Helix Packing of the Cardiac Na\(^{+}\)-Ca\(^{2+}\) Exchanger

PROXIMITY OF TRANSMEMBRANE SEGMENTS 1, 2, AND 6

Xiaoyan Ren, Debora A. Nicoll, and Kenneth D. Philipson

From the Departments of Physiology and Medicine and the Cardiovascular Research Laboratories, David Geffen School of Medicine at UCLA, Los Angeles, California 90095-1760

The cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger (NCX1) is a membrane protein that extrudes Ca\(^{2+}\) from cells using the energy of the Na\(^{+}\) gradient and is a key protein in regulating intracellular Ca\(^{2+}\) and contractility. Based on the current topological model, NCX1 consists of nine transmembrane segments (TMSs). The N-terminal five TMSs are separated from the C-terminal four TMSs by a large intracellular loop. Cysteine 768 is modeled to be in TMS 6 close to the intracellular surface. In this study, the proximity of TMS 6 to TMS 1 and 2 was examined. Insect High Five cells were transfected with cDNAs encoding mutant NCX1 proteins. Each mutant contained cysteine 768 and an introduced cysteine in TMS 1 or 2. Cross-linking between cysteines was determined after reaction with thiol-specific cross-linkers containing spacer arms of 6.5–12 Å. The data indicate that residues in TMSs 1 and 2 are close to cysteine 768 in TMS 6. Cysteine 768 cross-linked with residues at both ends of TMSs 1 and 2 and is likely located toward the middle of TMS 6. Based on these results, we present an expanded helix-packing model for NCX1.

Na\(^{+}\)-Ca\(^{2+}\) exchangers (NCX)\(^2\) are found in many tissues. The cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger (NCX1) plays a key role in myocardial contraction and relaxation. NCX exchanges three Na\(^{+}\) ions for one Ca\(^{2+}\) and can move Ca\(^{2+}\) either into or out of cells, depending on the electrochemical driving force. During the depolarization phase of the action potential, Ca\(^{2+}\) enters the cardiomyocyte primarily through L-type Ca\(^{2+}\) channels to trigger sarcoplasmic reticular Ca\(^{2+}\) release and to initiate contraction. During the relaxation phase, Ca\(^{2+}\) is transported back into the sarcoplasmic reticulum by an ATP-dependent Ca\(^{2+}\) pump and is extruded from the cell by NCX1 (1).

The current topological model of the NCX protein contains nine transmembrane segment (TMS) helices and two reentrant loops (Fig. 1). There is a large cytoplasmic loop between TMSs 5 and 6, and the N and C termini are located on the extracellular and intracellular sides, respectively. The native Na\(^{+}\)-Ca\(^{2+}\) exchanger protein has a molecular mass of 110 kDa based on amino acid sequence (2). Electrophoretic analysis of purified NCX1 displays three protein bands with molecular masses of 160, 120, and 70 kDa. The 70-kDa band is likely a proteolytic fragment (3, 4). Under reducing conditions, the 160-kDa band is weak, and the 120-kDa band is more intense. Under nonreducing conditions, the 120-kDa band disappears, and the 160-kDa band becomes prominent (3). This mobility shift is due to an intramolecular disulfide bond between cysteine 792 and either cysteine 14 or cysteine 20 as shown by mutagenesis and biochemical analysis (5). Thus, cross-linking between the N- and C-terminal halves of the exchanger results in decreased mobility on SDS-PAGE (6).

To understand the molecular mechanism of the Na\(^{+}\)-Ca\(^{2+}\) exchanger, it is necessary to learn about the three-dimensional arrangement of the TMSs within the plasma membrane. However, hydrophobic membrane proteins are difficult to crystalize, and a high resolution structure of NCX1 is not available. Qiu et al. (6) developed an alternative method for obtaining structural information about NCX1 by taking advantage of the shift in electrophoretic mobility when there is a cross-link between the two halves of the protein. A helix-packing model of TMSs 2, 3, 7, and 8 of NCX1 was formulated by introducing pairs of cysteine mutants into a cysteine-less background and testing for disulfide cross-link-induced mobility shifts.

