Formation of the Stable Structural Analog of ADP-sensitive Phosphoenzyme of Ca\(^{2+}\)-ATPase with Occluded Ca\(^{2+}\) by Beryllium Fluoride

**STRUCTURAL CHANGES DURING PHOSPHORYLATION AND ISOMERIZATION**

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As a stable analog for ADP-sensitive phosphorylated intermediate of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase E1PCa\(_2\)Mg, a complex of E1Ca\(_2\)-BeF\(_3\), was successfully developed by addition of beryllium fluoride and Mg\(^{2+}\) to the Ca\(^{2+}\)-bound state, E1Ca\(_2\). In E1Ca\(_2\)-BeF\(_3\), most probably E1Ca\(_2\)-BeF\(_3\), two Ca\(^{2+}\) are occluded at high affinity transport sites, its formation required Mg\(^{2+}\) binding at the catalytic site, and ADP decomposed it to E1Ca\(_2\), as in E1PCa\(_2\)Mg. Organization of cytoplasmic domains in E1Ca\(_2\)-BeF\(_3\) was revealed to be intermediate between those in E1Ca\(_2\)-AlF\(_4\)\(^{-}\)ADP (transition state of E1PCa\(_2\) formation) and E2-BeF\(_3\)\(^{-}\) (ADP-insensitive phosphorylated intermediate E2P-Mg). Trinitrophenyl-AMP (TNP-AMP) formed a very fluorescent (superfluorescent) complex with BeF\(_3\), which was stable. Tryptophan fluorescence revealed that the transmembrane structure of E1Ca\(_2\)-BeF\(_3\) mimics E1PCa\(_2\)Mg, and between those of E1Ca\(_2\)-AlF\(_4\)\(^{-}\)ADP and E2-BeF\(_3\). E1Ca\(_2\)-BeF\(_3\), at low 50–100 \(\mu\)M Ca\(^{2+}\), was converted slowly to E2-BeF\(_3\) releasing Ca\(^{2+}\), mimicking E1PCa\(_2\)Mg \(\rightarrow\) E2P-Mg + 2Ca\(^{2+}\). Ca\(^{2+}\) replacement of Mg\(^{2+}\) at the catalytic site at approximately millimolar high Ca\(^{2+}\) decomposed E1Ca\(_2\)-BeF\(_3\), to E1Ca\(_2\). Notably, E1Ca\(_2\)-BeF\(_3\) was perfectly stabilized for at least 12 days by 0.7 mM lumenal Ca\(^{2+}\) with 15 mM Mg\(^{2+}\). Stable E1Ca\(_2\)-BeF\(_3\) was produced from E2-BeF\(_3\) at 0.7 mM lumenal Ca\(^{2+}\) by binding two Ca\(^{2+}\) to lumenally oriented low affinity transport sites, as mimicking the reverse conversion E2P-Mg + 2Ca\(^{2+}\) \(\rightarrow\) E1PCa\(_2\)Mg.

Sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA1a), a representative member of the P-type ion transporting ATPases, catalyzes Ca\(^{2+}\) transport coupled with ATP hydrolysis (Fig. 1) (1–9). The enzyme forms phosphorylated intermediates from ATP or P\(_i\), in the presence of Mg\(^{2+}\) (10–13). In the transport cycle, the enzyme is first activated by cooperative binding of two Ca\(^{2+}\) ions at high affinity transport sites (E2 to E1Ca\(_2\), steps 1–2) (14) and autophosphorylated at Asp\(^{351}\) with MgATP to form the ADP-sensitive phosphoenzyme (E1P, step 3), which reacts with ADP to regenerate ATP in the reverse reaction. Upon this E1P formation, the two bound Ca\(^{2+}\) are occluded in the transport sites (E1PCa\(_2\)). Subsequent isomeric transition to the ADP-insensitive form (E2P-Ca\(_2\)), i.e. loss of ADP sensitivity at the catalytic site, results in rearrangement of the Ca\(^{2+}\) binding sites to deocclude Ca\(^{2+}\), reduce the affinity, and open the luminal gate, thus releasing Ca\(^{2+}\) into the lumen (E2P, steps 4–5). Finally Asp\(^{351}\)-acylphosphate in E2P is hydrolyzed to form the Ca\(^{2+}\)-unbound inactive E2 state (steps 6 and 7). Mg\(^{2+}\) bound at the catalytic site is required as a physiological catalytic cofactor in phosphorylation and dephosphorylation and thus for the transport cycle. The cycle is totally reversible, e.g. E2P can be formed from P\(_i\), in the presence of Mg\(^{2+}\) and absence of Ca\(^{2+}\), and subsequent Ca\(^{2+}\) binding at lumenerally oriented low affinity transport sites of E2P reverses the Ca\(^{2+}\)-releasing step and produces E1PCa\(_2\)Mg which is then decomposed to E1Ca\(_2\) by ADP.

Various intermediate structural states in the transport cycle were fixed as their structural analogs produced by appropriate ligands such as AMP-PCP (non-hydrolyzable ATP analog) or metal fluoride compounds (phosphate analogs), and their crystal structures were solved at the atomic level (15–22). The three cytoplasmic domains, N, P, and A, largely move and change their organization state during the transport cycle, and the changes are coupled with changes in the transport sites. Most remarkably, in the change from E1Ca\(_2\)-AlF\(_4\)\(^{-}\)ADP (the transition state for E1PCa\(_2\) formation, E1PCa\(_2\)-ADP-Mg\(^{3+}\)) to E2-BeF\(_3\) (the ground state E2P-Mg) (23–25), the A domain largely rotates by more than 90° approximately parallel to the membrane plane and associates with the P domain, thereby destroying the Ca\(^{2+}\) binding sites, and opening the luminal gate, thus releasing Ca\(^{2+}\) into the lumen (see Fig. 2). E1PCa\(_2\)Ca-AMP-PNP formed by CaAMP-PNP without Mg\(^{2+}\) is nearly the same as...
Structural Analog of $E1PCa_2\cdot Mg$ Intermediate of $Ca^{2+}$-ATPase

**EXPERIMENTAL PROCEDURES**

**Preparation of SR Vesicles and Treatment with BeFx, AlFₓ, and AlFₓ-ADP**—SR vesicles were prepared from rabbit skeletal muscle as described (28). The content of the phosphorylation site in the vesicles determined according to Barrabin et al. (29) was 4.49 ± 0.22 nmol/mg of vesicle protein ($n = 5$). The $Ca^{2+}$-dependent ATPase activity determined at 25 °C in a mixture containing 5 µg/ml microsomal protein, 1 mM ATP, 1.7 µM A23187, 7 mM MgCl₂, 0.1 mM KCl, 50 mM MOPS/Tris (pH 7.0), and 0.6 mM CaCl₂ with 0.5 mM EGTA (or 2 mM EGTA without added CaCl₂) was 1.87 ± 0.14 µmol/min/mg of vesicle protein ($n = 3$). The $Ca^{2+}$-ATPase was purified from the vesicles by deoxycholate as described (30, 31). The $E1Ca_2$ state ATPase was incubated with fluoride compounds, 2 mM potassium fluoride and 100 µM BeSO₄ or AlCl₃, at 25 °C for 30 min in the presence of 0.1 mM $Ca^{2+}$, 15 mM MgCl₂, 0.1 mM KCl, 30 mM MOPS/Tris buffer (pH 7.0), unless stated otherwise. $E1Ca_2$-AlFₓ-ADP was formed by including 50 µM ADP in the above AlF₃ incubation mixture as described (32). $E2$-BeF₅⁻, $E2$-AlF₄⁻, and $E2$-MgF₂⁺ were produced as described (23–25).

**Determination of $EP$—$EP$ formation was performed with 3 µM [γ-³²P]ATP in 100 (or 50) µM $Ca^{2+}$ at 0 °C for 3 s, and terminated by trichloroacetic acid containing carrier P;

**Ca²⁺ Binding and Occlusion**—$^{45}$Ca²⁺ binding and occlusion at the transport sites was determined at 25 °C with 2 ml of the SR vesicle mixture (0.2 mg/ml protein) with a 0.45-µm nitrocellulose membrane filter (Millipore) as described (31). In some cases, the vesicles on the filter were washed for 10 s by perfusion with 2 ml of a washing solution containing 5 mM EGTA. The amount of Ca²⁺ bound at the transport sites was obtained by subtracting the nonspecific Ca²⁺ binding level determined as described in the figure legends.

