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

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In a revised topological model of the cardiac Na\(^+\)-Ca\(^{2+}\) exchanger, there are nine transmembrane segments (TMSs) and two possible re-entrant loops (Nicoll, D. A., Ottolia, M., Lu, Y., Lu, L., and Philipson, K. D. (1999) J. Biol. Chem. 274, 910–917; Iwamoto, T., Nakamura, T. Y., Pan, Y., Uehara, A., Imanaga, L., and Shigekawa, M. (1999) FEBS Lett. 446, 264–268). The TMSs form two clusters separated by a large intracellular loop between TMS5 and TMS6. We have combined cysteine mutagenesis and oxidative cross-linking to study proximity relationships of TMSs in the exchanger. Pairs of cysteines were reintroduced into a cysteine-less exchanger, one in a TMS in the NH\(_2\)-terminal cluster (TMSs 1–5) and the other in a TMS in the COOH-terminal cluster (TMSs 6–9). The mutant exchanger proteins were expressed in HEK293 cells, and disulfide bond formation between introduced cysteines was analyzed by gel mobility shifts. Western blots showed that S117C/V804C, A122C/Y892C, and 120 kDa in the presence of reducing agents (16). The amino-terminal cluster is comprised of TMSs 1–5 with a possible re-entrant loop between TMSs 2 and 3, whereas the C-terminal cluster is comprised of TMSs 6–9 with a possible re-entrant loop between TMSs 7 and 8 (6, 7). Experimental evidence supports the extracellular localization of the amino terminus and loops c, e, g, and i and the intracellular localization of loops b, d, f, and h and the carboxyl terminus (6–13). From deletion experiments, it has been determined that the large cytoplasmic domain (loop f) is not essential for ion transport (12).

Inspection of the amino acid sequence of the exchanger also reveals that there are two homologous regions consisting of residues spanning portions of TMSs 2–3 and TMS7 plus part of loop h, respectively (shading in Fig. 1). These regions are designated as the α1 and α2 repeats and are conserved among all exchangers (14 and reviewed in Ref. 5). Extensive site-directed mutagenesis studies show that exchanger activity is highly sensitive to mutagenesis of residues in the α-repeats. Even conservative mutations alter or eliminate activity (15). The putative α-helices of the α-repeats (TMSs 2, 3, and 7) are amphipathic, and the hydrophilic faces of these helices may form a portion of the ion translocation pathway (15).

The electrophoretic mobility of the exchanger is different under reducing and nonreducing conditions. The exchanger proteins purified from canine cardiac sarcolemma migrate on SDS-PAGE as two bands with apparent molecular masses of 70 and 120 kDa in the presence of reducing agents (16). The 120-kDa protein species represents the mature protein, whereas the 70-kDa protein is an active proteolytic fragment of variable amount. Under nonreducing conditions, the apparent molecular mass of the 120-kDa protein shifts to 160 kDa (16). It has been shown recently that this mobility shift is due to an intramolecular disulfide bond between the cysteine at position 792 in loop g and the cysteine at either position 14 or 20 near the NH\(_2\) terminus of the exchanger (17). Disulfide bond formation apparently induces a significant conformational change in the exchanger protein under SDS-PAGE conditions.

The three-dimensional arrangement of the TMS helices of the Na\(^+\)-Ca\(^{2+}\) exchanger is unknown. Understanding the transport mechanism of the exchanger will require knowledge...
of the helix packing. It is not yet possible to crystallize the exchanger protein because of difficulties in producing a large amount of pure functional protein as well as difficulty in crystallizing membrane proteins in general. Alternative approaches to obtain structural information on membrane proteins are being developed. In this study, we employed an approach combining cysteine mutagenesis with disulfide cross-linking (18, 19) to analyze the arrangement of TMSs in the exchanger. Pairs of cysteines were reintroduced into a cysteine-less (cys-less) exchanger, and the mutant exchanger proteins were expressed in HEK cells. Disulfide cross-linking was detected by an electrophoretic mobility shift assay. Four cross-links have been identified, which provide initial information on the helix packing of the exchanger. Our data indicate that the same interface of TMS7 is close to TMS2 near the extracellular side but is adjacent to TMS3 near the intracellular side of the plasma membrane and that TMS2 adjoins TMS8. This suggests that the functionally important domains, the a 1 and a 2 repeats, are in the close proximity.

