Site-directed Disulfide Mapping of Helices M4 and M6 in the Ca\(^{2+}\) Binding Domain of SERCA1a, the Ca\(^{2+}\) ATPase of Fast Twitch Skeletal Muscle Sarcoplasmic Reticulum

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In an attempt to define the spatial relationships among SERCA1a transmembrane helices M4, M5, M6, and M8, involved in Ca\(^{2+}\) binding, all six cysteine residues were removed from predicted transmembrane sequences by substitution with Ser or Ala. The cysteine-depleted protein retained 44% of wild type Ca\(^{2+}\) transport activity. Pairs of cysteine residues were then reintroduced to determine whether their juxtaposition would result in the formation of disulfide cross-links between transmembrane helices. In initial studies designed to map the juxtaposition of Ca\(^{2+}\) binding residues, Cys was substituted for Glu\(^{309}\) or Gly\(^{310}\) in transmembrane sequence M4, in combination with the substitution of Cys for Glu\(^{771}\) in M5; for Asn\(^{796}\), Thr\(^{799}\), or Asp\(^{800}\) in M6; or for Glu\(^{808}\) in M8. These double mutants all retained the capacity to form a phosphoenzyme intermediate from P\(_i\), but not from ATP in the presence of Ca\(^{2+}\), and in all but mutants E309C/N796C and G310C/N796C, phosphoenzyme formation was insensitive to 100 \(\mu\)M Ca\(^{2+}\). These results support the view that both Glu\(^{309}\) and Asp\(^{800}\) contribute to Ca\(^{2+}\) binding site II, which is not required for conversion of E\(_s\), the substrate for P\(_i\) phosphorylation, to E\(_o\), Cross-linking in mutants E309C/N796C and G310C/L807C established reference points for the orientation of M4 and M6 relative to each other and provided the basis for the prediction of potential additional cross-links. Strong links were formed with the pairs T317C/A804C and T317C/L807C near the cytoplasmic ends of the two helices and with A305C/L792C and A305C/L793C near the luminal ends of these combined results support the conclusion that M4 and M6 form a right-handed coiled-coil structure that forms part of the pathway of Ca\(^{2+}\) translocation. In addition to providing a possible explanation for the mutation sensitivity of several pairs of residues in these helices, the proposed association of M4 and M6 supports a new model for the orientation of the two Ca\(^{2+}\) binding sites among transmembrane helices M4, M5, and M6.

The Ca\(^{2+}\) ATPase of rabbit fast twitch skeletal muscle sarcomplasmic reticulum (SERCA1a) is a 110-kDa transmembrane protein, which transports Ca\(^{2+}\) ions from the sarcoplasm to the lumen of the membrane system at the expense of ATP hydrolysis. We developed an expression/mutagenesis system for analysis of the functional consequences of site-specific mutagenesis of the Ca\(^{2+}\) ATPase (1) and used it to identify six amino acids, located in transmembrane sequences M4, M5, M6, and M8, which appeared to provide Ca\(^{2+}\) binding ligands (2, 3). These were Glu\(^{309}\) in M4; Glu\(^{771}\) in M5; Asn\(^{796}\), Thr\(^{799}\), and Asp\(^{800}\) in M6; and Glu\(^{808}\) in M8. Since Ca\(^{2+}\) binding to two sites in the ATPase is sequential and cooperative (4, 5), it is of interest to assign Ca\(^{2+}\) binding residues to each of the two sites. Extensive mutational analysis of the six Ca\(^{2+}\) binding residues has provided criteria for the assignment of Glu\(^{309}\), Thr\(^{799}\), and Glu\(^{808}\) to Ca\(^{2+}\) binding site I (the more luminal of the two stacked sites) and Glu\(^{309}\) and Asn\(^{796}\) to Ca\(^{2+}\) binding site II (the more cytoplasmic of the two stacked sites) (6–8). Asp\(^{800}\) has been proposed to contribute to both sites (8). Mutants in five of the six Ca\(^{2+}\) binding residues and in Gly\(^{310}\) lost their capacity to occlude Ca\(^{2+}\), but E908D, which had lost Ca\(^{2+}\) transport activity, retained the ability to occlude Ca\(^{2+}\) (8, 9), and E908A retained both Ca\(^{2+}\) occlusion and transport (10).

Scanning mutagenesis of transmembrane segments M4, M5, M6, and M8 revealed that M4 and M6 contain patches of mutation-sensitive residues on one face of the central tiers of each helix (11, 12). Helix M5, which runs antiparallel to M4 and M6, has a similar patch of mutation-sensitive residues. M4 and M6 share a sequence motif, (E/D)GLPA(V/T). The motif forms a prominent part of the mutation-sensitive patch, suggesting that it may be an important component of the Ca\(^{2+}\) binding pore. A reverse sequence in the mutation-sensitive patch of M5 shares many characteristics of the M4/M6 motif. In particular, the sequences EGL (M4), EGV (reverse in M5), and DGL (M6) share a negative charge, a Gly linked to an acidic residue and a hydrophobic residue. In contrast, transmembrane helix M8 contained only one mutation-sensitive residue, Glu\(^{808}\), and only vestiges of the motif observed in M4 and M6. These results suggest that Glu\(^{808}\) and, indeed, all of M8 is unlikely to play more than a peripheral role in Ca\(^{2+}\) binding and translocation.

