Glutamate 90 at the Luminal Ion Gate of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase Is Critical for Ca\textsuperscript{2+} Binding on Both Sides of the Membrane\textsuperscript{[5]}

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The roles of Ser\textsuperscript{72}, Glu\textsuperscript{90}, and Lys\textsuperscript{397} at the luminal ends of transmembrane helices M1, M2, and M4 of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase were examined by transient and steady-state kinetic analysis of mutants. The dependence on the luminal Ca\textsuperscript{2+} concentration of phosphorylation by P\textsubscript{i} (“Ca\textsuperscript{2+} gradient-dependent E\textsubscript{2P} formation”) showed a reduction of the apparent affinity for luminal Ca\textsuperscript{2+} in mutants with alanine or leucine replacement of Glu\textsuperscript{90}, whereas arginine replacement of Glu\textsuperscript{90} or Ser\textsuperscript{72} allowed E\textsubscript{2P} formation from P\textsubscript{i} even at luminal Ca\textsuperscript{2+} concentrations much too small to support phosphorylation in wild type. The latter mutants further displayed a blocked dephosphorylation of E\textsubscript{2P} and an increased rate of conversion of the ADP-sensitive E\textsubscript{1P} phosphoenzyme intermediate to ADP-insensitive E\textsubscript{2P} as well as insensitivity of the E\textsubscript{2P}-BeF\textsubscript{3} complex to luminal Ca\textsuperscript{2+}. Altogether, these findings, supported by structural modeling, indicate that the E\textsubscript{2P} intermediate is stabilized in the mutants with arginine replacement of Glu\textsuperscript{90} or Ser\textsuperscript{72}, because the positive charge of the arginine side chain mimics Ca\textsuperscript{2+} occupying a luminally exposed low affinity Ca\textsuperscript{2+} site of E\textsubscript{2P}, thus identifying an essential locus (a “leaving site”) on the luminal Ca\textsuperscript{2+} exit pathway. Mutants with alanine or leucine replacement of Glu\textsuperscript{90} further displayed a marked slowing of the Ca\textsuperscript{2+} binding transition as well as slowing of the dissociation of Ca\textsuperscript{2+} from Ca\textsubscript{2}E\textsubscript{1} back toward the cytoplasm, thus demonstrating that Glu\textsuperscript{90} is also critical for the function of the cytoplasmically exposed Ca\textsuperscript{2+} sites on the opposite side of the membrane relative to where Glu\textsuperscript{90} is located.

The Ca\textsuperscript{2+}-ATPase\textsuperscript{2} of sarco(endo)plasmic reticulum (1–3) is an ion-translocating ATPase of P-type that mediates active transport of Ca\textsuperscript{2+} from the cytoplasm to the endoplasmic reticulum lumen, thereby allowing rapid oscillation of Ca\textsuperscript{2+} during cellular activation events. Ca\textsuperscript{2+} pumping is achieved by means of a reaction cycle (Scheme 1) involving the formation and decomposition of an aspartyl-phosphorylated intermediate coupled to protein conformational changes that facilitate the binding of two Ca\textsuperscript{2+} ions at cytoplasmically facing high affinity binding sites and subsequent dissociation from luminally facing low affinity sites. Because of extensive efforts that in recent years have involved x-ray crystallography (4–7), an increasingly detailed picture of the structural changes relating to Ca\textsuperscript{2+} transport by the Ca\textsuperscript{2+} pump is steadily emerging. The Ca\textsuperscript{2+}-ATPase consists of 10 membrane-spanning helices (M1 through M10) connecting three major cytoplasmic domains named A (actuator), P (phosphorylation), and N (nucleotide binding) and some smaller luminal loops. Transmembrane helices M4-M6 and M8 contain the residues that coordinate the two Ca\textsuperscript{2+} ions bound side-by-side in a binding pocket in the Ca\textsubscript{2}E\textsubscript{1} and Ca\textsubscript{2}E\textsubscript{1P} states. A key element in the transport cycle is the action of the A domain, which is directly linked to M1-M3 via flexible linkers (see Fig. 1). Thus, occlusion of the Ca\textsuperscript{2+} ions at the high affinity sites of Ca\textsubscript{2}E\textsubscript{1} is achieved through an ATP-induced ~30° tilting of the A domain and accompanying partial unfolding and bending of the N-terminal part of M1 (3). After transfer of the γ-phosphate of ATP to Asp\textsuperscript{351}, a further ~90° rotation of the A domain during the Ca\textsubscript{2}E\textsubscript{1P} → Ca\textsubscript{2}E\textsubscript{2P} transition propagates to the transmembrane domain, disrupting the high affinity Ca\textsuperscript{2+} sites and exposing the Ca\textsuperscript{2+} ions to the lumen. The subsequent release of the Ca\textsuperscript{2+} ions to the lumen is succeeded by dephosphorylation of the aspartyl phosphate in E\textsubscript{2P}, catalyzed by the \textsuperscript{181}TGES phosphatase motif of the A domain (8) that has been brought into position in the catalytic site in E\textsubscript{2P} (5, 6). The cycle is completed by reversal of the A domain rotation and restoration of the cytoplasmically facing high affinity Ca\textsuperscript{2+} sites (E\textsubscript{2} → E\textsubscript{1} in Scheme 1).

The molecular nature and properties of the Ca\textsuperscript{2+} binding pocket in the membrane-spanning region of the Ca\textsubscript{2}E\textsubscript{1} and Ca\textsubscript{2}E\textsubscript{1P} states are known in great detail. Little, however, is known about the exact entry and exit pathways to and from the Ca\textsuperscript{2+} binding pocket and the structural elements involved in extrusion of the Ca\textsuperscript{2+} ions to the endoplasmic reticulum lumen. The crystal structure of Ca\textsuperscript{2+}-ATPase in the E\textsubscript{2P}-BeF\textsubscript{3} state (presumed E\textsubscript{2P} ground state analog) (7) provides some important clues. Thus, in the E\textsubscript{2P}-BeF\textsubscript{3} structure, part of the transmembrane domain near the lumen is in a considerably more open conformation compared with other crystal structures of the Ca\textsuperscript{2+}-ATPase. The M4-residue Glu\textsuperscript{399}, an essential residue for Ca\textsuperscript{2+} coordination in the occluded binding pocket of the
Ca₂E₁ and Ca₂E₁P states (4, 7, 9), is exposed to the lumen and associated with a Mg²⁺ ion in the E₂-BeF₃ structure (Fig. 1). The E₂-BeF₃ crystals were formed in the presence of a high concentration of Mg²⁺ (50 mM) and absence of Ca²⁺, and it may be speculated that the Mg²⁺ ion is bound in place of Ca²⁺ at a luminally facing low affinity Ca²⁺ site. Ser72 and Glu90 at the luminal ends of transmembrane helices M1 and M2, respectively, are positioned very close to Glu309 in E₂-BeF₃, and the Glu³⁰⁹ side-chain carboxylate seems to contribute to coordination of the luminal Mg²⁺ ion. In the Ca₂E₁ and Ca₂E₁P states, on the other hand, Ser²⁷ and Glu⁹⁰ are 10–20 Å apart from the Ca²⁺ sites due to translational movements of M1/M2 and M3/M4 relative to each other, and Ser²⁷ and Glu⁹⁰ instead interact with Lys²⁹⁷ at the luminal end of M4 (Fig. 1 and supplemental Table S1).

