Importance of Transmembrane Segment M3 of the Sarcoplasmic Reticulum Ca\(^{2+}\) -ATPase for Control of the Gateway to the Ca\(^{2+}\) Sites*

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The specific functional roles of various parts of the third transmembrane segment (M3) of the sarcoplasmic reticulum Ca\(^{2+}\) -ATPase were examined by functionally characterizing a series of mutants with multiple or single substitutions of M3 residues. Steady-state and transient kinetic measurements, assisted by computer simulation of the time and Ca\(^{2+}\) dependences of the phosphorylation level, were used to study the partial reaction steps of the enzyme cycle, including the binding and dissociation of Ca\(^{2+}\) at the high affinity cytoplasmically facing sites. The mutation Lys-Leu-Asp-Glu\(^{255}\) → Glu-Ile-Glu-His resulted in a conspicuous increase in the rate of Ca\(^{2+}\) dissociation as well as a displacement of the major conformational equilibria of the phosphoenzyme and dephosphoenzyme forms. The point mutant Phe\(^{256}\) → Ala also showed an increased rate of Ca\(^{2+}\) dissociation, whereas a conspicuous decrease both in the rate of Ca\(^{2+}\) dissociation and in the rate of Ca\(^{2+}\) binding was found for the mutant Gly-Glu-Glu-Leu\(^{260}\) → Ile-His-Leu-Ile. These findings suggest that the NH\(_2\)-terminal half of M3 is involved in control of the gateway to the Ca\(^{2+}\) sites. The main effect of two mutations to the COOH-terminal half of M3, Ser-Lys-Val-Ile-Ser\(^{265}\) → Thr-Gly-Val-Ala-Val and Leu-Ile-Cys-Val-Ala-Val-Trp-Leu-Ile\(^{274}\) → Phe-Leu-Gly-Val-Ser-Phe-Phe-Ile-Leu, was a block of the dephosphorylation.

The Ca\(^{2+}\) -ATPase\(^{1}\) of sarcoplasmic reticulum is a membrane-bound P-type ATPase that mediates active uptake of Ca\(^{2+}\) at a stoichiometry of two calcium ions transported for each ATP hydrolyzed. The translocation of Ca\(^{2+}\) is thought to occur by way of a series of conformational changes as indicated in Scheme 1, involving both the transmembrane and the cytoplasmic parts of the 110-kDa Ca\(^{2+}\) -ATPase polypeptide (1–6). The membrane domain consists of 10 transmembrane segments, M1–M10, whose secondary structure is mainly α-helical (4, 5). M1–M4 and M5 extend into a large cytoplasmic “head” containing the ATP binding site and the catalytic center for phosphorylation and dephosphorylation. The two high affinity Ca\(^{2+}\) binding sites are located in the membrane domain, and side chain and backbone oxygen atoms in M4–M6 and M8 serve as Ca\(^{2+}\) ligands (4–6). Furthermore, acidic residues in the cytoplasmic loop connecting M6 and M7 (L67) seem to play a role in determining the Ca\(^{2+}\) binding properties of the enzyme, possibly by directing the calcium ions toward the liganding residues in the membrane (7). Many more residues in the membrane and at the boundary between the cytoplasmic and membrane sectors could be of importance for the recognition and binding of Ca\(^{2+}\), since the pathway for migration of the calcium ions between the cytoplasm and the membrane-embedded ligands is unknown.

The specific inhibitor of the sarco(endo)plasmic reticulum Ca\(^{2+}\) -ATPases thapsigargin is of interest in relation to the understanding of the structure-function relationships of enzyme-Ca\(^{2+}\) interaction, since it has been shown to prevent high affinity Ca\(^{2+}\) binding (8, 9). The inhibitor may bind preferentially to a preexisting enzyme form with low affinity for Ca\(^{2+}\) (E\(_2\) state) and stabilize this form, or it may interact more directly with the Ca\(^{2+}\) binding pocket or with the gateway to this pocket. In studies of chimeric proteins made up from parts of the sarcoplasmic reticulum Ca\(^{2+}\) -ATPase and the thapsigargin-insensitive Na\(^{+}\)-K\(^{+}\) -ATPase, we previously demonstrated that transmembrane segment M3 of the Ca\(^{2+}\) -ATPase contains residues of crucial importance to thapsigargin inhibition (10). The effects of more discrete chimeric changes later indicated that the NH\(_2\)-terminal half of M3 is the more critical part with respect to thapsigargin sensitivity (11), and a conserved phenylalanine in this region, Phe\(^{256}\), was shown to undergo spontaneous mutations in cultured cells under selection pressure in the presence of thapsigargin (12). The residues Glu\(^{260}\) and Gly\(^{270}\) in the Ca\(^{2+}\) binding part of transmembrane segment M4 have also been shown to be important to thapsigargin sensitivity (6, 13), whereas the S4 stalk segment seems not to be involved (11).

The chimeras in which the transmembrane segment M3 of the Ca\(^{2+}\) -ATPase was replaced by M3 of the Na\(^{+}\)-K\(^{+}\) -ATPase not only displayed low sensitivity to thapsigargin but also a more than 100-fold reduction in the apparent Ca\(^{2+}\) affinity relative to wild type (10), suggesting a role for M3 in connection with Ca\(^{2+}\) binding. However, more detailed studies of the functional role of M3 in relation to the Ca\(^{2+}\) -ATPase reaction cycle have so far not been reported. M3 contains several polar and charged residues that are not conserved between the Ca\(^{2+}\) -ATPase and the Na\(^{+}\)-K\(^{+}\) -ATPase and which, therefore, are potential candidates for a role in determination of the specific

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The abbreviations used are: Ca\(^{2+}\) -ATPase, the sarco(endo)plasmic reticulum Ca\(^{2+}\) -transporting adenosine triphosphatase (EC 3.6.1.38); K\(_{50}\) ligand concentration giving half-maximum activity; M1–M10, the transmembrane segments numbered from the NH\(_2\)-terminal end of the Ca\(^{2+}\) -ATPase peptide chain; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; E\(_{\text{d}}\), enzyme form with cytoplasmically facing high affinity Ca\(^{2+}\) -binding sites; E\(_{\text{n}}\), enzyme form with low affinity for Ca\(^{2+}\); E\(_{\text{P}}\), ADP-sensitive phosphoenzyme intermediate; E\(_{\text{dP}}\), ADP-insensitive phosphoenzyme intermediate.

