Mutation of Amino Acid Residues in the Putative Transmembrane Segments of the Cardiac Sarcolemmal Na\(^+\)-Ca\(^{2+}\) Exchanger

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We have examined the role of conserved regions and acidic or basic residues located in the putative transmembrane segments of the cardiac sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchanger by site-directed mutagenesis. The \(\alpha-1\) and \(\alpha-2\) repeats are transmembrane regions of internal similarity, which are highly conserved among Na\(^+\)-Ca\(^{2+}\) exchangers. We find that Na\(^+\)-Ca\(^{2+}\) exchange activity is highly sensitive to mutagenesis in the \(\alpha\)-repeats. Mutation at residues Ser-109, Ser-110, Glu-113, Ser-139, Asn-143, Thr-810, Ser-811, Asp-814, Ser-818, or Ser-838 resulted in loss of exchanger activity. Mutation at residues Thr-103, Gly-108, Pro-112, Gly-809, Gly-837, and Asn-842 resulted in reduced exchanger activity, and altered current-voltage relationships were observed with mutations at residues Gly-138 and Gly-837. Only mutation at residue Ser-117 appeared to leave exchanger activity unaffected. Thus, the \(\alpha\)-repeats appear to be important components for ion binding and translocation. Another region implicated in exchanger function is a region of similarity to the Na\(^+\)/K\(^+\) pump (Nicoll, D. A., Longoni, S., Philipson, K. D. (1990) Science 250, 562-565). Mutations at two residues in the pump-like region, Glu-199 and Thr-203, resulted in nonfunctional exchangers, while mutation at two other residues, Glu-196 and Gly-200, had no effect. The role of acidic and basic residues in the transmembrane segments was also examined. Mutation of several basic residues (Arg-42, His-744, Lys-751, Lys-797, and His-858) did not affect exchange activity. Of the acidic residues located outside of the \(\alpha\)-repeat and pump-like regions (Asp-740, Asp-785, and Asp-798), only mutation at Asp-785 resulted in reduction of exchanger activity.

The plasma membrane Na\(^+\)-Ca\(^{2+}\) exchanger is an ion transport protein, which has been identified in several preparations including cardiomyocytes (1), squid axon (2), brain synaptosomes (3), renal tissue (4), and platelets (5). The primary function of the exchanger in the different cell types is to maintain Ca\(^{2+}\) homeostasis, especially in excitable cells where rapid and substantial Ca\(^{2+}\) fluxes are important in signaling pathways.

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The exchanger utilizes the Na\(^+\) electrochemical gradient to exchange 3 external Na\(^+\) ions for 1 internal Ca\(^{2+}\) ion. The exchanger is both electronic and reversible and exhibits several different regulatory properties. An example is regulation by intracellular Ca\(^{2+}\) (1, 6), i.e. there is a Ca\(^{2+}\) binding site at the intracellular surface that is distinct from the Ca\(^{2+}\) transport site. When Ca\(^{2+}\) is bound to the regulatory site, the exchanger is activated. We have previously identified the regulator Ca\(^{2+}\) binding site by deletion and site-directed mutagenesis (7-9). The exchanger is also regulated by ATP. In the cardiomyocyte, ATP regulation may act indirectly through an ATP-dependent phospholipid flipase, which is responsible for maintaining phospholipid asymmetry in the plasma membrane (10). In contrast, the exchanger in the squid axon appears to be regulated by direct phosphorylation (11). The exchanger can also be inhibited at the intracellular surface by a specific peptide, XIP

*1 The abbreviations used are: XIP, exchanger inhibitory peptide; NCKX1, Na\(^+\)-Ca\(^{2+}\) exchanger isoform 1; CalX, Drosophila type Na\(^+\)-Ca\(^{2+}\) exchanger; NCX2, Na\(^+\)-Ca\(^{2+}\) exchanger isoform 2; NCKX1, photoreceptor-type Na\(^+\)/(Ca\(^{2+}\)+K\(^+\)) exchanger; PBS, phosphate-buffered saline; IV, current-voltage; MES, 2-(N-morpholino)ethanesulfonic acid; TEA, tetraethylammonium.
Fig. 1. Model of the Na\(^{+}\)-Ca\(^{2+}\) exchanger (NCX1). Amino acid sequence of the proposed transmembrane segments is shown. Segments modeled to be outside the membrane are labeled a–l. Amino acids in the a–repeats and Na\(^{+}\), K\(^{+}\) pump region are in bold. The amino acids in loops h and j, which were initially proposed to be in transmembrane segments 8 and 9, are not given. The single glycosylation site at Asn-9 is shown (CH\(_{2}\)O).