In the present study we investigated the significance of the above-described luminal interaction networks seen in the various crystal structures of the Ca²⁺-ATPase. Thus, mutants with alterations to Ser72, Glu90, and Lys297 were compared with wild type Ca²⁺-ATPase by analyzing the partial reactions in transient and steady-state kinetic measurements. We found that the mutations to Glu⁹⁰ affect the Ca²⁺ binding properties profoundly both on the luminal side of the membrane, where Glu⁹⁰ is situated, as well as on the cytoplasmic side. Furthermore, single-substitution of either Ser²⁷ or Glu⁹⁰ with arginine gives rise to Ca²⁺ pumps with a remarkably stable E₂P state, compatible with the hypothesis that the positive charge of the arginine side chain interacts directly with the luminal Ca²⁺ outlet, possibly mimicking Ca²⁺ and thereby blocking further processing of the phosphoenzyme.

**EXPERIMENTAL PROCEDURES**

Site-directed mutagenesis of cDNA encoding the rabbit fast twitch muscle Ca²⁺-ATPase (SERCA1a isoform) inserted into the pMT2 vector (10) was carried out using the QuikChange site-directed mutagenesis kit (Stratagene), and the mutant cDNA was sequenced throughout. To express wild type or mutant cDNA, COS-1 cells were transfected using the calcium phosphate precipitation method (11). Microsomal vesicles containing either expressed wild type or mutant Ca²⁺-ATPase were isolated by differential centrifugation (12). The concentration of expressed Ca²⁺-ATPase was determined by an enzyme-linked immunosorbent assay (13) and by determination of the maximum capacity for phosphorylation with ATP or Pi (“active site concentration”; see Ref. 14). Transport of...
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<sup>46</sup>Ca<sup>2+</sup> into the microsomal vesicles and <sup>45</sup>Ca<sup>2+</sup> binding at 25 °C were measured by filtration, and the ATPase activity was determined by following the liberation of Pi (15) in the presence of 4 μM calcium ionophore A23187 to prevent inhibition caused by rebinding of Ca<sup>2+</sup> to the luminally facing Ca<sup>2+</sup> sites (14). Measurements of phosphorylation and dephosphorylation were generally carried out by manual mixing at 0 °C (14, 16). Transient state kinetics at 25 °C was analyzed using the Bio-Logic quench-flow module QFM-5 (Bio-Logic Science Instruments, Claix, France) with mixing protocols as previously described (17, 18). The determination of the phosphorylation level by acid quenching followed by acid SDS-polyacrylamide gel electrophoresis and quantification of the radioactivity associated with the Ca<sup>2+</sup>-ATPase band was carried out using the previously established procedures (14, 16).

The experiments were conducted at least twice, and average values are shown in Figs. 2–9. Generally, the complete data set (i.e. including all experimental points before averaging) was analyzed by nonlinear regression using the SigmaPlot program (SPSS, Inc.) with the equations described in the figure legends, giving the lines in the figures and the S.E. shown in the tables. To analyze the phosphorylation time courses in Fig. 2, the kinetic simulation software SimZyme was applied, allowing computation of the phosphorylation overshoot as detailed below and in previous publications (17, 18). The best fit was in this case determined manually by trial and error, comparing the computed time courses with the experimental data points for various choices of rate constants. For any choice of reaction cycle and rate constants, SimZyme solves the relevant differential equations using the fourth order Runge-Kutta numerical method and provides a graphical representation of the time dependence of the concentration of the reaction intermediates (17).

**RESULTS**

**Expression and Assays of Overall Function**—To study the potential roles of Glu<sup>90</sup> and Ser<sup>72</sup> for Ca<sup>2+</sup> interaction at the luminal ion gate in the E2P state as well as to address the significance of the Glu<sup>90</sup>-Lys<sup>297</sup>Ser<sup>72</sup> interaction network seen in the Ca<sub>2</sub>E1 structures, we produced six point mutants with alterations to Ser<sup>72</sup>, Glu<sup>90</sup>, and Lys<sup>297</sup> of Ca<sup>2+</sup>-ATPase. Glu<sup>90</sup> was substituted with alanine, leucine, and arginine, Ser<sup>72</sup> was substituted with alanine and arginine, and Lys<sup>297</sup> was substituted with alanine. The five mutants with alterations to Ser<sup>72</sup> or Glu<sup>90</sup> could be expressed in COS-1 cells to levels comparable with that obtained for the wild type, whereas the expression level in COS-1 cells of mutant K297A was generally only ∼30% that obtained with wild type (supplemental Table S2). A reduced expression level of mutants with alterations to Lys<sup>297</sup> has previously been reported by Chen et al. (19). Thus, K297G was not expressed (despite wild type-like levels of mRNA transcript in the cells), and K297F yielded low expression levels, whereas K297M, K297R, and K297E were expressed at wild type-like levels (19). Fortunately, the expression level of K297A was sufficiently high for us to carry out reliable functional measurements.

To assess the overall function of the mutants, the rate of ATP hydrolysis was measured at 37 °C in the presence of 5 mm MgATP and 3 μM free Ca<sup>2+</sup>. The catalytic turnover rates (ATP hydrolysis activity per enzyme molecule (14)) of S72A and K297A were wild type-like (80 and 87%, respectively, that obtained with wild type), whereas the remaining mutants displayed little or no ATPase activity (19% of the wild type rate for E90A and <10% for S72R, E90L, and E90R, supplemental Table S2). The results of measuring the rate of <sup>46</sup>Ca<sup>2+</sup> transport into the microsomal vesicles at 37 °C with 5 mM MgATP present were similar to those obtained in the ATPase activity assay, i.e. wild type-like Ca<sup>2+</sup> transport rates for S72A and K297A and much less for the remaining mutants (supplemental Table S2).

**Transient State Kinetics of Phosphorylation of Ca<sub>2</sub>E1 by ATP**—To determine the rate of phosphorylation of the Ca<sup>2+</sup>-bound enzyme from ATP and to obtain an initial overview of the effects of the mutants on the succeeding partial reactions of the pump cycle, we used rapid kinetic instrumentation at 25 °C to examine the transient state kinetics of phosphorylation at 5 μM [γ<sup>32</sup>P]MgATP at pH 7 of enzyme pre-equilibrated with Ca<sup>2+</sup> (Fig. 2). Under these conditions, the wild type and some of the mutants displayed overshoots of phosphorylation that could be reproduced by computation based on the simplified three-intermediate reaction cycle shown in the bottom right corner of Fig. 2 (for a detailed description of this approach as used previously in case of wild type and other mutants, see Refs. 17 and 18). We find that the best fit to the wild type data is obtained for the rate constants k<sub>a</sub> = 50 s<sup>−1</sup> for phosphorylation of Ca<sub>2</sub>E1, k<sub>b</sub> = 6 s<sup>−1</sup> for phosphoenzyme processing (i.e. Ca<sub>2</sub>E1P → Ca<sub>2</sub>E2P → E2P → E2), and k<sub>c</sub> = 20 s<sup>−1</sup> for the Ca<sup>2+</sup> binding transition of the dephosphoenzyme (i.e. E2 → Ca<sub>2</sub>E1). As seen in Fig. 2, for all six mutants a good fit to the data could be obtained with a phosphorylation rate constant (k<sub>a</sub>) similar to that of wild type. Mutants E90A and E90L displayed large overshoots of phosphorylation reflecting accumulation of a considerable amount of phosphoenzyme at steady state. In such cases where the phosphorylation overshoot is large, fairly accurate values of the rate constants k<sub>b</sub> and k<sub>c</sub> can be extracted from the computational analysis. Thus, the increased levels of dephosphoenzyme accumulating at steady state with E90A and E90L were found to result from 2-fold increased rates of phosphoenzyme processing (k<sub>b</sub>) combined with 10- and 20-fold reduced rates of the Ca<sup>2+</sup> binding transition of the dephosphoenzyme (k<sub>c</sub>), respectively (Fig. 2). Direct measurements of the rates of the partial reactions involved in phosphoenzyme and dephosphoenzyme processing are presented below.