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cation selectivities of these enzymes. In this paper, we describe the functional effects of four new chimeric mutations, A, B, C, and D, replacing Ca\textsuperscript{2+}-ATPase residues in M3 with the corresponding Na\textsuperscript{+}-K\textsuperscript{+}-ATPase residues (Table I). The mutants A and B were designed to separately test the functional roles of the four nonconserved residues immediately before and after the conserved Phe\textsuperscript{256} in the NH\textsubscript{2}-terminal half of M3. The COOH-terminal half of M3 was examined by mutations C and D. In addition, we have included as mutation F the point mutation Phe\textsuperscript{256} → Ala in our studies. Using both steady-state and transient kinetic measurements, we have determined the consequences of the mutations for the various partial reaction steps of the enzyme cycle shown in Scheme 1, including the steps associated with the binding and dissociation of Ca\textsuperscript{2+}. By computer simulations based on the reaction cycle in Scheme 1, we have obtained insight into the factors determining the apparent Ca\textsuperscript{2+} affinity and cooperativity observed in the steady-state phosphorylation experiments. Our results demonstrate for the first time that residues in the NH\textsubscript{2}-terminal half of M3 are crucial to the rates of dissociation and binding of Ca\textsuperscript{2+}, consistent with a role in the control of the gateway to the Ca\textsuperscript{2+} sites. Furthermore, the various parts of M3 play distinct roles in the major conformational changes and the dephosphorylation of the enzyme.

**EXPERIMENTAL PROCEDURES**

**General Methods**—The construction of mutant Ca\textsuperscript{2+}-ATPase cDNAs, the expression of wild type and mutants in COS-1 cells, and the isolation of microsomal membranes, as well as the procedures used in the functional assays, have previously been described (13-18), and further details of the functional assays are given in the figure legends. Time courses of phosphorylation at 25 °C were monitored using the Bio-Logic quench-flow module QFM-5 (Bio-Logic Instruments, Claux, France) as described (18). Phosphorylation and dephosphorylation at 0 °C was carried out using a manual mixing technique (13). Prior to use in the quench-flow module QFM-5 (Bio-Logic Instruments, Claux, France) as courses of phosphorylation at 25 °C were monitored using the Bio-Logic Phosphor System. Calculations of total and free calcium concentrations were carried out using the WEBMAXC program, available on the World Wide Web.

**Data Analysis**—All data presented are average values corresponding to 2–6 experiments. The SigmaPlot program (SPSS, Inc.) was used to analyze the data by nonlinear regression, and the best fits are shown as lines in the figures. The time courses of phosphorylation, dephosphorylation, and Ca\textsuperscript{2+} dissociation were analyzed by fitting mono- or biexponential functions. The Ca\textsuperscript{2+} concentration dependence of the amount of phosphorylase formed (EP) was analyzed using the Hill equation, which considers the fraction of the enzyme that has bound thapsigargin and therefore is inactive, $F_0 = 0.5 \cdot \left( R + R_0 + 1 - \left( R^2 + R_0^2 + 2 \cdot R \cdot R_0 + 2 \cdot R_0 - 2 \cdot R + 1 \right)^{0.5} \right)$ (Eq. 3).

To derive the latter equation, a simple one-site binding model is considered. $R$ is the molar ratio between the total thapsigargin concentration (free plus bound) and the enzyme concentration, $R_0$ is the ratio between the dissociation constant for the enzyme-thapsigargin complex and the enzyme concentration.

The rate constant for dissociation of the first calcium ion, $k_{-3}$ in Scheme 1, was calculated as previously explained (18) by numerically solving the equation,

$$\frac{EP_{ATP-EgTa}}{EP_{ATP}} = \frac{k_1}{k_1 + k_{-3} \left( 1 - e^{-k_1 \cdot t} \right)}$$

where $EP_{ATP-EgTa}$ is the amount of phosphorylase measured at $t = 34$ ns after the simultaneous addition of [γ-\textsuperscript{32}P]ATP and EGTA to Ca\textsuperscript{2+}-saturated enzyme, $EP_{ATP}$ is the amount of phosphorylase measured after a 34-ms incubation of the Ca\textsuperscript{2+}-saturated enzyme with [γ-\textsuperscript{32}P]ATP in the absence of EGTA, and $k_1$ is the pseudo-first order rate constant for phosphorylation of Ca\textsubscript{2}E\textsubscript{1}.

The kinetic simulation software SimZyme (18) was used to compute the amount of phosphorylase as a function of time or Ca\textsuperscript{2+} concentration on the basis of the reaction cycle shown as Scheme 1 with reactions 5 and 6 combined into a single dephosphorylation step. For any choice of rate constants and ligand concentrations, the program solves the relevant differential equations, using the 4th order Runge-Kutta numerical method and provides a graphical representation of the time dependence of the concentrations of the reaction intermediates that can be compared with experimental data points. To simulate the Ca\textsuperscript{2+} activation profiles, the steady-state concentration of phosphorylase was computed at a series of Ca\textsuperscript{2+} concentrations. The apparent affinity and cooperativity were then determined by fitting the Hill equation to the computation results by use of the SigmaPlot program.

**RESULTS**

**Mutants**—The helix corresponding to M3 starts at the cytoplasmic surface of the membrane at Pro\textsuperscript{248} and ends at the luminal surface at Ile\textsuperscript{274} (5). The mutants characterized in the present study extend from Lys\textsuperscript{255} to Ile\textsuperscript{274} (Table I). In mutants A, B, C, and D, four or more amino acid residues were replaced by those present at the corresponding positions in the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. The M3 segment is not very well conserved among the P-type ATPases, and in several cases these chimeric changes implied drastic substitutions such as for instance a positive residue with a negative or the reverse, a hydrophobic with a polar, or a small side chain with a bulky. In the point mutant

![Scheme 1. Ca\textsuperscript{2+}-ATPase reaction cycle.](image_url)

### Table I

<table>
<thead>
<tr>
<th>Mutants examined in the present study</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Lys-8-Leu-Asp-Glu\textsuperscript{255} → Glu-Ile-Glu-His\textsuperscript{a}</td>
</tr>
<tr>
<td>B</td>
<td>Gly-Glu-Gln-Asp\textsuperscript{260} → Ile-His-Ile-Ile</td>
</tr>
<tr>
<td>C</td>
<td>Ser-Lys-Val-Ile-Ser\textsuperscript{265} → Thr-Gly-Val-Ala-Val</td>
</tr>
<tr>
<td>D</td>
<td>Leu-Ile-Cys-Val-Ala-Val-Tyr-Leu-Ile\textsuperscript{271} → Phe-Leu-Gly-Val-Ser-Phe-Phe-Ile</td>
</tr>
<tr>
<td>F</td>
<td>Phe\textsuperscript{256} → Ala, point mutation of conserved Phe</td>
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</table>

\textsuperscript{a} The amino acid substitutions were chosen so that the resulting short sequence shown is identical to that present in the rat α1 isoform of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase.

