Mutations of Either or Both Cys\textsuperscript{876} and Cys\textsuperscript{888} Residues of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase Result in a Complete Loss of Ca\textsuperscript{2+} Transport Activity without a Loss of Ca\textsuperscript{2+}-dependent ATPase Activity

ROLE OF THE CYS\textsuperscript{876}-CYS\textsuperscript{888} DISULFIDE BOND

Disulfide-containing peptides in pepsin digest of sarcoplasmic reticulum vesicles were identified by using a fluorogenic thiol-specific reagent 4-fluoro-7-sulfamoylbenzofurazan and a reductant tributylphosphine. Sequencing of the purified peptides revealed the presence of a Cys\textsuperscript{876}-Cys\textsuperscript{888} disulfide bond on the luminal loop connecting the 7th and 8th transmembrane helices (loop 7–8) of the Ca\textsuperscript{2+}-ATPase (SERCA1a). We substituted either or both of these cysteine residues with alanine and made three mutants (C876A, C888A, C876A/C888A), in which the disulfide bond is disrupted. The mutants and the wild type were expressed in COS-1 cells, and functional analysis was performed with the microsomes isolated from the cells. Electrophoresis performed under reducing and non-reducing conditions confirmed the presence of Cys\textsuperscript{876}-Cys\textsuperscript{888} disulfide bond in the expressed wild type. All the three mutants possessed high Ca\textsuperscript{2+}-ATPase activity. In contrast, no Ca\textsuperscript{2+} transport activity was detected with these mutants. These mutants formed almost the same amount of phosphoenzyme in intermediate as the wild type from ATP and from Pi. Detailed kinetic analysis showed that the three mutants hydrolyze ATP in the mechanism well accepted for the Ca\textsuperscript{2+}-ATPase; activation of the catalytic site upon high affinity Ca\textsuperscript{2+} binding, formation of ADP-sensitive phosphoenzyme, subsequent rate-limiting transition to ADP-insensitive phosphoenzyme, and hydrolysis of the latter phosphoenzyme. It is likely that the pathway for delivery of Ca\textsuperscript{2+} from the binding sites into the lumen of vesicles is disrupted by disruption of the Cys\textsuperscript{876}-Cys\textsuperscript{888} disulfide bond, and therefore that the loop 7–8 having the disulfide bond is important for formation of the proper structure of the Ca\textsuperscript{2+} pathway.

The Ca\textsuperscript{2+}-ATPase of adult fast-twitch skeletal muscle sarcoplasmic reticulum (SERCA1a)\textsuperscript{1} is a 994-residue membrane-bound protein (1, 2) that catalyzes Ca\textsuperscript{2+} transport coupled to ATP hydrolysis (3, 4). In the catalytic cycle, the enzyme is activated by binding of two Ca\textsuperscript{2+} ions to the transport sites from the cytoplasmic side, and then γ-phosphoryl group of ATP is transferred to Asp\textsuperscript{351} (5–7) to form ADP-sensitive EP, which can react with ADP to form ATP (8–10). Upon formation of this EP, the two Ca\textsuperscript{2+} ions are occluded. A subsequent rate-limiting transition of ADP-sensitive EP to ADP-insensitive EP, which cannot react with ADP, results in release of the Ca\textsuperscript{2+} ions into the lumen. Finally, ADP-insensitive EP is hydrolyzed to form P\textsubscript{i} and the dephosphoenzyme. This EP can also be formed from P\textsubscript{i} in the absence of Ca\textsuperscript{2+} by reversal of its hydrolysis (11, 12). The Ca\textsuperscript{2+}-ATPase contains ten transmembrane-helices (M1 to M10), and the bound two Ca\textsuperscript{2+} ions are shown to be located side by side near the center of four helices, M4, M5, M6, and M8 in the crystal structure (13). The ATP binding site and phosphorylation site are located on the large cytoplasmic loop between M4 and M5 (13, 14). Luminal loops are short except for the one connecting M7 and M8, the loop 7–8 (approximately Ala\textsuperscript{553}Glu\textsuperscript{585}), which protrudes into the luminal space in the crystal structure (13). Possible roles of this loop have not yet been well understood. This loop contains two cysteine residues, Cys\textsuperscript{876} and Cys\textsuperscript{888}. These residues have been predicted to participate in disulfide bonds (15), although the exact disulfide structure has yet to be identified. Because mutations of these residues were reported to cause partial loss of function (14), the possible disulfide bonds formed with Cys\textsuperscript{876} and with Cys\textsuperscript{888} likely to be important for structure and function of the enzyme.

