Stable Structural Analog of Ca\textsuperscript{2+}-ATPase ADP-insensitive Phosphoenzyme with Occluded Ca\textsuperscript{2+} Formed by Elongation of A-domain/M1'-linker and Beryllium Fluoride Binding\textsuperscript{*}

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We have developed a stable analog for the ADP-insensitive phosphoenzyme intermediate with two occluded Ca\textsuperscript{2+} at the transport sites (E2PCa\textsubscript{2}) of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase. This is normally a transient intermediate state during phosphoenzyme isomerization from the ADP-sensitive to ADP-insensitive form and Ca\textsuperscript{2+} deocclusion/release to the lumen; E1PCa\textsubscript{2} \xrightarrow{\text{M}^2} E2PCa\textsubscript{2} \xrightarrow{\text{BeF}_3} E2P + 2Ca\textsuperscript{2+}. Stabilization was achieved by elongation of the Glu\textsubscript{40}-Ser\textsubscript{48} loop linking the Actuator domain and M1 (1st transmembrane helix) with four glycine insertions at Gly\textsubscript{46}/Lys\textsubscript{47} and by binding of beryllium fluoride (BeF\textsubscript{3}) to the phosphorylation site of the Ca\textsuperscript{2+}-bound ATPase (E1Ca\textsubscript{2}). The complex E2Ca\textsubscript{2}-BeF\textsubscript{3} was also produced by luminal Ca\textsuperscript{2+} binding to E2-BeF\textsubscript{3} (E2P ground state analog) of the elongated linker mutant. The complex was stable for at least 1 week at 25 °C. Only BeF\textsubscript{3}, but not AlF\textsubscript{3} or MgF\textsubscript{3}, produced the E2PCa\textsubscript{2} structural analog. Complex formation required binding of Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, or Ca\textsuperscript{2+} at the catalytic Mg\textsuperscript{2+} site. Results reveal that the phosphorylation product E1PCa\textsubscript{2} and the E2P ground state (but not the transition states) become competent to produce the E2PCa\textsubscript{2} transient state during forward and reverse phosphoenzyme isomerization. Thus, isomerization and luminal Ca\textsuperscript{2+} release processes are strictly coupled with the formation of the acyphosphate covalent bond at the catalytic site. Results also demonstrate the critical structural roles of the Glu\textsubscript{40}-Ser\textsubscript{48} linker and of Mg\textsuperscript{2+} at the catalytic site in these processes.

Sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA\textsubscript{1a})\textsuperscript{2} catalyzes Ca\textsuperscript{2+} transport coupled with ATP hydrolysis (Fig. 1) (1–9). In the catalytic cycle the enzyme is activated by two cytoplasmic Ca\textsuperscript{2+} ions binding to the transport sites. It is then autophosphorylated at Asp\textsubscript{351} by MgATP to produce the ADP-sensitive phosphoenzyme (E1P) that can react with ADP to regenerate ATP (steps 1–3). E1P formation results in Ca\textsuperscript{2+} occlusion at the transport sites (E1PCa\textsubscript{2}). Subsequent isomeric transition to an ADP-insensitive form (E2P), i.e. loss of ADP-sensitivity, results in Ca\textsuperscript{2+} deocclusion and release into the lumen (steps 4 and 5). This Ca\textsuperscript{2+}-release process is very rapid so that an E2PCa\textsubscript{2} intermediate state does not accumulate and in fact had never been found until we recently established its existence (10–13) and successfully trapped it for the first time (14). The Ca\textsuperscript{2+}-free E2P is finally hydrolyzed to the inactive E2 state (steps 6 and 7). Mg\textsuperscript{2+} as the physiological catalytic cofactor is required for both phosphorylation and hydrolysis. The transport cycle is reversible. Thus, E2P can be formed from P\textsubscript{i} in the presence of Mg\textsuperscript{2+} and absence of Ca\textsuperscript{2+}. Subsequent Ca\textsuperscript{2+} binding to luminal-oriented low affinity transport sites reverses the Ca\textsuperscript{2+}-releasing step and the E1P to E2P isomerization.

During EP isomerization/Ca\textsuperscript{2+}-release (E1PCa\textsubscript{2} \xrightarrow{\text{BeF}_3} E2P + 2Ca\textsuperscript{2+}), the A domain swings around parallel to the membrane plane (i.e. horizontal), whereas the A and P domains and M2 incline and tightly associate (Fig. 2) (15–25). We found that shortening of the A/M1'-linker by deletion of any single residue blocks E1PCa\textsubscript{2} \xrightarrow{\text{BeF}_3} E2PCa\textsubscript{2} isomerization and E2P hydrolysis (26). On the other hand, its elongation by two or more glycine insertions markedly accelerates the isomerization and blocks Ca\textsuperscript{2+} deocclusion/release (E2PCa\textsubscript{2} \xrightarrow{\text{BeF}_3} E2P + 2Ca\textsuperscript{2+}) (14). Thus, elongating the A/M1'-linker stabilized the normally transient intermediate state E2PCa\textsubscript{2} (i.e. ADP-insensitive EP with occluded Ca\textsuperscript{2+}) and showed that the length of this linker is critical for the structural changes that occur during E1PCa\textsubscript{2} \xrightarrow{\text{BeF}_3} E2PCa\textsubscript{2} \xrightarrow{\text{BeF}_3} E2P + 2Ca\textsuperscript{2+} and subsequent E2P hydrolysis (14, 26).

We have recently developed an E1Ca\textsubscript{2}-BeF\textsubscript{3} complex as a stable analog of E1PCa\textsubscript{2}-Mg\textsuperscript{2+} (E1PCa\textsubscript{2} with bound Mg\textsuperscript{2+} at the catalytic site) (27). Structural analysis of the analog and intermediate states suggests that formation of native E1PCa\textsubscript{2}-Mg\textsuperscript{2+} results in structural changes in the cytoplasmic and transmembrane domains due to configuration and ligation changes of the phosphate moiety (27). The Mg\textsuperscript{2+} bound at the catalytic site contributes to these structural changes (27). In fact, Ca\textsuperscript{2+} could not substitute for Mg\textsuperscript{2+} for formation of E1Ca\textsubscript{2}-BeF\textsubscript{3}, and an attempt to substitute Ca\textsuperscript{2+} for Mg\textsuperscript{2+} destroyed the complex (27). It is well known that Ca\textsuperscript{2+} substitution of Mg\textsuperscript{2+} at the catalytic site markedly retards E1PCa\textsubscript{2}-Ca isomerization (28, 29), a step that includes rotation of the A domain.

Further understanding of the mechanism of EP processing via the transient E2PCa\textsubscript{2} and of the critical roles of the A/M1'-linker is necessary in order to reveal the whole mechanism of EP processing.
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FIGURE 1. Ca2+-transport cycle of SERCA.

linker and catalytic Mg2+ requires detailed characterization of the development of E2PCa2, and of factors contributing to its possible stabilization. A great advance would be the finding of an analog stable enough for crystallographic studies.