In this study, we have attempted to identify regions of NCX1 that are involved in ion binding and transport by site-directed mutagenesis of amino acids modeled to be in transmembrane segments. Our initial mutations have included residues that are in highly conserved regions of the exchangers and in acidic and basic residues.

MATERIALS AND METHODS

Mutagenesis—The wild-type exchange cDNA was modified to allow the generation of 200–400 base-pair cassettes for mutagenesis as follows. The multiple cloning site of Bluescript (Stratagene) was removed and a BamH1 restriction site added at the 5’ end and a HindIII site at the 3’ end. The Pst1 site at nucleotide 40 and the EcoRI site at nucleotide 1800 were removed, and a SalI site was introduced at nucleotide 900 by silent mutagenesis. All mutations were generated in cassettes of DNA using either the method of Kunkel (19) or an Amer sham in vitro mutagenesis kit. Following mutagenesis reactions, the cassette DNA was sequenced using Sequenase (U. S. Biochemical Corp.) and then subcloned into the full-length exchange clone.

Expression in Oocytes—Full-length exchange cassettes were linearized with HindIII and RNA was synthesized using the T3 mMessage mMachine kit (Ambion). Unincorporated nucleotides were removed by gel filtration on Chromaspin-100 columns (Clontech).

Injection—The mRNA was then injected into oocytes from 5 to 50 pmol of Ca\(^{2+}\)/oocyte taken up in 10 min. Immunofluorescence—Fresh oocytes were gently placed on small (4 mm diameter) circles of hardened filter paper. They were quickly frozen by immersion in a slush of liquid propane maintained at liquid nitrogen temperature. 5–8 μm-thick cryosections were collected on slides for immunolabeling. The oocyte sections were fixed for 10 min in PBS containing 1% formaldehyde, then washed for 10 min in PBS with 2 mg/ml sodium borohydride and rinsed for 10 min in PBS. Sections were incubated for 1 h in blocking solution (5% goat serum, 3% bovine serum albumin in PBS) before adding the primary antibody. The primary antibody, R3F1 (21) at 16 μg/ml in rinse solution (1.5 dilution of blocking solution), was incubated with the sections for 2.5 h and then removed by rinsing for 1 h (12 changes) of rinse solution. The sections were then incubated with a secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse antibody, at a 1:500 dilution in rinse solution for 30 min. Unbound secondary antibody was removed by rinsing in PBS for 45 min.

Electrophysiological Techniques—The giant excised patch clamp technique of Hilgemann (22, 23) was used as described previously (7, 24). For all experiments, outward (reverse mode) Na\(^{+}\)-Ca\(^{2+}\) exchange currents were studied and patches were oriented in the inside-out configuration. Pipettes were pulled from borosilicate glass and polished to a final diameter of 15–30 μm. Pipettes were then coated with a paraffin-mineral oil mixture to enhance patch stability. Gigaohm seals were formed by gentle suction, and patches were excised by progressive movements of the pipette tip. Pipettes were filled with (in mM): 100 N-methyl-D-glucamine-MES, 30 HEPES, 30 TEA-Cl, 8 Ca\(_{2+}\), 6 Cs\(_{2+}\), 0.2 Ba(OH)\(_{2}\), 2 Mg(OH)\(_{2}\), 0.25 ouabain, 0.1 niflumic acid, 0.1 fluoramic acid, pH 7.0 (using MES). Outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents were elicited by switching from a Cs\(_{2+}\)-based solution containing (in mM): 100 Na\(^{+}\), 10 Cs\(_{2+}\), 30 HEPES, 30 TEA-Cl, 20 Na\(^{+}\)-based superfuse containing (in mM): 100 Na\(^{+}\)-Ca\(^{2+}\), 30 HEPES, 30 TEA-Cl, 20 Cs\(_{2+}\), 10 EGTA, 0–11 Ca\(_{2+}\), 1–1.5 Mg(OH)\(_{2}\), pH 7.0 (using MES). Ca\(^{2+}\) and Mg\(^{2+}\) amounts were adjusted to yield the indicated Ca\(^{2+}\) concentrations and maintain the free Mg\(^{2+}\) concentration at 1 mM. Ca\(^{2+}\) and Mg\(^{2+}\) concentrations were calculated using MAX-C software (25). Solution switching was computer-controlled using a custom-built 20-channel solution switcher. All experiments were conducted at 34 ± 1°C. IV relationships were obtained by ramp or step protocols using Axon Instruments (Foster City, CA) hardware and software.

