Val200 Residue in Lys189–Lys205 Outermost Loop on the A Domain of Sarcoplasmic Reticulum Ca2+-ATPase Is Critical for Rapid Processing of Phosphoenzyme Intermediate after Loss of ADP Sensitivity*

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Possible roles of the Lys189–Lys205 outermost loop on the A domain of sarcoplasmic reticulum Ca2+-ATPase were explored by mutagenesis. Both nonconservative and conservative substitutions of Val200 caused very strong inhibition of Ca2+-ATPase activity, whereas substitutions of other residues on this loop reduced activity only moderately. All of the Val200 mutants formed phosphoenzyme intermediate (EP) from ATP. Isomerization from ADP-sensitive EP (E1P) to ADP-insensitive EP (E2P) was not inhibited in the mutants, and a substantially larger amount of E2P actually accumulated in the mutants than in wild-type sarcoplasmic reticulum Ca2+-ATPase at steady state. In contrast, decay of EP formed from ATP in the presence of Ca2+ was strongly inhibited in the mutants. Hydrolysis of E2P formed from P(i) in the absence of Ca2+ was also strongly inhibited but was faster than the decay of EP formed from ATP, indicating that the main kinetic limitation of the decay comes after loss of ADP sensitivity but before E2P hydrolysis. On the basis of the well accepted mechanism of the Ca2+-ATPase, the limitation is likely associated with the Ca2+-releasing step from E2P-Ca2+. On the other hand, the rate of activation of dephosphorylated enzyme on high affinity Ca2+ binding was not altered by the substitutions. In light of the crystal structures, the present results strongly suggest that Val200 confers appropriate interactions of the Lys189–Lys205 loop with the P domain in the Ca2+-released form of E2P. Results further suggest that these interactions, however, do not contribute much to domain organization in the dephosphorylated enzyme and thus would be mostly lost on E2P hydrolysis.

Sarcolemmal reticulum Ca2+-ATPase (SERCA1a) is a 110-kDa membrane protein and a representative member of P-type ion-transporting ATPases. SERCA1a catalyzes Ca2+ transport coupled with ATP hydrolysis (Refs. 1 and 2, and for recent reviews, see Refs. 3 and 4). According to the E1/E2 transport mechanism (Fig. 1) (3–7), the enzyme is activated by the binding of two Ca2+ ions (E1Ca2+, steps 1–2) and then autophosphorylated by MgATP to form an ADP-sensitive phosphoenzyme (E1P, step 3). On formation of this EP, the bound Ca2+ ions are occluded in the transport sites. The subsequent isomeric transition to the ADP-insensitive form (E2P, step 4) will result in a reduction in affinity and a change in orientation of the Ca2+ binding sites and thus a Ca2+ release into lumen (step 5). Finally, hydrolysis takes place and returns the enzyme into an unphosphorylated and Ca2+-unbound form (E2, step 6). The main kinetic limitation in this cycle is associated with the mechanism of Ca2+ release before the hydrolysis of E2P (8, 9). E2P can also be formed from P(i) in the presence of Mg2+ and absence of Ca2+ by reversal of the hydrolysis of E2P.

The three-dimensional structure of Ca2+-ATPase with bound Ca2+ (E1Ca2+) and, very recently, the structure without bound Ca2+ and with bound thapsigargin (E2/TG), were solved by x-ray crystallography at the atomic level (10, 11). The enzyme has three cytoplasmic domains (A, N, and P) that are widely separated in E1-Ca2+ and associated in E2/TG. The modeling with a low resolution map of tubular crystals formed with decavanadate (E2V) revealed (10) that three cytoplasmic domains gather to form a more compactly organized single headpiece in E2V (see Fig. 7). Our previous limited and systematic proteolysis experiments showed (12, 13) that E2V is similar to the Ca2+-released form of E2P in the domain organization and that this EP is the intermediate with the most compactly organized headpiece in the catalytic cycle. The results further indicated that a large motion of the A domain (i.e. rotation by ~90° (10)) and the strong association of the A domain with the P and N domains most likely occur during the isomerization of EP and Ca2+ release and suggested that the stabilization energy provided by intimate contacts between all three cytoplasmic domains in E2P provides energy for moving transmembrane helices and releases the bound Ca2+ ions.