In this study we employed the mutant 4Gi-46/47 in which the A/M1’-linker is elongated by four glycine insertions at Gly46/Lys47 (14) and explored the formation of a stable structural analog of E2PCa2, using various configuration analogs of phosphate (BeF3/AlF4/MgF4) and catalytic cations (Mg2+/Mn2+/Ca2+). We found that BeF3 is uniquely efficacious and that both mutant E1Ca2,BeF3 and mutant E2,BeF3 are capable of producing mutant E2Ca2,BeF3, most probably E2Ca2,BeF3, and that Ca2+ can replace the catalytic Mg2+ when coming from the former species. The mutant complex E2Ca2,BeF3 is extremely stable even at 25 °C.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression—The pMT2 expression vector (30) carrying the mutant rabbit SERCA1a cDNA with four glycine residues inserted between Gly46 and Lys47 (4Gi-46/47) was constructed as described previously (14). Transfection of pMT2 DNA into COS-1 cells and preparation of microsomes from the cells were performed as described previously (31). The amount of expressed SERCA1a was quantified by a sandwich enzyme-linked immunosorbent assay (32). Expression levels of wild type SERCA1a and the mutants were 2–3% that of total microsomal proteins.

Metal Fluoride Treatment—Microsomes expressing the wild type or 4Gi-46/47 were treated at 25 °C for 30 min with BeF3, AlF3, and MgF4 as described previously (14, 23–25, 27, 33–36) and in the legends for Figs. 3–9 in detail.

Formation of EP—Phosphorylation of SERCA1a in microsomes with [γ-32P]ATP was performed under conditions described in the legends for Figs. 3–8. The reactions were quenched with ice-cold trichloroacetic acid containing P2, Precipitated proteins were separated by 5% SDS-PAGE at pH 6.0 according to Weber and Osborn (37). The radioactivity associated with the separated Ca2+-ATPase was quantified by digital autoradiography as described (38).

Ca2+ Oclusion in SERCA1a—Microsomes treated with metal fluoride were diluted with “washing solution” containing excess EGTA and then immediately filtered through a 0.45-μm nitrocellulose membrane filter (Millipore). The filter was washed extensively with the washing solution, and 45Ca2+ remaining on the filter was quantified. The amount of Ca2+ specifically bound to the transport sites of EP in the expressed SERCA1a was obtained by subtracting the amount of nonspecific Ca2+-binding, which was determined as described in the legends for Figs. 8 and 9. The Ca2+ occluded/mg of expressed SERCA1a protein was calculated from the amount of expressed SERCA1a and the amount of occluded Ca2+.

Limited Proteolysis and Western Blot Analysis—Major intermediates of the Ca2+-ATPase and their stable analogs were produced and subjected to structural analysis by limited proteolysis with trypsin and protease K (prtK) as described in the legends for supplemental Figs. S3 and S4. Proteolysis was terminated by 2.5% (v/v) trichloroacetic acid. The digests were subjected to SDS-PAGE (39) followed by Western blot analysis with IHH11 monoclonal antibody to the rabbit SERCA1a (Affinity Bioreagents), which recognizes an epitope between Ala199 and Arg505 as described (14).

Miscellaneous—Protein concentrations were determined by the method of Lowry et al. (40) 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 (41).

RESULTS

Inhibition of EP Formation by Metal Fluoride—The E1Ca2 state of wild type and mutant 4Gi-46/47 SERCA1a in 10 μM Ca2+ was treated with BeF3 or AlF3 and functionally analyzed. The ability to form EP from ATP (Fig. 3, A and C) and from P2 (data not shown) is almost completely lost in the presence of 15 mM Mg2+ but not in its absence. EP formation is not inhibited when F− treatment in 15 mM Mg2+ is made without Be2+ or Al3+. The results show that the E1Ca2 state of the mutant as well as of wild type forms stable complexes with BeF3 and AlF3 in the presence of Mg2+ but not with MgF4.

When the E2 state of wild type and mutant 4Gi-46/47 in the absence of Ca2+ was treated with BeF3, AlF3, and MgF4 in the absence of Be2+ and Al3+, the complexes E2,BeF3, E2,AlF3, and E2,MgF4, respectively, are produced (14, 25), and EP formation from ATP (Fig. 3, B and D, open bars) and from P2 (data not shown) is almost completely inhibited. These complexes were then treated with 10 mM Ca2+ for 1 h in the presence of Ca2+-ionophore A23187 (black bars in Fig. 3, B and D). In the case of wild type, the ability to form EP is restored, consistent with the previous observation (25, 36) that a high concentration of Ca2+ in the presence of A23187 restores Ca2+-ATPase activity by destroying the complexes and converting the enzyme to E1Ca2. In mutant 4Gi-46/47, the Ca2+-induced restoration of EP formation is observed with E2,MgF4 and E2,AlF3 but not at all with E2,BeF3. E2,BeF3 of the mutant is, thus, resistant to Ca2+. We previously found (14) that the transient intermediate E2PCa2 is produced and trapped in the mutant in the reverse direction of the pump cycle from E2P by Ca2+-binding from the luminal side as well as in the forward direction from E1Ca2 through
ATP-induced phosphorylation. Therefore, the complex produced in the mutant with BeFx is likely \(E_2\text{Ca}^{2+}/H_1\text{BeF}_3\)/H1, an analog of \(E_2\text{PCa}^{2+}\) (as is in fact shown later in the \(\text{Ca}^{2+}\)/H1 binding and structural analyses in Fig. 8 and supplemental Figs. S3 and S4).

**Kinetic Analysis of BeFx-induced Complex Formation**—The \(E_1\text{Ca}_2\) state of mutant 4Gi-46/47 was treated with various concentrations of \(\text{Be}^{2+}\) and 1 mM F\(^-\) in 10 \(\mu\text{M Ca}^{2+}\) and 15 mM Mg\(^{2+}\), and the resulting species was analyzed (Fig. 4A). The