RESULTS AND DISCUSSION

Selection of Residues to Mutate—The Na\(^{+}\)-Ca\(^{2+}\) exchanger has been modeled to contain 11 transmembrane segments...
based on hydropathy analysis (Fig. 1). The exchanger consists of three structural domains. In the amino terminus there is a domain containing five transmembrane segments. A large hydrophilic, cytoplasmic domain is between transmembrane segments 5 and 6. In the carboxyl terminus there is a domain containing six transmembrane segments. By deletion of the large cytoplasmic domain, we have determined that only the two transmembrane-containing domains are necessary for ion transport (7).

Ion binding is modeled to be voltage-dependent (26), and binding sites are likely to be in the transmembrane domains. Transport across the membrane must involve amino acids in the transmembrane segments. There are a total of 234 amino acid residues modeled to be in transmembrane segments (Fig. 1; the residues modeled to be in transmembrane segments 8 and 9 have been modified slightly from our initial proposal (13) to accommodate new observations regarding internal repeats). We made mutations to amino acids in three highly conserved regions of the exchanger. The conserved regions show similarities to NCKX1 (18) and the Na\textsuperscript{+},K\textsuperscript{+} pump (13). We also mutated acidic and basic residues, which have been modeled to be in transmembrane segments.

Fig. 3. Alignment of the exchangers with the Na\textsuperscript{+},K\textsuperscript{+} and Ca\textsuperscript{2+} pumps. Alignment of cardiac (NCX1), brain (NCX2), and Drosophila (CalX) exchangers with the Na\textsuperscript{+},K\textsuperscript{+} pump (NKP) and Ca\textsuperscript{2+} pump (CaP). Residues that, when mutated, produce functional exchangers are indicated with \(\ast\), and those that, when mutated, produce non-functional exchangers are indicated with \(-\). Residues that are identical to NCX1 are highlighted.

Fig. 4. Relative Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity of NCX1 exchangers with mutations in the proposed transmembrane segments. Activities are given as percent \((\pm\ S.E.,\ n = 2-11)\) of \(^{45}\text{Ca}^2+\) taken up into Na\textsuperscript{+}-loaded oocytes expressing mutant exchangers (in K\textsuperscript{+}-containing medium) relative to oocytes expressing wild-type exchanger. Mutants are divided into the regional categories: \(\alpha-1\), \(\alpha-1\) repeat; NKP, Na\textsuperscript{+},K\textsuperscript{+} pump-like region; AB, acidic and basic residues; \(\alpha-2 = \alpha-2\) repeat.

\[2\] E. Schwarz, and S. Benzer, personal communication.
adjusted the model from our initial proposal (13) accordingly. The adjustment does not change the number of proposed transmembrane segments or overall topology.

*Na<sup>+</sup>-K<sup>+</sup> Pump Region—The exchange also shares a region of sequence similarity with the Na<sup>+</sup>-K<sup>+</sup> pump (13), which spans 24 residues with 46% identity (Figs. 1 and 3). In the Na<sup>+</sup>-K<sup>+</sup> pump, the NH<sub>2</sub>-terminal amino acids, YTWLE, are modeled to be extracellular and to link transmembrane segments 3 and 4 (27). These amino acids have been suggested to be involved in ouabain sensitivity (28) and to serve as an antibody epitope (29). The remainder of the region is modeled to be a part of transmembrane segment 4 of the Na<sup>+</sup>-K<sup>+</sup> pump. The COOH-terminal amino acids, PEGLLA, are involved in ion binding. Mutations at the proline, glutamate, or the second leucine residue have been shown to alter the affinities for K<sup>+</sup> and Na<sup>+</sup> (30). In an analogous region of the sarcoplasmic reticular Ca<sup>2+</sup><sup>+</sup> pump, the residues EGL have been shown to be involved in Ca<sup>2+</sup><sup>+</sup> binding and transport and in the conformational changes associated with Ca<sup>2+</sup><sup>+</sup> transport (31).

