Mutational Analysis of the α-1 Repeat of the Cardiac Na\(^+\)-Ca\(^{2+}\) Exchanger*

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The Na\(^+\)-Ca\(^{2+}\) exchanger contains internal regions of sequence homology known as the α repeats. The first region (α-1 repeat) includes parts of transmembrane segments (TMSs) 2 and 3 and a linker modeled to be a reentrant loop. To determine the involvement of the reentrant loop and TMS 3 portions of the α-1 repeat in exchanger function, we generated a series of mutants and examined ion binding and transport and regulatory properties. Mutations in the reentrant loop did not substantially modify transport properties of the exchanger though the Hill coefficient for Na\(^+\) and the rate of Na\(^+\)-dependent inactivation were decreased. Mutations in TMS 3 had more striking effects on exchanger activity. Of mutations at 10 positions, 3 behaved like the wild-type exchanger (V137C, A141C, M144C). Mutants at two other positions expressed no activity (Ser139) or very low activity (Gly138). Six different mutations were made at position 143; only N143D was active, and it displayed wild-type characteristics. The highly specific requirement for an asparagine or aspartate residue at this position may indicate a key role for Asn\(^{143}\) in the transport mechanism. Mutations at residues Ala\(^{140}\) and Ile\(^{147}\) decreased affinity for intracellular Na\(^+\), whereas mutations at Phe\(^{145}\) increased Na\(^+\) affinity. The cooperativity of Na\(^+\) binding was also altered. In no case was Ca\(^{2+}\) affinity changed. TMS 3 may form part of a site that binds Na\(^+\) but not Ca\(^{2+}\). We conclude that TMS 3 is involved in Na\(^+\) binding and transport, but previously proposed roles for the reentrant loop need to be reevaluated.

Na\(^+\)-Ca\(^{2+}\) exchangers are found in a wide variety of tissues. These plasma membrane proteins are electrogenic transporters that utilize the electrochemical gradient of Na\(^+\) to exchange three extracellular Na\(^+\) ions for one intracellular Ca\(^{2+}\). As a Ca\(^{2+}\) efflux mechanism, the exchanger helps maintain intracellular Ca\(^{2+}\) homeostasis. Cloning of the Na\(^+\)-Ca\(^{2+}\) exchanger revealed a protein composed of 938 amino acids organized in nine membrane-spanning regions with the first five transmembrane segments (TMSs)\(^{1}\) separated from the four C-terminal TMSs by a large cytoplasmic loop (2–5).

Much progress has been made in understanding exchanger topology, function, and regulation. Modulation by intracellular factors such as Na\(^+\) and Ca\(^{2+}\) ions, ATP, and PIP\(_2\), have been extensively studied, and the domains involved in regulation have been localized to the large cytoplasmic loop (6). Less is known about the regions of the Na\(^+\)-Ca\(^{2+}\) exchanger involved in the translocation of Na\(^+\) and Ca\(^{2+}\). Two regions with similar sequences, called the α-1 and α-2 repeats, likely have key roles. The α-1 repeat consists of residues spanning TMSs 2 and 3 and an extracellular loop connecting the two TMSs. The homologous α-2 repeat encompasses TMS 7 and part of the following intracellular loop (Fig. 1). Several observations emphasize the functional importance of the α repeats. First, they are highly conserved among all Na\(^+\)-Ca\(^{2+}\) exchangers and are the only regions of sequence conservation between members of the NCX and NCKX families (7). Second, data suggest that the portions of the α repeats connecting TMSs 2–3 and TMS 7–8 form reentrant membrane loops (Fig. 1 and Ref. 2). Reentrant loops have been identified in ion channels ("P-Loops"), and in the aquaporin family of proteins and form a portion of the hydrophilic pathway for ions or other molecules to cross the membrane (8, 9). By analogy, the reentrant loops within the α repeats may be involved in the translocation pathway for Na\(^+\) and Ca\(^{2+}\). This hypothesis is supported by helix packing studies, which demonstrate that the two α repeats are in proximity (10). In addition, site-directed mutagenesis reveals that mutations in the α repeats drastically alter the transport of Na\(^+\) and Ca\(^{2+}\) (4, 11, 12), supporting a role in ion translocation.