Here, we extend the packing model of NCX1 to include TMSs 1 and 6. Cysteine mutations were introduced into the N or C terminus of TMSs 1 or 2 and then paired with cysteine 768 in TMS 6. cDNAs of NCX1 mutants were expressed in insect High Five cells. Intramolecular cross-linking was detected by mobility shifts on SDS-PAGE gels following treatment of intact cells with cross-linking agents. The results indicate that TMSs 1 and 2 are in proximity with TMS 6. Strikingly, cysteine 768 in TMS 6 could form cross-links with residues modeled to be near both the intracellular and the extracellular surfaces of TMS 1 and TMS 2. Cysteine 768 is likely near the center of TMS 6.

EXPERIMENTAL PROCEDURES

Construction of Exchanger Cysteine Mutants—The QuikChange site-directed mutagenesis method (Stratagene) was used to prepare mutants (7). Mutations were generated in 300–500-bp cassettes and verified by sequencing. Full-length exchangers with single or double mutations were constructed by subcloning the mutated cassettes into the cysteine-less exchanger.
Expression of the NCX1 Cysteine Mutants in Insect High Five Cells—A lepidopteran insect cell expression system, BTI-TN-5B1-4 (High Five, Invitrogen), was used for transient transfection of NCX1 cysteine mutants. High Five cells were cultured at 27 °C in Express Five SFM (Invitrogen) supplemented with 20 mM glutamine and 1% penicillin-streptomycin.

Mutant NCX1 cDNA was subcloned into the pIB/V5-His vector (Invitrogen) and transfected into High Five insect cells using lipid-mediated transfection with Cellfectin reagent (Invitrogen). 4 h after transfection, 0.1 mM cholesterol-cyclohextrin complex (Ch-MβCD) (8) was added to the transfected cells to increase exchanger protein expression. 24–48 h after transfection, Na⁺ gradient-dependent ⁴⁵Ca²⁺ uptake in transfected High Five insect cells was measured. Cells were harvested and washed twice with washing buffer plus 0.4 mM ouabain. Uptake was initiated by resuspending the cell pellet in assay medium: 10 mM MOPS (pH 7.4), 140 mM KCl and then loaded with Na⁺ by incubation with 10 mM MOPS (pH 7.4), 140 mM NaCl, 1 mM MgCl₂, 0.4 mM ouabain, and 25 μM nystatin for 10 min at room temperature. Nystatin was removed from the cells by two washes with washing buffer plus 0.4 mM ouabain. Uptake was initiated by resuspending the cell pellet in assay medium: 10 mM MOPS (pH 7.4), 140 mM KCl (or NaCl as control), 25 μM CaCl₂, 0.4 mM ouabain, and 5 μCi/ml ⁴⁵Ca²⁺. After 10 min, the reaction was stopped by adding 1 ml of ice-cold quenching solution (140 mM KCl, 1 mM EGTA) followed by two additional washes with quenching solution. Cell pellets were dissolved in 1 n NaOH at 60 °C for 30 min. Aliquots of samples were subjected to scintillation counting and protein assay (MicroBCA, Pierce).

Cross-linking Procedures—Intact cells were washed with washing buffer, and cross-linking was carried out at room temperature by the addition of oxidative reagent (CuSO₄/phenanthroline (CuPhe)), thiol-specific homobifunctional cross-linker, or methanethiosulfonate (MTS) cross-linkers (Toronto Research Chemicals) to the intact cell suspension. The final concentrations of reagents were 1 mM CuSO₄, 3 mM phenanthroline, or 0.5 mM N⁰, N⁰-o-phenylenedimaleimide (o-PDM) or 0.5 mM p-PDM, 0.5 mM 1,3-propanediyl bismethanethiosulfonate (M3M), or 0.5 mM 1,6-hexanediyl bismethanethiosulfonate (M6M). Some samples were preincubated with 10 mM [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET) or 10 mM N-ethylmaleimide (NEM). Reactions were terminated after 20 min by the addition of NEM (10 mM). Cells were lysed with 1% Triton X-100 plus protease inhibitors (Complete, EDTA-free, Roche Applied Science). Aliquots were subjected to 7.5% SDS-PAGE in the absence of reducing reagents, and immunoblot analysis was carried out with NCX1 antibody R3F1 (SWant).

