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Acid Residues in the Transmembrane Helices of the Na^+-Pumping NADH:Quinone Oxidoreductase from *Vibrio cholerae* Involved in Sodium Translocation[†]

Oscar Juárez, Kathleen Athearn, Portia Gillespie, and Blanca Barquera*

Department of Biology and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180

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ABSTRACT: *Vibrio cholerae* and many other marine and pathogenic bacteria possess a unique respiratory complex, the Na^+-pumping NADH:quinone oxidoreductase (Na^+-NQR), which pumps Na^+ across the cell membrane using the energy released by the redox reaction between NADH and ubiquinone. To function as a selective sodium pump, Na^+-NQR must contain structures that (1) allow the sodium ion to pass through the hydrophobic core of the membrane and (2) provide cation specificity to the translocation system. In other sodium-transporting proteins, the structures that carry out these roles frequently include aspartate and glutamate residues. The negative charge of these residues facilitates binding and translocation of sodium. In this study, we have analyzed mutants of acid residues located in the transmembrane helices of subunits B, D, and E of Na^+-NQR. The results are consistent with the participation of seven of these residues in the translocation process of sodium. Mutations at NqrB-D397, NqrD-D133, and NqrE-E95 produced a decrease of approximately ≥10-fold in the apparent affinity of the enzyme for sodium (Km_{app}^{Na+}), which suggests that these residues may form part of a sodium-binding site. Mutation at other residues, including NqrB-E28, NqrB-E144, NqrB-E346, and NqrD-D88, had a strong effect on the quinone reductase activity of the enzyme and its sodium sensitivity, but a weaker effect on the apparent sodium affinity, consistent with a possible role in sodium conductance pathways.

The sodium-pumping NADH:quinone oxidoreductase (Na^+-NQR)[1] is a unique prokaryotic respiratory enzyme capable of sustaining a sodium gradient across the plasma membrane, using the free energy released in the coupled oxidation of NADH and reduction of ubiquinone (*1*−*3*). Na^+-NQR is composed of six subunits (NqrA−F) and contains five cofactors involved in the internal electron transfer: a noncovalently bound FAD and a 2Fe-2S center located in NqrF (*4*−*8*), two covalently bound FMNs in NqrB and NqrC (*9*−*11*), which have been shown to give rise to two anionic flavosemiquinone radicals, observed in the partially and fully reduced forms of the enzyme (*12*), and a noncovalently bound riboflavin molecule that is found as a stable neutral flavosemiquinone radical in the oxidized state of the enzyme (*13*, *14*). This is notable because it is the only known instance in which riboflavin is present as a bona fide redox cofactor in any enzyme.

Topological analysis of the five membrane-bound subunits of Na^+-NQR from *Vibrio cholerae* (NqrB−F) has revealed the presence of 17 acid residues in the membrane-spanning segments of subunits B, D, and E (*15*). Most of these acid residues are conserved across many orthologs of the Na^+-NQR family, and eight of these are also conserved in the related protein RNF, which has recently been proposed to be a redox-driven sodium pump (*16*).

Ion transport by channels and pumps has at least two structural requirements. First, a substrate-binding site must provide a physiologically relevant affinity and specificity for transport. Second, a solute passageway must allow the movement of ions through the hydrophobic core of the membrane. If the substrate-binding site is deeply buried in the membrane, passageways will be needed to connect the binding site to the two opposite sides of the membrane. Additionally, enzymes that function as ion pumps must be able to use an energy source to drive transport, and a gating mechanism must be in place to ensure the unidirectional movement of ions.

The occurrence of charged residues is unusual in hydrophobic environments of proteins and represents only 1−3% of the amino acid composition of transmembrane helices. This presumably reflects the large energy cost of introducing a charge into the hydrophobic environment. Thus, the presence of charged residues in the membrane indicates an important role in protein function. In fact, these residues often form strong structural interactions between transmembrane segments, such as intramolecular helix−helix contacts or dimer associations (*17*). Alternatively, they may be parts of binding sites or pathways involved in the movement of solutes through the membrane, in ion pumps, or channels (*18*). In this respect, it has been proposed that these residues "prepay the Born energy" for the incorporation of ions into the membrane, which facilitates the transport process (*18*).

The identification of the sodium-binding sites in proteins has been challenging, mainly because the different families of sodium

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*To whom correspondence should be addressed: Room 2239, Center for Biotechnology and Interdisciplinary Studies, 110 Eight St., Troy, NY 12180. Phone: (518) 276-3861. Fax: (518) 276-2851. E-mail: barqub@rpi.edu.