The aim of this study was to further define the role of the α-1 repeat in ion translocation by combining mutational and biophysical analysis. Mutations were made in the first reentrant loop and in TMS 3. We show that mutations within the reentrant loop do not cause major perturbations in ion translocation; all mutations lead to active exchangers with little alteration in ion affinities or regulatory properties. These data seem to exclude the reentrant loop of the α-1 repeat as a key component of ion translocation. In contrast, residues within TMS 3 are important in determining Na\(^+\) affinity. Mutations of residues Ala\(^{140}\), Phe\(^{145}\), and Ile\(^{147}\) drastically alter the apparent affinity for Na\(^+\), without affecting the binding of transported Ca\(^{2+}\). Thus, TMS 3 is important in determining the ion dependence of the Na\(^+\)-Ca\(^{2+}\) exchanger.

EXPERIMENTAL PROCEDURES

Molecular Biology and 45Ca\(^{2+}\) Uptake Measurements—Mutations were generated in 200–400-bp cassettes from the full-length exchanger using QuikChange Mutagenesis (Stratagene). Cassettes were then se-

MES, 4-morpholinethanesulfonic acid; PIP\(_2\), phosphatidylinositol phosphate; TEAOH, tetraethylammonium hydroxide.

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The abbreviations used are: TMSs, transmembrane segments;
quenced and subcloned back into the full-length exchanger. RNA was synthesized using T3 mMessage mMachine (Ambion) after linearization with HindIII. RNA was injected into Xenopus oocytes and 45Ca2+ uptake measured as described previously (13). To measure extracellular Ca2+ dependence, mutants were transiently expressed in BHK or HEK cells, and 45Ca2+ uptake was measured at different [Ca2+]. Data were fit to the Michaelis-Menten equation using GraphPad Prism version 4.0 for Macintosh.

The dog/squid NCX chimera was generated by a combination of mutagenesis and subcloning. The squid exchanger has a BamHI site at TMSs 2 and 3.

**FIG. 1.** Membrane topology of the Na+-Ca2+ exchanger. The top panel shows a schematic representation of Na+-Ca2+ exchanger topology. Numbers indicate transmembrane segments while dark areas mark the α repeats. The alignment in the bottom panel shows the sequence similarity of the α-1 repeat within diverse members of the Na+-Ca2+ exchanger family. Residues marked with an asterisk were individually mutated and analyzed. The symbol † indicates that mutation of Ser 139 produced an inactive exchanger.

**RESULTS**

Electrophysiological Characterization of Reentrant Loop Mutants—We generated point mutants H124N, N125C, F126C, T127H, and D130C of the first reentrant loop of the Na+-Ca2+ exchanger and investigated biophysical properties. Mutants were expressed in Xenopus oocytes and analyzed by both 45Ca2+ uptake assays and by electrophysiology using the giant excised patch technique. In a typical excised patch experiment, Ca2+ is within the patch pipette at the extracellular surface. An outward Na+-Ca2+ exchange current is activated by the rapid application of Na+ to the intracellular surface. In this configuration, the excised patch technique allows assessment of two different regulatory properties (15). First, although Na+ provides substrate to activate transport, Na+ at the intracellular surface also induces a slow inactivation process known as $I_1$ (e.g., see Fig. 4 and Ref. 16). Second, intracellular Ca2+ binds to a regulatory site on the large intracellular loop to activate transport function (17, 18). To measure apparent ion affinities at transport sites without confounding effects of...

Exchanger inward currents were measured using the following solutions: pipette solution (mm): 100 NaOH, 10 HEPES, 20 TEAOH, 0.2 niflumic acid, 0.2 ouabain, 10 EGTA, 2 Mg(OH)2, pH 7 (using MES); bath solution (mm): 100 CsOH, 20 TEAOH, 10 HEPES, 10 EGTA, 0–10 Ca(OH)2, pH 7 (using MES). Free calcium concentrations were calculated according to the MAXc program.

