Importance of Transmembrane Segment M1 of the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase in Ca\(^{2+}\) Occlusion and Phosphoenzyme Processing*

Anja Pernille Einholm, Bente Vilsen, and Jens Peter Andersen‡

From the Institute of Physiology, University of Aarhus, Ole Worms Allé 160, DK-8000 Aarhus C, Denmark

The functional consequences of a series of point mutations in transmembrane segment M1 of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase were analyzed in steady-state and transient kinetic experiments examining the partial reaction steps involved in Ca\(^{2+}\) interaction and phosphoenzyme turnover. Arginine or leucine substitution of Glu\(^{31}\), Glu\(^{52}\), or Glu\(^{56}\), located in the N-terminal third of M1, did not affect these functions. Arginine or leucine substitution of Asp\(^{59}\), located right at the bend of M1 seen in the crystal structure of the thapsigargin-bound form, caused a 10-fold increase of the rate of Ca\(^{2+}\) dissociation toward the cytoplasmic side. Mutation of Leu\(^{62}\) to alanine or proline and of Val\(^{62}\) to alanine also enhanced Ca\(^{2+}\) dissociation, whereas an 11-fold reduction of the rate of Ca\(^{2+}\) dissociation was observed upon alanine substitution of Leu\(^{65}\), thus providing evidence for a relation of the middle part of M1 to a gating mechanism controlling the dissociation of occluded Ca\(^{2+}\) from its membranous binding sites. Moreover, phosphoenzyme processing was affected by some of the latter mutations, in particular leucine substitution of Asp\(^{59}\), and alanine substitution of Leu\(^{65}\) accelerated the transition to ADP-insensitive phosphoenzyme and blocked its dephosphorylation, thus demonstrating that this part of M1, besides being important in Ca\(^{2+}\) interaction, furthermore, is a critical element in the long range signaling between the transmembrane domain and the cytoplasmic catalytic site.

The Ca\(^{2+}\)-ATPase\(^{1}\) of sarcoplasmic reticulum is a membranous-bound enzyme that pumps Ca\(^{2+}\) from the cytosol to the lumen of the sarcoplasmic reticulum at the expense of chemical energy being released by ATP hydrolysis (1–4). It belongs to the family of P-type ATPases characterized by the formation of an ATPase, the sarco(endo)plasmic Ca\(^{2+}\)-transporting adenosine triphosphatase (EC 3.6.1.38); M1–M10, transmembrane segments numbered from the N terminus; MES, 2-[N-morpholino]ethanesulfonic acid; MOPS, 3-[N-morpholino]propanesulfonic acid; TES, N-(tris(hydroxymethyl)methyl)-2-aminoethane-sulfonic acid; SERCA, sarcoplasmic-endoplasmic reticulum calcium ATPase; Tg, thapsigargin.

* This study was funded in part by grants from the Danish Medical Research Council, the Nova Nordisk Foundation, the Lundbeck Foundation, and the Research Foundation of Aarhus University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 45-89-422-814; Fax: 45-86-129-065; E-mail: jpa@fi.au.dk.

Received for publication, January 7, 2004, and in revised form, January 27, 2004. Published, JBC Papers in Press, January 30, 2004, DOI 10.1074/jbc.M400158200

This paper is available online at http://www.jbc.org

Printed in U.S.A.
lipid bilayer. The other hydrophobic residues are embedded in the membrane, Leu60 below the level of the Ca\(^{2+}\) ions in the Ca\(^{2+}\)-bound structure. The effects of these mutations on the various partial reaction steps involved in Ca\(^{2+}\) binding and catalysis have been analyzed, using among other assays also transient kinetic methods that allow measurement of the rates of conformational changes and Ca\(^{2+}\) dissociation toward the cytoplasmic side.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Expression**—The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce the desired mutations directly into full-length cDNA encoding the rabbit fast twitch muscle Ca\(^{2+}\)-ATPase (SERCA1a isoform). The template for mutagenesis reaction consisted of SERCA1a cDNA inserted into the expression vector pMT2 (17). For expression of recombinant protein, pMT2 containing wild-type or mutant cDNA was transfected into mammalian COS-1 cells using the calcium phosphate precipitation method (18). The microsomal fraction containing expressed wild-type or mutant Ca\(^{2+}\)-ATPase was isolated by differential centrifugation (19), and the expression level was quantitated by a specific enzyme-linked immunosorbent assay (20).

**Functional Studies**—The ability of wild-type or mutant Ca\(^{2+}\)-ATPase to transport Ca\(^{2+}\) and hydrolyze ATP was determined by studying the Ca\(^{2+}\) uptake into microsomal membranes and the P\(_{i}\) liberation, respectively (21). The molecular turnover rate was calculated as the ratio between the specific Ca\(^{2+}\)-ATPase activity and the active-site concentration. The latter was determined by quantitation of phosphorylation from \([\gamma-32P]ATP\) at 0 °C in the presence of a saturating Ca\(^{2+}\) concentration. Samples of wild-type and mutant Ca\(^{2+}\)-ATPase were examined in parallel, and the molecular turnover rate corresponding to the mutants was expressed relative to that of the wild type. Partial reactions were studied according to previously described principles (20–23), taking advantage of the acid stability of the phosphoenzyme formed from \([\gamma-32P]ATP\) or \([\gamma-32P]P\), and details are given in the figure legends. A BioLogic quenched flow module QFM-5 or SFM-400/Q (Bio-Logic Science Instruments, Claix, France) was used for rapid mixing in experiments where the reaction time was below 1 s, applying the previously described protocols (22, 23). In all phosphorylation experiments, the reaction was quenched by addition of 0.5–2 volumes of 25% (w/v) trichloroacetic acid containing 100 mM H\(_3\)PO\(_4\). Subsequently, the acid-precipitated enzyme was washed by centrifugation, dissolved in loading buffer and subjected to separation by SDS-PAGE at pH 6.0 (24). The distribution of radioactivity associated with the gel was quantitated by electronic autoradiography of the dried gel using a Packard Cyclone\textsuperscript{TM} Storage Phosphor System.