RESULTS

Cysteine Residues Introduced into the Cysteine-less Na⁺-Ca²⁺ Exchanger—Fig. 1 shows residues mutated to cysteine in this study. The residues labeled with asterisks, 14, 20, 122, 768, and 792, are cysteines in the wild-type canine NCX1 but were mutated to alanine to produce the cysteine-less exchanger (7). The cysteine-less exchanger was used as our background protein for introduction of cysteines. Thus, each mutant contains only one or two cysteines as indicated. In the single-cysteine mutant A122C, for example, the alanine at position 122 in the cysteine-less exchanger has been mutated back to a cysteine. In addition to the five reintroduced cysteines, we also used cysteine mutants at positions 101 and 102, as described previously (9), and new cysteine introductions at positions 40, 41, 42, 45, 49, 59, and 765–767. Single cysteines or pairs of cysteines were introduced into the cysteine-less NCX1 background, and the proximity of the paired cysteine residues was then readily assayed by gel mobility shifts following treatment with disulfide or chemical cross-linking reagents (6).

Protein Expression and Na⁺ Gradient-dependent ⁴⁵Ca²⁺ Uptake of Cysteine Mutants in High Five Cells Increased by Applying Ch-MβCD—As a eukaryotic expression system, High Five cells provide similar posttranslational modifications as mammalian cell lines and have successfully been used to express the K⁺-dependent Na⁺-Ca²⁺ exchangers (10, 11). We found that transient transfection of High Five cells with the wild-type (WT) Na⁺-Ca²⁺ exchanger gave very consistent levels of NCX1 activity and immunoreactive protein (data not shown). Relative protein expression levels were determined by Na⁺-gradient-dependent ⁴⁵Ca²⁺ uptake into cells and immunoblot analysis and compared with expression levels of WT NCX1. The mutant proteins expressed a wide range of functional activities from almost none to about 95% of WT NCX1 (Table 1). Protein levels of mutants and WT NCX1, detected by immunoblot, showed a positive correlation with activity.

It has been reported that the level of cholesterol in the insect cell membrane is much lower than in mammalian cell membranes (8), and for some membrane proteins, expression is improved by increasing membrane cholesterol with Ch-MβCD (8). Since NCX1 activity is affected by cholesterol (12, 13), we reasoned that the low activity of some NCX1 mutants might be improved by increasing membrane cholesterol levels. We tested the effect on exchanger activity of adding Ch-MβCD to the culture medium after transfection. The activity of most mutants was substantially increased by the addition of Ch-MβCD (Table 1). Thus, all cross-linking studies were carried out using transfected cells treated with Ch-MβCD, and only active mutants were examined.
Helix Packing of NCX1

Cross-linking of TMSs 1 and 6 of the Na\(^{+}\)-Ca\(^{2+}\) Exchanger—To ascertain the proximity of cysteine residues, many cross-linkers are available, and their use is widespread for topology studies of membrane proteins. CuPhe is an oxidative reagent, which can catalyze disulfide formation between sulfhydryl groups. o-PDM and p-PDM are dimaleimide sulfhydryl cross-linkers with linker distances of 7.7 and 12 Å, respectively, and have been used successfully with NCX1 in a previous study (6). MTS cross-linkers react selectively with cysteines, resulting in a disulfide attachment of the spacer arm. We used the two different chemistries of MTS and PDM cross-linkers in this study. The MTS reagents we chose, M3M (6.5 Å) and M6M (10.4 Å), have similar cross-link sizes as o-PDM and p-PDM. All the cross-linkers chosen for this study are membrane-permeable, and the cross-linking experiments were carried out using intact cells.

