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Stable Structural Analog of Ca^{2+}-ATPase ADP-insensitive Phosphoenzyme with Occluded Ca^{2+} Formed by Elongation of A-domain/M1′-linker and Beryllium Fluoride Binding*[S]

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We have developed a stable analog for the ADP-insensitive phosphoenzyme intermediate with two occluded Ca^{2+} at the transport sites ($E2PCa_2$) of sarcoplasmic reticulum Ca^{2+}-ATPase. This is normally a transient intermediate state during phosphoenzyme isomerization from the ADP-sensitive to ADP-insensitive form and Ca^{2+} deocclusion/release to the lumen; $E1PCa_2 \rightarrow E2PCa_2 \rightarrow E2P + 2Ca^{2+}$. Stabilization was achieved by elongation of the Glu^{40}-Ser^{48} loop linking the Actuator domain and M1 (1st transmembrane helix) with four glycine insertions at Gly^{46}/Lys^{47} and by binding of beryllium fluoride (BeF_x) to the phosphorylation site of the Ca^{2+}-bound ATPase ($E1Ca_2$). The complex $E2Ca_2{\cdot}BeF_3^-$ was also produced by lumenal Ca^{2+} binding to $E2{\cdot}BeF_3^-$ ($E2P$ ground state analog) of the elongated linker mutant. The complex was stable for at least 1 week at 25 °C. Only BeF_x, but not AlF_x or MgF_x, produced the $E2PCa_2$ structural analog. Complex formation required binding of Mg^{2+}, Mn^{2+}, or Ca^{2+} at the catalytic Mg^{2+} site. Results reveal that the phosphorylation product $E1PCa_2$ and the $E2P$ ground state (but not the transition states) become competent to produce the $E2PCa_2$ transient state during forward and reverse phosphoenzyme isomerization. Thus, isomerization and lumenal Ca^{2+} release processes are strictly coupled with the formation of the acylphosphate covalent bond at the catalytic site. Results also demonstrate the critical structural roles of the Glu^{40}-Ser^{48} linker and of Mg^{2+} at the catalytic site in these processes.

Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA1a)[2] catalyzes Ca^{2+} transport coupled with ATP hydrolysis (Fig. 1) (1–9). In the catalytic cycle the enzyme is activated by two cytoplasmic Ca^{2+} ions binding to the transport sites. It is then autophosphorylated at Asp^{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^{2+} occlusion at the transport sites ($E1PCa_2$). Subsequent isomeric transition to an ADP-insensitive form ($E2P$), *i.e.* loss of ADP-sensitivity, results in Ca^{2+} deocclusion and release into the lumen (*steps 4* and *5*). This Ca^{2+}-release process is very rapid so that an $E2PCa_2$ 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^{2+}-free $E2P$ is finally hydrolyzed to the inactive $E2$ state (*steps 6* and *7*). Mg^{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_i in the presence of Mg^{2+} and absence of Ca^{2+}. Subsequent Ca^{2+} binding to lumenal-oriented low affinity transport sites reverses the Ca^{2+}-releasing step and the $E1P$ to $E2P$ isomerization.

During EP isomerization/Ca^{2+}-release ($E1PCa_2 \rightarrow E2P + 2Ca^{2+}$), 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 $E1PCa_2 \rightarrow E2PCa_2$ isomerization and $E2P$ hydrolysis (26). On the other hand, its elongation by two or more glycine insertions markedly accelerates the isomerization and blocks Ca^{2+} deocclusion/release ($E2PCa_2 \rightarrow E2P + 2Ca^{2+}$) (14). Thus, elongating the A/M1′-linker stabilized the normally transient intermediate state $E2PCa_2$ (*i.e.* ADP-insensitive EP with occluded Ca^{2+}) and showed that the length of this linker is critical for the structural changes that occur during $E1PCa_2 \rightarrow E2PCa_2 \rightarrow E2P + 2Ca^{2+}$ and subsequent $E2P$ hydrolysis (14, 26).

We have recently developed an $E1Ca_2{\cdot}BeF_3^-$ complex as a stable analog of $E1PCa_2{\cdot}Mg^{2+}$ ($E1PCa_2$ with bound Mg^{2+} at the catalytic site) (27). Structural analysis of the analog and intermediate states suggests that formation of native $E1PCa_2{\cdot}Mg^{2+}$ results in structural changes in the cytoplasmic and transmembrane domains due to configuration and ligation changes of the phosphate moiety (27). The Mg^{2+} bound at the catalytic site contributes to these structural changes (27). In fact, Ca^{2+} could not substitute for Mg^{2+} for formation of $E1Ca_2{\cdot}BeF_3^-$, and an attempt to substitute Ca^{2+} for Mg^{2+} destroyed the complex (27). It is well known that Ca^{2+} substitution of Mg^{2+} at the catalytic site markedly retards $E1PCa_2{\cdot}Ca$ isomerization (28, 29), a step that includes rotation of the A domain.

Further understanding of the mechanism of EP processing via the transient $E2PCa_2$ and of the critical roles of the A/M1′-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6 and Tables S1 and S2.

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[2] The abbreviations used are: SERCA1a, adult fast-twitch skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase; EP, phosphoenzyme; $E1PCa_2$, ADP-sensitive phosphoenzyme with occluded Ca^{2+}; $E2PCa_2$, ADP-insensitive phosphoenzyme with occluded Ca^{2+}; $E2P$, ADP-insensitive phosphoenzyme; TG, thapsigargin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; prtK, proteinase K; AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate.

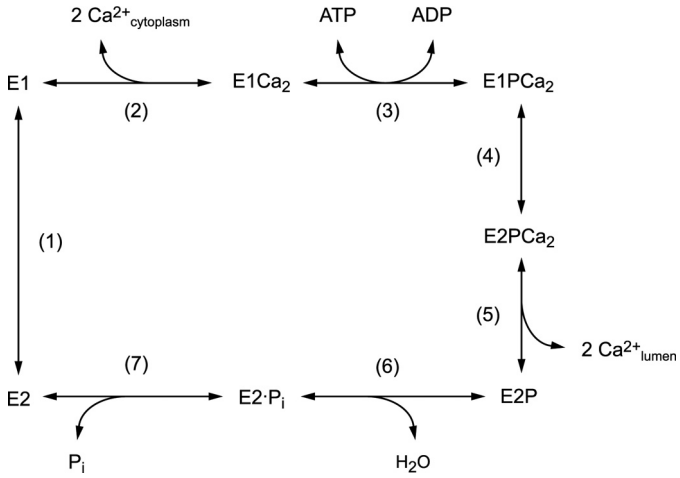

FIGURE 1. **Ca^{2+}-transport cycle of SERCA.**

linker and catalytic Mg^{2+} requires detailed characterization of the development of $E2PCa_2$ 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 Gly^{46}/Lys^{47} (14) and explored the formation of a stable structural analog of $E2PCa_2$ using various configuration analogs of phosphate (BeF_x/AlF_x/MgF_x) and catalytic cations (Mg^{2+}/Mn^{2+}/Ca^{2+}). We found that BeF_x is uniquely efficacious and that both mutant $E1Ca_2{\cdot}BeF_3^-$ and mutant $E2{\cdot}BeF_3^-$ are capable of producing mutant $E2Ca_2{\cdot}BeF_x$, most probably $E2Ca_2{\cdot}BeF_3^-$, and that Ca^{2+} can replace the catalytic Mg^{2+} when coming from the former species. The mutant complex $E2Ca_2{\cdot}BeF_3^-$ 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 Gly^{46} and Lys^{47} (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 BeF_x, AlF_x, and MgF_4^{2-} 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-^{32}P]ATP was performed under conditions described in the legends for Figs. 3–8. The reactions were quenched with ice-cold trichloroacetic acid containing P_i. Precipitated proteins were separated by 5% SDS-PAGE at pH 6.0 according to Weber and Osborn (37). The radioactivity associated with the separated Ca^{2+}-ATPase was quantified by digital autoradiography as described (38).