**EXPERIMENTAL PROCEDURES**

**Generation of Mutant Exchangers—**Clone I, a modified version of the cys-less exchanger (6), was used in this study for expression in HEK cells (see "Results"). Single or pairs of cysteines were reintroduced into the cys-less background. Mutations were generated in 300–500-base pair cassettes using the QuickChange site-directed mutagenesis kit (Stratagene) and verified by sequencing. Full-length exchangers were constructed by subcloning the cassettes carrying mutations into the cys-less exchanger.

**Expression of Exchanger Proteins in Xenopus Oocytes—**cDNA was prepared using the mMessage mMachine in vitro RNA synthesis kit (Ambion) and injected into Xenopus laevis oocytes. Na⁺-Ca²⁺ exchanger activity was measured as Na⁺ gradient-dependent Ca²⁺ uptake into oocytes as described previously (11).

**Na⁺ Gradient-dependent Ca²⁺ Uptake into Transfected HEK Cells—**Exchanger cDNAs were subcloned into pcDNA 3.1(−) vector (Invitrogen) and transfected into HEK293 cells using LipofectAMINE (Life Technologies, Inc.). 48–72 h post-transfection, cells were harvested and washed twice with washing buffer (10 mM MOPS (pH 7.4), 140 mM NaCl). Cells were 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 blank), 25 μM CaCl₂, 0.4 mM ouabain, and 0.4 mM MgCl₂. After incubation (typically 1–3 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 the quenching solution. Cell pellets were then dissolved in 1 ml NaOH at 80 °C for 20 min. Aliquots of samples were subjected to scintillation counting and protein assay.

**Crude Membrane Vesicle Preparation from Transfected HEK cells and Cross-linking Procedure—**Transfected cells were washed twice with washing buffer, resuspended in 20 mM MOPS (pH 7.4), 280 mM NaCl, and homogenized with 10 strokes in a Dounce homogenizer. After centrifugation at 14,000 × g for 5 min at 4 °C, the pellet was resuspended in washing buffer. The sample was then passed through a 20-gauge needle 20 times and centrifuged for 10 min at 4,000 × g at 4 °C to remove cell debris and nuclei. The supernatant was collected for cross-linking or stored at −80 °C. Cross-linking was carried out at 20 °C by adding oxidative reagent or thiol-specific homobifunctional cross-linker to the membrane preparation. The final concentrations of reagents were 3 mM CuSO₄, 9 mM phenanthroline, and 0.5 mM o-PDM or p-PDM. The reaction was terminated after 20 min by adding N-ethylmaleimide to a final concentration of 10 mM.

**SDS-PAGE and Western Blots—**48–72 h post-transfection, HEK cells were harvested and washed with washing buffer. Cells were lysed with radioimmuneprecipitation buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM EDTA) supplemented with 10 mM N-ethylmaleimide. After incubating on ice for 10 min, the samples were centrifuged for 15 min at 11,000 rpm at 4 °C. Supernatants were subjected to SDS-PAGE. Membrane vesicles or cross-linked samples were mixed with an equal volume of radiolmune precipitation buffer (plus 10 mM N-ethylmaleimide). Electrophoresis was performed on discontinuous 7% SDS-polyacrylamide gels. For reducing conditions, 2% β-mercaptoethanol was included in the sample buffer. Proteins in SDS-PAGE gels were transferred to nitrocellulose membrane (Bio-Rad). Blots were probed with exchanger-specific antibodies (C2C12 or R3F1) (13), and exchanger signals were detected by chemiluminescence (PerkinElmer Life Sciences).

**RESULTS**

**Expression of the Wild Type and Cys-less Exchangers in HEK Cells—**We have previously constructed a cys-less exchanger (clone H) with Na⁺ gradient-dependent Ca²⁺ uptake activity comparable with that of the wild type exchanger when expressed in Xenopus oocytes (6). However, clone H exchanger displayed no activity when expressed in HEK cells although exchanger protein was synthesized, as revealed by Western blot (data not shown). To construct a new version of the cys-less exchanger which would be active in HEK cells, we reintroduced each of the 15 native cysteines, one at a time, into the H background and expressed these mutant exchangers in HEK cells. Exchanger activity was restored to about 30% of the wild type activity when the cysteine at either position 151 or position 210 was present. In the H exchanger, both cysteines had been mutated to alanine. We tested whether conservative substitution of these cysteines with serine would restore exchanger activity. Indeed, a cys-less exchanger with a serine at position 210 (clone I) had 30% of wild type activity when expressed in HEK cells. Substitution of Cys-151 with serine did not rescue cys-less exchanger activity. Therefore, we used clone I as the background for these studies. Fig. 2 compares the activity of