$$EP = EP_{max} \cdot \left( [Ca^{2+}] / [K\textsubscript{a}^{2+} + [Ca^{2+}]] \right)$$ (Eq. 1)

The dependence of the phosphorylation level on the total thapsigargin concentration was analyzed using the equation,

$$EP = 100 \% \cdot \left( 1 - F_0 \right)$$ (Eq. 2)

where $F_0$ is the fraction of the enzyme that has bound thapsigargin and therefore is inactive.

$$F_0 = 0.5 \cdot \left( R + R_0 + 1 - \left( R^2 + R_0^2 + 2 \cdot R \cdot R_0 + 2 \cdot R_0 - 2 \cdot R + 1 \right)^{0.5} \right)$$ (Eq. 3)

The rate constant for dissociation of the first calcium ion, $k_{-3}$ in Scheme 1, was calculated as previously explained (18) by numerically solving the equation,

$$\frac{EP_{ATP-EgTa}}{EP_{ATP}} = \frac{k_1}{k_1 + k_{-3} \left( 1 - e^{-k_1 \cdot t} \right)}$$ (Eq. 4)}
Consistent with our previous findings, the chimera EL is seen which the entire transmembrane segment M3 is exchanged. F, the phenylalanine Phe^{256}, which is conserved between the sarco(endo)plasmic reticulum Ca^{2+}-ATPases and the Na\(^+\),K\(^+\)-ATPases, was replaced by alanine. These mutants could all be expressed in the COS cells at levels similar to that of the wild-type Ca^{2+}-ATPases, was replaced by alanine. These mutants could all be expressed in the COS cells at levels similar to that of the wild-type Ca^{2+}-ATPase.

**Thapsigargin Sensitivity**—Fig. 1 shows the sensitivity of the mutants to inhibition by thapsigargin. Like the wild type, the mutants were capable of forming a phosphoenzyme intermediate in the presence of Ca^{2+} and \(\gamma\)-32P-ATP, and the thapsigargin sensitivity could be determined by titration of the thapsigargin dependence of phosphoenzyme formation. The reason for presenting the data as a function of the total thapsigargin concentration instead of the free concentration of thapsigargin is that the latter is unknown as a consequence of the extremely high affinity displayed by the enzyme toward thapsigargin (\(K_d\) in the nanomolar range). To ensure sufficient time for the interaction between the inhibitor and the enzyme in the Ca^{2+}-free state thought to bind the inhibitor, the wild type and mutant Ca^{2+}-ATPases were preincubated for 15 min with the inhibitor in the absence of Ca^{2+} (presence of EGTA), prior to the addition of an excess of Ca^{2+} and \(\gamma\)-32P-ATP to initiate the phosphorylation. For comparison, we also included in these experiments the previously characterized chimera E\(^\ast\) (10), in which the entire transmembrane segment M3 is exchanged. Consistent with our previous findings, the chimera E\(^\ast\) is seen to display an affinity for thapsigargin 3 orders of magnitude lower than that of the wild type (Fig. 1). Likewise, mutant B displayed rather low thapsigargin affinity (about 100-fold reduced relative to wild type). The thapsigargin affinity of mutant F was found to be reduced about 10-fold relative to that of the wild type, a larger effect than observed by Yu et al. (19) in studies of thapsigargin inhibition of the ATPase activity in the same mutant. A smaller reduction in thapsigargin affinity, corresponding 4-fold relative to wild type, was found for mutant C. For mutant A, the best fit to the data indicated a 2-fold reduction in thapsigargin affinity relative to wild type, but this difference from wild type is too small to be considered significant. Furthermore, the thapsigargin affinity of mutant D was indistinguishable from that of the wild type (Fig. 1).

**ATP Hydrolysis**—The mutants A, B, C, D, and F were found to be able to hydrolyze ATP and accumulate \(4^{4}\)Ca^{2+} in the microsomal vesicles. The Ca^{2+} dependence of the overall functional performance was determined by measurement of the steady-state turnover rate for ATP hydrolysis at 37 °C in the presence of 5 mM ATP and various Ca^{2+} concentrations, together with Ca^{2+} ionophore to maintain the same Ca^{2+} concentration on both sides of the membrane (Fig. 2). The wild-type Ca^{2+}-ATPase is activated by submicromolar concentrations of Ca^{2+}, binding at the cytoplasmically facing high affinity sites. High Ca^{2+} concentrations are, on the other hand, inhibitory, because Ca^{2+} at the luminal low affinity sites drives the \(E_1P\) to \(E_2P\) conformational transition backwards (cf. Scheme 1) and because CaATP, present at a significant level at high Ca^{2+} concentrations, is being utilized more slowly than MgATP (20). For mutants A, B, C, and D, the maximum turnover rate determined at the optimum Ca^{2+} concentration was 39, 60, 12, and 19%, respectively, relative to wild type. For mutant F, the maximum turnover rate observed at a Ca^{2+} concentration between 1 and 10 \(\mu\)M was slightly higher than that of the wild type (reproduced in six independent sets of experiments), but at Ca^{2+} concentrations of \(\geq 100 \mu\)M there was no difference from wild type (Fig. 2). It can furthermore be seen in Fig. 2 that mutants B and F displayed a higher \(K_{0.5}\) value for Ca^{2+} activation than the wild type, corresponding to lower Ca^{2+} affinity. For mutant A, the \(K_{0.5}\) value for Ca^{2+} activation was wild type-like, but at Ca^{2+} concentrations between 1 and 10 \(\mu\)M, this mutant appeared more sensitive to Ca^{2+} inhibition than the wild type (Fig. 2).

**Dependence of Phosphorylation**—The activation by Ca^{2+} at the cytoplasmically facing high affinity sites was further studied in phosphorylation experiments (Fig. 3). At 25 °C (Fig. 3A), mutants B and F displayed reduced Ca^{2+} affinity in the activation of phosphorylation corresponding to \(K_{0.5}\) values 4- and 3-fold higher, respectively, relative to wild type, consistent with the ATP hydrolysis data in Fig. 2. The \(K_{0.5}\) value for Ca^{2+} activation of mutant A was found to be similar to that of the wild type, whereas mutants C and D showed increases in
conditions corresponding to Fig. 4A, the $E_1P$ to $E_2P$ conversion is the rate-limiting step in the dephosphorylation of the wild-type enzyme, and $E_1P$ accumulates at steady state, as seen by the very rapid (backward) dephosphorylation occurring upon the addition of ADP (solid diamond) and the somewhat slower dephosphorylation (in the forward direction) occurring upon the chase with nonradioactive ATP (open circles). In mutants A and B, the dephosphorylation in the forward direction occurred at a lower rate than in the wild type (solid circles and open triangles pointing upward), but in the presence of ADP there was no difference from wild type (solid diamond), suggesting that the $E_1P$ form accumulates in these mutants as well and that the reason for the reduced forward dephosphorylation rate is a reduced rate of the $E_1P$ to $E_2P$ conversion. In mutants C and D, that were found to hydrolyze ATP very slowly (cf. Fig. 2), the dephosphorylation rate was very low, both in the forward direction upon the addition of nonradioactive ATP (open triangles pointing downward and open squares) and in the backward direction with ADP (solid triangles and solid squares), suggesting that in these mutants the ADP-insensitive $E_2P$ form accumulates at steady state as a result of a block of the hydrolysis of $E_2P$. A comparison of Fig. 4A with Fig. 2 shows a semiquantitative correlation between the change in the rate of dephosphorylation in the forward direction and the change of the turnover rate for ATP hydrolysis, although these parameters were determined at widely different temperatures.