Na+ and Ca2+ activation curves were obtained by perfusing solutions with different ion concentrations. Currents were fitted to a Hill function with different ion concentrations. Currents were fitted to a Hill function and normalized to extrapolated maximum values. Values are mean ± S.E. Values were considered significantly different at a level of p < 0.05.

pCLAMP (Axon Instruments, Burlingame, CA) software was used for acquisition and analysis. Data were acquired on-line at 4 ms/point and filtered at 50 Hz using an 8-pole Bessel filter. Experiments were performed at 35°C and at a holding potential of 0 mV.
these regulatory processes, the exchanger can be first “deregulated” by treatment with chymotrypsin at the intracellular surface.

We first determined apparent affinities for Na\(^+\)/H\(^+\), measured at the cytoplasmic surface of deregulated mutants. Our data (see Figs. 2, left panels, and 8) show that the concentration of Na\(^+\) needed to activate 50% of the mutated exchangers slightly decreased in H124N and D130C and increased for mutant F126C. Replacement of Asn\(^{125}\) did not produce any apparent effect. Exchangers H124N, F126C, T127H, and D130C also showed a significant decrease in the Hill coefficient with the effects being most substantial for H124N and F126C (Fig. 8). The shallower course of the Na\(^+\)/H\(^+\) dependence for H124N and F126C allows these mutants to transport more efficiently at lower Na\(^+\)/H\(^+\) concentration than the wild type. For example, Fig. 2 shows that at 5 mM intracellular Na\(^+\)/H\(^+\), H124N and wild-type exchangers had about 23 and 6% of maximal activities, respectively.
To determine the affinity of the mutated exchangers for transported Ca\(^{2+}\), inward exchange currents were generated by application of different cytoplasmic Ca\(^{2+}\) concentrations with 100 mM extracellular Na\(^{+}\) within the pipette. We found no changes in the apparent Ca\(^{2+}\) affinity values for any reentrant loop mutant. However, F126C showed a shallower course of the Ca\(^{2+}\) dependence curve (Figs. 2 and 8).

Positions His\(^{124}\), Asn\(^{125}\), and Asp\(^{130}\) are of particular interest since a previous study showed that replacement of each of these residues with a cysteine decreased the apparent affinity of the external binding site for transported Ca\(^{2+}\) (4). In our hands, however, neither the N125C nor the D130C exchangers had altered apparent affinities for external Ca\(^{2+}\). The activities of N125C, D130C, and wild-type exchangers were measured at different extracellular Ca\(^{2+}\) concentrations as Na\(^{+}\) gradient-dependent \(^{45}\text{Ca}^{2+}\) uptake into intact cells. N125C and D130C behaved similarly to wild type (Fig. 3). This is consistent with the electrophysiological measurements on the apparent affinities for intracellular Ca\(^{2+}\).

We also examined the Na\(^{+}\)-dependent inactivation, \(I_1\), of the mutated exchangers (Fig. 4). Mutations H124N and D130C substantially slowed the time constant (\(\tau\)) of Na\(^{+}\)-dependent inactivation. Current decays were fitted to a single exponential to calculate time constants of 8.8 \(\pm\) 0.7 \(\mu\)s for H124N, 5.5 \(\pm\) 1.1 \(\mu\)s for D130C and 3.3 \(\pm\) 0.2 \(\mu\)s for the wild-type exchanger. Preliminary experiments also indicate that the mutant exchanger F126C had slowed inactivation (~5 \(\mu\)s, \(n = 2\)), while mutations at position 125 (\(n = 2\)) and 127 (\(n = 2\)) caused no change in the inactivation rate. We have previously reported on the inactivation of mutant H124N (3).

Overall, our results indicate that residues forming the putative reentrant loop connecting TMSs 2 and 3 do not set cytoplasmic affinities for transported ions but can alter the cooperativity of Na\(^{+}\) binding and inactivation rates.

**Functional Activity of the Squid Chimera Exchanger**—As shown in Fig. 1, the portion of the α-1 repeat that invaginates the membrane is highly conserved in the three mammalian Na\(^{+}\)-Ca\(^{2+}\) exchangers (NCX1, NCX2, and NCX3) but diverges in non-mammalian species such as the squid neuronal exchanger (NCX-SQ1). NCX-SQ1 has the following non-conservative changes: C122V, H124Q, N125K, T127E, and D130Q. Interestingly, NCX-SQ1 has biophysical properties similar to the mammalian NCX1 (19). If the reentrant loop is involved in ion transport, then perhaps other parts of the NCX-SQ1 protein compensate for the amino acid divergence within the reentrant loop. To explore this possibility, we replaced the α-1 reentrant loop of the mammalian Na\(^{+}\)-Ca\(^{2+}\) exchanger with that from NCX-SQ1 (squid chimera).