**The Ca<sub>2</sub>E1P → Ca<sub>2</sub>E2P Conformational Transition**—After the phosphorylation of Ca<sub>2</sub>E1 by ATP, the subsequent turnover of the phosphoenzyme occurs in at least three distinct steps comprising the Ca<sub>2</sub>E1P → Ca<sub>2</sub>E2P conformational transition, Ca<sup>2+</sup> dissociation from the Ca<sup>2+</sup> sites (now opening toward the luminal side and exhibiting low affinity), i.e. Ca<sub>2</sub>E2P → E2P, and the dephosphorylation of the Ca<sup>2+</sup>-free E2P intermediate (E2P → E2).

The time course of forward processing of the phosphoenzyme formed by phosphorylation of Ca<sup>2+</sup>-saturated enzyme with [γ<sup>32</sup>P]ATP was determined at 0 °C by chasing the accumulated phosphoenzyme with an excess of the Ca<sup>2+</sup> chelator EGTA (terminating phosphorylation by removal of Ca<sup>2+</sup> from
Ca$_2^+$E$_1$ followed by acid quenching at serial times (Fig. 3, open symbols). Mutants S72A and K297A displayed phosphoenzyme turnover rates differing less than 2-fold from that of the wild type, whereas the phosphoenzyme turnover in mutants S72R and E90R was extremely slow (no dephosphorylation detected within the time frame of the experiment). Similar direct measurements of phosphoenzyme turnover could not be performed for E90A and E90L because of their low steady-state phosphoenzyme levels (cf. Fig. 2), but as noted above, reliable rates of phosphoenzyme processing could be extracted by computational analysis of the time course of phosphorylation from ATP measured by rapid quench instrumentation at 25 °C (Fig. 2), showing that the phosphoenzyme processing was $\sim$2-fold accelerated for E90A and E90L relative to wild type, in sharp contrast to the block of phosphoenzyme turnover observed for S72R and E90R.

The phosphoenzyme intermediates E$_1$P and E$_2$P can be distinguished experimentally by their differential sensitivities to FIGURE 3. Rate of the Ca$_2^+$E$_{1P}$ → Ca$_2^+$E$_{2P}$ conformational transition. Mixing protocols are illustrated by the diagrams above the panels: A, shown is processing of phosphoenzyme accumulated by phosphorylation from ATP. The enzyme contained in microsomal vesicles was phosphorylated by incubation for 15 s at 0 °C in 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl$_2$, 1 mM EGTA, 0.955 mM CaCl$_2$ (giving a free Ca$^{2+}$ concentration of 10 μM during phosphorylation), 2 μM calcium ionophore A23187, and 5 μM [$\gamma$-32P]ATP. Dephosphorylation was then studied at 0 °C by the addition of excess EGTA (to remove Ca$^{2+}$ and, thus, terminate phosphorylation) followed by acid quenching at the indicated times (open symbols). The closed symbols represent experiments in which 1 mM ADP was added together with the EGTA chase medium. The symbol code is the same as for the corresponding open symbols. The lines show the best fits of a monoexponential decay function $E_{P} = E_{P_{max}} e^{-kt}$ (extracted rate constants are listed in Table 1 for the wild type, S72A, and K297A). The maximum phosphorylation level obtained in each case was taken as 100%.

Glu$^{90}$ of SR Ca$^{2+}$-ATPase

FIGURE 2. Transient kinetics of phosphorylation of Ca$_2^+$E$_{1}$ with [$\gamma$-32P]ATP. Using a QFM-5 quench-flow module at 25 °C, phosphorylation was carried out by mixing the enzyme contained in microsomal vesicles suspended in 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl$_2$, and 100 μM CaCl$_2$ with an equal amount of the same medium containing [$\gamma$-32P]ATP to produce a final concentration of 5 μM followed by acid quenching at the indicated time intervals. The lines show fits to the data obtained by computation based on the reaction scheme shown in the bottom right corner using the SimZyme program (17), giving the rate constants for Ca$_2^+$E$_{1}$ → Ca$_2^+$E$_{1P}$ ($k_A$), Ca$_2^+$E$_{1P}$ → E$_{2}$ ($k_B$), and E$_{2}$ → Ca$_2^+$E$_{1}$ ($k_C$) indicated in each panel as ($k_A, k_B, k_C$). The maximum phosphorylation level obtained in each case was taken as 100%.
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ADP, E1P being able to transfer the phosphoryl group back to ADP forming ATP and E2P being insensitive to added ADP and dephosphorylating only in the forward direction by hydrolysis of the phosphoryl bond. The ADP sensitivity of the phosphoenzyme accumulated at steady state was determined by a 5-s chase with excess EGTA and 1 mM ADP (Fig. 3A, closed symbols). For the wild type, the ADP chase resulted in a complete disappearance of the phosphoryl bond. The ADP sensitivity of the phosphoenzyme accumulated (Fig. 3A, open symbols), reflects the rate of the Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P transition. In contrast, the phosphoenzyme accumulated for S72R and E90R was almost exclusively ADP-insensitive (Fig. 3A, closed triangles), showing that the block of phosphoenzyme turnover in these two mutants results from a block of a step in the reaction sequence subsequent to the Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P conformational transition, i.e. a step in the sequence Ca$$^{2+}$$E2P → E2P → E2. For these two mutants it was feasible to determine the rate of Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P by following the formation of ADP-insensitive phosphoenzyme during incubation with [$\gamma$-$$^{32}$$P]ATP (Fig. 3B), assuming that Ca$$^{2+}$$E1P → Ca$$^{2+}$$E1P is as rapid as in the wild type (documented in Fig. 2, $k_s$) and, therefore, not rate-limiting for the reaction sequence Ca$$^{2+}$$E2P → E2P → E2. Hence, the data in Fig. 3B were obtained by incubating Ca$$^{2+}$$-saturated enzyme with [$\gamma$-$$^{32}$$P]ATP for varying time intervals under conditions similar to those used in Fig. 3A followed by determination of the amount of ADP-insensitive phosphoenzyme accumulated (i.e. the sum of Ca$$^{2+}$$E2P and E2P) by adding 1 mM ADP for 4 s to remove any Ca$$^{2+}$$E1P present before acid quenching. The rate of the Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P transition observed for S72R and E90R in this way was close to 10-fold faster than the rate of Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P obtained with the wild type as illustrated in Fig. 3A under the same buffer and temperature conditions. A summary of the relative Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P rate constants derived from the data of Figs. 2 and 3 is presented in Table 1.

