Importance of Stalk Segment S5 for Intramolecular Communication in the Sarcoplasmic Reticulum Ca$^{2+}$-ATPase*

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Sixteen residues in stalk segment S5 of the Ca$^{2+}$-ATPase of sarcoplasmic reticulum were studied by site-directed mutagenesis. The rate of the Ca$^{2+}$ binding transition, determined at 0 °C, was enhanced relative to wild type in mutants Ile743 → Ala, Val744 → Ala, Ala745 → Ala, Glu748 → Ala, Glu749 → Ala, Met753 → Gly, and Glu759 → Ala and reduced in mutants Asp737 → Ala, Asp738 → Ala, Ala752 → Leu, and Tyr754 → Ala. In mutant Arg762 → Ile, the rate of the Ca$^{2+}$ binding transition was wild type like at 0 °C, whereas it was 3.5-fold reduced relative to wild type at 25 °C. The rate of dephosphorylation of the ADP-insensitive phosphoenzyme was increased conspicuously in mutants Ile743 → Ala and Tyr754 → Ala (close to 20-fold in the absence of K$^+$) and increased to a lesser extent in Asn743 → Ala, Glu749 → Ala, Gly750 → Ala, Ala752 → Gly, Met753 → Gly, and Arg762 → Ile, whereas it was reduced in mutants Asp737 → Ala, Val744 → Ala, Gly750 → Ala, Val747 → Ala, and Ala752 → Leu. In mutants Ile743 → Ala, Tyr754 → Ala, and Arg762 → Ile, the apparent affinities for vanadate were enhanced 23-, 30-, and 18-fold, respectively, to wild type. The rate of Ca$^{2+}$ dissociation was 11-fold increased in Gly750 → Ala and 2-fold reduced in Val747 → Ala. Mutants with alterations to Arg751 either were not expressed at a significant level or were completely nonfunctional. The findings show that S5 plays a crucial role in mediating communication between the Ca$^{2+}$ binding pocket and the catalytic domain and that Arg751 is important for both structural and functional integrity of the enzyme.

The Ca$^{2+}$-ATPase of sarcoplasmic reticulum functions as a Ca$^{2+}$ pump that actively transports Ca$^{2+}$ against a concentration gradient in exchange for protons, utilizing energy derived from ATP (1–6). It belongs to the family of P-type ATPases in which the hydrolysis of ATP is linked with ion translocation through phosphorylation and dephosphorylation of an aspartic acid residue in the ATPase protein (Scheme 1). Transfer of the γ-phosphoryl group of ATP to the protein is activated by a series of protein conformational changes associated with the binding of two calcium ions at cytoplasmically facing sites in exchange for protons (“the Ca$^{2+}$ binding transition” H$_2$E$_2$ → CaE$_2$). The dephosphorylation (H$_2$E$_2$P → H$_2$E$_2$) is triggered by Ca$^{2+}$/H$^+$ exchange at luminally facing sites. Structural studies of the Ca$^{2+}$-ATPase have revealed a large cytoplasmic head, which through a stalk is connected with the transmembrane domain (7). The head is made up of two cytoplasmic loops of the peptide chain, and the phosphorylated aspartic acid residue (Asp351) resides in the largest of these. The membrane domain comprises ten transmembrane helices (M1–M10), encompassing sites for binding and translocation of Ca$^{2+}$ and H$^+$ (3, 8, 9). The stalk consists of extensions of some of the transmembrane helices continuing into the cytoplasmic head. The tight coupling between the catalytic and vectorial processes, associated with the cytoplasmic head and the membrane domain, respectively, is dependent on intramolecular communication through the interconnecting stalk. Mutations to residues in stalk segment S4 have been shown to impair the transition between the two major phosphoforms of the enzyme, Ca$_2$E$_2$P → H$_2$E$_2$P, suggesting an important role of this segment in transmission of ATP-derived energy required in the membrane domain for Ca$^{2+}$ translocation (3, 10, 11). Stalk segment S5 could be another important mediator of the intramolecular communication, but its role is less well understood compared with the role of S4. We have previously demonstrated that replacement of Lys758 in S5 with isoleucine leads to concurrent increase in the rate of dephosphorylation H$_2$E$_2$P → H$_2$E$_2$ and decrease in the rate of the Ca$^{2+}$ binding transition H$_2$E$_2$ → CaE$_2$ (12). Furthermore, the tight coupling between ATP hydrolysis and Ca$^{2+}$ translocation has been found to be abolished by replacement of Tyr763 at the M5S5 boundary with glycine (13). In the present study, we have inquired about the functional importance of many of the other residues in stalk segment S5. Would mutations to other S5 residues give rise to functional consequences similar to those observed for the Lys758 → Ile and the Tyr763 → Gly mutants, and do stalk segments S4 and S5 play separate roles in intramolecular signaling? In addition to Lys758, several other residues in the S5 region are well conserved among the P-type ATPases, such as the charged or polar residues Asp737, Asp738, Asn739, and Arg751, the aromatic residues Phe740 and Tyr754, the aliphatic residues Ile743, Val744, and Val747, and Gly750 (14). We have analyzed the importance of these and other residues located in S5 and at the M5S5 boundary (see

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Fig. 1), by studying the functional consequences of their replacement for the overall and partial reactions of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Expression**—The principles for construction of mutant Ca\(^{2+}\)-ATPase DNAs and expression of wild type and mutants in COS-1 cells have been described previously (15). Microsomes containing expressed wild-type or mutant Ca\(^{2+}\)-ATPase were harvested by differential centrifugation, and expression levels were quantified by a specific sandwich enzyme-linked immunosorbent assay (16), using as standard expressed wild type for which the concentration was determined by measurement of the capacity for phosphorylation by inorganic phosphate in the presence of 30% dimethyl sulfoxide (12). Total microsomal protein was determined by the dye binding method of Bradford (17).