**Data Analysis**—Using the Sigmaplot program (SPSS Inc.), time courses of phosphorylation and dephosphorylation were fitted according to first-order kinetics, whereas ligand concentration dependences were fitted by use of the Hill equation. Generally, the experiments were performed at least twice on different enzyme preparations, and average values are shown in the figures. Standard errors are shown in the tables except for a few cases, in which the experiment (giving a wild type-like result) was performed only once.

**RESULTS**

Site-directed Mutagenesis and Expression—Several Ca\(^{2+}\)-ATPase point mutants were designed to study the functional importance of transmembrane segment M1. An overview of these mutations is given in Table I. Two different strategies were used: (i) charge reversal or removal (Glu\(_{55}\), Glu\(_{55}\), Glu\(_{58}\), and Asp\(_{59}\)) and (ii) hydrophobic size reduction (Phe\(_{57}\), Leu\(_{60}\), Val\(_{62}\), and Leu\(_{65}\)) as part of a possible Ca\(^{2+}\) entry pathway and/or selectivity filter, we have mutated these amino acids individually to the neutral leucine and to the oppositely charged and rather bulky arginine, which would be expected to impose electrostatic and steric effects on Ca\(^{2+}\) migration, if positioned near the migration pathway. Moreover, we have substituted the hydrophobic amino acids Phe\(_{57}\), Leu\(_{60}\), Val\(_{62}\), and Leu\(_{65}\) to investigate the demands for bulkiness and hydrophobicity of these side chains. Phe\(_{57}\) is located on the hydrophobic surface of the bent part of M1 in the E\(_2\)-Tg structure and could be instrumental in attaching this part to the cytoplasmic side.
Importance of M1 of the SR Ca$^{2+}$-ATPase

**Table 1**

| ATP hydrolysis$^a$ | Ca$^{2+}$ transport$^b$ | $K_{m,5}$ for Ca$^{2+}$ activation of phosphorylation$^c$ | $t_{1/2}$ of Ca$^{2+}$ dissociation$^d$ | $k_{act}$ for $E_2 \rightarrow [Ca_{2+}]E_0^*$ |
|-------------------|--------------------------|-----------------------------------------------|---------------------------------|---------------------------------|
| Wild type         | 1                        | 1                                             | 1                              | 1                               |
| Glu$^{33}$ → Arg  | 0.84 ± 0.08              | 0.86 ± 0.02                                   | 0.92 ± 0.04                    | 1.27 ± 0.40                     | 0.79 ± 0.08                    |
| Glu$^{33}$ → Leu  | 1.08 ± 0.21              | 1.18 ± 0.25                                   | 0.80 ± 0.03                    | 0.86 ± 0.09                     | 0.78 ± 0.11                    |
| Glu$^{33}$ → Arg  | 1.00 ± 0.00              | 0.67 ± 0.05                                   | 0.79 ± 0.06                    | 1.14 ± 0.27                     | 0.80 ± 0.06                    |
| Glu$^{33}$ → Leu  | 0.87 ± 0.01              | 0.93 ± 0.10                                   | 0.89 ± 0.06                    | 0.77 ± 0.09                     | 0.97 ± 0.07                    |
| Phe$^{32}$ → Gin  | 0.94 ± 0.10              | 1.08 ± 0.07                                   | 1.20 ± 0.06                    | 0.73                            | 0.98 ± 0.07                    |
| Phe$^{32}$ → Ala  | 0.74 ± 0.01              | 0.98 ± 0.02                                   | 1.53 ± 0.09                    | 0.77                            | 0.88 ± 0.08                    |
| Glu$^{33}$ → Arg  | 1.03 ± 0.21              | 0.99 ± 0.09                                   | 0.84 ± 0.04                    | 0.95 ± 0.09                     | 0.84 ± 0.04                    |
| Glu$^{33}$ → Leu  | 0.99 ± 0.13              | 0.95 ± 0.06                                   | 0.76 ± 0.04                    | 1.14 ± 0.09                     | 0.98 ± 0.11                    |
| Asp$^{39}$ → Arg  | 0.97 ± 0.11              | 0.58 ± 0.11                                   | 0.49 ± 0.00                    | 0.86 ± 0.01                     | 1.06 ± 0.04                    |
| Asp$^{39}$ → Leu  | 0.15 ± 0.01              | 0.32 ± 0.01                                   | 1.01 ± 0.10                    | 0.10 ± 0.01                     | 1.01 ± 0.04                    |
| Asp$^{39}$ → Ala  | 0.74 ± 0.03              | 0.57 ± 0.01                                   | 0.91 ± 0.04                    | 0.91                            | 0.10 ± 0.04                    |
| Leu$^{60}$ → Ala  | 0.99 ± 0.08              | 1.19                                          | 3.07 ± 0.21                    | 0.50 ± 0.05                     | 0.76 ± 0.06                    |
| Leu$^{60}$ → Pro  | 1.01 ± 0.05              | 0.69 ± 0.07                                   | 1.85 ± 0.11                    | 0.55 ± 0.09                     | 0.83 ± 0.06                    |
| Val$^{62}$ → Ala  | 1.30 ± 0.03              | 0.92 ± 0.15                                   | 2.09 ± 0.08                    | 0.32 ± 0.01                     | 1.33 ± 0.11                    |
| Leu$^{60}$ → Ala  | 0.03 ± 0.01              | 0.08 ± 0.04                                   | 0.61 ± 0.04                    | 11.0 ± 1.1                      | 1.22 ± 0.11                    |

| $K_{m,5}$ for Ca$^{2+}$ activation of phosphorylation$^c$ | $t_{1/2}$ of Ca$^{2+}$ dissociation$^d$ | $k_{act}$ for $E_2 \rightarrow [Ca_{2+}]E_0^*$ |
|------------------------------------------------------------|---------------------------------|---------------------------------|
| 1                                                          | 1                              | 1                               |

$^a$ The maximum rate of Ca$^{2+}$-activated ATP hydrolysis was determined by measurement of Ca$^{2+}$-dependent P liberation (21) at 37 °C. The medium contained 50 mM TES/Tris (pH 7.0), 100 mM KCl, 5 mM ATP, 7 mM MgCl$_2$, 1 μM Ca$^{2+}$-ionophore A23187, 1 mM EGTA, and either no added CaCl$_2$ (control) or 0.9 mM CaCl$_2$ (giving a free Ca$^{2+}$ concentration of 3 μM). Following subtraction of background, the molecular turnover rate was calculated as the ratio between the rate of ATP hydrolysis and the active-site concentration, using as active-site concentration the maximum amount of phosphoenzyme that can be formed from ATP (cf. Fig. 1), and the resulting data are shown relative to wild type (turnover rate 110 s$^{-1}$).