**Proteolysis**—SR vesicles (0.45 mg/ml protein) were treated at 25 °C with trypsin (0.3 mg/ml) or proteinase K (0.1 mg/ml) as described (23, 24) and as noted in the figure legends. The samples were subjected to Laemmli SDS-PAGE (33) and densitometric analyses of the Coomassie Brilliant Blue R-250-stained gels (23, 24).

**Fluorescence Measurements**—The TNP-AMP fluorescence and intrinsic tryptophan fluorescence of $Ca^{2+}$-ATPase (0.06 mg/ml protein) were measured on a RF-5300PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths 408 and 540 nm for TNP-AMP (with bandwidth 1.5 and 5 nm), unless otherwise described (28).

**Miscellaneous**—Tryptsin (1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) and proteinase K were obtained from Sigma. TNP-AMP was synthesized according to Hiratsuka (34). Protein concentrations were determined by the method of Lowry et al. (35) with bovine serum albumin as a standard. Free Ca²⁺ concentrations were calculated by the Calco program. Data were analyzed by nonlinear regression using the program Origin (Microcal Software, Inc., Northampton.
MA). Three-dimensional models of the enzyme were reproduced by the program VMD (36).

RESULTS

Formation of E1PCa2 Analogs by Fluoride Compounds—The Ca2+-ATPase of SR vesicles in 100 μM Ca2+ and 15 mM Mg2+ was incubated with beryllium fluoride (BeFx) or aluminum fluoride (AlFx) for 30 min at 25 °C. The ability of EP formation from ATP was almost completely lost (actually within 1 min) (Fig. 3), showing the stable complex formation with BeFx and AlFx. No inhibition occurred with Mg2+ and fluoride without beryllium and aluminum (E1Ca2+(MgFx)). Thus, MgFx (MgF2−) was not able to produce a complex with E1Ca2, in contrast to E2-MgF2− formation from E2, the E2-Pi product analog in E2P hydrolysis (19, 25). This finding agrees with the in-line phosphorylation of E1Ca2 to E1PCa2 (37), in which there is no state with non-covalently bound Pi.

The binding of Mg2+ at the catalytic site as a physiological cation is nevertheless required for EP formation. Actually in Fig. 3, Mg2+ was required for complex formation with BeFx in 100 μM Ca2+. The apparent Mg2+ affinity (K0.5 of 5 mM, supplemental Fig. S1) was consistent with that of the catalytic site in phosphorylation from ATP or Pi (e.g. Refs. 11, 12, and 38–41). The BeFx-induced inhibition also occurred with Mn2+ with apparent affinity (K0.5 of 0.6 mM) significantly higher than that of Mg2+, as found in E1PCa2 formation and ATP hydrolysis with Mn2+ (40, 42).

When Ca2+ over millimolar amounts was added in place of Mg2+, the EP formation was not inhibited by BeFx (BeF6(Ca5)), Therefore, Ca2+ substitution probably at the catalytic Mg2+ site abolished complex formation with BeFx. Although CaATP as a substrate and Ca2+ bound at the catalytic site produce different structure from the Mg2+
bound structure (as in fact found, see below). The complex formation of E1Ca$_2$ by AlF$_x$ in 100 μM Ca$^{2+}$ also required Mg$^{2+}$ or Mn$^{2+}$ with somewhat higher apparent affinities than those for BeF$_x$-induced complex formation, and was abolished by Ca$^{2+}$ binding at the catalytic site.

Ca$^{2+}$ Binding and Occlusion at Transport Sites—In Fig. 4, Ca$^{2+}$ binding and occlusion in formation of the Ca$^{2+}$-ATPase complexes with fluoride compounds were determined in 15 mM Mg$^{2+}$ with and without a 10-s EGTA filter washing. In all the cases without washing, Ca$^{2+}$ was bound to the Ca$^{2+}$-ATPase with high affinities; $K_{0.5}$ at sub-micromolar to millimolar ranges, Hill coefficient $\sim$2, and maximum levels of 9–10 nmol/mg of protein, i.e. the stoichiometry of two Ca$^{2+}$ per phosphorylation site (inset at 50 μM Ca$^{2+}$). Therefore, E1Ca$_2$-BeF$_x$ and E1Ca$_2$-AlF$_x$-ADP/E1Ca$_2$-AlF$_x$ were produced by cooperative binding of two Ca$^{2+}$ ions at high affinity transport sites. This finding agrees with the property of the sites for Ca$^{2+}$ binding and the resulting enzyme activation for phosphorylation by ATP as nicely demonstrated for the first time by Inesi et al. (14). Upon the EGTA washing of E1Ca$_2$, that is complexed with BeF$_x$, the two bound Ca$^{2+}$ were not removed, and therefore occluded in the complex as “E1Ca$_2$-BeF$_x$.” The two Ca$^{2+}$ are occluded also in E1Ca$_2$-AlF$_x$-ADP and less strongly in E1Ca$_2$-AlF$_x$.

Note that the Ca$^{2+}$ affinity became 2–3-fold lower for BeF$_x$ and AlF$_x$. This may be because the Ca$^{2+}$-free E2 state produces E2-BeF$_x$ and E2-AlF$_x$ (25), and therefore competes with Ca$^{2+}$ binding for formation of E1Ca$_2$-BeF$_x$ and E1Ca$_2$-AlF$_x$. On the other hand, the observed ~3-fold Ca$^{2+}$-affinity increase in formation of E1Ca$_2$-AlF$_x$-ADP is probably brought about by the fact that ADP together with AlF$_x$ strongly stabilizes the cross-linked N-P domains (17, 18), which is unfavorable for formation of the Ca$^{2+}$-free E2 and E2-AlF$_x$ because for these structures the A domain should rotate into the opened space between the N and P domains and associate with them (19, 23, 24).

Cytoplasmic Structure in E1Ca$_2$-BeF$_x$ Is Intermediate between Those in E1Ca$_2$-AlF$_x$-ADP and E2-BeF$_x$—Proteolytic analysis was made to reveal the organization state of the cytoplasmic domains in the newly developed E1PCa$_2$-Mg analog E1Ca$_2$-BeF$_x$, and to compare with E1Ca$_2$-AlF$_x$-ADP/E1Ca$_2$-AlF$_x$ and E2-BeF$_x$ (E2P-Mg) (see the typical cleavage in supplemental Fig. S2). The initial rate of the “A1” appearance upon cleavage at the T2 site (Arg$^{198}$ on the Val$_{200}$ loop of the A domain) in E1Ca$_2$-BeF$_x$ was substantially slower than the rapid cleavage of E1Ca$_2$-AlF$_x$-ADP and E1Ca$_2$-AlF$_x$ as well as E1Ca$_2$ (Table 1). The slowed T2 cleavage was also observed when E1Ca$_2$-BeF$_x$ was formed with 3 mM Mn$^{2+}$ in place of 15 mM Mg$^{2+}$ (data not shown). Also important was the slow but definitely occurring T2 cleavage in E1Ca$_2$-BeF$_x$ in sharp contrast to its complete resistance in E2-BeF$_x$. Therefore, A-P domain organization at the Val$_{200}$ loop in E1Ca$_2$-BeF$_x$ is intermediate between those in E1Ca$_2$-AlF$_x$-ADP/E1Ca$_2$-AlF$_x$ and E2-BeF$_x$.

All complexes were almost completely resistant to proteinase K at the major site of Thr$^{242}$ on the A/M3-linker that produces the “p83” fragment. Therefore, in E1Ca$_2$-BeF$_x$, the A domain is rotated perpendicularly to the membrane plane from its position in E1Ca$_2$ thereby causing the A/M3-linker strain, as in E1Ca$_2$-CaAMP-PCP, E1Ca$_2$-AlF$_x$-ADP (18, 19, 24), and E1Ca$_2$-AlF$_x$.