In the absence of Ca$^{2+}$, the Ca$^{2+}$-ATPase can also be phosphorylated backwards with inorganic phosphate, resulting in the accumulation of $E_2P$ (cf. Scheme 1) (1). Fig. 4B shows the time course of dephosphorylation of the $E_2P$ phosphoenzyme formed in this way. The slow dephosphorylation observed for mutants C and D confirms that the hydrolysis of $E_2P$ is blocked in these mutants, and the rapid wild type like dephosphorylation seen for mutants A and B confirms that the reduced dephosphorylation rate shown for these mutants in Fig. 4A results from a reduced $E_1P$ to $E_2P$ transition rate rather than a reduced rate of hydrolysis of $E_2P$.

Rapid Kinetic Studies of Phosphorylation and the Ca$^{2+}$-binding Transition—The time course of the phosphorylation from [γ-32P]ATP was analyzed on a millisecond time scale at 25 °C, using the quench-flow technique described previously (18). Fig. 5 shows the data for mutants A, B, and C at pH 7.0. Two types of conditions were applied. To obtain information about the reaction sequence $E_2 → Ca_2E_2P$ (reactions 1–4 in Scheme 1), consisting of the $E_2$ to $E_1$ transformation (with associated proton dissociation; cf. Ref. 21), Ca$^{2+}$ binding in two steps, ATP binding, and subsequent phosphorlyl transfer, a protocol was used in which the enzyme is preincubated in the absence of Ca$^{2+}$ (presence of EGTA) followed by the simultaneous addition of Ca$^{2+}$ and [γ-32P]ATP (left panels of Fig. 5). Under these conditions, part of the enzyme pool has to undergo the $E_2$ to $E_1$ transformation before it can bind Ca$^{2+}$ and phosphorylate (how large a fraction depends on the preequilibrium between $E_2$ and $E_1$ in the absence of Ca$^{2+}$). To obtain specific information about the last part of the reaction sequence, $Ca_2E_2 → Ca_2E_2P$ (reaction 4 in Scheme 1), an alternative protocol was used in which [γ-32P]ATP was added to enzyme that had been preincubated in the presence of Ca$^{2+}$ (right panels of Fig. 5). For the wild type, we previously found a rate constant of 35 s$^{-1}$ for $Ca_2E_1 → Ca_2E_2P$, whereas the rate constant determined following preincubation in the absence of Ca$^{2+}$ was 21 s$^{-1}$, indicating the existence of a relatively slow step in the reaction sequence $E_2 → Ca_2E_1$ (the Ca$^{2+}$-binding transition; reactions 1–3 in Scheme 1) (18). These results with the wild type were obtained under exactly the same conditions as the results shown in Fig. 5 for the mutants A, B, and C and are highly reproducible. The data
FIG. 4. Dephosphorylation at 0 °C of phosphoenzyme formed from ATP (A) or P, (B). Measurements were carried out on the wild type (open circles and solid diamond) and mutants A (solid circles and solid diamond), B (open triangles pointing upward and solid diamond), C (triangles pointing downward), and D (squares). A, the microsomes were phosphorylated for 15 s at 0 °C in a medium containing 2 μM [γ-32P]ATP, 40 μM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl2, 0.1 mM CaCl2, and 1 μM calcium ionophore A23187, followed by the addition of either 0.6 mM nonradioactive ATP (open circles, open triangles, open squares, and open squares), or 1 μM ADP with 1 mM EGTA (solid diamond, solid triangle, and solid square) and acid quenching at the indicated time intervals. B, the microsomes were phosphorylated for 10 min at 25 °C in a medium containing 0.5 mM [γ-32P]P, 100 mM MES/Tris (pH 6.0), 10 mM MgCl2, 2 mM EGTA, and 30% (v/v) dimethyl sulfoxide. Subsequently, the reaction mixture was cooled to 0 °C and diluted 20-fold into ice-cold medium containing 50 mM TES/Tris (pH 7.0), 100 mM KCl, 2 mM MgCl2, 2 mM EGTA, and 5 mM nonradioactive P, followed by acid quenching at the indicated time intervals.

points for the wild type are therefore not shown again here, but for comparison the wild-type data obtained following preincubation in the absence of Ca2+ are represented by broken lines in the left panels of Fig. 5.

For all of the mutants, a monoexponential function could be fitted satisfactorily to the data obtained following preincubation in the absence of Ca2+, and the rate constants determined in this way are 35, 17, and 12 s−1 for A, B, and C, respectively (left panels). This should be compared with the value of 21 s−1 for the wild type (broken line). In all three mutants, the rate constant determined following preincubation in the presence of Ca2+ (right panels) was very similar to that obtained previously for the wild type (36, 31, and 32 s−1 for A, B, and C, respectively; compare with 35 s−1 for the wild type). For mutant A, the data obtained with and without Ca2+ in the preincubation medium are virtually indistinguishable, showing that in this mutant the Ca2+-binding transition, E −→ Ca,E, is no longer limiting for the phosphorylation rate. This indicates that in mutant A the E1-E2 equilibrium is displaced in favor of E2. Either the Ca2+-binding transition is severalfold enhanced in this mutant or the E1 to E2 conformational change in the reverse reaction sequence is slowed down so that the E1 form accumulates already during the preincubation in the absence of Ca2+. The Ca2+-binding transition of mutant B does not seem to differ from that of the wild type, whereas the rate constant of 12 s−1 determined for mutant C is significantly lower than that corresponding to wild type.

Similar experiments were conducted with mutants D and F, and the observed rate constants corresponding to preincubation in the absence of Ca2+ were 18 and 19 s−1, respectively, i.e., very similar to wild type. The rate constants corresponding to Ca,E −→ Ca,E,P in these mutants were likewise similar to that of the wild type (data not shown).