To substantiate such changes in cytoplasmic domain organization and their roles in the Ca2+ transport, it is essential to find the regions and residues involved in the domain-domain interactions and reveal their actual roles in the catalytic steps. The conserved outermost TGES loop (Thr181–Ser184) on the A domain is situated at the interface of the A and P domains in E2V. This loop was previously found to be essential for the isomerization of EP (14) and predicted by iron-catalyzed cleavage with Na+/K+-ATPase to participate in Mg2+ binding with specific residues in the conserved TGDGVND loop (starting

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from Thr$^{701}$ with Ca$^{2+}$-ATPase) on the P domain in E2P and E2 (15). One more outermost loop is located on the outer surface of the A domain in E1-Ca$^{2+}$, but situated at the A-P domain interface in E2Y, the Lys$^{188}$-Lys$^{205}$ loop that includes the tryptic T2 site (Arg$^{198}$) (see Fig. 7). We had actually indicated by site-directed chemical modification (16) and mutations (17) as well as by the limited proteolysis (12, 13) that Arg$^{198}$ comes very close to the phosphorylation site when E2P without bound Ca$^{2+}$ is formed and that the positive charge of this residue is important for its rapid hydrolysis. These findings suggest that the Lys$^{188}$-Lys$^{205}$ loop may have critical roles in domain organization, particularly in the Ca$^{2+}$-released form of E2P.

In the present study, we therefore further explored possible roles of the Lys$^{189}$-Lys$^{205}$ outermost loop by mutagenesis and found that Val$^{200}$ is critical for rapid processing of E2P, most likely in steps 5 and 6. With the crystal structures, our results suggest that Val$^{200}$ confers appropriate interactions between polar residues on the Lys$^{189}$-Lys$^{205}$ loop with those on the P domain in the Ca$^{2+}$-released form of E2P and, further, that these interactions would be mostly lost on the hydrolysis of E2P.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression—Overlap extension PCR (18) was used for the substitution of residues in the Lys$^{189}$-Lys$^{205}$ region in rabbit SERCA1a cDNA. The Apal-KpnI restriction fragments were excised from the PCR products and ligated back into the corresponding region in the full-length SERCA1a cDNA in the pMT2 expression vector (19). The pMT2 DNA was transfected into COS-1 cells by the liposome-mediated transfection method. Microsomes were prepared from the cells as described previously (20). The “control microsomes” were prepared from COS-1 cells transfected with the pMT2 vector containing no SERCA1a cDNA. The amount of expressed SERCA1a was quantified by a sandwich enzyme-linked immunosorbent assay as described previously (21). The expression levels of all the mutants in the microsomes were comparable with those of the wild type.

ATPase Activity—The rate of ATP hydrolysis was determined at 25 °C in the presence and absence of 0.5 μM thapsigargin in a mixture containing 20 μg/ml microsomal protein, 1 μM ATP, 7 mM MgCl$_2$, 0.1 mM KCl, 50 mM MOPS/Tris (pH 7.0), 0.55 mM CaCl$_2$, and 0.5 mM EGTA. The specific ATPase activity was measured activity of expressed SERCA1a was obtained by subtracting the background radioactivity with the control microsomes. This background was less than 5% of the radioactivity of EP formed with the expressed wild-type SERCA1a. The amount of EP/mg of SERCA1a protein was calculated from the amount of EP thus obtained and the amount of expressed SERCA1a.

Miscellaneous—Protein concentrations were determined by the method of Lowry et al. (24) with bovine serum albumin as a standard. Data were analyzed by nonlinear regression using the program Origin (Microcal Software, Inc., Northampton, MA). Three-dimensional models were reproduced by the program Swiss-PdbViewer (25).