**ATP Hydrolysis and Ca\(^{2+}\) Uptake**—For determination of Ca\(^{2+}\)-activated ATPase activity, microsomes were incubated for 10 min at 37 °C either in the presence or absence of 1 μm calcium ionophore A23187 in a medium containing 50 mM TES/Tris, pH 7.0, 100 mM KCl, 7 mM MgCl\(_2\), 5 mM ATP, 1 mM EGTA, and various concentrations of CaCl\(_2\) to set the desired free Ca\(^{2+}\) concentration, as calculated using the MAXC computer program and constants therein (18). The ATP hydrolysis catalyzed by the microsomes was determined by measuring the amount of Pi liberated (12) using the method of Baginski et al. (19). ATP hydrolysis referable to Ca\(^{2+}\)-ATPase activity was calculated following subtraction of the amount of Pi liberated in the presence of 4 mM EGTA without added Ca\(^{2+}\). The turnover rate was calculated as mol Pi liberated/mmol Ca\(^{2+}\)-ATPase/s. To study the vanadate inhibition of ATPase activity, the indicated concentrations of monovanadate were obtained from metavanadate as described previously (20).

**Ca\(^{2+}\) Uptake**—The Ca\(^{2+}\)-saturated form was studied in the presence of 0.5 mM Pi was added to increase the Pi affinity, thus ensuring that 0.5 mM Pi was saturating in the wild type (23, 24). To study dephosphorylation, the phosphorylated microsomes were cooled to 0 °C and subsequently diluted 20-fold into ice-cold medium containing 50 mM TES/Tris, pH 7.0, 2 mM MgCl\(_2\), 2 mM EGTA, and 5 mM nonradioactive Pb\(^{2+}\), with or without 100 mM KCl, followed by acid quenching at serial intervals (2, 4, 6, and 10 s after the dilution). The acid-quenched protein was analyzed as described above.

**Data Analysis and Presentation**—All data presented in the figures and table or described in the text are average values of 2–5 experiments. Standard deviations are given in the table and are shown in the figures when larger than the size of the symbol. Time courses of phosphorylation or dephosphorylation were either fitted to monoexponential functions corresponding to first-order kinetics, using the SigmaPlot program (SPSS, Inc.), or were fitted by computer simulation of a simplified reaction cycle as described previously (21).

**RESULTS**

**Expression**—Mutants analyzed in the present study are presented in Fig. 1. An expression level of 200–400 pmol Ca\(^{2+}\)-ATPase/mg of total microsomal protein was achieved for the wild-type Ca\(^{2+}\)-ATPase, i.e. several hundred-fold higher than that of the endogenous COS cell Ca\(^{2+}\)-ATPase. The various mutants represented by single-letter code without parentheses in Fig. 1 were expressed to wild-type level, whereas the expression levels were very low for the mutations shown in parentheses. The low levels of expression of mutants Phe\(^{720}\) → Ala, Ile\(^{723}\) → Gly, Val\(^{747}\) → Gly, and Gly\(^{750}\) → Leu precluded functional characterization but less drastic alterations to the same residues, mutations Phe\(^{720}\) → Leu, Ile\(^{723}\) → Ala, Val\(^{747}\) → Ala, and Gly\(^{750}\) → Ala resulted in expression to wild-type level, thereby enabling characterization of the functional importance of these residues. For Arg\(^{751}\), only the conservative replacement with lysine gave rise to a protein that could be expressed to a level comparable with the wild-type level, whereas insignificant expression was found for mutants with alanine, isoleucine, or glutamate substitutions, either by enzyme-linked immunosorbent assay or by Western blotting (Fig. 2). Two inversion mutants moving Arg\(^{751}\) one position in either direction were also produced, Gly\(^{750}\)Arg\(^{751}\) → Arg\(^{751}\)Gly\(^{750}\) and Arg\(^{751}\)Ala\(^{752}\) → Ala\(^{752}\)Arg\(^{751}\), but as illustrated in Fig. 2, only the Arg\(^{751}\)Ala\(^{752}\) → Ala\(^{752}\)Arg\(^{751}\) mutant could be expressed to a significant level.