Ca^{2+} Occlusion 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 $^{45}Ca^{2+}$ remaining on the filter was quantified. The amount of Ca^{2+} specifically bound to the transport sites of *E*P in the expressed SERCA1a was obtained by subtracting the amount of nonspecific Ca^{2+}-binding, which was determined as described in the legends for Figs. 8 and 9. The Ca^{2+} occluded/mg of expressed SERCA1a protein was calculated from the amount of expressed SERCA1a and the amount of occluded Ca^{2+}.

Limited Proteolysis and Western Blot Analysis—Major intermediates of the Ca^{2+}-ATPase and their stable analogs were produced and subjected to structural analysis by limited proteolysis with trypsin and proteinase 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 IIH11 monoclonal antibody to the rabbit SERCA1a (Affinity Bioreagents), which recognizes an epitope between $Ala^{199}$and Arg^{505} 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 $E1Ca_2$ state of wild type and mutant 4Gi-46/47 SERCA1a in 10 μM Ca^{2+} was treated with BeF_x or AlF_x and functionally analyzed. The ability to form *E*P from ATP (Fig. 3, *A* and *C*) and from P_i (data not shown) is almost completely lost in the presence of 15 mM Mg^{2+} but not in its absence. *E*P formation is not inhibited when F^- treatment in 15 mM Mg^{2+} is made without Be^{2+} or Al^{3+}. The results show that the $E1Ca_2$ state of the mutant as well as of wild type forms stable complexes with BeF_x and AlF_x in the presence of Mg^{2+} but not with MgF_x.

When the *E*2 state of wild type and mutant 4Gi-46/47 in the absence of Ca^{2+} was treated with BeF_x, AlF_x, and MgF_x (in the absence of Be^{2+} and Al^{3+}), the complexes $E2{\cdot}BeF_3^-$, $E2{\cdot}AlF_4^-$, and $E2{\cdot}MgF_4^{2-}$, respectively, are produced (14, 25), and *E*P formation from ATP (Fig. 3, *B* and *D*, *open bars*) and from P_i (data not shown) is almost completely inhibited. These complexes were then treated with 10 mM Ca^{2+} for 1 h in the presence of Ca^{2+} ionophore A23187 (*black bars* in Fig. 3, *B* and *D*). In the case of wild type, the ability to form *E*P is restored, consistent with the previous observation (25, 36) that a high concentration of Ca^{2+} in the presence of A23187 restores Ca^{2+}-ATPase activity by destroying the complexes and converting the enzyme to $E1Ca_2$. In mutant 4Gi-46/47, the Ca^{2+}-induced restoration of *E*P formation is observed with $E2{\cdot}MgF_4^{2-}$ and $E2{\cdot}AlF_4^-$ but not at all with $E2{\cdot}BeF_3^-$. $E2{\cdot}BeF_3^-$ of the mutant is, thus, resistant to Ca^{2+}. We previously found (14) that the transient intermediate $E2PCa_2$ is produced and trapped in the mutant in the reverse direction of the pump cycle from *E*2P by Ca^{2+} binding from the lumenal side as well as in the forward direction from $E1Ca_2$ through