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**FIGURE 2. Crystal structures of SERCA1a.** The coordinates for structures \(E_1\text{Ca}_2\cdot\text{AlF}_4^-\cdot\text{ADP}\) (the analog of the transition state of phosphorylation, left) and \(E_2\cdot\text{BeF}_3^-\) (the analog of the \(E_2\text{P}\) ground state, right) were obtained from Protein Data Bank (PDB accession code 1T5T (17) and 2ZBE (21), respectively). \(\alpha\), the cytoplasmic domains N (nucleotide binding), P (phosphorylation), A (actuator), 10 transmembrane helices (M1-M10), phosphorylation site Asp351, and TGES184 on the A domain are indicated. Cleavage sites by trypsin (T1 (Arg505) and T2 (Arg198 on the Val200 loop (DPRAVNQD203)) and by prtK (Leu119 on the top part of M2 and Thr122 on the A/M3-linker) are shown. Arrows indicate approximate motions of the A and P domains, M2, and M1′ from \(E_1\text{Ca}_2\cdot\text{AlF}_4^-\cdot\text{ADP}\) to \(E_2\cdot\text{BeF}_3^-\). Note the large rotation of the A domain and the inclination of the P and A domains and M2. In the \(E_2\text{P}\) state the A and P domains interact at three regions; at the \(\text{TGES}\) loop with the residues around Asp351, at the \(\text{Val}^{200}\) loop (Asp196-Asp203) with polar residues of the P domain, and at Leu119/Tyr122 on the top part of M2 with the A, P, and N domains. In \(E_2\cdot\text{BeF}_3^-\) without TG, the cluster structure is rather loose (as the side chains of Leu119/Tyr122 are pointing away from the hydrophobic cluster), but Leu119/Tyr122 produce a more extended interaction network involving Thr122 of the N domain and the hydrophobic cluster (see more details in supplemental Fig. S5). b, the catalytic site is enlarged, and the residues involved in the \(\text{Mg}^{2+}\) (site I) are depicted. The Val679-Lys686 region of the P domain is not depicted for simplicity (because it is positioned over the region of interest).
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FIGURE 3. Inhibition of EP formation from ATP by metal fluoride. A and C, microsomes expressing the wild type or mutant 4Gi-46/47 (0.35 mg/ml) were treated at 25 °C for 30 min with metal fluoride in the presence of 0.01 mM Ca2+ (0.01 mM CaCl2, without EGTA) in 3 mM KF plus 50 mM BeSO4 or AlCl3, 0.1 mM KCl, and 50 mM MOPS/Tris (pH 7) with (black bar) or without (white bar) 15 mM MgCl2. Subsequently, the samples were diluted 10-fold and phosphorylated at 0 °C for 15 s with 10 μM [γ-32P]ATP in 1 mM A23187, 0.1 mM Ca2+ (0.5 mM CaCl2 with 0.4 mM EGTA), 7 mM MgCl2, 0.1 mM KCl, and 50 mM MOPS/Tris (pH 7), and the amount of EP formed was determined. The amount of EP formed with the wild type in the control sample, i.e. incubated without the fluoride compounds and Mg2+ (4.7 nmol/mg of the expressed SERCA1a), was normalized to 100%. The amount of EP formed with the mutant 4Gi-46/47 in the control sample was almost the same as that of wild type. B and D, microsomes were treated with metal fluoride in the absence of Ca2+ (1 mM EGTA without added CaCl2) and in the presence of the indicated concentrations of MgCl2. Subsequently, the samples were diluted 2.5-fold with a solution containing 1 mM A23187, 0.1 mM KCl, 50 mM MOPS/Tris (pH 7), and EGTA (to give 1 mM white bar) or CaCl2 (to give 10 mM Ca2+, black bar) and incubated at 25 °C for 1 h. The samples were then further diluted 10-fold and phosphorylated with 10 μM [γ-32P]ATP and 0.1 mM Ca2+ as in A and C, and the amount of EP formed was determined.

FIGURE 4. Be2+ dependence of the rate of EP inhibition by BeF3- in 0.01 mM Ca2+. A, microsomes expressing the wild type or mutant 4Gi-46/47 were incubated for various periods in 0.01 mM Ca2+ and 1 mM KF with various concentrations of BeSO4 and otherwise as in Fig. 3. A and C, for BeF3- treatment. The samples were then diluted 10-fold and phosphorylated with 10 μM [γ-32P]ATP, and the amount of EP formed was determined, as in Fig. 3, A and C. Solid lines show the least squares fit to a single exponential. In B, the rate constants obtained in A were plotted versus the concentration of BeF3- added. The linear fit to the data gave a slope of 0.123 min−1 μmol−1.

Of inhibition of EP formation was determined. BeF3- induced inhibition is markedly accelerated with increasing Mg2+, giving a K0.5 value of 4.9 mM. The observed apparent Mg2+ affinity is consistent with those values obtained through phosphorylation of native Ca2+-ATPase (42–47) and for the formation of E1Ca2-BeF3- (E1PCa2-Mg2+ analog) (27), i.e. the Mg2+ binding affinity at the catalytic Mg2+ site (site I composed of Asp357/Thr357/Asp703 and the phosphate moiety (BeF3-)). Therefore, Mg2+ binding at site I is likely a prerequisite for BeF3- binding and complex formation.

In Figs. 6 and 7, we further observed that the BeF3- induced complex formation from E1Ca2 in the mutant occurs with Mn2+ or Ca2+ in place of Mg2+. The K0.5 values are 1.4 mM for Mn2+ and 0.76 mM for Ca2+ (supplemental Figs. S1 and S2) and are consistent with such values for binding to the catalytic Mg2+ site (46, 48). In wild type the BeF3- induced E1Ca2-BeF3- formation, which inhibits EP formation occurs with Mn2+ but not with 10 mM Ca2+ in place of Mg2+ (Figs. 6 and 7). Thus, the complex formed from E1Ca2 with BeF3- in the mutant 4Gi-46/47 (i.e. E2Ca2-BeF3-) is distinct from E1Ca2-BeF3- of wild type.

Interestingly, the Hill coefficient for the Mg2+ as well as Mn2+ and Ca2+ dependence for complex formation with BeF3- (E2Ca2-BeF3-) in the mutant is nearly 2 (Fig. 5 and supplemental Figs. S1 and S2), suggesting the involvement of more
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FIGURE 5. Mg\(^{2+}\) dependence of the rate of EP inhibition by BeF\(_x\). A, microsomes expressing the mutant 4Gi-46/47 were incubated for various periods in 0.01 mM Ca\(^{2+}\), 1 mM KF, 10 \(\mu\)M BeSO\(_4\), and various concentrations of MgCl\(_2\) and otherwise as in Fig. 3. Solid lines show the least squares fit to a single exponential. In B the rate constants obtained in A were plotted versus the concentration of Mg\(^{2+}\) added. \(K_a\) for the Mg\(^{2+}\) activation and Hill coefficient obtained by fitting to the Hill equation (solid line) were 4.9 mM and 2.3, respectively.

FIGURE 6. EP inhibition by Mn\(^{2+}\) and BeF\(_x\) in 0.01 mM Ca\(^{2+}\) without Mg\(^{2+}\). Microsomes expressing the wild type or mutant 4Gi-46/47 were treated with 1 mM F\(^{-}\) plus 50 \(\mu\)M Be\(^{2+}\) or Al\(^{3+}\) in 0.01 mM Ca\(^{2+}\) and in the absence (white bar) or presence (black bar) of 2 mM MnCl\(_2\) (in place of MgCl\(_2\)) and otherwise as in Fig. 3, A and C. The samples were then diluted 10-fold and phosphorylated with 10 \(\mu\)M \([\gamma-\text{P}]\text{ATP}\), and the amount of EP formed was determined as in Fig. 3, A and C.

FIGURE 7. EP inhibition by 10 mM Ca\(^{2+}\) and BeF\(_x\) without Mg\(^{2+}\) and Mn\(^{2+}\). Microsomes expressing wild type or mutant 4Gi-46/47 were treated with 1 mM F\(^{-}\) plus 50 \(\mu\)M Be\(^{2+}\) or Al\(^{3+}\) in 0.01 or 10 mM CaCl\(_2\) without Mg\(^{2+}\) and Mn\(^{2+}\) and otherwise as in Fig. 3, A and C. The samples were then diluted 10-fold and phosphorylated with 10 \(\mu\)M \([\gamma-\text{P}]\text{ATP}\), and the amount of EP was determined as in Fig. 3, A and C.

than one metal ion. This is in contrast to the value 1 for \(E1\text{Ca}_2\cdot\text{BeF}_3\) formation with Mg\(^{2+}\) and Mn\(^{2+}\) in wild type (see supplemental Fig. 1 in Ref. 27).

