Fast Kinetic Analysis of Conformational Changes in Mutants of the Ca\(^{2+}\)-ATPase of Sarcoplasmic Reticulum*

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Thomas Lykke-Møller Sørensen‡, Yves Dupont§, Bente Vilsen‡, and Jens Peter Andersen‡

From the ‡Department of Physiology, University of Aarhus, DK-8000 Aarhus C, Denmark and the §Laboratoire de Biophysique Moleculaire et Cellulaire, Centre d’Etudes Nucleaires de Grenoble, 38054 Grenoble, France

Rapid quench experiments at 25 °C were carried out on selected mutants of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase to assess the kinetics of the conformational changes of the dephosphoenzyme associated with ATP binding/phosphoryl transfer and the binding and dissociation of Ca\(^{2+}\) at the cytoplasmically facing transport sites. The mutants Glu\(^{233}\) → Glu, Gly\(^{233}\) → Val, Pro\(^{312}\) → Ala, Leu\(^{319}\) → Arg, and Lys\(^{604}\) → Arg differed conspicuously with respect to the behavior of the dephosphoenzyme, although they were previously shown to display a common block of the transformation of the phosphoenzyme from an ADP-sensitive to an ADP-insensitive form. The maximum rate of the ATP binding/phosphoryl transfer reaction was reduced 3.6-fold in mutant Gly\(^{233}\) → Glu and more than 50-fold in mutant Lys\(^{604}\) → Arg, relative to wild type. In mutant Leu\(^{319}\) → Arg, the rate of the Ca\(^{2+}\)-binding transition was reduced as much as 10–30-fold depending on the presence of ATP. In mutants Gly\(^{233}\) → Glu, Gly\(^{233}\) → Val, and Pro\(^{312}\) → Ala, the rate of the Ca\(^{2+}\)-binding transition was increased at least 2–3-fold at acid pH but not significantly at neutral pH, suggesting a destabilization of the protonated form. The rate of Ca\(^{2+}\) dissociation was reduced 12-fold in mutant Pro\(^{312}\) → Ala and 3.5-fold in Leu\(^{319}\) → Arg, and increased at least 4-fold in a mutant in which the putative Ca\(^{2+}\) binding pocket, Gly\(^{233}\) residue Glu\(^{109}\) was replaced by aspartate. The data support a model in which Pro\(^{312}\) and Leu\(^{319}\) are closely associated with the cation binding pathway between the ion-binding sites and the catalytic site, and Lys\(^{604}\) is an essential catalytic residue that may function in the same way as its counterpart in the soluble hydrolases belonging to the haloacid dehalogenase superfamily.

The challenge of understanding active ion transport across biological membranes is well illustrated by the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase.\(^{1}\) This enzyme is made up of a single 110-kDa peptide chain that catalyzes uphill Ca\(^{2+}\) transport at the expense of ATP being hydrolyzed (1–5). The coupling between energy utilization and ion movement depends on long-range functional linkages between cytoplasmic and transmembrane protein domains. ATP hydrolysis occurs through formation of an aspartyl-phosphorylated intermediate at Asp\(^{511}\) located in the large cytoplasmic domain (Fig. 1). The transfer of the γ-phosphoryl group of ATP to Asp\(^{511}\) requires the binding of two calcium ions at sites formed by residues located predominantly in the transmembrane segments M4, M5, and M6 (3–5). Before phosphorylation, Ca\(^{2+}\) has access to these sites from the cytoplasmic side of the membrane, but following the phosphorylation Ca\(^{2+}\) can only be released on the luminal side, in connection with or following the conversion of the phosphoenzyme from an ADP-sensitive form (“E\(_{2}\)-P”) to an ADP-insensitive form (“E\(_{2}\)-P”). Subsequently, the aspartyl phosphate bond is hydrolyzed with liberation of P\(_{i}\), and the dephosphoenzyme returns to a state that readily binds Ca\(^{2+}\) from the cytoplasmic side. The latter events are presumably accompanied by countertransport of protons (see reaction cycle in Fig. 1), and the dissociation of the translocated protons and binding of Ca\(^{2+}\) at the cytoplasmically facing sites of the dephosphoenzyme take place as a series of consecutive reaction steps involving enzyme conformational changes, which may collectively be denoted “the E\(_{2}\) to E\(_{1}\) transition” or “the Ca\(^{2+}\)-binding transition” (1, 4, 6, 7).