[1] Abbreviations: BCA, bicinchonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LB, Luria-Bertani; Na^+-NQR, Na^+-pumping NADH:quinone oxidoreductase; RNF, *Rhodobacter* nitrogen-fixing protein; Tris, tris(hydroxymethyl)aminomethane.

Table 1: Primers Designed for the Site-Directed Mutagenesis Used in This Work

subunit	mutation	primer sequence[a] (5′ → 3′)
B	E28A	GTGGTTTGCCCTGTAT**GCC**GCGGCGGCGACGCTG
	E144L	CTATCTACGCTACGGTGTTCATCGTCGGTGGTTTCTGG**CTC**GTGTTGTTCTGTATGGTGCGCAAGCATGAAGTCAAC
	D223A	GCACAGATCTCAGGA**GCT**CTAGTATGGACTGCGG
	E274A	GGTAACATCCCAGGTTCAATTGGC**GCC**GTGTCTACTCTGGCACTCATGATTG
	D346A	GGTATGTTCTTCATGGCGACG**GCG**CCATGGTCTGCGTCCTTCACC
	E380A	GTGAACCCGGCTTACCCA**GCC**GGCATGATGCTGGCGATC
	D397A	GCGAACCTATTTGCGCCACTGTTT**GCG**CATGTGGTTGTAGAGAAATATCAAG
D	D17A	GAGTGTGTTAGCCCCAGTGCTA**GCC**AACAACCCGATTGCACTGC
	E39L	GCGGTAACCACTAAG**CTG**TTAACGGCATTTGTTATGACGCTA
	D87L	TCGCTTCGTTAGTTATCGTGGTA**CTG**CAGATTCTGAAAGCGTATCTG
	E119A	CGTAATGGGTCGTGCC**GCG**GCGTTTGCAATGAAGTCTG
	D133A	TCTGAACCAATTCCTTCTTTCATT**GCC**GGCATTGGTAACGGTTTAGGA
	E153A	CTGATGACGGTTGGTTTCTTCCGA**GCT**CTTTTAGGCTCAGGTAAG
E	E15A	CTGCTGGTGAAATCGATTTTCATC**GCT**AACATGGCACTAAGCTTCTTCCTAGGG
	E95A	GCGGCATTATGACAGATTCTG**GCT**ATGATCCTCGATCGC
	E138A	CAGCGCGACTACAGCTTTGCT**GCT**AGCGTGGTATACGGTTTCGGTTC
	E162G	GTCGCTCTTGCAGGTATCCGC**GGT**AAGATGAAGTATTCAGAC

[a] The underlined bases indicate the sites that were modified to introduce the mutations.

transport proteins do not share conserved motifs. In many cases, the sodium-binding residues are separated by segments of variable length in the primary sequence. In some cases, carbonyl oxygens of the peptide backbone provide the ligands for sodium binding, which allows much more sequence variability than if the binding sites are in amino acid side chains. Moreover, water molecules can be ligands for sodium (*19*). The recognition of sodium-binding sites is not trivial, even with the availability of crystallographic data, because of the similar characteristics of sodium atoms and water molecules. Nayal and Di Cera (*20*) studied this question by means of valence calculations on protein crystallographic structures and proposed an algorithm to distinguish bound sodium ions from internal water molecules. They concluded that an optimal sodium-binding site consists of six oxygen atoms arranged in an octahedral geometry.

We have characterized mutants in which conserved glutamate and aspartate residues located in transmembrane helices of *V. cholerae* Na^+-NQR were substituted with nonpolar residues (A, G, or L). Mutation of some of these residues led to significant decreases in the sodium-dependent quinone reductase activity (Q_{red}), and thus in the coupled sodium pumping activity. We have identified seven acid residues, which appear to be of special importance for sodium translocation by the enzyme. Three of these mutations led to large decreases in the apparent affinity for sodium ($Km_{app}^{Na^+}$), consistent with a role as components of a sodium-binding site, although we cannot rule out the possibility that some of these mutations exert their effects indirectly through structural perturbation of the enzyme.

