P to E1P\(\text{Ca}_2\) in the wild type in the presence of K\(^+\) without ADP. A, the microsomes expressing the wild type were phosphorylated with \(^{32}\)P, in the presence of A23187 and absence of Ca\(^{2+}\) and chilled on ice, as described in Fig. 7. Subsequently, the phosphorylated sample was mixed at 0°C with a 20-fold volume of chase solution containing 105 mM KCl and various concentrations of CaCl\(_2\) without ADP, otherwise as described in Fig. 7. The final free Ca\(^{2+}\) concentrations were indicated in the figure. At the indicated time periods after this addition, the chase reaction was terminated by trichloroacetic acid, and the tions were indicated in the figure. At the indicated time periods after this addition, the chase reaction was terminated by trichloroacetic acid, and the tions were indicated in the figure. At the indicated time periods after this addition, the chase reaction was terminated by trichloroacetic acid, and the tions were indicated in the figure. At the indicated time periods after this addition, the chase reaction was terminated by trichloroacetic acid, and the tions were indicated in the figure.

**Between these forward and reverse rates of**

**As shown in Fig. 10 and supplemental Fig. S4, the extents of the mutational effects on the luminal Ca\(^{2+}\) affinities and on the**

**from E2PCa\(_2\) (reducing the Ca\(^{2+}\) affinity and opening the luminal gate), and formation of the E2P catalytic site for the subsequent Asp\(^{351}\)-acylphosphate hydrolysis. The formation of Y122-HC therefore functions critically for realizing and stabilizing the compactly organized and thus distorted structure of the Ca\(^{2+}\)-released form of E2P. The stabilization of this state is certainly important for making the time period long enough for Ca\(^{2+}\) release into lumen and likely for proton bindings to the empty Ca\(^{2+}\) sites, and for the fine rearrangement of the catalytic site for the subsequent Asp\(^{351}\)-acylphosphate hydrolysis.**

As shown in Fig. 10 and supplemental Fig. S4, the extents of the mutational effects on the luminal Ca\(^{2+}\) affinities and on the
E2P hydrolysis rates varied significantly among the seven Y122-HC mutants and depended on their positions. The residues of which mutation exhibited the strongest effects on increasing luminal Ca\textsuperscript{2+} affinity were Leu\textsuperscript{119}, Tyr\textsuperscript{122}, and Leu\textsuperscript{180}. This agrees with the critical role of M2 for rearrangement of the transmembrane helices for the Ca\textsuperscript{2+} release; i.e. the tight association of the top part of M2 with the largely rotating A domain in Y122-HC functions for the lever-like inclination of M2 to push the luminal part of M4 to open the luminal gate (14). In fact, in E2P\textsubscript{Ca\textsuperscript{2+}} trapped by the elongation of the A/M1-linker, the Leu\textsuperscript{119}/Tyr\textsuperscript{122} region on the top part of M2 is not involved fully in Y122-HC (9).

The residues of which mutations exhibited the strongest retardation of the E2P hydrolysis were Ile\textsuperscript{332} at the top part of the A/M3-linker and, again, Leu\textsuperscript{119} and Tyr\textsuperscript{122} on the A/M2-linker (top part of M2). Thus these residues on the linkers seem to contribute most critically to produce the proper configuration of the catalytic site. Consistently, the proteolytic cleavage at Leu\textsuperscript{119} on the A/M2-linker causes a marked inhibition of the E2P hydrolysis (43). The structural changes producing the Ca\textsuperscript{2+} release may be transmitted to the catalytic site via these residues of Y122-HC on the linkers, thereby ensuring the E2P hydrolysis to occur after the Ca\textsuperscript{2+} release. In any case, the different degree of the contributions of the seven residues of Y122-HC to the Ca\textsuperscript{2+} release and subsequent formation of the E2P catalytic site may suggest a possible sequential gathering of the seven residues. This possibility will be discussed more in the last section of “Discussion” in relation to the crystal structure E2-BeF\textsubscript{3}\textsuperscript{−} (17, 18).