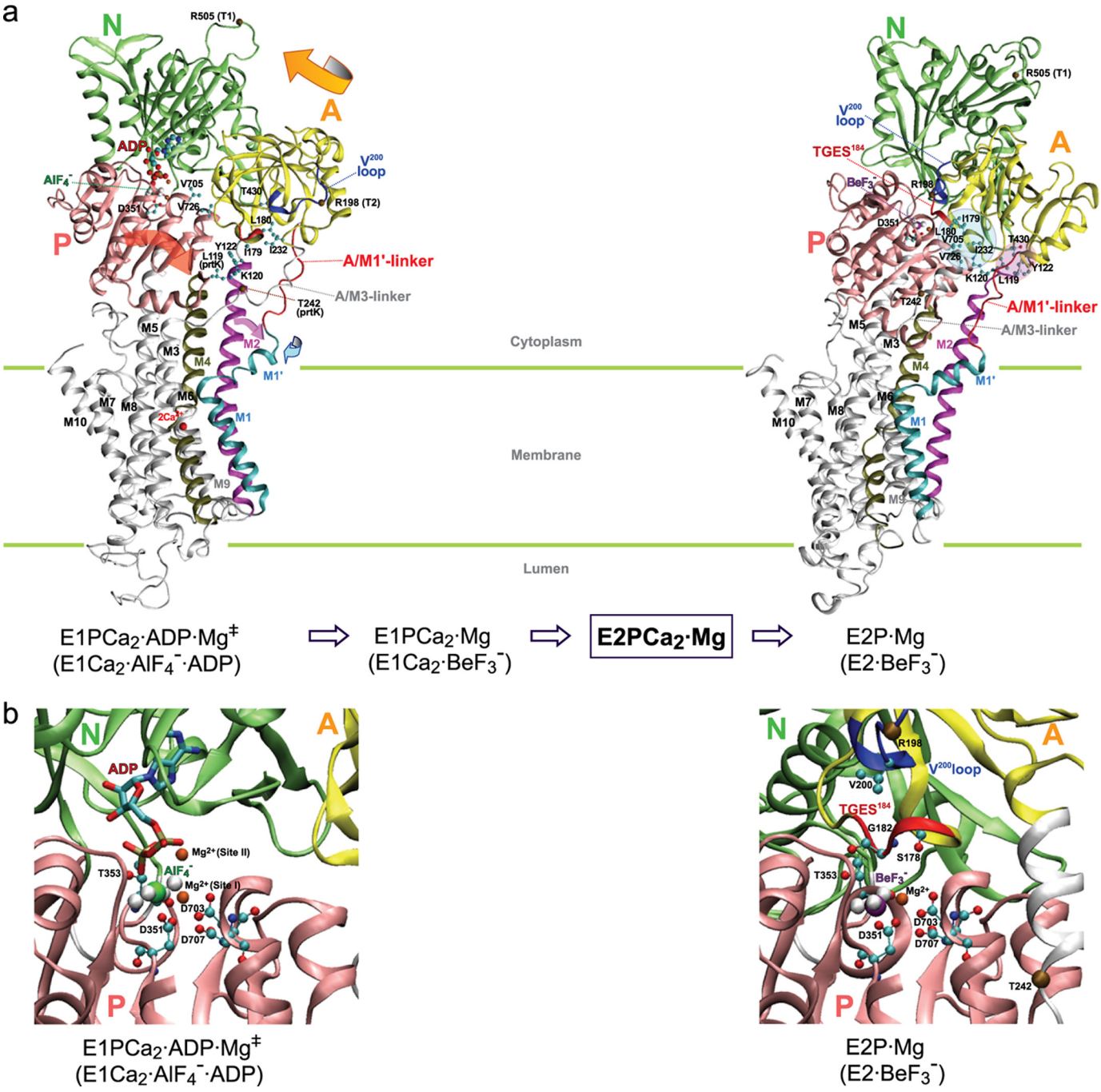

FIGURE 2. **Crystal structures of SERCA1a.** The coordinates for structures $E1Ca_2{\cdot}AlF_4^-{\cdot}ADP$ (the analog of the transition state of phosphorylation, *left*) and $E2{\cdot}BeF_3^-$ (the analog of the *E*2P ground state, *right*) were obtained from Protein Data Bank (PDB accession code 1T5T (17) and 2ZBE (21), respectively). *a*, the cytoplasmic domains N (nucleotide binding), P (phosphorylation), A (actuator), 10 transmembrane helices (M1-M10), phosphorylation site Asp^{351}, and $TGES^{184}$ on the A domain are indicated. Cleavage sites by trypsin (*T1* (Arg^{505}) and *T2* (Arg^{198} on the Val^{200} loop ($DP\underline{R}A\underline{V}NQD^{203}$)) and by prtK ($Leu^{119}$ on the top part of M2 and Thr^{242} on the A/M3-linker) are shown. *Arrows* indicate approximate motions of the A and P domains, M2, and M1′ from $E1Ca_2{\cdot}AlF_4^-{\cdot}ADP$ to $E2{\cdot}BeF_3^-$. Note the large rotation of the A domain and the inclination of the P and A domains and M2. In the *E*2P state the A and P domains interact at three regions; at the $^{181}TGES$ loop with the residues around Asp^{351}, at the Val^{200} loop (Asp^{196}-Asp^{203}) with polar residues of the P domain, and at Leu^{119}/Tyr^{122} on the top part of M2 with the A, P, and N domains. In $E2{\cdot}BeF_3^-$ (TG) (PDB accession code 2ZBF (21), supplemental Fig. S5), Leu^{119}/Tyr^{122} produce the Tyr^{122}-hydrophobic cluster with five other hydrophobic residues, $Ile^{179}/Leu^{180}/Ile^{232}$ of the A domain and Val^{705}/Val^{726} of the P domain. In $E2{\cdot}BeF_3^-$ without TG, the cluster structure is rather loose (as the side chains of Leu^{119}/Tyr^{122} are pointing away from the hydrophobic cluster), but Leu^{119}/Tyr^{122} produce a more extended interaction network involving Thr^{430} 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 Mg^{2+} (site I) are depicted. The Val^{679}-Lys^{686} region of the P domain is not depicted for simplicity (because it is positioned over the region of interest).

ATP-induced phosphorylation. Therefore, the complex produced in the mutant with BeF_x is likely $E2Ca_2{\cdot}BeF_3^-$, an analog of $E2PCa_2$ (as is in fact shown later in the Ca^{2+} binding and structural analyses in Fig. 8 and supplemental Figs. S3 and S4).

Kinetic Analysis of BeF_x-induced Complex Formation—The $E1Ca_2$ state of mutant 4Gi-46/47 was treated with various concentrations of Be^{2+} and 1 mM F^- in 10 μM Ca^{2+} and 15 mM Mg^{2+}, and the resulting species was analyzed (Fig. 4*A*). The

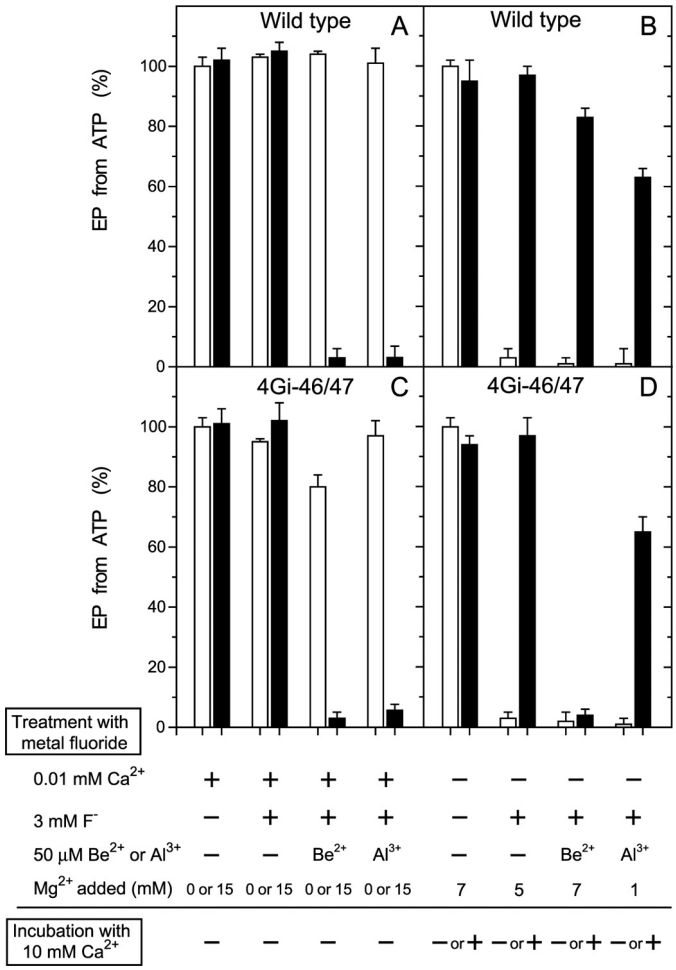

FIGURE 3. **Inhibition of *EP* formation from ATP by metal fluoride.** *A* and *C*, microsomes expressing the wild type or mutant 4Gi-46/47 (0.35 mg/ml) were treated at 25 °C for 30 min with metal fluoride in the presence of 0.01 mM Ca^{2+} (0.01 mM $CaCl_2$ without EGTA) in 3 mM KF plus 50 μM $BeSO_4$ or $AlCl_3$, 0.1 M KCl, and 50 mM MOPS/Tris (pH 7) with (*black bar*) or without (*white bar*) 15 mM $MgCl_2$. Subsequently, the samples were diluted 10-fold and phosphorylated at 0 °C for 15 s with 10 μM [γ-^{32}P]ATP in 1 μM A23187, 0.1 mM Ca^{2+} (0.5 mM $CaCl_2$ with 0.4 mM EGTA), 7 mM $MgCl_2$, 0.1 M 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 Mg^{2+} (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 Ca^{2+} (1 mM EGTA without added $CaCl_2$) and in the presence of the indicated concentrations of $MgCl_2$. Subsequently, the samples were diluted 2.5-fold with a solution containing 1 μM A23187, 0.1 M KCl, 50 mM MOPS/Tris (pH 7), and EGTA (to give 1 mM, *white bar*) or $CaCl_2$ (to give 10 mM Ca^{2+}, *black bar*) and incubated at 25 °C for 1 h. The samples were then further diluted 10-fold and phosphorylated with 10 μM [γ-^{32}P]ATP and 0.1 mM Ca^{2+} as in *A* and *C*, and the amount of *EP* formed was determined.