**Ca\(^{2+}\) and Ionophore Dependence of ATPase Activity**—To determine the overall functional consequences of the various amino acid substitutions, we measured the steady-state turnover number for ATPase activity at 37 °C in the presence of 5 mM MgATP and various Ca\(^{2+}\) concentrations, with and with-

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7 The abbreviations used are: TES, 2-[3-hydroxy-1,1-bis(hydroxy-

-ethyl)aminomethyl]ethanesulfonic acid; MES, 2-[(N-morpholino)-eth-

-anesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid.
out the calcium ionophore A23187 (25). The calcium ionophore allows passive efflux of Ca\(^{2+}\) that has been actively transported into the microsomal vesicles, and for the wild-type enzyme, this leads to a 2-fold increase in the maximum turnover rate, because the luminal Ca\(^{2+}\) concentration is diminished, thereby relieving the “back inhibition” of the rate-limiting Ca\(^{2+}\)P → H\(_{E2P}\)P transition caused by Ca\(^{2+}\) binding to luminal low affinity sites. All the mutants that could be expressed to a normal level, except Arg\(^{751}\) → Lys and the inversion mutant Arg\(^{751},\) Ala\(^{752} \rightarrow\) Ala\(^{753},\) Arg\(^{752}\), displayed ATPase activity, and the turnover rates determined at optimum Ca\(^{2+}\) concentration are given in Table I, both for the presence and absence of ionophore. The maximal turnover rates of mutants Val\(^{744} \rightarrow\) Gly, Val\(^{747} \rightarrow\) Ala, and Arg\(^{762} \rightarrow\) Ile were considerably lower than that of the wild type. Furthermore, mutants Val\(^{744} \rightarrow\) Gly, Val\(^{747} \rightarrow\) Ala, and Gly\(^{750} \rightarrow\) Ala showed diminished activation by ionophore, and for mutant Arg\(^{762} \rightarrow\) Ile we observed an anomalous, slightly inhibitory, effect of the ionophore on the ATPase activity, similar to that previously described for mutant Lys\(^{758} \rightarrow\) Ile (12).

The disappearance of the activating effect of the calcium ionophore could be the consequence of uncoupling of ATP hydrolysis from Ca\(^{2+}\) transport (13). To test for uncoupling, 45Ca\(^{2+}\) uptake in the microsomes was determined. All mutants displaying ATPase activity, including Val\(^{744} \rightarrow\) Gly, Val\(^{747} \rightarrow\) Ala, Gly\(^{750} \rightarrow\) Ala, and Arg\(^{762} \rightarrow\) Ile, were able to transport Ca\(^{2+}\) actively, and the ratios between Ca\(^{2+}\) transport and ATPase activity did not differ significantly among mutants and wild type (data not shown). Hence, there was no indication of an uncoupling of ATPase activity from Ca\(^{2+}\) transport in any of the mutants. In this situation, the reduced activation by the calcium ionophore may be explained by a shift of the rate-limiting step away from the Ca\(^{2+}\)P → H\(_{E2P}\)P transition sensitive to back inhibition by accumulated Ca\(^{2+}\). In fact, an inhibitory effect of the ionophore on the turnover rate, as observed for mutant Arg\(^{762} \rightarrow\) Ile, might be expected under conditions where the Ca\(^{2+}\) binding transition, H\(_{E2P}\) → Ca\(_{E2P}\), is rate-limiting, because the ionophore exerts a direct inhibitory effect on this step (12). When added in large doses, the ionophore causes strong inhibition of the Ca\(^{2+}\) binding transition of the wild type (26), and even the low ionophore concentration used here to make the membranes leaky should exert some inhibitory effect. In the wild type, the effect on the steady-state ATPase activity of this inhibition is masked because of the simultaneous relief of the back inhibition of the Ca\(_{E2P}\)P → H\(_{E2P}\)P transition, but if the Ca\(^{2+}\) binding transition has be-

![Summary of mutational effects on the overall and partial reactions](http://www.jbc.org/)
we studied the partial reactions. First, the Ca2+

of the various mutations on the overall enzyme function,
well as in the presence of 100 

100 

Ca2+

ATPase/s) are shown relative to the maximum turnover rate of the wild
type. The $K_m$ values determined from these data are given in Table I together with the results of similar measurements on the other mutants. The inhibitory effect of the ionophore should manifest itself as a decrease in the turnover rate, as actually seen for mutant Arg762 → Ile.

Examples of Ca2+ titration of the ATPase activity in the presence of ionophore are shown in Fig. 3, and the $K_m$ values for Ca2+ activation are indicated for all the mutants in Table I. The activation by Ca2+ reflects Ca2+ binding at the cytoplasmically facing high affinity sites, whereas the inhibition seen at high Ca2+ concentrations reflects in part the back inhibition by Ca2+ binding at the luminal sites and in part the effect of replacing MgATP by CaATP (27–29). In mutants Val744 → Gly, Val744 → Ala, and Val747 → Ala, the apparent affinity for Ca2+
at the activating sites was 1.5–2-fold increased ($K_m$ decreased) relative to wild type. By contrast, the apparent affinity was reduced 2- and 4-fold, respectively, in mutants Gly750 → Ala and Arg762 → Ile. In the other mutants, the $K_m$ values for Ca2+ activation did not deviate or deviated only insignificantly from wild type.