These analyses revealed that in the change E1Ca$_2$-AlF$_x$-ADP $\rightarrow$ E1Ca$_2$-BeF$_x$ (i.e. upon the ADP release from the transition state), the A domain moves, i.e. probably rotates to some extent parallel to the membrane plane likely due to the A/M3-linker strain, and thereby Arg$^{198}$ on the Val$_{200}$ loop comes close to the P domain. In the subsequent change, E1Ca$_2$-BeF$_x$ $\rightarrow$ E2-BeF$_x$, the A domain rotates further (by the A/M3-linker strain as predicted to be motive force (18, 19, 43, 44)) and pro-
Structural Analog of E1PCa\textsubscript{2}\textsuperscript{2+}Mg Intermediate of Ca\textsuperscript{2+}-ATPase

![Graph showing Ca\textsuperscript{2+} binding and occlusion at transport sites](image)

**FIGURE 4. Ca\textsuperscript{2+} binding and occlusion at transport sites.** The E1Ca\textsubscript{2} state ATPase of SR vesicles in various concentrations of 48Ca\textsuperscript{2+} and 15 mM MgCl\textsubscript{2} was incubated with BeF\textsubscript{x}, AlF\textsubscript{x}, and AlF\textsubscript{x}-ADP or without these compounds (E1Ca\textsubscript{2}) for 30 min at 25 °C. The amounts of bound (upper panel) and occluded (lower panel) 48Ca\textsuperscript{2+} were determined without and with the perfusion of the membrane filter with a 5 mM EGTA-containing washing solution (without CaCl\textsubscript{2} and fluoride compounds otherwise as the above incubation solution). The nonspecific Ca\textsuperscript{2+} binding was determined by including 10 mM thapsigargin before the addition of fluoride compounds, and subtracted. When ADP was used for E1Ca\textsubscript{2}-AlF\textsubscript{x}-ADP, 5 mM A23187 was included to avoid Ca\textsuperscript{2+} accumulation in the vesicles by ATP produced from ADP due to adenylate kinase in the vesicles. In the inset, the stoichiometries of bound Ca\textsuperscript{2+} (open bars) and occluded Ca\textsuperscript{2+} (closed bars) to the phosphorylation site (P-site) were determined at saturating 50 mM Ca\textsuperscript{2+}. Solid lines in the upper panel show the least squares fit to the Hill equation. K\textsubscript{D} of Ca\textsuperscript{2+} and Hill coefficients obtained were 1.3 μM and 1.7 (E1Ca\textsubscript{2}), 4.3 μM and 1.7 (E1Ca\textsubscript{2}-BeF\textsubscript{x}), 2.3 μM and 1.7 (E1Ca\textsubscript{2}AlF\textsubscript{x}), and 0.4 μM and 1.4 (E1Ca\textsubscript{2}-AlF\textsubscript{x}-ADP). In the lower panel, the values for E1Ca\textsubscript{2}-BeF\textsubscript{x} and E1Ca\textsubscript{2}-AlF\textsubscript{x}-ADP are essentially not altered by EGTA washing (4.7 μM and 1.9, and 0.4 μM and 1.9, respectively).

Ca\textsuperscript{2+} Ligation at the Catalytic Mg\textsuperscript{2+} Site—The proteolysis further revealed that E1Ca\textsubscript{2}-BeF\textsubscript{x} was not produced from E1Ca\textsubscript{2} in 5 mM Ca\textsuperscript{2+} without Mg\textsuperscript{2+} (E1Ca\textsubscript{2} + 5 mM Ca\textsuperscript{2+} + BeF\textsubscript{x} in Table 1), and that E1Ca\textsubscript{2}-BeF\textsubscript{x} produced in 15 mM Mg\textsuperscript{2+} and 50–100 μM Ca\textsuperscript{2+} was decomposed to the E1Ca\textsubscript{2} by 5 mM Ca\textsuperscript{2+} (E1Ca\textsubscript{2}-BeF\textsubscript{x} + 5 mM Ca\textsuperscript{2+}), as shown by the rapid cleavage can rotate into the space between the N and P domains to some extent thus resulting in partial T2 resistance (but not yet as rates at the T2 and proteinase K sites. In E1PCa\textsubscript{2}Mg and E1Ca\textsubscript{2} CaAMP-PCP formed in 5 mM Ca\textsuperscript{2+} without Mg\textsuperscript{2+} (Table 1), the T2 site was also rapidly cleaved, in contrast to its substantially slowed cleavage in E1Ca\textsubscript{2}-BeF\textsubscript{x} with Mg\textsuperscript{2+}. Thus, for organization of the cytoplasmic domains at the T2 site (Arg\textsuperscript{1096}). E1Ca\textsubscript{2}-CaAMP-PCP and E1PCa\textsubscript{2} are very similar to E1Ca\textsubscript{2}-AlF\textsubscript{x}-ADP, but differ from E1Ca\textsubscript{2}-BeF\textsubscript{x}. The close similarity between E1Ca\textsubscript{2}-CaAMP-PCP and E1Ca\textsubscript{2}-AlF\textsubscript{x}-ADP is in agreement with their nearly same atomic structures and previous observations (17, 18, 45). Also notably, structure E1PCa\textsubscript{2}-CaAMP-PN formed by CaAMP-PNP in 10 mM Ca\textsuperscript{2+} without Mg\textsuperscript{2+} (22) is almost identical with those of E1Ca\textsubscript{2}-CaAMP-PCP and E1Ca\textsubscript{2}-AlF\textsubscript{x}-ADP (see also Table 1).

In E1Ca\textsubscript{2}-CaAMP-PCP and E1PCa\textsubscript{2}-CaAMP-PN, the N-P domain cross-linked state is stabilized by Ca\textsuperscript{2+} bound at catalytic Mg\textsuperscript{2+} site I (Asp\textsuperscript{351}/Thr\textsuperscript{353}/Asp\textsuperscript{703} and the phosphate) and by the nucleotide to be nearly identical to the state stabilized with AlF\textsubscript{x} plus ADP in E1Ca\textsubscript{2}-AlF\textsubscript{x}-ADP (17, 18, 22). The results on E1PCa\textsubscript{2} further indicated that such an N-P domain closed state is stabilized solely by site I Ca\textsuperscript{2+} ligation without the nucleotide. The stabilization of this state in E1PCa\textsubscript{2} is consistent with its markedly retarded isomerization to E2P (27), because isomerization requires the A domain rotation into the space between the N and P domains. In E1Ca\textsubscript{2}-BeF\textsubscript{x} formed with Mg\textsuperscript{2+} at the catalytic site (site I), such a Ca\textsuperscript{2+} ligation effect is obviously not present. Therefore, the N and P domains are probably more easily separated from each other, and the A domain

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3 The proteolytic analysis of E1PCa\textsubscript{2} was made possible by its markedly retarded decay due to Ca\textsuperscript{2+} ligation at the catalytic site (27) and feedback inhibition by the high luminal Ca\textsuperscript{2+}. Analysis of E1PCa\textsubscript{2}-Mg formed from MgATP was not feasible because of its very rapid turnover, thus of a very rapid ATP exhaustion.
TABLE 1
Summary of fluorescence changes and proteolysis rates