Fig. 3 shows the alignment between the NCX1, NCX2, and CalX exchangers with the Na<sup>+</sup>-K<sup>+</sup> pump region (there is no analogous region in NCKX1).

Acidic and Basic Residues—The proposed transmembrane segments of the exchanger contain a number of acidic and basic residues as well as other hydrophilic residues. An ion conduction pathway is likely to contain hydrophilic residues, and charged residues may be especially important in ion binding and translocation. Charged residues have been shown to be involved in ion binding in several other proteins (32–34).

Activity of Mutants—Fig. 4 summarizes the transport activity of NCX1 with mutations in the putative transmembrane segments. Mutants were expressed by injecting cRNA into Xenopus oocytes. One to 5 days after injection, 45Ca<sup>2+</sup><sup>+</sup> uptake into Na<sup>+</sup>-loaded oocytes was measured. The results are presented as a percent of 45Ca<sup>2+</sup><sup>+</sup> uptake relative to that of oocytes expressing the wild-type exchanger. In cells expressing 10% or less activity, the amount of 45Ca<sup>2+</sup><sup>+</sup> uptake in the presence of extracellular K<sup>+</sup> was equivalent to the uptake in the presence of extracellular Na<sup>+</sup> (i.e. in the absence of a Na<sup>+</sup> gradient) and to cells injected with water only. Therefore, these cells do not express Na<sup>+</sup>-Ca<sup>2+</sup><sup>+</sup> exchange activity.

To verify that these mutants were indeed inactive, injections were repeated with several different batches of cRNA. We also verified synthesis of exchanger protein in oocytes by Western blot analysis (not shown), and immunofluorescence. All mutants were expressed at the cell surface at qualitatively similar levels. Fig. 5 shows representative immunofluorescent labelings of oocytes expressing wild-type or mutant exchangers. Fluorescence can be seen at the surface of wild-type and mutant-expressing cells (Fig. 5, a–c). In some instances fluorescence can also be detected in the cytoplasm of the cells (Fig. 5, b and c). Water-injected cells do not display any immunofluorescence (Fig. 5d).

Mutations in the α-Repeat Regions—Nineteen different residues (including Thr-103, just upstream of α-1) in the α-repeat regions were mutated (Figs. 4 and 6). Mutation at 10 of the 19 sites resulted in non-functional exchangers, and mutation at 7 of the sites resulted in exchangers with low to moderate (18–65% of wild-type) levels of activity. Only mutant S117A displayed wild-type levels of exchange activity. These observations indicate that the α-repeat regions are essential in exchanger function. In contrast, mutation at 7 of 12 sites in other regions of the putative transmembrane segments exhibited wild-type levels of activity; only 2 had reduced levels of activity and 3 were inactive.

At five of the sites in the α-repeats, more than one amino acid substitution was made. Ser-109 was initially mutated to another small amino acid, alanine. This mutant was non-functional. In NCKX1 there is a glycine residue at this location. Mutant S109G was constructed to mimic NCKX1 but was also non-functional. Likewise, residue Ser-110 was substituted with an alanine, cysteine, or threonine. Each of the three mutants was non-functional, indicating that conservation of neither size nor chemical nature at the site was sufficient to maintain activity. The glutamate at position 113 was conservatively mutated to either an aspartate or a glutamine and in either case a non-functional protein resulted, indicating that maintenance of an acidic residue or size was insufficient. Mutation at

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**Fig. 5.** Immunofluorescent labeling of oocytes expressing exchangers. Oocytes expressing wild-type Na<sup>+</sup>-Ca<sup>2+</sup><sup>+</sup> exchanger (a), mutant S110A (b), mutant T810A (c), and water-injected oocytes (d). Filled arrow shows labeling at the membrane surface. Open arrow shows labeling in the cytoplasm.