The WT NCX1 expressed in High Five insect cells migrates as a 120-kDa band in SDS-PAGE under reducing conditions (data not shown). Under nonreducing conditions, an additional band at 140 kDa is seen. For NCX1 expressed in other cell types (cardiomyocytes or HEK293 [human embryonic kidney cells]), the additional band is at 160 kDa. This discrepancy may be due to glycosylation differences (14). The 140-kDa (or 160-kDa) band results from an intramolecular disulfide bond between cysteine 792 and either cysteine 14 or cysteine 20 (5) and disappears under reducing conditions. Unlike in HEK293 cells, the exchanger protein is not seen in higher molecular weight aggregates on SDS-PAGE when expressed in High Five cells, thus making this a cleaner cell system to test for mobility shifts of the exchanger protein.

The general approach we took to identify interactions between TMSs was to first generate a set of consecutive single-cysteine mutants near the surface of a TMS (for example, in TMS 1, we made mutations at residues 40, 41, 42, and 43). Then, we paired each single-cysteine mutant with a second cysteine mutant in the other half of the exchanger and tested for cross-linking-induced gel shifts. We tested more than 20 double-cysteine mutants before finding one that showed a gel shift. There are numerous reasons why we might not detect cross-linking ranging from lack of proximity between TMSs, incorrect orientation of the TMSs, or simply an insufficient change in conformation in the cross-linked protein for detection on SDS-PAGE. A lack of gel shift for any given double mutant gives no information regarding helix packing, and null mutants were not pursued.

The first mutant we found positive for cross-linking was A41C/A768C. This indicated that residues located in TMSs 1 and 6 were close enough in the protein to result in cross-linking. The lowest concentration of M3M that induced a 140-kDa band was 100 μM. At 500 μM M3M, the maximal amount of

| Mutant | NCX activity | Fold increase |
|--------|--------------|---------------|
| WT     | -Ch-MRC    | +Ch-MRC        |
| Cysteine-less | 170 ± 30 | 1.7 |
| A14C/A20C/A768C  | 20 ± 12 | 5.0 |
| I40C/A792C       | 21 ± 6  | 3.0 |
| A41C/A768C       | 44 ± 33 | 7.3 |
| A41C/A767C       | 53 ± 21 | 5.3 |
| A41C/A766C       | 30 ± 4  | 5.0 |
| V45C/A768C       | 51 ± 40 | 3.4 |
| A49C/A768C       | 44 ± 24 | 5.5 |
| IS9C/A768C       | 27 ± 7  | 2.5 |
| IS9C/A767C       | 41 ± 27 | 3.4 |
| IS9C/A792C       | 12 ± 2  | 3.0 |
| N101C/A768C      | 32 ± 13 | 4.6 |
| L102C/A768C      | 22 ± 20 | 2.5 |
| A122C/A768C      | 95 ± 40 | 2.6 |
| A122C/A792C      | 47 ± 34 | 4.7 |
| A122C            | 87 ± 51 | 3.9 |
| A768C            | 63 ± 40 | 5.7 |

FIGURE 2. Cross-linking between residues in TMS 1 with a residue in TMS 6 (768). Double-cysteine mutants A14C/A768C (A), V45C/A768C (B, left panel), A49C/A768C (B, right panel), IS9C/A768C (C), A41C/A767C (D, left panel), and IS9C/A767C (D, right panel) were expressed in High Five Cells. 24 h after transfection, intact cells were treated with CuPhe (1 mM), o-PDM (0.5 mM), p-PDM (0.5 mM), M3M (0.5 mM) or M6M (0.5 mM), as indicated, for 20 min at room temperature. Proteins were separated by SDS-PAGE under nonreducing conditions, transferred to nitrocellulose membranes, and then probed with an anti-exchanger antibody. The upper panels show mobility shifts after cross-linking. The lower panels show the effects of preincubation with the membrane-permeable reagent NEM on cross-linking.