A limiting factor in our understanding of the mechanism of Ca\(^{2+}\) transport is our lack of knowledge of the detailed structure of the Ca\(^{2+}\) binding and translocation domain of SERCA1a. A method of site-directed disulfide mapping, in which cysteine residues are inserted in pairs into predicted transmembrane domains, followed by exposure to oxidant, has been used to map interactions between helices of several bacterial chemoreceptors (13–15). These proteins are homodimers,
with each monomer consisting of two membrane-spanning helices. Cross-links between monomers were detected by testing for anomalous migration of monomers in nonreducing SDS-PAGE. More recently, this method has been applied to eukaryotic multispanning membrane proteins such as rhodopsin (16) and P-glycoprotein (17). In the latter studies, the cDNA encoding a single large peptide chain was first divided into two fragments, and site-specific mutagenesis was used to introduce a single cysteine residue into each half. The two altered pieces were co-expressed and subjected to oxidizing conditions. Rejoining of the two expressed halves through disulfide bond formation was detected by SDS-PAGE.

Unlike P-glycoprotein and rhodopsin, expressed fragments of SERCA1 have not been shown to associate into an active enzyme complex. However, cleavage at the hypersensitive tryptic site, T1, located at Arg505 in the large cytoplasmic domain between transmembrane helices M4 and M5, divides the protein into two segments of nearly equal length (18, 19). Under conditions that stabilize the E2 conformation of the enzyme, this reaction is rapid, specific, and complete. Separation of the products by SDS-PAGE provides a convenient method of assay for cross-links formed between helix M4 and downstream helices. We have utilized this technique to explore the juxtaposition of the proposed Ca$^{2+}$ binding residues in the central tiers of helices M4, M5, M6, and M8, and we have extended the analysis to residues in the upper and lower tiers of the M4 and M6 helices, as illustrated in Fig. 1. The positions of cross-links demonstrate that M4 and M6 are likely to interact as a right-handed coiled-coil in the membrane. Insight into these structural arrangements has allowed us to propose a side-by-side arrangement of the Ca$^{2+}$ binding sites in the membrane (20).

EXPERIMENTAL PROCEDURES

Materials—Various enzymes for DNA manipulation were from New England Biolabs, Pharmacia Biotech Inc., Life Technologies, Inc., and Boehringer Mannheim GmbH. Tissue culture reagents were from Life Technologies, Inc. Trypsin, soybean trypsin inhibitor, NEM, PIPES, Tris, MOPS, bovine serum albumin, Tween 20, aprotinin, and 1,10-phenanthroline were from Sigma. $^{45}$Ca$^{2+}$ and $^{32}$P-ATP were from Amersham Corp. $^{32}$P was from Manzdel Scientific. Secondary antibodies were from Promega. Nicholson plastic models for helix modeling were from Labquip (Reading, UK).

Oligonucleotide-directed Mutagenesis, Expression of Mutant cDNA, and Preparation of Microsomes—Mutagenesis was carried out according to the method of Kunkel (21). Cell culture and transient transfections were performed as described previously (22). HEK-293 cells were harvested 40 h after transfection, and microsomes were prepared as described previously (1), with minor modifications made to accommodate the cross-linking assay. Briefly, cells were harvested 40 h after transfection, washed with phosphate-buffered saline, and frozen at 0 °C for 10 min in hypotonic solution. Aprotinin was added, and the cells were homogenized with 40 strokes in a glass Dounce homogenizer. The homogenate was diluted with an equal volume of solution A (0.5 m sucrose, 300 mM KCl, 10 mM Tris-HCl, pH 7.5, 40 mM CaCl$_2$, and 5 mM freshly dissolved glutathione) and homogenized with an additional 20 strokes. Mitochondria and nuclei were removed from the suspension by centrifugation at 10,000 × g for 30 min. The supernatant was made 0.6 M in KCl, and microsomes were pelleted by centrifugation at 180,000 × g for 1 h. The microsomal pellet was suspended in solution B (0.5 × solution A containing 10 mM Tris-HCl, pH 7.5). Microsomal protein was quantified (23) using bovine serum albumin as standard. Microsomes were flash-frozen in liquid N$_2$, and stored at −70 °C. Expression of mutant SERCA1a was verified by immunoblotting with monoclonal antibody A52 (24) and quantitated by an enzyme-linked immunosorbent assay (25).

$^{1}$ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; NEM, N-ethylmaleimide; E$_p$, ADP-sensitive phosphoenzyme intermediate; E$_{p}$P, ADP-insensitive phosphoenzyme intermediate; TBS, Tris-buffered saline, pH 7.5; DTT, dithiothreitol; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); MOPS, 3-(N-morpholino)propane-sulfonic acid.

