Critical Role of Glu\textsuperscript{40}–Ser\textsuperscript{48} Loop Linking Actuator Domain and First Transmembrane Helix of Ca\textsuperscript{2+}-ATPase in Ca\textsuperscript{2+} Deocclusion and Release from ADP-insensitive Phosphoenzyme\textsuperscript{*}

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Takashi Daiho, Kazuo Yamasaki, Stefania Danko, and Hiroshi Suzuki
From the Department of Biochemistry, Asahikawa Medical College, Asahikawa 078-8510, Japan

...The functional importance of the length of the A/M1 linker (Glu\textsuperscript{40}–Ser\textsuperscript{48}) connecting the actuator domain and the first transmembrane helix of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase was explored by its elongation with glycine insertion at Pro\textsuperscript{42}/Ala\textsuperscript{43} and Gly\textsuperscript{46}/Lys\textsuperscript{47}. Two or more glycine insertions at each site completely abolished ATPase activity. The isomerization of phosphoenzyme (EP) intermediate from the ADP-sensitive form (E1P) to the ADP-insensitive form (E2P) was markedly accelerated, but the decay of EP was completely blocked in these mutants. The E2P accumulated was therefore demonstrated to be E2PCa\textsubscript{2}, possessing two occluded Ca\textsuperscript{2+} ions at the transport sites, and the Ca\textsuperscript{2+} deocclusion and release into lumen were blocked in the mutants. By contrast, the hydrolysis of the Ca\textsuperscript{2+}-free form of E2P produced from P\textsubscript{i} without Ca\textsuperscript{2+} was as rapid in the mutants as in the wild type. Analysis of resistance against trypsin and proteinase K revealed that the structure of E2PCa\textsubscript{2} accumulated is an intermediate state between E1PCa\textsubscript{2} and the Ca\textsuperscript{2+}-released E2P state. Namely in E2PCa\textsubscript{2}, the actuator domain is already largely rotated from its position in E1PCa\textsubscript{2} and associated with the phosphorylation domain as in the Ca\textsuperscript{2+}-released E2P state; however, in E2PCa\textsubscript{2}, the hydrophobic interactions among these domains and Leu\textsuperscript{119}/Tyr\textsuperscript{122} on the top of second transmembrane helix are not yet formed properly. This is consistent with our previous finding that these interactions at Tyr\textsuperscript{122} are critical for formation of the Ca\textsuperscript{2+}-released E2P structure. Results showed that the EP isomerization/Ca\textsuperscript{2+}-release process consists of the following two steps: E1PCa\textsubscript{2} → E2PCa\textsubscript{2} → E2P + 2Ca\textsuperscript{2+}; and the intermediate state E2PCa\textsubscript{2} was identified for the first time. Results further indicated that the A/M1 linker with its appropriately short length, probably because of the strain imposed in E2PCa\textsubscript{2}, is critical for the correct positioning and interactions of the actuator and phosphorylation domains to cause structural changes for the Ca\textsuperscript{2+} deocclusion and release.

Sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA1a)\textsuperscript{2} is a representative member of P-type ion transporting ATPases and catalyzes Ca\textsuperscript{2+} transport coupled with ATP hydrolysis (Fig. 1) (Refs. 1, 2 and for recent reviews see Refs. 3–7). In the catalytic cycle, the enzyme is activated by binding of two Ca\textsuperscript{2+} ions to the transport sites (E2 to E1Ca\textsubscript{2}, steps 1 and 2) and then autophosphorylated at Asp\textsuperscript{351} with MgATP to form the ADP-sensitive phosphoenzyme (E1P, step 3), which can react with ADP to regenerate ATP in the reverse reaction. Upon formation of this EP, the bound Ca\textsuperscript{2+} ions are occluded in the transport sites (E1PCa\textsubscript{2}). The subsequent isomeric transition to the ADP-insensitive form (E2P), i.e. the loss of the ADP sensitivity at the catalytic site, results in rearrangements of the Ca\textsuperscript{2+}-binding sites to deoclude Ca\textsuperscript{2+}, reduce the affinity, open the luminal gate, and thus release Ca\textsuperscript{2+} into the lumen (steps 4 and 5). As an intermediate state in the EP isomerization/Ca\textsuperscript{2+}-release process, E2PCa\textsubscript{2} has been postulated (e.g. see Ref. 8), although this state has never been identified. Finally, the E2P hydrolysis takes place and returns the enzyme into an unphosphorylated and Ca\textsuperscript{2+}-unbound form (E2, steps 6 and 7). The transport cycle is totally reversible, e.g. E2P can be formed from P\textsubscript{i} in the presence of Mg\textsuperscript{2+} and the absence of Ca\textsuperscript{2+} by reversal of its hydrolysis, and the subsequent addition of high concentrations of Ca\textsuperscript{2+} to E2P reverse the Ca\textsuperscript{2+}-releasing step and the E1P to E2P isomerization.

The enzyme has three cytoplasmic domains as follows: the nucleotide binding (N), phosphorylation (P), and actuator (A) domains, and 10 transmembrane helices M1–M10 (Fig. 2). During the EP isomerization/Ca\textsuperscript{2+}-release E1PCa\textsubscript{2} → E2P + 2Ca\textsuperscript{2+}, the A domain largely rotates (by ~110°) parallel to the membrane and associates with the P domain (see Refs. 9–17) (see E1/AlF\textsubscript{4}–ADP (the E1PCa\textsubscript{2}/ADP analog) → E2–MgF\textsubscript{4}\textsuperscript{2+} (the E2-P analog) in Fig. 2). The interactions of the A domain with the P domain in the E2P state occur at three regions (Fig. 2, semitransparent purple, blue, and orange on E2–MgF\textsubscript{4}\textsuperscript{2+}: i.e. at the T181GES loop with the residues of the P domain around Asp\textsuperscript{351}; at the V1100 loop (Asp\textsuperscript{196}–Asp\textsuperscript{203}) with the polar residues of the P domain (Arg\textsuperscript{678}/Glu\textsuperscript{680}/Asp\textsuperscript{656}/Asp\textsuperscript{660}); and at the

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\textsuperscript{1} To whom correspondence should be addressed: Dept. of Biochemistry, Asahikawa Medical College, Midorigaoka-higashi, Asahikawa, 078-8510, Japan. Tel.: 81-166-68-2350; Fax: 81-166-68-2359; E-mail: daiho@asahikawa-med.ac.jp.

\textsuperscript{2} The abbreviations used are: SERCA1a, adult fast-twitch skeletal muscle sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase; SR, sarcoplasmic reticulum; EP, phosphoenzyme; E1P, ADP-sensitive phosphoenzyme; E2P, ADP-insensitive phosphoenzyme; Tg, thapsigargin; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; V, orthovanadate; PrtK, proteinase K.


**ADP-insensitive Phosphoenzyme with Occluded Ca\(^{2+}\) in SERCA1a**

Tyr\(^{122}\)-hydrophobic cluster formed by seven hydrophobic residues gathered from the A domain (Ile\(^{279}\)/Leu\(^{180}\)/Ile\(^{332}\)), the P domain (Val\(^{195}\)/Val\(^{276}\)), and the top part of M2 (the A/M2 linker region, Leu\(^{119}\)/Tyr\(^{122}\)). The formation of the A-P domain interaction at the T\(^{181}\)GES loop has been predicted to be critical for the loss of ADP sensitivity at the catalytic site, i.e. the E1P to E2P isomerization, by the structural and mutation studies (18–20). The mutations at the latter two interaction regions were shown not to inhibit the E1P to E2P isomerization but to markedly retard the subsequent EP decay (19, 21, 22). Its kinetics were consistent with the view that there is a Ca\(^{2+}\)-releasing step from E2PCa\(^{2+}\) (E2PCa\(^{2+}\) → E2P + 2Ca\(^{2+}\)) before the E2P hydrolysis and that this Ca\(^{2+}\)-releasing step is blocked and became the kinetic limit for the EP decay by the disruption of the A-P domain interactions at each of the latter two regions (19, 21, 22). It is therefore very interesting to know how the motions and interactions of the A and P domains progress during the postulated successive steps E1PCa\(^{2+}\) → E2PCa\(^{2+}\) → E2P + 2Ca\(^{2+}\) as the key structural events in the energy coupling between the cytoplasmic and transmembrane domains. In this respect, it is also critical to clarify and distinguish the structural roles of the three linkers connecting the A domain with M1/M1, M2, and M3 (A/M1, A/M2, and A/M3 linkers). Tyr\(^{122}\)/Leu\(^{119}\) involved in the aforementioned Tyr\(^{122}\)-hydrophobic cluster is at the A/M2 linker region. The A/M3 linker, because of its strain, has been predicted to be important for the large rotation of the A domain in the EP isomerization (12, 13).

![FIGURE 1. Ca\(^{2+}\) transport cycle of SERCA.](image)

**FIGURE 2. Structure of SERCA1a. a**, structural model for the change E1PCa\(^{2+}\) → E2P + 2Ca\(^{2+}\) was depicted with E1-AIF\(_2\)-ADP (E1PCa\(^{2+}\)-ADP analog) and E2-MgF\(^{2-}\) (E2-P analog) (Protein Data Bank accession codes 1WPE and 1WPG, respectively; see Refs. 13 and 17). The cytoplasmic three domains N, P, and A are green, pink, and yellow. The transmembrane helices (M1–M10) are numbered, and some are colored. The two structures were manually fitted with M8–M10, which virtually do not move in the two structures. In E1-AIF\(_2\)-ADP, the two Ca\(^{2+}\) ions bound at their binding (transport) sites are depicted (red spheres); the sites consist of the residues on M4, M5, M6, and M8. The linkers connecting the A domain with M1 (A/M1 linker with red), with M2 (A/M2 linker with green), and with M3 (A/M3 linker with gray) are indicated. The arrows on E1-AIF\(_2\)-ADP with yellow, pink, and blue show the movements of the A domain, the P domain, and M1, respectively, in the change E1-AIF\(_2\)-ADP → E2-MgF\(^{2-}\). The phosphorylation site Asp\(^{351}\), AIF\(_2\), and MgF\(^{2-}\) are shown, but ADP was not depicted for simplicity. The specific cleavage sites for trypsin (I2, Arg156 on the Val200 loop (Asp196–Asp203); T1, Arg505) and proteinase K (Prk; Leu145) are indicated on E1-AIF\(_2\)-ADP. The semitransparent spheres on E2-MgF\(^{2-}\) with purple, blue, and orange indicate the three contact regions between the A and P domains, i.e. at the T\(^{181}\)GES loop (purple loop with TGES) with the residues around Asp\(^{351}\), at the Val\(^{562}\) loop, including Arg\(^{196}\) (dark blue loop) with the polar residues of the P domain (Arg\(^{379}\)/Glu\(^{370}\)/Arg\(^{556}\)/Asp\(^{568}\), and at Tyr\(^{122}\)/Leu\(^{119}\), Tyr\(^{132}\) and Leu\(^{119}\) on the top part of M2 (A/M2 linker region) form the interaction network “Tyr\(^{122}\)-hydrophobic cluster” with Ile\(^{119}\)/Leu\(^{119}\/Ile\(^{122}\) of the A domain and Val\(^{395}\)/Val\(^{256}\) of the P domain (see Fig. 13). Details of the other two interaction regions are also not depicted for simplicity (see supplemental Figs. II and III in Ref. 19 for the details). b, in E2-MgF\(^{2-}\), the regions at the A/M1 linker (Glu\(^{402}\)-Ser\(^{448}\) red), M1’ (Leu\(^{429}\)-Asp\(^{429}\) blue), M1 (blue), and the N-terminal neighbor of the A/M1 linker (yellow) are depicted. The sites for the insertion mutations made in this study are at Gly\(^{46}\)/Lys\(^{47}\) (between Gly\(^{46}\) and Lys\(^{47}\)) and Pro\(^{22}/\)Ala\(^{23}\) on the A/M1 linker and at Thr\(^{32}/\)Gly\(^{33}\) (as indicated with the α-carbons in pink spheres), and at Val\(^{37}/\)Ile\(^{38}\) on M1’.

![FIGURE 2. Structure of SERCA1a. a](image)
Regarding the A/M1 linker, we recently found (23) that its shortening by deletions of any single residues within this linker (Glu^{40}–Ser^{48}) blocks the E1P to E2P isomerization and the hydrolysis of the Ca^{2+}-free form of E2P, whereas substitutions of any residues in this linker do not inhibit the function. Our results indicated that the A/M1 linker with its correct length critically contributes to the E1P isomerization/Ca^{2+} release and to the E2P hydrolysis, and we pointed out the possible importance of this linker in the proper positioning of the A and P domains for their motions and association during these processes. Therefore, in this study, we further explored the structural roles of this linker and the structural events occurring in the processes by elongating this linker with insertion of glycines (see Fig. 2).