Structural Mechanism Involving Y122-HC and Other Critical Elements—In E1Ca\textsubscript{2+}AlF\textsubscript{4−}-ADP → E2-MgF\textsubscript{2−} as an overall structural change, including the EP isomerization and Ca\textsuperscript{2+} release (supplemental Fig. S5A), the A domain largely rotates and M2 largely inclines. Also the P domain markedly inclines toward the lower side of the A domain and rotates by ~20° around the phosphorylation site (Asp\textsuperscript{351}) parallel to the membrane and in the opposite direction of the A-domain rotation. These motions involve (can be dissected into) the horizontal and vertical factors, parallel and perpendicular to the membrane plane. As a consequence of the motions, the A and P domains and M2 will come to their appropriate positions producing their tight association at Y122-HC. At the A-P domain interface in the E2P analog structures, there is another interaction network between these domains at the Val\textsuperscript{200} loop, Asp\textsuperscript{196}→Asp\textsuperscript{203} of the A domain (Fig. 1, and see supplemental Fig. S3 in Ref. 23 for the details of the interactions and central role of Val\textsuperscript{200}). Our previous mutations of Val\textsuperscript{200} showed (24) that this A-P domain interaction is critical for Ca\textsuperscript{2+} release from E2P\textsubscript{Ca\textsuperscript{2+}} and for formation of the E2P catalytic site, thus very similarly to Y122-HC. Then note that, in the E2P analog structures (see supplemental Fig. S5A for E2-MgF\textsubscript{2−}), the two networks at Y122-HC and at Val\textsuperscript{200} are located at each side of the A-P domain interface on its top view and at the bottom and upper parts of the interface, respectively on its side view. Thus the two are situated horizontally and vertically with the specific relative positioning. It is very likely that this positioning of the two is most efficiently functioning to realize and stabilize the compactly organized and distorted structure of the Ca\textsuperscript{2+}-released E2P: i.e. the interactions at the two positions are most appropriate to produce the horizontal and vertical motions of the P and A domains and M2 required for Ca\textsuperscript{2+} release from E2P\textsubscript{Ca\textsuperscript{2+}} and to stabilize the Ca\textsuperscript{2+}-released E2P state. Certainly these motions cause the rearrangements in the transmembrane helices for Ca\textsuperscript{2+} release: e.g. the P-domain inclination with slight rotation is directly associated with the bending and slight rotation of connected M4/M5 and downward movement of M4, thus their twisting-like motion. The largely inclining M2 pushes the luminal part of M4 (supplemental Fig. S5B). Hence the Ca\textsuperscript{2+} sites are destroyed, and the luminal gate is opened.

It should be noted that, for the loss of the ADP sensitivity E1P\textsubscript{Ca\textsuperscript{2+}} → E2P\textsubscript{Ca\textsuperscript{2+}}, the large rotation of the A domain and its docking onto the P domain should occur so as to bring the T\textsuperscript{185}GES loop above Asp\textsuperscript{351}-acylphosphate to block the ADP access from the N domain. As the motive force of this large A-domain rotation approximately parallel to membrane plane, the strain imposed on the A/M3-linker in E1P\textsubscript{Ca\textsuperscript{2+}} was predicted to be critical (13, 14, 20, 44). Also, the sufficiently long length of the A/M1-linker was revealed to be critical for this EP isomerization, in this case, probably for realizing the E2P\textsubscript{Ca\textsuperscript{2+}} structure, in which the A domain is positioned above the P domain (9, 45). For the subsequent Ca\textsuperscript{2+} release in E2P\textsubscript{Ca\textsuperscript{2+}} → E2 + 2Ca\textsuperscript{2+}, the A/M1-linker with its appropriately short length (therefore its strain) is critical (9). Actually, the elongation of this linker blocks completely Ca\textsuperscript{2+} deocclusion/release from E2P\textsubscript{Ca\textsuperscript{2+}}, thus trapping this E2P\textsubscript{Ca\textsuperscript{2+}} state in which Y122-HC is not properly formed yet in contrast to its proper formation in the Ca\textsuperscript{2+}-released form of E2P with the luminally opened normal Ca\textsuperscript{2+} release pathway (9). The results clearly demonstrated that the native and appropriately short length of A/M1-linker functions critically in inducing the motions from the E2P\textsubscript{Ca\textsuperscript{2+}} state, especially inclination of the A and P domains and M2, to accomplish the Y122-HC formation and the Ca\textsuperscript{2+} deocclusion/release from E2P\textsubscript{Ca\textsuperscript{2+}}. During the Y122-HC formation, the interaction force being produced in Y122-HC will likely function to induce the final process of the vertical and horizontal motions of the P and A domain and M2 to realize and stabilize the Ca\textsuperscript{2+}-released E2P structure (supplemental Fig. S6). Importantly also, the E2P catalytic site is produced by these rearrangements. In this mechanism, a possible hydrolysis of Asp\textsuperscript{351}-acylphosphate without releasing Ca\textsuperscript{2+} will be avoided; thereby the ordered reaction sequence of the Ca\textsuperscript{2+} release from E2P\textsubscript{Ca\textsuperscript{2+}} and the subsequent E2P hydrolysis will be accomplished for the energy coupling.