3 Null mutants included: 40/788, 40/789, 40/892, 40/907, 40/909, 40/912, 40/923, 41/788, 41/789, 41/892, 41/907, 41/909, 41/912, 41/923, 42/789, 42/892, 42/907, 42/923, 59/892, 59/907, 59/909, 59/912, 59/923.
NCX1 protein in the 140-kDa band was observed (about 50%). This level of cross-linking was observed within 1 min of exposure to M3M. We were surprised that residues 41 and 768 cross-linked since they were modeled to be near opposite sides of the membrane (Fig. 1).

To further explore the relationship between TMSs 1 and 6, we paired A768C with residues 45, 49, and 59, all modeled to be on the same face of TMS 1 as residue 41 (see Fig. 7). Residues 45 and 49 are modeled to be toward the center of TMS 1, whereas residue 59 is modeled to be at the intracellular surface. Mobility shifts in SDS-PAGE under nonreducing conditions following application of cross-linkers were observed for mutants V45C/A768C (Fig. 2B, left panel) and I59C/A768C (Fig. 2C, upper panel) but not for mutant A49C/A768C (Fig. 2B, right panel) indicating that residue 768 can react with residues along the entire length of TMS 1. We also found that mutants A41C/A767C and I59C/A767C showed evidence of cross-linking (Fig. 2D). Mobility shifts could be prevented by incubating the cells with 10 mM NEM before application of cross-linkers (Fig. 2, lower panels).

On a helical wheel projection of TMS 1, residues 41, 45 and 59 are within a few degrees of each other, whereas residue 49 is slightly offset (see Fig. 7), suggesting that residues outside of the packing faces would not cross-link. Therefore, we tried pairing A768C with I40C and R42C, residues within one turn of A41C. However, neither of these double-cysteine mutants displayed NCX activity. Conversely, we also paired A41C with G765C, W766C, and A767C. Neither NCX1 protein nor activity could be detected with mutant A41C/G765C. Mutant A41C/W766C is active but showed no mobility shift upon application of cross-linkers (data not shown). However, mutant A41C/A767C both is active and demonstrates cross-linker-induced gel shifts (Fig. 2D, left panel). Similarly, we paired I59C with G765C, W766C, and A767C. No protein expressed in the mutants I59C/G765C and I59C/W766C. Mutant I59C/A767C showed activity and cross-linker-induced mobility shift in SDS-PAGE (Fig. 2D, right panel). These data confirm that TMSs 1 and 6 interact along one face of their respective helices.

Cross-linking of TMSs 2 and 6 of the Na\(^{+}\)-Ca\(^{2+}\) Exchanger—

We also tested for proximity of residue 768 to residues on either end of TMS 2. In the wild-type NCX1, there is a cysteine at position 122. Cysteine scanning mutagenesis studies indicate that this residue is located in TMS 2 or the first reentrant loop close to the extracellular surface (7, 15). We reintroduced the two native cysteine residues 122 and 768 into the cysteine-less background and transfected the cDNA into High Five insect cells. The A122C/A768C mutant migrated as a 120-kDa band in SDS-PAGE under nonreducing conditions (Fig. 3A, first lane). Both 120-kDa and 140-kDa bands were observed upon application of CuPhe or the cross-linkers o-PDM or M3M. The 140-kDa band was especially intense with CuPhe (Fig. 3A, second lane). CuPhe catalyzes formation of a disulfide bond between adjacent cysteines. Thus, residues 122 and 768 are close enough to form a disulfide bond. Pretreatment with either 10 mM NEM (Fig. 3A, bottom panel) or 10 mM MTSET (data not shown) does not fully prevent cross-linking of residues 122 and 768. Thus, residues 122 and 768 may be buried in lipid or protein and is not readily accessible to NEM.