As an approach toward a detailed characterization of the transport-associated conformational changes we have used site-directed mutagenesis analysis to map several critical residues. Beginning with the Gly\(^{233}\) (8) and Pro\(^{312}\) (9), about 20 residues have now been shown to be crucial to the E\(_{2}\)-P to E\(_{2}\)-P transformation of the phosphoenzyme (for reviews, see Refs. 4, 5, and 10). This step is the slowest in the cycle and can be studied at 0 °C using a manual mixing technique (8, 9). The residues critical to the E\(_{2}\)-P to E\(_{2}\)-P conversion are located in various regions of the protein, including the large cytoplasmic loop that makes up the catalytic site, the fourth stalk segment that links the catalytic site to transmembrane segment M4, the β-strand domain that interconnects the transmembrane segments M2 and M3, and, to a lesser extent, the transmembrane segments.

Much less is known about the positions of residues that participate in the conformational changes of the dephosphoenzyme. These conformational changes can be analyzed by fast kinetic studies of phosphorylation. In classic rapid quench experiments with the Ca\(^{2+}\)-ATPase isolated from rabbit muscle, the time course of the Ca\(^{2+}\)-binding transition of the dephosphoenzyme was monitored by following the phosphorylation upon addition of Ca\(^{2+}\) and [γ-\(^{32}\)P]ATP to enzyme initially present in the Ca\(^{2+}\)-deprived form (11–15). Likewise, the time course of Ca\(^{2+}\) dissociation from the dephosphoenzyme has been examined in the muscle enzyme by following the loss of phosphorylation activity upon removal of Ca\(^{2+}\).
Fast Kinetics of Ca\(^{2+}\)−ATPase Mutants

Lys684

The phosphorylation kinetics of the enzyme was quantified by a specific sandwich enzyme-linked immunoassay procedure (22) and microsomal membranes were harvested by differential centrifugation as before (8, 9). The amount of expressed phosphate was determined by "imaging" using a Packard Cyclone Storage Phosphor System.

**Experimental Procedures**

**Expression**

The previously described mutant cDNAs (8, 9, 18, 19, 21), derived from the cDNA encoding the rabbit fast-twitch muscle Ca\(^{2+}\)−ATPase (SERCA1a isoform), were transfected into COS-1 cells by the calcium phosphate procedure (22) and microsomal membranes were harvested by differential centrifugation as before (8, 9). The amount of expressed enzyme was quantified by a specific sandwich enzyme-linked immunosorbent assay (18) using as standard expressed wild type for which the concentration was determined by measurement of the capacity for phosphorylation by inorganic phosphate in the presence of 30% dimethyl sulfoxide (23). The enzyme-linked immunosorbent assay was usually supplemented with a determination of the capacity for phosphorylation from ATP ("active site concentration"). The expression levels varied between 200 and 400 pmol of Ca\(^{2+}\)−ATPase/mg of total microsomal protein. This is several hundredfold higher than the level of endogenous Ca\(^{2+}\)−ATPase in the COS-1 cells and the contribution of the endogenous enzyme to the phosphorylation signals is, thus, negligible.

**Quench-flow Instrumentation**

All fast kinetic experiments were performed using a Bio-Logic quench-flow module QFM-5 (Bio-Logic Science Instruments, Claix, France). The complete experimental setup also included a control unit/pow supply (MPS-5) and a PC equipped with an RS-232 serial interface. The apparatus is designed to work with reaction volumes in the microliter range and can perform both single and double mixing experiments using three or four reaction syringes, respectively. In addition, a fifth waste syringe allows a design of the instrument that makes no contact with the flow line and can perform both single and double mixing experiments.

**Curve Fitting and Simulation**

The phosphorylation time courses were fitted to a monoexponential time function corresponding to first-order kinetics, using the SigmaPlot program (SPSS, Inc.), and when good fits could be obtained, the extracted rate constants were used to characterize the data. In many cases, this procedure was feasible, and for further analysis we refrained from using more complex descriptions with several parameters.

In cases where there was an obvious deviation from monophasic kinetics, the data were fitted by computer simulation of the simplified reaction cycle in Scheme 1 using a kinetic simulation software, SimZyme, developed in this laboratory. SimZyme is based on the principle of simulating the reaction cycle step by step and comparing the calculated rate constants with the experimental data.
FIG. 1. Schematic presentation of the primary and probable secondary structure of the Ca$^{2+}$-ATPase with indication of the mutants studied and a minimum reaction cycle. The structural model is based on evidence reviewed in Ref. 3 and the homology of the phosphorylation domain with haloacid dehalogenases. Amino acid residues are represented by circles containing the single-letter code. α-helical structure is shown as diagonal rows of three or four residues and β-strands as a ladder-type of arrangement of the residues. M1-M10 denote the membrane-spanning helices. Amino acid substitutions described in the present study are indicated by inverted color and include Gly$^{233}$ → Val, Gly$^{233}$ → Glu, Glu$^{309}$ → Asp, Pro$^{312}$ → Ala, Leu$^{319}$ → Arg, and Lys$^{684}$ → Arg. The minimum reaction cycle shown in the upper left corner is based on the original proposal in Ref. 1, using the $E_1$-$E_2$ notation and taking into account recent evidence for countertransport of protons.