Possible Structural Role of K\textsuperscript{+} for Reducing Ca\textsuperscript{2+} Affinity and Luminal Gating—K\textsuperscript{+} is known to markedly accelerate the E2P hydrolysis (35, 36) and also to modulate the E2 to E1Ca\textsubscript{2+} transition in the non-phosphorylated Ca\textsuperscript{2+}-ATPase (46, 47). In the present study, we further found that the K\textsuperscript{+} binding is important for reducing the affinity for Ca\textsuperscript{2+} and luminal gating thus for Ca\textsuperscript{2+} release from E2P\textsubscript{Ca\textsuperscript{2+}}. In the crystal structure E1Ca\textsubscript{2+}AlF\textsubscript{4−}-ADP, K\textsuperscript{+} is situated at the bottom part of the P domain and coordinated by the backbone carbonyls of the loop Leu\textsuperscript{711}−Glu\textsuperscript{715} and by the Glu\textsuperscript{737} side chain (Fig. 11). The K\textsuperscript{+} binding at this site was indeed previously found by the mutations to be critical for the stimulation of the E2P hydrolysis (37). In the structures E2P analogs and E2(TG), this K\textsuperscript{+} site of the P
Tyr$^{122}$-hydrophobic Cluster of SERCA1a for Ca$^{2+}$ Release

**FIGURE 11. Bound K$^+$ and Tyr$^{122}$-hydrophobic cluster in crystal structures.** The part of structures $E1Ca_2AlF_4$·ADP (E1·PCa$_2$·ADP analog, left) and $E2AlF_4^-$ (E2·P analog, right) around Y122-HC and the bound K$^+$ ion are shown in schematic models (PDB codes: 1T5T and 1XP5 (12, 15)). The two structures were manually aligned with M8–M10 helices, which do not move virtually in the two. K$^+$ bound in these structures is shown by a yellow van der Waals sphere. Gln$^{244}$ on the A/M3-linker at the immediate vicinity of the bound K$^+$ in $E2AlF_4^-$ is indicated by ball and stick model.

domain comes very close to the A/M3-linker, and actually K$^+$ at this site is further coordinated by the Gln$^{244}$ side chain on the A/M3-linker (see $E2AlF_4^-$ in Fig. 11). Because the alanine substitution of Gln$^{244}$ and those of Glu-Gln-Asp$^{245}$ gave virtually no effect on Ca$^{2+}$ transport activity (48), K$^+$ at this region may be coordinated by their neighboring residues or backbone carbonyls on the A/M3-linker and thereby perform a structural function. In the present study, we found that the lack of K$^+$ binding has the consequences very similar to those of the mutations at Y122-HC. It is therefore possible that the K$^+$ binding functions with similar structural effects as Y122-HC to produce the proper structure of the Ca$^{2+}$-released form of E2P.

Then note that the K$^+$ site of the P domain in $E1Ca_2AlF_4$·ADP is situated at much higher position from the membrane plane than the Gln$^{244}$ region on the A/M3-linker (Fig. 11) and that, in the change $E1Ca_2AlF_4$·ADP $\rightarrow$ $E2AlF_4^-$ (or $E2BeF_3^-$ and $E2MgF_4^{2-}$), the P domain with connected M4/M5 largely inclines toward the A domain, hence the K$^+$ site with bound K$^+$ on the P domain moves down to the Gln$^{244}$ region on the A/M3-linker to make contact. The interactions between the bottom part of the P domain and the A/M3-linker via bound K$^+$ thus produced would likely cross-link them and hence contribute to formation and stabilization of this compactly organized Ca$^{2+}$-released structure of E2P with the reduced Ca$^{2+}$ affinity and luminaly opened gate. Alternatively, it is also possible that the appropriate P-domain structure produced by K$^+$ binding on this domain solely contributes to the formation of the Ca$^{2+}$-released E2P structure.