Ca$^{2+}$ Dependence of Ca$^{2+}$ Transport Activity and Analysis of Phosphoenzyme Intermediates—Ca$^{2+}$ uptake activity was assayed as described previously (11). Free Ca$^{2+}$ concentrations were calculated using the computer program of Fabiato (26). Assays of phosphoenzyme formation from ATP and stability of E$_p$ or E$_{p}$P in nontransporting mutants were carried out as described previously (2, 27). Phosphorylation from P$_i$ was performed in a buffer containing 100 mM MOPS/Tris, pH 7.0, 10 mM MgCl$_2$, 0.5 mM $^{32}$P$_i$, 20% (v/v) Me$_2$SO, and either 100 μM CaCl$_2$ or 2 mM EGTA. Approximately 10 μg of microsomes were phosphorylated for 10 min at 22 °C before quenching with 10 volumes of ice-cold 7% trichloroacetic acid. Sample preparation and polyacrylamide gel electrophoresis were performed as described previously (2, 27).

Dissolute Cross-linking and Trypsin Digestion—Microsomes were.thawed, diluted to 1 μg of microsomal protein/ml, and reduced by the addition of 1% volume of 20 mM freshly dissolved glutathione. After a 5-min incubation at room temperature, samples were oxidized by the addition of an equal volume of a buffer containing copper phenanthroline as an oxidant and EGTA and vanadate, which are likely to promote the E$_p$ conformation. The final incubation conditions were 140 mM KCl, 5 mM MgCl$_2$, 5 mM EGTA, 400 μM sodium vanadate, 25 mM PIPES, pH 7, 150 mM sucrose, 1.75 mM glutathione, 0.3 mM CuSO$_4$, and 0.9 mM 1,10-phenanthroline. Following a 10-min incubation at room temperature, trypsin, at a concentration of 1 mg/ml, was added to a final ratio of 1:20 (trypsin/microsomal protein). After 4 min at room temperature, an equal volume of 2 x loading buffer (28), containing 25 mM NEM and 245 μg/ml soybean trypsin inhibitor in place of reducing agent, was added.

Glutaraldehyde Cross-linking—Glutaraldehyde cross-linking was performed by a modification of the method of Ross and McIntosh (29). Briefly, microsomes of SERCA1 were incubated with 20 μg of protein/ml were warmed to 22 °C. Cross-linking was initiated by the addition of 2 μl of 125 mM glutaraldehyde, followed by incubation for 3 min at 22 °C. Tryptic digestion was initiated by the addition of 1 μl of 0.5 μM EGTA, followed by 2 μl of trypsin (1 mg/ml), and incubation for 3 min at 22 °C. The reaction was stopped by the addition of 40 μl of nonreducing loading buffer.

Electrophoresis and Immunoblotting of Proteins—For nonreducing gels, samples were loaded on 8% polyacrylamide gels (37.5:1 acrylamide: bisacrylamide) and run according to standard protocols (28). For reducing gels, DTT was added to 200 μM, and samples were incubated at 37 °C for 5 min prior to loading on 10% acrylamide gels. Prestained standards (Bio-Rad) were used in both cases to visualize bands during the run. Nonstained standards (New England Biolabs) were loaded on nonreducing gels for more accurate determination of molecular weight. In both cases, loading volumes were adjusted so that approximately equal amounts of expressed SERCA1 were present in each lane. A semidry electroblot apparatus was used to transfer proteins to nitrocellulose membranes (30), and Ponceau red stain was used to visualize transferred protein. Membranes were blocked for 1 h in a solution of Tris-buffered saline, pH 7.5 (TBS), containing 0.5% (w/v) powdered milk and incubated with mouse monoclonal antibody A25 (N-terminal specific) or A52 (C-terminal specific) (24) at a concentration of 1 μg/ml in TBS containing 0.1% (w/v) powdered milk for an additional 60 min at room temperature. Membranes were washed twice with TBS containing 0.5% Tween 20 for 15 min and incubated in TBS with horseradish peroxidase-conjugated anti-mouse secondary antibody (Promega) according to the manufacturer’s instructions. Membranes were again washed in TBS/Tween, and expressed SERCA1 protein was detected with an enhanced chemiluminescence kit (Pierce SuperSignal). Bands were visualized on Kodak X-ray film. Quantitation of band intensities was done by analysis of computer-scanned images of the autoradiographs using NIH Image version 1.59 software.