Previous studies demonstrated (15–17) that totally 3 or 4 disulfide bonds are present in the Ca\textsuperscript{2+}-ATPase. We showed (16) that all of the disulfide bonds in the enzyme of the SR vesicles were not readily reduced with dithiothreitol even at high concentrations but can be readily reduced if both Ca\textsuperscript{2+} and a purine nucleotide are present. We further revealed that the reduction time courses of the disulfide bonds were not well separated from each other and thus that selective reduction of the disulfide bonds in the enzyme is impossible (16). The functional roles of the disulfide bonds can be explored, therefore, only by the site-directed mutagenesis of the cysteine residues identified as to form the disulfide bond of interest.

In the present study, we have explored first the exact disulphide bonds: FCR, polymerase chain reaction; MOPS, 3-(N-morpholino)propane sulfonic acid; MES, 2-(N-morpholinoethanesulfonic acid; ABDF, 4-fluoro-7-sulfamoylbenzofurazan; ABD, 7-sulfamoylbenzofurazan-4-yl; TBP, tributylphosphine; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

\textsuperscript{1} The abbreviations used are: SERCA1a, adult fast-twitch skeletal muscle sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase; SR, sarcoplasmic reticulum; ATP, adenosine triphosphate; EP, ADP-sensitive enzyme; P, the two Ca\textsuperscript{2+} ions are occluded. A subsequent rate-limiting transition of ADP-sensitive EP to ADP-insensitive EP, which cannot react with ADP, results in release of the Ca\textsuperscript{2+} ions into the lumen. Finally, ADP-insensitive EP is hydrolyzed to form P\textsubscript{i} and the dephosphoenzyme. This EP can also be formed from P\textsubscript{i} in the absence of Ca\textsuperscript{2+} by reversal of its hydrolysis (11, 12).

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\textsuperscript{d} The abbreviations used are: SERCA1a, adult fast-twitch skeletal muscle sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase; SR, sarcoplasmic reticulum; ATP, adenosine triphosphate; EP, ADP-sensitive enzyme; P, the two Ca\textsuperscript{2+} ions are occluded. A subsequent rate-limiting transition of ADP-sensitive EP to ADP-insensitive EP, which cannot react with ADP, results in release of the Ca\textsuperscript{2+} ions into the lumen. Finally, ADP-insensitive EP is hydrolyzed to form P\textsubscript{i} and the dephosphoenzyme. This EP can also be formed from P\textsubscript{i} in the absence of Ca\textsuperscript{2+} by reversal of its hydrolysis (11, 12).

\textsuperscript{e} This paper is available on line at http://www.jbc.org
fide structure of Cys876 and Cys888 on the loop 7–8, and then to identify the possible role of the disulfide bond. Sequencing of the disulfide-containing peptides purified from the pepsin digest of SR vesicles showed that a disulfide bond is formed between Cys876 and Cys888 of the Ca2+-ATPase. We have then substituted either or both of these residues with alanine and made three SERCA1α mutants (C876A, C888A, and C876A/C888A), in which the disulfide bond is disrupted. All the three mutants hydrolyzed ATP at high rates in the mechanism well accepted for the Ca2+-ATPase. In contrast, none of the mutants could transport Ca2+ at a detectable rate. Results indicate that the loop 7–8 having the disulfide bond is important for formation of the proper structure of the Ca2+-release pathway.

EXPERIMENTAL PROCEDURES

Preparation of SR Vesicles—SR vesicles were prepared from rabbit skeletal muscle as described previously (18). The content of phosphorylation site determined with [γ-32P]ATP according to Barrabrin et al. (19) was 4.1 ± 0.1 nmol/mg of SR protein (n = 6). The number of disulfide bonds in the Ca2+-ATPase of SR vesicles estimated with ABD-F and TBP according to Kirley (20) was 2.4 ± 0.4 disulfide bonds (n = 4), and agreed closely with the number determined by Thorley-Lawson et al. (21), who sequenced three disulfide bonds of the Ca2+-ATPase.