$^b$ Measurement of ATP-driven Ca$^{2+}$ transport was carried out by filtration following incubation for 5 min at 37 °C in a medium containing 20 mM MOPS/Tris (pH 6.8), 100 mM KCl, 5 mM ATP, 5 mM MgCl$_2$, 0.5 mM EGTA, 0.45 mM CaCl$_2$, and 5 mM potassium oxalate to trap Ca$^{2+}$ in the lumen of the microsomal vesicles. To correct for expression level, the data were related to the active-site concentration as described above for ATP hydrolysis, and the results are shown relative to wild type.

$^c$ Data obtained as for Fig. 1. $K_{m,5}$ values are shown relative to that of the wild type determined in the same series of experiments (0.89 ± 0.02 μM).

$^d$ Data obtained as for Fig. 2. $t_{1/2}$ values are shown relative to that of the wild type determined in the same series of experiments (0.22 ± 0.03 s).

$^e$ Data obtained as for Fig. 3. $k_{act}$ values are shown relative to that of the wild type determined in the same series of experiments (9.0 ± 0.5 s$^{-1}$).

Examination of the mutational effect on Ca$^{2+}$ titration of phosphorylation therefore provides information about the Ca$^{2+}$-binding properties. The Ca$^{2+}$ dependence of steady-state phosphorylation was studied at 0 °C, where phosphoenzyme accumulation is favored. Fig. 1 shows the titration curves for some of the mutants, and Table I summarizes the data for all mutants. Substitution of Glu$^{51}$, Glu$^{55}$, or Glu$^{58}$ by either leucine or arginine had no significant effect on the apparent Ca$^{2+}$ affinity, as exemplified by Glu$^{58}$ → Arg in Fig. 1 (cf. Fig. 1 and Table I). In contrast, arginine substitution of Asp$^{39}$ caused a 5-fold reduction of apparent Ca$^{2+}$ affinity (increase of $K_{m,5}$) compared with wild type, whereas the apparent affinity for Ca$^{2+}$ was unaffected by leucine or alanine substitution of this residue. These results are in accordance with a previous study in which the Ca$^{2+}$ dependence of Ca$^{2+}$ transport was found normal in a cluster mutant with alanine substitutions of Glu$^{55}$, Glu$^{56}$, Glu$^{58}$, and Asp$^{39}$ (25). The mutants Phe$^{37}$ → Ala, Leu$^{60}$ → Ala/Pro, and Val$^{62}$ → Ala, with alterations to hydrophobic residues, displayed moderate reductions of apparent Ca$^{2+}$ affinity (1.5- to 3-fold increase of $K_{m,5}$). On the contrary, Leu$^{65}$ → Ala displayed higher affinity for Ca$^{2+}$ than wild type, the $K_{m,5}$ being reduced to 61% of the corresponding value for the wild type (Fig. 1 and Table I).

Rate of Ca$^{2+}$ Dissociation toward the Cyttoplasmic Side—To determine more directly the effects of the mutations on Ca$^{2+}$ binding, we examined the rate of Ca$^{2+}$ dissociation toward the cytoplasmic side of the [Ca$^{2+}]E_0$ form at pH 6.0, 25 °C (Fig. 2), using a previously described quenched flow technique (23). Mutant or wild-type enzyme pre-equilibrated with saturating amounts of Ca$^{2+}$ was treated with an excess of the Ca$^{2+}$ chelator EGTA for various time intervals prior to a 34-ms incubation with [$\gamma$-32P]ATP testing the ability to phosphorylate. Because occupancy of the Ca$^{2+}$ binding sites is required to render the enzyme phosphorylatable from ATP, the time course of disappearance of the ability to phosphorylate reflects the dissociation of Ca$^{2+}$. Because the enzyme is not phosphorylated at the time when Ca$^{2+}$ dissociates, the dissociation must occur.
toward the cytoplasmic side of the membrane. It is believed that the ability to phosphorylate disappears with the dissociation of the first Ca2+ ion in a sequential mechanism (26). Fig. 2 shows such time courses for some of the mutants, and Table I summarizes the results for all mutants in terms of the half-life of the Ca2+-bound enzyme form capable of undergoing phosphorylation. The half-lives corresponding to mutants Glu51→Arg/Leu, Glu55→Arg/Leu, Glu56→Arg/Leu, and Asp59→Ala were indistinguishable from that of the wild type, whereas a decrease of around 10-fold was determined for Asp59→Arg/Leu, corresponding to a 10-fold higher rate of Ca2+ dissociation (Fig. 2 and Table I). Furthermore, the Ca2+ dissociation kinetics was quite sensitive to the size of the hydrophobic side chains in the middle part of M1. Hence, Leu60→Ala/Pro and Val62→Ala displayed markedly reduced half-lives relative to wild type, i.e. corresponding to a 2- to 3-fold increase of the Ca2+ dissociation rate. Most conspicuously, alanine substitution of Leu60 resulted in an 11-fold increase of the half-life (reduction of the rate of Ca2+ dissociation). Mutation of Phe57→Val resulted in a 10-fold higher rate of Ca2+ dissociation (Fig. 2 and Table I). The (relative) $K_{d,2}$ values are listed in Table I together with the corresponding values for other mutants obtained in the same way.

**Fig. 1. Ca2+ dependence of phosphorylation from $\gamma^{32P}$ATP.** Phosphorylation was performed at 0 °C for 15 s in 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl2, 1 mM EGTA, 5 μM $\gamma^{32P}$ATP, and CaCl2 to obtain the indicated concentrations of free Ca2+. The Hill equation, $[Ca^{2+}]_E = [Ca^{2+}]_I + [Ca^{2+}]_I^*/[Ca^{2+}]^*$, was fitted to the data, and the results were normalized separately for wild type and each mutant, taking $ET_{max}$ as 100%. The (relative) $K_{d,2}$ values are listed in Table I together with the corresponding values for other mutants obtained in the same way.