Results demonstrated that the elongation of the linker markedly accelerates the E1PCa_{2} to E2PCa_{2} isomerization, strongly stabilizes E2PCa_{2} that possesses two occluded Ca^{2+} ions at the transport sites, and blocks the Ca^{2+} deoclusion and release from E2PCa_{2}. Thus, for the first time, the intermediate state E2PCa_{2} was identified and trapped in this study. We were then able to characterize the structure of this state. Results revealed that the correct length of the A/M1 linker is critical for structural events in each of successive steps in E1PCa_{2} → E2PCa_{2} → E2P + 2Ca^{2+} and E2P + H_{2}O → E2 + P_{i}, and they further suggested how the motions and interactions of the properly positioned A and P domains progress with the critical contribution of the linker to accomplish the successive structural events in these steps. Our study also revealed the importance of M1’ directly connected with the A/M1 linker likely for forming the base of this linker.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Expression**—The QuickChange™ site-directed mutagenesis method (Stratagene, La Jolla, CA) was utilized for the insertions and substitutions of residues in the rabbit SERCA1α cDNA. The Apal-KpnI restriction fragments with the desired mutation were excised from the plasmid and ligated back into the corresponding region in the full-length SERCA1α cDNA in the pMT2 expression vector (24). The pMT2 DNA was transfected into COS-1 cells by the liposome-mediated transfection method. Microsomes were prepared from the cells as described previously (25). The "control microsomes" were prepared from COS-1 cells transfected with the pMT2 vector containing no SERCA1α cDNA. The amount of expressed SERCA1α was quantified by a sandwich enzyme-linked immunosorbent assay as described previously (26). Expression levels of the wild-type SERCA1α and mutants examined in this study were 2–3% of total microsomal proteins, except a mutant 4950525354S for M1’ with serine substitutions of Leu^{59}/Trp^{60}/Leu^{92}/Val^{102}/Ile^{103}, which showed markedly reduced expression (only ~15–20% of the wild type).

**Ca^{2+}-ATPase Activity**—The rate of ATP hydrolysis was determined at 25 °C in a mixture containing 1 μg of microsomal protein, 0.1 mm [γ-^{32}P]ATP, 1 mm EGTA, 0.1 M KCl, 7 mm MgCl_{2}, 0.55 mm CaCl_{2}, 0.5 mm MgCl_{2}, and 50 mm MOPS/Tris (pH 7.0). The Ca^{2+}-ATPase activity was obtained by subtracting the Ca^{2+}-independent ATPase activity, which was determined in the presence of 5 mm EGTA without added CaCl_{2}, otherwise as above. The ATPase activity/mg of expressed SERCA1α protein was calculated from the amount of expressed SERCA1α and the Ca^{2+}-ATPase activity of expressed SERCA1α, which was obtained by subtracting the Ca^{2+}-ATPase activity of the control microsomes from that of the microsomes expressing SERCA1α. This background level with the control microsomes was as low as 3% of the activity of microsomes expressing the wild-type SERCA1α.

**Formation and Hydrolysis of EP**—Phosphorylation of SERCA1α in microsomes with [γ-^{32}P]ATP or ^{32}P, and dephosphorylation of ^{32}P-labeled SERCA1α 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 as above. The precipitated proteins were separated by 5% SDS-PAGE at pH 6.0 according to Weber and Osborn (28). The radioactivity associated with the separated Ca^{2+}-ATPase was quantitated by digital autoradiography as described (29). The amount of EP formed with the expressed SERCA1α 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 SERCA1α. The amount of EP/mg of SERCA1α protein was calculated from the amount of EP thus obtained and the amount of expressed SERCA1α.

**Ca^{2+} Occlusion in EP**—As described in Fig. 8 legend, the expressed mutant SERCA1α in microsomes was phosphorylated with ATP and 45CaCl_{2}, and then the mixture was diluted by a “washing solution” containing excess EGTA and immediately filtered through a 0.45-μm nitrocellulose membrane filter (Millipore). The filter was washed four times with 2 ml of the washing solution, and 45Ca^{2+} remaining on the filter was quantitated. The amount of Ca^{2+} specifically bound to the transport sites of EP in the expressed SERCA1α was obtained by subtracting the amount of nonspecific Ca^{2+} binding, which was determined by including 1 μm thapsigargin (TG) in the phosphorylation mixture, otherwise as above. This background subtraction is ensured by the fact that TG inhibits the Ca^{2+} binding at the transport sites and the EP formation (30). The background level thus determined was ~60% of the total amount of 45Ca^{2+} remaining on the filter when the maximum amount of EP was present (*i.e.* at the zero time of EP decay in Fig. 8). It should be noted that the specifically bound Ca^{2+} in EP thus determined represents the occluded one because it is not released even after the extensive washing by EGTA. The Ca^{2+} occluded/mg of expressed SERCA1α protein was calculated from the amount of expressed SERCA1α and the amount of occluded Ca^{2+}. The Ca^{2+} occlusion resulted from Ca^{2+} binding to E2P in the reverse reaction of the Ca^{2+}-release process was also determined. In this case, E2P was first formed from P_{i} in the absence of Ca^{2+}, and 45Ca^{2+} was then added to E2P otherwise as described in Fig. 10 legend, and the amount of occluded 45Ca^{2+} was determined as above.

**Limited Proteolysis of Major Intermediates and Western Blot Analysis**—Major intermediates and its stable analogs of the Ca^{2+}-ATPase were produced and subjected to the structural analysis by limited proteolysis with trypsin and proteinase K (PrtK) as described in Fig. 12 legend. The digests were separated by 10.5 or 7.5% SDS-PAGE, according to Laemmli (31), and
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blotted onto a polyvinylidene fluoride membrane and then incubated with IIH11 monoclonal antibody to the rabbit SERCA1a (Affinity Bioreagents), which recognizes an epitope between Ala\(^{199}\)–Arg\(^{200}\). After incubation with secondary antibody (goat anti-mouse IgG-horseradish peroxidase-conjugated), the bound proteins were probed using an enhanced chemiluminescence-linked detection system (ECL Plus, GE Healthcare).

Miscellaneous—Protein concentrations were determined by the method of Lowry et al. (32) 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 (33).

RESULTS

Ca\(^{2+}\)-ATPase Activity—The specific Ca\(^{2+}\)-ATPase activity of the expressed SERCA1a mutants was determined at saturating 50 μM Ca\(^{2+}\) and 25 °C and compared with that of the wild type (Fig. 3). Insertion of one glycine between Gly\(^{46}\) and Lys\(^{47}\) (1Gi-46/47) and between Pro\(^{42}\) and Ala\(^{43}\) (1Gi-42/43) within the A/M1 linker slowed the ATPase activity by ~50%. Insertion of two or more glycines at each of these sites abolished the activity almost completely (2Gi-46/47 to 6Gi-46/47 and 4Gi-42/43). Thus, the elongation of the linker by the glycine insertion at the two different positions within the A/M1 linker exhibited the same inhibitory effects on the ATPase, indicating the importance of the correct length of this loop in the function.\(^3\)

We then examined the possible effects of the 4-amino acid-insertion in the C- and N-terminal regions of the A/M1 linker: between Val\(^{53}\) and Ile\(^{54}\) on the helix M1’ and between Thr\(^{22}\) and Gly\(^{23}\) in the immediate vicinity of the Thr\(^{25}\)–Tyr\(^{36}\) helix (see Fig. 2b). In the mutants 4Ai-53/54 and 4Ai-22/23, alanines were inserted (for M1’, we intended to minimize possible disruption of the helical structure). These mutants exhibited the high ATPase activity (Fig. 3) and the Ca\(^{2+}\) transport coupled with the ATP hydrolysis (data not shown). Thus the insertions at the adjacent regions of the A/M1 linker did not inhibit the activity. We inserted amino acids also in the immediate N-terminal region of the A/M1 linker, for example at His\(^{32}\)–Leu\(^{33}\) and at Gly\(^{37}\)–His\(^{38}\); however, the protein expression levels of these mutants were extremely low (less than ~10% of the wild type); therefore, their functional analysis was not possible.

We also investigated the possible importance of amphipathic property of the helix M1’ (Trp\(^{50}\)–Glu\(^{58}\) in E1-AlF•·ADP or Leu\(^{49}\)–Gln\(^{66}\) in E2-MgF\(^{2-}\)), which is directly connected with the A/M1 linker and formed by kinking of M1. M1’ lies on the membrane surface, having hydrophilic residues aligned on the membrane side (Leu\(^{49}\)/Trp\(^{50}\)/Val\(^{51}\)/Ile\(^{52}\)) and the polar residues (Glu\(^{51}\)/Glu\(^{55}\)/Gln\(^{56}\)/Glu\(^{58}\)) on the cytoplasmic side (Fig. 2b). Therefore, the hydrophobic interactions of M1’ with the membrane core and/or its hydrophilic interactions at the membrane surface may possibly be important for function (13). Previously the mutations of the single residues on the M1’ region were found to have almost no or only a slight effect on the activity (23, 34). In this study, we therefore introduced the extensive nonconservative substitutions as follows: the serine substitution of all Leu\(^{49}\)/Trp\(^{50}\)/Leu\(^{52}\)/Val\(^{53}\)/Ile\(^{54}\) (4950525354S) and the alanine substitution of all Glu\(^{51}\)/Glu\(^{55}\)/Gln\(^{56}\)/Glu\(^{58}\)/Asp\(^{59}\) (5155565859A). The mutant 4950525354S exhibited the markedly reduced ATPase activity (17% that of the wild type), whereas the mutant 5155565859A exhibited a fairly high activity (70% that of the wild type). The results indicate that the hydrophobic interaction of M1’ with the membrane core may be important.

EP Formation from ATP and the E2-E1Ca\(^{2+}\) Transition—The amount of EP formed from ATP at saturating 50 μM Ca\(^{2+}\) was determined at steady state and 0 °C with 10 μM ATP under the

\(^3\) In this regard, we should describe the reasons why we chose the positions 42/43 and 46/47 for the insertions. First of all, to explore the importance of the length of the linker and to assign the effects of the insertions straightforwardly as those of the elongation of the linker, it is necessary or better to have insertions at two (at least) different positions. Then for the choice of the two, the positions approximately at one-third and at two-thirds of the length of the linker would be reasonable because the A/M1 linker, Glu\(^{46}\)–Ser\(^{49}\) loop, is an extended loop without helical structure (see Fig. 2) and has no extensive interactions with other parts of the ATPase molecule (actually the substitutions of any single residues in this loop did not impair the function (23)). Also, the two positions should be not too close to the junctions of the A/M1 linker with the A domain and with M1’ (and to the membrane lipids). This is to avoid possible disruption of the structures at the junctions. With these reasons, we chose the two insertion positions at 42/43 and 46/47, and we therefore distributed the two on the linker but did not position them very close to each other at the middle part or at either side of the linker. As a reason for the choice of glycine(s) as the amino acid for the insertions, we intended to minimize possible disruption of the extended loop structure of the linker and to avoid possible formation of side-chain interactions. The results in Fig. 3 of the mutants with the insertions at the different positions therefore agree with the view that the observed effects are because of the elongation of the A/M1 linker. The kinetic analysis shown in this study further revealed that the properties of the mutants with the insertions at the two different positions are essentially the same.
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The affinity of the transport sites for Ca\(^{2+}\) in the presence of various concentrations of Ca\(^{2+}\) under the conditions otherwise as described in the legend to Fig. 4 and by the least squares fit to the Hill equation. The rates of the E2 to E1Ca\(_2\) transition in steps 1 and 2 were determined by the EP1Ca\(_{2}\) formation from the E2 state upon the simultaneous addition of saturating 100 \(\mu\)M Ca\(^{2+}\) and ATP at pH 6.0 under the conditions otherwise as in Fig. 4. In this EP formation, the E2 to E1Ca\(_2\) transition is rate-limiting. The rates for the other steps were obtained at 0 °C in the experiments in Fig. 5, A and C (EP1Ca\(_{2}\) to E1Ca\(_{2}\) in step 3, Fig. 5, B and D (loss of ADP sensitivity (i.e. accumulation of ADP-insensitive EP from ADP-sensitive EP in step 4)), Fig. 6 (decay of EP formed from ATP in the presence of Ca\(^{2+}\) (EP\(_{1}\)Ca\(_{2}\)), and Fig. 7 (hydrolysis of EP2 formed from P in the absence of Ca\(^{2+}\) (EP2P\(_{2}\)) in steps 6 and 7). In parentheses, the values obtained with the wild type are normalized to 100%. For E2 to EP1Ca\(_{2}\) or E1Ca\(_{2}\) the accessibility of luminal Ca\(^{2+}\) to the transport sites of EP2 was assessed at 0 °C in Fig. 10 by determining the affinity for Ca\(^{2+}\) (K\(_{Ca}\)) and the Hill coefficient (n) in the reverse reaction, i.e., upon the addition of Ca\(^{2+}\) to EP2 and the consequent formation of EP2Ca\(_{2}\) or EP1Ca\(_{2}\) (see “Results” with Fig. 10 for details of the formation of these stable EP species in the reverse reaction).