RESULTS

Construction and Analysis of Serca1aC—The deduced transmembrane sequences of wild type SERCA1a contain six cysteine residues. Mutant Serca1aC was constructed by mutating Cys$^{70}$ to Ser and Cys$^{268}$, Cys$^{318}$, Cys$^{774}$, Cys$^{910}$, and Cys$^{938}$ to Ala. The locations of Cys$^{70}$, Cys$^{268}$, Cys$^{318}$, Cys$^{774}$, and Cys$^{910}$ are indicated in Fig. 1. Cys$^{774}$ and Cys$^{938}$, predicted to lie in a large luminal loop and to form a disulfide cross-link (31), could not be mutated because substitution of either of these residues reduced expression dramatically. Attempts at simultaneous removal of cytoplasmic cysteine residues C-terminal to the T$_2$ site also decreased expression, although none of them appear, individual...
Packing of SERCA1 Helices M4 and M6

**FIG. 1. Location of cysteine substitutions in transmembrane helices M4, M5, M6, and M8 of SERCA1α, displayed as helical nets (12).** Three lightly shaded residues, Cys316 in M4, Cys774 in M5, and Cys910 in M8, were mutated to Ala. Additional mutations, not indicated, were Cys597 to Ser in M1, Cys606 to Ala in M3, and Cys820 to Ala in M9. Darkly shaded residues were mutated from the residue indicated to cysteine. For cross-linking experiments, mutation to cysteine was carried out in pairs, as indicated under “Results.”

usually, to be essential to function.2

Serca1αC, expressed in HEK-293 cells, transported Ca2+ with activity equivalent to 44% of wild type (Table I). Apparent Ca2+ affinity, measured as Ca2+ dependence of Ca2+ uptake, was increased slightly, from pCa 6.5 in wild type to pCa 6.74 in Serca1αC (Table I). Since gross perturbations of the structure of the mutant protein would manifest as either loss of expression or loss of function, these data indicate that the overall structure of the Serca1αC mutant was not significantly different from that of the wild type enzyme.

Replacement of Ca2+ Binding Ligands with Cysteine—Our initial strategy was to introduce cysteine residues, one pair at a time, into different helices in the Ca2+ binding domain. Cys was substituted for either Glu309 or Gly310, in combination with a second substitution of Cys for either Glu771, Asn796, Thr799, Asp800, or Glu908. These residues, identified as probable Ca2+ binding ligands (2), were expected to cluster close to each other in the transmembrane domain. In later mutants, a series of residues in M4 and M6 were replaced with cysteine, on the basis of models that allowed us to predict which residues might lie close to each other. The locations of all of the residues that were changed to Cys in different experiments are shown in Fig. 1.

**Enzymatic Activity of the Cysteine-substituted Serca1αC—**All of the doubly cysteine-substituted mutants were expressed in HEK-293 cells at levels similar to the wild type enzyme, as judged by Western blotting. None of the mutants in which Ca2+ binding ligands were replaced had Ca2+ uptake activities above that of untransfected cells, and none of them formed a high energy phosphoenzyme intermediate (E2P) by phosphorylation from ATP in the presence of Ca2+ (data not shown). As shown in Fig. 2, all of these mutants formed a low energy phosphoenzyme intermediate (E2P) from P in the absence of Ca2+ and, in all but mutants E309C/N796C and G310C/N796C, E2P formation was insensitive to 100 μM Ca2+.

The observation that double mutant E309C/N796C showed no Ca2+ activation of phosphoenzyme formation from ATP but normal Ca2+ inhibition from P at neutral pH is consistent with the hypothesis that Glu309 and Asn796 both contribute to Ca2+ binding site II, occupation of which is not required for conversion of E2 to E2P conformations of the ATPase (32). The presence of 100 μM Ca2+ resulted in partial inhibition of P phosphorylation of the double mutant G310C/N796C. This type of inhibition is consistent with an alteration of Ca2+ affinity at Ca2+ binding site I. Thus, an increase in the size of the residue (Gly to Cys) at position 310 appears to perturb site I, but not to disrupt it. Single mutations of Gly310 to Val or Pro had a similar effect (11, 33).

Detection of Disulfide Cross-links in the Central Tiers of M4 and M6—Microsomal vesicles containing mutant ATPases were reduced with glutathione, a soluble, membrane-impermeant reducing agent, to prevent the formation of disulfide bonds between endogenous Cys residues located in the cytoplasmic domain. They were then incubated in a buffer containing EGTA and vanadate under conditions known to promote and maintain the E2 conformation of the enzyme. Cu(II) (1,10-phenanthroline)2 was present in this buffer as a membrane-permeant oxidant. After a 10-min incubation, trypsin was added for 4 min before loading buffer containing soybean trypsin inhibitor was added to stop the digestion. In this incubation buffer, trypsin cut predominantly and nearly completely at the T1 site (Arg259), as illustrated in Figs. 3–6, demonstrating that the enzyme was in the E2 conformation. The loading buffer also contained NEM to block free cysteines and prevent spurious cross-linking in denatured SERCA1. Following electrophoresis and blotting onto nitrocellulose membranes, SERCA1 fragments were detected immunologically.