Because the rate of the Ca2+-binding transition may be limited by proton dissociation, it was also of interest to examine the time course of phosphorylation at a lower pH, where the E2 form of the wild type is thought to be fully protonated (21).
Data obtained in the same way as for Fig. 5 (left panels), but at pH 6.0, are shown in Fig. 6. For comparison, the wild-type data obtained previously under identical conditions (18) are indicated by broken lines. The observed rate constants were 11, 4.4, 2.4, and 4.8 s⁻¹ for mutants A, B, C, and F, respectively, which should be compared with the value of 3.8 s⁻¹ obtained for the wild type. Since the rate constants observed at pH 6.0 following preincubation in the presence of Ca²⁺, i.e. corresponding to Ca₂⁺E₁ → Ca₃EP₁, were higher than 24 s⁻¹ for all four mutants (data not shown), the slow step limiting the phosphorylation rate in Fig. 6 must in all four mutants be the Ca²⁺-binding transition and not ATP binding or phosphoryl transfer. Therefore, it may be concluded that at pH 6.0 the rate of the Ca²⁺-binding transition is about 3-fold enhanced in mutant A relative to wild type, whereas it is wild type-like in mutants B and F and 1.6-fold reduced in mutant C.

Phosphorylation From P₁—To further study the equilibrium between E₁ and E₂ in mutant A at pH 7.0, the backward phosphorylation with inorganic phosphate was determined as a function of the P₁ concentration. As seen in Fig. 7, the apparent affinity for P₁ is reduced in mutant A relative to wild type (about 6-fold). Since only the E₂ form and not E₁ is able to react with P₁ (cf. Scheme 1), this result is in line with the above described findings indicating that the E₁-E₂ equilibrium is displaced in favor of E₁ in this mutant. Another possibility would be that the equilibrium E₂ + P₁ ⇌ E₃P₁ is displaced toward the left. The data in Fig. 4B show, however, that the phosphorylation rate of mutant A is wild type-like.

Ca²⁺ Dissociation—To study the dissociation of Ca²⁺ bound at the cytoplasmically facing sites of the E₁ form, we took advantage of the dependence of the phosphorylation reaction on the occupancy of the Ca²⁺ sites. When a Ca²⁺ chelator such as EGTA is added to Ca²⁺-saturated enzyme, the two calcium ions dissociate sequentially, and the ability to phosphorylate disappears at a rate corresponding to the first Ca²⁺ dissociation step, because both Ca²⁺ sites must be occupied to allow phosphoryl transfer from ATP (22). If [γ⁻³²P]ATP is added simultaneously with EGTA, the enzyme partitions between phosphorylation and dissociation of Ca²⁺⁺, and the amount of phosphoenzyme formed depends on the balance between the rate constant for the first Ca²⁺⁺ dissociation step and the rate constant for phosphorylation (here denoted k₋ and k₊, respectively, by reference to Scheme 1). ² We have previously (18) described a simple approach in which k₋ is calculated from k₊ and the ratio between EPT⁺ + EGTA and EPT⁺, where EPT⁺ + EGTA is the amount of phosphoenzyme measured 34 ms after the simultaneous addition of [γ⁻³²P]ATP and EGTA and EPT⁺ is the amount of phosphoenzyme measured after 34-ms incubation of the Ca²⁺⁺-saturated enzyme with [γ⁻³²P]ATP in the absence of EGTA. The rate constant k₊ is the one obtained from the data corresponding to the right panels in Fig. 5. Results obtained in this way at pH 7.0 and 25 °C are displayed in Fig. 8. Interestingly, we found substantial changes in the rate of Ca²⁺⁺ dissociation in some of the mutants relative to wild type. A 5–6-fold increase in k₋ was observed for mutant A, and a 4-fold decrease in k₋ for mutant B. In mutants C and D, k₋ was only 1.5-fold lower than in the wild type. In mutant F, k₋ was enhanced about 3-fold.

This encouraged us to study the time course of Ca²⁺⁺ dissociation in more detail for mutants A, B, and F, as shown in Fig. 9. In these experiments, we found it advantageous to change the pH to 6.0 to reduce the rate of Ca²⁺⁺ dissociation in the mutants as well as the wild type (23, 24), thereby allowing the full time course to be monitored. A double mixing procedure was used in which EGTA is added to enzyme preincubated with Ca²⁺⁺, followed by the addition of [γ⁻³²P]ATP at the indicated time interval, t, and acid quenching 34 ms later. Under these conditions, the amount of phosphoenzyme measured at t + 34 ms must follow the same function of t as the disappearance of ATP-reactive enzyme (cf. Ref. 25), reflecting the dissociation of the calcium ion, which is first to leave in the sequential mechanism, corresponding to k₋. As seen in Fig. 9, reasonable fits of monoeponential functions to the data could be obtained, and there were conspicuous differences between the rate constants of wild type and mutants. The Ca²⁺⁺ dissociation rate was found to be enhanced 17-fold in mutant A, relative to wild type, and 4-fold in mutant F, whereas the rate was reduced 23-fold in mutant B. These data extend and corroborate the above described findings at pH 7.0.

Single Turnover Experiment—To further examine the partial reactions at pH 7.0 and 25 °C, the development of the phos-
The phosphorylation level was determined following 34-ms exposure to \([\gamma-32P]ATP\) at 25 °C under two different sets of conditions (\(EP_{\text{ATP} + \text{EGTA}}\) and \(EP_{\text{ATP}}\)), using the quench-flow module QFM-5. To determine \(EP_{\text{ATP} + \text{EGTA}}\), the enzyme was preincubated in a medium containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl₂, and 0.1 mM CaCl₂, and this solution was mixed with an equal volume of 40 mM MES/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl₂, 4 mM EGTA, and 10 mM \([\gamma-32P]ATP\), followed by acid quenching 34 ms later. To determine \(EP_{\text{ATP}}\), the enzyme was preincubated in a medium containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl₂, and 0.1 mM CaCl₂, and this solution was mixed with an equal volume of the same medium containing 10 mM \([\gamma-32P]ATP\), followed by acid quenching 34 ms later. The columns show the ratio between \(EP_{\text{ATP} + \text{EGTA}}\) and \(EP_{\text{ATP}}\), and to the right are shown the rate constants for \(Ca^{2+}\) dissociation (\(k_{-3}\)), calculated as described under “Experimental Procedures.” Error bars on the columns indicate S.D.