### Table 1

**Kinetic parameters determined for partial reaction steps**

| Ca\(^{2+}\) affinity K\(_{Ca}\) | Rate | Loss of ADP sensitivity | Decay of \(EP_{ATP}\) | Hydrolysis of \(EP_{2P_2}\) | \(Ca^{2+}\) affinity K\(_{Ca}\) |
|---|---|---|---|---|---|
| \(\mu M\) | \(\%\) | \(\%\) | \(\%\) | \(\%\) | \(\mu M\) | \(\%\) |
| **Wild type** | 0.16 | 1.87 | 0.213 (100) | 1.37 (100) | 0.34 (100) | 0.0390 (100) | 0.64 (100) | 1.4 | 1.5 |
| 4Gi-53/54 | 0.16 | 2.05 | 0.103 (100) | 1.50 (100) | 0.36 (100) | 0.0613 (157) | 1.07 (169) | 1.2 | 1.4 |
| 4Gi-42/43 | 0.16 | 0.58 | 0.242 (137) | 1.30 (96) | 0.53 (156) | 0.247 (63) | 1.63 (162) | 2.3 | 2.5 |
| 4Gi-42/43 | 0.16 | 2.69 | 0.102 (100) | 1.50 (100) | 0.53 (156) | 0.247 (63) | 1.63 (162) | 2.3 | 2.5 |
| 4Gi-42/43 | 0.16 | 1.74 | 0.147 (69) | 1.09 (80) | 0.24 (95) | 0.219 (110) | 0.62 (98) | 1.4 | 1.5 |
| 2Gi-46/47 | 0.13 | 1.68 | 0.208 (97) | 1.30 (95) | 0.54 (138) | 0.0613 (157) | 1.07 (169) | 2.3 | 2.5 |
| 3Gi-46/47 | 0.13 | 2.50 | 0.239 (112) | 1.80 (131) | 2.83 (170) | 0.247 (63) | 1.63 (162) | 2.3 | 2.5 |
| 4Gi-46/47 | 0.14 | 0.14 | 0.226 (106) | 1.94 (142) | 2.71 (170) | 0.0613 (157) | 1.07 (169) | 2.3 | 2.5 |
| 4Gi-42/43 | 0.15 | 1.48 | 0.136 (64) | 4.20 (307) | 4.37 (1054) | 0.0613 (157) | 1.07 (169) | 2.3 | 2.5 |
| 4Gi-53/54 | 0.29 | 1.70 | 0.428 (201) | 2.26 (165) | 1.08 (170) | 0.0613 (157) | 1.07 (169) | 2.3 | 2.5 |
| 4Gi-53/54 | 0.29 | 1.70 | 0.428 (201) | 2.26 (165) | 1.08 (170) | 0.0613 (157) | 1.07 (169) | 2.3 | 2.5 |

* The rate of the EP1Ca\(_{2}\) formation from E1Ca\(_{2}\) in the presence of K\(^{+}\) was very similar to that in the absence of K\(^{+}\). * (see Fig. 5, A and C) and therefore not shown for simplicity.

* Not determined because the accumulation of ADP-insensitive EP was low.

* The rate of the EP isomerization (loss of the ADP sensitivity) must be faster because almost all of EP formed had become already ADP-insensitive during the EP formation (see Fig. 5).

* Not determined because the EP formation from E1Ca\(_{2}\) was slow (see Fig. 5A).

* The apparent slow rate is probably due to the slowed EP formation from E1Ca\(_{2}\) and ATP. The rate of the EP isomerization (loss of the ADP sensitivity) must be faster because a very large fraction of EP formed had become already ADP-insensitive during the EP formation (see Fig. 5).
ADP-insensitive Phosphoenzyme with Occluded Ca\textsuperscript{2+} in SERCA1α

FIGURE 5. Time course of accumulation of ADP-insensitive EP. Microsomes expressing the wild-type or mutant SERCA1α were phosphorylated with \( \gamma\textsuperscript{32P} \)ATP at 0 °C for various periods as indicated on the abscissa in 50 µl of a mixture containing 1 µg of microsomal protein, 10 µM \( \gamma\textsuperscript{32P} \)ATP, 1 µM A23187, 7 mM MgCl\textsubscript{2}, 0.55 mM CaCl\textsubscript{2}, 0.5 mM EGTA, and 50 mM MOPS/Tris (pH 7.0) in the presence of 0.1 M LiCl without added KCl (A and B) or 0.1 M LiCl without LiCl (C and D). The reaction was quenched by acid, and the total amount of EP (A and C) was determined. For determination of ADP-insensitive EP (B and D), an equal volume (50 µl) of a mixture containing 1 µM A23187, 7 mM MgCl\textsubscript{2}, 10 mM EGTA, 50 mM MOPS/Tris (pH 7.0), and 0.1 M LiCl without KCl (B) or 0.1 M LiCl without LiCl (D) was added to the above phosphorylation mixture at the indicated time. At 1 s after the addition, the reaction was quenched by acid. Solid lines show the least squares fit to a single exponential, and the apparent rates to reach the steady-state level are given in Table 1. The maximum values of the total amount of EP obtained at infinite time in the fitting are normalized to 100% (A and C), and the amounts of the ADP-insensitive EP are shown as percentages of the maximum value of the total amount of EP (B and D).

4950525354S (Fig. 5A). The accumulation of ADP-insensitive EP apparently proceeded with first-order kinetics. The rate and extent of the accumulation in the mutants 1Gi-46/47, 2Gi-46/47 (Fig. 5B), and 1Gi-42/43 (data not shown) in the absence of K\textsuperscript{+} were comparable with those of the wild type (see Table 1 for the rates). The accumulation of ADP-insensitive EP became extremely rapid in the mutants with three or more glycine insertions, 3Gi-46/47 and 4Gi-46/47. Actually, during the time course of EP formation in these mutants, nearly all of EP formed was already ADP-insensitive. Essentially the same results were obtained with the mutant 4Gi-42/43 as with these mutants (data not shown, but see Table 1). Thus in the mutants with the elongated A/M1 linker with the three or more glycine insertions, the EP isomerization was markedly accelerated, and the ADP-insensitive EP (E2P) accumulated exclusively. In these mutants, the rates of EP formation and the EP isomerization in the presence of K\textsuperscript{+} (Fig. 5, C and D) were almost the same as those in the absence of K\textsuperscript{+} (Fig. 5, A and B). In the mutant 2Gi-46/47, the fair amount of ADP-insensitive EP accumulated rapidly even in the presence of K\textsuperscript{+} (see Figs. 4 and 5D).

It should be noted that the mutant 4950525354S exhibited the almost exclusive accumulation of ADP-insensitive EP both in the absence (Fig. 5B) and presence (Fig. 4) of K\textsuperscript{+} at steady state. In this regard, the removal of the hydrophobic property of M1\textsuperscript{+} by the serine substitutions (removal of the likely hydrophobic interaction with the membrane core) caused the same consequence as that of the elongation of the A/M1 linker. Besides, the observed slow EP formation from E1Ca\textsubscript{2} and ATP in this mutant 4950525354S (Fig. 5A) suggests the importance of the hydrophobic property of this region in rapid structural changes for ATP binding and phosphorylation. The mutants 5155658589A on M1\textsuperscript{+} and 4Ai-22/23 and 4Ai-53/54 adjacent to the A/M1 linker exhibited the extent and rate of the accumulation of ADP-insensitive EP almost the same as those of the wild type (see Table 1 for the rates, Figs. 4 and 5B for the extent).

Decay of EP Formed from ATP and Ca\textsuperscript{2+}—The decay of EP formed from ATP and Ca\textsuperscript{2+} was determined at 0 °C in the presence of K\textsuperscript{+} and is shown with the representative mutants in Fig. 6. The decay time courses were fitted well with a single exponential (Fig. 6A), and the rates were summarized in Table 1. The fraction of the ADP-insensitive EP (E2P) remaining in
the decay course was also determined (Fig. 6B). In the wild type, the EP remaining was exclusively the ADP-sensitive EP (E1P), and this is consistent with the well known rate-limiting E1P to E2P transition in the ATPase cycle (36, 37). The EP decay was slightly slowed in the single glycine-insertion mutants 1Gi-46/47 (Fig. 6A) and 1Gi-42/43 (see Table 1), being consistent with the slight reduction in the ATPase activity in these mutants (cf. Fig. 3). The EP decay was almost completely blocked in the mutants with two or more glycine insertions in the A/M1 linker, 2Gi-46/47 to 4Gi-46/47 (Fig. 6A) and 4Gi-42/43 (see Table 1). This is consistent with the complete loss of the ATPase activity in these mutants. EP present at the start and course of the decay reaction was exclusively the ADP-sensitive one (E2P) in the mutants 3Gi-46/47 and 4Gi-46/47, and ~50% in the mutant 2Gi-46/47 (Fig. 6B, as also shown in Figs. 4 and 5D). In the mutants with the elongated A/M1 linker with the two or more glycine insertions, these results show that the E1P-E2P isomerization was strongly shifted toward the ADP-insensitive EP (E2P) and that the decay of the ADP-insensitive EP was blocked. In the mutant 4950525354S on M1′, the EP decay was markedly slowed (Fig. 6A), and the EP remaining was almost exclusively the ADP-insensitive EP during the decay reaction (data not shown, but see Fig. 4). Thus in this mutant, the decay of the ADP-insensitive EP was markedly retarded. The mutants 5155565859A (Fig. 6A), 4Ai-22/23, and 4Ai-53/54 (Table 1) showed the rapid EP decay as the wild type, being consistent with their high ATPase activities.

Hydrolysis of E2P Formed from P_i without Ca^{2+}—The observed block of decay of the ADP-insensitive EP (E2P) formed from ATP and Ca^{2+} in the mutants with the elongated A/M1 linker and 4950525354S might possibly be due to the block of hydrolysis of the Ca^{2+}-free form of E2P. Therefore, in Fig. 7, the E2P hydrolysis was directly examined by first phosphorylating the enzyme with 32P, in the absence of Ca^{2+} and K+ and the presence of 35% (v/v) Me2SO, which extremely favors the E2P formation in the reverse reaction (38), and then by diluting the phosphorylated sample at 0 °C with a large volume of solution containing nonradioactive P_i and K+ without Ca^{2+}. The conditions for the hydrolysis were thus otherwise made the same as those for the decay of E2P formed from ATP with Ca^{2+} in Fig. 6. Hydrolysis of 32P-labeled E2P proceeded with first-order kinetics as shown with the representative mutants, and the rates obtained were summarized in Table 1. To our surprise, in all the mutants with the elongated A/M1 linker and 4950525354S, 4Ai-22/23, and 4Ai-53/54 also exhibited the rapid E2P hydrolysis as the wild type.

**Ca^{2+} Occlusion in Stable E2P**—The observed block of the decay of ADP-insensitive EP (E2P) formed from ATP with Ca^{2+} (Fig. 6) and the rapid hydrolysis of E2P formed from P_i without Ca^{2+} (Fig. 7) in the mutants indicate that there may be a kinetic limit for the decay of E2P formed with Ca^{2+} before the hydrolysis of E2P without bound Ca^{2+}. This limiting step is possibly the Ca^{2+}-releasing step from E2PCa_2: E2PCa_2 →...
ADP-insensitive Phosphoenzyme with Occluded Ca\(^{2+}\) in SERCA1a

**FIGURE 8. Occlusion of Ca\(^{2+}\) in EP formed from ATP and Ca\(^{2+}\).** A, microsomes expressing the mutant 4Gi-46/47 were phosphorylated for 10 s with ATP and 45Ca\(^{2+}\) at 25 °C in 50 μl of a mixture containing 2 μg of microsomal protein, 1 μM ATP, 10 μM 45CaCl\(_2\), 0.1 M KCl, 7 mM MgCl\(_2\), and 30 mM MOPS/Tris (pH 7.0) in the presence (△) or absence (○) of 1 μM A23187. Then a small volume of EGTA was added to give 2 mM to terminate the phosphorylation. At the indicated time after this EGTA addition, the mixture was diluted at 25 °C by 2 ml of a washing solution containing 5 mM A23187, 0.1 M KCl, 7 mM MgCl\(_2\), 2 mM EGTA, and 50 mM MOPS/Tris (pH 7.0) and immediately filtered through a 0.45-μm nitrocellulose membrane filter. The samples on the filter were rapidly washed four times by 2 ml of the above washing solution at 25 °C. The amount of 45Ca\(^{2+}\) specifically bound to the expressed SERCA1a, i.e. the occluded Ca\(^{2+}\) in SERCA1a, was determined after this extensive washing by subtracting the amount of nonspecific Ca\(^{2+}\) binding, which was determined by including 1 μM TG in the above phosphorylation mixture, otherwise as described under “Experimental Procedures.” The values are shown as pmol per mg of SERCA1a applied on the filter (△, △). By using [γ-32P]ATP and nonradioactive CaCl\(_2\), the total amount of EP present in the above EGTA-added phosphorylation mixture was determined at the indicated time by acid quenching (○, ○). EP present was found to be exclusively the ADP-insensitive one (E2P) as also demonstrated with this mutant in Figs. 4–6. The phosphorylated samples were filtered at the indicated time with and without addition of ADP immediately before the filtration. Approximately 50% of the total amount of E2P + 2Ca\(^{2+}\) and E2PCa\(_{2}\) may be stabilized and accumulated in the mutants. In Fig. 8, this possibility was directly examined by the determination of 45Ca\(^{2+}\) occlusion in E2P accumulated from ATP and 45Ca\(^{2+}\). With the representative mutants 4Gi-46/47 (Fig. 8, A and C) and 2Gi-46/47 (Fig. 8, B and C), EP was first formed from ATP and 45Ca\(^{2+}\) at steady state in the absence or presence of the Ca\(^{2+}\) ionophore A23187, and then the EP decay was initiated by the addition of excess EGTA. The amount of occluded 45Ca\(^{2+}\) was determined at the indicated time by membrane filtration with an extensive washing with a solution containing EGTA and A23187. The total amount of EP and the fraction of ADP-insensitive EP (E2P) was determined by the use of [γ-32P]ATP and nonradioactive Ca\(^{2+}\).