**The Stability of E2P and Ca$$^{2+}$$E2P—**All six mutants were able to form E2P phosphoenzyme by phosphorylation of Ca$$^{2+}$$-deprived enzyme from P$_i$ in the backward direction of normal turnover and displayed apparent affinities for P$_i$ at least as high as that of the wild type (supplemental Fig. S1). To measure the rate of E2P dephosphorylation (i.e. the forward reaction E2P → E2), E2P was formed in the presence of a saturating concentration of $^{32}$P$_i$ and subsequently chased by dilution into a buffer containing excess nonradioactive P$_i$, followed by acid quenching at serial times (Fig. 4). Mutants S72A and K297A displayed dephosphorylation rates 1.4- and 3.4-fold lower than that of the wild type, respectively, whereas the dephosphorylation rates of mutants E90A and E90L were enhanced (2.8- and 1.8-fold, respectively) relative to wild type. Importantly, the dephosphorylation of E2P was completely blocked in mutants S72R and E90R, with no phosphoenzyme decay detected within the time frame of the experiment (Fig. 4 and Table 1).

Such a block of dephosphorylation of E2P has previously been noted for mutations of some of the residues involved in Ca$$^{2+}$$ binding from the cytoplasmic side in the E1 conformation, which in E2P are candidates for luminal interaction sites for Ca$$^{2+}$$ or protons to be countertransported such as Glu$^{309}$ (9, 20, 21). For direct comparison with S72R and E90R, we also conducted a dephosphorylation experiment with mutant E309Q under the presently applied conditions (Fig. 4). In accordance with the previous result (20, 21), the dephosphorylation of E2P was also markedly slowed in E309Q, although not quite as much as seen for S72R and E90R (Fig. 4 and Table 1).

For S72R and E90R, the results summarized in Table 1 demonstrate that the first and the last reaction step in the sequence Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P → E2P → E2 is enhanced and inhibited, respectively. It is, however, unclear from these data how the intermediate reaction step Ca$$^{2+}$$E2P → E2P, i.e. the dissociation of Ca$$^{2+}$$ from the luminally exposed Ca$$^{2+}$$ sites, is affected by the mutations. To answer the question of whether the inserted arginine residue inhibits the luminal Ca$$^{2+}$$ dissociation such that Ca$$^{2+}$$E2P → E2P contributes to rate limitation of phosphoenzyme turnover, we examined whether Ca$$^{2+}$$ remained tightly bound ("occluded") in the accumulated phosphoenzyme (Fig. 5). A mutant previously studied by Daiho et al. (22), with four glycines inserted in the A/M1 linker between Gly$^{46}$ and Lys$^{37}$ (4G46/46/47), was included as a positive control in which the luminal Ca$$^{2+}$$ dissociation step Ca$$^{2+}$$E2P → E2P is blocked. For reference we first demonstrated the ability of the mutants

| TABLE 1 Summary of relative rate constants of the phosphoenzyme processing steps |
|---------------------------------|---------------------------------|---------------------------------|
| Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P | Ca$$^{2+}$$E2P → E2P             |
| Wild type                       | 1.0 ± 0.05 (n = 40, 52)          | 1.0 ± 0.04 (n = 89)             |
| S72A                           | 0.74 ± 0.04 (n = 16)            | 0.71 ± 0.06 (n = 18)           |
| S72R                           | 11 ± 0.6 (n = 17)               | -0 (n = 25)                    |
| E90A                           | 2.2 (n = 54)                    | 2.8 ± 0.16 (n = 18)            |
| E90L                           | 1.7 (n = 54)                    | 1.8 ± 0.09 (n = 18)            |
| E90R                           | 7.8 ± 0.6 (n = 17)              | -0 (n = 26)                    |
| K297A                          | 1.6 ± 0.03 (n = 16)             | 0.29 ± 0.01 (n = 18)           |
| E309Q                          | ND (n = 54)                     | 0.065 ± 0.001 (n = 17)         |

*Rate constant of dephosphorylation of phosphorylation with P$_i$ (experimental details are in the legend to Fig. 4) relative to that obtained with wild type (k = 0.11 s$^{-1}$).

*Rate constant of dephosphorylation of Ca$$^{2+}$$E1P formed by phosphorylation of Ca$$^{2+}$$E1P with ATP (at 0 °C; experimental details are in the legend to Fig. 3A) relative to that obtained with wild type (k = 0.10 s$^{-1}$).

*For mutants E90A and E90L, the rate constant of Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P was extracted by analysis of the time course of phosphorylation by ATP (at 25 °C, experimental details in the legend to Fig. 2) using the SimZyme simulation software (see "Experimental Procedures" and Ref. 17). Because the dephosphorylation of E2P in mutants E90A and E90L is at least as fast as that of wild type (cf. Fig. 4 and the right column of this table), it can be assumed that Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P is rate limiting for the phosphoenzyme turnover. The rate constants listed are relative to that obtained with the wild type (k = 6 s$^{-1}$). Because SimZyme does not provide a regression analysis, no S.E. is shown in this case.

*For mutants S72R and E90R the rate constant of Ca$$^{2+}$$E1P → Ca$$^{2+}$$E2P was determined by measuring the rate of loss of ADP sensitivity of the phosphoenzyme formed by phosphorylation with ATP (at 0 °C; experimental details are in the legend to Fig. 3B). The rate constants listed in the table are relative to that obtained with wild type in the experiments corresponding to Fig. 3A (directly comparable to those of Fig. 3B because the conditions during phosphoenzyme processing are identical in the two experimental setups).

*ND, not determined, because E309Q does not undergo phosphorylation under the present conditions for reaction with ATP (21).
occlude Ca$^{2+}$ in the stable Ca$_2$E1P-like Ca$_2$E1:AlF$_3$:ADP complex. The enzyme was incubated with 45Ca$^{2+}$ in the presence of AlF$_3$ and ADP followed by a chase with excess EGTA, removing free Ca$^{2+}$ from the medium (on both sides of the membrane due to the presence of calcium ionophore). At varying time intervals after the chase, the samples were rapidly filtered and washed to determine the amount of 45Ca$^{2+}$ associated with the Ca$_2$E1P:ATPase. As seen in Fig. 5, open symbols, S72R, E90R, and 4Gi-46/47 were all able to form a stable Ca$_2$E1P:AlF$_3$:ADP complex similar to the wild type (23, 24). Then in another set of experiments without AlF$_3$ and ADP, the enzyme was phosphorylated by ATP in the presence of 45Ca$^{2+}$ followed by the same EGTA chase and filtration procedure as described above (closed symbols in Fig. 5). In parallel experiments using [$\gamma$-32P]ATP, the phosphoenzyme decay was followed under comparable conditions at 25 °C (supplemental Fig. S2). In the wild type, the phosphoenzyme decayed too rapidly for any Ca$^{2+}$ occlusion in the phosphoenzyme to be detected by the manual filtration technique used here. However, in mutants 4Gi-46/47 and E90R, the phosphoenzyme was very stable even at 25 °C (supplemental Fig. S2), and reliable measurements of Ca$^{2+}$ occlusion in the phosphoenzyme could easily be performed. For 4Gi-46/47, a high level of 45Ca$^{2+}$ occlusion similar to that obtained in the presence of ADP-AlF$_3$ was seen after the phosphorylation by ATP, in accordance with the previously described block of luminal Ca$^{2+}$ dissociation from the Ca$_2$E2P state in this mutant (22). Notably, the E2P phosphoenzyme of E90R was not in the 45Ca$^{2+}$-occluded state, unlike that of 4Gi-46/47 (Fig. 5), thus indicating that in E90R the luminal Ca$^{2+}$ dissociation does not contribute to the rate limitation in the Ca$_2$E2P → E2P → E2 reaction sequence. Likewise, no 45Ca$^{2+}$ occlusion could be detected in S72R during the phosphorylation experiment, but in the latter case the conclusion is uncertain, because much of the phosphoenzyme may have disappeared during the measurement due to the higher dephosphorylation rate of S72R (compare Fig. 5 with supplemental Fig. S2).