**Fig. 2. Time course of Ca2+ dissociation toward the cytoplasmic side determined by loss of ability to phosphorylate.** The kinetics of Ca2+ dissociation toward the cytoplasmic side was monitored at 25 °C by the loss of ability to undergo phosphorylation from $\gamma^{32P}$ATP, using a Bio-Logic quenched flow module SFM-400/Q and the previously described mixing protocol (23). Wild-type or mutant Ca2+-ATPase preincubated in 40 mM MES/Tris (pH 6.0), 80 mM KCl, 5 mM MgCl2, and 100 μM CaCl2 was mixed with an equal volume of 40 mM MES/Tris (pH 6.0), 80 mM KCl, 5 mM MgCl2, and 4 mM EGTA, followed by the addition of the double volume of 40 mM MES/Tris (pH 6.0), 80 mM KCl, 5 mM MgCl2, 2 mM EGTA, and 10 μM $\gamma^{32P}$ATP at the times indicated on the abscissa, and acid quenching 34 ms later. A mono- or bi-exponential function was fitted to the data and the half-life ($t_{1/2}$) determined. The (relative) $t_{1/2}$ values are listed in Table I together with the corresponding values for other mutants obtained in the same way.

**Vanadate Dependence of Inhibition of Phosphorylation from $\gamma^{32P}$ATP—Vanadate (VO4$^-$), acting as an analogue of the phosphoryl group in the transition state during dephosphorylation, binds preferentially to the $E_2$ form (28, 29). Hence, the apparent affinity for vanadate can be used to quantify mutational effects on the equilibrium between $E_2$ and $E_1$ conformations, provided that the intrinsic affinity of the $E_2$ form for vanadate is not affected by the mutations. We have previously devised an assay in which the enzyme is equilibrated with various concentrations of vanadate at 25 °C in the absence of
Ca\(^{2+}\) and ATP, followed by determination of the amount of phosphoenzyme formed upon the addition of Ca\(^{2+}\) and \(\gamma\text{-}[\text{P}]\text{ATP}\) at 0°C (23, 30). Because vanadate binding and phosphorylation are competitive, and vanadate dissociation is very slow at 0°C, the amount of phosphoenzyme formed from ATP under these conditions reflects the proportion of enzyme phosphotyrosine. Consequently, vanadate dissociation is consistent with a displacement of the \(E_2\text{-}E_1\) transition rate in asp\(^{59}\) to Arg caused by an enhanced rate of the \(E_2\text{-}E_1\) conformational change of \(Ca^{2+}\)-free enzyme (Reaction 1 in Scheme 1). Among the other mutants, Leu\(^{60}\) → Ala and Val\(^{62}\) → Ala showed a slight (2-fold) increase in vanadate affinity, suggesting that there could be a displacement of the \(E_2\text{-}E_1\) equilibrium in favor of \(E_2\).

Time Course of Dephosphorylation of Phosphoenzyme Formed from \(\gamma\text{-}[\text{P}]\text{ATP}\)—To study the effects of the mutations on the processing of the phosphoenzyme, we first followed the decay of phosphoenzyme formed from \(\gamma\text{-}[\text{P}]\text{ATP}\) at pH 7.0, 0°C, presence of K\(^+\), i.e. conditions where it is well recognized that the \(Ca^{2+}\text{E}_1\) transition (cf. Reaction 4 in Scheme 1) is the major rate-limiting step during phosphoenzyme turnover of the wild-type enzyme (31, 32). To observe the dephosphorylation, phosphoenzyme from \(\gamma\text{-}[\text{P}]\text{ATP}\) was terminated by simultaneous addition of EGTA and excess non-labeled ATP. Fig. 5 shows the phosphoenzyme decay for some of the mutants, and Table II summarizes the results for all mutants in terms of the rate constants obtained by fitting a monoexponential decay function to the data. The rate of disappearance of phosphoenzyme was wild type-like in mutants Glu\(^{51}\) → Arg, Leu, and Glu\(^{53}\) → Leu, and asp\(^{59}\) → Arg, whereas asp\(^{59}\) → Ala, in contrast, displayed a strikingly reduced appearance of K\(^+\) i.e. conditions where it is well recognized that the \(Ca^{2+}\text{E}_1\) transition (cf. Reaction 1 in Scheme 1), was observed for Leu\(^{66}\) → Ala, whereas Leu\(^{66}\) → Ala and Val\(^{62}\) → Ala were slightly affected in the opposite direction (1.7-fold increase).

To distinguish between effects on Reactions 4–6 in Scheme 1, we determined the ADP sensitivity of the phosphoenzyme intermediate accumulated after phosphorylation with \(\gamma\text{-}[\text{P}]\text{ATP}\), i.e. its ability to react with ADP and donate the phosphonyl group back to ADP, forming ATP. Only \(Ca^{2+}\text{E}_1\) is able to undergo this reaction.

![Fig. 3. Time course of phosphorylation of asp\(^{59}\) → Arg and wild type following addition of \(\gamma\text{-}[\text{P}]\text{ATP}\) and \(Ca^{2+}\) to Ca\(^{2+}\)-depleted enzyme in \(E_2\) form.](image-url)