| ATPase state (→ consequent state, if altered) | Ca²⁺ (mm)/Mg²⁺ (mm) | Relative TNP-AMP fluorescence intensity, see Fig. 5A and supplemental Fig. S5 | Change in tryptophan fluorescence from E1Ca²⁺ (or from another state), supplemental Fig. S3 | Relative digestion rate | Tryptophan (T2), supplemental Fig. S2 | Proteinase K, supplemental Fig. S2 |
|---------------------------------------------|----------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|------------------------|---------------------------------------|----------------------------------|
| E1Ca²⁺ | 0.1/15 | 7 | (3.27 ↑ from E2) | 100 | 100 |
| E1Ca²⁺MgAMP-PCP | 0.1/15 | 5/0 | 0.80 ↑ | 64 | 25 |
| E1Ca²⁺CaAMP-PCP | 5/0 | 0.84 ↑ | 61 | 9 |
| E1Ca²⁺AIF₃-ADP | 0.1/15 | 0.77 ↑ | 65 | 4 |
| E1PCa²⁺CaAMP-PCP | 5/0 | 0.69 ↓ | 80 | 6 |
| E1PCa²⁺Mg | 0.1/15 | 1.18 ↓ | 35 | 13 |
| E1PCa²⁺BeF₃ + 5 mM Ca²⁺ (→ partially E1Ca²⁺) | 0.1/15 + 5 mM Ca²⁺ | 30 | 70 | 32 |
| E1Ca²⁺ + 5 mM Ca²⁺ + BeF₃ | 5/0 | 16 | 0 | 116 | 90 |
| E2-BeF₃ | 0/15 | 100 | (0.66 ↑ from E2) | 0 | 0 |
| E1Ca²⁺BeF₃ + TG (→ E2-BeF₃TG) | 0.1/15 | 64 | (5.35 ↓ from E1Ca²⁺BeF₃) | 0 | 0 |
| E2-BeF₃ + TG (→ E2-BeF₃TG) | 0/15 | 65 | (4.62 ↓ from E2-BeF₃ TG) | 0 | 0 |
| E1Ca²⁺BeF₃ + A23187 (→ E2-BeF₃) | 0.1/15 | 100 | 10 | 0 |
| E1PCa²⁺Mg (0.7 mM Ca²⁺) | 0.1/15 | 1.19 ↓ | 0 | 0 |
| E1PCa²⁺BeF₃ (0.7 mM Ca²⁺) | 0.1/15 | 75 | 1.27 ↓ | 39 | 15 |
| E1PCa²⁺BeF₃ (0.7 mM Ca²⁺ + A23187) | 0.1/15 | 75 | 32 | 8 |
| E2-BeF₃ + 0.7 mM Ca²⁺ | 0/15 + 0.7 mM Ca²⁺ | 100 | (0 from E2-BeF₃) | 0 | 2 |
| E2-BeF₃ + 0.7 mM Ca²⁺ + A23187 | 0/15 + 0.7 mM Ca²⁺ | 75 | 38 | 16 |

Note also that partially E1PCa²⁺Mg complex was formed in 15 mM Mg²⁺ and 100 mM Ca²⁺ and then incubated with the subsequently added 5 mM Ca²⁺ for 3 h; E1Ca²⁺ + 5 mM Ca²⁺ + BeF₃, the E1Ca²⁺BeF₃ complex was formed in 15 mM Mg²⁺ and 100 mM Ca²⁺ and then incubated with BeF₃ for 10 min in the presence of 5 mM Ca²⁺ without Mg²⁺.

ATPase state (→ consequent state, if altered)

Ca²⁺ (mm)/Mg²⁺ (mm) | Relative TNP-AMP fluorescence intensity, see Fig. 5A and supplemental Fig. S5 | Change in tryptophan fluorescence from E1Ca²⁺ (or from another state), supplemental Fig. S3 | Relative digestion rate |

E1Ca²⁺ | 0.1/15 | 7 | (3.27 ↑ from E2) | 100 | 100 |
E1Ca²⁺MgAMP-PCP | 0.1/15 | 5/0 | 0.80 ↑ | 64 | 25 |
E1Ca²⁺CaAMP-PCP | 5/0 | 0.84 ↑ | 61 | 9 |
E1Ca²⁺AIF₃-ADP | 0.1/15 | 0.77 ↑ | 65 | 4 |
E1PCa²⁺CaAMP-PCP | 5/0 | 0.69 ↓ | 80 | 6 |
E1PCa²⁺Mg | 0.1/15 | 1.18 ↓ | 35 | 13 |
E1PCa²⁺BeF₃ + 5 mM Ca²⁺ (→ partially E1Ca²⁺) | 0.1/15 + 5 mM Ca²⁺ | 30 | 70 | 32 |
E1Ca²⁺ + 5 mM Ca²⁺ + BeF₃ | 5/0 | 16 | 0 | 116 | 90 |
E2-BeF₃ | 0/15 | 100 | (0.66 ↑ from E2) | 0 | 0 |
E1Ca²⁺BeF₃ + TG (→ E2-BeF₃TG) | 0.1/15 | 64 | (5.35 ↓ from E1Ca²⁺BeF₃) | 0 | 0 |
E2-BeF₃ + TG (→ E2-BeF₃TG) | 0/15 | 65 | (4.62 ↓ from E2-BeF₃TG) | 0 | 0 |
E1Ca²⁺BeF₃ + A23187 (→ E2-BeF₃) | 0.1/15 | 100 | 10 | 0 |
E1PCa²⁺Mg (0.7 mM Ca²⁺) | 0.1/15 | 1.19 ↓ | 0 | 0 |
E1PCa²⁺BeF₃ (0.7 mM Ca²⁺) | 0.1/15 | 75 | 1.27 ↓ | 39 | 15 |
E1PCa²⁺BeF₃ (0.7 mM Ca²⁺ + A23187) | 0.1/15 | 75 | 32 | 8 |
E2-BeF₃ + 0.7 mM Ca²⁺ | 0/15 + 0.7 mM Ca²⁺ | 100 | (0 from E2-BeF₃) | 0 | 2 |
E2-BeF₃ + 0.7 mM Ca²⁺ + A23187 | 0/15 + 0.7 mM Ca²⁺ | 75 | 38 | 16 |

The fluorescence level after the decrease of the transient superfluorescence of E1Ca²⁺BeF₃ was somewhat higher than the non-superfluorescent low level of TNP-AMP bound to E1Ca²⁺ especially at 25°C. We obtained the results indicating that a small fraction of E2-BeF₃ was produced even in the presence of 50 mM Ca²⁺ (more at 25 than at 4°C) after the TNP-AMP-induced E1Ca²⁺BeF₃ decomposition to E1Ca²⁺, and therefore remained somewhat superfluorescence (data not shown).
Ca\(^{2+}\) (see Fig. 8 and Table 1). Also, inclusion of 5 mM Ca\(^{2+}\) without Mg\(^{2+}\) in the E1Ca\(_2\)-BeF\(_x\) formation mixture abolished the superfluorescence (Table 1). The results agree with the above findings that E1Ca\(_2\)-BeF\(_x\) is not produced from and decomposed to E1Ca\(_2\) by Ca\(^{2+}\) ligation at the catalytic Mg\(^{2+}\) site (site 1).

**Transmembrane Domain Structure**—The 12 tryptophan residues among 13 in the Ca\(^{2+}\)-ATPase are located at the transmembrane region. The tryptophan fluorescence changes in fact reflect the transmembrane domain structural changes, i.e. rearrangements of the transmembrane helices upon Ca\(^{2+}\) binding to the high affinity transport sites and during the transport cycle (28, 51, 52) as found originally by Dupont and Leigh (53). As summarized in Table 1 with typical fluorescence traces in supplemental Fig. S3, the fluorescence changes were determined at 4 °C upon formation of the E1PCa\(_2\) analogs by the addition of fluoride compounds to E1Ca\(_2\) in 15 mM Mg\(^{2+}\) and 100 \(\mu\)M Ca\(^{2+}\). E1Ca\(_2\)-BeF\(_x\) formation decreased fluorescence by 1.3% very similar to the decrease in E1PCa\(_2\)-Mg formation from E1Ca\(_2\) by MgATP, i.e. in E1Ca\(_2\)-MgATP \(\rightarrow\) E1PCa\(_2\)-Mg (52). In contrast, E1Ca\(_2\)-AlF\(_x\) formation did not cause any change. The E1Ca\(_2\)-AlF\(_x\)-ADP formation increased the fluorescence by 0.8%. (F\(^-\) alone and ADP alone did not cause any change, except the dilution (F\(^-\)) and absorption of excitation light (ADP).) Thus the transmembrane structure of E1Ca\(_2\)-BeF\(_x\) mimics that of E1PCa\(_2\)-Mg, but those of E1Ca\(_2\)-AlF\(_x\)-ADP and E1Ca\(_2\)-AlF\(_x\) differ substantially although the Ca\(^{2+}\) ions are occluded at the transport sites (or less strongly in E1Ca\(_2\)-AlF\(_x\), Fig. 4). This observation is consistent with the finding in proteolysis and TNP-AMP superfluorescence that organization of the cytoplasmic domains and structure at the catalytic site in E1Ca\(_2\)-BeF\(_x\) substantially differ from those in E1Ca\(_2\)-AlF\(_x\)-ADP and E1Ca\(_2\)-AlF\(_x\).