The extremely slow EP decay was observed at 25 °C with the mutant 4Gi-46/47 (Fig. 8A). At the zero time of the decay reaction, the amount of occluded Ca\(^{2+}\) and that of EP were ~9 and 4 ~ 4.5 nmol/mg of expressed SERCA1a protein, respectively, both in the presence and absence of A23187. These values gave the stoichiometry “two occluded Ca\(^{2+}\) ions per one EP.” The amount of 45Ca\(^{2+}\) remaining on the membrane filter decreased concomitantly with the EP decay, and therefore the stoichiometry was always found to be “two” during the EP decay course (Fig. 8C). This stoichiometry is in complete agreement with the presence of two Ca\(^{2+}\)-binding (transport) sites in the ATPase molecule. Importantly, EP remaining during the decay time course was exclusively the ADP-insensitive one (E2P) in this mutant 4Gi-46/47 (data not shown, but see Fig. 6B). Thus, the results clearly demonstrated that EP accumulated in the mutant is the ADP-insensitive EP that possesses two occluded Ca\(^{2+}\) ions, i.e. E2PCa\(_{2}\), and the Ca\(^{2+}\) deocclusion from E2P Ca\(_{2}\) is extremely slowed by the elongation of the A/M1 linker, and therefore E2P Ca\(_{2}\) is accumulated exclusively. The decay of E2P Ca\(_{2}\) became faster by A23187 (although only slightly), being consistent with the mechanism that the Ca\(^{2+}\) release occurs into lumen from E2P Ca\(_{2}\). It should be noted that this type of experiment was not possible with the wild type because its EP decay is extremely rapid and completed during the EGTA washing (actually within 1 s).

In Fig. 8, B and C, the experiments were performed with the mutant 2Gi-46/47 in the presence of A23187 at 10 °C. At this temperature, the EP decay was most conveniently followed in this mutant. The phosphorylated samples were filtered at the indicated time with and without addition of ADP immediately before the filtration. Approximately 50% of the total amount of employed throughout the experiments in A and B for convenience. B, microsomes expressing the mutant 2Gi-46/47 were phosphorylated with ATP and 45Ca\(^{2+}\) in the presence of 1 μM A23187 at 10 °C otherwise as described in A. The phosphorylation was terminated by the EGTA addition as in A, and at the indicated time after this EGTA addition, the mixture was diluted at 10 °C by 2 ml of a washing solution containing 1 μM A23187, 0.1 M KCl, 7 mM MgCl\(_2\), 2 mM EGTA, and 50 mM MOPS/Tris (pH 7.0) in the presence (●) or absence (○) of 1 mM A23187. Then a small volume of EGTA was added to give 2 mM to terminate the phosphorylation. At the indicated time after this EGTA addition, the mixture was diluted at 25 °C by 2 ml of a washing solution containing 5 mM A23187, 0.1 M KCl, 7 mM MgCl\(_2\), 2 mM EGTA, and 50 mM MOPS/Tris (pH 7.0) and immediately filtered through a 0.45-μm nitrocellulose membrane filter. The samples on the filter were rapidly washed four times by 2 ml of the above washing solution at 25 °C. The amount of 45Ca\(^{2+}\) specifically bound to the expressed SERCA1a, i.e. the occluded Ca\(^{2+}\) in SERCA1a, was determined after this extensive washing by subtracting the amount of nonspecific Ca\(^{2+}\) binding, which was determined by including 1 μM TG in the above phosphorylation mixture, otherwise as described under “Experimental Procedures.” The values are shown as pmol per mg of SERCA1a applied on the filter (●, ●). By using [γ-32P]ATP and nonradioactive CaCl\(_2\), the total amount of EP present in the above EGTA-added phosphorylation mixture was determined at the indicated time by acid quenching (●, ●). EP present was found to be exclusively the ADP-insensitive one (E2P) as also demonstrated with this mutant in Figs. 4–6. The phosphorylated samples were filtered at the indicated time with and without addition of ADP immediately before the filtration. Approximately 50% of the total amount of E2P + 2Ca\(^{2+}\) and E2P Ca\(_{2}\) may be stabilized and accumulated in the mutants. In Fig. 8, this possibility was directly examined by the determination of 45Ca\(^{2+}\) occlusion in E2P accumulated from ATP and 45Ca\(^{2+}\). With the representative mutants 4Gi-46/47 (Fig. 8, A and C) and 2Gi-46/47 (Fig. 8, B and C), EP was first formed from ATP and 45Ca\(^{2+}\) at steady state in the absence or presence of the Ca\(^{2+}\) ionophore A23187, and then the EP decay was initiated by the addition of excess EGTA. The amount of occluded 45Ca\(^{2+}\) was determined at the indicated time by membrane filtration with an extensive washing with a solution containing EGTA and A23187. The total amount of EP and the fraction of ADP-insensitive EP (E2P) was determined by the use of [γ-32P]ATP and nonradioactive Ca\(^{2+}\).

The extremely slow EP decay was observed at 25 °C with the mutant 4Gi-46/47 (Fig. 8A). At the zero time of the decay reaction, the amount of occluded Ca\(^{2+}\) and that of EP were ~9 and 4 ~ 4.5 nmol/mg of expressed SERCA1a protein, respectively, both in the presence and absence of A23187. These values gave the stoichiometry “two occluded Ca\(^{2+}\) ions per one EP.” The amount of 45Ca\(^{2+}\) remaining on the membrane filter decreased concomitantly with the EP decay, and therefore the stoichiometry was always found to be “two” during the EP decay course (Fig. 8C). This stoichiometry is in complete agreement with the presence of two Ca\(^{2+}\)-binding (transport) sites in the ATPase molecule. Importantly, EP remaining during the decay time course was exclusively the ADP-insensitive one (E2P) in this mutant 4Gi-46/47 (data not shown, but see Fig. 6B). Thus, the results clearly demonstrated that EP accumulated in the mutant is the ADP-insensitive EP that possesses two occluded Ca\(^{2+}\) ions, i.e. E2P Ca\(_{2}\), and the Ca\(^{2+}\) deocclusion from E2P Ca\(_{2}\) is extremely slowed by the elongation of the A/M1 linker, and therefore E2P Ca\(_{2}\) is accumulated exclusively. The decay of E2P Ca\(_{2}\) became faster by A23187 (although only slightly), being consistent with the mechanism that the Ca\(^{2+}\) release occurs into lumen from E2P Ca\(_{2}\). It should be noted that this type of experiment was not possible with the wild type because its EP decay is extremely rapid and completed during the EGTA washing (actually within 1 s).

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ADP-insensitive Phosphoenzyme with Occluded Ca^{2+} in SERCA1a

![Diagram](image)

**FIGURE 9.** Formation of stable E2P in the reverse reaction by addition of a high concentration of Ca^{2+} to E2P formed from P, without Ca^{2+}. Microsomes expressing the wild-type or mutant SERCA1a were phosphorylated with^{32}P, at 25°C for 10 min in 2.5 μl of a mixture containing 1 μg of microsomal protein, 0.1 mM ATP, 1 μM A23187, 1 mM EGTA, 10 mM MgCl₂, 50 mM MOPS/Tris (pH 7.0), and 35% (v/v) Me₂SO. The mixture was cooled and diluted at 0°C with 22.5 μl of the “Ca solution” containing 22.3 mM CaCl₂ (to give 20 mM Ca^{2+}), 1 μM A23187, 111 mM KCl, 7 mM MgCl₂, and 50 mM MOPS/Tris (pH 7.0) and incubated for 1 min. This mixture was then further diluted 10-fold at 0°C by the addition of 225 μl of the “EGTA solution” containing 44.4 mM EGTA, 1 μM A23187, 0.1 mM KCl, 7 mM MgCl₂, and 50 mM MOPS/Tris (pH 7.0). At different times after this Ca^{2+}-removal, the EP decay was quenched by acid, and the amounts of EP remaining were determined (A). The amounts of EP obtained at zero time (i.e., immediately before the addition of the EGTA solution) are normalized to 100%. It should be noted that the amount of E2P formed from P, (at zero time) was comparable with that of EP formed from ATP and Ca^{2+} shown in Fig. 4 in the wild type and in each of the mutants (e.g., in the wild type, E2P formed from P, was 3.45 ± 0.22 nmol/mg of SERCA1a protein (n = 4) and EP formed from ATP was 3.31 ± 0.14 nmol/mg of the SERCA1a protein (n = 4)). For determination of the ADP-insensitive EP, an equal volume (250 μl) of a mixture containing 4 mM ADP, 1 μM A23187, 0.1 mM KCl, 7 mM MgCl₂, 10 mM EGTA, and 50 mM MOPS/Tris (pH 7.0) was added to the above EGTA-diluted phosphorylation mixture at the indicated time. At 1 s after this addition, the reaction was quenched by acid. The amount of the ADP-insensitive EP determined at each of the indicated time is shown as percentage of the total amount of EP determined at zero time.

EP was ADP-insensitive EP (E2P) throughout the decay reaction (compare open and closed squares in Fig. 8B, also see Fig. 6B). The total amount of EP, the amount of ADP-insensitive EP, and the amounts of occluded Ca^{2+} determined without and with the ADP addition decreased very slowly and concomitantly (Fig. 8B). Thus, as plotted in Fig. 8C, the stoichiometry of the occluded Ca^{2+} was always found to be two in the total amount of EP (E1P plus E2P) and in the amount of ADP-insensitive EP (E2P) throughout the decay reaction. The results show that two Ca^{2+} ions are occluded in both the ADP-sensitive EP, i.e., E1PCa₂, and the ADP-insensitive EP, i.e., E2PCa₂, in the mutant 2Gi-46/47. The results are consistent with the view that the E1PCa₂-E2PCa₂ equilibrium was largely shifted to E2PCa₂, and the Ca^{2+} deocclusion from E2PCa₂ was blocked in this mutant. It should be mentioned for the mutant 495025354S that, as indicated by the kinetic analyses in Figs. 4–7, E2PCa₂ is probably accumulated in this mutant from ATP and Ca^{2+} as in the mutant 4Gi-46/47. The Ca^{2+}-binding experiments were not possible, however, with this mutant because of its very low protein expression level (only ~15–20% that of 4Gi-46/47 and 2Gi-46/47 or wild type).

### Formation of Stable E2PCa₂ from E2P and Ca^{2+} in Reverse Reaction

As demonstrated with SR Ca^{2+}-ATPase (39), the E1P to E2P transition and the Ca^{2+} release into lumen can be reversed by the low affinity Ca^{2+} binding from the luminal side to the transport sites of the Ca^{2+}-free form of E2P. In Fig. 9, we examined with the representative mutants whether the stable E2PCa₂ can be produced in the reverse reaction. In the experiments, E2P was first formed from^{32}P, without Ca^{2+} in the presence of A23187 and 35% (v/v) Me₂SO that strongly favors E2P (38), and subsequently the phosphorylation mixture was diluted 10-fold with a solution containing CaCl₂ to give a very high (saturating) Ca^{2+} concentration of 20 mM. After 1 min of incubation with Ca^{2+}, the mixture was further diluted 10-fold with a solution containing excess EGTA and 0.1 mM KCl, and the decay of EP was followed at 0°C. Thus, the final conditions for this EP decay in Fig. 9 were made to be essentially the same as those for the decay of EP formed from ATP and Ca^{2+} in the forward reaction in Fig. 6. We observed actually the same time courses in Fig. 9 as those in Fig. 6 for each of the representative mutants as well as for the wild type. Most importantly, in the mutants with the two or more glycine insertion in the A/M1 linker (2Gi-46/47, 3Gi-46/47, and 4Gi-46/47), the decay of EP formed from E2P with the subsequently added Ca^{2+} was nearly completely blocked. Furthermore, EP remaining during the decay course was exclusively the ADP-insensitive one in the mutants 3Gi-46/47 and 4Gi-46/47, and ~50% in the mutant 2Gi-46/47 (Fig. 9B). The results show that the stable E2P, probably E2PCa₂, is formed in these mutants in the reverse reaction from E2P and Ca^{2+} as well as in the forward reaction with ATP and Ca^{2+}. In the wild type, E2P formed from P, became exclusively the ADP-sensitive one (E1PCa₂) upon the Ca^{2+} addition, being consistent with the previous observation (39).