**FIG. 1.** Topological model of the Na⁺-Ca²⁺ exchanger. TMSs are represented by cylinders and are numbered. The α-repeats are shaded. All labeled residues within the transmembrane regions were used in this study. This includes native cysteines (reintroduced into the cys-less exchanger) and residues mutated to cysteines. Also shown are Cys-20 and Cys-792, the two cysteines that form an endogenous disulfide bond in the wild type protein. The extracellular surface is at the top. Extramembrane segments are labeled a through j. CH₂O denotes the protein glycosylation site.
post-transfection, the Na\(^+\)-gradient dependent uptake of \(^{45}\)Ca\(^2+\) into the cells was determined (described under “Experimental Procedures”). Clones H and I are both cys-less exchangers. In clone H, Cys-210 has been mutated to alanine and in clone I, position 210 is a serine. Values are the average of five independent experiments. Error bars show standard errors.

It is unknown why the alanine to serine substitution restored activity.

**Mobility Shift of Mutant Exchangers with Substituted Cysteine Pairs**—We have previously identified an intramolecular disulfide bond between cysteines 20 and 792 in the exchanger that induces a mobility shift on SDS-PAGE under nonreducing conditions (17). These two cysteines are located in extracellular segments connecting TMSs in the NH\(_2\)-terminal and COOH-terminal TMS clusters, respectively. We reasoned that other cross-links between substituted cysteines in TMSs in the NH\(_2\)-terminal cluster (TMSs 1–5) to those in the COOH-terminal cluster (TMSs 6–9) might also give rise to an electrophoretic mobility shift. To test this possibility, we reintroduced the native cysteine residue Cys-151 in TMS3 into the cys-less background. This cysteine was then paired with a series of cysteines (native or substituted) in the TMSs in the COOH-terminal cluster (Table I). The mutant exchangers carrying cysteine pairs were expressed in HEK cells and analyzed by Western blot under reducing and nonreducing conditions (Fig. 3). In mutants A151C/T815C and A151C/A821C, the majority of the protein migrated as the 120-kDa band, whereas a fraction (10–15% of total protein mass) had an apparent molecular mass of 160 kDa under nonreducing conditions. The two mutant proteins migrated as a 120-kDa band when the reducing reagent \(\beta\)-mercaptoethanol was present. These results suggest that Cys-151 can form disulfide bonds with either Cys-815 or Cys-821 to give rise to a 160-kDa species as previously observed with mutants carrying Cys-20 and Cys-792. This partial disulfide bond formation between Cys-151 and Cys-815 or Cys-821 also suggests that TMS3 and TMS7 are within close proximity.

**Screening of Mutant Exchangers for Mobility Shifts**—To further analyze proximities between different TMSs, we constructed a series of cysteine substitutions at sites modeled to be in the TMSs. Mutants carrying a single cysteine substitution in the cys-less background were first assayed for exchanger activity in oocytes using Na\(^+\) gradient-dependent \(^{45}\)Ca\(^2+\) uptake (Refs. 6, 11, and 15 and data not shown). Only active cysteine substitutions were then paired, and mutant exchangers carrying substituted cysteine pairs were expressed in HEK cells and analyzed for electrophoretic mobility on SDS-PAGE. Cysteine substitutions were tested in each of the TMSs. Pairings were based on the current topological model such that paired cysteines were modeled to reside within the TMSs near the same side of the plasma membrane. Table I summarizes all the cysteine pair mutants that have been tested for activity and mobility shift. Among the 66 cysteine pairs, four active mutants displayed mobility shifts (Table I) and were further analyzed using cross-linking techniques.