AlF\(_x\) produces the complex with the \(E1\text{Ca}_2\cdot\text{AlF}_x\) state of the mutant 4Gi-46/47 as well as of wild type (\(E1\text{Ca}_2\cdot\text{AlF}_x\)) with Mg\(^{2+}\) and Mn\(^{2+}\) but not with Ca\(^{2+}\) at the catalytic Mg\(^{2+}\) site (Figs. 5, 6, and 7). Therefore, in the mutant the complex with AlF\(_x\) (\(E1\text{Ca}_2\cdot\text{AlF}_x\)) is distinct from that with BeF\(_x\) (\(E2\text{Ca}_2\cdot\text{BeF}_x\)) with respect to the strict preference of the divalent cation at the catalytic Mg\(^{2+}\) site.

Ca\(^{2+}\) Occlusion in the Mutant Complexed with BeF\(_x\).—In Fig. 8A, the \(E1\text{Ca}_2\) state of the mutant 4Gi-46/47 in 10 \(\mu\)M 4Ca\(^{2+}\) and 15 mM Mg\(^{2+}\) was complexed with BeF\(_x\) at a low concentration of BeF\(_x\) (1 \(\mu\)M) with 1 mM F\(^{-}\) to slow complex formation. The amount of occluded \(45\text{Ca}^{2+}\) was determined at various periods by membrane filtration with extensive washing with a solution containing excess EGTA and A23187. The loss of EP-forming ability with ATP decreases reciprocally and linearly with an increase in the amount of occluded Ca\(^{2+}\) (see the inset). The amount of occluded \(45\text{Ca}^{2+}\) at the intercept of the abscissa, i.e. when all the ATPases are complexed with BeF\(_x\) is 8.4 nmol/mg of expressed SERCA1a mutant protein. The stoichiometry of the occluded Ca\(^{2+}\) is nearly 2 per phosphorylation site, which is 4.3 nmol/mg as determined from the intercept on the ordinate. Therefore, the complex formed with BeF\(_x\) has two occluded Ca\(^{2+}\). When the mutant was incubated for 15 min with BeF\(_x\) and 1.5 mM Mn\(^{2+}\) in place of Mg\(^{2+}\) under otherwise identical conditions, EP formation was completely inhibited, and the amount of occluded \(45\text{Ca}^{2+}\) was 8.3 nmol/mg of expressed SERCA1a mutant protein, giving a stoichiometry of 2 per phosphorylation site (data not shown).

In Fig. 8B, we examined whether the complex \(E2\text{Ca}_2\cdot\text{BeF}_3\) can be produced from \(E2\text{BeF}_3\) by lumenal Ca\(^{2+}\) binding, mimicking the reverse conversion \(E2P + 2\text{Ca}^{2+} \rightarrow E2\text{PCa}_2\) (14). \(E2\text{BeF}_3\) was first formed in the mutant in the absence of Ca\(^{2+}\).
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![Diagram](image)

**FIGURE 8. Ca\(^{2+}\) occlusion in E2Ca\(_2\)-BeF\(_x\) of the mutant 4Gi-46/47 formed from E1Ca\(_2\), (A) and from E2-BeF\(_x\), (B).** A, microsomes (0.2 mg/ml) expressing the mutant 4Gi-46/47 were incubated for various periods at 25 °C in 10 μl of a mixture containing 0.01 mM [\(^{45}\)Ca\(_{1}\)], 1 mM KF, 1 μM BeSO\(_4\), 50 mM MgCl\(_2\), 0.1 mM KCl, 50 mM MOPS/Tris (pH 7). The mixture was then diluted 200-fold at 0 °C with a washing solution containing 2 mM EGTA, 5 μM A23187, 0.1 mM KCl, 7 mM MgCl\(_2\), and 50 mM MOPS/Tris (pH 7), subjected to membrane filtration, and washed rapidly with 6 ml of the washing solution for 45 s at 0 °C. For determination of EP, the above BeF\(_x\)-incubation was made with [\(^{45}\)Ca\(_2\)] instead of [\(^{32}\)P]ATP at 0 °C for 15 s as in Fig. 3C. The sample was then further diluted 10-fold at 0 °C with the washing solution, immediately filtered as above, and washed rapidly with ice-cold trichloracetic acid containing P. The EP level was not changed during the above sample handling because the decay of EP (E2PCa\(_2\)) is almost completely blocked in the mutant (14). The amount of \(^{45}\)Ca\(_2\) specifically bound and occluded (■) and that of E2P (○) in the expressed SERCA1a mutant were obtained by subtracting the background values determined by including 1 μM Tg in the BeF\(_x\) incubation mixture. The values presented are the mean ± S.D. (n = 5). Inset, the amount of EP formed was replotted versus that of occluded Ca\(_{2+}\) with the BeF\(_x\) treatment. The solid line represents the linear least squares fit. The y and x intercepts gave 4.3 and 8.4 nmol/mg of the expressed SERCA1a for the amounts of EP and of Ca\(_{2+}\) occluded, respectively. B, for formation of E2-BeF\(_x\), microsomes (1 mg/ml) expressing the mutant 4Gi-46/47 were incubated at 25 °C for 30 min with 1 mM KF and 20 μM BeSO\(_4\), in 1 mM EGTA, 7 mM MgCl\(_2\), 50 mM LiCl, and 50 mM MOPS/Tris (pH 7). Then the mixture was diluted 2.5-fold with a solution containing 7 mM MgCl\(_2\), 50 mM LiCl, 50 mM MOPS/Tris (pH 7), 5 μM Ca\(_{2+}\) ionophore A23187, and various concentrations of 4CaCl\(_2\) to give the indicated final \(^{45}\)Ca\(_{2+}\) concentrations. After incubating at 25 °C for 1 min, the mixture was further diluted with 400-fold of the washing solution containing the excess EGTA, filtered, and washed with the washing solution as above. The amount of \(^{45}\)Ca\(_{2+}\) specifically bound and occluded in the SERCA1a was obtained by subtracting the nonspecific Ca\(_{2+}\) binding, which was determined without KF in the BeF\(_x\) treatment and then incubated for 1 min at 25 °C with various concentrations of \(^{45}\)Ca\(_{2+}\) in the presence of Ca\(_{2+}\) ionophore A23187. The amount of occluded \(^{45}\)Ca\(_{2+}\) was determined after a large dilution followed by filtration and extensive EGTA washing. The maximum amount of occluded \(^{45}\)Ca\(_{2+}\) is 7.7 nmol/mg of mutant SERCA1a protein and 1.8 times that of the phosphorylation site (4.3 nmol/mg), giving a stoichiometry of nearly 2. Thus, mutant E2Ca\(_2\)-BeF\(_x\) is produced from mutant E2-BeF\(_x\) by the addition of Ca\(_{2+}\) in the presence of A23187.