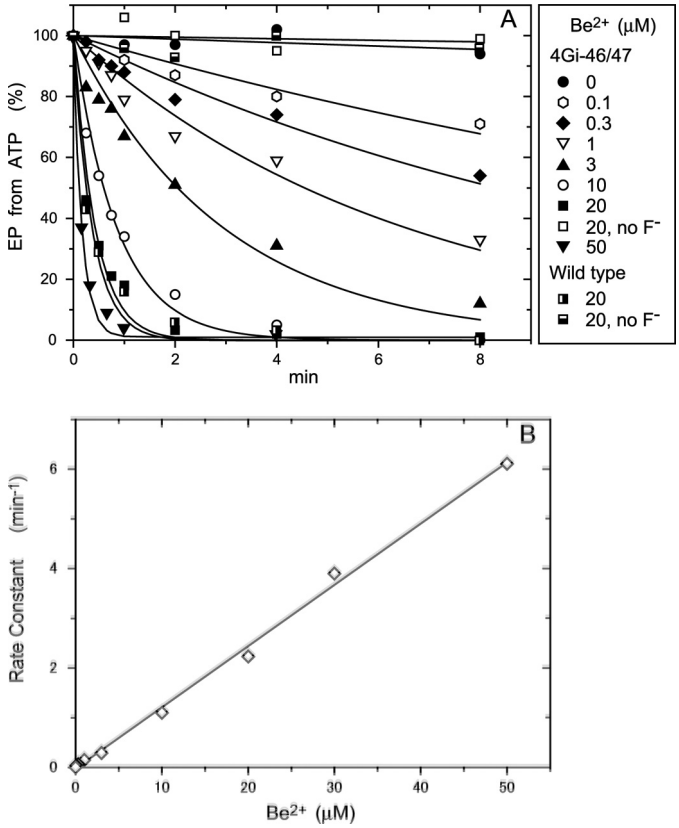

FIGURE 4. **Be^{2+} dependence of the rate of *EP* inhibition by BeF_x in 0.01 mM Ca^{2+}.** *A*, microsomes expressing the wild type or mutant 4Gi-46/47 were incubated for various periods in 0.01 mM Ca^{2+} and 1 mM KF with various concentrations of $BeSO_4$ and otherwise as in Fig. 3, *A* and *C*, for BeF_x-treatment. The samples were then diluted 10-fold and phosphorylated with 10 μM [γ-^{32}P]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 Be^{2+} added. The linear fit to the data gave a slope of 0.123 $min^{-1}\mu M^{-1}$.

presence of both Be^{2+} and F^- (BeF_x) but not F^- without Be^{2+} or Be^{2+} (20 μM) without F^- inhibits *E*P formation. The time courses of BeF_x-induced inhibition follow first order kinetics. A plot of the inhibition rate constants *versus* Be^{2+} (BeF_x) concentration is a straight line with no evidence of saturation within the experimental range, indicating that BeF_x binding is the rate-determining step in the inhibition process (Fig. 4*B*). BeF_x inhibits wild type at nearly the same rate as it does the mutant as seen at a representative 20 μM Be^{2+} with 1 mM F^-.

In Fig. 5, the mutant $E1Ca_2$ state in 10 μM Ca^{2+} was incubated with BeF_x at various Mg^{2+} concentrations, and the level of inhibition of *E*P formation was determined. BeF_x-induced inhibition is markedly accelerated with increasing Mg^{2+}, giving a $K_{0.5}$ value of 4.9 mM. The observed apparent Mg^{2+} affinity is consistent with those values obtained through phosphorylation of native Ca^{2+}-ATPase (42–47) and for the formation of $E1Ca_2{\cdot}BeF_3^-$ ($E1PCa_2{\cdot}Mg^{2+}$ analog) (27), *i.e.* the Mg^{2+} binding affinity at the catalytic Mg^{2+} site (site I composed of Asp^{351}/Thr^{353}/Asp^{703} and the phosphate moiety (BeF_3^-)). Therefore, Mg^{2+} binding at site I is likely a prerequisite for BeF_x binding and complex formation.

In Figs. 6 and 7, we further observed that the BeF_x-induced complex formation from $E1Ca_2$ in the mutant occurs with Mn^{2+} or Ca^{2+} in place of Mg^{2+}. The $K_{0.5}$ values are 1.4 mM for Mn^{2+} and 0.76 mM for Ca^{2+} (supplemental Figs. S1 and S2) and are consistent with such values for binding to the catalytic Mg^{2+} site (46, 48). In wild type the BeF_x-induced $E1Ca_2{\cdot}BeF_3^-$ formation, which inhibits *E*P formation occurs with Mn^{2+} but not with 10 mM Ca^{2+} in place of Mg^{2+} (Figs. 6 and 7). Thus, the complex formed from $E1Ca_2$ with BeF_x in the mutant 4Gi-46/47 (*i.e.* $E2Ca_2{\cdot}BeF_3^-$) is distinct from $E1Ca_2{\cdot}BeF_3^-$ of wild type.

Interestingly, the Hill coefficient for the Mg^{2+} as well as Mn^{2+} and Ca^{2+} dependence for complex formation with BeF_x ($E2Ca_2{\cdot}BeF_3^-$) in the mutant is nearly 2 (Fig. 5 and supplemental Figs. S1 and S2), suggesting the involvement of more

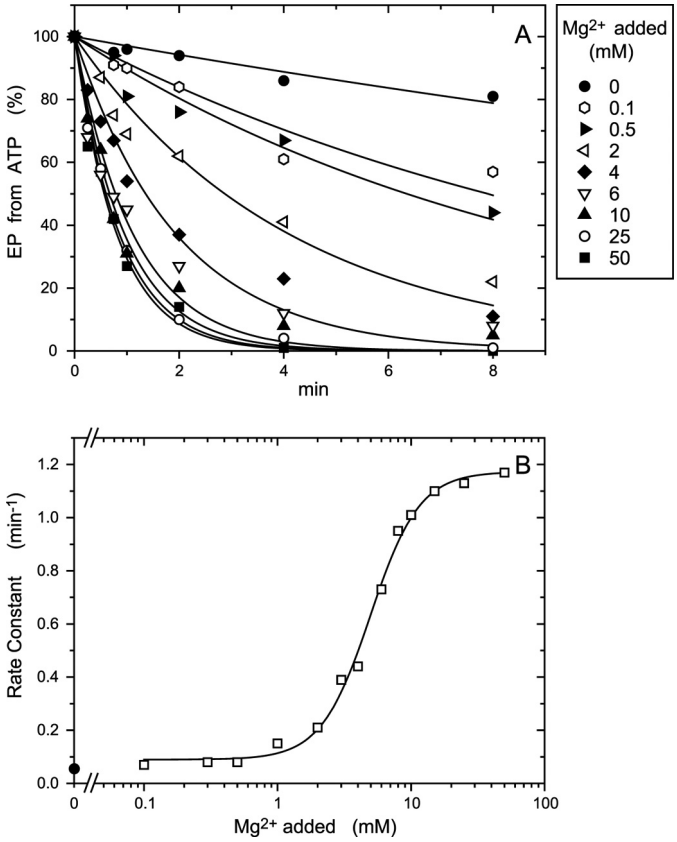

FIGURE 5. **Mg^{2+} dependence of the rate of *E*P inhibition by BeF_x in 0.01 mM Ca^{2+}.** *A*, microsomes expressing the mutant 4Gi-46/47 were incubated for various periods in 0.01 mM Ca^{2+}, 1 mM KF, 10 μM $BeSO_4$, and various concentrations of $MgCl_2$ and otherwise as in Fig. 3, *A* and *C*, for BeF_x treatment. The samples were then diluted 10-fold and phosphorylated with 10 μM [γ-^{32}P]ATP, and the amount of *E*P 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 Mg^{2+} added. $K_{0.5}$ 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.