Identification of Disulfide Bonds—SR vesicles (1 mg/ml) in 12 mm HCl (pH 2.0) were degassed and flushed with nitrogen, and then digested with pepsin (0.1 mg/ml) under nitrogen at 37 °C for 1 h under acidic conditions that were previously shown to prevent possible disulfide interchanges (21) and oxidation of cysteine residues (22, 23). After centrifugation, the supernatant was subjected to reversed phase HPLC performed as previously described (24). All the elution buffers were acidic (pH below 4) and degassed with an on-line degasser to prevent the possible oxidation and disulfide interchanges (21–23). Aliquots of each fraction were assayed for disulfides by simultaneous reduction and labeling of disulfides with a fluorogenic thiol-specific reagent ABD-F and a reductant TBP. ABD-F reacts with neither disulfide interchanges (21) and oxidation of cysteine residues (22, 23), and in solutions de-gassed or flushed with nitrogen prior to and during use (see “Experimental Procedures”). When SR vesicles were digested extensively with pepsin, 78% of disulfides in the whole digest were acidic (pH below 4), which prevent possible disulfide interchanges or oxidation of cysteine residues (21–23), and in solutions de-gassed or flushed with nitrogen prior to and during use (see “Experimental Procedures”). When SR vesicles were digested extensively with pepsin, 78% of disulfides in the whole digest was recovered in the supernatant. The supernatant was subjected to the first reversed phase HPLC, and aliquots of each fraction were assayed for disulfides (Fig. 1, A–C). Peptides containing disulfides were present in a major sharp peak, fraction I, and two minor broad peaks, fraction II and fraction III (Fig. 1C).

In the second reversed phase HPLC, fraction I gave a single disulfide-containing peak (peak Ia, Fig. 1, D–F). Peptides in this peak were further purified in the third reversed phase HPLC, and aliquots of each fraction were assayed for disulfides (Fig. 1, G–I). Peptides containing disulfides were present in a major sharp peak, fraction Ia, and one minor (peak Ia*) and one minor (peptide Ia**) fluorescent peaks of bound ABD, which gave the sequences Met774-Glu889 and Met774-Cys888, respectively, of SERCA1α (Table I). These peptides contained only two cysteine residues, Cys876 and Cys888, and both of these cysteine residues had been labeled with ABD-F only in the presence of TBP to generate ADB-cysteine (Fig. 1E). The results show that a disulfide bond(s) is formed with these cysteine residues. Furthermore, we found in SDS-PAGE performed under non-reducing conditions that the observed molecular mass of the Ca2+-ATPase corresponded to its monomeric form but not to a possible dimeric or higher oligo-linked detection system (Amersham Pharmacia Biotech).
FIG. 1 Peptide mapping of pepsin digests of SR vesicles and purification of peptides containing disulfides. All the procedures for identification of disulfide bonds were carried out under conditions that prevent possible oxidation and disulfide interchanges, as described under “Experimental Procedures.” A–C, SR vesicles were digested with pepsin and subjected to the first reversed phase HPLC. Elution was performed with an acetonitrile gradient in 0.1% trifluoroacetic acid. The absorbance of peptides was monitored at 214 nm (A195). Fluorescence intensity of bound ABD was measured after incubating an aliquot of each fraction with ABD-F in the presence (○) or absence (■) of TBP (B), and the difference in the intensity (△) was plotted (C). Fractions containing disulfides, indicated by horizontal bars I, II, and III, were pooled separately and subjected to the second reversed phase HPLC in D–F, G–I, and J–L, respectively, with an acetonitrile gradient in 15 mm ammonium acetate (pH 4.0). Fluorescence intensity of bound ABD was measured after the incubation in the presence (○) or absence (■) of TBP as above (E, H, and K), and the difference in the intensity (△) was plotted (F, I, and L). Fractions containing disulfides, indicated by horizontal bars Ia, Ib, IIIa, IIIb, IIIc, and IIId, were pooled separately for further purification.

FIG. 2. Disulfide Bond in SR Ca2+/ATPase. In the second reversed phase HPLC, fraction II and fraction III gave two (peak IIa and peak IIb) (Fig. 1, G–I) and four (peak IIIa, peak IIIb, peak IIIc, and peak IIId) (Fig. 1, J–L) disulfide-containing peaks, respectively. The disulfide-containing peptides in these peaks were further purified and labeled with ABD-F in the presence of TBP as above. The peptide from peak IIIc gave a sequence Met874–Phe891 of SERCA1a, in which Cys876 and Cys888 had been labeled with ABD only in the 160-kDa glycoprotein (and its splice variant 53-kDa glycoprotein) of SERCA1a as in the SR Ca2+/ATPase. In Fig. 2, the microsomes, SR vesicles, and their tryptic digests were either unreduced or reduced in SDS with 2-mercaptoethanol at a high concentration (0.7 M), and then subjected to SDS-PAGE and immunoblotting.