EXPERIMENTAL PROCEDURES

Mutant Construction. Mutants were constructed with the Quikchange site-directed mutagenesis kit (Stratagene), using as a template the wild-type *nqr* operon from *V. cholerae* cloned into the pBAD vector (Invitrogen), as reported previously (*6*, *7*). Primers were designed to replace the acid residues with small nonpolar amino acids, such as A, G, or L, and to simultaneously incorporate new restriction sites to aid in identification of mutants. Generally, alanine residues were introduced, but in some cases, it was necessary to use glycine or leucine instead to create the restriction site. A list of the primers used in this study is found in Table 1. Mutations were verified by restriction digestion analysis and by direct sequencing of the plasmids. Mutant plasmids were introduced by electroporation into the Δnqr *V. cholerae* strain.

Cell Culture. Δnqr *V. cholerae* cells containing the wild-type or mutant *nqr* operon on the pBAD plasmid were grown at 37 °C in LB medium in New Brunswick BioFlo-5000 fermentors under constant aeration and agitation. Arabinose was added as the inducer of expression, as described previously (*6*, *7*).

Protein Purification. Protein purification was performed using Ni-NTA (Qiagen) affinity chromatography (*6*), and protein concentrations were measured with the BCA assay (Pierce). Flavin content was measured spectrophotometrically at 450 nm ($\varepsilon_{FAD} = 12.1$ mM^{-1} cm^{-1}) in 6 M guanidine hydrochloride (*6*). In all of the cases, the flavin/protein molar ratio varied from 3.1 to 3.6, consistent with the presence of four flavin molecules in the enzyme. This argues against the possibility that some of the acid residue mutations affect the binding of flavin cofactors.

Activity Measurements. Specific activities were measured spectrophotometrically as reported previously (*6*). Measurements were taken in buffer [50 mM Tris-HCl (pH 8), 1 mM EDTA, 5% (v/v) glycerol, and 0.05% (w/v) *n*-dodecyl *β*-maltoside] containing 150 *μ*M K_2-NADH and 50 *μ*M Q-1. The NADH dehydrogenase (NADH-DH) activity was measured at 340 nm ($\varepsilon_{NADH} = 6.22$ mM^{-1} cm^{-1}). The NADH-dependent quinone reductase activity (Q_{red}) was measured at 282 nm, using the molar absorptivities for quinol ($\varepsilon = 2.7$ mM^{-1} cm^{-1}) and quinone ($\varepsilon = 14.5$ mM^{-1} cm^{-1}) in an aqueous environment. The NADH oxidase (NADHox) activity was calculated as the difference between the NADH dehydrogenase and NADH-dependent quinone reductase activities, which presumably corresponds to the rate of electron branching to oxygen. In fact, under anaerobic conditions, the rate of the NADH oxidase reaction decreased by more than 95% for the wild-type enzyme (not shown).

Enzyme Reconstitution. Wild-type and mutant enzymes were reconstituted into liposomes following the general recommendations of Rigaud et al. (*21*). Two milligrams of purified enzyme was added to a total *Escherichia coli* phospholipid extract (15 mg/mL, Avanti lipids) in buffer containing 25 mM *n*-octyl glucoside (detergent/phospholipid molar ratio of 1.3), 100 mM HEPES, 150 mM KCl, 1 mM NaCl, and 1 mM EDTA (pH 7.5). The detergent was slowly removed via addition of small amounts

Table 2: Sequence Alignments of the Transmembrane Segments of Na^+-NQR Subunits B, D, and E, versus the Homologous Subunits of RNF[a]

Transmembrane helices	**NqrB *vs* RnfD**	**NqrD *vs* RnfE**	**NqrE *vs* RnfA**

I	RnfD ---------------MAFFIA
	NqrB KWFALYEAAATLFYTPGLVTK
	                     .:.
		RnfE MWNNNPALVQLLGLCPLLAV
		NqrD VLDNNPIALQVLGVCSALAV
		     : :*** :*:**:*. ***
			RnfA LVNNFVLVKFLGLCPFMGVSK
			NqrE FIENMALSFFLGMCTFLAVSK
			     :::*:.* ***:*.*:.*

II	RnfD VMRWVLACALPGLIAQTYFFG
	NqrB IMIMVWLAVFPAMFWGMYNAG
	     :* * ..:*.:: * *
		RnfE TNALGLGIATLLVLVGSNVTVS
		NqrD ETAFVMTLAVMFVTALSNFFVS
		     .*: : :*.:: * . **. **
			RnfA IGMGLATTFVLTLASVCAYLV
			NqrE FGLGIAVIVVLTISVPVNNLV
			     :*:*:*. .***:: **