Previously we have shown that the outward current from the squid chimeric exchanger possesses both Na\(^{+}\)- and Ca\(^{2+}\)-dependent inactivation processes (3). We now performed kinetic analysis of the \(I_1\) regulatory properties of the squid chimera. At a cytoplasmic Ca\(^{2+}\) concentration of 1 \(\mu\)M, the time constant for inactivation of squid chimera exchangers was modestly increased as compared with the wild-type cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger (4.1 \(\pm\) 0.2 s, \(n = 3\) versus 3.3 \(\pm\) 0.2 s, \(n = 23\)); Fig. 4). The affinities of the squid chimera

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**Fig. 3.** Exchangers N125C, D130C and wild type have similar apparent affinity for external Ca\(^{2+}\). Panel A shows averaged Na\(^{+}\) gradient-dependent \(^{45}\text{Ca}^{2+}\) uptake for each exchanger. Data were normalized and fit to the Michaelis-Menten equation. In panel B, the \(K_{DP}\) is plotted for each mutant. The number of experiments is indicated in each bar.

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**Fig. 4.** Time constants for Na\(^{+}\)-dependent inactivation of wild-type and Na\(^{+}\)-Ca\(^{2+}\) exchanger mutants. Shown are the time constant (\(\tau\)) values of current decay due to the presence of high intracellular Na\(^{+}\) (Na\(^{+}\)-dependent inactivation) for wild type exchanger and mutants H124N, D130C, and the squid chimera. Typical traces recorded from each indicated construct are also shown. Currents were measured in the presence of 100 mM cytoplasmic Na\(^{+}\) and 1 \(\mu\)M cytoplasmic Ca\(^{2+}\).
exchanger for transported ions (measured at the cytoplasmic surface) were determined after the removal of the Na\(^+\) and Ca\(^{2+}\)-dependent inactivation processes (I\(_1\) and I\(_2\)) using chymotrypsin. As shown in Figs. 5A and 8, the squid chimera displayed a slight but significant increase in Na\(^+\)/H\(^+\) motrypsin. As shown in Figs. 5A and 8, the squid chimera displayed a slight but significant increase in Na\(^+\)/H\(^+\) motrypsin.

The fit of the Na\(^+\) dependence curves to a Hill function yielded apparent affinities values of 16.6 \pm 1.2 mM (n = 6) for the wild type and 13.4 \pm 0.7 mM (n = 6) for the squid chimera, while inward currents (B) were obtained with 100 mM Na\(^+\) in the pipette and varying cytoplasmic Na\(^+\), while inward currents (B) were obtained with 100 mM Na\(^+\) in the pipette and varying cytoplasmic Na\(^+\), while inward currents (B) were obtained with 100 mM Na\(^+\) in the pipette and varying cytoplasmic Na\(^+\), while inward currents (B) were obtained with 100 mM Na\(^+\) in the pipette and varying cytoplasmic Na\(^+\). The Ca\(^{2+}\) concentration dependence curves for the transport site on the cytoplasmic surface was also investigated and no significant differences were found between the canine cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (wild type) and the exchanger containing residues 122 to 130 of the squid isoform. Panel B of Fig. 5 shows examples of inward currents and the corresponding Ca\(^{2+}\) concentration response curve (see Fig. 8 for values).

The results indicate that replacement of the extracellular portion of the \(\alpha\)-1 repeat does not cause major perturbations of Na\(^+\) or Ca\(^{2+}\) transport. There were minor effects on the apparent Na\(^+\) affinity and more substantial effects on the cooperativity of Na\(^+\) binding.