Effects of 2-Mercaptoethanol on Mobilities of SERCA1a and Its Tryptic Fragments in SDS-PAGE—To explore possible roles of the Cys876–Cys888 disulfide bond, we made three SERCA1a substitution mutants, C876A, C888A, and C876A/C888A, in which the disulfide bond of interest is disrupted, and expressed in COS-1 cells. The microsomes were prepared from the cells in the presence of 3 mM 2-mercaptoethanol to prevent possible formation of non-native disulfide bonds in the wild type and mutants. We first confirmed the presence of the intramolecular Cys876–Cys888 disulfide bond in the expressed wild type SERCA1a as in the SR Ca2+/ATPase. In Fig. 2, the microsomes, SR vesicles, and their tryptic digests were either unreduced or reduced in SDS with 2-mercaptoethanol at a high concentration (0.7 M), and then subjected to SDS-PAGE and immunoblotting.

When reduced, all the SR Ca2+/ATPase, wild type, and three mutants migrated at 110 kDa (Fig. 2A, dotted lines), which agrees with the molecular mass (1, 2). When unreduced, the SR Ca2+–ATPase migrated at a substantially faster rate (95 kDa) (Fig. 2A, arrows), but the three mutants migrated only very slightly faster than the reduced 110-kDa ATPase chains. The unreduced wild type SERCA1a migrated at exactly the same rate (95 kDa) as the unreduced SR Ca2+–ATPase. These results together with the above sequencing results show that the expressed wild type SERCA1a as well as the SR Ca2+–ATPase...
The disulfide-containing peptides purified from the pepsi digest of SR vesicles were labeled with 0.2 mM ABD-F in the presence of 0.5 mM TBP then purified by the fourth reversed phase HPLC and sequenced.

which were prepared from the COS-1 cells transfected with the vector containing no SERCA1a cDNA. This background level was as low as 4% of the activity of microsomes expressing the wild type SERCA1a. The specific transport rate/mg of the wild type SERCA1a increased with increasing Ca\(^{2+}\) concentration, and reached the maximal level at 10 \(\mu M\) Ca\(^{2+}\) with the half-maximal rate at 0.4 \(\mu M\) Ca\(^{2+}\) (Fig. 3B). In contrast, none of the mutants C876A, C888A, and C876A/C888A was able to transport Ca\(^{2+}\) at a detectable rate at all the Ca\(^{2+}\) concentrations examined.

ATP hydrolysis was assayed with 0.1 mM \([\gamma-\text{P}]\text{ATP}\) in the presence of Ca\(^{2+}\) ionophore A23187 under conditions otherwise similar to those for the Ca\(^{2+}\) transport assay. In sharp contrast to non-detectable Ca\(^{2+}\) transport activity, fairly high thapsigargin-sensitive ATPase activities were observed with the microsomes expressing the mutants over the background level of the control microsomes at 10 \(\mu M\) Ca\(^{2+}\) (Fig. 4A). This background level was 8% of the activity of microsomes expressing the wild type SERCA1a. The specific ATPase activity/mg of SERCA1a protein increased with increasing Ca\(^{2+}\) concentration, and reached the maximal level at 10 \(\mu M\) Ca\(^{2+}\) with the half-maximal activity at 1.0–1.2 \(\mu M\) Ca\(^{2+}\) for the three mutants and 0.4 \(\mu M\) Ca\(^{2+}\) for the wild type (Fig. 4B). The maximal specific activities of the three mutants agreed closely with each other, and ~1.5 times higher than that of the wild type.

**Ca\(^{2+}\) Concentration Dependence of Phosphorylation with ATP and with \(P_i\)**—EP was formed from 10 \(\mu M\) ATP at 0 °C under conditions otherwise similar to those for the ATPase assay (Fig. 5A). The amounts of EP formed with the mutant and wild type SERCA1a at steady state increased with increasing Ca\(^{2+}\) concentration and reached the maximal level at 10 \(\mu M\) Ca\(^{2+}\). The half-maximal EP formation was obtained at 0.8–1.0 \(\mu M\) Ca\(^{2+}\) for the three mutants and 0.4 \(\mu M\) Ca\(^{2+}\) for the wild type. The maximal EP levels of the three mutants were almost the same as that of the wild type.