**Function of the Luminally Exposed Low Affinity Ca$^{2+}$ Sites—**

To examine the accessibility and affinity of the luminally exposed Ca$^{2+}$ sites in E2P, we applied a recently devised assay (25) that takes advantage of the well known Ca$^{2+}$ gradient-dependent formation of E2P from P$_i$ (26, 27). Microsomal vesicles containing wild type or mutant Ca$^{2+}$:ATPase were loaded passively with various Ca$^{2+}$ concentrations by overnight incubation and then diluted into a medium containing $^{32}$P$_i$ and EGTA. The latter chelator was present to remove Ca$^{2+}$ in the medium outside the vesicles, thereby preventing Ca$^{2+}$ binding from the
markedly reduced apparent Ca\textsuperscript{2+} affinities (K\textsubscript{0.5} increased ~3- and ~5-fold, respectively). Mutant 4Gi-46/47 displayed a 9-fold increased apparent Ca\textsuperscript{2+} affinity (K\textsubscript{0.5} reduced) relative to wild type (Fig. 6 and Table 2), which likely is associated with the high stability of the Ca\textsubscript{2P} state in this mutant (22). Remarkably, S72R and E90R were maximally phosphorylated even at 0.01 mM luminal Ca\textsuperscript{2+}, where almost no phosphoenzyme is obtained with wild type, and remained maximally phosphorylated throughout the range of luminal Ca\textsuperscript{2+} concentrations applied in the experiment (Fig. 6). As further seen in Fig. 6, E309Q likewise showed a rather constant phosphorylation level independent of the luminal Ca\textsuperscript{2+} concentration. The phosphorylation level of E309Q was lower than that of S72R and E90R, consistent with the slightly higher rate of dephosphorylation of E2P in E309Q as compared with S72R and E90R (Fig. 4).

The phosphoenzyme that accumulates for the wild type upon phosphorylation of Ca\textsuperscript{2+}-loaded vesicles with P\textsubscript{i} is ADP-sensitive Ca\textsubscript{2P,E1P}, due to the equilibrium E2P + 2Ca\textsubscript{2+}\textsubscript{lum} ⇌ Ca\textsubscript{2P,E2P} ⇌ Ca\textsubscript{2P,E1P} (25). In contrast, as demonstrated by the experiment shown in the bottom panel of Fig. 6, the phosphoenzyme accumulated for mutants S72R, E90R, and 4Gi-46/47 remained ADP-insensitive even when the vesicles had been loaded with high amounts of Ca\textsuperscript{2+}, implying either a block of Ca\textsuperscript{2+} binding at the luminal Ca\textsuperscript{2+} sites and/or of the Ca\textsubscript{2P,E2P} → Ca\textsubscript{2P,E1P} transition.

The Stability and Ca\textsuperscript{2+} Sensitivity of E2P-like Analog States—We furthermore studied the properties of E2 in complex with the two inhibitory phosphoryl analogs BeF\textsubscript{3} and vanadate. E2-BeF\textsubscript{3} is believed to mimic the E2P ground state (7, 28), whereas vanadate generally thought to capture the ATPase in a state similar to the transition state of E2P dephosphorylation (29). Both inhibitors bind to the Ca\textsuperscript{2+}-deprived E2 state in a slow reaction that requires Mg\textsuperscript{2+}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.pdf}
\caption{Function of the luminal Ca\textsuperscript{2+} sites of the E2P state. Microsomal vesicles containing wild type or mutant Ca\textsuperscript{2+}-ATPase were loaded with Ca\textsuperscript{2+} in the lumen by incubation overnight (~18 h) on ice in 150 mM MOPS/Tris (pH 7.0), 125 mM sucrose, 75 mM KCl, and the indicated CaCl\textsubscript{2} concentration ([Ca\textsuperscript{2+}]\textsubscript{lum}). The phosphorylation by P\textsubscript{i} was determined at 25 °C by a 50-fold dilution of the Ca\textsuperscript{2+}-loaded vesicles into a phosphorylation medium containing 150 mM MOPS/Tris (pH 7.0), 125 mM sucrose, 77 mM KCl, 10 mM EGTA (removing Ca\textsuperscript{2+} from the medium outside the vesicles), and 0.5 mM \textsuperscript{32}P-generating 1 min later. The ordinate shows the results as percentage of the maximal phosphorylation level (max EP) obtained by phosphorylation with P\textsubscript{i} in the absence of a Ca\textsuperscript{2+} gradient, and EP\textsubscript{min} representing the extrapolated phosphorylation level at infinite intravesicular Ca\textsuperscript{2+} (K\textsubscript{0.5} (Ca\textsuperscript{2+})). The K\textsubscript{0.5} values obtained are listed in Table 2. The bottom panel shows \textsuperscript{32}P-phosphorylated Ca\textsuperscript{2+}-ATPase bands on SDS-polyacrylamide gel separation gels in experiments assessing the ADP sensitivity of the phosphoenzyme accumulated with wild type and mutants S72R, E90R, and 4Gi-46/47 either under conditions of maximal EP formation in the presence of dimethyl sulfoxide (max EP) or after phosphorylation of the Ca\textsuperscript{2+}-loaded vesicles in the medium without dimethyl sulfoxide as described above. The ADP sensitivity was studied by a 2-s incubation with 1 mM ADP at 25 °C before the acid quenching (ADP +). For comparison, the phosphorylation of samples that have not been incubated with ADP is also shown (ADP –). The samples indicated by bg represent background phosphorylation obtained in the presence of excess Ca\textsuperscript{2+} (absence of EGTA) in the phosphorylation medium.
\end{figure}
TABLE 2
Summary of apparent affinities and rate constants relating to Ca2+-
interaction

|                     | $K_{o.5}$ (Ca2+-ATP) | Rate constant of Ca2+-
|                     |                     | binding transition $E_2 \rightarrow Ca_2E_1$ |
|                     |                     | Rate constant of Ca2+-
|                     |                     | dissociation from Ca_2E_1$^d$ |
| Wild type           | 1.0 ± 0.04 ($n = 62$) | 1.0 ± 0.05 ($n = 62$) |
| S72A                | 0.9 ± 0.11 ($n = 16$) | 1.2 ± 0.08 ($n = 32$) |
| S72R                | 2.6 ± 0.46 ($n = 24$) | 0.055 ± 0.011 ($n = 32$) |
| E90A                | 0.38 ± 0.05 ($n = 27$) | 0.060 ± 0.007 ($n = 9$) |
| E90L                | 5.9 ± 0.43 ($n = 18$) | 0.18 ± 0.014 ($n = 34$) |
| E90R                | 3.9 ± 0.28 ($n = 18$) | 2.4 ± 0.17 ($n = 35$) |
| K297A               | 1.8 ± 0.07 ($n = 18$) | 0.35 ± 0.014 ($n = 18$) |
| 4Gi-46/47           | 0.11 ± 0.01 ($n = 32$) | ND$^d$ |
| E309Q               | 0.16 ($n = 16$) | ND$^d$ |

*a $K_{o.5}$ values for Ca2+ activation from the luminal side of the membrane of phosphorylation from $P_i$ relative to that obtained with wild type (7.5 mM). For experimental details, see the legend to Fig. 6.