Phosphorylation by [γ-32P]ATP—Having established the impact of the various mutations on the overall enzyme function, we studied the partial reactions. First, the Ca2+-activated phosphorylation by [γ-32P]ATP was determined after 15 s of incubation with 2 μM [γ-32P]ATP in the presence of 100 μM Ca2+ at 0 °C. All the mutants exhibiting ATPase activity were able to form a phosphoenzyme intermediate under these conditions, whereas the two expressed mutants that were inactive with respect to ATP hydrolysis, Arg751 → Lys and Arg753, Ala752 → Ala751, Arg752, showed no measurable phosphorylation. Further examination showed that the latter mutants were unable to phosphorylate also in the presence of 10 mM Ca2+ at 0 °C as well as in the presence of 100 μM or 10 mM Ca2+ at 25 °C (data not shown).

The kinetics of the reaction sequence consisting of dissociation of protons from H2E2, Ca2+, and ATP binding, and subsequent phosphorylation to form Ca2+E2P and H2E2P (Scheme 1) were studied at 0 °C at pH 7.0. The enzyme was preincubated in the absence of Ca2+ (presence of EGTA), and phosphorylation was initiated by the simultaneous addition of CaCl2 and [γ-32P]ATP to yield final concentrations of 100 μM Ca2+ and 2 μM [γ-32P]ATP. Examples of the data are shown in Fig. 4. The time courses of phosphorylation could be fitted to a monoeponential function, and the derived rate coefficient is given in Table I for all the mutants. A significant enhancement of the rate of phosphorylation relative to wild type was found for six mutants, more than 2-fold in mutants Val747 → Ala (shown in Fig. 4) and Met757 → Gly and somewhat less in Ile743 → Ala, Glu746 → Ala, Glu746 → Ala, and Glu750 → Ala. In another four mutants, Asp737 → Ala, Asp738 → Ala, Ala752 → Leu (shown in Fig. 4), and Tyr754 → Ala, a significant decrease in the phosphorylation rate was found (in Asp738 → Ala and Ala752 → Leu the rate was about half that of wild type).

Under the experimental conditions applied here, the phosphorylation rate of the wild type is limited by one or more of the steps in the Ca2+ binding transition (H2E2 → Ca2+E2) and not by ATP binding or phosphoryl transfer (Ca2+E2P → Ca2+E1P) (12). Therefore, it may be concluded that the rate of the Ca2+ binding transition is enhanced relative to wild type in mutants Ile743 → Ala, Val747 → Ala, Gly746 → Ala, Glu746 → Ala, and Met757 → Gly, and Glu750 → Ala.

To locate the step limiting the rate in the slowly phosphorylating mutants, Asp737 → Ala, Asp738 → Ala, Ala752 → Leu, and Tyr754 → Ala, these mutants were further examined in experiments where 2 μM [γ-32P]ATP was added to enzyme preincubated with 100 μM Ca2+ (i.e., initially present in the Ca2+-saturated Ca2+E1 form), under conditions otherwise similar to those in Fig. 4. As a consequence of the preincubation with Ca2+, the rate of phosphorylation increased considerably, so that more than 80% of the maximal phosphorylation level was reached after 1 s, the lower time limit for data collection by the manual mixing technique (data not shown). Under these conditions, the Ca2+ binding transition is excluded from the reaction sequence studied, and it appears, therefore, that the slow step limiting the phosphorylation rate of these mutants in the experiments carried out after preincubation in the absence of Ca2+ must be the Ca2+ binding transition and not ATP binding or phosphoryl transfer.

Dephosphorylation of Phosphoenzyme Formed from [γ-32P]ATP—The Ca2+E1P phosphoenzyme intermediate can dephosphorylate either by donation of the bound phosphoryl group back to ADP, producing ATP or through the Ca2+E1P → H2E2P transition and subsequent hydrolysis of the aspartyl phosphoryl bond, H2E2P → H2E2 (Scheme 1). The H2E2P phospho-enzyme intermediate reacts with water liberating P1, but cannot react with ADP. We examined the dephosphorylation of phosphoenzyme formed in the presence of [γ-32P]ATP under conditions (0 °C, presence of K+ neutral pH) where the Ca2+E1P form tends to accumulate in the wild type as a consequence of a relatively slow Ca2+E1P → H2E2P transition (15, 28–30). The fraction of phosphoenzyme remaining 5 s after chase with an excess of either nonradioactive ATP or ADP was determined and the derived rate constants for the two types of chase are...
and pH 7.0 in the absence of K$^+$, the mixture was cooled to 0 °C, and dephosphorylation was studied at 0 °C in the absence of K', by a 20-fold dilution of the phosphorylated enzyme into an ice-cold medium, using a manual mixing procedure (see “Experimental Procedures”). The data were analyzed as monoexponential decays, and the extracted rate coefficients are shown in Table I together with the results of similar measurements on the other mutants.

Table I

shown in Table I. It can be seen that the dephosphorylation of the wild type occurred more rapidly upon addition of ADP as compared with ATP, consistent with the presence of a large fraction of the wild-type phosphoenzyme as ADP-sensitive CaE1P. For some of the mutants, notably Val744 → Gly and Val747 → Ala, the data shown in Table I indicate a significant reduction of the dephosphorylation rate relative to wild type, in the presence of ATP as well as in the presence of ADP, suggesting that the reaction $H_E E_P \rightarrow H_E$ occurs at a reduced rate with resulting accumulation of the ADP-insensitive $H_E E_P$ form. None of the mutants showed the combination of a significantly reduced dephosphorylation rate in the presence of ATP and a normal or increased dephosphorylation rate in the presence of ADP that would be consistent with a block of the CaE1P→H_EE_P transition (cf. Refs. 10, 11 and 15).