K\(_{0.5}\) and the Hill coefficient observed in Fig. 8B are 0.1 mM and ~2, respectively, i.e. very similar values to those observed during E2PCa\(_2\) formation from E2P and Ca\(_{2+}\) in the mutant (14). The observed low Ca\(_{2+}\) affinity is in agreement with the wild type property (25, 49) that E2-BeF\(_x\) as well as E2P have low affinity Ca\(_{2+}\) binding sites; that is, the luminal-oriented transport sites. Importantly, E2Ca\(_2\)-BeF\(_x\)/E2PCa\(_2\) formed in the mutant (either from E1Ca\(_2\), or from E2-BeF\(_x\)/E2P) is remarkably stable and virtually not in equilibrium with E1Ca\(_2\)-BeF\(_x\)/E1PCa\(_2\) or E2-BeF\(_x\)/E2P, i.e. their formation is almost irreversible, as shown previously (14) and in this study. When Ca\(_{2+}\) comes from the cytoplasmic side for E2PCa\(_2\) formation from E1Ca\(_2\) with ATP (via E2 → E1Ca\(_2\) → E1PCa\(_2\) → E2PCa\(_2\)) in the mutant, the apparent Ca\(_{2+}\) affinity is very high, with K\(_{0.5}\) = 0.14 μM (14), equal to the value for cytoplasmic Ca\(_{2+}\) binding at the transport sites in wild type. Also in the case of mutant E2Ca\(_2\)-BeF\(_x\) formation from E1Ca\(_2\) with BeF\(_x\) in Fig. 8A, 0.1 μM Ca\(_{2+}\) is obviously enough to saturate (even 1 μM Ca\(_{2+}\) saturates (data not shown)), suggesting a similar high Ca\(_{2+}\) affinity as in E2PCa\(_2\) formation from E1 + 2Ca\(_{2+}\).

**Structures of Complexes Formed from E1Ca\(_2\) with Metal Fluoride**—During the Ca\(_{2+}\) transport cycle, the A, P, and N domains move and reorganize substantially. These changes can be monitored by proteolytic patterns and resistance against trypsin and prtK (23, 24). Therefore, we applied proteolytic analyses to mutant E2Ca\(_2\)-BeF\(_x\) to reveal the position of the domains and to establish whether it is a true structural E2Ca\(_2\) analog (supplemental Figs. S3 and S4 and Tables S1 and S2 and Refs. 54 and 55). All the various major intermediates and their analogs were formed from E1Ca\(_2\) in the mutant and wild type and then subjected to proteolyses. The results show that mutant E2Ca\(_2\)-BeF\(_x\) has the same structure as that of mutant E2PCa\(_2\) and that this structural state is intermediate between wild type E1PCa\(_2\) (wild type E1Ca\(_2\)-BeF\(_x\)) and Ca\(_{2+}\)-free E2P (wild type as well as mutant E2-BeF\(_x\)) as described below. In mutant E2Ca\(_2\)-BeF\(_x\) and in mutant E2PCa\(_2\), the T2 site Arg\(_{2198}\) on the Val\(_{200}\) loop is completely resistant to trypsin, as in wild type E2P (E2-BeF\(_x\)), showing that the A domain has rotated from its position in E1PCa\(_2\) (E1Ca\(_2\)-BeF\(_x\)) of wild type and is associated with the P domain at Arg\(_{2198}\) of the Val\(_{200}\) loop.

In both wild type E1Ca\(_2\)-BeF\(_x\) (E1PCa\(_2\)) and wild type and mutant E2-BeF\(_x\) (E2P), Leu\(_{119}\) on the upper portion of M2 is completely resistant to prtK attack and is, thus, sterically protected as found previously (Refs. 25 and 27; see a detailed mixture. In fitting to the Hill equation (solid line), the maximum amount of occluded Ca\(_{2+}\), K\(_{0.5}\), for the Ca\(_{2+}\) activation, and Hill coefficient were obtained as 7.7 nmol/mg of the expressed SERCA1a, 0.1 μM, and 1.6, respectively. The values presented are the mean ± S.D. (n = 7).
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description and reasons for protection in supplemental Fig. S5 and Ref. 56). By contrast, in mutant E2Ca\(_2\)-BeF\(_3\), and mutant E2PCa\(_2\), the prtK-site Leu\(^{119}\) is rapidly cleaved and, thus, exposed. Evidently Leu\(^{119}/\)Tyr\(^{122}\) on M2 in mutant E2Ca\(_2\)-BeF\(_3\) and mutant E2PCa\(_2\) have moved from their hidden position in E1PCa\(_2\) (E1Ca\(_2\)-BeF\(_3\)) but are not yet buried again through interaction with the A and P domains as in E2P (E2-BeF\(_3\)), suggesting an intermediate structure. The results also reveal how critical the native length of the A/M1’-linker is for moving M2 and the A and P domains to realize the Ca\(^{2+}\)-free state E2P (E2-BeF\(_3\)).

The proteolyses also reveal that wild type and mutant E1Ca\(_2\)-AlF\(_x\) are not structurally similar to wild type E1Ca\(_2\)-BeF\(_3\) (E1PCa\(_2\)) and mutant E2Ca\(_2\)-BeF\(_3\) (E2PCa\(_2\)). Interestingly, the rate of cleavage at the T2 site of mutant E1Ca\(_2\)-AlF\(_x\) is intermediate between that of wild type transition state (E1Ca\(_2\)-BeF\(_3\)/E1Ca\(_2\)-AlF\(_x\)/ADP) and that of the E1PCa\(_2\) product state (E1Ca\(_2\)-BeF\(_3\)), suggesting that the structure is also intermediate. Thus, elongation of the A/M1’-linker brought the E1Ca\(_2\)-AlF\(_x\) structure closer to that of wild type E1Ca\(_2\)-BeF\(_3\). Only BeF\(_x\) and not AlF\(_x\) produces a species analogous to the E2Ca\(_2\) structural state (E2Ca\(_2\)-BeF\(_3\) via E1Ca\(_2\)-BeF\(_3\)). This means that the phosphorylation reaction must have passed through the transition state to progress to the isomerization step.

In the mutant and wild type, the prtK-site Thr\(^{242}\) on the A/M3-linker is completely resistant in all the states E1Ca\(_2\)-AlF\(_x\)/ADP/E1Ca\(_2\)-AlF\(_x\)/E1Ca\(_2\)-BeF\(_3\) (E1PCa\(_2\)), E2Ca\(_2\)-BeF\(_3\) and E2PCa\(_2\), and E2-BeF\(_3\)/E2-BeF\(_3\)/E2-BeF\(_3\)/E2-MgF\(_4\)\(^-\) (as shown previously with sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (23, 24)). The result indicates that in both mutant and wild type, the A/M3-linker is strained by the A-domain rotation perpendicular to the membrane plane upon E1PCa\(_2\) formation from E1Ca\(_2\) and remains taut during E\(_{P}\) processing.