The results of experiments with cross-linking of Ca2+ binding residues are presented in Fig. 3 and quantitated in Table II. The immunoblot in Fig. 3A was probed with monoclonal antibody A25, specific to the N-terminal fragment, and the immunoblot in Fig. 3B was probed with antibody A52, specific to the C-terminal fragment. All lanes in Fig. 3, A and B, contained heavy bands at 55 kDa, and double mutants E309C/N796, E309C/D800C, and G310C/D800C contained an additional band corresponding to a mass of 152 kDa. A very weak 152-kDa band was also observed with E309C/T799C. The 152-kDa band was detected with both antibodies and appeared with the same intensity relative to the 55-kDa band in all cases, demonstrating that both N- and C-terminal fragments were present in equal proportions (Table II). Although the anomalous mobility of the 152-kDa band suggested that its mass might approach that of three half molecules, antibody staining data are consistent only with a dimeric complex between the two halves of the molecule.

On the basis that the 152-kDa band represents a dimeric complex between the two halves of the cleaved SERCA1α molecule, cross-linking experiments were performed for the two mutants E309C/N796C and E309C/D800C, and a trace cross-linking experiment was performed for E309C/T799C (Fig. 3 and Table II). Cross-links were not formed in the double mutants E309C/E771C, G310C/E771C, G310C/N796C, G310C/T799C, E309C/E908C, or G310C/E908C. These observations suggest that Ca2+ binding residues in M4 are juxtaposed with Ca2+ binding residues in M6 and possibly in M5, but not with Ca2+ binding residues in M8 in the E2 conformation.

Fig. 4 shows a second gel electrophoretic pattern in which the

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*2 D. M. Clarke, T. W. Loo, and D. H. MacLennan, unpublished observations.*

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TABLE I

Properties of mutants retaining Ca2+ transport function

| Alteration | \( V_{\text{max}} \) | % of wild type |
|------------|-----------------|--------------|
| Wild type  | 100             | 5.00±0.01    |
| Serca1αC  | 44±6            | 6.74±0.04    |
| I298C/T788C | 10±1          | 6.66±0.09    |
| A301C/T788C | 23±7          | 6.52±0.06    |
| A303C/L792C | 34±9          | 6.04±0.05    |
| A303C/V795C | 49±16         | 6.44±0.05    |
| L321C/G808C | 11±2           | 6.53±0.04    |
same proteins examined in Fig. 3 were separated under reducing conditions. The addition of DTT to the loading buffer resulted in the disappearance of both the 152-kDa band and the small amount of a 110-kDa residual band. This degree of sensitivity to reducing agents demonstrates that both the 152- and 110-kDa bands are formed by disulfide cross-links between the two halves of the cleaved SERCA1a molecule. We propose that the 110-kDa band represents cross-links very near to the T1 cleavage site, leading to only minor changes in mobility, while the 152-kDa band represents cross-links in the transmembrane domain of the molecule, leading to more pronounced changes in mobility.

Cross-linking in Upper and Lower Tiers of M4 and M6 Helices—Since cross-links were abundant in the Ca\textsuperscript{2+} binding tiers of the M4/M6 helices, it was of interest to determine whether cross-links might form in the tiers above and below the central tiers. Earlier analysis of mutation-sensitive residues in M4 and M6 helices—

**TABLE II**

Quantitation of cross-links by densitometric scanning of Western blots

Cross-linking, where evident in Figs. 2 and 4, was quantitated by densitometry as described under “Experimental Procedures.” Although not shown, the 110-kDa band, present in every lane, accounted for less than 5% of total intensity in all cases. The 60-kDa band, present in only a few lanes, accounted for between 5 and 10% of the total intensity.

| Double mutant | Cross-link | A25 | A52 | Ratio (A25/A52) |
|---------------|------------|-----|-----|-----------------|
| I298C/I788C  | <4         | <4  | ND  |                 |
| A301C/I788C  | <4         | <4  | ND  |                 |
| A305C/L792C  | 14.7       | 14.2| 1.03|                 |
| A305C/L793C  | 15.1       | 15.3| 0.99|                 |
| E309C/N796C  | <4         | <4  | ND  |                 |
| E309C/T799C  | <1         | <1  | ND  |                 |
| E309C/D800C  | <4         | <4  | ND  |                 |
| G310C/D800C  | 4.0        | 4.1 | 0.96|                 |
| T317C/A804C  | 74.4       | 72.4| 1.03|                 |
| T317C/L807C  | 34.0       | 29.0| 1.17|                 |
| L321C/G808C  | 45.7       | 50.6| 0.90|                 |

* ND, not determined.
M6 revealed that T317D and A804V were conformational change mutants (12, 34). On the basis that these residues, located at a distance from the Ca\textsuperscript{2+} binding residues, might interact in a “knob-in-hole” fashion, we tested whether they might cross-link. As demonstrated in Fig. 3 and quantitated in Table II, the pair T317C/A804C formed a strong cross-link. Modeling of the two central cross-linking mutants, E309C/N796C and G310C/D800C, together with the distal cross-linking mutant, T317C/A804C, using Nicholson plastic model sets, allowed us to predict that other cross-links might occur along a line that represented crossing of the M4/M6 helices at an angle of about 40°. On the basis of these predictions, a second round of cysteine substitutions was performed on residues in the upper and lower tiers of helices M4 and M6. Cross-linking results are presented as immunoblots in Figs. 5 and 6, and quantitation is presented in Table II.