It is concluded that the transmembrane structure with the occluded Ca\(^{2+}\) adopts not simply one state, but changes with the change in the cytoplasmic region during phosphoryl transfer and ADP release (see the diagram of tryptophan fluorescence change in supplemental Fig. S3E (with Ref. 54)).

Upon formation of E1Ca\(_2\)-CaAMP-PCP and E1PCa\(_2\)-CaAMP-PNP by CaAMP-PCP and CaAMP-PPN, respectively, the fluorescence increased by 0.8% equal to that upon E1Ca\(_2\)-AlF\(_x\)-ADP formation (Table 1), in agreement with their essentially identical structures with occluded Ca\(^{2+}\) (17, 18, 22, 45). By contrast, the fluorescence did not change upon formation of the E1Ca\(_2\)-MgAMP-PCP, which is the Ca\(^{2+}\)-unoccluded state (28, 45), and in rapid equilibrium with E1Ca\(_2\).

Upon the exclusive accumulation of E1PCa\(_2\)-Ca by CaATP without Mg\(^{2+}\), tryptophan fluorescence decreased by 0.9%, being slightly less than that by formation of E1PCa\(_2\)-Mg and E1Ca\(_2\)-BeF\(_x\) (Table 1). Thus in the overall structure,
E1PCa2-Ca may be between E1PCa2-CaAMP-PCP and E1PCa2-Mg (E1Ca2-BeFx), and closer to the latter state. Although Ca2+ ligation at catalytic Mg2+ site I in E1PCa2-Ca favors the N-P domain closed state, similar to E1Ca2-CaAMP-PCP, the absence of the N-P domain cross-linking nucleotide in E1PCa2-Ca likely altered the overall structure slightly.

Upon formation of E1Ca2-BeFx from E2 by BeFx and Mg2+ without Ca2+, the fluorescence increased by 0.7%, mimicking the change upon E2P-Mg formation from E2 with P1 and Mg2+, and reflecting the opening of the luminal gate from the closed state (25). As a consequence, the fluorescence of E1Ca2-BeFx was definitely higher by ~1.3% than that of E2-BeFx, showing their distinct difference in the transmembrane structure. In agreement, the previous kinetic analysis have shown (28) that tryptophan fluorescence decreases by ~1% in the isomerization/Ca2+ release, E1PCa2-Mg → E2P-Mg + 2Ca2+, reflecting the transmembrane structural change from the Ca2+-occluded state to the Ca2+-released and luminaly opened state.

Upon the addition of thapsigargin (TG) to E1Ca2-BeFx and E2-BeFx, tryptophan fluorescence decreased rapidly by 5.4 and 4.6%, respectively, and reached the level of E2-BeFx with bound TG (E2-BeFx (TG), see Table 1). TNP-AMP superfluorescence (supplemental Fig. S5, A and B) and proteinolysis (Table 1) also demonstrated that E1Ca2-BeFx was converted by TG to E2-BeFx (TG). Importantly, as described under supplemental Fig. S4, two Ca2+ occluded in E1Ca2-BeFx are most likely released into the lumen by the TG-induced structural perturbation and trapped in the lumen by the bound TG, as TG fixes the luminal gate in the closed state and suppresses Ca2+ leakage (16, 55).

E1Ca2-BeFx is ADP-sensitive—In Fig. 6, two 45Ca2+ occluded in E1Ca2-BeFx were rapidly removed by washing with 1 mM ADP, whereas the occluded 45Ca2+ remained completely without ADP. Thus ADP caused the loss of Ca2+ occlusion. In agreement, ADP binding to E1Ca2-BeFx increased tryptophan fluorescence to the E1Ca2 level, and resulted in the tryptic T2 site cleavage as E1Ca2 with bound ADP (data not shown). By contrast, ADP binding to E2-BeFx did not alter its structure (data not shown). The ADP-induced decomposition of E1Ca2-BeFx to E1Ca2 was also demonstrated with the ADP-induced loss of TNP-AMP superfluorescence, in contrast to normal superfluorescence development in E2-BeFx after ADP incubation (data not shown). Thus E1Ca2-BeFx is ADP-sensitive as E1PCa2-Mg, and E2-BeFx is ADP-insensitive as E2P-Mg.

Conversion of E1Ca2-BeFx to E2-BeFx at 50 mM Ca2+—In Fig. 7, E1Ca2-BeFx was first formed in SR vesicles with BeFx at 25 °C in 50 mM Ca2+ and 15 mM Mg2+, then further incubated at 25 and 4 °C in the presence of these ligands. The amount of bound and occluded Ca2+ was lost slowly (τ1/2 ~ 2 h at 25 °C and ~7 h at 4 °C). TNP-AMP superfluorescence (Fig. 8) and tryptic and proteinase K proteinolyses (data not shown) revealed that E1Ca2-BeFx turned to E2-BeFx with Ca2+ loss. Thus E1Ca2-BeFx proceeded its spontaneous slow conversion to E2-BeFx, as the autoisomerization of E1PCa2-Mg to E2P-Mg. The Ca2+ ions released into the lumen may leak out during such long periods. In E1Ca2-AlF4− and E1Ca2-AlF4−·ADP, the amount of bound (occluded) Ca2+ was not decreased during the above 10-h incubation at 25 °C (data not shown). The proteinolysis showed that these complexes were not converted to the Ca2+-released forms, E2-AlF4− (E2-AlF4−) with and without ADP (data not shown). The results indicate that the product state E1PCa2-Mg in the phosphoryl transfer acquires the structure ready for autoisomerization to E2P-Mg releasing Ca2−, whereas the transition state structure is not yet fully prepared for autoisomerization to the Ca2+-released E2P form. Interestingly, as described in supplemental Figs. S4 and S5 (with Refs. 56 and 57), the conversion E1Ca2-BeFx → E2-BeFx was markedly accelerated by the transmembrane structural perturbation with hydrophobic reagents such as A23187, lasalocid, and C12E8, as
well as TG. In contrast, E1Ca₂⁺AlF₃ and E1Ca₂⁺AlF₃⁺ADP were resistant against these reagents.

**E1Ca₂⁺BeF₃ Is Perfectly Stabilized at 0.7 mM Ca²⁺**—As found here, Ca²⁺ binding at high affinity transport sites in E1Ca₂⁺ is obligatorily required for E1Ca₂⁺BeF₃ formation, whereas millimolar high Ca²⁺ (Ca²⁺ ligation at the catalytic Mg²⁺ site I) decomposes this complex to E1Ca₂⁺. Furthermore, E1Ca₂⁺BeF₃ at 50 μM Ca²⁺ is spontaneously and slowly converted to E2-BeF₃ releasing Ca²⁺, and the conversion is markedly accelerated by transmembrane perturbation with hydrophobic reagents such as C₁₀E₆ and A23187 (see supplemental materials). The results showed that E1Ca₂⁺BeF₃ as the E1PCa₂⁺Mg analog possesses the structure prepared for its isomerization to E2-BeF₃ with Ca²⁺ release as E1PCa₂⁺Mg → E2P-Mg + 2Ca²⁺. On the other hand, it is essential for crystallographic studies to find conditions to perfectly stabilize the E1Ca₂⁺BeF₃ complex. In Fig. 8A, E1Ca₂⁺BeF₃ was first formed in 0.1 mM Ca²⁺ and 15 mM Mg²⁺, then further incubated with various concentrations of Ca²⁺ with and without A23187. The structural state was monitored by TNP-AMP superfluorescence (Fig. 8), proteolysis, and tryptophan fluorescence (see Table 1 for representative data). Then we successfully found that Ca²⁺ at a very narrow concentration range, 0.7 mM, perfectly stabilizes E1Ca₂⁺BeF₃ and maintains this complex for at least 12 days at 25 °C (and 4 °C) even in the presence of A23187. The ⁴⁵Ca²⁺ binding measurements on E1Ca₂⁺BeF₃ in 0.7 mM ⁴⁵Ca²⁺ demonstrated that two Ca²⁺ ions are bound and occluded in this complex (Fig. 9A).