E2Ca\(_2\)-BeF\(_3\): Formation from E2-BeF\(_3\) by Lumenal Ca\(^{2+}\) Binding—The Ca\(^{2+}\)-free complexes E2-BeF\(_3\), E2-AlF\(_x\), and E2-MgF\(_4\)\(^-\) (the analogs of the E2P ground state, transition state, and product complex of E2P hydrolysis, respectively (25)) were first formed in mutant 4Gi-46/47 and wild type, with Mg\(^{2+}\) bound at the catalytic site, and subsequent proteolyses were performed with and without a 10 mM Ca\(^{2+}\) treatment in the presence of ionophore A23187 (supplemental Fig. S4 and Table S2). Under these conditions Ca\(^{2+}\)-treated mutant E2-BeF\(_3\) exhibits complete resistance at the trypptic T2 site Arg\(^{198}\) and a fairly rapid prtK cleavage at Leu\(^{119}\) on the top of M2, exactly as in mutant E2PCa\(_2\) and E2Ca\(_2\)-BeF\(_3\) produced from E1Ca\(_2\). These results agree with those in Fig. 3D where it is found that the ability to form E\(_{P}\) is not restored by Ca\(^{2+}\) treatment of E2-BeF\(_3\). Thus, E2Ca\(_2\)-BeF\(_3\), as the E2PCa\(_2\) analog, is produced from both E2-BeF\(_3\) and from E1Ca\(_2\) (mimicking luminal Ca\(^{2+}\) binding to E2P in the reverse direction of the pump cycle and the forward ATP-induced E\(_{P}\) formation and isomerization, respectively). On the other hand, mutant and wild type complexes E2-AlF\(_x\) and E2-MgF\(_4\)\(^-\) and wild type E2-BeF\(_3\) are destroyed by Ca\(^{2+}\) treatment as found previously with sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (25, 27).

Stability of Complex E2Ca\(_2\)-BeF\(_3\)—In Fig. 9, E2Ca\(_2\)-BeF\(_3\) was first produced from mutant E1Ca\(_2\) with BeF\(_x\) in 50 mM 45Ca\(^{2+}\) and 15 mM Mg\(^{2+}\), then further incubated at 25 °C in the presence of these ligands, and the amount of occluded 45Ca\(^{2+}\) was determined. The results show that the complex E2Ca\(_2\)-BeF\(_3\) of the mutant is perfectly stable even after 1 week. Proteolysis confirms that the structure remains unchanged during the incubation (data not shown). The stability of the complex was further tested by diluting into an EGTA-containing solution without BeF\(_x\) and the incubation was continued at 25 °C (see the inset). Ca\(^{2+}\) is slowly released with a rate constant of 7.0 h\(^{-1}\). The addition of thapsigargin (TG) to the diluent only doubles the rate of release, indicating that the transmembrane domain is fairly resistant to TG-induced structural perturbation. These decay rates are very similar to those of mutant E2PCa\(_2\) without and with TG addition, 9.7 and 27.3 h\(^{-1}\), respectively (14). Thus, in this respect also, mutant E2Ca\(_2\)-BeF\(_3\) is analogous to mutant E2PCa\(_2\).

**DISCUSSION**

**Mutant E2Ca\(_2\)-BeF\(_3\) as an Analog of Native Transient State E2PCa\(_2\) Using our elongated A/M1’-linker mutant, we have developed the complex E2Ca\(_2\)-BeF\(_3\), most probably E2Ca\(_2\)-BeF\(_3\) as a stable structural analog of the native transient state E2PCa\(_2\) (ADP-insensitive E\(_{P}\) with two Ca\(^{2+}\) at the transport sites), an intermediate in E\(_{P}\) isomerization and Ca\(^{2+}\) decoupling/release. The complex E2Ca\(_2\)-BeF\(_3\) has two occluded Ca\(^{2+}\) and is produced from both mutant E1Ca\(_2\) and mutant E2Ca\(_2\)-BeF\(_3\), mimicking native E2PCa\(_2\) formation from E1Ca\(_2\) after ATP-induced forward phosphorylation via E1PCa\(_2\) isomerization.
and in the reverse direction from E2P after lumenal Ca\(^{2+}\) binding. Mutant E2Ca\(_2\)BeF\(_3\) formation requires Mg\(^{2+}\) at the catalytic site as in native ATP- and P\(_i\)-induced E\(_P\) formation. The disposition of the cytoplasmic domains in mutant E2Ca\(_2\)BeF\(_3\) is equivalent to that in E2PCa\(_2\) trapped with the mutant and intermediate between native E1PCa\(_2\)Mg\(^{2+}\) (E1Ca\(_2\)-BeF\(_3\) of wild type) and native E2PCa\(_2\Mg\(^{2+}\) (E2BeF\(_3\) of wild type and mutant). All these properties of mutant E2Ca\(_2\)BeF\(_3\) meet the requirements of a native E2PCa\(_2\) analog.

Importantly, AlF\(_3\) and MgF\(_4\) are not able to produce this E2PCa\(_2\) analog either from mutant E1Ca\(_2\) or from mutant E2-AlF\(_3\) and E2-MgF\(_4\). Thus, BeF\(_3\) is unique in this regard. The coordination chemistry of the beryllium in BeF\(_3\) (BeF\(_3\)) allows it to directly ligate the aspartyl oxygen, thereby producing the same tetrahedral geometry as the covalent Asp\(^{351}\), acylphosphate, as seen in the atomic structure of the E2P ground state analog E2-BeF\(_3\) (21, 22). On the other hand, AlF\(_3\) (AlF\(_3\)-AlF\(_3\)) mimics the transition state of phosphorylation and dephosphorylation as seen in structures E1Ca\(_2\)-AlF\(_3\)-ADP and E2-AlF\(_3\) (17, 19, 22). MgF\(_4\) mimics P\(_i\) in the product complex E2P, after E2P hydrolysis as seen in structure E2-MgF\(_4\) (19). Our results taken together with the coordination chemistry show that the structural changes for E\(_P\) isomerization and Ca\(^{2+}\) deocclusion/release in the forward and reverse reactions are strictly coupled with the particular configuration of the acylphosphate after formation of the covalent bond within the catalytic site. The product E1PCa\(_2\) state and the E2P ground state are ready for the changes, but the transition state structures are not.