III	RnfD AVAIPPLSPWWVVVIGLIFAI
	NqrB GATYFLPIYATVFIVGGFWEV
	     ..: *.::* :: :
		RnfE VRIPVFVMIIASLVTCVQ
		NqrD VRIIVQMAIIASLVIVVD
		     *** * : ****** *:
			RnfA LRTMSFILVIAVVVQFTEMVV
			NqrE LNFITFIGVIAALVQILEMIL
			     :*. ::** ***.:**: **::

IV	RnfD PFNPAMIAYVVLLISFPVQMT
	NqrB FFVTSILFALIVPPTLPLWQA
	      * .::: ::: ::*: :
		RnfE LGIFIPLIVTNCIIIGRAEAF
		NqrD LSVFVGLIITNCIVMGRAEAF
		     .:: **:****::******
			RnfA RLLGIFLPLITTNCAVLGVALL
			NqrE NALGIFLPLITVNCAIFGGVSF
			     . *********.***::* . :

V	RnfD IFGG------FDSDGLSLQQI
	NqrB ALAGRAFLFFAYPAQISGDLV
	      :.* . :* : :
		RnfE DFWMGLGMTSVLVVLGSLREI
		NqrD DIGNGLGYGFVLMTVGFFREL
		     : *** **:.:* :**::
			RnfA QSIIYGFGAAVGFSLVLILFASMRE
			NqrE ESVVYGFGSGVGWMLAIVALAGIRE
			     :*::****:.**: *.:: :*.:**

VI	RnfD WEWVNIAYLLGGLILLKLRII
	NqrB GEVSTLALMIGAAFIVYMGIA
	       * .:* ::*. ::: : *
		RnfE SAFLLALLPPGAFIGVGFLIAA
		NqrD QPNGLMLLAPSAFFLIGFMIWA
		     .. * **.*.**: :**:* *
			RnfA GASIAMITAGLMSLAFMGFT
			NqrE GLGITFITAGLMALGFMSFS
			     * .*::******:*.**.*:

VII	RnfD WHIPMAMLAGLVFTALLAQLF NqrB WRIIGGVMIGMILLSTLFNVI *:* .:: *::: : * :::		
VIII	RnfD -MIHLLSGATMLGAFFIATDP NqrB WHWHLVLGGFAFGMFFMATDP **: *. :* **:****		
IX	RnfD DGVAFAVLLANMCVPLIDYYT NqrB EGMMLAILFANLFAPLFDHVV :*: :*:*:**: .**:*: .		

[a] Conserved acidic residues are indicated in white on black. The transmembrane segments of each subunit were obtained with the algorithms used previously (*15*). In cases where a consensus transmembrane helix was not well-defined, all possible amino acids were included in the alignment, resulting in some helices appearing longer than others.

of SM2 Biobeads (Bio-Rad), following the protocol described by Verkhovskaya et al. (*22*).

Formation of Membrane Potential ($\Delta\Psi$). $\Delta\Psi$ was measured spectrophotometrically at 625 nm minus 587 nm in the preparations of enzyme reconstituted in proteoliposomes, using Oxonol VI (*23*, *24*). The reaction buffer contained 200 μM NADH, 100 μM Q-1, 100 mM NaCl, 100 mM HEPES, 150 mM KCl, and 1 mM EDTA (pH 7.5). Calibration of the spectrophotometric signal of Oxonol VI was performed by varying the diffusion potential of potassium (*23*).

RESULTS

Topological studies of Na^+-NQR from *V. cholerae* have found 17 negatively charged amino acid residues in the transmembrane α-helices of subunits B, D, and E, most of which are conserved across members of the Na^+-NQR family (*15*). Many of these residues are also conserved in the related enzyme RNF, which has a cofactor and subunit composition similar to those of Na^+-NQR. RNF has also been proposed to function as a redox-driven sodium pump (*16*, *25*). We compared the sequences of the transmembrane segments of Na^+-NQR with the corresponding regions of the homologous subunits of RNF (Table 2). Eight of the 17 acidic residues in Na^+-NQR are completely conserved in RNF, while an additional six are semiconserved. Conservation in the more distantly related RNF is an additional indication of the functional importance of these amino acid residues.