The rate of dephosphorylation is reduced 20–100-fold or more, relative to the wild type in the mutants Gly$^{233}$ → Glu, Gly$^{233}$ → Val, Pro$^{312}$ → Ala, Leu$^{319}$ → Arg, and Lys$^{684}$ → Arg, as a consequence of the block of the $E_1$-$P$ to $E_2$-$P$ conversion (8, 9, 18, 19). In mutant Glu$^{309}$ → Asp, the dephosphorylation rate is also very low, due to a block of the hydrolysis of the $E_2$-$P$ intermediate (21). For each mutant, the appearance of phosphoenzyme following addition of ATP to the Ca$^{2+}$-saturated enzyme could be fitted to a monoeponential function, as shown by the lines in Fig. 2 for mutants Gly$^{233}$ → Glu, Gly$^{233}$ → Val, Pro$^{312}$ → Ala, Leu$^{319}$ → Arg, and Lys$^{684}$ → Arg. The rate coefficient determined in this way corresponds to that termed $k_4$ for the wild type, since $k_2$ (the rate coefficient corresponding to dephosphorylation) is insignificant for the mutants. The apparent monophasic time course may suggest that under the present conditions a single rate-limiting step exists in the reaction path consisting of ATP binding and phosphoryl transfer to the Ca$^{2+}$-saturated enzyme. However, it is impossible to completely rule out the existence of a small lag phase indicative of two or more consecutive slow reactions (17). For the mutants Gly$^{233}$ → Val, Pro$^{312}$ → Ala, and Leu$^{319}$ → Arg, the fits of the data to monoeponential functions gave rate coefficients of 40 s$^{-1}$, 38 s$^{-1}$, and 34 s$^{-1}$, respectively, i.e., indistinguishable from the value $k_2 = 35$ s$^{-1}$ determined for the wild type. A lower rate coefficient of 25 s$^{-1}$ was found for the Gly$^{233}$ → Glu mutant. The significance of this difference was attested by its consistency over a range of ATP concentrations (not shown, but see, Fig. 3).

As further seen in Fig. 2, the Lys$^{684}$ → Arg mutant phosphorylated with a rate coefficient of only 1.2 s$^{-1}$, i.e. an almost 30-fold reduction compared with the wild type. This feature of the Lys$^{684}$ → Arg mutant indicates that Lys$^{684}$ plays an important role in ATP binding and/or catalysis. The Glu$^{309}$ → Asp mutant was found to phosphorylate with a rate coefficient about 8-fold lower than that of the wild type (4.3 s$^{-1}$, data not...
ATP Dependence of Wild-type and Mutants Gly<sup>233</sup>→Glu and Lys<sup>684</sup>→Arg—To further analyze the mechanism behind the reduction of the rate constant for phosphorylation in the mutants Gly<sup>233</sup>→Glu and Lys<sup>684</sup>→Arg, we investigated the ATP concentration dependence. At each ATP concentration tested, the phosphorylation rates of the Gly<sup>233</sup>→Glu and Lys<sup>684</sup>→Arg mutants were lower than that of the wild type. Fig. 3 shows double reciprocal plots of the initial phosphorylation rate versus ATP concentration (1, 2, 5, and 10 μM). The initial rates are in units of s<sup>−1</sup>, because the absolute rates have been divided by the enzyme concentration to obtain the rate per ATPase molecule (“molecular rate”). For the wild type as well as the mutants, the data could be fitted satisfactorily by a straight line in accordance with the results obtained with the purified muscle enzyme (26). The extrapolated maximum rate of 225 s<sup>−1</sup> corresponding to an infinite ATP concentration and the K<sub>m</sub> value of 13 μM determined for the wild type are fairly consistent with the literature on the purified muscle enzyme (12, 14). As illustrated by the lines in Fig. 4, good fits to the data to a monoeponential function could be obtained for the wild type as well as the mutants. The wild-type enzyme phosphorylated with an apparent rate constant of 21 s<sup>−1</sup> corresponding to a 1.7-fold reduction relative to the value found when phosphorylation was initiated after preincubation with Ca<sup>2+</sup>. This shows that a relatively slow step had indeed been introduced by omitting the pre-equilibration with Ca<sup>2+</sup>. For mutant Leu<sup>319</sup>→Arg, the rate coefficient observed after preincubation without Ca<sup>2+</sup> was only 0.70 s<sup>−1</sup>, an almost 50-fold reduction compared with the rate coefficient determined after preincubation in Ca<sup>2+</sup>-containing medium and 30-fold lower than that of the wild type. This is a very significant effect showing that the introduction of an arginine as substitute for Leu<sup>319</sup> severely impairs the ability of the enzyme to undergo one or more steps in the Ca<sup>2+</sup>-binding transition.
Fast Kinetics of Ca\(^{2+}\)-ATPase Mutants