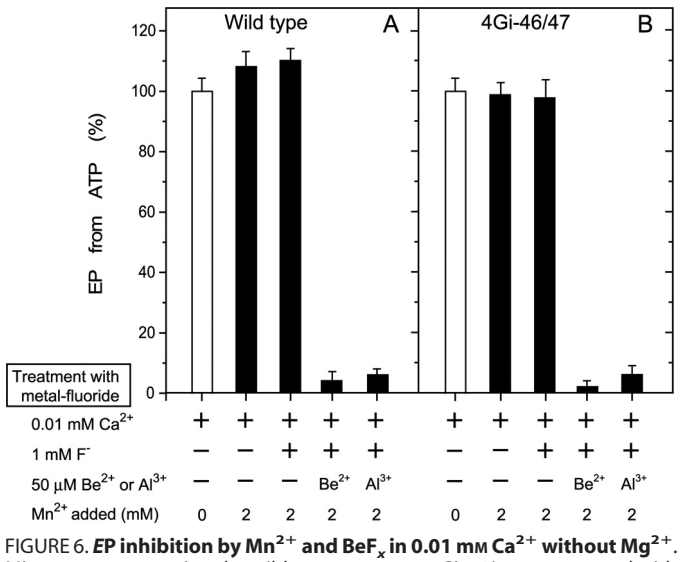

FIGURE 6. ***E*P inhibition by Mn^{2+} and BeF_x in 0.01 mM Ca^{2+} without Mg^{2+}.** Microsomes expressing the wild type or mutant 4Gi-46/47 were treated with 1 mM F^- plus 50 μM Be^{2+} or Al^{3+} in 0.01 mM Ca^{2+} and in the absence (*white bar*) or presence (*black bar*) of 2 mM $MnCl_2$ (in place of $MgCl_2$) and otherwise as in Fig. 3, *A* and *C*. The samples were then diluted 10-fold and phosphorylated with 10 μM [γ-^{32}P]ATP, and the amount of *E*P formed was determined as in Fig. 3, *A* and *C*.

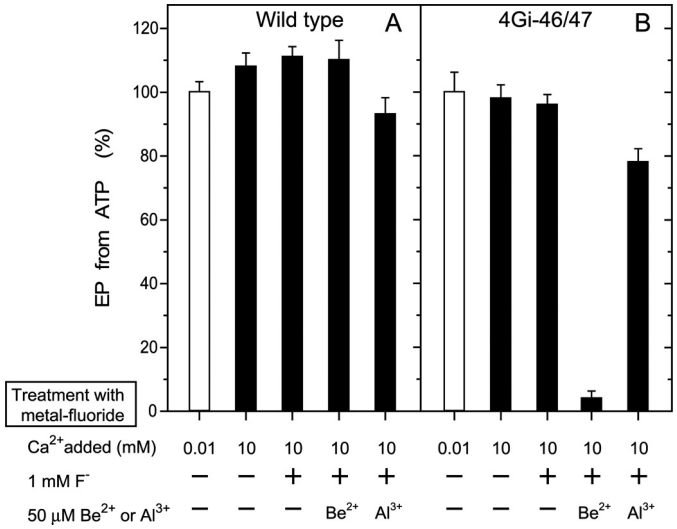

FIGURE 7. ***E*P inhibition by 10 mM Ca^{2+} and BeF_x without Mg^{2+} and Mn^{2+}.** Microsomes expressing wild type or mutant 4Gi-46/47 were treated with 1 mM F^- plus 50 μM Be^{2+} or Al^{3+} in 0.01 or 10 mM $CaCl_2$ without Mg^{2+} and Mn^{2+} and otherwise as in Fig. 3, *A* and *C*. The samples were then diluted 10-fold and phosphorylated with 10 μM [γ-^{32}P]ATP, and the amount of *E*P was determined as in Fig. 3, *A* and *C*.

than one metal ion. This is in contrast to the value 1 for $E1Ca_2{\cdot}BeF_3^-$ formation with Mg^{2+} and Mn^{2+} in wild type (see supplemental Fig. 1 in Ref. 27).

AlF_x produces the complex with the $E1Ca_2$ state of the mutant 4Gi-46/47 as well as of wild type ($E1Ca_2{\cdot}AlF_x$) with Mg^{2+} and Mn^{2+} but not with Ca^{2+} at the catalytic Mg^{2+} site (Figs. 3, 6, and 7). Therefore, in the mutant the complex with AlF_x ($E1Ca_2{\cdot}AlF_x$) is distinct from that with BeF_x ($E2Ca_2{\cdot}BeF_3^-$) 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. 8*A*, the $E1Ca_2$ state of the mutant 4Gi-46/47 in 10 μM $^{45}Ca^{2+}$ and 15 mM Mg^{2+} was complexed with BeF_x at a low concentration of Be^{2+} (1 μM) with 1 mM F^- to slow complex formation. The amount of occluded $^{45}Ca^{2+}$ was determined at various periods by membrane filtration with extensive washing with a solution containing excess EGTA and A23187. The loss of *E*P-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 $^{45}Ca^{2+}$ 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, *E*P formation was completely inhibited, and the amount of occluded $^{45}Ca^{2+}$ was 8.3 nmol/mg of expressed SERCA1a mutant protein, giving a stoichiometry of 2 per phosphorylation site (data not shown).

In Fig. 8*B*, we examined whether the complex $E2Ca_2{\cdot}BeF_3^-$ can be produced from $E2{\cdot}BeF_3^-$ by lumenal Ca^{2+} binding, mimicking the reverse conversion $E2P + 2Ca^{2+} \rightarrow E2PCa_2$ (14). $E2{\cdot}BeF_3^-$ was first formed in the mutant in the absence of Ca^{2+}