*b $K_{o.5}$ values for Ca2+ activation from the cytoplasmic side of the membrane of phosphorylation from ATP, relative to that obtained with wild type (0.9 mM). For experimental details, see the legend to Fig. 2.

c Rate constants relative to that obtained with wild type (0.9 s$^{-1}$). For experimental details, see the legend to Fig. 9, left panels.

d Rate constants relative to that obtained with wild type (2.7 s$^{-1}$). For experimental details, see the legend to Fig. 9, right panels.

Functional of the Cytoplasmically Facing High Affinity Ca2+-
Sites—Phosphorylation of the Ca2+-ATPase by ATP depends on the binding of Ca2+ at the high affinity cytoplasmically exposed sites of the E1 form. Fig. 8 shows the Ca2+ concentration dependence of the steady-state level of phosphorylation from ATP. Mutants S72A, S72R, and K297A deviated less than 2-fold from the wild type with respect to the $K_{o.5}$ of Ca2+ activation. The apparent Ca2+ affinities of E90A, E90L, and E90R were more markedly affected, being 6-, 8-, and 4-fold reduced, respectively, relative to wild type ($K_{o.5}$ increased, see Table 2).

The left panels of Fig. 9 show the kinetics of the Ca2+ binding transition measured by use of rapid kinetic instrumentation at 25 °C. This transition comprises Ca2+ binding to the dephosphoenzyme with accompanying enzyme conformational changes, i.e. reactions $E_2 \rightarrow E_1 \rightarrow CaE_1 \rightarrow Ca_2E_1$ in Scheme 1. The assay takes advantage of the fact that only the Ca2E1 state is able to be phosphorylated by ATP (31). Ca2+-deprived enzyme is incubated with Ca2+ for varying time intervals (t in the mixing protocol at the top left of Fig. 9), and the amount of phosphorylatable CaE1 is determined for each time interval by a 34-ms incubation with [gamma-32P]ATP followed by acid quenching (32). The mutations S72R and E90R reduced the rate of the Ca2+ binding transition 2-fold, whereas mutation K297A led to a 2-fold enhanced rate. Mutation S72A displayed a wild type-like rate of Ca2+ binding. Importantly, the rate of the Ca2+ binding transition was extremely slow in mutants E90A and E90L, being reduced by more than 2 orders of magnitude relative to wild type. This is in keeping with the large phosphorylation overshoots seen for E90A and E90L in Fig. 2 as a result of the accumulation of dephosphoenzyme at steady state and with the resulting small values for the derived rate constants $k_{p}$. It should be emphasized that the rate constant of the Ca2+ binding transition extracted from the data in Fig. 9 is independent of the rate of phosphorylation of Ca2E1 (32). The data in Fig. 2 indicate that the phosphorylation of Ca2E1, unlike the Ca2+ binding transition, proceeds with roughly the same rate constant ($k_{a}$ ~50 s$^{-1}$) in mutants and wild type. To examine whether this is the case for E90A and E90L also under the conditions (pH 6.0) applied for Fig. 9, we repeated for these mutants the phosphorylation of Ca2E1 corresponding to Fig. 2 but this time at pH 6.0. The result was again that $k_{a}$ ~ 50 s$^{-1}$ for mutants as well as wild type (supplemental Fig. S3).

The dissociation of Ca2+ from the high affinity sites of the Ca2E1 state back toward the cytosol was also examined by the rapid-quench technique (Fig. 9, right panels). Again, the assay uses advantage of the dependence of the reaction with ATP on Ca2+ occupancy of the Ca2+ sites, as illustrated by the mixing protocol in the top right of Fig. 9. Thus, when an excess of EGTA is added to Ca2+-saturated enzyme, the ability to become phosphorylated by ATP will disappear at a rate corresponding to the rate of Ca2+ dissociation from the enzyme. After incubation with EGTA for varying time intervals, the amount of enzyme still in the phosphorylatable Ca2E1 state is determined by a 34-ms incubation with [gamma-32P]ATP before acid quenching. Moderate deviations from wild type of the rate of Ca2+ dissociation from the high affinity Ca2+ sites of the Ca2E1 state were seen with mutants S72R, S72A, and E90R (1.5- and 2-fold reduced rates and 2.4-fold enhanced rate, respectively, Table 2). K297A displayed an ~3-fold slowing of Ca2+ dissociation. Again the mutations E90A and E90L proved most detrimental, reducing the rates of Ca2+ dissociation from Ca2E1 24- and 6-fold, respectively, relative to wild type. Thus, mutations E90A and E90L markedly inhibit the Ca2+ binding transition as well as the dissociation of Ca2+ from the high affinity cytoplasmically exposed Ca2+ sites. Ca2+ dissociation is also slowed by mutation K297A, but unlike E90A and E90L, K297A enhances the Ca2+ binding transition slightly. These data on Ca2+ interaction are summarized in Table 2. Caution is generally required in the interpretation of the $K_{o.5}$ values determined at steady state (Fig. 8) because they depend not only on the rate constants of Ca2+ binding and Ca2+ dissociation but also on the rate constants of all the other partial reaction steps in the Ca2+ transport cycle, as previously discussed in detail.
Our functional analysis of mutants with alterations to Glu90 implicates this residue as a critical element at the luminal ion gate for extrusion of the Ca2+ ions from the E2P state to the endoplasmic reticulum lumen. It is furthermore of note that some of the mutations to Glu90 also had a pronounced impact on the function of the cytoplasmically exposed high affinity Ca2+ sites of the E1 state, i.e. events that take place at the opposite side of the membrane relative to where Glu90 is located.

**Glu90 and Glu309 Provide Ligands for Luminal Ca2+ Binding**—The propagation of the movements of the A domain to the transmembrane region during the Ca2E1 → Ca2E1P → Ca2E2P → E2P transitions leads to a considerable displacement of the transmembrane hairpins M1/M2 and M3/M4 relative to each other (cf. the arrows in Fig. 1). Consequently, in the presumed structural analog of the Ca2+-free E2P ground state, the E2-BeF3 crystal structure (7), Ser72 and Glu90 are positioned near Glu309, which is known as an essential Ca2+ binding residue in Ca2E1 and Ca2E1P and probably plays a central role as well in the countertransport of protons (9, 20, 21). An issue of great importance for the understanding of the transport mechanism is where in the enzyme Ca2+ is bound before it leaves from E2P in exchange for the protons to be countertransported. In E2-BeF3 the luminally protruding end of the transmembrane domain is in an open configuration compared with other crystal structures of the Ca2+-ATPase, with Glu309 exposed to the lumen and associated with a Mg2+ ion (7). The hypothesis that this Mg2+ is bound in place of Ca2+ at a luminally facing low affinity Ca2+ site is consistent with the observation that under certain condi-