**Analysis of partial reaction steps involved in phosphoenzyme processing and vanadate binding**

| \(K_{0.5}\) for VO\(^{-}\) inhibition of phosphorylation | \(k_{dephos}\) for dephosphorylation of phosphoenzyme formed from ATP | EP remaining 5 s after ADP addition | \(K_{0.5}\) for P\(_i\) phosphorylation | \(k_{dephos}\) for dephosphorylation at pH 6.0 | \(k_{dephos}\) for dephosphorylation at pH 7.0 | \(k_{dephos}\) for \(Ca^{2+}\text{E}_1\) → \(Ca^{2+}\text{E}_2\) |
|---|---|---|---|---|---|---|
| Wild type | 1 | 1 | 1.0 ± 0.3 | 1 | 1 | 1 |
| Glu\(^{51}\) → Arg | ND | ND | 0.75 ± 0.07 | 1.6 | 1.06 ± 0.14 | ND | ND |
| Glu\(^{53}\) → Leu | ND | ND | 0.84 ± 0.09 | 3.4 | 1.22 ± 0.15 | 0.07 | ND |
| Glu\(^{55}\) → Arg | ND | ND | 0.84 ± 0.05 | 4.2 | 1.17 ± 0.16 | ND | ND |
| Glu\(^{58}\) → Leu | ND | ND | 0.77 ± 0.05 | 3.6 | 1.34 ± 0.18 | ND | ND |
| Phe\(^{61}\) → Glu | 0.85 ± 0.10 | 0.84 ± 0.11 | 1.6 | 1.83 ± 0.24 | 1.87 ± 0.27 | ND | ND |
| Phe\(^{63}\) → Ala | 0.75 ± 0.20 | 0.75 ± 0.07 | 5.5 | 1.95 ± 0.24 | 1.95 ± 0.27 | ND | ND |
| Phe\(^{65}\) → Arg | ND | ND | 0.82 ± 0.02 | 1.4 | 0.94 ± 0.14 | 0.72 ± 0.10 | ND |
| Phe\(^{68}\) → Leu | ND | ND | 0.77 ± 0.07 | 1.5 | 0.84 ± 0.20 | 0.72 ± 0.10 | ND |
| Asp\(^{59}\) → Arg | 4.70 ± 0.60 | 0.93 ± 0.11 | 4.1 ± 0.4 | 6.10 ± 1.59 | 8.00 ± 0.67 | ND | ND |
| Asp\(^{59}\) → Leu | 0.90 ± 0.15 | 0.17 ± 0.02 | 7.2 ± 7.0 | 0.65 ± 0.07 | 0.23 ± 0.03 | 0.10 ± 0.01 | 3.42 ± 0.42 |
| Asp\(^{59}\) → Ala | 0.85 ± 0.25 | 0.68 ± 0.07 | 1.4 | 1.22 ± 0.24 | 0.72 ± 0.07 | ND | ND |
| Leu\(^{60}\) → Ala | 0.55 ± 0.05 | 1.73 ± 0.23 | 4.3 ± 0.3 | 1.09 ± 0.15 | 1.48 ± 0.14 | 0.70 ± 0.13 | ND |
| Leu\(^{62}\) → Pro | 0.75 ± 0.10 | 0.95 ± 0.09 | 3.0 ± 0.7 | 4.02 ± 0.37 | 4.95 ± 0.40 | 4.26 ± 0.43 | ND |
| Val\(^{62}\) → Ala | 0.55 ± 0.05 | 0.68 ± 0.14 | 3.5 ± 0.7 | 1.83 ± 0.12 | 2.34 ± 0.14 | ND | ND |
| Leu\(^{65}\) → Ala | 0.15 ± 0.20 | 0.09 ± 0.02 | 1.0 | 0.37 ± 0.04 | 0.03 ± 0.01 | 0.03 ± 0.00 | 4.16 ± 0.32 |

1 Data obtained as for Fig. 4. \(K_{0.5}\) values are shown relative to that of the wild type determined in the same series of experiments (0.20 ± 0.02 μM).
2 Data obtained as for Fig. 5. \(k_{dephos}\) values are shown relative to that of the wild type determined in the same series of experiments (0.44 ± 0.06 s\(^{-1}\)).
3 Following phosphorylation from \(\gamma\text{-}[\text{P}]\text{ATP}\) under conditions identical to those described for Fig. 5, 1 mm ADP was added and allowed to react for 5 s. The percentage of phosphoenzyme remaining after 5 s is shown.
4 Phosphorylation was carried out for 10 min at 25°C in a buffer containing 100 μM MES-Tris (pH 6.0), 2 μM EGTA, 30% (v/v) MeSO, 10 mM MgCl\(_2\), and varying concentrations of \(32P\). The Hill equation was fitted to the data, and \(K_{0.5}\) values are shown relative to that of the wild type determined in the same series of experiments (8.22 ± 0.62 μM).
5 Data obtained as for Fig. 7. \(k_{dephos}\) values are shown relative to that of the wild type determined in the same series of experiments (0.47 ± 0.03 s\(^{-1}\)).
6 Data obtained as for Fig. 8. \(k_{dephos}\) values are shown relative to that of the wild type determined in the same series of experiments (0.19 ± 0.02 s\(^{-1}\)).
7 ND, not determined.
8 Data obtained as for Fig. 6. \(k_{dephos}\) values are shown relative to that of the wild type determined in the same series of experiments (0.015 ± 0.001 s\(^{-1}\)).
9 Data obtained as for Fig. 6. \(k_{dephos}\) values are shown relative to that of the wild type determined in the same series of experiments (0.029 ± 0.003 s\(^{-1}\)).
reaction. Following phosphorylation of wild-type and mutant Ca\(^{2+}\)-ATPase under conditions identical to those described for Fig. 5, 1 mM ADP was added and allowed to react for 5 s. For the wild type, only about 1% of the original amount of phosphoenzyme remained after this incubation, thus demonstrating the accumulation of the \([\text{Ca}^{2+}]E_1P\) form. For Asp\(^{59}\) \(\rightarrow\) Leu and Leu\(^{60}\) \(\rightarrow\) Ala, as much as 72 and 82% of the phosphoenzyme remained after treatment with ADP for 5 s, respectively (Table II), indicating accumulation of ADP-insensitive \(E_1P\) phosphoenzyme occurred (or possibly \(\text{Ca}_2E_2P\), which is thought to be rather unstable in the wild type, but might be stabilized by mutation).

For all the other mutants, the ADP sensitivity was wild-type-like, and the ADP-insensitive \(E_1P\) form (Figs. 6 and 7), as well as the rate of dephosphorylation of \(E_1P\) phosphoenzyme at pH 7.0, indicating that \([\text{Ca}^{2+}]E_1P\) accumulated, and in these cases the dephosphorylation rate observed in the experiments corresponding to Fig. 5 reflects the \([\text{Ca}^{2+}]E_1P \rightarrow \text{Ca}_2E_2P\) transition. The anomalous accumulation of ADP-insensitive phosphoenzyme seen for Asp\(^{59}\) \(\rightarrow\) Leu and Leu\(^{60}\) \(\rightarrow\) Ala upon phosphorylation with ATP could in principle result from an enhanced rate of the \([\text{Ca}^{2+}]E_1P \rightarrow \text{Ca}_2E_2P\) transition (Reaction 4 in Scheme 1) or from a reduced rate of the subsequent steps (Reactions 5 and 6 in Scheme 1). The reduced rate of phosphoenzyme decay observed for these mutants in Fig. 5 suggests the latter possibility. To clarify this issue further, we measured directly the dephosphorylation of the \(E_1P\) form (Figs. 6 and 7), as well as the rate of
Importance of M1 of the SR Ca\(^{2+}\)-ATPase