**Phosphorylation with \(P_i\)** was performed in the presence of 35% (v/v) Me\(_2\)SO, which greatly favors EP formation (39). The three mutants and the wild type formed almost the same maximal amounts of EP at pCa 9–7 (Fig. 5B). The EP formation was almost completely inhibited at 100 \(\mu M\) Ca\(^{2+}\) with the half-maximal inhibition at 2.3–2.9 \(\mu M\) Ca\(^{2+}\).
Formation of ADP-sensitive EP and ADP-insensitive EP from ATP—EP was formed from ATP in the presence and absence of added KCl at 0°C and 50 μM Ca²⁺ under conditions otherwise similar to those for the ATPase assay, and the total amount of EP and amount of ADP-insensitive EP were determined at steady state. In the presence of KCl, which strongly accelerates hydrolysis of ADP-insensitive EP and thus suppresses its accumulation (40), only a very small fraction of EP was ADP-insensitive in the three mutants as well as in the wild type (Fig. 6A). On the other hand, in the absence of KCl, EP formed was largely ADP-insensitive in the three mutants as well as in the wild type (Fig. 6B).

Dephosphorylation of EP—Dephosphorylation of ADP-sensitive EP in the presence of K⁺ was examined by first phosphorylating the Ca²⁺-ATPase with [γ-³²P]ATP at 50 μM Ca²⁺ under the conditions in Fig. 6A, in which EP formed at steady state is almost completely ADP-sensitive and then terminating phosphorylation by adding excess EGTA to allow dephosphorylation of ³²P-labeled EP (Fig. 7A). The dephosphorylation of the ADP-sensitive EP in the three mutants and in the wild type proceeded with first-order kinetics. The results are consistent with the view that the transition of EP from ADP-sensitive to ADP-insensitive form is rate-limiting for the dephosphorylation in the presence of K⁺. Dephosphorylation rates of the three mutants agreed well with each other, and ~1.7 times higher than that of the wild type.

Hydrolysis of ADP-insensitive EP was examined in the absence of K⁺ by first phosphorylating the Ca²⁺-ATPase with ³²P and then chasing with an excess of non-radioactive Pᵢ (Fig. 7B). Hydrolysis of the ³²P-labeled ADP-insensitive EP in the three mutants and in the wild type proceeded with first-order kinetics. The hydrolysis rates of the three mutants were almost the same as that of the wild type.

DISCUSSION

The present study shows that an intramolecular disulfide bond is formed between Cys⁸⁷⁶ and Cys⁸⁸⁸ on the luminal loop of SERCA1a and expressed wild type SERCA1a, and that the disruption of this disulfide bond by the mutations results in a complete loss of the Ca²⁺ transport activity without a loss of the Ca²⁺-dependent ATP hydrolysis activity. It is likely that the pathway for delivery of Ca²⁺ from the binding sites into the lumen of vesicles is disrupted by disruption of the
Disulfide Bond in SR Ca\(^{2+}\)-ATPase

FIG. 5. Dependence of phosphorylation of SERCA1a with [\(^{\gamma-}\)\(^{32}\)P]ATP or \(^{32}\)P on Ca\(^{2+}\) concentration. A, microsomes expressing the wild type or mutant SERCA1a were phosphorylated with [\(^{\gamma-}\)\(^{32}\)P]ATP at 0 °C for 15 s in 100 μl of a mixture containing 10 μg of microsomal protein, 1 μM A23187, 10 μM [\(^{\gamma-}\)\(^{32}\)P]ATP, 7 mM MgCl\(_2\), 0.1 mM KCl, 50 mM MOPS/NaOH (pH 7.0), 0.5 mM EGTA, and various concentrations of CaCl\(_2\). B, the microsomes were phosphorylated with \(^{32}\)P, at 25 °C for 10 min in 100 μl of a mixture containing 10 μg of microsomal protein, 0.1 mM \(^{\gamma-}\)32P, 10 mM MgCl\(_2\), 35% (v/v) Me\(_2\)SO, 100 mM MOPS/Tris (pH 7.0), 0.5 mM EGTA, and various concentrations of CaCl\(_2\). The amount of EP/mg of SERCA1a protein was determined. •, wild type; □, C876A; △, C888A; ▽, C876A/C888A. The values presented are the mean ± S.D. of four independent measurements.