RESULTS

Effects of Substitutions in Lys$^{189}$–Lys$^{205}$ Loop on ATP Hydrolysis—The specific Ca$^{2+}$-ATPase activities of the expressed mutant and wild-type SERCA1a were determined at 25 °C (Fig. 2). The substitution of Val$^{200}$ with Ala resulted in very strong inhibition of the activity, whereas nonconservative substitutions of Lys$^{189}$ His$^{190}$ Thr$^{191}$ Pro$^{193}$ Val$^{194}$ Arg$^{198}$ Ala$^{199}$ Glu$^{202}$ Asp$^{203}$ Lys$^{204}$, and Lys$^{205}$ caused only slight or moderate reduction, and those of Asp$^{196}$ Pro$^{197}$, and Asn$^{201}$ caused almost no change or rather a slight increase. Moderate effects of substitutions of Asp$^{196}$ Arg$^{198}$ Glu$^{202}$, and Asp$^{203}$ on the activity were also reported previously (4, 17, 26), and the results are in essential agreement. Substitutions of Glu$^{202}$ and Pro$^{195}$ caused no loss of function (4). We then focused on Val$^{200}$, of which substitutions gave distinguishably strong inhibition, and substituted with various amino acids including nonpolar and polar (positively charged, negatively charged, and uncharged) amino acids (Ala, Ile, Arg, Lys, Asp, Thr, and Glu). The activity was strongly inhibited in the mutants V200I, V200D, V200Q, and V200K (Fig. 3).

Formation of EP from ATP—We then performed kinetic analysis on the formation and decay of EP with these Val$^{200}$ mutants at 0 °C under conditions otherwise similar to those for the ATPase assay. All of the mutants possessed the ability to form EP, and the amount formed was comparable with that of wild type (Table I). As shown in Fig. 4, the time course of E2P formation from ATP was examined in the presence and absence of K$^+$. Under both sets of conditions, the total amount of EP reached its maximum level within 1 s after the addition of ATP and remained unchanged for the time periods of observation (120 s) (data not shown). Thus, the time course actually reflects E2P accumulation from E1P. In the presence of K$^+$, the amount of accumulated E2P at steady state in wild type was very low (Fig. 4A), in agreement with the previous observations on the
null
substitutions. The apparent decay rate ($k_{\text{decay}}$) was strongly reduced in V200D, V200Q, V200T, and V200I to 0.5–20% of that of the wild type and was further reduced to ~10% in V200A, V200K, and V200R (Table II). It should be noted that the reported value of $k_{\text{decay}}$ agreed well with that of inhibition of ATPase activity (cf. Table II and Fig. 3). Also, in each mutant the $k_{\text{decay}}$ value is much lower than the $k_{\text{formation}}$ value.

Hydrolysis of E2P Formed from $P_i$. To examine the hydrolysis of E2P without bound Ca$^{2+}$ (step 6), the enzyme was first phosphorylated by $^{32}$P, without Ca$^{2+}$ in the absence of K$^+$ and the presence of 35% (v/v) Me$_2$SO, which extremely favors E2P formation (28), and then the phosphorylated samples were diluted with a large volume of solution containing K$^+$ and non-radioactive P$_i$ (Fig. 6). The conditions were thus made otherwise identical to those used for the E2P formation from ATP in Fig. 4A and the decay of EP formed from ATP in Fig. 5.

The hydrolysis of $^{32}$P-labeled E2P proceeded with first-order kinetics, and its rate ($k_{\text{hydrolysis}}$) was summarized in Table II. The rate was moderately reduced in V200D and largely reduced in V200T and V200Q (8 and 7% that of wild type, respectively). It was further reduced in V200A, V200I, V200K, and V200R (less than 3% that of wild type).

Kinetic Limitation in the Mutants for Decay of EP Formed from ATP. For the mutants, the above kinetic results show that the decay of EP formed from ATP in the presence of Ca$^{2+}$ is much slower than the accumulation of E2P from ATP and also that the EP decay is substantially slower than the E2P hydrolysis in the absence of Ca$^{2+}$ (as is most clearly shown with V200K, V200D, V200T, and V200Q). The results indicate for the mutants that the main kinetic limitation (although not the only one) in the decay of EP formed from ATP comes after loss of ADP sensitivity (step 4) but before E2P hydrolysis (step 6). On the basis of the well accepted mechanism of the Ca$^{2+}$-ATPase (Fig. 1), it is therefore likely that step 5 associated with Ca$^{2+}$ release is greatly slowed by the Val$^{200}$ substitutions and is the main kinetic limitation in the mutants.²