Y122-HC in Crystal Structure of E2BeF$_3^-$—The crystal structures of E2BeF$_3^-$, the analog for the E2P ground state (21), were solved at the atomic level very recently with and without bound thapsigargin, TG (E2BeF$_3^-$ and E2BeF$_3^-$·TG) (17, 18). Surprisingly, in this crystallized E2BeF$_3^-$, the side chains of Ile$^{119}$ and Tyr$^{122}$ are somewhat pointing away from the clustered other five residues on the A and P domains (Ile$^{179}$/Leu$^{180}$/Ile$^{232}$ and Val$^{705}$/Val$^{726}$), although all these seven residues are closely located in the E2BeF$_3^-$ structures of both 2ZBE (17) and 3B9B (18). On the other hand, Y122-HC is formed fully from all these seven residues in E2BeF$_3^-$·TG as well as in the other E2P analogous structures, E2·AlF$_4^-$ and E2·MgF$_4^{2-}$. Thus, the assembling manner of the seven residues in the crystal structure E2·BeF$_3^-$ seemingly conflicts with our results that the gathering of all the seven residues, including Tyr$^{122}$/Ile$^{119}$ in Y122-HC, is required for producing the Ca$^{2+}$-released E2P. Furthermore, Tyr$^{122}$ and Ile$^{119}$ on the top part of M2 (A/M2-linker) are likely most critical in Y122-HC and play central roles (Fig. 10). Our previous biochemical structural analysis of SR Ca$^{2+}$-ATPase in solution by the proteolysis and the luminal Ca$^{2+}$ accessibility demonstrated (21) that, in E2·BeF$_3^-$ without TG, Leu$^{119}$/Tyr$^{122}$ are surely gathered and involved in Y122-HC, and thereby the luminal gate is opened and the luminal Ca$^{2+}$ is accessible to the transport sites. Thus the crystal structure E2·BeF$_3^-$ seems to conflict also with these biochemical results obtained in solution.

Nevertheless, as a comprehensive idea, the crystal structure of E2·BeF$_3^-$ may be consistent with (or indicative of) the view that the gathering of the seven residues to form Y122-HC upon motions of the A and P domains and M2 (A/M2-linker) occurs in some ordered sequence but not necessarily at once (see Fig. 10 and under “Discussion”). Most peculiar to us is that Tyr$^{122}$ and Leu$^{119}$, of which mutations exhibited the most inhibitory effects, are not involved yet in the hydrophobic cluster in the crystal structure E2·BeF$_3^-$·TG. Here note that, in the structure E2·BeF$_3^-$·Al$^{3+}$ ion is bound near the Ca$^{2+}$ binding sites in the transmembrane domain because an extremely high Mg$^{2+}$ concentration employed for crystallization (18), or protonation on the residues of transmembrane helices, including Ca$^{2+}$ ligands, must have occurred as in low pH for crystallization (17). Thus, these ligations are probably involved critically in the stabilization of the transmembrane helices for the crystallization. This might mean that the transmembrane structure thus stabilized differs from that without any ligations, i.e. the state immediate after the Ca$^{2+}$ release (the empty Ca$^{2+}$ sites) that is realized by the contribution of Y122-HC. Therefore, Y122-HC is, in return, disrupted or not properly produced yet in the crystal structure E2·BeF$_3^-$ as it occurs with the luminal Ca$^{2+}$ binding in the E2PCa$_2$ state as postulated in this study. Therefore the following sequential gathering of the seven residues to produce Y122-HC can be speculated: The five hydrophobic residues on the A domain (Ile$^{179}$/Leu$^{180}$/Ile$^{232}$) and P domain (Val$^{705}$/Val$^{726}$) are first gathered through the motions of the A and P domains and top part of M2 (A/M2-linker), and subsequently, the top part of M2, including Ile$^{119}$ and Tyr$^{122}$, makes further motions during the final process of the M2 inclination to join them and produce the fully assembled Y122-HC, thereby to realize and stabilize fully the gathered state of the A and P domains and top part of M2 (A/M2-linker) as in the Ca$^{2+}$-released form of E2P. Being in agreement with this view, in E2PCa$_2$ trapped by the elongation of the A/M1-linker, Leu$^{119}$/
Tyr$^{122}$ on the top part of M2 is not fully involved yet in Y122-HC (9). The Ca$^{2+}$-released and empty Ca$^{2+}$ sites (without any protonation and stabilization immediately after the Ca$^{2+}$ release) will be subsequently protonated producing the E2P ground state for its hydrolysis.

Alternatively, if a possible contribution of such ligation in the transmembrane domain (Mg$^{2+}$ or protonation) should not be concerned in the crystallization of E2P, the following might be possible: first of all, the arrangements of helices of the Ca$^{2+}$-released empty transport sites must be unstable, for example, due to possible repulsions between the negative charges of the Ca$^{2+}$ ligands. Then to relieve the instability, the most effective gathering of Tyr$^{122}$/Leu$^{119}$ in Y122-HC, which produces and stabilizes the Ca$^{2+}$-released empty state, might possibly be disrupted; thereby the helices may be rearranged so as to form the more stabilized arrangements that can be crystallized.

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 are also grateful to Dr. Chikashi Toyoshima, University of Tokyo, for helpful discussions.

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