The mutants Gly\(^{233}\) → Glu, Gly\(^{233}\) → Val, and Pro\(^{312}\) → Ala displayed rate coefficients of 28 s\(^{-1}\), 24 s\(^{-1}\), and 27 s\(^{-1}\), respectively. Although these values appear slightly higher than that of 21 s\(^{-1}\) determined for the wild type, the increase cannot be considered significant given the scatter of the experimental points. The rate coefficient of 28 s\(^{-1}\) for the Gly\(^{233}\) → Glu mutant is indistinguishable from the value observed for the same mutant following pre-equilibration with Ca\(^{2+}\) (25 s\(^{-1}\), see above). Thus, in the Gly\(^{233}\) → Glu mutant the Ca\(^{2+}\)-binding transition does not seem to be rate-limiting for the reaction sequence leading to phosphorylation. For the Lys\(^{664}\) → Arg mutant, the rate coefficient observed after preincubation in the absence of Ca\(^{2+}\) was 0.74 s\(^{-1}\), i.e. even lower than the 1.2 s\(^{-1}\) described above for preincubation with Ca\(^{2+}\).

The Ca\(^{2+}\)-Binding Transition in the Leu\(^{319}\) → Arg Mutant in the Absence of ATP—Because even micromolar concentrations of ATP accelerate a slow step associated with Ca\(^{2+}\) binding in the wild-type enzyme (15), we inquired whether the low rate of the Ca\(^{2+}\)-binding transition in the Leu\(^{319}\) → Arg mutant might be a consequence of a defect in this ATP modulation. To examine the rate of the Ca\(^{2+}\)-binding transition in the absence of ATP, we performed a series of measurements using the double mixing technique described by Guillian et al. (15). This approach is feasible when the phosphorylation rates measured with and without pre-equilibration with Ca\(^{2+}\) differ. The enzyme is preincubated in the absence of Ca\(^{2+}\) as in the above described experiments, but instead of adding \([\gamma^3P]ATP\) and Ca\(^{2+}\) simultaneously, Ca\(^{2+}\) binding is initiated by addition of Ca\(^{2+}\) without ATP. After a variable time interval, \(t\), \([\gamma^3P]ATP\) is included to assess the amount of enzyme that during \(t\) has acquired the ability to react with ATP and become phosphorylated (i.e. which has bound two calcium ions), and acid quenching is performed 34 ms later. As discussed by Guillian et al. (15), the amount of phosphoenzyme measured at \(t + 34\) ms follows the same function of \(t\) as the appearance of ATP-reactive enzyme, and, hence, this protocol allows determination of the basic rate of the Ca\(^{2+}\)-binding transition in the absence of ATP modulation. The results are shown in Fig. 5. The data could be fitted to a monoexponential time function, but in the case of the wild type with an offset indicating that part of the enzyme was initially able to react with Ca\(^{2+}\) and become phosphorylated during the 34-ms incubation with ATP. The data are consistent with a rate coefficient of 4.3 s\(^{-1}\) for the Ca\(^{2+}\)-binding transition in the wild type, i.e. 5-fold lower than the ATP-modulated transition rate of 21 s\(^{-1}\). For mutant Leu\(^{319}\) → Arg, the data are consistent with a rate coefficient of 0.43 s\(^{-1}\), i.e. 10-fold lower than that of the wild type and 1.6-fold lower than the ATP-modulated rate of 0.70 s\(^{-1}\) in the mutant. Thus, both the basic rate of the Ca\(^{2+}\)-binding transition measured in the absence of ATP and the modulatory effect of ATP are much reduced in the mutant compared with the wild type.