**Cross-linking of Exchanger Mutants**—The proximity relationships between selected cysteine residues were further analyzed in cross-linking experiments using the oxidative reagent CuPhe or the homobifunctional thiol-specific linkers \(\alpha\)-PDM and \(p\)-PDM. CuPhe catalyzes oxidation of adjacent thiol groups to promote the disulfide bond formation between cysteine residues. \(\alpha\)-PDM and \(p\)-PDM are noncleavable, rigid homobifunctional reagents in which the maleimido groups are coupled to a benzene ring in the \(\alpha\) or \(p\) para position at fixed distances of 6 or 10 Å, respectively (19). Membrane vesicles prepared from transfected cells expressing mutant exchanger were subjected to cross-linking, SDS-PAGE, and immunoblot analysis. For all cysteine pairs that showed mobility shifts (151/815, 151/821, 117/804, and 122/892), treatment with CuPhe and \(p\)-PDM enhanced the conversion of the 120-kDa band to the 160-kDa band under nonreducing conditions (Fig. 4 and data not shown). Total exchanger signal (120- + 160-kDa bands) was reduced in samples from mutant A151C/T815C upon treatment with CuPhe or \(p\)-PDM (not shown). Apparently this mutant protein was prone to aggregation in the presence of cross-linking reagents. In samples treated with \(p\)-PDM, only a small increase of the 160-kDa species was observed (Fig. 4). The data are quantified in Table II. No 160-kDa band was ever seen with the cys-less exchanger (Fig. 4). Under reducing conditions, the presence of \(\beta\)-mercaptoethanol converted the 160-kDa band to the 120-kDa band in untreated and CuPhe-treated samples. A fraction of the 160-kDa exchanger protein was resistant to \(\beta\)-mercaptoethanol in \(p\)-PDM- or \(p\)-PDM-treated samples (Fig. 4). In some samples, an additional band with an apparent molecular mass of 140 kDa was present (Fig. 4). The origin of this band is unknown, and it has been observed in our previous studies (16, 17). Although the exact position and intensity of this band vary in different sample preparations, the band was not affected by the presence or absence of reducing reagents as also noted previously (17) or by cross-linking reagents. Taken together, these results indicate that cysteine pairs within each of the three mutants (A151C/A821C, S117C/V804C, A122C/Y892C) are close to each other (within a distance of 6 Å).

In other studies using similar approaches, it has been demonstrated that ligand binding leads to an altered cross-linking pattern in lactose permease and P-glycoprotein (19, 20). We tested whether the presence or absence of Na\(^+\) and/or Ca\(^{2+}\), the two ligands for the exchanger, has any effect on cross-linking. Membrane vesicles were prepared under different conditions in which buffers containing Na\(^+\) only, Na\(^+\) plus Ca\(^{2+}\), K\(^+\) only, or K\(^+\) plus Ca\(^{2+}\) were used. No significant difference in cross-linking with different vesicle preparations was observed by Western blot analysis (data not shown).

**Effect of Cysteine Disulfide Bond Formation on Exchanger Activity**—Each individual cysteine substitution mutant has been tested in *Xenopus* oocytes and showed Na\(^+\) gradient-dependent \(^{45}\)Ca\(^2+\) uptake activity. To study the effects of disulfide bond formation on exchanger activity, HEK cells transfected with exchanger constructs carrying selected cysteine pair mutants were assayed for exchanger activity. As shown in Fig. 5, A151C/T815C and A151C/A821C mutants have an increased exchanger activity, whereas activity in other mutants maintained the same level as that of the cys-less exchanger, including mutants S117C/V804C and A122C/Y892C, which displayed mobility shifts. Perhaps the disulfide bond between Cys-151 and Cys-815 or Cys-821 facilitates a more active conformation of the exchanger or enhances the cell surface expression of the exchanger protein. In contrast, mutant S117C/K909C was inactive (Fig. 5) although Western blot experiments showed that...
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TABLE I

Disulfide cross-linking of exchanger mutants

Intensities of the 120- and 160-kDa exchanger protein bands on immunoblots were quantified by densitometry using an AlphaImager system. Data are the percentage of the 160-kDa band compared to total exchanger protein bands (120-160 kDa). Transmembrane segment numbers are indicated by roman numerals.

Residues near the extracellular side of the membrane

| COOH-cluster | NH\(_2\)-cluster |
|--------------|-----------------|
| S788C (VI)   | A43C (I)        |
| V804C (VII)  | S177C (II)      |
| Y892C (VIII) | A122C (II)      |
| K909C (IX)   | S188C (IV)      |
| S58C (I)     | 0.8 ± 0.5\(^*\) |
| S210C (V)    | 1.2 ± 0.7       |
| S210C (V)    | 2.6 ± 0.3       |
| S188C (IV)   | 0.7 ± 0.3       |
| S188C (IV)   | 5.3 ± 3.5\(^*\) |
| A122C (II)   | 0.7 ± 0.3       |
| A122C (II)   | 1.4 ± 0.4       |