Cys\(^{876}\)-Cys\(^{888}\) disulfide bond, and therefore that the loop 7–8 having the disulfide bond is important for formation of the proper structure of the Ca\(^{2+}\) pathway.

It is unlikely that this disulfide bond is a non-native one formed by oxidation or disulfide interchanges during the proteolysis and chromatographic isolation procedures, because all the procedures were carried out under the conditions that prevent oxidation and disulfide interchanges (21–23). It should also be noted that the disulfide bond is present in the expressed wild type SERCA1a prepared in the presence of 2-mercaptoethanol (Fig. 2). This result is consistent with our previous finding that the disulfide bonds in the enzyme are not readily reducible (see Ref. 16 and the introduction). The observed identical behavior of the wild type SERCA1a and the SR Ca\(^{2+}\)-ATPase in SDS-PAGE performed under reducing and non-reducing conditions (Fig. 2), therefore, also indicates that the Cys\(^{876}\)-Cys\(^{888}\) disulfide bond is present in the native enzyme.

Previous studies by Green and co-workers (15, 17) as well as ours (16) have demonstrated that totally three or four disulfide bonds are present in the enzyme molecule. The Cys\(^{876}\)-Cys\(^{888}\) bond has been considered as a most likely candidate for one of the disulfide bonds (15, 32). Close location of Cys\(^{876}\) to Cys\(^{888}\) (at 7.7 Å between their α-carbons) in the crystal structure (13) suggests formation of the Cys\(^{876}\)-Cys\(^{888}\) disulfide bond being potentially possible. However, no disulfide bonds are seen in the crystal structure (13). It may be possible that disulfide bonds of the enzyme in the crystal had been reduced by dithiothreitol during the purification and dialysis for crystallization of the enzyme. We think this is quite possible, because all the disulfide bonds in the enzyme can not be readily reduced by dithiothreitol but can be readily reduced if both Ca\(^{2+}\) and ADP are present (16) and because, in fact, Ca\(^{2+}\), ADP, and dithiothreitol were all present during the purification with a Red-agarose column chromatography and dialysis (in its initial period) employed for crystallization of the enzyme (13).

The requirements for the disulfide bond would be demonstrated unambiguously if it were possible to obtain functional alternations by treating the native enzyme with reductants and thus by selective reduction of the disulfide bond of interest. However, our previous finding showed that such selective reduction is impossible to achieve (see Ref. 16 and the introduc-
The characteristics of the mutants in the ATP hydrolysis reaction are in essential agreement with those of the wild type. The disulfide bonds with only a very slightly faster reduction of the three mutants C876A, C888A, and C876A/C888A, in which the Cys876-Cys888 disulfide bond is disrupted, hydrolyzed Ca\(^{2+}\)-dependent ATP hydrolysis is likely caused by disruption of the pathway for delivery of Ca\(^{2+}\) from the binding sites into the lumen of vesicles, or possibly by increased rate of passive efflux through the long-range interaction (41, 42) was not inhibited in the mutants (Figs. 4 and 5). The observed predominant accumulation of ADP-sensitive EP in the presence of K\(^+\) is in harmony with the presence of the rate-limiting transition of EP from ADP-sensitive form to ADP-insensitive form in the ATPase cycle (Fig. 6). In fact, the decay rate of the accumulated ADP-sensitive EP and the specific ATPase activity were changed to almost the same extent (1.5–1.7 times increase) by the mutations (Figs. 4B and 7A). Hydrolysis of the ADP-insensitive EP in the mutants was directly demonstrated to take place by the EP formation from P\(_i\), in the reversal of its hydrolysis (Fig. 5B) and by dephosphorylation of this EP occurring with almost the same rate as the wild type (Fig. 7B). Collectively, the results show that the Ca\(^{2+}\)-activated mutants hydrolyze ATP through formation of ADP-sensitive EP, subsequent rate-limiting transition of this EP to ADP-insensitive EP, and hydrolysis of the latter EP, in the mechanism well accepted for the Ca\(^{2+}\)-ATPase. It should be noted that the high affinity ATP binding at the catalytic site is also not impaired in the mutants, because the level of EP formed from ATP as low as 10 \(\mu\)M was almost the same as that of the wild type (Fig. 5A).