The Ca\(^{2+}\)-Binding Transition at pH 6.0—It has previously been demonstrated that the rate of the Ca\(^{2+}\)-binding transition in the purified muscle Ca\(^{2+}\)-ATPase depends strongly on the pH (7, 14, 29, 30). The reduction in the rate observed when the pH is lowered is probably a consequence of accumulation of an enzyme form ("EH" or "E") with protons bound at the transport sites, perhaps in an occluded state (cf. Refs. 2, 4, 6, 7, and "Discussion"). Fig. 6 shows the results of experiments in which the Ca\(^{2+}\)-binding transition of the expressed wild type and mutants Gly\(^{233}\) → Glu, Gly\(^{233}\) → Val, and Pro\(^{312}\) → Ala was examined at pH 6.0, using an experimental protocol otherwise similar to that described for Fig. 4 (preincubation in the absence of Ca\(^{2+}\) followed by simultaneous addition of Ca\(^{2+}\) and...
Fig. 6. Time course of phosphorylation by \(\gamma^{32}\text{P}]\text{ATP}\) of enzyme preincubated in the absence of Ca\(^{2+}\) at pH 6.0. Phosphorylation of wild-type and the Gly\(^{233}\) → Val, Gly\(^{233}\) → Glu, and Pro\(^{312}\) → Ala mutants was carried out as described for Fig. 4, except that pH was 6.0 (see “Experimental Procedures”). The data for the wild type and each mutant were normalized separately taking the maximum level of phosphorylation as 100%. The lines show the best fits to a monoexponential function, giving the following rate constants: wild type, 3.8 s\(^{-1}\); Gly\(^{233}\) → Val, 9.8 s\(^{-1}\); Gly\(^{233}\) → Glu, 7.8 s\(^{-1}\); Pro\(^{312}\) → Ala, 8.1 s\(^{-1}\).

[\(\gamma^{32}\text{P}]\text{ATP}\). As illustrated by the line, the apparent rate constant, \(k_{\text{app}}\), determined for the wild type by fitting the data in Fig. 6 to a monoexponential time function, is 3.8 s\(^{-1}\), i.e., 5–6-fold lower than that determined at pH 7.0 (cf. Fig. 4). For the mutants Gly\(^{233}\) → Glu, Gly\(^{233}\) → Val, and Pro\(^{312}\) → Ala, the fits to monoexponential functions illustrated in Fig. 6 gave apparent rate constants of 7.8 s\(^{-1}\), 9.9 s\(^{-1}\), and 8.1 s\(^{-1}\), respectively, i.e., 2–3-fold higher values compared with wild type. When experiments at pH 6.0 were carried out according to the same protocol as in Fig. 2 (i.e., preincubation in the presence of Ca\(^{2+}\)), the rate constants for phosphorylation did not differ significantly from those determined at pH 7.0 (\(k_A = 35\) s\(^{-1}\); \(k_B = 2\) s\(^{-1}\); and \(k_C = 2\) s\(^{-1}\), not shown), but in the mutants, where the dephosphorylation is significant (the rate constants for phosphorylation did not differ significantly from ours (12, 17, 31)).

The differences between the wild type and the mutants seen in Fig. 6 can be ascribed to mutational effects on the "true" rate coefficient \(k_C\) for the Ca\(^{2+}\)-binding transition, which is because \(k_{\text{app}}\) describing the approach to steady state, approximately equals the sum of \(k_A\) and the rate coefficient of dephosphorylation, \(k_B\). Thus, \(k_{\text{app}}\) is higher than \(k_C\) in the wild type where the dephosphorylation is significant (the rate coefficients determined by computer simulation of the data for the wild type at pH 6.0 are \(k_A = 35\) s\(^{-1}\); \(k_B = 2\) s\(^{-1}\); and \(k_C = 2\) s\(^{-1}\), not shown), but in the mutants, where the dephosphorylation is negligible, \(k_{\text{app}}\) is very close to \(k_C\). Therefore, the enhancement of \(k_C\) induced by the mutations may actually amount to as much as 4–5-fold.

**Dissociation of Ca\(^{2+}\)**—Finally, we focused on the rate of Ca\(^{2+}\) dissociation at the cytoplasmically facing transport sites of the dephosphoenzyme. When a Ca\(^{2+}\) chelator such as EGTA is added to Ca\(^{2+}\)-saturated enzyme, the ability to phosphorylate will disappear at a rate corresponding to the dissociation of the calcium ion that is first to leave in the sequential mechanism, because both Ca\(^{2+}\) sites must be occupied to allow phosphoryl transfer from ATP (17). It is therefore possible to determine the rate of this Ca\(^{2+}\) dissociation step by rapid-quench phosphorylation measurements (11, 12, 17). We have used an experimental setup where the Ca\(^{2+}\)-saturated enzyme is mixed simultaneously with [\(\gamma^{32}\text{P}]\text{ATP}\) and excess EGTA at pH 7.0. When free Ca\(^{2+}\) in the medium is removed with EGTA, the enzyme partitions between phosphorylation and dissociation of Ca\(^{2+}\), as illustrated in Scheme 2, and, hence, the amount of phosphoenzyme formed will depend on the rate constants of both processes.