Double mutants T317C/L807C and L321C/G808C formed very strong cross-links. Double mutants A305C/L792C and A305C/L793C formed weaker cross-links. Double mutants I298C/I788C and A301C/I788C formed weak cross-links. Two of these, A301C/I788C and A305C/L792C, had a slightly higher mobility in the gel than the others (Figs. 3 and 5). Double mutants A303C/L792C, A303C/V795C, A306C/L792C, A306C/V795C, and A313C/D800C did not form cross-links. Fig. 6 demonstrates that all proposed cross-links are sensitive to reducing conditions. These mutants illustrate that cross-links do occur along a line representing crossing of the M4/M6 helices at an angle of about 40°.

The group of double mutants outside of the proposed Ca\textsuperscript{2+} binding cavity was assayed for Ca\textsuperscript{2+} transport activity, and those that transported Ca\textsuperscript{2+} were also assayed for apparent Ca\textsuperscript{2+} affinity, measured as Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} transport (25). Functional data, summarized in Table I, show that those mutants that retained low Ca\textsuperscript{2+} transport activities had apparent Ca\textsuperscript{2+} affinities similar to that of the wild type enzyme.

Transport-negative mutants were assayed for phosphoenzyme formation from ATP and were tested for blocks in the Ca\textsuperscript{2+} transport cycle. Those mutants in which Ca\textsuperscript{2+} binding ligands were unaltered were phosphorylated by ATP in the presence of Ca\textsuperscript{2+} (Fig. 7, lane 1). In Fig. 7, lanes 2 and 3, it is apparent that all of the Ca\textsuperscript{2+} transport-deficient double mutants were blocked at the E\textsubscript{2}P dephosphorylation step, although the kinetics of the reaction differed among mutants. This phenotype has been identified in several single mutations involving residues in each of the helices M4, M5, and M6 (11, 12, 33). In these earlier studies, alteration of Ala305, Ala 306, Gly310, or Ala804 to Val resulted in a blockage of E\textsubscript{2}P dephosphorylation, while the mutant T317D was blocked in the E\textsubscript{1}P to E\textsubscript{2}P step (34).

**DISCUSSION**

In this study, disulfide cross-linking was used to gain insight into the structural relationships that exist among the three or four helices (M4, M5, M6, and possibly M8) that contribute residues to the two Ca\textsuperscript{2+} binding and translocation sites in SERCA1a. Data obtained from the formation of site-directed cross-links demonstrate the juxtaposition of Ca\textsuperscript{2+} binding ligands between M4 and M6, but not between M4 and M5 or between M4 and M8. Cross-links formed at both ends of transmembrane helices M4 and M6 provide evidence that the interaction between these helices occurs throughout their length. The association of Ca\textsuperscript{2+} binding ligands in M4 and M6 provides valuable experimental evidence for modeling of the Ca\textsuperscript{2+} binding sites.

**Identification of Cross-linked Bands—**Disulfide cross-linking of Serca1aΔC, followed by tryptic cleavage near the center of the 994-residue molecule, resulted in the formation of four types of bands. A major band at 55 kDa in all lanes undoubtedly represented the two cleaved fragments. In some lanes, an additional band appeared at 60 kDa, but, unlike the other bands, it was only detected with the N-terminal antibody. Since it was sensitive to reducing conditions, it may result from a cytoplasmic disulfide linkage formed within the N-terminal half of SERCA1a.

A weak band at 110 kDa appeared in all lanes. Since this band was sensitive to reducing conditions, it is most likely that...
it resulted from disulfide linkage and not from incomplete tryptic digestion. Only in lane 1 of Fig. 4 and lane 12 of Fig. 6 was a small amount of undigested protein observed. SERCA1a contains 18 cysteine residues in the cytoplasmic domain, and it is possible that some of these are sufficiently close to each other to form disulfide linkages under oxidizing conditions. Some cysteine residues lie near the tryptic cleavage site, and disulfide bonds there, unlike those formed in the transmembrane domain, could preserve the linear structure of the peptide, providing it with a mobility in SDS-PAGE similar to that of the native enzyme. In all cases, the intensity of this band accounted for less than 5% of the total protein (Table II).