For the mutants, the actual rate of formation of E2P from E1P on isomerization ($k_{\text{isomerization}}$) in the presence of K$^+$ can be estimated by assuming the equilibrium in step 4 without further processing of EP (because of the subsequent very slow processing as described above for the mutants). The $k_{\text{isomerization}}$ value thus estimated for the mutants (s$^{-1}$) by an equation, $k_{\text{isomerization}} = k_{\text{formation}} \times (\text{fraction of E2P at steady state})$, was 0.063 (V200A), 0.108 (V200I), 0.011 (V200R), 0.013 (V200K), 0.032 (V200D), 0.036 (V200T), and 0.084 (V200Q). These values are in fact substantially larger than the $k_{\text{decay}}$ values (as most clearly shown with V200A, V200I, V200R, V200T, and V200Q).

Transition from E2 to E1-Ca$^{2+}$-Ca$^{2+}$ dependence of phosphorylation with ATP was determined in the presence of K$^+$ at various concentrations of Ca$^{2+}$ under conditions as described in the legend to Table I and thus similar to those for the ATPase assay. The experiments were performed with the four representative mutants (V200A, V200I, V200R, V200T) and wild-type SERCA1a. The dissociation constant for Ca$^{2+}$ and Hill coefficient obtained by least square fits for these mutants (0.20–0.29 μM and 1.8–2.5, respectively) were nearly the same as those for wild type (0.27 μM and 1.9, respectively).

To compare the rate of the E2 to E1-Ca$^{2+}$ transition, the four mutants and wild type were preincubated in the absence of Ca$^{2+}$ at pH 6, where equilibrium between E1 and E2 is most shifted to E2 (30) and then phosphorylated by the simultaneous addition of saturating concentrations of Ca$^{2+}$ and ATP under otherwise identical conditions as described above. The time course of EP formation was well described by the first order kinetics (data not shown). The rates (s$^{-1}$) obtained with the mutants (0.178 (V200A), 0.122 (V200I), 0.140 (V200R), and 0.204 (V200T)) were almost the same as or only slightly lower than that of the wild type (0.204). When ATP was added to the wild-type and mutant enzymes were repreincubated with Ca$^{2+}$, the EP formation was much faster and reached its maximal level within 1 s (data not shown). These results show that the Ca$^{2+}$-induced E2 to E1-Ca$^{2+}$ transition, which is rate-limiting for the EP formation, takes place at almost the same rate in the mutants and wild type.

²The results with the wild type (the very low accumulation of E2P in spite of the slow $k_{\text{isomerization}}$) are accounted for by the equilibrium in step 4 favoring E1P-Ca$^{2+}$ (29). To account for this very low E2P accumulation, it is also possible to assume the rate-limiting E1P to E2P isomerization (step 4) followed by rapid Ca$^{2+}$ release (step 5) and hydrolysis (step 6).

In any case, however, the same conclusion as described in text will be made for the mutants that the step after loss of ADP sensitivity but before hydrolysis of E2P is greatly slowed by the Val$^{200}$ substitutions.
In the present study, we explored possible roles of the Lys189-Lys205 loop on the A domain by mutagenesis and found that Val200 is critical for rapid processing of E2P, most likely in step 5, and also for rapid hydrolysis of E2P in step 6 in the Ca\textsuperscript{2+} transport cycle (Fig. 1). During isomerization of EP and Ca\textsuperscript{2+} release, a large movement of the A domain (i.e. rotation by \textasciitilde 90° (10)) and its strong association with the P and N domains occurs to form the most compactly organized cytoplasmic domains in E2P without bound Ca\textsuperscript{2+} (12, 13). In this E2P, a hydrophobic atmosphere (28, 31–33) is thus realized around the phosphorylation site, and a specific water molecule can now attack the acylphosphate bond. It is likely that the Lys189–Lys205 loop, especially Val200, is critical for the appropriate domain interactions and proper structure in E2P without bound Ca\textsuperscript{2+}.