Residues Ser-100 and Leu-102 were previously shown to be near the intracellular surface of TMS 2 since the activities of mutants S100C and L102C were inhibited by intracellular applications of MTSET (9). We paired A768C with either N101C or L102C on the cysteine-less background and examined the effect of cross-linking reagents M3M or M6M on SDS-PAGE mobility (Fig. 3, B and C). For both mutants, cross-linker-induced mobility shifts, which could be inhibited by preincubation with NEM, were observed.

We were unable to examine the effect of disulfide cross-linking on the activity of exchanger mutants expressed in High Five cells as the activity of the cysteine-less exchanger itself was inhibited in a concentration-dependent manner by cross-linking reagents. This may be secondary to alterations of other cellular constituents by the cross-linking reagents.

Evidence to Support the Location of Cysteines 40, 59, 122, and 768—Many data support the topological model presented in Fig. 1 (7, 9, 15). However, details, such as the precise beginning and ending points for TMSs, are uncertain. Thus, we undertook experiments to verify the proposed positions of cysteines at positions 40, 59, 122, and 768. Cysteines at positions 40, 59, and 122 were paired with native cysteine 792, which has been shown to be at the extracellular side of the membrane (5). Residues 40 and 122, modeled to be at the extracellular surfaces of TMS 1 and 2, respectively, demonstrated mobility shifts when paired with A792C and treated with cross-linkers (Fig. 4, A and B). On the other hand, when residue 59, modeled to be at the intracellular surface of TMS 1, was paired with A792C, no cross-linker-induced mobility shifts were seen (Fig. 4C). To determine the location of Cys-768, it was combined with native cysteines Cys-14 and Cys-20. These residues are both located at the N-terminal, extracellular surface of NCX1 (5, 7). As shown in Fig. 4D, no mobility shift was observed for this mutant follow-
ing cross-linking, thus suggesting that Cys-768 is not accessible to the extracellular surface.

To obtain more information on the location of Cys-768, we examined the effect of sulfhydryl-modifying reagents on the activity of the single-cysteine mutant Cys-768 expressed in Xenopus oocytes. We have used this approach previously to determine NCX1 topology (7, 9). cRNA of NCX1 exchanger mutant A768C was injected into Xenopus laevis oocytes, and exchanger activity was measured following treatment with the sulfhydryl reagents MTSET or NEM. MTSET is a cationic, membrane-impermeable reagent, and NEM is membrane-permeable. Both form covalent bonds with accessible cysteines. MTSET or NEM were either applied extracellularly, by incubating oocytes in medium containing 10 mM reagent, or applied intracellularly, by injecting MTSET solution into the oocyte for an estimated cytoplasmic concentration of 10 mM (Fig. 5). Extracellular application of impermeant MTSET had no significant effect on mutant A768C activity. On the other hand, intracellular application of MTSET or extracellular application of permeant NEM inhibited the activity of A768C. The results verify that residue 768 is more accessible from the cytoplasmic side of the membrane. The results also suggest that residue 768 may be in a functionally significant region because exchange activity was inhibited by sulfhydryl modifications. Interestingly, intracellular application of MTSET had no effect on the activity of WT NCX1 (9), which also contains a cysteine at position 768. Perhaps labeling of other native cysteines blocks accessibility to Cys-768, or Cys-768 forms an undetected disulfide bond with another native cysteine. The latter seems less likely since Cys-768 is accessible to the intracellular milieu and thus probably is in a reducing environment.

Co-transfection of Single-cysteine Mutants—To distinguish between cross-linking derived from intermolecular versus intramolecular interactions, single-cysteine mutants (I40C, A41C, I59C, A122C, N101C, L102C, A768C, and A792C) were co-transfected into High Five cells. 24 h after transfection, the cross-linking experiments were carried out as described in the legend for Fig. 2.
tein mutants was due to intramolecular, and not intermolecular, interactions.