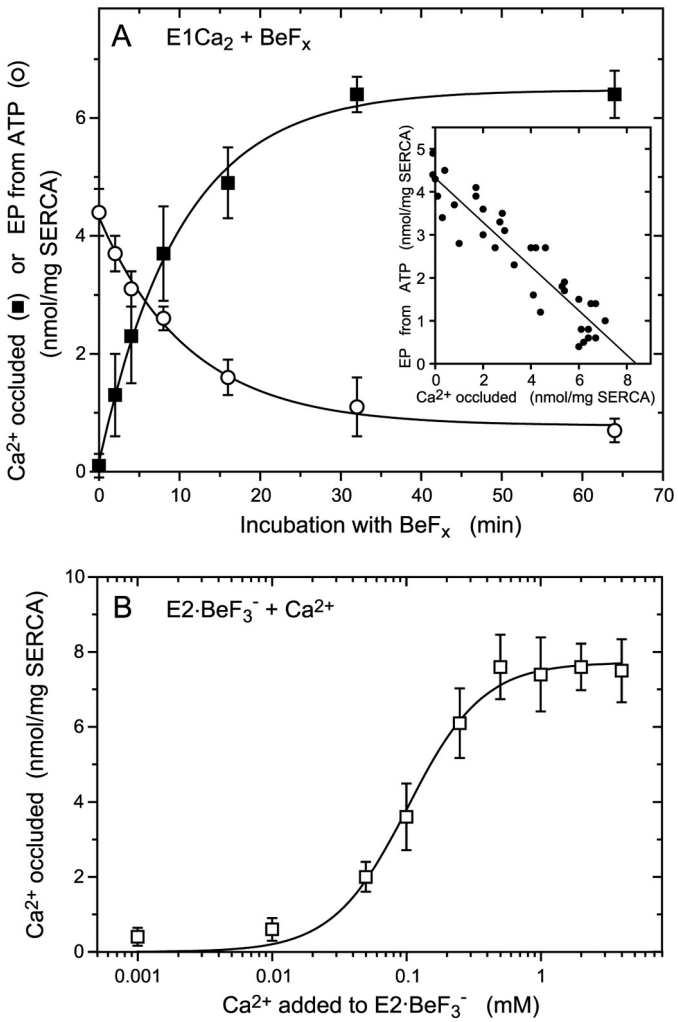

FIGURE 8. **Ca^{2+} occlusion in $E2Ca_2{\cdot}BeF_3^-$ of the mutant 4Gi-46/47 formed from $E1Ca_2$ (*A*) and from $E2{\cdot}BeF_3^-$ (*B*).** *A*, microsomes (0.2 mg/ml) expressing the mutant 4Gi-46/47 were incubated for various periods at 25 °C in 10 μl of a mixture containing 0.01 mM $^{45}CaCl_2$, 1 mM KF, 1 μM $BeSO_4$, 15 mM $MgCl_2$, 0.1 M 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 M KCl, 7 mM $MgCl_2$, and 50 mM MOPS/Tris (pH 7.0), subjected to membrane filtration, and washed rapidly with 6 ml of the washing solution for 4 s at 0 °C. For determination of *E*P, the above BeF_x-incubation was made with $^{40}Ca^{2+}$ instead of $^{45}Ca^{2+}$ otherwise as above, and the sample was diluted 10-fold and phosphorylated with 10 μM [γ-^{32}P]ATP at 0 °C for 15 s as in Fig. 3*C*. The sample was then further diluted 20-fold at 0 °C with the washing solution, immediately filtered as above, and washed rapidly with ice-cold trichloroacetic acid containing P_i. The *E*P level was not changed during the above sample handling because the decay of *E*P ($E2PCa_2$) is almost completely blocked in the mutant (14). The amount of $^{45}Ca^{2+}$ specifically bound and occluded (■) and that of $E^{32}P$ formed (○) in the expressed SERCA1a mutant were obtained by subtracting the background levels 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 *E*P 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 *E*P and of Ca^{2+} occluded, respectively. *B*, for formation of $E2{\cdot}BeF_3^-$, 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 $^{45}CaCl_2$ 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 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 mM, and 1.6, respectively. The values presented are the mean ± S.D. (n = 7).

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 $E2Ca_2{\cdot}BeF_3^-$ is produced from mutant $E2{\cdot}BeF_3^-$ by the addition of Ca^{2+} in the presence of A23187.

$K_{0.5}$ and the Hill coefficient observed in Fig. 8*B* are 0.1 mM and ~2, respectively, *i.e.* very similar values to those observed during $E2PCa_2$ formation from *E*2P and Ca^{2+} in the mutant (14). The observed low Ca^{2+} affinity is in agreement with the wild type property (25, 49) that $E2{\cdot}BeF_3^-$ as well as *E*2P have low affinity Ca^{2+} binding sites; that is, the lumenal-oriented transport sites. Importantly, $E2Ca_2{\cdot}BeF_3^-/E2PCa_2$ formed in the mutant (either from $E1Ca_2$ or from $E2{\cdot}BeF_3^-/E2P$) is remarkably stable and virtually not in equilibrium with $E1Ca_2{\cdot}BeF_3^-/E1PCa_2$ or $E2{\cdot}BeF_3^-/E2P$, *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 $E2 \rightarrow E1Ca_2 \rightarrow E1PCa_2 \rightarrow E2PCa_2$) 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 $E2Ca_2{\cdot}BeF_3^-$ formation from $E1Ca_2$ with BeF_x in Fig. 8*A*, 10 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 $E2Ca_2{\cdot}BeF_3^-$ to reveal the position of the domains and to establish whether it is a true structural $E2PCa_2$ 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 $E2Ca_2{\cdot}BeF_3^-$ has the same structure as that of mutant $E2PCa_2$ and that this structural state is intermediate between wild type $E1PCa_2$ (wild type $E1Ca_2{\cdot}BeF_3^-$) and Ca^{2+}-free *E*2P (wild type as well as mutant $E2{\cdot}BeF_3^-$) as described below. In mutant $E2Ca_2{\cdot}BeF_3^-$ and in mutant $E2PCa_2$, the T2 site Arg^{198} on the Val^{200} loop is completely resistant to trypsin, as in wild type *E*2P ($E2{\cdot}BeF_3^-$), showing that the A domain has rotated from its position in $E1PCa_2$ ($E1Ca_2{\cdot}BeF_3^-$ of wild type) and is associated with the P domain at Arg^{198} of the Val^{200} loop.

In both wild type $E1Ca_2{\cdot}BeF_3^-$ ($E1PCa_2$) and wild type and mutant $E2{\cdot}BeF_3^-$ (*E*2P), 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

description and reasons for protection in supplemental Fig. S5 and Ref. 56). By contrast, in mutant $E2Ca_2{\cdot}BeF_3^-$ and mutant $E2PCa_2$, the prtK-site Leu^{119} is rapidly cleaved and, thus, exposed. Evidently Leu^{119}/Tyr^{122} on M2 in mutant $E2Ca_2{\cdot}BeF_3^-$ and mutant $E2PCa_2$ have moved from their hidden position in $E1PCa_2$ ($E1Ca_2{\cdot}BeF_3^-$) but are not yet buried again through interaction with the A and P domains as in $E2P$ ($E2{\cdot}BeF_3^-$), 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 $E2P$ ($E2{\cdot}BeF_3^-$).

The proteolyses also reveal that wild type and mutant $E1Ca_2{\cdot}AlF_x$ are not structurally similar to wild type $E1Ca_2{\cdot}BeF_3^-$ ($E1PCa_2$) and mutant $E2Ca_2{\cdot}BeF_3^-$ ($E2PCa_2$). Interestingly, the rate of cleavage at the T2 site of mutant $E1Ca_2{\cdot}AlF_x$ is intermediate between that of wild type transition state ($E1Ca_2{\cdot}AlF_x$/$E1Ca_2{\cdot}AlF_4^-{\cdot}ADP$) and that of the $E1PCa_2$ product state ($E1Ca_2{\cdot}BeF_3^-$), suggesting that the structure is also intermediate. Thus, elongation of the A/M1′-linker brought the $E1Ca_2{\cdot}AlF_x$ structure closer to that of wild type $E1Ca_2{\cdot}BeF_3^-$. Only BeF_x, and not AlF_x, produces a species analogous to the $E2PCa_2$ structural state ($E2Ca_2{\cdot}BeF_3^-$ via $E1Ca_2{\cdot}BeF_3^-$). 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 $E1Ca_2{\cdot}AlF_4^-{\cdot}ADP/E1Ca_2{\cdot}AlF_x$, $E1Ca_2{\cdot}BeF_3^-$ ($E1PCa_2$), $E2Ca_2{\cdot}BeF_3^-$ and $E2PCa_2$, and $E2{\cdot}BeF_3^-/E2{\cdot}AlF_4^-/E2{\cdot}MgF_4^{2-}$ (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 EP processing.