With the representative mutant 4Gi-46/47, we then actually determined the Ca^{2+} occlusion in the stable E2P formed from E2P with the subsequently added 3 mM Ca^{2+} (rather than 20 mM because of the experimental limitation), otherwise as above. We found that the amounts of stable E2P formed and the

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4 The details of the experimental conditions are as follows. E2P was first formed from nonradioactive P, in 35% (v/v) Me₂SO in the absence and presence of 1 μM TG, otherwise as in Fig. 9. Subsequently, the mixture was diluted 10-fold at 0°C with a solution containing 3 mM CaCl₂, 1 μM A23187, 0.1 mM KCl, 7 mM MgCl₂, and 50 mM MOPS/Tris (pH 7.0), and incubated for 1 min. This mixture was further diluted 10-fold with a washing solution containing 20 mM EGTA, 1 μM A23187, 0.1 mM KCl, 7 mM MgCl₂, and 50 mM MOPS/Tris (pH 7.0) and incubated for 3 min for hydrolyzing the Ca^{2+}-bound fraction of E2P (see Figs. 7 and 10) and for reducing non-specifically bound Ca^{2+}. The mixture was then subjected to the membrane filtration with the extensive washing by the above EGTA-containing washing solution, otherwise as described in Fig. 8.
occluded Ca$^{2+}$ were 1.65 ± 0.13 and 3.33 ± 0.25 nmol/mg of expressed SERCA1a protein ($n = 4$), respectively, and thus the stoichiometry of the occluded Ca$^{2+}$ in E2P was 2.02. The results demonstrated that two Ca$^{2+}$ ions are occluded in the stable E2PCa$_2$, which is formed in the reverse reaction as in the forward reaction. The results also indicated that the reverse transition from E2PCa$_2$ to E1PCa$_2$ was dramatically retarded (blocked) in the mutants 3Gi-46/47 and 4Gi-46/47.

**Accessibility of Luminal Ca$^{2+}$ to Transport Sites of E2P Formed from P$_i$**—We then measured the Ca$^{2+}$ concentration dependence of the Ca$^{2+}$-induced formation of the stable EP from E2P and Ca$^{2+}$ in the reverse reaction (Fig. 10). In the experiments, E2P was first formed from P$_i$ without Ca$^{2+}$ in the presence of A23187, and subsequently the phosphorylation mixture was largely diluted with a solution containing CaCl$_2$ to give the free Ca$^{2+}$ concentrations indicated in Fig. 10. Immediately after this Ca$^{2+}$ addition, the EP decay was followed without removing Ca$^{2+}$. As typically shown in Fig. 10, A and B, for the wild type and the mutant 2Gi-46/47, the decay of EP proceeded with two phases as shown previously in this type of experiment with the Ca$^{2+}$-ATPase (40). The first and rapid phase corresponds to the hydrolysis of E2P without bound Ca$^{2+}$. In the wild type, the second and very slow phase corresponds to the forward decay of E1PCa$_2$ that is formed from E2P and Ca$^{2+}$, as well documented previously (39, 40). In fact, nearly all the EP remaining in the decay course was the ADP-sensitive one (E1P) in the wild type (data not shown).

In the mutants 2Gi-46/47 (Fig. 10B) and 3Gi-46/47 and 4Gi-46/47 (data not shown), the EP decay in the second phase was extremely slow, and actually almost no decay occurred during the period of observation. The decay of EP in these mutants was extremely slow even after removal of Ca$^{2+}$ as shown in Fig. 9. The EP remaining in this slow phase was almost exclusively the ADP-insensitive one (E2P) in the mutants 3Gi-46/47 and 4Gi-46/47 even at the highest Ca$^{2+}$ concentration 20 mM and ~50% in the mutant 2Gi-46/47 at 20 mM Ca$^{2+}$ (see Fig. 9).

The content of EP in the second and slow phase was obtained by extrapolating to the zero time and plotted versus the Ca$^{2+}$ concentrations (Fig. 10C). The content increased with the increase in the Ca$^{2+}$ concentration and was nearly saturated at ~10 mM Ca$^{2+}$. $K_{0.5}$ was estimated to be 1.4 mM in the wild type and similarly 0.9–1.3 mM in the mutants in Fig. 10 and other mutants as well (see Table 1). These values are actually consistent with the high Ca$^{2+}$ concentrations required for the Ca$^{2+}$-induced reverse reaction from E2P determined previously with SR Ca$^{2+}$-ATPase as the access of luminal Ca$^{2+}$ to the transport sites of E2P (41–44). The results indicate that in these mutants with the elongated A1/M1 linker, the luminal Ca$^{2+}$ can access and bind to the transport sites of E2P as in the wild type, and the mutants produce the stable ADP-insensitive EP, i.e., E2PCa$_2$.

In this context, it should be noted that the Ca$^{2+}$-dependent increase in the stable EP in Fig. 10C reflects mostly the relative values between the Ca$^{2+}$-dependent increasing rate of the formation of the stable EP versus the rate of the hydrolysis of the Ca$^{2+}$-bound E2P. Namely, the curve reflects mostly the relative rates between E2P + 2Ca$^{2+}$ → E2PCa$_2$ (or further to E1PCa$_2$) versus E2P + H$_2$O → E2 + P$_i$, rather than the relative rates between the reverse and forward reactions in E2P + 2Ca$^{2+}$ → E2PCa$_2$ (or E1PCa$_2$) (i.e. the Ca$^{2+}$ affinity). This is because the curve in Fig. 10C is the plot of the amount of EP stably remaining after the hydrolysis of the Ca$^{2+}$-bound E2P, and because the amount of the remaining EP is dependent on the rate of its formation relative to the rate of the E2P hydrolysis. Furthermore, the decay of the remaining EP in the second phase was extremely slow in the wild type as well as in the mutants, and thus virtually negligible as compared with its
rapid formation and the rapid hydrolysis of the Ca\textsuperscript{2+}-unbound E2P both in the wild type and mutants.\textsuperscript{5} Importantly, the E2P hydrolysis rate was found to be essentially the same between the wild type and the mutants (Fig. 7). Therefore, the very similar Ca\textsuperscript{2+} dependence curves of the wild type and mutants in Fig. 10C indicate that the rates of the formation of the stable EP upon the luminal Ca\textsuperscript{2+} binding to E2P are very similar between them. Thus we concluded that E2P formed from P\textsubscript{i} without Ca\textsuperscript{2+} in the mutants possesses the luminally opened Ca\textsuperscript{2+} release pathway as in the wild type.

**Figure 11. Thapsigargin accelerates the decay of E2P\textsubscript{Ca} accumulated with the mutant.**

| EP from ATP, None | EP from ATP, TG | EP from P\textsubscript{i}, None | EP from P\textsubscript{i}, TG |
|------------------|-----------------|-------------------|----------------------------|
| 0 | 0 | 100 | 0 |
| 200 | 40 | 80 | 20 |
| 400 | 60 | 60 | 40 |
| 600 | 80 | 80 | 60 |


\textsuperscript{5} Regarding the wild type, the observed biphasic behavior in the EP decay after the addition of the high concentrations of Ca\textsuperscript{2+} in Fig. 10A may not be accounted for if we assume a rapid equilibrium in the reaction E1PCa\textsubscript{2} ⇌ E2PCa\textsubscript{2} ⇌ E2P + 2Ca\textsuperscript{2+} under these conditions, i.e., in the presence of high Ca\textsuperscript{2+} concentrations (because if in the rapid equilibrium, a slowed (but still) single exponential decay after the addition of Ca\textsuperscript{2+} may be expected). In this regard, it may be of interest to note that in the previous analysis with SR Ca\textsuperscript{2+}-ATPase by Nakamura (41) and Inesi and co-workers (42) for the decay of E1PCa\textsubscript{2} formed from ATP, a biphasic E1PCa\textsubscript{2} decay with a markedly slower decay in the first phase, the cytoplasmic three domains, N, P, and A largely move into the first time.

**Structure of E2PCa\textsubscript{2} Revealed by Proteolytic Analysis as an Intermediate State between E1PCa\textsubscript{2} and E2P**—In the transport cycle, the cytoplasmic three domains, N, P, and A largely move and change their organization states (7, 9–17). These changes are definitely monitored as the changes in the resistance of the specific cleavage sites against trypsin and PrtK (15–17). As one of most notable examples, the tryptic T2 site Arg\textsuperscript{198} on the outermost Val\textsuperscript{200} loop (Asp\textsuperscript{196}–Asp\textsuperscript{203}) of the A domain is rapidly cleaved in E1PCa\textsubscript{2}, by contrast, it is completely resistant in E2P (15–17). This is because the A domain largely rotates parallel to the membrane plane by \( \sim \)110°, and the Val\textsuperscript{200} loop, including Arg\textsuperscript{198}, associates with the P domain by forming an ionic interaction network and thus blocks sterically against the tryptic attack, as seen in E1-AlF\textsubscript{4}–ADP \( \rightarrow \) E2-MgF\textsubscript{2}\textsuperscript{2–} (or E2-AlF\textsubscript{4}–) (see Refs. 11–14) (see Figs. 2 and 13).

In Fig. 12A, upper panel, the tryptophan proteolysis was performed with the wild type for the major intermediates, and their structural analogs were stabilized by the appropriate ligands according to previous findings (15–17). The ATPase chain and its fragments were immunodetected with a monoclonal antibody that recognizes Ala\textsuperscript{199}–Arg\textsuperscript{505} (the tryptic fragment “A1”) of SERCA1a. In all the structural states, the T1 site (Arg\textsuperscript{505}) on the outermost loop of the N domain was very rapidly cleaved to produce the fragment “A” (Met\textsuperscript{1}–Arg\textsuperscript{505}, as immunodetected) and the fragment “B” (Ala\textsuperscript{506} to the C terminus Gly\textsuperscript{994}, not immuno-monitored). In the structural states E2, E1Ca\textsubscript{2},
FIGURE 12. Structural analysis of major intermediates and E2PCa₂ by limited proteolysis. The major intermediates of the transport cycle and their structural analogs were produced with the wild type (upper panels) and the mutant 4Gi-46/47 (lower panels) in the microsomes (0.1 mg/ml), under the conditions described below, and subjected to the limited proteolysis with 0.5 mg/ml trypsin (A) or PrtK (B) at 25 °C for the indicated time periods. The proteolysis was terminated by 2.5% (v/v) trichloroacetic acid, and the digests were subjected to Laemmli SDS-PAGE. The ATPase chain and its fragments separated on the gel were blotted onto a polyvinylidene fluoride membrane and visualized by immunodetection with a monoclonal antibody that recognizes the Ala₁⁹⁹–Arg₅₀₅ peptide (tryptic fragment "A1") of SERCA1a, as described under "Experimental Procedures." The E₂ state was produced in 5 mM EGTA, 0.1 M KCl, 7 mM MgCl₂, 1 μM A₂₃₁₈₇, and 50 mM MES/Tris (pH 6.0). The E₁Ca₂ state was produced in 0.1 mM CaCl₂, 0.1 M KCl, 7 mM MgCl₂, 1 μM A₂₃₁₈₇, and 50 mM MOPS/Tris (pH 7.0). To produce the E₁–AlF₄⁻/ADP complex (E₁PCa₂–ADP analog), the microsomes were incubated at 25 °C for 40 min in 50 μM AlCl₃, 3 mM KF, 0.1 mM ADP, 0.1 mM KCl, 0.1 mM CaCl₂, 7 mM MgCl₂, 1 μM A₂₃₁₈₇, and 50 mM MOPS/Tris (pH 7.0), and then the protease was added. For the EP formation from ATP, the microsomes were incubated at 25 °C for 10 s in 0.5 mM ATP, 0.1 M KCl, 7 mM MgCl₂, 1 mM EGTA, 7 mM MgCl₂, 1 μM A₂₃₁₈₇, and 50 mM MOPS/Tris (pH 6.0), and then the protease was added. In the wild type, EP accumulated was exclusively E₁PCa₂, and its decay was extremely slowed during the proteolysis period because of the feedback inhibition by the high concentration of Ca²⁺. In the mutant, EP accumulated was exclusively E₂PCa₂, and its decay was extremely slow, as demonstrated in Figs. 6 and 8. The same results were obtained in 0.1 mM Ca²⁺ as in 5 mM Ca²⁺ in the mutant (data not shown). To produce the E₂–AlF₄⁻ complex (the transition state analog of the E₂P hydrolysis (17)), the microsomes were preincubated at 25 °C for 40 min in 50 μM AlCl₃, 3 mM KF, 0.1 M KCl, 5 mM EGTA, 7 mM MgCl₂, 1 μM A₂₃₁₈₇, and 50 mM MOPS/Tris (pH 6.0). For producing E₂V₁ (the E₂P analog (15)), the microsomes were preincubated with 0.1 mM orthovanadate in 50 mM MOPS/Tris (pH 7.0) instead of 50 mM AlCl₃, 3 mM KF in 50 mM MES/Tris (pH 6.0), otherwise as above. The tryptic fragments: A, Met₁–Arg₅₀₅; A₁, Ala₂⁹⁹–Arg₅₀₅. The fragments formed by PrtK: p95, Lys₁²⁶–Gly₉₉₄; p81, Met₁–Met₇₃₃; and p83, Glu₂₄₃–Gly₉₉₄ (48, 49). The positions of the Ca²⁺–ATPase chain and its fragments and those of the molecular mass markers are indicated on the left and right margins, respectively. Note also that the antibody immunodecorated trypsin (A) and PrtK (B), in addition to the Ca²⁺–ATPase fragments.
**E1·AlF₄⁻·ADP (E1PCa₂·ADP analog), and E1PCa₂ accumulated exclusively with the wild type from ATP at a very high concentration (5 mM) of Ca²⁺, and the T2 site was rapidly cleaved to produce the fragment A1 as immunodetected and the fragment “A2” (Met¹⁻Arg¹⁹⁸, not immuno-monitored). By contrast, in the structural states of E2P without bound Ca²⁺ stabilized by orthovanadate (E₂V₄) and by Al³⁺/F⁻ (E₂·AlF₄⁻), the transition state analog of the E₂P hydrolysis (17)), the T2 site was completely resistant. The complete resistance was also found with E₂·MgF₄⁻ and E₂·BeF₄⁻, the E₂P analog, and the E₂P ground state analog, respectively (17) (data not shown). These results with the wild type agree with the previous demonstration with SR Ca²⁺-ATPase (15–17).