Phosphorylation was performed at 0 °C for the indicated time intervals in 40 mM TES/Tris (pH 8.0), 80 mM LiCl, 10 mM MgCl\(_2\), 50 mM CaCl\(_2\), 10 mM calcium ionophore A23187, and 5 μM [γ-\(^{32}\)P]ATP. At the indicated time intervals after initiation of phosphorylation, an equal volume of 40 mM TES/Tris (pH 8.0), 80 mM LiCl, 10 mM MgCl\(_2\), 10 mM EGTA, and 2 mM ADP was added to remove ADP-sensitive phosphoenzyme, followed by acid quenching 4 s later. A monoeXponential function was fitted to the data, and the results were normalized separately for wild type and mutant, taking the phosphorylation corresponding to infinite time as 100%. The (relative) rate constant is shown in Table II together with the corresponding value for Asp\(^{59}\) → Leu obtained in the same series of experiments.

**DISCUSSION**

The present results provide the first functional evidence that transmembrane segment M1 of the Ca\(^{2+}\)-ATPase is critical to Ca\(^{2+}\) interaction and to phosphoryzyme turnover. During active Ca\(^{2+}\) transport, the Ca\(^{2+}\)-ATPase binds Ca\(^{2+}\) from the cytoplasmic side of the membrane, transfers the ions across the membrane, and releases them on the luminal side. It is generally believed that the translocation of Ca\(^{2+}\) across the membrane involves an “occluded” state, where the ions are inaccessible to the medium on either side of the membrane and their dissociation restricted by structural components acting as gates. In the occluded state, Ca\(^{2+}\) is bound by residues in M4, M5, M6, and M8 (8, 10–12). The Ca\(^{2+}\) ions may become occluded within the protein concomitantly with phosphorylation from ATP (5, 33). Evidence has, however, been presented that the Ca\(^{2+}\) ions are not only occluded in the phosphoryzyme, but also most of the time in the non-phosphorylated Ca\(^{2+}\)-bound form (6), as indicated by the *brackets* in Scheme 1. This seems to agree with the crystal structure of the Ca\(^{2+}\)-ATPase with bound Ca\(^{2+}\), revealing no obvious Ca\(^{2+}\) entry or exit pathways (8). Furthermore, the fact that Ca\(^{2+}\) can dissociate only very slowly from the enzyme complex with CrATP, even though the γ-phosphoryl group is not transferred to the enzyme in this complex, indicates that phosphorylation is not required for occlusion of Ca\(^{2+}\) (7). The present findings are consistent with the existence of a Ca\(^{2+}\)-occluded non-phosphorylated state, whose stability depends on the structural properties of M1. Our measurements indicate a 10-fold increase of the rate of Ca\(^{2+}\) dephosphorylation.
dissociation from this state toward the cytoplasmic side is in mutants Asp59 → Arg and Asp59 → Leu, whereas Ca2+ dissociation was wild type-like in the mutants with arginine or leucine substitution of Glu51, Glu55, or Glu58 (Fig. 2 and Table I). The Phe57 mutants also displayed wild type-like Ca2+ dissociation, whereas Leu50 → Ala/Pro and Val62 → Ala caused an acceleration of Ca2+ dissociation, although to a lesser extent than the Asp59 mutations. Moreover, a remarkable 11-fold reduction of the rate of Ca2+ dissociation toward the cytoplasmic side was observed for Leu60 → Ala. This demonstration of the role of M1 in Ca2+ occlusion was made feasible by the quenched flow technique that allows measurement of Ca2+ dissociation from the [Ca2+]E1 form toward the cytoplasmic side, even with the relatively small amounts of enzyme that can be harvested from the cell culture. Thus, the rate of Ca2+ dissociation from the [Ca2+]E1 form can be both increased and decreased by mutation within the middle part of M1 (C-terminal to the bend of M1 seen in the E2-Tg crystal structure), and our results suggest that the middle part of M1, but not the most N-terminal part containing Glu51, Glu55, and Glu58, is important in control of the gates at the Ca2+ occlusion sites.

As regards the proposed role of Glu51, Glu55, and Glu58 in the Ca2+ entry pathway (14), the results reported here clearly argue against any involvement of these glutamate side chains in Ca2+ recognition and binding, and it is not likely that they contribute to a Ca2+ selectivity filter. Furthermore, the finding that substitution of Glu58 by arginine left the Ca2+ binding properties of the Ca2+-ATPase unaffected questions the existence of a close interaction between Glu58 and Glu309 at Ca2+ binding site II, such as seen in the published crystal structure of the Ca2+-bound enzyme (8). A possible reason for this apparent discrepancy could be a high degree of thermal mobility of the Glu58 side chain. It is also possible that the [Ca2+]E1 form adopted in the native state differs more profoundly from the crystal structure.

The reduced apparent affinity of mutant Asp59 → Arg for Ca2+ activation of phosphorylation (Fig. 1) reflects the enhanced rate of Ca2+ dissociation from [Ca2+]E1, and not a displacement of the E2-E1 equilibrium in favor of the low affinity E1 form. In fact, the data in Figs. 3 and 4 suggest that mutation Asp59 → Arg enhances the rate of the E2 → E1 conformational change, leading to accumulation of E1 with resulting low sensitivity to vanadate inhibition of phosphoenzyme formation. Because release of counter-transported protons from E2 may be rate-limiting for the E2 → E1 transition (27), the enhancement of this transition suggests that proton dissociation from the transport sites, like Ca2+ dissociation, is facilitated by the Asp59 → Arg mutation.