Phosphoenzyme Formed from $^{32}$P—All the mutants exhibiting ATPase activity and phosphorylation from ATP were able to form a phosphoenzyme also in the backward reaction with $^{32}$P in the absence of Ca$^{2+}$ (the ADP-insensitive $H_E E_P$ intermediate; Scheme 1), whereas no phosphorylation from $^{32}$P could be detected for the two expressed mutants that were inactive with respect to ATP hydrolysis and phosphorylation from ATP, Arg751 → Lys and Arg751-Val752 → Ala753-Arg752 (data not shown).

The dephosphorylation of the phosphoenzyme formed from $^{32}$P was studied upon a 20-fold dilution of the phosphorylation mixture into a chase medium containing nonradioactive P. For many of the mutants the dephosphorylation was examined both in the presence and absence of K' in the chase medium (Table I). K' is known to stimulate dephosphorylation in the wild-type Ca$^{2+}$-ATPase by binding to sites facing the cytoplasmic side of the membrane without being transported (31, 32). The data could be fitted to monoexponential decay functions as illustrated by a few examples in Fig. 5, and the derived rate constants are given in Table I. The smaller rate constants relative to wild type determined for Val744 → Gly and Val747 → Ala in the presence of K' confirm that the reason for the reduced ADP sensitivity of the phosphoenzyme of these mutants noted above is a reduced rate of dephosphorylation of $H_E E_P$ with resulting accumulation of this intermediate. Likewise, the dephosphorylation rate was found to be somewhat reduced relative to wild type in mutants Asp739 → Ala, Val744 → Ala, and Ala762 → Leu in the presence of K' (Table I), also consistent with the data for the ADP sensitivity of the phosphoenzyme formed from ATP.

The mutants Ile743 → Ala and Tyr754 → Ala displayed a conspicuous increase of the dephosphorylation rate relative to wild type both in the presence and absence of K'. In the presence of K', the rate was too high for accurate measurement in these mutants, but in the absence of K' the rate constants could be determined and were close to 20-fold higher than that corresponding to wild type (Table I and Fig. 5). In the absence of K', the rate constants corresponding to mutants Asn739 → Ala, Gly750 → Ala, and Arg762 → Ile were found to be 2–10-fold higher than that of the wild type. For Glu749 → Ala, Gly750 → Ala, Met757 → Gly, and Arg762 → Ile, the rate constant was determined in the presence of K', as well, and under these conditions it was 1.5–2-fold enhanced relative to wild type (Table I).

Rapid Kinetic Studies of the Ca$^{2+}$-Binding Transition—Because the simplest explanation of the anomalous ionophore effect described above for mutant Arg762 → Ile is a shift of the rate-limiting step away from the CaE1P→H_EE_P transition to the Ca$^{2+}$-binding transition, we were puzzled over the apparently normal rate of the Ca$^{2+}$ binding transition determined for this mutant (15 s$^{-1}$; Table I). Because this result had been obtained at 0 °C, the kinetics of the Ca$^{2+}$ binding transition in mutant Arg762 → Ile and other selected mutants were further analyzed at 25 °C, using the quench flow technique described previously (21). Except for the higher temperature and a higher ATP concentration of 5 μM instead of 2 μM, the experimental protocol for the left panels of Fig. 6 was similar to that used for Fig. 4 and Table I, phosphorylation being initiated by simultaneous addition of Ca$^{2+}$ and $[γ^{32}$P]ATP to Ca$^{2+}$-deprived enzyme. By fitting the data in the

![Graph](http://www.jbc.org/)

**Fig. 5.** Dephosphorylation of phosphoenzyme formed in the presence of $^{32}$P. Wild type (●) and mutants Ile743 → Ala (△), Tyr754 → Ala (○), and Arg762 → Ile (▽) were phosphorylated for 10 min at 25 °C in the presence of 0.5 mM $^{32}$P. Subsequently, the phosphorylation mixture was cooled to 0 °C, and dephosphorylation was studied at 0 °C and pH 7.0 in the absence of K', by a 20-fold dilution of the phosphorylated enzyme into an ice-cold medium, using a manual mixing procedure (see "Experimental Procedures"). The data were analyzed as monoexponential decays, and the extracted rate coefficients are shown in Table I together with the results of similar measurements on the other mutants.