**Fig. 6.** Relative Na<sup>+</sup>-Ca<sup>2+</sup><sup>+</sup> exchange activity of NCX1 exchangers with mutations in the α-repeat regions. The amino acid sequences of α-1 and α-2 are aligned, and the activity of parallel mutants indicated above the alignment for α-1 and below the alignment for α-2. The amount of 45Ca<sup>2+</sup><sup>+</sup> taken up into oocytes expressing wild-type exchanger in K<sup>+</sup>-containing medium is 100%. 

site Gly-138 was of interest because when the glycine was substituted with an alanine, the mutant displayed a moderate level of activity. However, when the glycine was substituted with a serine, there was a significant reduction in the level of activity. Mutants at this residue or its complement in the α-2 repeat, Gly-837, displayed an altered current-voltage relationship (see below), attesting to the importance of this region in exchanger function. Residue Asn-842 was mutated to either an aspartate or a valine, and in either case, the same moderate levels of activity were observed.

Another interesting aspect of mutations in the α-repeat regions was the parallel nature of effects of mutagenesis in the two regions (Fig. 6). Mutations were made at a number of the homologous sites in the α-repeats and the levels of activity compared. For example, residues Gly-108 and Gly-809 are located at homologous sites, and both exhibit moderate levels of $^{45}\text{Ca}^{2+}$ uptake when mutated to alanine. Similarly, neither mutant S109A nor T810A expressed exchanger activity. The only exceptions to this rule are for the pairs Ser-117/Ser-818 and Asn-143/Asn-842. S117A expressed wild-type levels of activity, while a mutation at the homologous residue, S818A, yielded an inactive exchanger. Mutant N143V was inactive, and mutant N842V expressed reduced levels of exchanger activity.

The presence of the α-repeats in the exchanger, which have apparently risen from a gene duplication event, and the parallel effect of mutations at homologous sites suggest that the transmembrane segments are arranged in a dimer-like fashion with each of the dimer halves functioning similarly though not necessarily independently. However, the fact that mutations at the pairs Ser-117/Thr-810 and Asn-143/Asn-842 do not have parallel effects indicates that, while the α-repeats are homologous, their functions in the exchanger are not identical.

Mutations in the Na$^+$,K$^+$ Pump Region—Four of the residues in the region of similarity to the Na$^+$,K$^+$ pump, Glu-196, Glu-199, Gly-200, and Thr-203, were mutated. Glu-196 is modeled in the exchanger to be near the extracellular face of transmembrane segment 5. Mutation of Glu-196 to the neutral, polar amino acid glutamine had no apparent effect on exchanger activity. In keeping with this result, the glutamate at position 196 of NCX1 is not conserved among the other exchangers. In NCX2 there is a glutamine, and in CalX there is a leucine at the equivalent position.

Unlike residue Glu-196, Glu-199 appears to be of functional importance. Glu-199 is conserved among the three NCX-type exchangers, and the analogous glutamate in the Na$^+$,K$^+$, and Ca$^{2+}$ pumps is involved in ion binding and translocation (30, 31). Conservative mutations at Glu-199 to either glutamine or aspartate resulted in non-functional exchangers.

In the Na$^+$,K$^+$, and Ca$^{2+}$ pumps the glycine residue, which is analogous to Gly-200 of NCX1, has been shown to be functionally important. However, in the exchangers, glycine is not conserved at this position. In NCX2 and CalX, an alanine replaces glycine. Mutation of Gly-200 in NCX1 to an alanine results in a functionally active exchanger as determined by $^{45}\text{Ca}^{2+}$ influx measurements. Thus residue Gly-200 does not appear to have an essential role in exchanger function. More subtle effects of the mutation might be observable upon analysis of the G200A mutant with the giant excised patch technique or by a less conservative amino acid substitution.

Residue Thr-203 is conserved in all the NCX-type exchang-
ers but the analogous residues in Na\textsuperscript{+},K\textsuperscript{+} and Ca\textsuperscript{2+} pumps are both alanines. Mutation of Thr-203 to a valine results in an inactive exchanger.

In summary, mutations in the pump-like region of the exchanger suggest that this part of the exchanger is also important in transport function.

Acidic and Basic Residues—Three of the eight acidic residues which have been modeled to be in transmembrane segments are in the \( \omega \)-repeats (Glu-113, Glu-120, and Asp-814). Residues Glu-113 and Asp-814 are conserved among all of the exchangers, while residue Glu-120 is conserved in all except NCX1 where the homologous residue is a glycine. Mutations at conserved residues Glu-113 and Asp-814 eliminate exchanger activity, while mutation at residue Glu-120 does not appear to affect exchanger activity. Likewise for the two acidic residues in the Na,K pump-like region, Glu-196 and Glu-199, Glu-196 is conserved neither among the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers nor the pumps and can be mutated without affecting exchanger activity, while mutation at residue Glu-196, which is conserved among the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers and pumps, eliminates exchanger activity.