In the lower panel of Fig. 12A for the representative mutant 4Gl-46/47, the distinct finding was obtained with EP accumulated from ATP and Ca²⁺, which was exclusively E₂PCa₂ as demonstrated in Figs. 6 and 8. In E₂PCa₂, the T2 site was completely resistant to tryptic attack as clearly shown by the exclusive accumulation of the fragment A without any further cleavage (i.e. without any formation of A1). This is in sharp contrast to its rapid cleavage in E₁PCa₂ accumulated from ATP with the wild type and in the E₁PCa₂ structural state of the mutant and wild type stabilized as E₁·AlF₄⁻·ADP. In the Ca²⁺-bound E₂P structural analogs (E₂V₄, E₂·AlF₄⁻, E₂·MgF₄⁻, and E₂·BeF₄⁻), the T2 site in the mutant was completely resistant as in the wild type. The results show that in E₂PCa₂ accumulated with the mutant, the A domain is already largely rotated from its position in E₁PCa₂ and associated with the P domain at the T2 site region (Val⁷⁰⁵/Val⁷²⁶) as in the Ca²⁺-bound E₂P state. This agrees with the fact that E₂PCa₂ accumulated is ADP-insensi-

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**FIGURE 13. Detailed structural model for the change E₁PCa₂ → E₂P + ²Ca²⁺.** The coordinates for the structures E₁·AlF₄⁻·ADP and E₂·MgF₄⁻ were obtained from the Protein Data Bank (access code 1WPE and 1WPG, respectively (13)). The two structures were manually fitted with M₈–M₁₀, which do not move virtually in the two structures. The A and P domains are yellow and pink, respectively. The N domain and ADP are not depicted for simplicity. The junctions of the N domain with the P domain are indicated by To N and From N. M₄C and M₄L are the cytoplasmic and luminal parts of M₄, respectively. The A/M₁ linker (Glü₁⁴⁰–Ser₁⁴⁸) is red. The phosphorylation site (Asp₁³⁵) and the bound Pi analogs AlF₄⁻ and MgF₄⁻ (blue spheres; fluoride) with white or magenta sphere (aluminum or magnesium); in E₁·AlF₄⁻·ADP and E₂·MgF₄⁻ are shown. Yellow solid arrow on E₁·AlF₄⁻·ADP indicates the movement of the A domain in the change E₁·AlF₄⁻·ADP → E₂·MgF₄⁻ (i.e. rotation by ~110° approximately parallel to the membrane plane). Dashed arrows with red, blue, and green on E₂·MgF₄⁻ indicate the approximate change of the position of the P, analog (red), rotation of M₁¹ (blue), and tilting of M₂ (green) in the change E₁·AlF₄⁻·ADP → E₂·MgF₄⁻. Note that the P domain tilts significantly with the connected M₄/M₅ toward the bottom part of the A domain and that the V-shaped rigid body structure formed by M₁⁰/M₁ and M₂ largely tilts. These rearrangements of the transmembrane helices were predicted to result in the Ca²⁺ release into lumen (13). T2, the tryptic site Arg₁⁹⁸ on the Val⁷⁰⁵ loop (Asp₁⁹⁶, Asp₂₀³, blue); Ptk, the sites for proteinase K (Leu₁₁⁹ on the top part of M₂ (A/M₂ linker region), Thr²⁴² on the A/M₃ linker, and Ala₇₄₆ on M₅). In E₂·MgF₄⁻, the A and P domains are associated by their interactions at three regions (semi-transparent purple, blue, and orange spheres) as also depicted in Fig. 2. The Tyr¹²¹, hydrophobic cluster (orange region) is formed from the A domain (Ile¹⁷⁹/Leu₁₸⁰/Ile₃₃₂), the P domain (Val⁷⁰⁵/Val⁷₂⁶), and the top part of M₂ (Tyr¹²¹/Leu¹₉₀). The F₁⁴⁰·GES loop (purple region) forms a hydrogen-bonding network with the residues of the P domain around Asp₁³⁵; the Val⁷⁰⁵ loop (blue region) forms an ionic and hydrogen-bonding network with the polar residues of the P domain (Arg⁹⁷/Glu₁⁴⁰/Arg₁⁹⁸/Asp₂₀³). Details of these interaction networks are not depicted for simplicity (see supplemental Figs. in Ref. 19 for the detailed structures of the interaction networks). Note that the interactions between the A and P domains at the Val⁷⁰⁵ loop and at the Tyr¹²¹-hydrophobic cluster are definitely monitored by the resistance because of steric hindrance of Arg¹⁹⁸ against trypsin and that of Leu¹₉₀ against proteinase K, respectively (15–17).
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tive, because the loss of the ADP sensitivity is brought about by the association of the largely rotated A domain with the P domain (13–17, 21) (Fig. 2 and Fig. 13).

In Fig. 12B, the same set of experiments was performed with PrtK. As demonstrated previously with SR Ca$^{2+}$-ATPase (48, 49), in the E2 state, PrtK cleaved at Leu$^{119}$ on the top part of M2 (A/M2 linker region, see Figs. 2 and 13) producing the fragment “p95,” and more slowly at Thr$^{242}$ on the A/M3 linker and Ala$^{176}$ on M5 producing the fragments “p81/83.” The Leu$^{119}$ site became resistant in E1Ca$_2$ and all the sites were nearly completely resistant in E1P + Ca$^{2+}$ accumulated from ATP with the wild type and in the E1P + ADP structural state (E1-AlF$_3$-ADP) produced with the wild type and with the mutant. The ATPase chain was also completely resistant in the Ca$^{2+}$-bound E2P structural analogs E2V$_{2}$, E2-AlF$_3$ (Fig. 12B), E2-MgF$_2$, and E2-BeF$_4$ (data not shown). These observations in the wild type and the mutant are in complete agreement with the previous demonstration with SR Ca$^{2+}$-ATPase (15–17). Distinct finding was obtained with E2P + Ca$^{2+}$ accumulated with the mutant. Namely, a fairly rapid cleavage at Leu$^{119}$ occurred in E2P + Ca$^{2+}$ to produce p95, and was in sharp contrast to its resistance in the E1P + Ca$^{2+}$ state and the Ca$^{2+}$-unbound E2P state. The results show that the structure at Leu$^{119}$ in E2P + Ca$^{2+}$ differs distinctly from those in E1P + Ca$^{2+}$ and in the Ca$^{2+}$-free form of E2P.

In this regard, it is essential to note that the structural bases rendering the complete resistance at Leu$^{119}$ are totally different between E1P + Ca$^{2+}$ and the Ca$^{2+}$-free form of E2P, as clearly seen in the structures E1-AlF$_3$-ADP and E2-MgF$_2$ (see Fig. 13). The resistance in E1P + Ca$^{2+}$ (E1-AlF$_3$-ADP) is most likely because of the steric blocking against PrtK brought about by van der Waals contacts of the top part of M2, including Leu$^{119}$ with the top part of M4 (Asn$^{330}$/Ile$^{332}$). On the other hand, in the Ca$^{2+}$-free form of E2P (E2-MgF$_2$ and E2-AlF$_3$), the resistance is most likely because of the steric blocking brought about by van der Waals contacts of Leu$^{119}$ with the hydrophobic residues in the interaction network “Tyr$^{122}$-hydrophobic cluster” formed from the A and P domains and the top part of M2 (A/M2 linker region). This cluster actually consists of Ile$^{179}$/Leu$^{180}$/Ile$^{322}$ at the bottom part of the A domain, Val$^{305}$/Val$^{276}$ of the P domain, and Leu$^{119}$/Tyr$^{122}$ at the top part of M2. Then a structural basis for the observed rapid cleavage at Leu$^{119}$ in E2P + Ca$^{2+}$, the intermediate state between E1P + Ca$^{2+}$ and E2P, can be deduced from the structural change E1-AlF$_3$-ADP $\rightarrow$ E2-AlF$_3$ (or E2-AlF$_3$), which is the presently available model for the overall change in E1P + Ca$^{2+}$ $\rightarrow$ E2P + 2Ca$^{2+}$ (Fig. 13). During this change, the A domain largely rotates, and M2 largely inclines away from its original position in E1P + Ca$^{2+}$. Also the P domain inclines toward the bottom part of the A domain and associates with the A domain and top part of M2. Hence, the Leu$^{119}$ region on the top part of M2 widely separates from M4 and comes to Ile$^{179}$/Leu$^{180}$/Ile$^{322}$ of the rotated A domain, and Val$^{305}$/Val$^{276}$ of the P domain moves down to these residues to form the Tyr$^{122}$-hydrophobic cluster. It is therefore very likely that Leu$^{119}$ will become sterically available to the PrtK attack in the intermediate structural state during this change, especially if such state is trapped and stably accumulated. Such a case was probably achieved in E2P + Ca$^{2+}$ accumulated with the mutants with the elongated A/M1 linker. It is concluded that the Tyr$^{122}$-hydrophobic cluster is not formed properly yet in E2P + Ca$^{2+}$, although in this state the A domain is already largely rotated and associated with the P domain at the Val$^{200}$ loop (the T2 site region).

**DISCUSSION**

In this study, we explored the functional importance of the length of the A/M1 linker (Glu$^{40}$–Ser$^{48}$) loop and found that its elongation markedly accelerates the loss of the ADP sensitivity in EP (the E1P to E2P isomerization) but blocks almost completely the decay of the ADP-insensitive EP accumulated. This EP was demonstrated to be E2P + Ca$^{2+}$, having two occluded Ca$^{2+}$ ions at the transport sites, and the Ca$^{2+}$-deocclusion and release from E2P + Ca$^{2+}$ were blocked. On the other hand, the hydrolysis of the Ca$^{2+}$-free form of E2P produced from the E2 state with P, in the mutants was as rapid as in the wild type. The stable E2P + Ca$^{2+}$ of the mutants was also produced in the reverse reaction upon addition of high concentrations of Ca$^{2+}$ to E2P formed from P without Ca$^{2+}$, and the reverse isomerization from E2P + Ca$^{2+}$ to E1P + Ca$^{2+}$ was blocked in the mutants.