**Roles of A/M1’-linker and Structural Changes during E\(_P\) Formation and Processing**—The transient E2PCa\(_2\) state formed during E\(_P\) processing and its analog E2Ca\(_2\)BeF\(_3\) were trapped and stabilized by elongation of the A/M1’-linker. As revealed by the proteolyses, in mutant E2Ca\(_2\)-BeF\(_3\) and mutant E2PCa\(_2\), the A domain has already rotated parallel to membrane from its position in E1Ca\(_2\)-BeF\(_3\) (E1PCa\(_2\)-Mg) and has associated with the P domain at the Val\(^{200}\) loop. Because mutant E2PCa\(_2\) is ADP-insensitive (14), the outermost loop TGES\(^{184}\) of the A domain is most probably docked onto the Asp\(^{351}\) region, thereby blocking ADP access to the Asp\(^{351}\) acylphosphate (19). Thus, in mutant E2Ca\(_2\)-BeF\(_3\) and mutant E2PCa\(_2\), the A domain is positioned above the P domain. On the other hand, the proteolyses also show that the spatial relationship of the top part of M2 (Leu\(^{119}\)/Tyr\(^{122}\)) with the P and A domains in mutant E2Ca\(_2\)-BeF\(_3\) (equivalent to native E2PCa\(_2\)-Mg) is intermediate between those of the wild type E1Ca\(_2\)-BeF\(_3\) (native E1PCa\(_2\)-Mg) and the wild type and mutant E2BeF\(_3\) (native E2P-Mg). Thus, Leu\(^{119}\) (the prtK site) on the top part of M2 has broken its van der Waals contact with upper M4 seen in E1PCa\(_2\) but has not yet reached the P and A domains to form their interaction network at Leu\(^{119}\)/Tyr\(^{122}\), i.e. the Tyr\(^{122}\) hydrophobic cluster has not formed (see supplemental Fig. S5 for its structure). This interaction network formed from Ile\(^{179}\)/Leu\(^{180}\)/Ile\(^{232}\) of the A domain, Val\(^{705}\)/Val\(^{726}\) of the P domain, and Tyr\(^{122}\)/Leu\(^{119}\) of M2 is actually critical for the E2P structure (11–13). Therefore, in E2Ca\(_2\)-BeF\(_3\) and E2PCa\(_2\), stabilized by elongation of the A/M1’-linker, the inclining motions of domains and helix are not yet advanced enough to reach the E2P structure.

Deletion of any single residue in the A/M1’-linker, i.e. shortening it, completely blocks E1PCa\(_2\) isomerization to E2PCa\(_2\) (26). By contrast, its elongation markedly accelerates the isomerization and greatly stabilizes E2PCa\(_2\) blocking Ca\(^{2+}\) deocclusion/release from this transient state (14). These findings suggest that formation of the transient E2PCa\(_2\) state (mutant E2Ca\(_2\)-BeF\(_3\)) from E1PCa\(_2\) (E1Ca\(_2\)-BeF\(_3\)), strains the A/M1’-linker with the wild type/native length due to rotation and positioning of the A domain above the P domain, which in turn causes further movements of the A and P domains facilitating Ca\(^{2+}\) deocclusion/release (14) (see the schematic model in supplemental Fig. S6). The A and P domains incline more, as will M1/M2 and M4/M5 connected with these domains, favoring release of the Ca\(^{2+}\). This view agrees with the structural changes required for Ca\(^{2+}\) release described by Toyoshima et al. (19); the bending and movement of M4/M5 by inclination of the P domain is predicted to destroy the Ca\(^{2+}\) binding sites, and the inclination of M2 and M1 (as a V-shaped rigid body) will push the lower part of M4 via M1 and open the luminal gate.

These domain and segmental motions associated with Ca\(^{2+}\) release will establish the interaction network at Leu\(^{119}\)/Tyr\(^{122}\), the Tyr\(^{122}\) hydrophobic cluster, and stabilize the E2P structure with the luminal gate open (11–13). The position of the two A/P-domain interaction networks, with Leu\(^{119}\)/Tyr\(^{122}\) at the lower part and Val\(^{200}\) loop on the upper part of the interface, seems particularly appropriate to stabilize the inclined A and P domains and helices and, therefore, the gate in an open state.

These cluster formations are also critical for producing the E2P catalytic site with hydrolytic activity (11–13). Therefore, in this mechanism E2P hydrolysis can only occur after Ca\(^{2+}\) release, ensuring energy coupling. The relative stability of native E2P may function as a brake to allow enough time for releasing Ca\(^{2+}\) and for refining the catalytic site for subsequent hydrolysis, e.g. appropriate positioning of TGES\(^{184}\) and Glu\(^{183}\)-coordinated attacking water molecule.

**Ca\(^{2+}\) Substitution of Mg\(^{2+}\) at the Catalytic Site**—In the elongated A/M1’-linker mutant, Ca\(^{2+}\) as well as Mg\(^{2+}\) bound at the catalytic Mg\(^{2+}\) site is able to produce E2Ca\(_2\)-BeF\(_3\) from E1Ca\(_2\) via E1Ca\(_2\)-BeF\(_3\). This binding of Ca\(^{2+}\) is also found when mutant E2PCa\(_2\) is formed from CaATP in the absence of Mg\(^{2+}\) (14). This is in sharp contrast to the situation in the wild type, where Ca\(^{2+}\) cannot substitute for Mg\(^{2+}\) at the catalytic site for E1Ca\(_2\)-BeF\(_3\) formation. An attempt to substitute Ca\(^{2+}\) for Mg\(^{2+}\) actually destroys wild type E1Ca\(_2\)-BeF\(_3\) (27). The extremely rapid isomerization of E\(_P\) with bound Ca\(^{2+}\) at the Mg\(^{2+}\) site in the elongated A/M1’-linker mutant (E1Ca\(_2\) Ca \(\rightarrow\) E2PCa\(_2\)Ca) is again very different to the markedly retarded E1PCa\(_2\)-Ca isomerization in wild type (14). The atomic structures provide insights into why elongation of the linker allows Ca\(^{2+}\) to replace Mg\(^{2+}\) at the catalytic site.

In the atomic structures of E1Ca\(_2\)-CaAMPPCP and E1Ca\(_2\)-AlF\(_3\)-ADP described by Toyoshima et al. (18, 19), Mg\(^{2+}\) or Ca\(^{2+}\) ligation at the catalytic Mg\(^{2+}\) site (Asp\(^{351}\)/Thr\(^{353}\)/Asp\(^{703}\) of the P domain and the phosphate moiety (or its analog); see Fig. 2) induces the P domain to bend and, thereby, the A domain to rotate upward, perpendicular to the membrane plane (see
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Figs. 4 and 5 in Ref. 18 and the schematic in supplemental Fig. S6). This A-domain rotation raises its junctions with the A/M1’-linker and the A/M3-linker. The strain imposed on the A/M3-linker in E1PCI\(_2\) apparently drives the large horizontal rotation of the A domain during E1PCI\(_2\) to E2P isomerization (18, 19, 50, 51). In the stringent coordination chemistry, the ligation length is shorter in Mg\(^{2+}\) than in Ca\(^{2+}\) typically by 0.2 Å (e.g. 2.1 versus 2.3 Å (52, 53)). Therefore, Mg\(^{2+}\) ligation probably induces more P-domain bending and in consequence more upward swinging of the A domain, leading to a stronger pull from the A/M3-linker to effect the horizontal rotation of the A domain (27). This is substantiated by the finding that in wild type, E1PCI\(_2\)-Mg\(^{2+}\) is rapidly isomerized, whereas in E1PIC\(_2\)-Ca it is markedly retarded (28, 29).