The fourth band was a DTT-sensitive, variable band of 152 kDa. This band is likely to be formed from cross-linking of the two fragments in the transmembrane sequence, leading to their anomalous mobility in SDS-PAGE. When cross-linked soluble proteins are separated by SDS-PAGE under nonreducing conditions, they often migrate with higher mobility than the native protein, presumably due to a more compact structure. This is not necessarily true of membrane proteins. Although no unexpected mobility shift was found for cross-linked fragments of rhodopsin (16), disulfide cross-linking of chemo-receptor dimers decreased their mobility, giving them apparent masses 2–3 times greater than the monomer, depending on where the linkage occurred (14, 15, 35). When disulfide cross-links were introduced into the multispansing transmembrane domain of the P-glycoprotein, its mobility corresponded to a higher apparent mass (17). For SERCA1a (29), a glutaraldehyde cross-link in the cytoplasmic domain between Lys492 and Arg878, followed by tryptic cleavage at Arg990, decreased the mobility of the cross-linked protein so that its apparent mass corresponded to 135 kDa. In our hands, the apparent mass was 140 kDa. On the basis of these examples of anomalous mobility, it is reasonable to conclude that cross-linked, cleaved Serca1aΔC could move in SDS-PAGE with a mobility corresponding to 152 kDa.

Further support for this view is provided by the observation that the ratio between the stain incorporated into the 152- and 55-kDa bands was the same when the samples were stained with either the N-terminal or C-terminal specific monoclonal antibodies, demonstrating that the 152-kDa bands contain both halves of the molecule in an equal ratio (Table II). These observations lead us to conclude that the 152-kDa bands result from disulfide cross-links formed between residues in M4 and M6, rejoining the two halves of the protein that were cleaved by tryptic digestion.

We have considered the possibility that cross-linking results from misfolding of the expressed protein, since many of the constructs were unable to transport Ca2+. An increased sensitivity to proteases is a clear sign of misfolding, and our tryptic digests did not show this for any of the mutants in this study. Another indication of misfolding is extremely low expression levels, and, again, all of the mutants expressed at least 30% of wild type levels. In addition, all mutants showed phosphoenzyme formation, indicating that the cytoplasmic domains were intact in all cases. Accordingly, we conclude that misfolding did not contribute to the results obtained in this study.

Structural Predictions—The numerous cross-links formed between M4 and M6 establish that there is close contact between these helices under conditions that would normally favor the E2 conformation. Maintenance of the protein in a single conformation would minimize helix movements, which might otherwise be an important factor, since each link was formed in a separate experiment. Movement of membrane-spanning helices during conformational changes has been described in the case of the aspartate receptor (36). In light of our proposed mechanism of Ca2+ transport (20), it is likely that helix movements do occur as the enzyme moves among the different conformations that are critical to Ca2+ transport, although these will be restrained by close packing (36).

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Fig. 7. Phosphoenzyme formation and decay of inactive mutants. Microsomal fractions from transfected HEK-293 cells were phosphorylated with [γ-32P]ATP in the presence of 100 μM Ca2+ for 7 s. All reactions were done on ice. Samples were then either exposed to 1 mM EGTA to block forward phosphorylation and permit forward dephosphorylation or exposed to 1 mM EGTA in the presence of 1 mM ADP to allow dephosphorylation through ATP formation. After the reaction, samples were quenched with ice-cold 7% trichloroacetic acid. Lane 1, samples were quenched immediately following phosphorylation. Lane 2, EGTA and ADP were added 10 s before quenching. Lane 3, EGTA was added 30 s before quenching. Following quenching, samples were treated as described in the legend to Fig. 2.

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panels A and D). Our linkage data are therefore, most consistent with a 4–4 crossing interaction. Since this interaction continues throughout the length of both helices, the two helices must form a right-handed coiled-coil. Maintenance of the large crossing angle required by 4–4 packing would require significant bending of straight helices. This may be correlated with the presence of three conserved proline and two conserved glycine residues in M4 and M6. Recently published structures of the water channel aquaporin-1 have shown precisely this sort of interaction between transmembrane helices (41, 42).

Implications of Cross-linking for Ca2+ Binding Sites—The results of this study allow us to reconcile functional data obtained through mutagenesis with physical Ca2+ binding data to form a consistent model of how the Ca2+ binding pore is formed. SERCA1 binds two Ca2+ ions, and the bound ions can be distinguished kinetically. Ca2+ binding to the second site is cooperative (43), so that site I must be filled before site II. Binding of Ca2+ to site II prevents the cytoplasmic exchange of Ca2+ bound to site I, providing evidence that the two sites are "stacked" (4), with site I being more lumenal than site II. Although the two ions are randomized in the occluded state, there is evidence that stacking is regained once they face the lumen (5). Most models, therefore, illustrate the two sites with one stacked above the other.

Experiments involving ATP and P1 phosphorylation of constructs containing mutations of the proposed Ca2+ binding ligands have allowed their assignment to sites I and II (6–8). The formation of the high energy phosphoenzyme intermediate, E1P, is dependent on occupation of both Ca2+ binding sites (44). By contrast, formation of a low energy phosphoenzyme intermediate, E3P, from P1 is inhibited by binding of Ca2+ to a single site, because occupation of site I alone is sufficient to convert the P1-reactive E3P to the P1-nonreactive E1P (45). Since mutations of Glu771 and Thr799 prevent both E3P formation and inhibition of E1P formation, these residues were proposed to contribute to site I (8). On the basis that mutations of Glu309 and Asn796 have limited ability to prevent Ca2+ inhibition of phosphoenzyme formation from P1, but profound effects on phosphorylation from ATP, they were assigned to site II (8). Direct measurement of Ca2+ binding to the E309Q mutant expressed in Sf9 cells demonstrated the loss of a single Ca2+ binding site. The remaining site was accessible from the luminal side at pH 6.4, but not from the cytoplasmic side, supporting the view that Glu779 contributes to site II (7). Intermediate effects on phosphorylation suggested that Asp308 contributes to both sites (6, 8, 10).