The results indicate that the EP isomerization/Ca$^{2+}$-release process described as a single step, E1P + Ca$^{2+}$ $\rightarrow$ E2P + 2Ca$^{2+}$, consists of or can be dissected into the two successive steps E1P + Ca$^{2+}$ $\rightarrow$ E2P + Ca$^{2+}$, i.e. the loss of ADP sensitivity at the catalytic site (the EP isomerization) and the subsequent Ca$^{2+}$ release into lumen. This mechanism actually agrees with the one previously postulated on SR Ca$^{2+}$-ATPase, although the intermediate state E2P + Ca$^{2+}$ has never been identified and its mere presence has been questioned (e.g. Ref. 50). In this regard, we make the following two additional discussions in relation to previously proposed mechanisms. First, the standard view on the reaction mechanism with the sequential occurrence of E1P and E2P in the linear kinetic model (see Fig. 1) has been challenged, and instead an “out-of-phase coupling of the catalytic reactions” in the interacting ATPase molecules in their dimer was postulated (58, 59). In this model, one ATPase molecule in the dimer precedes the catalytic steps one step ahead of the other, and therefore the presence of equimolar steady-state concentrations of E1P + Ca$^{2+}$ and E2P + Ca$^{2+}$ are expected to be observed. On the other hand, in this study with the mutants with the elongated A/M1 linker, we observed clearly the exclusive accumulation of E2P + Ca$^{2+}$ (E2P possessing the stoichiometric amount of occluded Ca$^{2+}$) among all the EP present at the steady state with no E1P + Ca$^{2+}$. As shown in more detail in Figs. 4–6 and 8, almost all of the EP accumulated at steady state with the wild type was E1P + Ca$^{2+}$, and the accumulation of the stable E2P + Ca$^{2+}$ increased with the increase in the number of the inserted glycines in the A/M1 linker (i.e. 50% E2P + Ca$^{2+}$, with 50% E1P + Ca$^{2+}$ in 2G4-6/47, and nearly 100% E2P + Ca$^{2+}$ in the mutants with three or more glycine insertions). Thus, at least in our system in this study, the postulated out-of-phase coupling of the catalytic reactions is unlikely, or it might be possible that the postulated interactions between the ATPase molecules are disrupted in the mutants. As the second point of the additional discussions, the presence of a slow step in the reverse transition E2P + Ca$^{2+}$ to E1P + Ca$^{2+}$, upon the luminal Ca$^{2+}$ binding to E2P has also been a subject of controversy, and the mere presence of E2P + Ca$^{2+}$ has been questioned (50). Myung and Jencks (50) concluded on the basis of the observed very rapid reverse transition that there is no kinetic barrier in this reversal E2P + 2Ca$^{2+}$ $\rightarrow$ E1P + Ca$^{2+}$, and that there is only one phosphorylated intermediate with bound Ca$^{2+}$ in the transport cycle (i.e. E1P + Ca$^{2+}$). This reversal in the wild type upon the luminal Ca$^{2+}$ binding to E2P is certainly very rapid (as the kinetic results in Fig. 10A with the wild type indicated). On the other hand, in this study of the mutants with the elongated A/M1 linker, we also found the exclusive accumulation of E2P + Ca$^{2+}$ in the reverse reaction from E2P + Ca$^{2+}$ as well as in the forward reaction with ATP and Ca$^{2+}$. Thus, for the first time, we were able to trap the inter-
this study, we could successfully trap and thus identify this E2PCa2 state for the first time. E2PCa2, accumulated was actually shown to possess the structural feature as the intermediate state between E1PCa2 and the Ca<sup>2+</sup>-released form of E2P (Fig. 12), and the results revealed the critical importance of the length of the A/M1 linker in the Ca<sup>2+</sup> deocclusion and release from E2PCa2.

Importantly, our previous study showed (23) that the substitutions of any residues in the A/M1 linker do not inhibit the reaction cycle, whereas the shortening of the linker by a deletion of any single residue in this linker almost completely blocks the E1PCa2 to E2PCa2 isomerization and the hydrolysis of the Ca<sup>2+</sup>-free form of E2P. Thus, the length of this linker is obviously critical for each of the successive three steps, E1PCa2 → E2PCa2 → E2P + 2Ca<sup>2+</sup> and E2P + H<sub>2</sub>O → E2 + P<sub>i</sub>; and the shortening and the elongation of the linker both cause the severe defects but in distinct steps. Based on the results and the crystal structures, we discuss below the structural roles of the A/M1 linker in each of the three steps and dissect the structural events occurring in these steps.

Loss of ADP Sensitivity: the E1PCa2 to E2PCa2 Isomerization—

The loss of ADP sensitivity at the catalytic site (the EP isomerization) involves the large rotation of the A domain approximately parallel to the membrane plane and its association with the P domain (see E1-ADP → E2-MgF<sub>4</sub> in Figs. 2 and 13). Therefore, the outermost T<sup>181</sup>GES loop of the A domain comes above and docks onto the phosphorylation site Asp<sup>351</sup> by forming an extensive hydrogen-bonding network with the residues of the P domain around Asp<sup>351</sup>, and thus the T<sup>181</sup>GES loop sterically blocks the access of the ADP β-phosphate to the Asp<sup>351</sup>-acylphosphate (13). In E2PCa<sub>2</sub>, accumulated in the mutants with the elongated A/M1 linker, the rotation of the A domain and its docking with the P domain for the loss of the ADP sensitivity must already be achieved as it is ADP-insensitive. Such a structural state in E2PCa<sub>2</sub> was in fact clearly demonstrated by the observation that the tryptic T2 site Arg<sup>198</sup> on the Val<sup>200</sup> loop (Asp<sup>196</sup>–Asp<sup>203</sup>), another outermost loop of the A domain juxtaposed to the T<sup>181</sup>GES loop, is completely resistant against tryptic attack as in the E2P state without bound Ca<sup>2+</sup> (Fig. 12).

The elongation of the A/M1 linker markedly accelerated the E1PCa<sub>2</sub> to E2PCa<sub>2</sub> isomerization to cause the exclusive accumulation of E2PCa<sub>2</sub> (Figs. 4 and 5), and in contrast, its shortening by a deletion of any single residue blocked this isomerization (23). Therefore, the length of the A/M1 linker needs to be sufficiently long for the E1PCa<sub>2</sub> to E2PCa<sub>2</sub> isomerization. The A/M1 linker is probably critical for the positioning of the A domain relative to the P domain, i.e. the height from the membrane plane for their docking. Actually in the structural analogs for the ADP-insensitive EP (E2-MgF<sub>4</sub> and E2-AlF<sub>4</sub>), the largely rotated A domain is positioned above about half of the P domain, including Asp<sup>351</sup>, i.e. about half of the P domain is located underneath the A domain (Figs. 2 and 13). Hence, the T<sup>181</sup>GES loop at the lower part of the A domain comes above Asp<sup>351</sup> and blocks the access of ADP bound on the N domain to the Asp<sup>351</sup>-acylphosphate (13).

For realizing the A-P domain association, the P domain also should move significantly from the original position in E1PCa<sub>2</sub>, i.e. incline to the underneath of the A domain. Such motion of the P domain, as well as the large rotation of the A domain, is probably achieved to produce E2PCa<sub>2</sub> and at least to some extent to cause their docking and thus the loss of ADP sensitivity. (These motions are still not enough for the subsequent Ca<sup>2+</sup>-deocclusion/release from E2PCa<sub>2</sub> (see below for the Ca<sup>2+</sup> release.).

As the nature of the wild type, it is known that the EP isomerization is a very slow process thus having a kinetic barrier, and this slow process is followed by a very rapid Ca<sup>2+</sup> release in the scheme in Fig. 1 (actually the isomerization is rate-limiting in the whole Ca<sup>2+</sup> transport cycle). This means that the isomerization from E1PCa<sub>2</sub> to E2PCa<sub>2</sub> is accompanied by some structural distortion or strain. Our findings show that such structural restriction for the EP isomerization is markedly relieved by the elongation of the A/M1 linker (this study) but markedly enhanced by its shortening (23). The findings further suggest that structural distortion or strain imposed in E2PCa<sub>2</sub> upon the EP isomerization in the wild type benefits to the work for the subsequent Ca<sup>2+</sup> release from E2PCa<sub>2</sub> (see below). This is because virtually no Ca<sup>2+</sup>-deocclusion/release from E2PCa<sub>2</sub> occurred in the mutants with the elongated A/M1 linker. It is tempting to speculate that the largely rotated A domain may be pushed (or even moved) upward upon its positioning above and docking onto the P domain and that such mounting motion could be the structural restriction related to the length of the A/M1 linker.

As a summary of this section, the sufficiently long length of the A/M1 linker is probably critical for the proper motions and...
positioning of the A and P domains and their docking to produce the E2PCa2 structure in the E1PCa2 to E2PCa2 isomerization. The structural events are markedly accelerated by the elongation of the A/M1 linker, and the structural state of E2PCa2 thus produced with the mutants is very stable; therefore, the Ca\(^{2+}\)-deocclusion/release from E2PCa2 and the reverse change from E2PCa2 to E1PCa2 are blocked.

Ca\(^{2+}\) Release from E2PCa2 after the E1PCa2 to E2PCa2 Isomerization—After the E1PCa2 to E2PCa2 isomerization, our results showed that further structural changes should take place with a critical contribution of the A/M1 linker to rearrange the transmembrane helices and thereby deocclude Ca\(^{2+}\) ions at the transport sites and release into lumen (E2PCa2 → E2P + 2Ca\(^{2+}\)). Because the elongation of the A/M1 linker blocked the Ca\(^{2+}\) deocclusion and release, it is clear that this linker needs to be appropriately short for this event. The structural requirement of the length of the linker is hence in sharp contrast to that for the preceding E1PCa2 to E2PCa2 isomerization, in which the linker needs to be sufficiently long. Our present and previous results (23) clearly showed the following: 1) the shortening of the A/M1 linker blocks the E1PCa2 to E2PCa2 isomerization; 2) its elongation on the other hand markedly accelerates this isomerization; and 3) its elongation blocks the subsequent Ca\(^{2+}\)-deocclusion and release from E2PCa2. These results strongly suggest that the A/M1 linker with its native and correct length in the wild type will be strained upon the motion of the A domain and its positioning above half the P domain in the E1PCa2 to E2PCa2 isomerization, and the strain thus imposed in E2PCa2 will be utilized to cause the subsequent structural changes for the Ca\(^{2+}\)-deocclusion and release, E2PCa2 → E2P + 2Ca\(^{2+}\). Then the question is what structural changes are produced by critical contributions of the A/M1 linker with its correct length, i.e. its strain, and how the changes are transmitted to the transmembrane region to rearrange the helices to deocclude and release Ca\(^{2+}\) by the contribution of the A/M1 linker.

The essential changes of the transmembrane helices for the Ca\(^{2+}\)-release related with the motions of the cytoplasmic domains are seen in E1·AlF4-·ADP → E2·MgF42- as the model for the overall structural change in the EP isomerization/Ca\(^{2+}\)-release process E1PCa2 → E2P + 2Ca\(^{2+}\). As described in detail by Toyoshima and co-workers (13, 51), the inclination of the P domain (i.e. its moving and tilting to the A domain) causes the sideward shift of the cytoplasmic part of M4 (M4C), downward movement of M4, bending of M5C, and also rotation of M6; these changes would destroy the Ca\(^{2+}\)-binding sites. Also critical is the tilting of M2, which forms a V-shaped rigid body structure with M1’/M1 by van der Waals contacts. This rigid body moves (M1’ rotates and M2/M1 tilts) as the A domain largely rotates (see Fig. 13 and Fig. 4 in Ref. 13). Especially, the top part of M2 at its junction with the A domain largely moves outward, and thus M2 largely tilts and pushes against the luminal half of M4 via M1 to open the luminal gate (13).

Because the A/M1 linker connects directly to the A domain with M1’/M1, it would be easy at first glance to assume that this linker functions for transmitting the motion of the A domain directly to the M1’/M1/M2 rigid body to cause its motion, and such linkage would be impaired by the elongation of the A/M1 linker. In more detail in the change E1·AlF4-·ADP → E2·MgF42-, M1’ rotates away from its original position as the A domain rotates, and the top part of M2 comes to the position where M1’ was originally positioned, and the top part of M1 also moves sideward (see Fig. 4 in Ref. 13). If the A/M1 linker is elongated, the linker, for example, cannot pull M1’/M1, and the coordination between the motion of the A domain and that of M1’/M1/M2 would be impaired or not enough for the Ca\(^{2+}\)-deocclusion and release.

Nevertheless, to further clarify the critical structural contributions of the A/M1 linker in the Ca\(^{2+}\)-deocclusion/release process, it is necessary to take into account the actual structural difference revealed between E2PCa2 and the Ca\(^{2+}\)-released form of E2P. Namely, in E2PCa2, the Tyr122-hydrophobic cluster is not properly formed yet as demonstrated by the rapid PrtK cleavage at Leu1119 in sharp contrast to its complete resistance with the properly formed cluster in the Ca\(^{2+}\)-released form of E2P (Fig. 12). Most importantly, this cluster is produced by the largely moved three structural elements and their appropriate positioning, i.e. by the largely rotated A domain, the largely tilted M2, and the largely inclined P domain (see E1·AlF4-·ADP → E2·MgF42- in Fig. 13). Therefore, in the mutants with the elongated A/M1 linker, the motions of these elements are not yet achieved enough to form the cluster, and they are not stabilized at the appropriate positions because of the lack of the proper cluster. In this context, it should be noted that our present observations are in accord with the previous mutation studies on this cluster (19, 22) that the formation of this cluster and resulting strong interactions of the three structural elements are critical for the Ca\(^{2+}\)-released structure of E2P. Actually, in the mutants with the elongated A/M1 linker, the Ca\(^{2+}\)-released form of E2P or its structural analogs were shown to possess the proper Tyr122-hydrophobic cluster (Fig. 12) and the luminally opened Ca\(^{2+}\)-release pathway (Fig. 10).