**Discussion**

Here we have investigated the interaction network involving Ser72, Glu90, and Lys297 at the luminal protruding ends of the transmembrane helices M1, M2, and M4 of the Ca2+-ATPase.
FIGURE 9. Kinetics of binding and dissociation of Ca²⁺ at cytoplasmically facing sites. Quench-flow experiments were carried out with mixing protocols as illustrated by the diagrams above the panels using a QFM-5 module at 25 °C. Left panels, the Ca²⁺ binding transition taking place in the presence of 40 mM MES/ Tris (pH 6.0), 80 mM KCl, and 2 mM EGTA (to accumulate E₂), was mixed with an equal volume of 40 mM MES/Tris (pH 6.0), 80 mM KCl, and 1.2 mM CaCl₂. At the indicated time intervals, the amount of phosphorylatable Ca₂⁺E₁ was determined by adding the double volume of 40 mM MES/Tris (pH 6.0), 80 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 μM [γ-³²P]ATP, and 1.1 mM CaCl₂, followed by acid quenching 34 ms later. To obtain the point corresponding to zero time, the enzyme was preincubated in 40 mM MES/Tris (pH 6.0), 80 mM KCl, and 2 mM EGTA and mixed with an equal volume of 40 mM MES/Tris (pH 6.0), 80 mM KCl, 10 mM MgCl₂, 10 μM [γ-³²P]ATP, and 2.2 mM CaCl₂ followed by acid quenching 34 ms later. To obtain the point corresponding to zero time, the enzyme was preincubated in 40 mM MES/Tris (pH 6.0), 80 mM KCl, and 100 μM CaCl₂ (to accumulate E₁), was mixed with an equal volume of 40 mM MES/Tris (pH 6.0), 80 mM KCl, 5 mM MgCl₂, and 4 mM EGTA to initiate Ca²⁺ dissociation. At the indicated time intervals, the amount of phosphorylatable Ca₂⁺E₁ remaining was determined by adding the double volume of 40 mM MES/Tris (pH 6.0), 80 mM KCl, 5 mM MgCl₂, 2 mM EGTA, and 10 μM [γ-³²P]ATP followed by acid quenching 34 ms later. To obtain the point corresponding to zero time, 4 mM EGTA was replaced by 100 μM CaCl₂. The lines show the best fits of a monoeponential rise to maximum function \( EP = EP_{\text{max}} + (EP_{\text{max}} - EP_{\text{min}})(1 - e^{-kt}) \), giving the rate constants of E₂ → Ca₂⁺E₁ listed in Table 2. In each case the EPmax extracted from the fit was taken as 100%. Right panels, Ca²⁺ dissociation from the high affinity Ca²⁺ sites of Ca₂⁺E₁ back toward the cytoplasm is shown. The enzyme, preincubated in 40 mM MES/Tris (pH 6.0), 80 mM KCl, 10 mM MgCl₂, 10 μM [γ-³²P]ATP, and 2.2 mM CaCl₂, followed by acid quenching 34 ms later. The lines represent the best fits of a monoeponential rise to maximum function with an initial offset, \( EP = EP_{\text{max}} + (EP_{\text{max}} - EP_{\text{min}})(1 - e^{-kt}) \), giving the rate constants of E₁ → Ca₂⁺E₃ listed in Table 2. In each case the EPmax extracted from the fit was taken as 100%.

The close proximity of the side chains of Glu⁹⁰ and Ser⁷² suggests a more peripheral position of Ser⁷² relative to the luminal Ca²⁺ site in accordance with the 5.9 Å distance to the Mg²⁺ ion in the E₂-BeF₃ crystal structure. When, however, Ser⁷² or Glu⁹⁰ was replaced by an arginine, possessing a longer and positively charged side chain, high amounts of phosphoenzyme were obtained even at very low luminal Ca²⁺ concentrations, i.e. conditions where little or no phosphoenzyme was formed in the wild type or any of the other mutants examined here, except E309Q (Fig. 6). Like S72R and E90R, E309Q also displayed considerable activation of phosphor-
phorylation of E2P during the normal Ca\(^{2+}\) transport cycle does not take place until the translocated Ca\(^{2+}\) has dissociated from the luminally exposed site(s).

It should be noted that E90A and E90L differ from E90R by not displaying a reduced rate of dephosphorylation of E2P (Fig. 4). The apparent affinity for luminal Ca\(^{2+}\) was nevertheless found markedly reduced in these mutants relative to wild type (Fig. 6), thus indicating that the reduced luminal Ca\(^{2+}\) affinity is not an indirect consequence of E2P stabilization. This supports the hypothesis that Glu\(^{90}\) is directly involved in luminal Ca\(^{2+}\) binding. Furthermore, because E2P is stabilized in E309Q, as in E90R, it is likely that neutralization of the negative charge of Glu\(^{90}\), similar to what is obtained by luminal Ca\(^{2+}\) binding, is the reason for the E2P stabilization in E90R. This fits well with the hypothesis that in the normal Ca\(_{E2P}\) state Glu\(^{90}\) and Glu\(^{309}\) bind a Ca\(^{2+}\)-ion that through this interaction prevents dephosphorylation, probably through substantial rearrangements of the transmembrane domain, resulting in long-range effects of the altered positions of the transmembrane helices on the insertion of the TGES phosphate motif of the A domain into the catalytic site (5-7).

When phosphorylation was carried out with ATP in the forward-running mode of the Ca\(^{2+}\)-ATPase cycle, the E2P phosphoenzyme accumulated for E90R (Fig. 3A) was Ca\(^{2+}\)-free (Fig. 5), which excludes the possibility that the stabilization of E2P seen under these conditions is due to hindrance of luminal Ca\(^{2+}\) dissociation by the arginine side chain. This contrasts with the 4Gi-46/47 mutant that displayed a very stable E2P state with occluded Ca\(^{2+}\) due to impaired Ca\(^{2+}\) dissociation (Fig. 5 and Ref. 22). Like 4Gi-46/47, the S72R and E90R mutants were unable to form ADP-sensitive Ca\(_{E1P}\) backward from the P\(_{i}\)-phosphorylated enzyme (bottom panel of Fig. 6). In S72R and E90R the reason may be the arginine guanidinium group occupying a Ca\(^{2+}\)-site of E2P without being able to fulfill a similar role in E1P, because of the large distance between Ser\(^{72}\)/Glu\(^{90}\) and Glu\(^{309}\) in this conformation (cf. Fig. 1). The hypothesis that E2P is stabilized by insertion of the arginine guanidinium group into a luminally exposed Ca\(_{E2P}\) site is also consistent with the highly accelerated loss of ADP sensitivity of the phosphoenzyme of E90R and S72R (Fig. 3B and Table 1), because the measured rate of Ca\(_{E1P}\) → Ca\(_{E2P}\) is a net rate that depends both on the forward rate as well as the rate of the reverse reaction, which according to our hypothesis could be greatly reduced as a consequence of the guanidinium group interfering with luminal Ca\(^{2+}\) binding in E2P, thereby enforcing Ca\(^{2+}\) dissociation and depriving Ca\(_{E2P}\). E90A and E90L likewise showed some acceleration of Ca\(_{E1P}\) → Ca\(_{E2P}\), although not to the extent of S72R and E90R (Table 1), which may again be explained by a reduced rate of the reverse reaction due to enhanced Ca\(^{2+}\) dissociation, as predicted from the reduced affinity of these mutants for luminal Ca\(^{2+}\) (Fig. 6 and Table 2).