Because the substitution of either or both of the two residues resulted in the complete loss of the Ca\(^{2+}\)-transport activity (Fig. 3), the loss is most likely a consequence of the disruption of the Cys\(^{876}\)-Cys\(^{888}\) disulfide bond. The observation that the three mutants are identical to each other also in the altered kinetic properties in the ATP hydrolysis is consistent with the view that each of the two cysteine residues has a specific function in the formation of the disulfide bond. However, it cannot be excluded that the effect of mutations may be due to other effects such as steric hindrance in the site caused by the different side chain.

The complete loss of Ca\(^{2+}\) transport without a loss of normal Ca\(^{2+}\)-dependent ATP hydrolysis is likely caused by disruption of the pathway for delivery of Ca\(^{2+}\) from the binding sites into the lumen of vesicles, or possibly by increased rate of passive efflux of transported Ca\(^{2+}\) through the mutants. Cys\(^{876}\) and Cys\(^{888}\) are located on the long luminal loop 7–8, which is directly connected to M7 and M8. In the crystal structure (13), M7 is in close contact with M5 near the luminal surface and the likely Ca\(^{2+}\)-outlet to lumen is predicted to be in the area surrounded by M5, M4, and M3. M8 is directly involved in the formation of the Ca\(^{2+}\) channel (13). It is possible that the tertiary structure of loop 7–8 stabilized by the Cys\(^{876}\)-Cys\(^{888}\) disulfide bond is essential for proper orientation of M7 and M8 relative to other helices, conferring appropriate packing of the helices required for the delivery of Ca\(^{2+}\) into the lumen and prevention of passive Ca\(^{2+}\) efflux. Interestingly, mutations of the residues on M5, Val\(^{772}\), Cys\(^{774}\), and Ile\(^{775}\), which are located at the position in close contact with M7 in the crystal structure (13), were reported to cause inhibition of Ca\(^{2+}\) uptake with little or no inhibition of ATPase activity, i.e. the uncoupling effect (43). In agreement with the view that local interference with proper packing of the helices results in the uncoupling, several other mutations within M4, M5, M6, or M8 were also reported to cause the uncoupling (43, 44). A possible role in sealing the lumen and preventing passive Ca\(^{2+}\) efflux was predicted (43) for Cys\(^{776}\) located at the luminal end of M4.

Alternatively, the loop 7–8 may directly be involved in the Ca\(^{2+}\) release process. Kinetic studies on the effect of luminal Ca\(^{2+}\) on EP formation from P\(_i\) by Myung and Jencks (45) predicted a second set of Ca\(^{2+}\) binding sites on the luminal surface, possibly on the loop 7–8 (46), through which Ca\(^{2+}\) is released into the lumen. If such sites exist on this loop, it is possible that disruption of the Cys\(^{876}\)-Cys\(^{888}\) disulfide bond.
could result in the loss of the Ca\textsuperscript{2+} transport activity.

The loop 7–8 regions of the Ca\textsuperscript{2+}-ATPases in the SERCA family have high homology to each other, and both Cys\textsuperscript{875} and Cys\textsuperscript{888} are conserved in this family (1, 2, 47–49). On the other hand, other members of P-type ATPases, plasma membrane Ca\textsuperscript{2+}-ATPase (50), Na\textsuperscript{+}K\textsuperscript{+}-ATPase (51, 52), and H\textsuperscript{+}K\textsuperscript{+}-ATPase (53) contain no cysteine residues (or only one in human gastric H\textsuperscript{+}K\textsuperscript{+}-ATPase (54)) on the loop 7–8 region. It is possible that the role of the disulfide bond on the loop 7–8 found in this paper is specific to the SERCA family.

Recently, Darier disease, a human autosomal dominant skin disorder, was shown to be caused by mutations in the SERCA2b gene (55), and a single missense mutation at Cys\textsuperscript{875} (C875G) was found in one of the Darier-disease pedigrees (56). Therefore, Cys\textsuperscript{875} of SERCA1a, our present observations suggest that the mutation i.e. disruption of the Cys\textsuperscript{875}-Cys\textsuperscript{888} disulfide bond in SERCA2b results in the loss of the Ca\textsuperscript{2+} transport and therefore causes perturbation in calcium homeostasis and the disease.

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