Residues near the intracellular side of the membrane

| COOH-cluster | NH\(_2\)-cluster |
|--------------|-----------------|
| S58C (I)     | S106C (II)      |
| A151C (III)  | T172C (IV)      |
| A854C (P)    | S210C (V)       |
| A151C (III)  | 0.8 ± 0.7       |
| A151C (III)  | 14.5 ± 3.8\(^*\) |
| A151C (III)  | 2.5 ± 0.6       |
| A151C (III)  | 0.4 ± 0.2       |
| A151C (III)  | 1.4 ± 0.7       |
| A151C (III)  | 0.5 ± 0.3       |
| A151C (III)  | 0.9 ± 0.5       |
| A151C (III)  | 0.6 ± 0.3       |

\(^*\) Mean ± S.E.; \(n = 3\).
\(^*\) Quantification was carried out on duplicate samples and in both blots the percentage of the 160-kDa band was under 2%.
\(^*\) Mutant displayed mobility shift but no exchanger activity.
\(^*\) Visible 160-kDa band was readily discernible; at percentages below 5% no visible band was seen. P, P-loop-like region between TMSs 7 and 8.

FIG. 3. Immunoblots of mutant proteins under reducing (R) or nonreducing (NR) conditions. Cysteines (labeled on top of each lane) were introduced into a cys-less exchanger, and mutant proteins were analyzed. Positions of protein standards (in kDa) are indicated on the left. Partial mobility shifts in samples from A151C/T815C and A151C/A821C are indicated by roman numerals. The diffuse appearance of nonreduced samples from A20C/A792C and wild type exchangers is due to a higher level of protein expression in cells transfected with these exchanger cDNAs. Under nonreducing conditions, A20C/A792C and the wild type exchangers appeared predominantly as a 160-kDa band when less protein was loaded onto the SDS-gel and in previous studies (17).

it displayed partial mobility shift under nonreducing conditions (Table I). This inhibition could not be reversed by treatment with \(\beta\)-mercaptoethanol. Disulfide bond formation in mutant S117C/K909C may disrupt trafficking of exchanger to the plasma membrane or constrain conformational flexibility for active ion transport. As with the data shown in Fig. 2, empty vector transfected cells showed no significant Na\(^{+}\) -Ca\(^{2+}\) exchange activity.

DISCUSSION

A number of membrane proteins have been reported to have a retarded electrophoretic mobility in SDS-PAGE under nonreducing conditions (20, 21). This is in contrast to what has been observed for many soluble proteins in which preserving intramolecular disulfide bonds during SDS-PAGE generally leads to an increased mobility. It has been further noted that for the mobility shift of membrane proteins to occur in the absence of reducing agents, residues involved in disulfide bond formation or cross-linking must be distantly spaced (20). We have reported that purified cardiac Na\(^{+}\) -Ca\(^{2+}\) exchanger proteins migrate differently in SDS-PAGE depending on the redox environment: a 120-kDa band in the presence and a 160-kDa band in the absence of dithiothreitol (16). By systematically removing each of the 15 endogenous cysteine residues, Santana-Tolosa et al. (17) determined that the mobility shift to 160 kDa under nonreducing conditions is primarily due to the presence of an intramolecular disulfide bond between residues Cys-20 and Cys-792. Residues Cys-20 and Cys-792 are located in extracellular segments connecting TMSs in the NH\(_{2}\)-terminal and COOH-terminal clusters, respectively. Between the two clusters is a large intracellular loop (loop f about 550 amino...
acid residues in length) that is capable of undergoing dramatic conformational changes (22). The conformation of loop f may contribute to the variation in apparent molecular mass of the exchanger. Under reducing conditions, when there is no disulfide bond connecting the two TMS clusters, loop f may form a more compact structure, and the exchanger migrates as a 120-kDa band. When a disulfide bond between the two TMS clusters brings helices into a more tightly packed bundle, loop f may extend further from the membrane. This “parachute” effect may result in the mobility shift to 160 kDa under nonreducing conditions.

By introducing paired cysteine residues back into the cys-less background, one in each half of the protein, we show here that a number of exchanger mutants also displayed a mobility shift under nonreducing conditions (Fig. 3). This implies that spontaneous disulfide bond formation occurs between the introduced paired cysteines. Treatment with CuPhe significantly enhanced the mobility shift. Thus, the mobility shift provides a useful tool to study proximity relationships of transmembrane helices in the exchanger.