The three remaining acidic residues which have been modeled to be in transmembrane segments are Asp-740, Asp-785, and Asp-798. Asp-740 is modeled to be near the cytoplasmic surface of transmembrane segment 6. Asp-740 is not conserved among exchangers; in CalX the homologous residue is a serine. Asp-740 can be mutated to an asparagine with no apparent effect on exchanger activity. Asp-798 is modeled here to be in cytoplasmic loop 8 though previously (13) was modeled to be intramembrane. While Asp-798 is conserved in all the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers, mutation of the residue to asparagine does not decrease exchanger activity.

Asp-785 is modeled to be near the cytoplasmic surface of transmembrane segment 7. Asp-785 is also conserved among all the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers. Mutation to glutamate reduces the exchanger activity to 39% of wild-type activity, and mutation to asparagine reduces the activity further to 11% of wild-type activity. These results suggest that Asp-785 is involved in some aspect of exchanger function.

We mutated five of the six basic residues which have been modeled to be in transmembrane segments. Arg-42 is modeled to be in transmembrane segment 1, and His-744 and Lys-751 are modeled to be in transmembrane segment 6. Lys-797 and His-858 were initially modeled to be in transmembrane segment 8 but, in the model used here, have been moved to intracellular loop 8. All five residues are conserved among the NCX-type exchangers except His-858. In NCX2, a tryptophan substitutes for the histidine. Mutation of His-858 to tryptophan in NCX1 results in an exchanger that exhibits wild-type levels of activity. The other basic residue mutations that have been constructed are R42Q, H744W, K751Q, and K797Q. All exhibit wild-type levels of activity. It thus appears that none of these basic residues play a role in exchanger function.

Electrophysiological Analysis of the Exchangers—A few of the mutant exchangers were studied electrophysiologically in giant excised patches. One particularly interesting group of mutants was G138A, its \( \omega \)-2 repeat homolog, G837A, and a double mutant construct containing G138A, and G837A. For these mutants the current-voltage (IV) relationship was altered relative to the wild-type exchanger (Fig. 7). For the wild-type exchanger, the IV relationship is nearly linear. The IV relationships for each of the mutants, on the other hand, are much more curved, especially at more positive potentials.

Models of the exchanger reaction mechanism place the bulk of the voltage dependence in the Na\textsuperscript{+} translocation steps (26), particularly at the extracellular side of the translocation path-way. Significantly, residues Gly-138 and Gly-837, which play a role in the voltage dependence of the exchanger, are modeled to be near to the extracellular surface of the exchanger. Perhaps mutation of these residues alters the Na\textsuperscript{+} binding or occlusion process at the extracellular surface. More detailed mutational analysis of this region should be informative.

Conclusions—Keeping in mind the caveat that mutations may result in secondary conformational changes, which can alter function, the \( \omega \)-repeat regions of the exchanger appear to be important in the ion transport mechanism. These regions are highly conserved among different exchangers and between one another. The \( \omega \)-repeat regions are very sensitive to mutagenesis; mutations at 18 of 19 residues resulted in protein with decreased or no activity. Mutation at two residues in the \( \omega \)-repeat regions altered the IV relationship.

The region of similarity to the Na\textsuperscript{+},K\textsuperscript{+} pump also appears to be involved in exchanger function. Mutation at two highly conserved amino acids in this region results in loss of activity.

Acidic residues appear to be important in exchanger function when located in the \( \omega \)-repeat or Na\textsuperscript{+},K\textsuperscript{+} pump-like region. Mutation of only one acidic residue outside of the \( \omega \)-repeat and Na\textsuperscript{+},K\textsuperscript{+} pump regions resulted in altered activity following mutation. None of the basic residues appeared to be involved in exchanger function.

Strikingly, exchanger activity was especially sensitive to mutation of hydroxyl-containing residues. Mutants at only 2 of the 10 hydroxyl-containing residues examined displayed activity. Thus, the exchanger ion conduction pathway is likely to be lined with acidic- and hydroxyl-containing residues, many of which are in the \( \omega \)-repeat regions. We suggest that at least a portion of the ion translocation pathway is lined with residues from transmembrane segments 2, 3, 8, and 9.

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