The NqrB subunit contains a total of seven acid residues, one in each transmembrane helices I (E28), III (E144), V (D224), VI (E274), and VIII (D346) and two in helix IX (E380 and D397). Three of these are conserved in the corresponding Rnf subunit D (E274, D346, and D397); two are present as semiconservative substitutions (D224 → Q and E380 → D), and two are not conserved (E28 and E144). NqrD contains a total of six acid residues, one in each transmembrane helices I (D17), II (E39), III (D87), and IV (E119) and two in helix V (D133 and E153). Three of these residues are conserved in the corresponding RnfE (E119, D133, and E153); two are present as semiconservative substitutions (D17 → N and D87 → Q), and one (E39) is not conserved. NqrE contains a total of four acid residues, one in each transmembrane helices I (E15) and III (E95) and two in helix V (E138 and E162). Two of these residues are conserved in the corresponding RnfA (E95 and E162), and the other two are present as semiconservative substitutions (E15 → N and E138 → Q) (Table 2).

Thus, all but three of the acid groups in the transmembrane helices of Na^+-NQR are either conserved in RNF or present as semiconservative substitutions, in which the new amino acid still contains an oxygen atom in the side chain (glutamine, asparagine, or threonine). This suggests that a full or partial negative charge is required for the interactions in which these amino acids participate.

Activity Measurements. We have analyzed the catalytic activity of wild-type Na^+-NQR and the acid group mutants and studied the effect of sodium concentration on these reactions. The physiological redox reaction of Na^+-NQR is the transfer of electrons from NADH to quinone, which drives the pumping of sodium. This reaction can be measured by following either the

Table 3: Steady-State Activities[a] of Wild-Type and Acid Mutants of Na^+-NQR in the Presence and Absence of Saturating Concentrations of Sodium (100 mM NaCl)

			without Na^+			with Na^+			
subunit	enzyme species	conserved in RNF	$NADH_{DH}$	Q_{red}	$NADH_{ox}$	$NADH_{DH}$	Q_{red}	$NADH_{ox}$	Q_{red}^{Na+}/Q_{red}
	wild type		437.3	65.4	374.9	557.7	521.4	74.1	8.0
B	E28A		370.0	37.2	332.8	474.8	69.7	405.0	1.9
	E144L		431.0	46.4	384.6	529.1	115.0	414.1	2.5
	D224A	[c]	334.1	58.5	275.6	522.4	226.5	295.9	3.9
	E274A	[b]	378.6	70.2	308.5	517.2	315.9	201.3	4.5
	D346A	[b]	433.4	57.4[d]	406.3	535.8	66.3[d]	500.6	1.2
	E380A	[c]	326.7	64.9	261.9	396.9	254.0	142.9	3.9
	D397A	[b]	331.7	53.2[d]	312.4	366.8	58.9[d]	349.5	1.1
D	D17A	[c]	365.2	58.1	307.1	477.7	303.4	174.4	5.2
	E39L		334.9	60.4	274.5	417.2	244.9	172.3	4.1
	D88L	[c]	477.8	31.5	446.3	533.6	58.9	474.7	1.9
	E119A	[b]	365.6	54.2	311.4	466.4	312.6	153.8	5.8
	D133A	[b]	486.0	25.4	460.6	545.1	42.3	502.8	1.7
	E153A	[b]	397.5	67.4	330.1	567.4	300.7	266.7	4.5
E	E15A	[c]	421.3	70.6	350.7	612.8	350.4	262.5	5.0
	E95A	[b]	505.3	50.2[d]	474.3	544.8	69.6[d]	504.7	1.4
	E138A	[c]	367.1	64.3	302.8	488.5	383.0	105.5	6.0
	E162G	[b]	366.1	62.5	303.6	561.4	290.2	271.3	4.6

[a] Steady-state activity determination of wild-type and Na^+-NQR mutants was performed in the presence and absence of saturating concentrations of sodium (100 mM NaCl). [b] Completely conserved in RNF. [c] Semiconserved. Activities were measured as described in Experimental Procedures. $NADH_{DH}$, NADH dehydrogenase activity; Q_{red}, quinone reductase activity; $NADH_{ox}$, NADH oxidase activity. All activities are expressed as turnover rates (s^{-1}). [d] The data indicated were obtained with enzyme that have been further purified by gel filtration chromatography (HiLoad 16/60 Superdex 200 column) using buffer containing 50 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 0.1% (w/v) *n*-dodecyl *β*-maltoside, and 5% (v/v) glycerol.

oxidation of NADH at 340 nm (NADH dehydrogenase = $NADH_{DH}$) or the reduction of quinone at 282 nm (Q_{red}). However, as a side reaction the enzyme can also donate electrons to dioxygen, producing superoxide (*26*−*28*) (NADH oxidase activity = $NADH_{ox}$). This side reaction is often observed in the isolated enzyme, resulting in a rate of quinone reduction (Q_{red}) that is lower than the rate of NADH consumption ($NADH_{DH}$) in the presence of oxygen. Here, the $NADH_{ox}$ activity was calculated from the difference between $NADH_{DH}$ and Q_{red} activities, which is presumed to reflect the rate of leakage of electrons to oxygen.