Phosphoenzyme with time was monitored following the simultaneous addition of \([\gamma-32P]ATP\) and EGTA to \(Ca^{2+}\)-saturated enzyme (i.e. the same condition as used for Fig. 8). The data obtained for mutants A, B, and F are shown in Fig. 10. The corresponding data for the wild type obtained under identical conditions have previously been presented and analyzed (18) and are only shown here as broken lines for comparison. The phosphorylation reaches a peak value and then tends toward zero after a few hundred milliseconds, because the removal of \(Ca^{2+}\) by EGTA prevents rephosphorylation after the dephosphorylation has occurred. The magnitude of the peak value depends on the balance between the rate constants for \(Ca^{2+}\) dissociation (\(k_{-3}\)) and phosphorylation (\(k_{3}\)), as described for Fig. 8, whereas the slope of the decay occurring after the peak depends on the \(E_1P\) to \(E_2P\) conversion and the dephosphorylation of \(E_2P\). For the wild type and mutants A and B, the decay rate is limited mainly by the \(E_1P\) to \(E_2P\) conversion (the dephosphorylation of \(E_2P\) is known to be as fast as 60 s⁻¹ for the wild type under conditions similar to those used here (26)). In Fig. 10, the lines show computations of the expected time course based on Scheme 1 with reactions 5 and 6 combined into a single dephosphorylation step with the rate constant \(k_{-3}\). It can be seen that the experimental data in Fig. 10 could be reproduced using \(k_{3}\) values of 1.6, 3.5, and 9 s⁻¹ for mutants A, B, and F, respectively, together with the values for \(k_{-3}\) and \(k_{4}\) determined in the experiments corresponding to Fig. 8 and Fig. 5 (right panels), respectively. The \(k_{3}\) value determined previously for the wild type is 5 s⁻¹ (18) corresponding to the broken line. Hence, these data confirm the values for \(k_{-3}\) and \(k_{4}\) determined in the experiments corresponding to Fig. 8 and Fig. 5 (right panels) and add rate constants for the \(E_1P\) to \(E_2P\) conversion at 25 °C. The \(E_1P\) to \(E_2P\) conversion rate seems to be reduced to approximately one-third that of the wild type in mutant A and to two-thirds in mutant B, consistent with the observations at 0 °C (Fig. 4A). For mutant F, the \(k_{3}\) value giving the best fit to the data in Fig. 10 (9 s⁻¹) is almost 2-fold higher than that corresponding to the wild type (5 s⁻¹), a finding that may be related to the slightly higher maximum steady-state turnover rate for ATP hydrolysis in this mutant relative to wild type (Fig. 2).

Experiments similar to those described for Fig. 10 were also conducted with mutants C and D. For these mutants, there was no measurable decay of the phosphoenzyme within 500 ms, consistent with the very low rate of dephosphorylation observed at 0 °C (Fig. 4), thus precluding an accurate determination of the rate constant (data not shown).

**DISCUSSION**

The present study reveals the importance of transmembrane segment M3 of the \(Ca^{2+}\)-ATPase for the kinetics of \(Ca^{2+}\) interaction at the cytoplasmically facing sites and for the major conformational changes and the dephosphorylation of the enzyme.

The data in Figs. 8 and 9 directly demonstrate that the rate of \(Ca^{2+}\) dissociation from the \(Ca_2E_1\) form is enhanced relative to wild type in mutants A and F and reduced in mutant B. Furthermore, as explained below, the reduced apparent affinity of mutant B for \(Ca^{2+}\) seen in the steady-state phosphorylation experiment in Fig. 3A indicates that in this mutant there is a substantial reduction of the rate of \(Ca^{2+}\) binding as well.

The changes in the maximum steady-state ATP hydrolysis rate, seen in Fig. 2, can be explained by the mutational effects on the \(E_1P\) to \(E_2P\) transition and the dephosphorylation of \(E_2P\). In the wild-type enzyme, the \(E_1P\) to \(E_2P\) transition is one of the major rate-limiting steps for ATP hydrolysis under the experimental conditions corresponding to Fig. 2 (27). Accordingly, in mutants A and B there seems to be a nearly perfect match between the decrease in the steady-state ATP hydrolysis rate and the decrease in the \(E_1P\) to \(E_2P\) transition rate (both being reduced to approximately one-third in mutant A and two-thirds in mutant B, relative to wild type). The conspicuous reduction of the rate of dephosphorylation of \(E_2P\) observed for mutants C and D should make the dephosphorylation rate-limiting for ATP hydrolysis in these mutants, and very low rates of ATP hydrolysis were indeed found. Furthermore, the slightly enhanced maximum steady-state rate of ATP hydrolysis seen for mutant F in Fig. 2 is in accordance with the enhanced \(E_1P\) to
is possible that the dephosphorylation block is mediated through a change in the position of M4, since some of the residues replaced in mutants C and D are in rather close contact with M4 residues in the wild-type enzyme (5).

The finding that the apparent affinity for Ca\(^{2+}\) is lower in mutant B than in mutant A at 25 °C (Fig. 3A), although the rate of Ca\(^{2+}\) dissociation is about 20-fold lower in mutant B compared with mutant A under the same conditions (Fig. 8), calls for some explanation. Furthermore, it is not straightforward to understand the higher apparent cooperativity (Hill number) of mutant A relative to the wild type that makes the curves intersect in Fig. 3A, or the reversal of the apparent Ca\(^{2+}\) affinities of mutants A and B occurring when the temperature is lowered to 0 °C (Fig. 3B). These problems encouraged us to perform a series of computations of the predicted relationship between the Ca\(^{2+}\) concentration and the phosphorylation level, using the software SimZyme described under “Experimental Procedures.” In Table II is shown the \(K_{0.5}\) value and the apparent cooperativity (Hill number, \(n_{H}\)) of Ca\(^{2+}\) activation of phosphorylation predicted for various choices of the rate constants in Scheme 1. Selected examples of the complete computed activation profiles are shown in Fig. 11. Simulation 1, which results in \(K_{0.5} = 2.3\times10^{-7}\) m and \(n_{H} = 1.39\), is seen to reproduce the data for the wild type in Fig. 3A (\(K_{0.5} = 2.3\times10^{-7}\) m, \(n_{H} = 1.48\)) rather satisfactorily. As explained in a footnote to Table II, this simulation is based on the rate constants determined in the transient kinetic experiments carried out with the wild type at pH 7.0 and 25 °C. The other simulations in Table II demonstrate the effects of varying several of the rate constants in the enzyme cycle one by one and in combination, thus providing insight into the factors determining the apparent Ca\(^{2+}\) affinity and cooperativity. As expected, an increase of the rate of Ca\(^{2+}\) dissociation without a change in the rate of Ca\(^{2+}\) binding (i.e. a reduced Ca\(^{2+}\) affinity) results in a right shift of the activation curve corresponding to an increased \(K_{0.5}\) value (reduced “apparent Ca\(^{2+}\) affinity”); see simulations 6 and 7. However, we had not expected that the enhanced Ca\(^{2+}\) dissociation in addition would give rise to an increase of the Hill number (from 1.39 to 1.69 for a 10-fold increase in Ca\(^{2+}\) dissociation rate). It was furthermore somewhat surprising that a combined 10-fold reduction in both the “off” and the “on” rate constant for Ca\(^{2+}\) (i.e. retaining a normal Ca\(^{2+}\) affinity) results in a right shift of the activation curve corresponding to a 6-fold decrease in the apparent affinity (simulation 10). Changes to the \(E_{1}E_{2}\) equilibrium (simulations 2–5) result in changes to the \(K_{0.5}\) value but not in substantial changes to the apparent cooperativity. A 10-fold increase of the phosphorylation rate has very little influence on the \(K_{0.5}\) value or the apparent cooperativity (simulation 12). On the other hand, a 10-fold lowering of the dephosphorylation rate results in a conspicuous increase of both the apparent Ca\(^{2+}\) affinity and the Hill number (simulation 13). This is a purely kinetic effect, unrelated to the Ca\(^{2+}\) binding properties of the enzyme, explaining very well the left shift of the activation curve, relative to wild type, observed in Fig. 3A for the very slowly dephosphorylating mutants C and D.