The results of such experiments with the wild type are illustrated in the upper panel of Fig. 7, where the phosphoenzyme level at various time intervals after addition of [\(\gamma^{32}\text{P}]\text{ATP}\) together with EGTA is shown as percentage of the maximum level reached in the presence of Ca\(^{2+}\) in the medium (the 100% value in Fig. 2). As a consequence of dissociation of part of the bound Ca\(^{2+}\) during the incubation with [\(\gamma^{32}\text{P}]\text{ATP}\), the phosphorylation reaches a lower peak value relative to that seen in the presence of Ca\(^{2+}\) in the medium (compare Fig. 7 with Fig. 2). Furthermore, the phosphorylation level tends toward zero after a few hundred milliseconds, because the removal of Ca\(^{2+}\) prevents rephosphorylation after the dephosphorylation has occurred. As shown by the line, the data in the upper panel of Fig. 7 could be reproduced satisfactorily by computer simulation of Scheme 2, using the rate constants \(k_A = 35\) s\(^{-1}\) and \(k_B = 5\) s\(^{-1}\), determined in Fig. 2, together with a value of \(k_{-C\text{a}} = 27\) s\(^{-1}\) for the rate constant corresponding to Ca\(^{2+}\) dissociation, thus demonstrating that Scheme 2 provides a sound description of the phosphorylation under these conditions. The value of \(k_{-C\text{a}} = 27\) s\(^{-1}\) giving the best fit to the data lies within the range of 15–55 s\(^{-1}\) reported in the literature for the purified muscle enzyme under conditions that differ only slightly from ours (12, 17, 31).

On the basis of the above described principles, we designed a similar approach in which \(k_{-C\text{a}}\) is determined from the ratio \(E\text{P}_{\text{ATP} \cdot \text{EGTA}}/E\text{P}_{\text{ATP}}\) obtained corresponding to a single time point of 34 ms. \(E\text{P}_{\text{ATP} \cdot \text{EGTA}}\) is the amount of phosphoenzyme measured 34 ms after simultaneous addition of [\(\gamma^{32}\text{P}]\text{ATP}\) and EGTA, whereas \(E\text{P}_{\text{ATP}}\) is the amount of phosphoenzyme measured after 34 ms incubation of the Ca\(^{2+}\)-saturated enzyme with [\(\gamma^{32}\text{P}]\text{ATP}\) in the absence of EGTA. The time point of 34 ms was selected, because it is convenient that the dephosphorylation is negligible, so that the ratio \(E\text{P}_{\text{ATP} \cdot \text{EGTA}}/E\text{P}_{\text{ATP}}\) can be translated to a value for \(k_{-C\text{a}}\) by numerically solving Equation 1,

\[
\frac{E\text{P}_{\text{ATP} \cdot \text{EGTA}}}{E\text{P}_{\text{ATP}}} = \frac{k_A + k_{-C\text{a}}}{k_A} \left(1 - e^{-k_A t} \right) \left(1 - e^{-k_{-C\text{a}} t} \right)
\]

where \(k_A\) is known from the data in Fig. 2. This equation has been derived from Scheme 2 under the assumption that \(k_A|Ca\text{PATP}) = 0\). To examine whether the dephosphorylation can actually be considered insignificant under the present conditions, we have in the lower panel of Fig. 7 for \(t = 34\) ms and various values of \(k_{-C\text{a}}\) compared the expected ratio \(E\text{P}_{\text{ATP} \cdot \text{EGTA}}/E\text{P}_{\text{ATP}}\) calculated by using Equation 1 and \(k_{-C\text{a}} = 35\) s\(^{-1}\) (line indicated "calculated") with the corresponding ratios generated by computer simulation under the assumption that \(k_A = 35\) s\(^{-1}\) and \(k_B = 5\) s\(^{-1}\) (line indicated "simulated"). It can be seen that the two lines are indistinguishable, thus indicating that it is legitimate to neglect the dephosphorylation.
for times $\leq 34$ ms, when $k_B$ is $5 \text{s}^{-1}$ (corresponding to wild type) or lower (corresponding to mutants).

Fig. 8 shows experimental data obtained for the wild type and all the mutants at $t = 34$ ms and the corresponding $k_{-\text{Ca}}$ values derived using Equation 1. The $k_{-\text{Ca}}$ value of $27 \text{s}^{-1}$ for the wild type is identical to that obtained independently in Fig. 7, hence validating the simpler approach based on a single time point at 34 ms. It is also seen in Fig. 8 that $k_{-\text{Ca}}$ is reduced as much as 12-fold in mutant Pro312→Ala relative to wild type. A significant reduction (3.5-fold) was also found for mutant Leu319→Arg, whereas the mutants with alterations to Gly233 or Lys684 displayed $k_{-\text{Ca}}$ values indistinguishable from that of the wild type. For comparison, we also included the Glu239→Asp mutant in these experiments. This mutant has previously been shown to be defective with respect to Ca$^{2+}$-occlusion (21) and in line with this finding the rate of Ca$^{2+}$ dissociation determined in Fig. 8 was conspicuously increased (at least 4-fold) relative to wild type.