The perfectly stable E1Ca₂⁺BeF₃ was produced from E1Ca₂⁺ even in the presence of A23187 if 0.7 mM Ca²⁺ was included before BeF₃⁻ addition (Fig. 8, B and F, and Table 1). Also, E1Ca₂⁺BeF₃ was successfully produced with the Ca²⁺-ATPase purified from SR vesicles by depolymerization with A23187 (30); in this case again, by including 0.7 mM Ca²⁺ before BeF₃⁻ addition. E1Ca₂⁺BeF₃ thus produced with the purified and depolymerized Ca²⁺-ATPase was perfectly stable at least for 12 days at 4 and 25 °C in 0.7 mM Ca²⁺ (Fig. 8, B and G, at 25 °C).

**E1Ca₂⁺BeF₃ Is Produced from E2-BeF₃⁻ by Lumenal Ca²⁺ Binding**—We successfully found also that E1Ca₂⁺BeF₃ (E1Ca₂⁺BeF₃⁻) can be produced from E2-BeF₃⁻ by lumenal Ca²⁺ binding, as mimicking the luminal Ca²⁺-induced reverse transition, E2P-Mg + 2Ca²⁺ → E1PCa₂⁺Mg. In Fig. 10, we added...
various concentrations of Ca$^{2+}$ to E2-BeF$_3$ formed in SR vesicles in 15 mM Mg$^{2+}$ without Ca$^{2+}$ in the presence and absence of A23187, then at 10 s after Ca$^{2+}$ addition the structural state was examined by TNP-AMP superfluorescence. With increasing Ca$^{2+}$ to 0.7 mM in the presence of A23187, the stable superfluorescence of E2-BeF$_3$ was converted to the transient and slightly lower superfluorescence characteristic of E1Ca$_2$-BeFx with K$_{0.5}$ of 0.4 mM Ca$^{2+}$ and a Hill coefficient of 4 (Fig. 10, A and D). A further Ca$^{2+}$ increase in the millimolar range caused the marked loss of superfluorescence with K$_{0.5}$ of 1.7 mM and a Hill coefficient of 1 (Fig. 10, B and D). The proteolysis also clearly showed that E2-BeF$_3$ was converted to E1Ca$_3$-BeFx by 0.7 mM Ca$^{2+}$ (Table 1), and this complex was further decomposed to E1Ca$_3$ by 10 mM Ca$^{2+}$ (data not shown). In Fig. 9B, two 45Ca$^{2+}$ were shown to be bound producing E1Ca$_2$-BeFx, when 0.75 mM Ca$^{2+}$ was added to E2-BeF$_3$ in the presence of A23187. In contrast, in the absence of A23187, E2-BeFx was neither converted to E1Ca$_2$BeFx nor decomposed to E1Ca$_2$ even at 10 mM Ca$^{2+}$ (Figs. 9B and 10, C and E, and Table 1 (proteolysis at 0.7 mM Ca$^{2+}$)).

The results demonstrated that E1Ca$_2$-BeFx, most probably E1Ca$_2$-BeFx, was produced from E2-BeFx by the luminal Ca$^{2+}$ binding at the lumenerally oriented low affinity transport sites, and further that Ca$^{2+}$ substitution of Mg$^{2+}$ at the catalytic site in E1Ca$_2$-BeFx produced from E2-BeFx caused its decomposition to E1Ca$_2$, therefore as the change E2-BeFx $\rightarrow$ E1Ca$_2$-BeFx $\rightarrow$ E1Ca$_2$. Note that Mg$^{2+}$ bound at the catalytic site in E2P-Mg is occluded, whereas it is not and therefore is exchangeable in E1PCa$_2$Mg (42). Thus, these two distinctly different states of the ligated Mg$^{2+}$ at the catalytic site (site I) in E2P-Mg and E1PCa$_2$Mg are obviously mimicked here by the respective analogs E2-BeFx$_3$ and E1Ca$_2$-BeFx$_3$. The perfect stabilization of E1Ca$_2$-BeFx achieved by 0.7 mM Ca$^{2+}$ (Fig. 10) obviously involves luminal Ca$^{2+}$ binding and prevention of the Ca$^{2+}$ release into the lumen. The stabilization by 0.7 mM Ca$^{2+}$ in the absence of A23187 is probably due to Ca$^{2+}$ moved passively into the vesicle lumen during the long incubation periods. All these findings show that the forward and reverse transition, E1PCa$_2$-Mg $\leftrightarrow$ E2P-Mg + 2Ca$^{2+}$, is mimicked by the forward and reverse conversion, E1Ca$_2$-BeFx$_3$ $\leftrightarrow$ E2-BeFx$_3$ + 2Ca$^{2+}$.

It is of interest to note the Hill coefficient of 4 in the luminal Ca$^{2+}$-induced reverse conversion, E2-BeFx$_3$ + 2Ca$^{2+}$ $\rightarrow$ E1Ca$_2$-BeFx$_3$ at 0.1–0.7 mM Ca$^{2+}$ in Fig. 10A. This might be indicative of the existence of luminal Ca$^{2+}$ access sites in addition to transport sites and their possible cooperative involvement in luminal Ca$^{2+}$ access to the transport sites. In fact, two such sites besides the two transport sites have been suggested by the kinetics and protein-chemical study on the luminal loops (58, 59).

**DISCUSSION**

*Formation of E1Ca$_2$BeFx$_3*—As a structural analog of the physiological intermediate E1PCa$_2$-Mg, the E1Ca$_2$-BeFx complex was successfully produced by BeFx binding to the E1Ca$_2$ state Ca$^{2+}$-ATPase and from E2-BeFx$_3$ by luminal Ca$^{2+}$ binding to the lumenerally oriented low affinity transport sites. All the revealed properties of E1Ca$_2$-BeFx met the requirements for the E1PCa$_2$-Mg analog: i.e. two Ca$^{2+}$ occluded at the transport sites, Mg$^{2+}$ bound (but not occluded) at the catalytic site, the ADP-released but still ADP-sensitive state, and its isomerization to the ADP-insensitive Ca$^{2+}$-released state E2P-Mg (E2-BeFx$_3$) and reversal by luminal Ca$^{2+}$ binding, E1PCa$_2$-Mg $\leftrightarrow$ E2P-Mg + 2Ca$^{2+}$.

Furthermore, the coordination chemistry of beryllium fluoride, actually BeFx, fulfills the requirement of E1Ca$_2$-BeFx$_3$ as the E1PCa$_2$-Mg analog. In chemistry, beryllium fluoride compounds are known to adopt tetrahedral geometry with the Be-F 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60). Moreover, because of the high charge density due to the small size, beryllium is able to occupy the 1.55 Å bond length, thereby making them strictly isomorphous to the tetrahedral phosphate group (60).
logs agree with the conclusion that E1Ca2-BeF3 is the analog for E1PCa2-Mg, and BeF3 is most probably BeF3−, i.e. E1Ca2-BeF3−.

Here note that the replacement of phosphate with BeF3− produces stabilization of the E1PCa2-Mg structure with the same geometry of BeF3− as phosphate, and therefore probably with the same binding residues for them within the catalytic site. The E1Ca2-Mg stability is likely brought about by the specific chemical nature of fluoride. Namely, it possesses a significantly higher electronegativity than oxygen (actually the highest in the periodic table) and a small size, therefore producing stronger BeF3− binding in the catalytic site and fixing the intermediate structure.