We screened a total of 66 exchanger mutants with double cysteines (Table I). Four pairs induced mobility shifts that were enhanced with cross-linking reagents (Figs. 3 and 4). Based on these data, we propose an initial packing model for TMSs 2, 3, 7, and 8 of the exchanger (Fig. 6). Disulfide bond formation between S117C and V804C indicates that TMS7 is in close proximity to TMS2 at the extracellular side of the membrane. At the intracellular side of the membrane, TMS7 is in the vicinity of TMS3 as indicated by cross-linking of A151C/A821C. Furthermore, residues Cys-804 and Cys-821 in TMS7, which are adjacent to TMS2 and TMS3, are located on the same surface of TMS7 (Fig. 6) according to helical wheel modeling. This suggests that the same surface of TMS7 interacts with TMS2 at the extracellular and TMS3 at the intracellular side of the membrane. Possibly TMS7 tilts in the membrane between TMSs 2 and 3 or has a bend. A proline residue at position 813 in the center of TMS7 may facilitate a nonhelical structure and/or a bend. On this surface of TMS7, mutations S811T (15),

![Fig. 4. Cross-linking of exchanger mutants displaying mobility shifts.](image)

![Fig. 5. Exchanger activity of selected cysteine mutants.](image)

![Fig. 6. Model of the TMS packing in the cardiac Na+-Ca2+ exchanger.](image)

**Table II**

Quantification of cross-linking efficiency

Intensities of the 120- and 160-kDa exchanger protein bands on immunoblots (Fig. 4, nonreducing conditions) were quantified by densitometry using the Alphalager system. Data (mean ± S.E.; n = 3) are the percentage of the 160 kDa band compared to total exchanger protein bands (120 + 160 kDa).

| Mutants          | Nontreated | CuPhe | o-PDM | p-PDM |
|------------------|------------|-------|-------|-------|
| S117C/V804C      | 13.6 ± 4.3 | 21.3 ± 3.5 | 34.5 ± 8.9 | 14.9 ± 4.6 |
| A122C/Y892C      | 5.3 ± 3.5 | 37.2 ± 5.8 | 48.3 ± 12.4 | 21.6 ± 4.5 |
| A151C/A821C      | 14.5 ± 3.8 | 61.2 ± 11.4 | 63.3 ± 7.8 | 29.2 ± 6.6 |
| Cys-less         | 0.9 ± 0.5 | 0.8 ± 0.8 | 0.5 ± 0.4 | 0.8 ± 0.3 |
S818A (15), and S818C (data not shown) inhibited exchanger activity when the exchangers were expressed in oocytes. Our model (Fig. 6) places TMS2 between TMSs 7 and 8. This requires a reasonable length for the loop connecting TMSs 7 and 8. Significantly, loop h connecting TMSs 7 and 8 is modeled to be 49 amino acid residues in length and includes a speculative P-loop-like region (Ref. 6 and Fig. 1). Also, there is experimental evidence that loop c connecting TMSs 2 and 3 re-enters the membrane from the extracellular side of the membrane (7). Thus, the two proposed re-entrant loops may be constrained to be in close proximity.

It has been suggested previously that the $\alpha_1$ and $\alpha_2$ repeats, comprising parts of TMSs 2, 3, and 7 and loop h, form a portion of the ion translocation pathway (14, 15). Significantly, our packing model now indicates that the $\alpha$-repeats are in close proximity. Within the $\alpha$-repeat regions, we have previously identified mutations in TMSs 2, 3, and 7, which either abolish or lead to a decreased exchanger activity, including mutations at Ser-109, Ser-110, Glu-113, and Glu-120 (TMS2); Ser-139 and Asn-143 (TMS3); Ser-811, Asp-814, and Ser-818 (TMS7) (Ref. 15 and Qiu et al.2). Strikingly, these residues line the helical surfaces that face one another according to our initial helix packing model. Therefore, our data provide structural evidence for the earlier suggestion that hydrophilic faces of amphipathic TMSs 2, 3, and 7 form a portion of the ion translocation pathway.

Also, our data support recent topology models (6, 7) that suggest that residues 804 and 821 of TMS7 must be near the extracellular and intracellular surfaces, respectively. In initial models (23), this would not be possible as the orientation of TMS7 (TMS8 of earlier models) would be reversed. This information will be helpful in designing future experiments to further elucidate helical interactions within the exchanger. More importantly, accumulation of structural information will greatly facilitate understanding of the mechanism of ion translocation.

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