**DISCUSSION**

In the present work, we have used the quench flow technique to analyze mutational effects on reactions associated with the dephosphoenzyme in mutants showing a common block of the $E_1$-$P$ to $E_3$-$P$ transition of the phosphoenzyme. As summarized in Table I, our analysis reveals a surprising variability among these mutants with respect to the behavior of their depho-

**Fig. 8. Rate of Ca$^{2+}$ dissociation.** For the wild type and each mutant the phosphoenzyme level measured after 34 ms exposure of Ca$^{2+}$-saturated enzyme to excess EGTA and [γ-32P]ATP according to Protocol 4 (see "Experimental Procedures") is shown as percentage of the amount of phosphoenzyme determined after 34 ms incubation with [γ-32P]ATP in the presence of Ca$^{2+}$ without EGTA (i.e. according to Protocol 1). The rate constants for Ca$^{2+}$ dissociation, determined by numerically solving Equation 1 given under "Results," are shown to the right.

The Ca$^{2+}$-binding transition was conspicuously slowed down in mutant Leu319→Arg relative to wild type and accelerated in mutants Gly233→Glu, Gly233→Val, and Pro312→Ala at pH 6.0, but not significantly at pH 7.0. It has been proposed that before Ca$^{2+}$ binding the wild-type enzyme exists in a pH-dependent pre-equilibrium of two conformational states, $E_1$ and $E_2$, of which only $E_1$ is able to bind Ca$^{2+}$ with high affinity and become phosphorylated by ATP (1, 7, 14, 29). According to the detailed analysis by Forge et al. (6, 30), there are as many as three Ca$^{2+}$ free species with different degrees of protonation, $E_{H_3}$ (equivalent to $E_2$), EH, and $E$ (together constituting $E_1$). EH can bind one calcium ion, but only the fully deprotonated form, $E$, is able to bind both calcium ions as required for phosphorylation by ATP (6, 30). The deprotonation steps are relatively slow (30) and may represent conformational changes associated with "deocclusion" of the Ca$^{2+}$-reactive EH and/or $E$ forms. In the $E_1$-$E_2$ notation, this would correspond to a displacement of the $E_1$-$E_2$ pre-equilibrium in favor of $E_1$. This hypothesis predicts that the accelerating effect of the mutations on the Ca$^{2+}$-binding transition should be much less pronounced at pH 7.0 compared with pH 6.0, because at pH 7.0 the $E_1$ forms accumulate in the wild type as well. The actual finding is in accordance with the prediction.
The severely reduced rate of the Ca\(^{2+}\)-binding transition observed for mutant Leu\(^{319}\) → Arg could be due to a stabilization of the protonated E\(_{3}\) species (E\(_{2}\)). It is possible that the ATP-induced acceleration of the deprotonation (32) is defective in this mutant, since the rate coefficients determined in the presence and absence of ATP differed much less in the mutant than in the wild type (compare Figs. 4 and 5). An alternative interpretation would be that another partial reaction step in the Ca\(^{2+}\)-binding transition, not modulated by ATP, is slowed down by the mutation so that it contributes more significantly to rate limitation in the mutant than in the wild type. In this connection it is interesting to note that mutant Leu\(^{319}\) → Arg in addition to the reduced rate of the Ca\(^{2+}\)-binding transition displayed a 3.5-fold reduced rate of Ca\(^{2+}\) dissociation (Fig. 8). Either observation would be explained if the bulky and positively charged side chain of arginine imposes steric or electrostatic hindrance to Ca\(^{2+}\) diffusion to and from the binding sites or to the local conformational changes associated with opening and closure of the binding pocket in the E\(_{1}\) form (33). This would imply that Leu\(^{319}\) is rather close to the cytoplasmic inlet to the ion binding pocket, which is not unrealistic given the structural model in Fig. 1 where Leu\(^{319}\) is located right at the boundary between the fourth transmembrane segment and its cytoplasmic extension.

An even more pronounced reduction (12-fold) of the rate of Ca\(^{2+}\) dissociation from the dephosphoenzyme was found for the Pro\(^{312}\) → Ala mutant (Fig. 8). Such a low rate of Ca\(^{2+}\) dissociation under conditions where the rate of Ca\(^{2+}\) binding appears to be normal (Fig. 4) suggests that the stability of the Ca\(^{2+}\) bound state, perhaps the occluded form, is increased in the mutant relative to the wild type. The effect of the Pro\(^{312}\) → Ala mutation on Ca\(^{2+}\) dissociation is most likely associated with the position of Pro\(^{312}\) in the M4 helix right above one of the putative Ca\(^{2+}\) liganding residues, Glu\(^{309}\) (Fig. 1). Presumably, the alanine substituent stabilizes the Ca\(^{2+}\) bound state by removing the kink of the M4 helix introduced by the proline.