![Graph](http://www.jbc.org/)

**Fig. 6.** Phosphorylation by $[γ^{32}$P]ATP at 25 °C and pH 7.0. Phosphorylation of wild-type enzyme and mutants Val744 → Ala, Ala762 → Leu, and Arg762 → Ile was carried out in the presence of 5 μM $[γ^{32}$P]ATP, using a Bio-Logic quench flow module (see "Experimental Procedures"), following preincubation either in the absence of Ca$^{2+}$ (to study H_E E_P → Ca_E_P, left panels) or in the presence of 100 μM Ca$^{2+}$ (to study CaE1P → E2P, right panels). The time course of phosphorylation of enzyme preincubated in the absence of Ca$^{2+}$ was fitted to a monoexponential function with rate coefficients of 21, 19, 11, and 6 s$^{-1}$ for the wild type and mutants Val747 → Ala, Ala762 → Leu, and Arg762 → Ile, respectively. The time course of phosphorylation of enzyme preincubated in the presence of Ca$^{2+}$ was fitted by computer simulation of a simplified reaction cycle as described previously (21). In each case the maximum level of phosphorylation was set to 100%.
left panels to monoexponential functions, rate constants of 21, 19, 11, and 6 s−1 were obtained for the wild type and mutants Val747 → Ala, Ala752 → Leu, and Arg762 → Ile, respectively. Hence, the observed rate constant for mutant Arg762 → Ile was 3.5-fold lower than that of the wild type at 25 °C and 5 μM ATP (Fig. 6, left panel), whereas it was wild type like at 0 °C and 2 μM ATP (Table I). The observed rate constant for mutant Val747 → Ala was wild type like at 25 °C and 5 μM ATP (Fig. 6, left panels), whereas it was more than 2-fold enhanced relative to wild type at 0 °C and 2 μM ATP (Table I). For mutant Ala752 → Leu, the observed rate constant was about half that of the wild type under both sets of conditions.

The right panels in Fig. 6 show the results of initiating the phosphorylation by [γ-32P]ATP addition to enzyme preincubated in the presence of Ca2+, under conditions otherwise similar to those applied corresponding to the left panels. After the preincubation with Ca2+, the initial rates of phosphorylation were very similar for wild type and mutants and higher than the rates observed following preincubation in the absence of Ca2+. As already noted in connection with Fig. 4, this means that the time courses observed following preincubation in the absence of Ca2+ (i.e. those displayed in the left panels of Fig. 6) reflect the Ca2+ binding transition and not ATP binding or phosphoryl transfer. The right panels of Fig. 6 furthermore show an initial overshoot of phosphorylation. This is caused by the occurrence of a relatively slow step between dephosphorylation and repolymerization. The overshoot is seen to be largest for mutant Arg762 → Ile, consistent with a very low rate of the Ca2+ binding transition in this mutant.

Additional phosphorylation experiments conducted at 25 °C as in Fig. 6 (left panels) but in the presence of 2 μM [γ-32P]ATP instead of 5 μM demonstrated that the difference between Fig. 4/Table I and Fig. 6 (left panels) with respect to the rates of mutants Val747 → Ala and Arg762 → Ile relative to wild type is caused by the temperature difference and not by the difference in ATP concentration (data not shown).

Hence, it may be concluded that the temperature dependence of the rate of the Ca2+ binding transition is less steep for mutants Val747 → Ala and Arg762 → Ile compared with the wild type and mutant Ala752 → Leu. This would be explained if the energy of activation of a rate-limiting substep is changed by the mutation or if a different substep has taken over the role of being rate-limiting. According to Forge et al. (5), the Ca2+ binding transition of the wild type comprises at least four substeps between H2E2 and Ca2E1 in a branched scheme containing three or more Ca2+-free species with different degrees of protonation. At room temperature in the absence of Ca2+, almost all the enzyme resides in the H2E2 form at pH 6.0, whereas HE1 prevails at neutral pH (5). To look for possible changes to the proton dissociation steps in the Val747 → Ala mutant, we examined the kinetics of the wild type and the Val747 → Ala mutant at pH 6.0 under conditions otherwise similar to those corresponding to the left panels of Fig. 6, and the rate coefficients were 3.8 and 8.4 s−1, respectively (data not shown), which should be compared with the corresponding values of 21 and 19 s−1 obtained at pH 7.0 (see above). This demonstrates that the Val747 → Ala mutant is less sensitive to the pH change than the wild type, which is reminiscent of the lower temperature sensitivity of the Val747 → Ala mutant relative to the wild type. The finding that for mutant Val747 → Ala the rate of the Ca2+ binding transition is wild-type like at 25 °C at pH 7.0, whereas it is enhanced relative to wild type both at 0 °C at pH 7.0 and at 25 °C at pH 6.0, would be consistent with the hypothesis that the same step (possibly H2E2 → HE1; cf. Ref. 5) limits the rate of the Ca2+ binding transition in the latter two conditions and that this step is accelerated in the mutant relative to wild type, whereas a different step, which is unaffected by the mutation, is rate-limiting at 25 °C at pH 7.0 both in wild type and mutant.