For mutants Leu60 → Ala/Pro and Val62 → Ala, the enhanced rate of Ca2+ dissociation likewise seems to result in a reduced apparent affinity for Ca2+ activation of phosphorylation. In addition, there could be a contribution to the reduced apparent affinity for Ca2+ from displacement of the E2-E1 equilibrium in favor of the low affinity E2 form in Leu60 → Ala and Val62 → Ala, because the vanadate affinity was slightly increased in these mutants (Table II). The apparent affinity for Ca2+ inactivation of phosphorylation was normal in Asp59 → Leu, despite the increased Ca2+ dissociation rate (Figs. 1 and 2). This may be accounted for by a considerably reduced rate of dephosphorylation (Figs. 5–7). As previously reported, the effect of a low rate of phosphoenzyme turnover on the apparent affinity for Ca2+ activation of phosphorylation can be understood on the basis of computer simulations of the Ca2+-ATPase reaction cycle (34). According to the computational analysis, an increased apparent affinity for Ca2+ is actually expected when the rate of phosphoenzyme turnover is reduced, because a lower phosphorylation rate (i.e. Ca2+ saturation) is required to maintain a certain level of phosphorylation under these conditions. Hence, for Asp59 → Leu, the increased rate of Ca2+ dissociation and the decreased rate of phosphoenzyme turnover act in opposite directions, thereby masking the effects on the apparent Ca2+ affinity.

The importance of the middle part of M1 in the gating at the Ca2+ occlusion sites may be related to the presence of a water-accessible channel leading between M1 and M3 in the crystal structure of Ca2+-ATPase in the Ca2+-free E2-Tg form. This channel has the potential of a Ca2+-entry port and is apparently opened by the bending and partial unfolding of the helical structure of M1 at Asp59 (9). A similar channel may exist in the native non-crystalline protein and function as a migration pathway for Ca2+ in one or more of the conformations that precede the occluded [Ca2+]E1 form in the process of Ca2+ binding (E3, E1, and CaE). Because the channel leads to Glu509 at Ca2+ site II, it may provide a passage for the Ca2+ ion that binds at site II (i.e. the one that binds last in the sequential mechanism). In line with this assignment, the presently observed mutational effects on the rate of disappearance of ability to phosphorylate from ATP upon addition of EGTA (Fig. 2) reflect changes of the rate of dissociation of the Ca2+ ion that leaves first in the sequential mechanism, i.e. the one that was bound last (26, 35). The Ca2+ ion that binds first (at site I, cf. Ref. 12) might enter (and leave upon dissociation) by a different route. Some evidence has actually been presented suggesting the existence of a pre-binding site for Ca2+ in the loop between transmembrane segments M6 and M7 (“L6–7”), which could be related to the entry port for the Ca2+ ion binding at site I (36).

Comparison of the two crystal structures of the Ca2+-ATPase indicates that the transition from [Ca2+]E1 to E2-Tg is accompanied by a lateral and upward (toward the cytoplasmic side) movement of M1 in the membrane, and bending of the helix at Asp59, which probably is caused by steric collision with M3 (9). In E2-Tg, the N-terminal part of M1 containing Glu51, Glu55, and Glu58 is bent away from M3, and their side chains define the hydrophilic surface of the amphiphilic section. These residues are therefore peripherally located with respect to the proposed Ca2+ entrance. A similar position of this part of M1 in the native non-crystalline protein could explain the lack of importance of the glutamate side chains for Ca2+ binding. Asp59, on the other hand, is located right at the bending point of M1. Thus, the dramatic effect on Ca2+ dissociation of substitution of Asp59 by leucine or arginine may result from direct interference with Ca2+ interaction or interference with the movement of M1 that occludes Ca2+, a movement that conceivably is facilitated by flexibility at Asp59. The finding that alanine substitution of Asp59 is tolerated stresses the requirement for a small side chain that allows movement. The negative charge of the Asp59 side chain, which might have been expected to participate in directing Ca2+ to the binding sites, seems not to be required for normal Ca2+ interaction.

Among the hydrophobic side chains substituted in the present study, Phe57 seems less important for the Ca2+-binding properties and the E2 → E1 conformational transition than expected if it were crucial to the attachment of the bent part of M1 to the lipid bilayer and, thereby, to the positioning of this part of M1. On the other hand, the lack of a significant effect on Ca2+ binding of substitution of Phe57 may be considered consistent with its location away from the proposed Ca2+ inlet, on the hydrophobic surface of the bent part of M1, and it is conceivable that the removal of a single anchoring side chain is not enough to disrupt the attachment to the lipid bilayer. As regards Leu60, Val62, and Leu65, which are embedded within the membrane, their bulky hydrophobic side chains appear to form
an integral part of the wall lining the water-accessible channel seen in the $E_2$-Tg structure, with Leu$^{65}$ at the bottom. Hence, our finding of significant changes of the Ca$^{2+}$ dissociation rate upon substitution of these residues with the smaller alanine seems to be compatible with the hypothesis that this channel serves as a migration pathway for Ca$^{2+}$. The effects of hydrophobic size reduction in this part of M1 could simply be due to local changes in the proportions of the channel, providing a more free passage for the dissociating Ca$^{2+}$ ions in case of Leu$^{60}$ → Ala/Pro and Val$^{62}$ → Ala and collapsing the channel in case of the Leu$^{65}$ → Ala mutation. Of course, it cannot be excluded that changes to M1 exert more distant effects on a Ca$^{2+}$ migration pathway located elsewhere in the protein.

In the crystal structure of the Ca$^{2+}$-bound enzyme, Leu$^{65}$ is below the level of the Ca$^{2+}$ ions (i.e. closer to the luminal surface), and it seems that its bulky side chain may contribute to form a luminal gate, preventing the immediate transport of the ions to the lumen upon binding from the cytoplasm. A premature transfer of the Ca$^{2+}$ ions to the lumen caused by a defective luminal gate in Leu$^{65}$ → Ala (i.e. rapid formation of an enzyme form somewhat similar to Ca$^{2+}$-E2P) could account for the rapid disappearance of ADP sensitivity of the phosphoenzyme (Table II), which again might indicate premature Ca$^{2+}$ transfer to luminal facing sites. In addition to the enhanced rate of disappearance of ADP sensitivity of the phosphoenzyme (Table II), these two mutations also affected the processing of the phosphoenzyme (Reactions 4 and 5), which again might indicate premature Ca$^{2+}$ transfer to the catalytic site in these forms (9, 37–39).}

In conclusion, our results provide evidence for a relation of the middle part of M1 to a gating mechanism controlling the dissociation of Ca$^{2+}$ from its luminal bound sites. Our results further indicate that these residues are also critical to the proper processing of the phosphoenzyme and, thus, to the long range transmission of conformational changes to the cytoplasmic catalytic site.