**Structure of E1Ca2-BeF3−—**Then with the newly developed E1Ca2-BeF3−, we explored its structural properties and uncovered the hitherto unknown nature of the physiological intermediate E1PCa2-Mg and structural changes during the phosphoryl transfer/ADP release and subsequent EP isomerization/Ca2+ release. The observed proteinase K resistance of Thr242 on the A/M3-linker revealed that, in E1PCa2-Mg (E1Ca2-BeF3−), the A domain is already rotated perpendicular to the membrane plane from the position in E1Ca2+, thereby bringing up its junction with the A/M3-linker and imposing a strain on this linker, similarly to E1Ca2-AlF4−-ADP and E1Ca2-PCP (17, 18).

As described for the E1Ca2-PCP structure (18), the A/M3-linker strain, i.e. the A domain perpendicular rotation is brought about by bending the P domain due to binding of the phosphate moiety and Ca2+ at the catalytic site (Mg2+−site I, Asp351/Thr353/Asp703) on the P domain (see Figs. 4 and 5 in Ref. 18).5 Our results revealed that such a strained state is achieved even without the N-P domain cross-linking nucleotide but solely with BeF3− and Mg2+ binding at the catalytic site, and therefore remains in E1PCa2-Mg after ADP release.

The strain of the A/M3-linker thus imposed has been predicted with the atomic structure (18, 19) to function as a motive force for the A domain rotation parallel to the membrane in the E1P to E2P isomerization. The partial resistance at T2 site Arg198 in E1Ca2-BeF3− (as compared with the rapid cleavage in E1Ca2-PCP/PCP/E1Ca2-AlF4−-ADP and E1Ca2-AlF3−) further indicated that in E1PCa2-Mg, the A domain is already likely

5 As depicted in Figs. 4 and 5 by Toyoshima et al. (18) for the change E1Ca2→E1Ca2-PCP, the top part of the first half of the P domain (Pβ1–Pβ4) moves together as a result of γ-phosphate and Ca2+ (Mg2+−) binding, because Thr242 just above Pβ1 coordinates to both ligands. Furthermore, Pβ5 twists upon binding of Ca2+ (Mg2+−) because of the coordination by Asp353, which causes P5→P2 tilting. Thus the P domain is bent. This bending causes the perpendicular A domain rotation because the P7 helix moves upwards and tilts so that Gly198-Lys198 on the A domain is brought up as they are in contact with Ala251-Val256 on top of the P7 helix.
rotated parallel to the membrane to some extent—from the position in $E1Ca_{2}\text{MgATP}/E1PCa_{2}\text{ADP-Mg}$. Thus, the A/M3-linker strain is likely functioning for this partial A domain rotation during the phosphoryl transfer/ADP release to produce $E1PCa_{2}\text{Mg}$, and further for the large and complete rotation to achieve the tight A-P domain association at Arg$^{108}$ on the Val$^{200}$ loop in the Ca$^{2+}$-released state $E2P\text{-Mg} (E2\text{-BeF}_3)$. The A-P domain interaction at the Val$^{200}$ loop is actually critical for formation of the proper Ca$^{2+}$-released structure, $E2P\text{-Mg}$ and its analog $E2\text{-BeF}_3$ (25, 62, 63).

Here, it is of interest to note that residues Asp$^{351}$, Thr$^{353}$, and Asp$^{703}$ ligating Mg$^{2+}$ and phosphate will come more proximate to each other during $E1PCa_{2}\text{-ADP-Mg} \rightarrow E1PCa_{2}\text{Mg} + ADP (E1Ca_{2}\text{AlF}_3\text{-ADP/E1Ca}_{2}\text{AlF}_3 \rightarrow E1Ca_{2}\text{BeF}_3)$ as a consequence, a further P domain bending and more strain for the A/M3-linker will likely be induced by this coordination-chemical change, thereby contributing to inducing the A domain rotations during $E1PCa_{2}\text{-Mg}$ formation and subsequent isomerization to $E2PCa_{2}\text{Mg}$ (besides the release of the N-P domain cross-linking nucleotide ADP). In any case, our results show that $E1PCa_{2}\text{Mg} (E1Ca_{2}\text{BeF}_3)$ as the product of the phosphorodiation reaction acquires the structure ready for isomerization and Ca$^{2+}$ deocclusion/release (i.e. ready for the large A domain rotation to produce $E2P\text{-Mg} (E2\text{-BeF}_3)$), whereas the transition state structure in the phosphorylation ($E1Ca_{2}\text{AlF}_3\text{-ADP/E1Ca}_{2}\text{AlF}_3$) is not yet fully prepared. Note again that the $E1PCa_{2}\text{Mg}$ structure before such motions for its isomerization to $E2P\text{-Mg}$ is stabilized with replacement of phosphate with BeF$_3$ in $E1Ca_{2}\text{BeF}_3$.

Important also, we found that the Ca$^{2+}$-occluded transmembrane structure adopts not simply one state, but will proceed through changes during the phosphoryl transfer and ADP release to form $E1PCa_{2}\text{Mg}$ (see supplemental Fig. S3E). The structural change is probably coupled with the above described motions of the P and A domains (more bending and rotation) during this process. In the subsequent Ca$^{2+}$ deocclusion/release in $E1PCa_{2}\text{-Mg} \rightarrow E2P\text{-Mg} + 2\text{Ca}^{2+}$, the transmembrane structure changes further (52), which was also clearly mimicked here in the change $E1Ca_{2}\text{BeF}_3 \rightarrow E2\text{BeF}_3$. Thus, the structures of the transmembrane domain as well as the cytoplasmic domains in $E1Ca_{2}\text{BeF}_3$ ($E1PCa_{2}\text{Mg}$) are intermediate between those of $E1Ca_{2}\text{AlF}_3\text{-ADP/E1PCa}_{2}\text{ADP-Mg}$ and $E2\text{-BeF}_3$ ($E2P\text{-Mg}$).

$\text{Mg}^{2+}$ as Physiological Catalytic Cation—Important questions regarding the $E1Ca_{2}\text{BeF}_3$ structure are why Ca$^{2+}$-coordinated at the catalytic Mg$^{2+}$ site (site I, Asp$^{351}$/Thr$^{353}$/Asp$^{703}$) is absolutely unfavorable for $E1Ca_{2}\text{BeF}_3$ formation, and why the Mg$^{2+}$-coordinated structure $E1Ca_{2}\text{BeF}_3$ differs from Ca$^{2+}$-coordinated $E1PCa_{2}\text{-Ca, E1Ca}_{2}\text{CaAMP-PCP}$, and $E1PCa_{2}\text{CaAMP-PN}$ structures as well as from $E1Ca_{2}\text{AlF}_3\text{-ADP/E1Ca}_{2}\text{AlF}_3$, especially in A domain positioning. These questions may be relevant to the questions of why forward isomerization of $E1Ca_{2}\text{Ca}$ to $E2P$ is markedly retarded in contrast to $E1PCa_{2}\text{Mg}$ and thus why Mg$^{2+}$ is preferred as the catalytic cation. In stringent coordination chemistry, the coordination distance of Mg$^{2+}$ is shorter than Ca$^{2+}$, typically by 0.2 Å (e.g. 2.1 versus 2.3 Å (64, 65)). As a consequence, in the case of $E1Ca_{2}\text{CaAMP-PCP}$, the distance between the $\gamma$-phosphate and Asp$^{351}$-oxygen becomes 3.24 Å, being greater by 0.3 Å than that predicted in $E1Ca_{2}\text{MgAMP-PCP}$. Therefore MgAMP-PCP (MgATP) binding would result in steric clash, and $E1Ca_{2}\text{CaAMP-PCP}$ is more stable than $E1Ca_{2}\text{MgAMP-PCP}$, and therefore has less tendency to decompose to $E1Ca_{2}$ (also in the forward direction to the $E\text{P}$ formation and its decay in the case of $E1Ca_{2}\text{CaATP}$) (45). In $E1Ca_{2}\text{-BeF}_3$ formed here with Mg$^{2+}$, the direct coordination between Asp$^{351}$ and the berylillium and their proximate positioning would probably favor the closely positioned ligand residues (Thr$^{353}$/Asp$^{703}$/Asp$^{351}$) for BeF$_3$ and Mg$^{2+}$ but not for Ca$^{2+}$. Therefore Ca$^{2+}$ substitution of Mg$^{2+}$ probably disrupted the precise geometry and decomposed the $E1Ca_{2}\text{BeF}_3$ complex. Also, a possible difference in the coordination number might be involved; Mg$^{2+}$ prefers definitely six, whereas Ca$^{2+}$ can accommodate seven or eight ligands (65—69).