DISCUSSION

A previous helix-packing study of NCX1 demonstrated that TMSs 2, 3, 7, 8, and their associated reentrant loops are in proximity (6). The results of this current study extend these observations to include TMS 1 and 6. Our data show that residue 768 in TMS 6 is in proximity to residues in TMS 1 and 2. A new helical wheel projection of NCX1 that includes TMSs 1 and 6 along with TMSs 2, 3, 7, and 8 is shown in Fig. 7. This figure summarizes the results obtained in the present study and also includes the results from Qiu et al. (6). For this model, it was assumed that each TMS spans the membrane uninterrupted by discontinuities and that each of the indicated residues is in a helical part of the TMS and not in connecting loops. This model should provide guidelines and predictions for further experimentation and model refinement.

The only TMSs not yet accounted for in our helix-packing model are TMSs 4, 5, and 9. Most of the loops connecting consecutive TMSs appear to be relatively short, with the notable exception of the large loop connecting TMSs 5 and 6. It has also been noted that consecutive TMSs tend to be near one another in packed structures of membrane proteins (16). Therefore, it seems likely that TMSs 4 and 5 will reside in the vicinity of TMS 3. Likewise, TMS 9 is likely to be near TMS 8. Qiu et al. (6) reported cross-linking in the nonfunctional mutant S117C/K909C, which also supports placing TMS 9 into the area bounded by TMSs 8, 2, and 7.

The most striking experimental result presented here indicates that residues Cys-768 and Ala-767 are accessible to residues modeled to be near both the extracellular and the intracellular sides of TMSs 1 and 2 (Figs. 2 and 3). Cys-768 has previously been modeled to be in TMS 6 toward the intracellular surface of the membrane (Fig. 1) (17). However, the position of Cys-768 can just as easily be modeled to be more toward the center of TMS 6 without significantly reducing hydrophobicity of the TMS. Our results strongly indicate that this is the case. Nevertheless, accessibility of Cys-768 to both sides of the membrane is still surprising. If TMS 1 and TMS 2 are both continuous α-helices, then the residues that interact with Cys-768 would be separated by five turns or a distance of about 27 Å. Assuming a static interaction between the helices, a single residue in the middle of TMS 6 would require a cross-linker that is longer than 10 Å to reach both residues at either side of the membrane. However, cross-linking was observed with M3M, a 6.4 Å cross-linker. There are a number of possibilities for how this might occur. For example, TMS 1 and 2 may not be continuous helices. There is a growing body of x-ray structures of membrane proteins in which TMSs are not continuous α-helices (18, 19). If there are nonhelical sections of TMSs 1 and 2, then residues modeled to be on opposite sides of the membrane could be closer than estimated from the rise of a single helix. TMS 2 does have a proline at residue 112, about half the distance between residues 101 and 122, that might induce a kink in the helix. Also, there are several tyrosines (weak helix breakers) and a glycine (strong helix breaker) in TMS 1, although these residues are not consecutive. Alternatively, the residues that are modeled to be within a transmembrane-spanning region may be in a loop connecting TMSs. Loops could be flexible and reach in toward residue 768 if there is a vestibule-like structure in NCX to allow accessibility. Finally, there may be flexibility in the exchanger protein such that TMS 6 can move depending on

FIGURE 7. Packing model for NCX with summary of the interactions between residues in TMSs 1, 2, 3, 6, 7, and 8. a, view from above the membrane surface. Experimental data presented in this study (heavy lines) are combined with previous studies (light lines) to show a model for the arrangement of TMSs. Residues 41 and 59 are predicted to be at the same angle but separated by five full turns of the helix. b, sideways view of TMSs. Heavy lines indicate cross-linking between TMS 6 and 2. Light lines indicate cross-linking between TMS 6 and 1. For simplicity, only cross-links determined in this work are indicated with lines.
the conformation of the protein. This might allow Cys-768 to alternately interact with residues at the intracellular or extracellular surface. A precedent is the S4 segment of ion channels, which moves outward in response to membrane depolarization (20–22).