Interestingly, simulation 15 shows that it is feasible to combine the various changes to the partial reactions observed experimentally for mutant A into an activation profile (\(K_{0.5} = 2.7\times10^{-7}\) m, \(n_{H} = 1.70\)), matching rather accurately the experimental activation curve for this mutant in Fig. 3A (\(K_{0.5} = 2.7\times10^{-7}\) m, \(n_{H} = 1.90\)). As illustrated in Fig. 11, both the increased Hill number, which makes the curve corresponding to mutant A intersect the wild-type curve, and the almost normal apparent Ca\(^{2+}\) affinity seen for mutant A, despite the increased rate of Ca\(^{2+}\) dissociation, are reproduced satisfacto-

**FIG. 10. Single turnover experiment.** The panels represent mutants A, B, and F as indicated by the lettering. The time course of phosphorylation and dephosphorylation was monitored at 25 °C, using the quench-flow module QFM-5. The enzyme was preincubated in a medium containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl\(_2\), and 0.1 mM CaCl\(_2\), and this solution was mixed with an equal volume of 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl\(_2\), and 0.1 mM CaCl\(_2\), and EGTA, and 10 μM [γ-\(^{32}\)P]ATP, followed by acid quenching at the indicated time intervals. The phosphorylation level is shown as a percent of the maximum level reached in the presence of 100 μM Ca\(^{2+}\) (corresponding to the 100% value in Fig. 5). The solid lines show computations of the time course on the basis of Scheme 1 with reactions 5 and 6 combined into a single dephosphorylation step with the rate constant \(k_{s}\), which was varied to obtain the best fit to the data points, giving \(k_{s} = 1.6, 3.5, \) and 9 s\(^{-1}\) for mutant A, B, and F, respectively. Additional rate constants used in the computation were \(k_{a} = 36,\) 31, and 35 s\(^{-1}\) and \(k_{s} = 150, 7,\) and 77 s\(^{-1}\) for mutants A, B, and F, respectively, as obtained from Figs. 5 and 8. The other rate constants in Scheme 1 are unimportant for this analysis, since the enzyme initially is present as the \(CaE_{1}\) form, and the concentrations of free Ca\(^{2+}\) and ADP are 0. The broken lines indicate the wild type data (\(k_{s} = 5\) s\(^{-1}\)) obtained previously under identical conditions (18).
The degree of cooperativity observed in the phosphorylation experiment is similar to that reported for equilibrium Ca\(^{2+}\) activation of phosphorylation for various choices of rate constants in the enzyme cycle in Scheme 1.

For each set of rate constants, the steady-state phosphorylation level was computed at a series of Ca\(^{2+}\) concentrations using the SimZyme simulation software (16). The apparent Ca\(^{2+}\) affinity (\(K_{0.5}\)) and cooperativity (Hill number, \(n_H\)) was then determined by fitting the Hill equation to the computer-generated data.

<table>
<thead>
<tr>
<th>Simulation</th>
<th>Rate constants(^a)</th>
<th>Apparent Ca(^{2+}) affinity</th>
<th>Hill number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (^b)</td>
<td>(k_{i,j} = 5/25, k_{j,i} = 1.2 \cdot 10^7/13.5, k_{i,j} = 1.2 \cdot 10^7/27),</td>
<td>2.3\cdot10^{-7}</td>
<td>1.39</td>
</tr>
<tr>
<td>2.</td>
<td>(k_1 = 250)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>(k_2 = 2.5)</td>
<td>1.7\cdot10^{-7}</td>
<td>1.36</td>
</tr>
<tr>
<td>4.</td>
<td>(k_1 = 500)</td>
<td>4.4\cdot10^{-7}</td>
<td>1.33</td>
</tr>
<tr>
<td>5.</td>
<td>(k_2 = 1)</td>
<td>8.8\cdot10^{-7}</td>
<td>1.23</td>
</tr>
<tr>
<td>6.</td>
<td>(k_1 = 135, k_2 = 270)</td>
<td>1.5\cdot10^{-7}</td>
<td>1.28</td>
</tr>
<tr>
<td>7.</td>
<td>(k_{i,j} = 75, k_{j,i} = 150)</td>
<td>1.2\cdot10^{-6}</td>
<td>1.69</td>
</tr>
<tr>
<td>8.</td>
<td>(k_{i,j} = 1.35, k_{j,i} = 2.7)</td>
<td>7.1\cdot10^{-7}</td>
<td>1.65</td>
</tr>
<tr>
<td>9.</td>
<td>(k_2 = 1.2 \cdot 10^7, k_2 = 1.2 \cdot 10^7)</td>
<td>1.4\cdot10^{-7}</td>
<td>1.10</td>
</tr>
<tr>
<td>10.</td>
<td>(k_{i,j} = 1.2 \cdot 10^7/13.5, k_{i,j} = 1.2 \cdot 10^7/2.7)</td>
<td>2.4\cdot10^{-7}</td>
<td>1.38</td>
</tr>
<tr>
<td>11.</td>
<td>(k_{i,j} = 1.2 \cdot 10^7/13.5, k_{i,j} = 1.2 \cdot 10^7/27)</td>
<td>1.4\cdot10^{-6}</td>
<td>1.11</td>
</tr>
<tr>
<td>12.</td>
<td>(k_{i,j} = 350)</td>
<td>1.2\cdot10^{-7}</td>
<td>1.67</td>
</tr>
<tr>
<td>13.</td>
<td>(k_1 = 0.5)</td>
<td>2.1\cdot10^{-7}</td>
<td>1.34</td>
</tr>
<tr>
<td>14.</td>
<td>(k_2 = 50)</td>
<td>6.2\cdot10^{-6}</td>
<td>1.71</td>
</tr>
<tr>
<td>15.</td>
<td>(k_{i,j} = 250, k_{j,i} = 75, k_{j,i} = 150, k_1 = 1.6)</td>
<td>5.7\cdot10^{-7}</td>
<td>1.24</td>
</tr>
<tr>
<td>16.</td>
<td>(k_{i,j} = 1.5 \cdot 10^7/3.5, k_{j,i} = 1.5 \cdot 10^7/7, k_2 = 3.5)</td>
<td>2.7\cdot10^{-7}</td>
<td>1.70</td>
</tr>
</tbody>
</table>

\(^a\)For simulations 2–16, only the rate constants differing from those used in simulation 1 are indicated. The rate constants are designated as units of mol\(^{-1}\) liter\(^{-1}\) s\(^{-1}\). Being second order rate constants, \(k_1\) and \(k_2\) are in units of mol\(^{-1}\) liter\(^{-1}\) s\(^{-1}\), while all the other rate constants are in units of s\(^{-1}\) (\(k_{i,j}\) and \(k_{j,i}\) being treated as pseudo-first order rate constants).