Inhibition by Vanadate—As a transition state analog of the phosphoryl group, vanadate binds preferentially to the H3E2 form of the enzyme (20, 33), and the apparent affinity for vanadate depends on the concentration of H3E2 relative to the other enzyme intermediates, as well as on the intrinsic affinity of H3E2 for vanadate. The intrinsic affinity is related to the stability of the transition state in the H3E2P → H3E2 dephosphorylation reaction and is expected to increase under conditions where the rate of dephosphorylation is enhanced (34–36). Hence, an increased dephosphorylation rate or a reduced rate of the Ca2+ binding transition (resulting in accumulation of H3E2) would both be expected to be associated with an increased apparent affinity for vanadate. To obtain further information about these partial reactions in selected mutants at 37 °C under the conditions used to study the overall functional performance of the enzyme, the vanadate affinity was examined by titrating the vanadate inhibition of ATPase activity, and the results are shown in Fig. 7. In mutants Ile743 → Ala, Tyr754 → Ala, and Arg762 → Ile, the apparent affinities for vanadate were enhanced 23-, 30-, and 18-fold, respectively, relative to wild type, consistent with stabilization of the transition state in the dephosphorylation reaction and/or accumulation of H3E2 in these mutants at 37 °C. Only a 2-fold increase in apparent vanadate affinity relative to wild type was found for the Ala752 → Leu mutant, which could reflect a balance between effects related to a reduced dephosphorylation rate (Table I) and a reduced rate of the Ca2+ binding transition (Fig. 6). For the Val747 → Ala mutant, the apparent vanadate affinity was 1.4-fold reduced relative to wild type, possibly reflecting a reduced dephosphorylation rate (Table I).

Dissociation of Ca2+—To understand the basis for the altered apparent affinity for Ca2+ observed for mutants Val747 → Ala, Gly750 → Ala, and Arg762 → Ile in the Ca2+ titrations of ATPase activity (Fig. 3), we analyzed the rate of Ca2+ dissociation in these mutants. Upon addition of EGTA to Ca2+-saturated enzyme, the ability to phosphorylate disappears at a rate corresponding to the dissociation of the calcium ion that is first to leave toward the cytoplasmic side in the sequential mechanism (37), and it is therefore possible to determine this rate by phosphorylation measurements (37, 38). We have previously described a simple approach in which the apparent rate constant for Ca2+ dissociation (κ−C ) is calculated from the ratio

Fig. 7. Vanadate inhibition of Ca2+-ATPase activity. The rate of ATP hydrolysis of wild type and mutant enzymes in the presence of 5 μM ATP, 100 μM Ca2+, and calcium ionophore A23187 at pH 7.0 and 37 °C was measured as described under “Experimental Procedures” in the presence of various concentrations of vanadate as indicated on the abscissa. The 100% value corresponds to the activity in the absence of vanadate. The data were fitted to the Hill equation with K(V ) values as indicated in parentheses: ( ), wild type (18 μM); ( △), Ile743 → Ala (0.8 μM); (○), Val747 → Ala (26 μM); (□), Ala752 → Leu (7.8 μM); (■), Tyr754 → Ala (0.6 μM); (▲), Arg762 → Ile (1 μM).
Fig. 8. Rate of Ca\textsuperscript{2+} dissociation. For the wild type and each mutant, the phosphoenzyme level measured at 25 °C after 34 ms of exposure of Ca\textsuperscript{2+}-saturated enzyme to excess EGTA, and [γ-32P]ATP as described under “Experimental Procedures” is shown as the percentage of the amount of phosphoenzyme determined after 34 ms of incubation with [γ-32P]ATP in the presence of Ca\textsuperscript{2+} without EGTA. To the right are shown the rate constants for Ca\textsuperscript{2+} dissociation (k_{Ca}), calculated as described previously (21). The calculation was based on a rate constant of 35 s\textsuperscript{-1} for the Ca\textsubscript{a}E\textsubscript{1} → Ca\textsubscript{a}E\textsubscript{1}p reaction in wild type as well as mutants (21), because experiments performed as in Fig. 6 (right panels) showed no significant differences between the initial phosphorylation rates.

| Mutant   | k_{Ca} (s\textsuperscript{-1}) |
|----------|---------------------------------|
| Wild type| 27                              |
| Val\textsuperscript{747}→Ala | 15                              |
| Gly\textsuperscript{750}→Ala | 300                             |
| Arg\textsuperscript{762}→Ile | 25                              |

DISCUSSION

In the present study, we have found that various mutations to residues in stalk segment S5 of the Ca\textsuperscript{2+}-ATPase have effects on the rate of the reaction sequence H\textsubscript{a}E\textsubscript{2} → Ca\textsubscript{a}E\textsubscript{1} associated with Ca\textsuperscript{2+} binding at the cytoplasmically facing sites (the Ca\textsuperscript{2+} binding transition) and on the rate of the dephosphorylation of the ADP-insensitive phosphoenzyme, H\textsubscript{a}E\textsubscript{1}p → H\textsubscript{a}E\textsubscript{2}, normally triggered by Ca\textsuperscript{2+}/H\textsuperscript{+} exchange at the luminal sites. Both accelerating and decelerating effects on these two reaction sequences were observed. At 0 °C, the rate of the Ca\textsuperscript{2+} binding transition was enhanced in six mutants (more than 2-fold in Val\textsuperscript{747} → Ala and Met\textsuperscript{757} → Gly), whereas a decrease in the rate was noted in another four mutants (most significant in Asp\textsuperscript{309} → Ala and Ala\textsuperscript{742} → Leu). In mutant Arg\textsuperscript{762} → Ile, the rate of the Ca\textsuperscript{2+} binding transition was wild type like at 0 °C, whereas it was as much as 3.5-fold reduced relative to wild type at 25 °C, and given this less steep temperature dependence relative to wild type, the rate of the Ca\textsuperscript{2+} binding transition at 37 °C may be considerably reduced in the Arg\textsuperscript{762} → Ile mutant compared with wild type. This would also be consistent with our observation of an anomalous, slightly inhibitory, effect of the calcium ionophore on the ATPase activity of this mutant.