In our experiments, the double mutant E309C/N796C was not phosphorylated from ATP, but was phosphorylated by P1, (Fig. 2). At pH 7.0, phosphorylation from P1 was inhibited by 100 μM Ca2+. This observation is fully consistent with the contribution of both Glu309 and Asn796 to site II. The double mutant G310C/N796C was not phosphorylated from ATP but was phosphorylated from P1. In this mutant, 100 μM Ca2+ caused partial inhibition of P1 phosphorylation. This result is consistent with loss of site II, together with a decrease in the Ca2+ affinity of site I. Since mutation of Gly310 to Val or Pro resulted in a block of E2P dephosphorylation, in a decrease in Ca2+ affinity (as assayed by P1 phosphorylation (11, 33)), and even in loss of Ca2+ occlusion for the G310P mutant (9), it now seems probable that Gly310 is located in a position that impinges on site I, so that an increase in its side chain length will affect the Ca2+ affinity of site I. The loss of Ca2+ sensitivity of P1 phosphorylation for double mutants E309C/E771C, E309C/T799C, E309D/E800C, and E309C/E908C is consistent with contributions of Glu771, Thr799, Asp800, and Glu908 to site I, as proposed earlier (6, 8).

A problem with the stacking of site II directly above site I is that Asn796 in M6, assigned to site II, is closer to the lumen than Thr799, assigned to site I, the more luminal of the sites. To resolve this problem, one model proposes that Asn796 is not a Ca2+ ligand at all (47, 48). This proposal seems unlikely, since Asn796 is required for Ca2+ occlusion (8). Andersen and Vilsen (49) suggested that M6 is not fully helical but rather loops back on itself. However, there is both biochemical and structural evidence for 10 transmembrane helices (50, 51). The cross-linking data presented in this paper provide the basis for a
“side-by-side” model for the Ca\(^{2+}\) binding site that accommodates the structural problems that arise with the “stacked sites” model (20). If M4 and M6 are oriented to optimize cross-links between them, as indicated in Fig. 9a, then Glu\(^{771}\), Asn\(^{796}\), and Asp\(^{800}\) would be positioned near the M4/M6 contact, while Thr\(^{799}\) would lie to one side of this contact. If M5 were placed with Glu\(^{309}\) apposed to Thr\(^{799}\) in M6, then the ligands between M4 and M6 would be those assigned to site II (Glu\(^{309}\), Asn\(^{314}\)), while the ligands between M5 and M6 would be those assigned to site I (Glu\(^{271}\), Thr\(^{799}\)). Asp\(^{800}\) in M6 would be in a position to contribute to both sites (Fig. 9b). This side-by-side sites model, more fully illustrated in Ref. 20, has the advantage that Asn\(^{796}\) can contribute to site II without distortion of helix M6. This model also places Gly\(^{310}\) in a position in which alteration in its size would impinge on Ca\(^{2+}\) binding site I, rather than Ca\(^{2+}\) binding site II. In a stacked model, Gly\(^{310}\) would contribute only to site II, unless helix M4 were distorted.

**Implications for Mutation Sensitivity outside the Ca\(^{2+}\) Binding Cavity**—The strong cross-links found at the top and bottom of M4 and M6 provide possible explanations for previous mutagenesis data. Single mutants T317D and A804V are nonfunctional, conformation change mutants, while mutant L807A has a significantly lower Ca\(^{2+}\) affinity than wild type (12, 34). These functional consequences of mutagenesis might be explained in structural terms. The space between the hydrophobic Ala\(^{800}\) and Leu\(^{793}\) in M6 might better accommodate a polar Thr residue in M4 than a negatively charged Asp. Ala\(^{800}\) might be small enough to provide space for the \(\beta\)-branched Thr\(^{797}\), while Leu\(^{793}\) might help lock it into place. At the bottom of M4, mutation of Ala\(^{305}\) to Val was found to result in an E\(_P\) dephosphorylation block (11). Mutation of either Leu\(^{792}\) or Leu\(^{793}\) in M6 to Ala or Ser decreased Ca\(^{2+}\) transport to less than 50% of wild type, although the slightly smaller Val was tolerated at either position (12, 48). Ca\(^{2+}\) affinity was not altered significantly by either of these two mutations in M6. Positioning of the small Ala\(^{305}\) in M4 between the bulky Leu\(^{792}\) and Leu\(^{793}\) residues in M6 provides a possible explanation for the sensitivity to residue size at position 305.

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