$E2Ca_2{\cdot}BeF_3^-$ Formation from $E2{\cdot}BeF_3^-$ by Lumenal Ca^{2+} Binding—The Ca^{2+}-free complexes $E2{\cdot}BeF_3^-$, $E2{\cdot}AlF_4^-$, and $E2{\cdot}MgF_4^{2-}$ (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 $E2{\cdot}BeF_3^-$ exhibits complete resistance at the tryptic 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 $E2Ca_2{\cdot}BeF_3^-$ produced from $E1Ca_2$. These results agree with those in Fig. 3*D* where it is found that the ability to form EP is not restored by Ca^{2+} treatment of $E2{\cdot}BeF_3^-$. Thus, $E2Ca_2{\cdot}BeF_3^-$, as the $E2PCa_2$ analog, is produced from both $E2{\cdot}BeF_3^-$ and from $E1Ca_2$ (mimicking lumenal Ca^{2+} binding to $E2P$ in the reverse direction of the pump cycle and the forward ATP-induced EP formation and isomerization, respectively). On the other hand, mutant and wild type complexes $E2{\cdot}AlF_4^-$ and $E2{\cdot}MgF_4^{2-}$ and wild type $E2{\cdot}BeF_3^-$ are destroyed by Ca^{2+} treatment as found previously with sarcoplasmic reticulum Ca^{2+}-ATPase (25, 27).

Stability of Complex $E2Ca_2{\cdot}BeF_3^-$—In Fig. 9, $E2Ca_2{\cdot}BeF_3^-$ was first produced from mutant $E1Ca_2$ with BeF_x in 50 μM $^{45}Ca^{2+}$ and 15 mM Mg^{2+}, then further incubated at 25 °C in the presence of these ligands, and the amount of occluded $^{45}Ca^{2+}$ was determined. The results show that the complex $E2Ca_2{\cdot}BeF_3^-$ 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 $E2Ca_2{\cdot}BeF_3^-$ is analogous to mutant $E2PCa_2$.

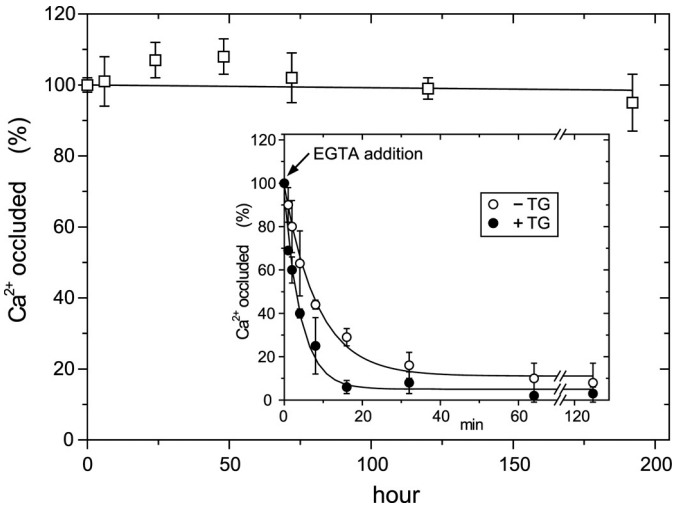

FIGURE 9. **Stability of $E2Ca_2{\cdot}BeF_3^-$ of the mutant 4Gi-46/47.** The complex $E2Ca_2{\cdot}BeF_3^-$ was produced with the mutant 4Gi-46/47 for 30 min at 25 °C in 0.05 mM $^{45}CaCl_2$, 1 mM KF, 50 μM $BeSO_4$, and 15 mM $MgCl_2$ and otherwise as in Fig. 8*A*. Then a small volume of A23187 was added to give 1 μM, and the incubation was further continued at 25 °C. At various times, the amount of $^{45}Ca^{2+}$ specifically bound and occluded in the mutant was measured after an EGTA wash and by subtracting the background levels determined in the absence of F^- in the incubation mixture and otherwise as in Fig. 8*A*. *Inset*, after the formation of $E2Ca_2{\cdot}BeF_3^-$ as above, the sample was diluted 100-fold at 25 °C with a solution containing 1 μM A23187, 0.1 M KCl, 7 mM $MgCl_2$, 2 mM EGTA, and 50 mM MOPS/Tris (pH 7.0) (without BeF_x) in the absence (○) or presence (●) of 1 μM TG and incubated for various periods, and the amount of $^{45}Ca^{2+}$ specifically bound and occluded in the mutant was obtained as above. The values presented are the mean ± S.D. ($n = 7$). *Solid lines* in the *inset* show the least squares fit to a single exponential, and the decay rate constants thus obtained are 7.0 (○) and 14.0 (●) h^{-1} without and with TG, respectively. In the *main panel* and *inset*, the amount of Ca^{2+} occluded in the complex $E2Ca_2{\cdot}BeF_3^-$ at time 0 (immediately before starting the long incubation or the dilution) was normalized to 100%.

DISCUSSION

Mutant $E2Ca_2{\cdot}BeF_3^-$ as an Analog of Native Transient State $E2PCa_2$—Using our elongated A/M1′-linker mutant, we have developed the complex $E2Ca_2{\cdot}BeF_x$, most probably $E2Ca_2{\cdot}BeF_3^-$, as a stable structural analog of the native transient state $E2PCa_2$ (ADP-insensitive EP with two Ca^{2+} at the transport sites), an intermediate in EP isomerization and Ca^{2+} deocclusion/release. The complex $E2Ca_2{\cdot}BeF_3^-$ has two occluded Ca^{2+} and is produced from both mutant $E1Ca_2$ and mutant $E2{\cdot}BeF_3^-$, mimicking native $E2PCa_2$ formation from $E1Ca_2$ after ATP-induced forward phosphorylation via $E1PCa_2$ isomerization

and in the reverse direction from *E*2P after lumenal Ca^{2+} binding. Mutant $E2Ca_2{\cdot}BeF_3^-$ 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 $E2Ca_2{\cdot}BeF_3^-$ is equivalent to that in $E2PCa_2$ trapped with the mutant and intermediate between native $E1PCa_2{\cdot}Mg^{2+}$ ($E1Ca_2{\cdot}BeF_3^-$ of wild type) and native $E2P{\cdot}Mg^{2+}$ ($E2{\cdot}BeF_3^-$ of wild type and mutant). All these properties of mutant $E2Ca_2{\cdot}BeF_3^-$ meet the requirements of a native $E2PCa_2$ analog.

Importantly, AlF_x and MgF_x are not able to produce this $E2PCa_2$ analog either from mutant $E1Ca_2$ or from mutant $E2{\cdot}AlF_4^-$ and $E2{\cdot}MgF_4^{2-}$. Thus, BeF_x is unique in this regard. The coordination chemistry of the beryllium in BeF_x (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 *E*2P ground state analog $E2{\cdot}BeF_3^-$ (21, 22). On the other hand, AlF_x (AlF_3 or AlF_4^-) mimics the transition state of phosphorylation and dephosphorylation as seen in structures $E1Ca_2{\cdot}AlF_4^-{\cdot}$ADP and $E2{\cdot}AlF_4^-$ (17, 19, 22). MgF_4^{2-} mimics P_i in the product complex $E2{\cdot}P_i$ after *E*2P hydrolysis as seen in structure $E2{\cdot}MgF_4^{2-}$ (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 *E*2P ground state are ready for the changes, but the transition state structures are not.