\(^b\)Simulation 1 represents the wild type under our standard phosphorylation conditions in the presence of 5 \(\mu\)M ATP, 5 \(\mu\)M Mg\(^{2+}\), and 80 \(\mu\)M K\(^+\) at pH 7.0 and 25 °C. The basis for our assignment of the rate constants is as follows. The values \(k_1 = 25\) s\(^{-1}\), \(k_2 = 35\) s\(^{-1}\), and \(k_5 = 5\) s\(^{-1}\) were previously estimated from data obtained with the wild type (18) in experiments similar to those corresponding to Figs. 5 and 10 in the present paper. The value \(k_{i,j} = 27\) s\(^{-1}\) was obtained from Fig. 8 (for further consolidation, see Ref. 18). Assuming that the two calcium ions dissociate through a common gateway, \(k_{i,j} = 0.5 \cdot k_{i,j} (23, 24)\). The value \(k_{i,j} = 50\) s\(^{-1}\) was assigned to reproduce as accurately as possible the apparently monoeponential time dependence of phosphorylation starting from the Ca\(^{2+}\)-deprived enzyme (Fig. 4 in Ref. 18, the wild type data corresponding to Fig. 5 (left panels) of the present paper). The rate constants for Ca\(^{2+}\) binding, \(k_1\) and \(k_2\), were varied until the best possible fit to the wild-type data in Fig. 3A was obtained. To reduce the radius, \(k_2\) and \(k_3\) were assumed to be equal. A further helpful constraint for the wild type is that the degree of cooperativity observed in the phosphorylation experiment is similar to that reported for equilibrium Ca\(^{2+}\) binding in the absence of ATP (Hill number 1.3–1.4) (21), which is fulfilled for the indicated assignments of the rate constants corresponding to reactions 1, 2, and 3. The pseudo-first order rate constants \(k_{i,j}\) and \(k_{j,i}\) were given very small values in consideration of the essentially irreversible nature of these reactions under our experimental conditions (no added ADP or P\(_i\); Ca\(^{2+}\) concentration ≥0.1 mM).

On the basis of the computations, we may also offer a tentative explanation of the reversal of the apparent Ca\(^{2+}\) affinities of mutants A and B seen when the temperature is lowered to 0 °C (Fig. 3B). The right shift of the titration curve observed at 0 °C for mutant A would be explained if the rate constants associated with Ca\(^{2+}\) dissociation, \(k_{i,j}\) and \(k_{j,i}\), had a less steep temperature dependence in mutant A than in the wild type, corresponding to a lower Arrhenius activation energy of the conformational change opening the pathway between the Ca\(^{2+}\) sites and the cytoplasm. The reason for the left shift of the activation curve observed for mutant B at low temperature could, on the other hand, be that the temperature dependence of the Ca\(^{2+}\) dissociation rate is steeper in this mutant than in the wild type, corresponding to increased activation energy of the gate-opening conformational change.

The mutational effects on the kinetics of Ca\(^{2+}\) interaction observed here point clearly to a direct or indirect role of the NH\(_2\)-terminal part of M3 in control of the gateway to the Ca\(^{2+}\) binding.
sites. The recently published high resolution crystal structure of the Ca\(^{2+}\)-ATPase in Ca\(_2\)E\(_1\) form (5) does not reveal the entrance pathway to the Ca\(^{2+}\) sites, presumably because it normally closes following the binding of the calcium ions (“occlusion”). However, the residues substituted in mutants A, B, and F could be intimately related to such a pathway, since they are at the same time located near the cytoplasmic surface of the protein and rather close (12–25 Å) to one of the Ca\(^{2+}\) sites.

The cytoplasmic loop connecting M6 and M7 (L67) has been suggested to play an important role in the coordination of conformational changes in different parts of the enzyme, and a movement of this loop could possibly affect the Ca\(^{2+}\) conformational changes in different parts of the enzyme, and a suggested to play an important role in the coordination of E binding of thapsigargin (11, 19). A critical factor could be the substitution in M3 might represent an indirect effect rather than the removal of side chains directly participating in the binding of thapsigargin resulting from amino acid substitutions in M3 might represent an indirect effect rather than the removal of side chains directly participating in the binding of thapsigargin (11, 19). A critical factor could be the E\(_1\)-E\(_2\) equilibria of the phosphoenzyme and dephosphoenzyme forms.

The importance of M3 for thapsigargin sensitivity seems to be associated particularly with residues located in the short segment between Phe\(^{256}\) and Leu\(^{260}\), substituted in mutants F and B (Fig. 1). It has previously been discussed that the reduced sensitivity to thapsigargin resulting from amino acid substitutions in M3 might represent an indirect effect rather than the removal of side chains directly participating in the binding of thapsigargin. A critical factor could be the E\(_1\)-E\(_2\) conformational equilibrium, since it has been proposed that thapsigargin reacts preferentially with the E\(_2\) form (9, 28). The lack of correlation between the mutational effects on the sensitivity to thapsigargin and the displacement of the E\(_1\)-E\(_2\) equilibrium found in the present study is therefore noteworthy. That mutants B and F display a much more substantial reduction of the thapsigargin sensitivity than mutant A, in which the E\(_1\)-E\(_2\) equilibrium is displaced in favor of E\(_1\), shows that other factors are more critical and raises the question of whether in mutant A the E\(_1\) form in its Ca\(^{2+}\)-free state might actually bind thapsigargin with high affinity. The concurrence of very low thapsigargin sensitivity with a reduced rate of Ca\(^{2+}\) binding in mutant B is striking and may suggest that the same factors are involved in preventing Ca\(^{2+}\) and thapsigargin from entering into their binding sites. It is possible that thapsigargin binding requires an open state of the entrance pathway to the Ca\(^{2+}\) sites.

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