The titration of the Ca\textsuperscript{2+} dependence of the turnover rate for ATP hydrolysis demonstrated an increased apparent Ca\textsuperscript{2+} affinity relative to wild type in mutants Val\textsuperscript{744} → Gly, Val\textsuperscript{744} → Ala, and Val\textsuperscript{747} → Ala, whereas the apparent Ca\textsuperscript{2+} affinity was reduced in Gly\textsuperscript{750} → Ala and Arg\textsuperscript{762} → Ile. For mutants Val\textsuperscript{747} → Ala, Gly\textsuperscript{750} → Ala, and Arg\textsuperscript{762} → Ile, we determined the rate of Ca\textsuperscript{2+} dissociation (Fig. 8) and in case of Val\textsuperscript{747} → Ala and Gly\textsuperscript{750} → Ala the result of this measurement, showing a reduced and an increased rate, respectively, explains the change in apparent Ca\textsuperscript{2+} affinity. For Arg\textsuperscript{762} → Ile, the Ca\textsuperscript{2+} dissociation rate was found to be normal, but the reduced rate of the Ca\textsuperscript{2+} binding transition explains the reduced apparent Ca\textsuperscript{2+} affinity.

For mutants Ile\textsuperscript{743} → Ala, Tyr\textsuperscript{754} → Ala, and Arg\textsuperscript{762} → Ile, we found significant increases in the apparent affinity for vanadate determined under steady-state ATP hydrolysis conditions at 37 °C (Fig. 7). Because vanadate binds preferentially to the H\textsubscript{a}E\textsubscript{2} form of the enzyme as a transition state analog of the phosphoryl group, the data are consistent with the increased dephosphorylation rate seen particularly for Ile\textsuperscript{743} → Ala and Tyr\textsuperscript{754} → Ala and with the reduced rate of the Ca\textsuperscript{2+} binding transition of Arg\textsuperscript{762} → Ile.

Although the functional effects of several hundred different mutations to the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase have been examined, the combination of changes to the Ca\textsuperscript{2+} binding transition of the dephosphoenzyme and the dephosphorylation of H\textsubscript{a}E\textsubscript{2}p, as observed in the present study, has only been previously reported for another S5 mutant, Lys\textsuperscript{758} → Ile (12), and for the mutant Gly\textsuperscript{309} → Asp, in which the critical Ca\textsuperscript{2+} liganding residue in M4 is altered (39). Certain mutations in other regions such as the smaller cytoplasmic domain (“β-domain”) and stalk segment S4 have been shown to affect the rate of the Ca\textsuperscript{2+} binding transition, but in these cases there was a simultaneous block of the Ca\textsubscript{a}E\textsubscript{1}p → H\textsubscript{a}E\textsubscript{2}p transition (21). In the light of the previous findings of reduced Ca\textsubscript{a}E\textsubscript{1}p → H\textsubscript{a}E\textsubscript{2}p transition rates in mutants with alterations to residues in stalk segment S4 (3, 10, 11), it is remarkable that no block of...
the Ca$_2^+$E$_3$P → H$_3$E$_3$P transition was seen for any of the S5 mutants examined in the present study. Instead, the rate of dephosphorylation of H$_3$E$_3$P was altered in the S5 mutants, and in several cases it was enhanced. This difference points to a mechanism in which S4 and S5 play distinct roles in energy transduction. S4 takes part in the conformational changes associated with the Ca$_2^+$E$_3$P → H$_3$E$_3$P transition, whereas S5 may be instrumental in transmitting conformational changes from the Ca$^{2+}$-/H$^+$-binding sites in the membrane sector to the phosphorylation domain, controlling phosphorylation and dephosphorylation.

One S5 residue, Arg$^{751}$, appeared to be crucial both to the structural and the functional integrity of the enzyme. Arg$^{751}$ is very highly conserved within the P-type ATPase family and is important for the expression of the ATPase protein in the COS cells. Probably, the arginine is needed to attain the conformation required for proper membrane insertion. Only the conservative substitution Arg$^{751} \rightarrow$ Lys and the inversion mutation Arg$^{751}$Ala$^{752} \rightarrow$ Ala$^{751}$Arg$^{752}$ were compatible with expression at a significant level, and even these mutants were unable to hydrolyze ATP or become phosphorylated by ATP or Pi. Also the substitution of glycine and alanine at positions Lys and the inversion mutation Arg$^{751}$Ala$^{752}$ were compatible with expression at a significant level, and even these mutants were unable to hydrolyze ATP or become phosphorylated by ATP or Pi. Instead, the rate of dephosphorylation of H$_3$E$_3$P mutants examined in the present study. Instead, the rate of dephosphorylation of H$_3$E$_3$P.

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Importance of Stalk Segment S5 for Intramolecular Communication in the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase

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