In previous work, we have demonstrated that Glu\(^{309}\) is involved in the binding of the most superficially located calcium ion (the one that binds last in the sequential binding process) (4), and measurements of Ca\(^{2+}\) occlusion following incubation with the \(\beta,\gamma\)-bidentate chromium(III) complex of ATP and removal of non-occluded Ca\(^{2+}\) by gel filtration in detergent solution suggested that the bound Ca\(^{2+}\) does not become properly occluded in the Glu\(^{309}\) → Asp mutant (21). This hypothesis seems to be further substantiated by the present finding of a conspicuous enhancement by the Glu\(^{309}\) → Asp mutation of the rate of Ca\(^{2+}\) dissociation from the dephosphoenzyme in the presence of the natural substrate MgATP and in the absence of the perturbing influence of detergent. The side chain of Glu\(^{309}\), which is shortened by one methylene group in this mutant, may possibly constitute an essential element in a molecular gate at the entrance to the Ca\(^{2+}\) binding pocket.

Contrary to the Glu\(^{309}\), Pro\(^{312}\), and Leu\(^{319}\) mutants, the mutants with alteration to Gly\(^{233}\) or Lys\(^{684}\) showed no significant change in the rate of Ca\(^{2+}\) dissociation relative to wild type. This is consistent with the proposed location of the latter two residues outside the membrane far from the ion-binding sites (Fig. 1). The above discussed effect of the Gly\(^{233}\) mutations on the E\(_{1}\)→E\(_{2}\) conformational equilibrium suggests a role of the glycine in long-range conformational changes. It is noteworthy in this connection that Gly\(^{233}\) is highly conserved among P-type ATPases with various cation specificities, and studies of proteolytically cleaved Na\(^{+}\),K\(^{-}\)-ATPase have suggested that inactness of the peptide segment right on the C-terminal side of the glycine is important for the coordination between structural changes of the catalytic site and the ion-binding sites (34, 35). Interestingly, the present results demonstrate a specific effect of the Gly\(^{233}\) → Glu mutation on ATP binding/phosphoryl transfer. In the Gly\(^{233}\) → Glu mutant, the phosphorylation rate measured after pre-equilibration with Ca\(^{2+}\) was significantly lower than that of the wild type over a range of ATP concentrations, and our analysis indicates that the maximal rate of phosphorylation is lower than one-third that of the wild type. This effect, which may be due to the negative charge of the glutamate as it was not observed with valine as substituent, is in line with the hypothesis that the glycine is part of a long-range signal transmission pathway between the ion-binding sites and the catalytic site.

Finally, our fast kinetic studies reveal the importance of Lys\(^{684}\) for the ATP-binding/phosphoryl transfer reactions. This lysine is also highly conserved among the P-type ATPases and on the basis of sequence and structural alignment studies it was recently suggested to be equivalent to a lysine that plays a central role in the catalytic mechanism of soluble hydrolases belonging to the haloacid dehalogenase superfamily (20, 36). We previously found that the ability of the Ca\(^{2+}\) -ATPase to undergo phosphorylation by ATP is retained following substitution of Lys\(^{684}\) with arginine, and the most noticeable characteristic of the Lys\(^{684}\) → Arg mutant observed previously was its inability to form the E\(_{1}\)-P phosphoenzyme intermediate, both in the forward direction of the reaction cycle, from E\(_{1}\)-P, as well as in the backward reaction with P\(_{i}\) (18). The present investigation has, however, revealed that the maximal rate of phosphorylation is reduced more than 50-fold relative to wild type. This finding is in accordance with a critical role for Lys\(^{684}\) in stabilization of the excess of negative charge developed in the transition state complex between the active-site aspartate and the γ-phosphoryl group of ATP, as predicted on the basis of the catalytic mechanism of the haloacid dehalogenases (20). Hence, a structural and functional homology between the P-type ATP-
Pases and the soluble hydrolases belonging to the haloacid dehalogenase superfamily seems to be corroborated by the present data and it is likely that the side chain of Lys$^{684}$ is close to that of Asp$^{351}$ in the three-dimensional structure. In the light of this information, the inability of the Lys$^{684}$ → Arg mutant to form $E_2$-P may as well be explained by a requirement for the lysine side chain to participate in the interaction with the phosphoryl group.

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Thomas Lykke-Møller Sørensen, Yves Dupont, Bente Vilsen and Jens Peter Andersen

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