Notable also is the interaction between residues 122 and 768. In the wild-type NCX1, both of these residues are cysteines. Residue 122 is near the interface between the extracellular end of TMS 2 and the reentrant loop of the α-1 repeat, a region of functional importance (23). There had been no previous indication of interaction between Cys-122 and Cys-768 in the WT NCX1 (5). However, a disulfide bond formed between residues Cys-768 and Cys-122 in the presence of CuPhe (Fig. 3A) indicates a short distance between these two cysteine residues. Proximity of the two residues is consistent with a functional importance for TMS 6.

Crystallization of membrane proteins is a difficult process. The NCX1 protein has not yet been crystallized, and large quantities of purified functional protein are not available. Hopefully, our studies on helix packing of NCX1 may help provide initial insight on the mechanism of action of this ion transporter and lead to the design of additional studies.

REFERENCES
1. Bers, D. M. (2001) Excitation-Contraction Coupling and Cardiac Contractile Force, Second Edition, pp. 133–160; Kluwer Academic Publishers, Norwell, MA
2. Nicoll, D. A., Longoni, S., and Philipson, K. D. (1990) Science 250, 562–565
3. Philipson, K. D., Longoni, S., and Ward, R. (1988) Biochim. Biophys. Acta 945, 298–306
4. Durkin, J. T., Ahrens, D. C., Pan, Y. C., and Reeves, J. P. (1991) Arch Biochem. Biophys. 290, 369–375
5. Santacruz-Toloza, L., Ottolia, M., Nicoll, D. A., and Philipson, K. D. (2000) J. Biol. Chem. 275, 182–188
6. Qiu, Z., Nicoll, D. A., and Philipson, K. D. (2001) J. Biol. Chem. 276, 194–199
7. Nicoll, D. A., Ottolia, M., Lu, L., Lu, Y., and Philipson, K. D. (1999) J. Biol. Chem. 274, 910–917
8. Gimpi, G., Klein, U., Reilander, H., and Fahrenholz, F. (1995) Biochemistry 34, 13794–13801
9. Doering, A. E., Nicoll, D. A., Lu, Y., Lu, L., Weiss, J. N., and Philipson, K. D. (1998) J. Biol. Chem. 273, 778–783
10. Szerencsei, R. T., Tucker, J. E., Cooper, C. B., Winkfein, R. J., Farrell, P. J., Iatrou, K., and Schnetkamp, P. P. (2000) J. Biol. Chem. 275, 669–676
11. Winkfein, R. J., Szerencsei, R. T., Kinjo, T. G., Kang, K., Perizzolo, M., Eisner, L., and Schnetkamp, P. P. (2003) Biochemistry 42, 543–552
12. Veturi, R., and Philipson, K. D. (1988) Biochim. Biophys. Acta 937, 258–268
13. Veturi, R., and Philipson, K. D. (1989) J. Biol. Chem. 264, 8680–8685
14. Tomiya, N., Narang, S., Lee, Y. C., and Betenbaugh, M. J. (2004) Glycoconj. J. 21, 343–360
15. Iwamoto, T., Nakamura, T. Y., Pan, Y., Uehara, A., Imanaga, I., and Shigekawa, M. (1999) FEBS Lett. 464, 264–268
16. Bowie, J. U. (1997) J. Mol. Biol. 272, 780–789
17. Philipson, K. D., Nicoll, D. A., Ottolia, M., Quednau, B. D., Reuter, H., John, S., and Qiu, Z. (2002) Ann. N. Y. Acad. Sci. 976, 1–10
18. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kabaek, H. R., and Iwata, S. (2003) Science 301, 610–615
19. Hunte, C., Screpanti, E., Venturi, M., Rimon, A., Padan, E., and Michel, H. (2005) Nature 435, 1197–1202
20. Yang, N., George, A. L., Jr., and Horn, R. (1996) Neuron 16, 113–122
21. Yang, N., George, A. L., Jr., and Horn, R. (1997) Biophys. J. 73, 2260–2268
22. Grottesi, A., Domene, C., Hall, B., and Sansom, M. S. (2005) Biochemistry 44, 14586–14594
23. Ottolia, M., Nicoll, D. A., and Philipson, K. D. (2005) J. Biol. Chem. 280, 1061–1069