Furthermore, the difference in A domain positioning between the Mg$^{2+}$-coordinated state $E1Ca_{2}\text{BeF}_3$ and the Ca$^{2+}$-coordinated states may be reasonably understood by the consequence of the stringent coordination chemistry. Namely, because the shorter coordination distance of Mg$^{2+}$, P domain bending, and the resulting A domain rotation perpendicular to the membrane will be greater in the Mg$^{2+}$-coordinated state. Therefore the strain of the A/M3-linker and A domain rotation parallel to the membrane will be more in the Mg$^{2+}$ state $E1Ca_{2}\text{BeF}_3$. In this context, it is also reasonable that $E1PCa_{2}\text{Mg}$ is more rapidly isomerized to $E2P$ with less energy barrier for the large A domain rotation, in contrast to the retarded isomerization in $E1PCa_{2}\text{Ca}$ that is stabilized by the likely conformational inadequacy. Here note that the cause of the $E1PCa_{2}\text{Ca}$ stabilization is obviously different from that of $E1PCa_{2}\text{Mg}$ stabilization produced by replacement of phosphate with BeF$_3$ (see the above discussion for $E1Ca_{2}\text{BeF}_3$ stabilization).

Previously it was documented (45, 64, 70) that destabilization of the non-covalent complex $E1Ca_{2}\text{-MgATP}$ by Mg$^{2+}$ (as found with MgAMP-PCP versus CaAMP-PCP) together with stabilization of the transition state by Mg$^{2+}$ (as found with $E1Ca_{2}\text{AlF}_3\text{-ADP}$ bound Mg$^{2+}$ at both sites I and II) leads to a decrease of the activation energy and a rapid phosphoryl transfer. As another critical reason for Mg$^{2+}$ preference for catalysis, we predict here by exploring the property of the $E1Ca_{2}\text{BeF}_3$ that the Mg$^{2+}$ bound at the catalytic site produces the proper $E1PCa_{2}$ structure, which is ready for rapid transition to $E2P$ in this rate-limiting process of the transport cycle.
Structural Analog of E1PCa$_2$Mg Intermediate of Ca$_{2+}$-ATPase

hydrophobic closed catalytic site is accomplished by the direct coordination and close proximity of the beryllium with Asp$^{351}$-oxygen and by the specific coordination of the tetrahedral -O-Be$_3^-$, i.e. Asp$^{351}$-acylphosphate within the catalytic site. This is obviously not the case in AlF$_4^{-}$ (the penta-coordinated phos-}

phorus of the transition states) and MgF$_4$ attack of nonspecific water molecules on the Asp$^{351}$-acylphosphate thus accomplishing Ca$_2^+$ release. Whether E1PCa$_2$Mg develops the superfluorescence had been controversial. In addition to the obvious problem of TNP-AMP competition against ATP for phosphorylation, the observed TNP-AMP-induced decomposition of E1Ca$_2$Be$_3^-$ further revealed that the E1PCa$_2$Mg structure may be similarly disrupted rapidly by TNP-AMP binding, therefore making it virtually impossible to examine the superfluorescence development in E1PCa$_2$Mg. The TNP-AMP-induced E1Ca$_2$Be$_3^-$ decomposition might have occurred by means of a similar structural change as the ADP-induced one, i.e. disruption of the cytoplasmic domain organization and possible Be$_3^-$ release. The most important conclusion here is that the hydrophobic and closed property of the phosphorylated catalytic site both in E1PCa$_2$Mg and E2P-Mg may be requisite to avoid a possible attack of nonspecific water molecules on the Asp$^{351}$-acylphosphate thus accomplishing Ca$_{2+}$ release into the lumen and energy coupling.

Formation of E1Ca$_2$Be$_3^-$ from E2-Be$_3^-$ and Perfect Stabilization of E1Ca$_2$Be$_3^-$—E1Ca$_2$Be$_3^-$ was produced also from E2-Be$_3^-$ by binding two luminal Ca$_{2+}$ to the lumenally oriented low affinity transport sites at 0.7 mM Ca$^{2+}$ and 15 mM Mg$^{2+}$, as mimicking the reverse transition E2P-Mg + 2Ca$^{2+}$ → E1PCa$_2$Mg. At the critical concentration of 0.7 mM Ca$^{2+}$ in 15 mM Mg$^{2+}$, E1Ca$_2$Be$_3^-$ is perfectly stabilized without decomposition to E1Ca$_2$ or conversion to E2-Be$_3^-$. The perfect E1Ca$_2$Be$_3^-$ stabilization is obviously achieved by preventing Ca$_{2+}$ release into the lumen and by avoiding the absolutely unfavorable Ca$_{2+}$ substitution of Mg$^{2+}$ in site I at the most appropriately balanced concentrations of Ca$_{2+}$ and Mg$^{2+}$. As noted in the last paragraph under “Results,” stabilization of E1Ca$_2$Be$_3^-$ might possibly involve luminal Ca$_{2+}$ access at the putative luminal gating sites besides the transport sites. If this is the case, the gate-opening and Ca$_{2+}$ release into the lumen takes place when the luminal Ca$_{2+}$ is low enough to avoid the possible luminal Ca$_{2+}$ access to the gate.

Integrated Picture of EP Processing—Recently, we successfully identified and trapped for the first time the intermediate state E2PCa$_2$Mg, ADP-insensitive EP with two Ca$^{2+}$ occluded at transport sites, by elongating the A/M1’-linker (71), and revealed that the proper length of this linker is critical for inducing structural changes for Ca$_{2+}$ deocclusion and release from E2PCa$_2$Mg. This dependence on the length of the linker is probably because the length controls the extent of strain between the A domain and M1’, which causes motions of the cytoplasmic A and P domains thereby transmitting the structural signal to the transmembrane transport sites. In trapped E2PCa$_2$Mg, the A domain is already largely rotated, and A-P domain associations at Val$^{200}$ and TGES$^{184}$ loops are already produced, although the interaction network is not produced properly at the Tyr$^{122}$-hydrophobic cluster (71), which is critical for Ca$_{2+}$ deocclusion/release and E2P hydrolysis (72–74). In the Ca$_{2+}$-released E2P-Mg, this cluster is formed from seven residues of the A (Ile$^{177}$/Leu$^{180}$/Ile$^{232}$) and P (Val$^{205}$/Val$^{206}$) domains and the top part of M2 (Leu$^{119}$/Tyr$^{122}$) (see Fig. 2).

The results indicated that the successive structural changes take place as follows: in E1PCa$_2$Mg → E2PCa$_2$Mg, the A domain rotates largely (further from the position in E1PCa$_2$Mg) into the space between the N and P domains and docks onto the Asp$^{351}$-acylphosphate of the P domain, thereby causing loss of ADP sensitivity and also the strain of the A/M1’-linker (because the A domain is brought above the P domain). The strain thus imposed will cause inclinations of the A and P domains and the connected M2 and M4/M5 thereby rearranging the helices to destroy Ca$_{2+}$ sites and open the lumenal gate thus to release Ca$_{2+}$. Upon these motions, the Tyr$^{122}$-hydrophobic cluster is produced from the inclined A and P domains and M2. Hence, interactions at this cluster and at the Val$^{200}$ loop stabilize the Ca$_{2+}$-released structure E2P-Mg, and also produce the catalytic site for the acylphosphate hydrolysis to occur after Ca$_{2+}$ release, ensuring energy coupling (63, 72–74). Atomic level structural studies of E1Ca$_2$Be$_3^-$ as E1PCa$_2$Mg and the trapped intermediate state E2PCa$_2$Mg will contribute to further understanding of EP processing, Ca$_{2+}$ handling, and E2P hydrolysis.

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