All these findings directed us that for understanding the Ca\(^{2+}\)-release process from E2PCa2, it is necessary to further consider and dissect the possible roles of the A/M1 linker in the motions and proper positioning of these three structural elements to form the cluster. This implies even a possible contribution of the A/M1 linker to the motion of the P domain from E2PCa2 to E2P. Note that the rearrangements of M4/M5 are essential for the Ca\(^{2+}\)-deocclusion/release, and they must be linked with the motion of the P domain from the E2PCa2 state because M4/M5 is directly connected with the P domain. As discussed above for the E1PCa2 to E2PCa2 isomerization, in E2PCa2 half of the P domain is probably positioned already underneath the A domain and associated with this domain by forming the two interaction networks at the Val1200 loop and at the T181GES loop. For this A-P domain association in the E2P isomerization, the P domain may be inclined to some extent (as also described in Footnote 8). It is possible that the A/M1 linker with its strain likely functions to cause a further motion of the P domain from E2PCa2 via the associated A domain, thereby accomplishing the rearrangements of M4/M5 required for the Ca\(^{2+}\)-deocclusion/release from E2PCa2. It is tempting to speculate that the A domain is pulled by the strain of the A/M1 linker and pushes down the P domain located underneath the A domain. Thereby the P domain inclines further from the
E2PCa\textsubscript{2} state, and the residues of this domain (Val\textsuperscript{105}/Val\textsuperscript{206}) come to the position to form the Tyr\textsubscript{122}-hydrophobic cluster with the A domain (Ile\textsuperscript{179}/Leu\textsuperscript{180}/Ile\textsuperscript{285}) and the top part of M2 (Tyr\textsubscript{122}/Leu\textsuperscript{117}). Consequently, M4/M5 connected with the P domain will be rearranged to finally destroy the Ca\textsuperscript{2+} sites and release Ca\textsuperscript{2+}. The formation of the Tyr\textsubscript{122}-hydrophobic cluster at the bottom part of the A-P domain interface would stabilize this inclined state of the P domain, i.e. the Ca\textsuperscript{2+}-released form of E2P. The two interaction networks at the upper part of the A-P domain interface (at the Val\textsuperscript{200} loop and at the T\textsuperscript{181}GES loop) may function in E2PCa\textsubscript{2} \rightarrow E2P + 2Ca\textsuperscript{2+} as a base for such final adjustment of the interface of the A and P domains by the contribution of the A/M1 linker.

Regarding the motion of M1'/M1/M2 to achieve the Ca\textsuperscript{2+} release from E2PCa\textsubscript{2}, the structural state of E2PCa\textsubscript{2} accumulated without having the proper Tyr\textsubscript{122}-hydrophobic cluster, which is consistent with the view that M2 is not yet tilted enough in E2PCa\textsubscript{2} for the Ca\textsuperscript{2+} release and/or that the tilted state (i.e. the Ca\textsuperscript{2+}-released state) is not stabilized. Note that Leu\textsuperscript{119} (PrtK site) and Tyr\textsubscript{122} on the top part of M2 are involved in formation of this cluster (Fig. 13). As an additional mechanism to accomplish the tilting of M2 for the Ca\textsuperscript{2+} release besides the rotation of the A domain, the inclination of the P domain toward and its colliding with the top part of M2 (thus producing the Tyr\textsubscript{122}-hydrophobic cluster) might possibly be involved. Such changes would push the top part of M2 outward to cause further its tilting to finally achieve the Ca\textsuperscript{2+} release. As for the critical structural role of the long helix M2, it is important to state that its top part is fixed by the firm interactions with the A and P domain at the Tyr\textsubscript{122}-hydrophobic cluster, and therefore M2 can function for transmitting the motions of the cytoplasmic domains to the transmembrane domain by its large tilting, like a lever arm.

In this section, we dissected the possible contributions of the A/M1 linker with its strain to the further motions of the three structural elements from the E2PCa\textsubscript{2} state for producing the Ca\textsuperscript{2+}-released structure of E2P with the Tyr\textsubscript{122}-hydrophobic cluster. Through these changes in the cytoplasmic region, the A/M1 linker likely contributes not only to the motions of M1'/M1/M2 but also those of M4/M5 to open the luminal gate and release Ca\textsuperscript{2+}. Consequently, M4/M5 connected with the P domain will be rearranged to finally destroy the Ca\textsuperscript{2+} sites and release Ca\textsuperscript{2+}. The elongation of the A/M1 linker probably diminishes its strain and disrupted these structural events and the proper formation of the Tyr\textsubscript{122}-hydrophobic cluster that stabilizes the Ca\textsuperscript{2+}-released state. E2PCa\textsubscript{2} is therefore trapped in the mutants with the elongated linker. As for the two-step mechanism E1PCa\textsubscript{2} \rightarrow E2PCa\textsubscript{2} \rightarrow E2P + 2Ca\textsuperscript{2+}, it is of interest to know in future structural studies how far the rearrangements in the cytoplasmic and transmembrane regions actually occur in the first step and further in the second to finally deocclude and release Ca\textsuperscript{2+} from E2PCa\textsubscript{2}. In the wild type, however, all the structural changes in these processes may be occurring as a series of successive events by the critical contribution of the A/M1 linker, and E2PCa\textsubscript{2} may not be trapped as a distinct intermediate.

**Possible Role of M1' in Ca\textsuperscript{2+} Release from E2PCa\textsubscript{2}**—As indicated by the kinetic results, the nonconservative serine substitution of the hydrophobic residues Leu\textsuperscript{49}/Trp\textsuperscript{50}/Leu\textsuperscript{52}/Val\textsuperscript{53}/Ile\textsuperscript{54} on the amphipathic helix M1' (4950525354S) causes the strongly favored E2PCa\textsubscript{2} accumulation (Figs. 4 and 5) and blocks the E2PCa\textsubscript{2} decay (Figs. 6 and 7). In this regard, the consequence of this mutation is very similar to that of the elongation of the A/M1 linker. M1' is directly connected with the A/M1 linker and lying on the membrane surface, and the hydrophobic residues aligned on M1' probably form interactions with the membrane core thus anchoring M1' to the membrane (12, 13) (see Fig. 2). Our results therefore suggest that the hydrophobic interactions are important for fixing M1' at the membrane surface and thus for forming a base for the A/M1 linker to restrict its possible dislocation; therefore, the strain will be imposed on the linker in E2PCa\textsubscript{2} and can be utilized for the Ca\textsuperscript{2+} deocclusion and release from E2PCa\textsubscript{2}.

**E2P Hydrolysis after Ca\textsuperscript{2+} Release**—The elongation of the A/M1 linker does not inhibit the rapid hydrolysis of the Ca\textsuperscript{2+}-free form of E2P (Fig. 7), and in contrast, the shortening of the linker by the deletion of any single residues blocks the hydrolysis as shown previously (23). Therefore, the length of the A/M1 linker needs to be sufficiently long for the Asp\textsuperscript{351}-acylphosphate hydrolysis. The catalytic site for the E2P hydrolysis consists of the P and A domains, and the A domain is positioned above about half of the P domain (see Fig. 13, E2-MgF\textsubscript{4} \textsuperscript{2-}). Hence, the T\textsuperscript{181}GES loop of the A domain is situated above Asp\textsuperscript{351}, and a water molecule coordinated by Glu\textsuperscript{183} can attack the acylphosphate (13, 14, 52). It is therefore very likely that the A/M1 linker of its sufficiently long length is critical for formation of the correct configuration of the catalytic site from the properly positioned A and P domains, and the shortening of the linker by the deletion of even a single residue disrupted the catalytic site.

**Energy Coupling**—Regarding the coupling between ATP hydrolysis and Ca\textsuperscript{2+} transport, it is important to note that the Asp\textsuperscript{351}-acylphosphate of the mutants with the elongated A/M1 linker (i.e. with the sufficiently long linker) was not hydrolyzed in E2PCa\textsubscript{2}; by contrast, it was rapidly hydrolyzed in the Ca\textsuperscript{2+}-released form of E2P produced from P without Ca\textsuperscript{2+} (Figs. 6 and 7). The results indicate that some rearrangement in the A and P domains should take place during the Ca\textsuperscript{2+} release from E2PCa\textsubscript{2} to produce the catalytic site with the hydrolytic function. As described above, the structural difference between these two states is that the Tyr\textsubscript{122}-hydrophobic cluster is not formed properly yet in E2PCa\textsubscript{2} but is present in the Ca\textsuperscript{2+}-released E2P state. These observations are actually in complete agreement with the previous finding by the mutation studies (19, 22) that the formation of the Tyr\textsubscript{122}-hydrophobic cluster is critical for formation of the catalytic site for the acylphosphate hydrolysis in E2P. In fact, the T\textsuperscript{181}GES loop in the catalytic site is at the immediate C terminus of Ile\textsuperscript{179}/Leu\textsuperscript{180} of the Tyr\textsubscript{122}-hydrophobic cluster, and the DGVND loop (where underline indicates valine at position 705) that coordinates catalytic Mg\textsuperscript{2+} has Val\textsuperscript{705} of the cluster (for details of the structure, see supplemental Fig. II in Ref. 19). As described above, the formation of this cluster is most probably critical also for the Ca\textsuperscript{2+}-deocclusion/release from E2PCa\textsubscript{2}. In fact, the Ca\textsuperscript{2+}-released form of E2P produced from P\textsubscript{i} in the mutants was shown to possess the luminaly opened Ca\textsuperscript{2+}-release pathway (Fig. 10) as
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well as the catalytic site with the normal hydrolytic activity (Fig. 7). Thus, the Tyr\(^{122}\)-hydrophobic cluster formed with the critical contribution of the A/M1 linker functions both for the Ca\(^{2+}\)-deocclusion/release at the transport sites and for the formation of the catalytic site with the hydrolytic activity. Most importantly, as a consequence of this structural mechanism, a possible acylphosphate hydrolysis in E2PcA\(_{2}\) without releasing Ca\(^{2+}\) will be avoided, and hence the reaction sequence for the Ca\(^{2+}\) release and subsequent acylphosphate hydrolysis will be ensured, thus accomplishing the energy coupling in the Ca\(^{2+}\) transport.

During the E2P hydrolysis to E2, the luminal Ca\(^{2+}\) gate should be closed, and the ATPase prevents possible Ca\(^{2+}\) leakage from the lumen. On the basis of the structural change E2MgF\(_{4}\)\(^{2-}\) \(\rightarrow\) E2(TG), it was predicted (13) that the luminal gate closure involves the release of the A domain from the P domain (the loss of the interactions at the T\(^{181}\)GES loop and Val\(^{200}\) loop regions) and a tilting of the A domain upon the dephosphorylation (see Fig. 6 in Ref. 13). An interesting and specific question for us here was whether the strain of the A/M1 linker possibly contributes to the tilting of the A domain during the E2P hydrolysis and thus to the closure of the luminal gate. But this seems unlikely. This is because in the rate in the E2P hydrolysis and the Ca\(^{2+}\) affinity and rate in the E2 to E1Ca\(_{2}\) transition in the mutants with the elongated A/M1 linker are normal (Fig. 7 and Table 1), and therefore the normal structural changes probably take place during the E2P hydrolysis to E2 in the mutants as in the wild type.

Finally, it is worth noting that the strain of the A/M3 linker has been predicted to be important for the large rotation of the A domain in the EP isomerization (13, 53). The A/M1 linker, the A/M2 linker (top part of M2), including Tyr\(^{122}\)/Leu\(^{119}\), and the A/M3 linker therefore play distinct structural roles for the successive structural events in the E1PcA\(_{2}\) to E2PcA\(_{2}\) isomerization, the Ca\(^{2+}\)-deocclusion/release, and the E2P hydrolysis. In the transport cycle, the energy gained by the Ca\(^{2+}\) and ATP bindings will be transformed into the structural states of the phosphorylated intermediates producing the strain of the linkers and the motions and strong interactions of the A and P domains, and the conformational energy thus produced is utilized for the structural changes to deoccluded and release Ca\(^{2+}\) into lumen. The usefulness for the Ca\(^{2+}\)-ATPase having flexible links between cytoplasmic domains and the transmembrane domain is also discussed extensively in the very recent paper by Toyoshima and co-workers (51).

In summary, our studies revealed the structural requirements of the length of the A/M1 linker. The linker needs to be as follows: 1) sufficiently long for the E1PcA\(_{2}\) to E2PcA\(_{2}\) isomerization, 2) appropriately short for the subsequent Ca\(^{2+}\)-deocclusion and release, E2PcA\(_{2}\) \(\rightarrow\) E2P + 2Ca\(^{2+}\), and 3) then again sufficiently long for the E2P hydrolysis, E2P + H\(_{2}\)O \(\rightarrow\) E2 + P, \(_{\gamma}\)P. The native length of the A/M1 linker in the wild type is therefore precisely designed for inducing and coordinating the successive structural events in these steps and for the energy coupling. On the basis of the results and structures, we dissected the structural events for these processes and the functions of the structural elements involved in the processes.

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