**Acknowledgments**—We thank Karin Kracht and Lene Jacobsen for expert technical assistance and Dr. J. D. Clausen (University of Aarhus) for discussion and help with some experiments. Dr. C. Toyoshima (University of Tokyo) is thanked for discussion on several occasions and Dr. R. J. Kaufmann (Genetics Institute, Boston) for the gift of the expression vector pMT2.

**REFERENCES**

1. de Meis, L., and Vianna, A. L. (1979) *Annu. Rev. Biochem.* 48, 275–292
2. Lehnert, G., Blotta, S., and Kortylewski, M. R. (1990) *Biochem. Biophys. Res. Commun.* 170, 749–760
3. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) *J. Biol. Chem.* 272, 26815–26818
4. Stokes, D. L., and Green, N. M. (2000) *Annu. Rev. Biophys. Biomol. Struct.* 32, 445–468
5. Dupont, Y. (1980) *Eur. J. Biochem.* 109, 231–238
6. Mantsch, H. H., and Schürmann, M. (1991) *Biochemistry* 30, 352–361
7. Vilsen, B. (1995) *Acta Physiol. Scand.* 154, suppl. 624, 1–146
8. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) *Nature* 405, 457–455
9. Toyoshima, C., and Nomura, H. (2002) *Nature* 418, 605–611
10. Clarke, D. M., Luo, T. W., Inesi, G., and MacLennan, D. H. (1989) *Nature* 339, 476–478
11. Vilsen, B., and Andersen, J. P. (1992) *J. Biol. Chem.* 267, 25739–25743
12. Andersen, J. P., and Vilsen, B. (1994) *J. Biol. Chem.* 269, 15931–15936
13. Dahlöf, T., Yamazaki, K., Wang, G., Danko, S., Izuka, H., and Suzuki, H. (2003) *J. Biol. Chem.* 278, 39197–39204
14. Lee, A. G., and East, J. M. (2001) *J. Biol. Chem.* 276, 7361–7366
15. Lipkind, G. M., and Fozzard, H. A. (2000) *J. Biol. Chem.* 275, 6766–6794
16. Leitner, G., and Jencks, W. P. (1988) *Biochemistry* 27, 5553–5564
17. Forge, V., Mintz, E., and Guillain, F. (1993) *J. Biol. Chem.* 268, 8161–8170
18. Lipkind, G. M., and Fozzard, H. A. (2001) *Biochemistry* 40, 6755–6759
19. Kaufman, R. J., Davies, M. V., Pathak, V. K., and Hershey, J. W. (1989) *Mol. Cell. Biol.* 9, 946–958
20. Chen, C., and Okaya, H. (1987) *Mol. Cell. Biol.* 7, 2745–2752
21. Maruyama, K., and MacLennan, D. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 3314–3318
22. Vilsen, B., Andersen, J. P., and MacLennan, D. H. (1991) *J. Biol. Chem.* 266, 16157–16164
23. Sorensen, T., Vilsen, B., and Andersen, J. P. (1997) *J. Biol. Chem.* 272, 30244–30250
24. Sorensen, T. L., Dupont, Y., Vilsen, B., and Andersen, J. P. (2000) *J. Biol. Chem.* 275, 5404–5408
25. Clausen, J. D., and Andersen, J. P. (2003) *Biochemistry* 42, 2565–2594
26. Andersen, J. P., Vilsen, B., Leberer, E., and MacLennan, D. H. (1989) *J. Biol. Chem.* 264, 21018–21023
27. Clarke, D. M., Maruyama, K., Luo, T. W., Leberer, E., Inesi, G., and MacLennan, D. H. (1989) *J. Biol. Chem.* 264, 11246–11251
28. Pitotchy, J. R., and Jencks, W. P. (1988) *Biochemistry* 27, 5553–5564
29. Forge, V., Mintz, E., and Guillain, F. (1993) *J. Biol. Chem.* 268, 10961–10968
30. Cantley, L. C., Jr., Cantley, L. G., and Josephson, L. (1978) *J. Biol. Chem.* 253, 7361–7368
31. Pick, U. (1982) *J. Biol. Chem.* 257, 611–619
32. Dole, L., Vilsen, B., Van Baelen, K., Waytack, F., Clausen, J. D., and Andersen, J. P. (2002) *J. Biol. Chem.* 277, 45579–45591
33. Lund, S., and Moller, J. V. (1988) *J. Biol. Chem.* 263, 1654–1664
34. Andersen, J. P., Lassen, K., and Moller, J. V. (1985) *J. Biol. Chem.* 260, 371–380
35. Nakamura, H., and Makinose, M. (1981) *Nature* 290, 271–273
36. Andersen, J. P., Sorensen, T. L., Povlsen, K., and Vilsen, B. (2001) *J. Biol. Chem.* 276, 23312–23321
37. Inesi, G. (1987) *J. Biol. Chem.* 262, 16338–16342
38. Mung, T., Corre, F., Juul, B., Bouneau, L., Latifte, D.,errick, P. J., Sharma, P. S., Folsom, P., Levine, B. A., Moller, J. V., and Le Maire, M. (2002) *J. Biol. Chem.* 277, 13560–13564
39. Patchornik, G., Goldshleger, R., and Karslish, S. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 11954–11959
40. Diakon, S., Yamazaki, K., Dahlöf, T., Suzuki, H., and Toyoshima, C. (2001) *FEBS Lett.* 505, 129–135
41. Toustrup-Jensen, M., and Vilsen, B. (2003) *J. Biol. Chem.* 278, 11402–11410
Importance of Transmembrane Segment M1 of the Sarcoplasmic Reticulum Ca\textsuperscript{2+}\textsuperscript{-}ATPase in Ca\textsuperscript{2+} Occlusion and Phosphoenzyme Processing
Anja Pernille Einholm, Bente Vilsen and Jens Peter Andersen

J. Biol. Chem. 2004, 279:15888-15896. doi: 10.1074/jbc.M400158200 originally published online January 30, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400158200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 23 of which can be accessed free at http://www.jbc.org/content/279/16/15888.full.html#ref-list-1