**Biophysical Properties of Cardiac Na\(^+\)/Ca\(^{2+}\) Exchangers Mutated within TMS 3**—We made point mutations at residues 137–145 and 147. Effects of some mutations at three of the residues (Gly\(^{138}\), Ser\(^{139}\), and Asn\(^{143}\)) have been reported previously (11). Conservative mutations of Gly\(^{138}\) to an alanine, serine, or cysteine have resulted in low activity and were not reexamined here. Mutants S139A and N143V are inactive (11), and we examined the effects of additional mutations at position Asn\(^{143}\). Replacement of Asn\(^{143}\) with a cysteine, alanine, glutamate, or serine also all produced inactive exchangers. Surprisingly, however, the mutant N143D produced an exchanger with normal Na\(^+\) affinity and activity level (Fig. 6). The N143D exchanger also displayed normal regulatory properties. Both Na\(^+\) - and Ca\(^{2+}\)-dependent inactivations are demonstrated in Fig. 6C. Na\(^+\)-dependent inactivation is removed by high intracellular Ca\(^{2+}\), and activity disappears upon the removal of regulatory Ca\(^{2+}\) as also occurs with the wild-type exchanger.

Of the remaining residues mutated, three (V137C, A141C, and M144C) displayed robust Na\(^+\)/Ca\(^{2+}\) exchange currents and had biophysical properties similar to wild type (Figs. 6A and 8). Mutations at positions Ala\(^{140}\), Phe\(^{145}\), and Ile\(^{147}\), however, displayed interesting characteristics.

Mutation of residues Ala\(^{140}\), Phe\(^{145}\), and Ile\(^{147}\) in the \(\alpha\)-1 region of TMS 3 caused substantial alterations in the apparent affinity for intracellular Na\(^+\). As shown in Fig. 7A, mutation of alanine at position 140 or isoleucine at position 147 decreased the Na\(^+\) affinity of the exchanger. In contrast, mutant exchanger F145C had an increased Na\(^+\) affinity. The apparent
Na⁺/H⁻ affinities were: 37.1 ± 2.6 mM (n = 5) for A140C, 9.9 ± 0.9 mM (n = 6) for F145C and 88.5 ± 2.3 mM (n = 4) for I147S. The value for the wild-type exchanger was 16.6 ± 1.2 mM (n = 6).

The extent of the decrease in the Na⁺ affinity caused by the replacement of alanine 140 correlated with the size of the residue inserted (Figs. 7B and 8). The smallest residue, glycine, yielded the highest affinity for Na⁺ (13.7 mM) and the largest, asparagine, yielded the lowest affinity (60.2 mM).

Replacement of phenylalanine 145 produced two effects: an increase in the apparent Na⁺ affinity and a decrease in the Hill coefficient for both Na⁺ and Ca²⁺. The increase in Na⁺ affinity was prominent in mutants F145C and F145L and did not correlate with either hydrophobicity or size of the introduced residue (see Figs. 7C and 8). The shift in the shape of the Na⁺ dependence curve was prominent in mutants F145C and F145S and may correlate with either hydrophobicity or size. Serine...
and cysteine are of comparable size and are smaller than leucine or phenylalanine and are also both less hydrophobic than leucine or phenylalanine. Thus, the slope of the Na⁺ dependence may be set by either the size or the hydrophobicity of the amino acid at position 145. A mutation at a nearby position, F145C, also had a decreased Hill coefficient for Na⁺ activation though, in this case, apparent Na⁺ affinity was unchanged (Fig. 8).

Fig. 8. Properties of the mutated Na⁺-Ca²⁺ exchangers. Values are given as mean ± S.E. Numbers of experiments are indicated inside bars. Values statistically different from wild type are marked with an asterisk (p < 0.05).