The observed formation of E2Ca\(_2\)-BeF\(_3\) and E2PCI\(_2\) (via very rapid E1PCI\(_2\) isomerization) from mutant E1Ca\(_2\) with Ca\(^{2+}\) or Mg\(^{2+}\) at the catalytic Mg\(^{2+}\) site shows that the poor Ca\(^{2+}\) effect on the A-domain upward rotation and subsequent horizontal rotation is relieved by elongation of the A/M1’-linker. Note again that the A-domain junction with the A/M1’-linker is raised by the upward movement of the A domain. It is, therefore, likely that in wild type, the A/M1’-linker is strained to some extent by this movement of the A domain on formation of E1PCI\(_2\). This possible strain is evidently not deleterious for wild type, but it becomes a serious energy barrier when the A/M1’-linker is shortened by deletion of any single residue as the deletions completely block E1PCI\(_2\) to E2P isomerization (26). Strain in the wild type A/M1’-linker in E1PCI\(_2\) is likely to be important as a build up to generating stronger strain during E1PCI\(_2\) to E2PCI\(_2\) isomerization. Thus, the strain of the A/M1’-linker seems to be imposed increasingly during E1PCI\(_2\) formation and the subsequent isomerization to E2PCI\(_2\), and this energy finally could be used for inducing structural changes for Ca\(^{2+}\) deocclusion and release.

_E1Ca\(_2\)_-

**E1Ca\(_2\)-AlF\(_3\) Formed from E1Ca\(_2\) in the Elongated A/M1’-linker Mutant**—The proteolytic analyses reveal that in wild type organization of the cytoplasmic domains of the transition state analog E1Ca\(_2\)-AlF\(_x\) is identical to that of E1Ca\(_2\)-AlF\(_x\)-ADP and has obviously not yet reached the product E1PCI\(_2\) state E1Ca\(_2\)-BeF\(_3\). Namely, during the reaction E1Ca\(_2\)-AlF\(_x\)-ADP/E1Ca\(_2\)-AlF\(_x\) → E1Ca\(_2\)-BeF\(_3\), the A domain rotates partially in a horizontal direction and comes close to the P domain at tryptic T2 site Arg\(_{198}\) but is not completely engaged, so that it cannot produce the E2Ca\(_2\)-BeF\(_3\) and E2BeF\(_3\) states (Ref. 27 and see the schematic in supplemental Fig. S6). On the other hand, in the elongated A/M1’-linker mutant, the structure of E1Ca\(_2\)-AlF\(_x\) is intermediate between those of E1Ca\(_2\)-AlF\(_x\)-ADP and E1Ca\(_2\)-BeF\(_3\) of wild type as judged from the intermediate tryptic cleavage rate at Arg\(_{198}\). Thus, elongation of the A/M1’-linker partly relieves barriers to A-domain rotation, bringing the structure of E1Ca\(_2\)-AlF\(_x\) closer to that of E1Ca\(_2\)-BeF\(_3\). The finding agrees with our above postulate that the A/M1’-linker is strained by the A-domain upward movement during E1PCI\(_2\) (E1Ca\(_2\)-BeF\(_3\)) formation from the transition state (E1Ca\(_2\)-AlF\(_x\)). In fact, because the length of the Asp\(^{351}\)-phosphate bond in the transition state (as mimicked by AlF\(_x\)) is obviously longer than that of the covalent acylphosphate bond (as mimicked by BeF\(_3\)), the transition state (AlF\(_x\)) must exhibit less P-domain bending.

**Luminal Ca\(^{2+}\)-induced E2Ca\(_2\)-BeF\(_3\) Formation from E2BeF\(_3\)—**The observed reverse formation of E2Ca\(_2\)-BeF\(_3\) (native E2PCI\(_2\)) from mutant E2BeF\(_3\) (E2P) through Ca\(^{2+}\) binding from the lumen shows that the luminal gate (Ca\(^{2+}\) releasing pathway) is open in E2BeF\(_3\) (E2P ground state immediately before Ca\(^{2+}\) binding). This is in contrast to the closed gate in E2-BeF\(_3\) and E2-MgF\(_2\) (25). Thus, luminal gating is strictly coupled with the conformation change in the phosphate during E2P hydrolysis, thereby avoiding possible Ca\(^{2+}\) leakage (25). Note that in wild type, E2-BeF\(_3\) (open luminal gate) formed with Mg\(^{2+}\) is converted to E1Ca\(_2\)+ BeF\(_3\) by Ca\(^{2+}\), because cycle reversal and subsequent Ca\(^{2+}\) substitution of Mg\(^{2+}\) at the catalytic site destabilizes E1Ca\(_2\)-BeF\(_3\) as previously demonstrated (27). E2-BeF\(_3\) and E2-MgF\(_2\) (gates closed) in wild type and mutant were also decomposed to E1Ca\(_2\) by Ca\(^{2+}\) but probably by the high Ca\(^{2+}\) concentration disrupting the luminal and transmembrane regions, thereby destabilizing AlF\(_x\) and MgF\(_2\) ligation at the catalytic site.

**Mg\(^{2+}\) Dependence of E2Ca\(_2\)-BeF\(_3\) Formation from E1Ca\(_2\)—**The Mg\(^{2+}\) as well as Mn\(^{2+}\) or Ca\(^{2+}\) dependence of E2Ca\(_2\)-BeF\(_3\) formation from mutant E1Ca\(_2\) (Fig. 5 and supplemental Figs. S1 and S2) exhibited a Hill coefficient of 2, which is in contrast to the value of 1 for wild type E1Ca\(_2\)-BeF\(_3\) formation from E1Ca\(_2\) (27). The results suggest that one or more Mg\(^{2+}\) besides the one at catalytic Mg\(^{2+}\) site I is involved cooperatively in the E2Ca\(_2\)-BeF\(_3\) formation from E1Ca\(_2\). In the atomic structures of E1Ca\(_2\)-CaAMPPCP and E1Ca\(_2\)-AlF\(_x\)-ADP, only one Mg\(^{2+}\) (or Ca\(^{2+}\)) at site I is seen (in addition to the one coordinated with the nucleotide, which was predicted to aid phosphoryl transfer). Also, in the structures of E2-BeF\(_3\), E2-AlF\(_x\), and E2-MgF\(_2\), only one Mg\(^{2+}\) is seen (at site I). Therefore, in E2Ca\(_2\)-BeF\(_3\) (E2P) formation a second (or more) Mg\(^{2+}\) may possibly be required only transiently and, together with the catalytic ion, aids the motions of N, P, and A domains and their gathering during the E1PCI\(_2\) isomerization to E2PCI\(_2\). In summary, our previous (14, 26) and present studies show that the A/M1’-linker should be appropriately long for the E1Ca\(_2\) to E2P isomerization then short enough for the Ca\(^{2+}\) deocclusion/release from E2PCI\(_2\) and again appropriately long for E2P hydrolysis. Thus, the length of the A/M1’-linker in wild type is naturally designed to induce successive structural changes and motions of the cytoplasmic and transmembrane domains for these processes. These functions of the A/M1’-linker act in concert with the changing configuration of the phosphate and catalytic Mg\(^{2+}\) and the Asp\(^{351}\)-phosphate bond length, with strength being critical in the formation of E2PCI\(_2\), a species poised to deliver Ca\(^{2+}\) to the lumen. The stable analogs, E1Ca\(_2\)-BeF\(_3\) (27) and E2Ca\(_2\)-BeF\(_3\) (this study) with bound Mg\(^{2+}\) could be critically important for obtaining atomic models of E1PCI\(_2\)-Mg\(^{2+}\) and the hitherto elusive transient E2PCI\(_2\)-Mg\(^{2+}\) intermediate for further understanding of the transport mechanism.

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