In E2V that is very similar to E2P without bound Ca\textsuperscript{2+} in the cytoplasmic domain organization (12, 13), the Lys189-Lys205 loop forms an A-P domain interface (Fig. 7). The side chain of Val200 protrudes toward the P domain and is in van der Waals contact with the side chain hydrocarbon parts of Arg198, Gln202, and Asp203, which interact with polar residues on the P domain by hydrogen bonding or salt bridge formation (broken green lines in Fig. 7). It is likely that Val200 coordinates spatial arrangement of these surrounding residues by the nonpolar interactions so as to produce the most favorable configuration for appropriate interactions at the terminal N and O atoms with the polar residues on P domain and thus for concurrent contribution of these residues to intimate contact between the A and P domains in E2P without bound Ca\textsuperscript{2+}.

In agreement with this view, the substitutions of Val200 by any examined conservative or nonconservative amino acids resulted in strong inhibition of the Ca\textsuperscript{2+}-ATPase activity because of inhibition of the EP decay, whereas substitutions of each of the above surrounding three residues caused only moderately reduced activity (Fig. 2 and Refs. 17 and 26). Actually, the positive charge of Arg198 was previously shown to be important for rapid E2P hydrolysis, and its nonconservative substitutions partially reduce the \( k_{\text{hydrolysis}} \) value (by 62%) (17). Mutations of Glu\textsuperscript{680} (4) and of Arg\textsuperscript{176} on the P domain (see Fig. 7) also cause only partial loss of function.\textsuperscript{3} It is likely that the substitutions at Val200 would seriously affect coordination of the surrounding residues and thus appropriate interactions of the Lys189–Lys205 loop with the P domain.

The observed high Ca\textsuperscript{2+} affinity and rate of the Ca\textsuperscript{2+}-induced activation in the Val200 mutants being comparable with those in the wild type (see "Results") indicate that the interactions between the Lys189–Lys205 loop and the P domain likely do not contribute much to cytoplasmic domain organization in the unphosphorylated states and thus would be mostly lost on E2P hydrolysis. In fact, in E2(TG) that is very similar to E2 in the cytoplasmic domain organization (12), no hydrogen bonds or salt bridges are found between the Lys189–Lys205 loop and the P domain (Fig. 7). Consistently, the enzyme in E2 state is rapidly attacked and degraded by proteinase K and V8 protease, whereas E2P without bound Ca\textsuperscript{2+} is completely resistant to all proteinase K, V8 protease, and trypsin at the T2 site (Arg159) (12, 13), E2 is, however, partially resistant to tryptic attack at the T2 site, indicating that the A domain in this state is still partially associated with the P and N domains and has not yet completely rotated back to the widely separated state found in E1-Ca\textsubscript{2} (12, 13), as is in fact seen in the E2(TG) structure. Possible interactions between the A and N domains and between the TGES loop and the P domain (possibly through ligation of Mg\textsuperscript{2+} (15)) may be significant for holding the A domain in such an organized state in E2. On hydrolysis of E2P to E2, the atmosphere around the phosphorylation site again becomes hydrophilic (28, 31–33). It is possible that loss of the interactions between the Lys189–Lys205 loop and the P domain contribute at least in part to such changes in hydrophobicity.

In summary, we conclude that the Lys189–Lys205 loop, especially Val200, is critical for the intimate contact between the A and P domains and the appropriate cytoplasmic domain organization in E2P without bound Ca\textsuperscript{2+}. Although the structure of E2P-Ca\textsubscript{2} (ADP-insensitive EP with occluded Ca\textsuperscript{2+} (8)) is not yet characterized, the final process of appropriate gathering and intimate contact of the A domain with the P and N domains will likely be accomplished in step 5 after the loss of ADP sensitivity, and this change is likely essential for Ca\textsuperscript{2+} release into lumen.

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\textsuperscript{3} K. Yamasaki, T. Daiho, S. Kato, and H. Suzuki, unpublished observations with Arg\textsuperscript{176} substitutions.
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