We also investigated the response of mutant exchangers A140C, F145C, and I147S to cytoplasmic Ca²⁺ (Fig. 7D). We observed no significant shift in the apparent affinities for Ca²⁺. However, mutation of phenylalanine 145 to cysteine modestly reduced the Hill coefficient for the binding of cytoplasmic Ca²⁺; Hill coefficients were 1.3 ± 0.1 and 1.1 ± 0.1 (n = 5) for wild-type and F145C exchangers, respectively.
The functional importance of TMS 2 and the linker between TMS 2 and 3 has been extensively investigated through mutagenesis studies (2, 4, 5, 11, 13) with results emphasizing the critical role of TMS 2 in ion translocation. In contrast, TMS 3 has received relatively little attention. Nicoll et al. (11) showed that exchange activity is highly sensitive to mutation of Gly^{138}, Ser^{139}, or Asn^{143} within TMS 3. Also, recent data place this portion of the exchanger in contact with TMS 2, 7, and 8 to form a three-dimensional structure that may define a pathway for ion movement (10). These data and the high conservation of this region suggest an involvement in the movement of ions. To examine whether TMS 3 plays a role in ion selectivity and transport, we investigated the effects of mutations within TMS 3. We mutated each of the residues belonging to the α repeat of TMS 3 and measured the effects of these mutations on Na\(^+\) and Ca\(^{2+}\) translocation. Several mutations produced prominent effects. First, as reported previously (11), mutants G138A and G138S have low activity and S139A has no activity. Of the active mutant exchangers, replacement of alanine 140, phenylalanine 145, and isoleucine 147 produced the most prominent changes in the biophysical properties of the Na\(^+\)-Ca\(^{2+}\) exchanger. Mutations at these sites altered the apparent affinity of the exchanger for Na\(^+\), with the most prominent effect at position 147. The effects of mutations at position 140 may be sensitive to the size of the replacement residue. Substitution with a large amino acid (asparagine) produced the largest decrease in the Na\(^+\) dependence, while insertion of a small residue (glycine) did not modify transport. There is no simple pattern to explain the effects of mutations at position 145.

Since Ca\(^{2+}\) and Na\(^+\) are thought to bind at the same sites, mutations that alter Na\(^+\) dependence might also be expected to modify the apparent affinity for transported Ca\(^{2+}\). However, the apparent affinity for Ca\(^{2+}\) of mutant exchangers A140C, F145C, and I147S was not significantly different from that measured for the wild type exchanger. It has previously been proposed (26, 27) that the Na\(^+\)-Ca\(^{2+}\) exchanger has two classes of cation binding sites. One class of sites binds either Ca\(^{2+}\) or two Na\(^+\) and the second class binds one Na\(^+\). Residues 140, 145, and 147 may be involved in the second class of sites, and thus mutations affect Na\(^+\) but not Ca\(^{2+}\), affinity. There is little previous information on the location of Na\(^+\) binding sites within the exchanger. The only TMS mutation previously reported to alter Na\(^+\) affinity is T103V, near the intracellular surface of TMS2 (13). In this case, the cooperativity of Na\(^+\) transport was also reduced.

Particular attention must be given to the asparagine at position 143 in TMS 3. Replacement of this residue with an alanine, cysteine, serine, valine, or glutamate residue totally abolished exchange activity. However, replacement of Asn^{143} with an aspartate residue produced an exchanger indistin-
guishable from wild type. It is quite striking that introduction of an aspartate leaves properties unaltered while introduction of any of five other residues abolishes activity. The sensitivity of this position to mutations may indicate the direct involvement of this residue in ion transport and also indicates a strict requirement for specific amino acids (asparagine or aspartate) at this site. Aspartate, asparagine, and valine are all of similar size but only aspartate and asparagine are hydrophilic. Thus, not only size but also degree of hydrophilicity at this position is essential in maintaining exchanger activity. It may seem surprising that introduction of a negatively charged aspartate residue into the center of a transmembrane segment does not disrupt transport function. However, in a hydrophobic environment this aspartate may remain in an undissociated uncharged state.

The three Na\(^+\)-Ca\(^{2+}\) exchanger mutations in TMS 3 that do not perturb wild type properties (A141C, M144C, and V137A) can all be modeled to be on the same face of a transmembrane \(\alpha\)-helix. All the remaining mutations that alter exchanger function are excluded from this face. These sites may face TMSs 2, 7, and 8 that have been modeled to interact with TMS 3 and form an ion translocation pathway (10).

Taken together, our data indicate that residues forming the first reentrant membrane loop of the Na\(^+\)-Ca\(^{2+}\) exchanger do not have a key role in ion transport but could be involved in movements associated with Na\(^+\)-dependent inactivation and in cooperativity between Na\(^+\) binding sites. In contrast, at least six amino acid residues within the extracellular half of TMS 3 are important components of exchanger function and some may be involved in the binding of Na\(^+\). The Na\(^+\) binding site associated with TMS 3 may be a unique site that does not participate in Ca\(^{2+}\) binding. The data confirm the importance of TMS 3 and the \(\alpha\)-1 repeat in exchanger function.

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