Roles of A/M1′-linker and Structural Changes during EP Formation and Processing—The transient $E2PCa_2$ state formed during *E*P processing and its analog $E2Ca_2{\cdot}BeF_3^-$ were trapped and stabilized by elongation of the A/M1′-linker. As revealed by the proteolyses, in mutant $E2Ca_2{\cdot}BeF_3^-$ and mutant $E2PCa_2$, the A domain has already rotated parallel to membrane from its position in $E1Ca_2{\cdot}BeF_3^-$ ($E1PCa_2{\cdot}Mg$) 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 $E2Ca_2{\cdot}BeF_3^-$ 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 $E2Ca_2{\cdot}BeF_3^-$ (equivalent to native $E2PCa_2{\cdot}Mg$) is intermediate between those of the wild type $E1Ca_2{\cdot}BeF_3^-$ (native $E1PCa_2{\cdot}Mg$) and the wild type and mutant $E2{\cdot}BeF_3^-$ (native $E2P{\cdot}Mg$). 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 *E*2P structure (11–13). Therefore, in $E2Ca_2{\cdot}BeF_3^-$ 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 *E*2P 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 $E2Ca_2{\cdot}BeF_3^-$) from $E1PCa_2$ ($E1Ca_2{\cdot}BeF_3^-$), 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 lumenal 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 *E*2P structure with the lumenal 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 *E*2P catalytic site with hydrolytic ability (11–13). Therefore, in this mechanism *E*2P hydrolysis can only occur after Ca^{2+} release, ensuring energy coupling. The relative stability of native *E*2P 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 $E2Ca_2{\cdot}BeF_3^-$ from $E1Ca_2$ via $E1Ca_2{\cdot}BeF_3^-$. 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 $E1Ca_2{\cdot}BeF_3^-$ formation. An attempt to substitute Ca^{2+} for Mg^{2+} actually destroys wild type $E1Ca_2{\cdot}BeF_3^-$ (27). The extremely rapid isomerization of *E*P with bound Ca^{2+} at the Mg^{2+} site in the elongated A/M1′-linker mutant ($E1PCa_2{\cdot}Ca \rightarrow E2PCa_2{\cdot}Ca$) is again very different to the markedly retarded $E1PCa_2{\cdot}Ca$ 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 $E1Ca_2{\cdot}$CaAMPPCP and $E1Ca_2{\cdot}AlF_4^-{\cdot}$ADP described by Toyoshima *et al.* (18, 19), Mg^{2+} or Ca^{2+} ligation at the catalytic Mg^{2+} site I (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

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 $E1PCa_2$ probably drives the large horizontal rotation of the A domain during $E1PCa_2$ 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, $E1PCa_2{\cdot}Mg^{2+}$ is rapidly isomerized, whereas in $E1PCa_2{\cdot}Ca$ it is markedly retarded (28, 29).

The observed formation of $E2Ca_2{\cdot}BeF_3^-$ and $E2PCa_2$ (via very rapid $E1PCa_2$ 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 $E1PCa_2$. 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 $E1PCa_2$ to $E2PCa_2$ isomerization (26). Strain in the wild type A/M1′-linker in $E1PCa_2$ is likely to be important as a build up to generating stronger strain during $E1PCa_2$ to $E2PCa_2$ isomerization. Thus, the strain of the A/M1′-linker seems to be imposed increasingly during $E1PCa_2$ formation and the subsequent isomerization to $E2PCa_2$, and this energy finally could be used for inducing structural changes for Ca^{2+} deocclusion and release.

$E1Ca_2{\cdot}AlF_x$ 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 $E1Ca_2{\cdot}AlF_x$ is identical to that of $E1Ca_2{\cdot}AlF_4^-{\cdot}ADP$ and has obviously not yet reached the product $E1PCa_2$ state $E1Ca_2{\cdot}BeF_3^-$. Namely, during the reaction $E1Ca_2{\cdot}AlF_4^-{\cdot}ADP/E1Ca_2{\cdot}AlF_x \rightarrow E1Ca_2{\cdot}BeF_3^-$, 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 $E2Ca_2{\cdot}BeF_3^-$ and $E2{\cdot}BeF_3^-$ 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 $E1Ca_2{\cdot}AlF_x$ is intermediate between those of $E1Ca_2{\cdot}AlF_4^-{\cdot}ADP$ and $E1Ca_2{\cdot}BeF_3^-$ 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 $E1Ca_2{\cdot}AlF_x$ closer to that of $E1Ca_2{\cdot}BeF_3^-$. The finding agrees with our above postulate that the A/M1′-linker is strained by the A-domain upward movement during $E1PCa_2$ ($E1Ca_2{\cdot}BeF_3^-$) formation from the transition state ($E1Ca_2{\cdot}AlF_x$). In fact, because the length of the Asp^{351} O-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.

Lumenal Ca^{2+}-induced $E2Ca_2{\cdot}BeF_3^-$ Formation from $E2{\cdot}BeF_3^-$—The observed reverse formation of $E2Ca_2{\cdot}BeF_3^-$ (native $E2PCa_2$) from mutant $E2{\cdot}BeF_3^-$ ($E2P$) through Ca^{2+} binding from the lumen shows that the lumenal gate (Ca^{2+} releasing pathway) is open in $E2{\cdot}BeF_3^-$ ($E2P$ ground state immediately before Ca^{2+} binding). This is in contrast to the closed gate in $E2{\cdot}AlF_4^-$ and $E2{\cdot}MgF_4^{2-}$ (25). Thus, lumenal gating is strictly coupled with the configuration change in the phosphate during $E2P$ hydrolysis, thereby avoiding possible Ca^{2+} leakage (25). Note that in wild type, $E2{\cdot}BeF_3^-$ (open lumenal gate) formed with Mg^{2+} is converted to $E1Ca_2$ + BeF_x by Ca^{2+}, because cycle reversal and subsequent Ca^{2+} substitution of Mg^{2+} at the catalytic site destabilizes $E1Ca_2{\cdot}BeF_3^-$ as previously demonstrated (27). $E2{\cdot}AlF_4^-$ and $E2{\cdot}MgF_4^{2-}$ (gates closed) in wild type and mutant were also decomplexed to $E1Ca_2$ by Ca^{2+} but probably by the high Ca^{2+} concentration disrupting the lumenal and transmembrane regions, thereby destabilizing AlF_4^- and MgF_4^{2-} ligation at the catalytic site.

Mg^{2+} Dependence of $E2Ca_2{\cdot}BeF_3^-$ Formation from $E1Ca_2$—The Mg^{2+} as well as Mn^{2+} or Ca^{2+} dependence of $E2Ca_2{\cdot}BeF_3^-$ 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 $E1Ca_2{\cdot}BeF_3^-$ 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 $E2Ca_2{\cdot}BeF_3^-$ formation from $E1Ca_2$. In the atomic structures of $E1Ca_2{\cdot}CaAMPPCP$ and $E1Ca_2{\cdot}AlF_4^-{\cdot}ADP$, 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 $E2{\cdot}BeF_3^-$, $E2{\cdot}AlF_4^-$, and $E2{\cdot}MgF_4^{2-}$, only one Mg^{2+} is seen (at site I). Therefore, in $E2Ca_2{\cdot}BeF_3^-$ ($E2PCa_2$) 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 $E1PCa_2$ isomerization to $E2PCa_2$.

In summary, our previous (14, 26) and present studies show that the A/M1′-linker should be appropriately long for the $E1PCa_2$ to $E2PCa_2$ isomerization then short enough for the Ca^{2+} deocclusion/release from $E2PCa_2$ 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 $E2PCa_2$, a species poised to deliver Ca^{2+} to the lumen. The stable analogs, $E1Ca_2{\cdot}BeF_3^-$ (27) and $E2Ca_2{\cdot}BeF_3^-$ (this study) with bound Mg^{2+} could be critically important for obtaining atomic models of $E1PCa_2{\cdot}Mg^{2+}$ and the hitherto elusive transient $E2PCa_2{\cdot}Mg^{2+}$ intermediate for further understanding of the transport mechanism.

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