The interaction of the arginine side chain with the luminally exposed Ca\(_{E2P}\) site in the E2P state of S72R and E90R is further reflected by the results shown in Fig. 7 in which the Ca\(^{2+}\) sensitivity of the E2P analog states E2-BeF\(_{3}\) and E2-vanadate were examined. Ca\(^{2+}\)-induced dissociation of E2-BeF\(_{3}\) has been shown to result from Ca\(^{2+}\) binding at a luminally exposed Ca\(^{2+}\)-site (28), in accordance with the idea that E2-BeF\(_{3}\) represents an E2P ground state-like conformation. The dissociation is most likely a three-step process in which Ca\(^{2+}\) first binds at the luminally exposed Ca\(^{2+}\)-sites, forming Ca\(_{2}\)E2-BeF\(_{3}\) (Ca\(_{2}\)E2P analog) followed by a slow conformational transition to Ca\(_{2}\)E1-BeF\(_{3}\) (Ca\(_{2}\)E1P analog) and subsequent BeF\(_{3}\) release from Ca\(_{2}\)E1-BeF\(_{3}\) (36). E2-vanadate is generally believed to represent an E2P transition state-like conformation (29), and its dissociation is triggered by Ca\(^{2+}\) binding at the high affinity Ca\(^{2+}\)-sites on the cytoplasmic side of the membrane allowed by the equilibrium of E2 with E1 (30). Accordingly, the Ca\(^{2+}\)-induced dissociation of E2-BeF\(_{3}\) was very slow in mutant 4Gi-46/47 (Fig. 7C), reflecting the high stability of the occluded Ca\(_{2}\)E2P state in this mutant (22), whereas the Ca\(^{2+}\)-induced dissociation of the E2-vanadate complex was rapid (Fig. 7D), reflecting the more...
wild type-like characteristics of the E2P transition state of 4Gi-46/47, allowing formation of E2 from E2-vanadate at a normal rate and subsequent transition to E1 (22). In contrast, the dissociation of E2-BeF$_3^-$ as well as that of E2-vanadate were very slow in both S72R and E90R (Fig. 7, C and D), thus reflecting the stabilization of a Ca$^{2+}$-free E2P-like state with the arginine side chain located in the luminal Ca$^{2+}$ site.

**Importance of Glu$^{90}$ for Cytoplasmic Ca$^{2+}$ Binding**—The results of Fig. 9 and Table 2 show that Glu$^{90}$ is an important player not only at the luminal ion gate but is required for normal function of the cytoplasmic Ca$^{2+}$ sites as well.

The rate of Ca$^{2+}$ dissociation back toward the cytosol from Ca$_2$E1 was markedly slowed by mutations E90A and E90L. Because Glu$^{90}$ is located at the luminal side of the membrane and, in the Ca$_2$E1 state, some 15–20 Å below the Ca$^{2+}$ ions (Fig. 1 and supplemental Table S1), any direct interaction with the cytoplasmic Ca$^{2+}$ sites seems to be excluded. In all the Ca$_2$E1 crystal structures (with or without bound nucleotide or phosphorlyl analogs), the side-chain carboxylate of Glu$^{90}$ is closely associated with the side-chain amino group of Lys$^{297}$ (Fig. 1 and supplemental Table S1). This led us to speculate that a Glu$^{90}$-Lys$^{297}$ ion bond might be involved in control of cytoplasmic Ca$^{2+}$ binding, and we, therefore, included mutation K297A in the study. The reduced rate of Ca$^{2+}$ dissociation seen for K297A supports the hypothesis that the presence of an ion bond between Glu$^{90}$ and Lys$^{297}$ influences the dissociation of Ca$^{2+}$ from Ca$_2$E1. The finding that E90R displayed a 2-fold enhanced rate of Ca$^{2+}$ dissociation indicates that a surplus of positive charge in this area will destabilize the Ca$_2$E1 state, thus pointing to a possible role for Glu$^{90}$ in neutralizing the positive charge of Lys$^{297}$ in Ca$_2$E1. This influence on the cytoplasmically exposed Ca$^{2+}$ sites of E1 must be exerted by long-range effects involving repositioning of the transmembrane helices.

The Ca$^{2+}$ binding properties of E90A and E90L were further profoundly affected by a more than 2 orders of magnitude-slowing of the Ca$^{2+}$ binding transition E2 → Ca$_2$E1. Because K297A showed a 2-fold-enhanced rate of E2 → Ca$_2$E1 (Table 2), the slow rates of E2 → Ca$_2$E1 in E90A and E90L seem to be unrelated to the disruption of the ion bond between Glu$^{90}$ and Lys$^{297}$ in Ca$_2$E1. Moreover, E90R displayed only a 2-fold reduction of E2 → Ca$_2$E1 relative to the wild type, implying that the very slow rate of this transition in E90A and E90L is related to the hydrophobic nature of the side chain in these mutants. The various structures of the Ca$^{2+}$-ATPase crystallized in E2 state (37–41) provide a possible explanation. Thus, common to these structures is that the Glu$^{90}$ side chain is located inside a hydrophobic pocket consisting of Val$^{300}$ of M4 and Ile$^{798}$, Pro$^{798}$, and Val$^{798}$ of M6 (Fig. 11). It is, therefore, quite conceivable that substitution of Glu$^{90}$ with a hydrophobic residue such as alanine or leucine would strengthen the interaction between M2 and M4/M6, hindering the displacement of M1/M2 relative to M3/M4 during the E2 → Ca$_2$E1 transition, thus effectively locking the enzyme in the E2 conformation.

**Importance of Lys$^{297}$**—In a previous mutational study predating the high resolution crystal structures of the Ca$^{2+}$-ATPase, Chen et al. (19) carried out functional analysis of Ca$^{2+}$ pumps with other mutations to Lys$^{297}$ than the K297A mutation studied here and found that mutants K297M and K297F displayed slow E2P dephosphorylation, leading to the suggestion that Lys$^{297}$ seals the luminal gate of the Ca$^{2+}$ transport pathway (19). In the present study mutant K297A displayed a 3.4-fold reduction of the rate of E2P dephosphorylation (Fig. 4 and Table 1), resembling the effects observed by Chen et al. (19) with K297M and K297F. However, the apparent affinity of the E2 state of K297A for Ca$^{2+}$ binding at the luminal sites was indistinguishable from that of the wild type (Fig. 6 and Table 2), making it unlikely that Lys$^{297}$ is associated with the luminal ion gate of E2P. Rather, the effect of mutations to Lys$^{297}$ on E2P stability could be due to critical ion bonding between Lys$^{297}$ and the residue(s) in the loop connecting M1 and M2, one likely interaction partner candidate being Glu$^{90}$ (Fig. 1).

**Conclusion and Perspective**—In conjunction, all the data presented here seem to point to Glu$^{90}$ as a residue that participates together with Glu$^{309}$ in Ca$^{2+}$ binding at a luminally exposed site in the E2P state. This finding, together with previous functional (9) and structural (3, 7) evidence for an alternating exposure of Glu$^{309}$ at the two sides of the membrane during the pump cycle is consistent with a mechanism in which Glu$^{309}$ carries one of the two Ca$^{2+}$ ions along from the cytoplasmic side to a leaving site, where Ca$^{2+}$ is received by Glu$^{90}$ before the final exit to the lumen. Because the present data do not allow a distinction between the two Ca$^{2+}$ ions, it still remains to be clarified how the other Ca$^{2+}$ ion is translocated; that is, whether it also uses Glu$^{90}$ or takes a different exit pathway from the binding pocket?

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