In the absence of sodium, the consumption of NADH by wild-type Na^+-NQR was 6−7 times faster than the reduction of quinone, which indicates that 80−90% of the electrons from NADH flow to oxygen ($NADH_{ox}/NADH_{DH} = 0.85$) (Table 3). Addition of sodium produced a reproducible 20−40% increase in $NADH_{DH}$ activity but also led to a 6−8-fold increase in the rate of Q_{red} and, consequently, a large effect on the electron branching between the $NADH_{ox}$ and Q_{red} reactions, in which approximately 90% of the electrons go to the Q_{red} activity ($Q_{red}^{Na+}/NADH_{DH}^{Na+} = 0.86$).

Table 3 shows the results of activity measurements on wild-type Na^+-NQR and the acid group mutants in the presence and absence of sodium. In the wild-type enzyme, sodium accelerates Q_{red} activity 7−8-fold. This effect was attenuated in the acid mutants. In some mutants such as NqrB-D397A, NqrB-D346A, and NqrE-E95A, the effect of sodium on Q_{red} activity was almost eliminated, while in other mutants, a weaker effect was observed. Figure 1 shows the relationship between the sodium sensitivity of the Q_{red} activity (Q_{red}^{Na+}/Q_{red}), the net Q_{red} activity, and the fraction of electrons from NADH that flow to oxygen. Mutants with the least sodium sensitivity showed both the lowest Q_{red} activity and the greatest electron leak to oxygen (in the presence of sodium). These results are expected if the acid group mutants restrict the access of sodium to the enzyme.

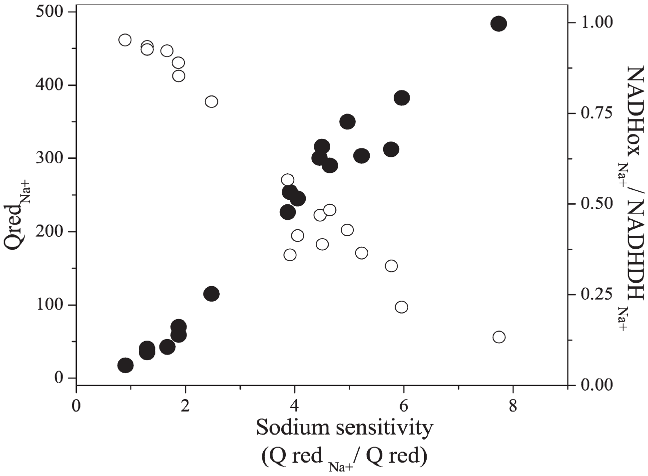

FIGURE 1: Correlation between the sodium sensitivity (Q_{red}^{Na+}/Q_{red}) of the wild-type and mutant Na^+-NQR with the quinone reductase activity [Q_{red}^{Na+} (●)] and the fraction of electrons leaking to oxygen [$NADH_{ox,Na+}/NADH_{DH}^{Na+}$ (○)] in the presence of sodium.

Some of the mutants (especially those with the least sodium sensitivity) displayed an apparent decrease in activity, compared to that of the wild type, even in the absence of sodium. Since the determination of the concentration of enzyme was performed spectrophotometrically, it was possible that contaminants in some mutant preparations might have increased the apparent concentration of enzyme in the sample and therefore have decreased the apparent specific activity. To check for this possibility, mutants NqrB-D346A, NqrB-D397A, and NqrE-E95A were further purified by gel filtration chromatography. As shown in Table 3, these purified mutant enzymes had Q_{red} activity values very similar to that of wild-type Na^+-NQR, in the absence of sodium (data marked with footnote b). These results are consistent with the idea

Table 4: Effects of Sodium and Potassium on the Activity of Wild-Type and Acid Mutants of Na^+-NQR

		activity with cation/activity without cation[a]					
		$\mathrm{NADH_{DH}}$		Q_{red}		$\mathrm{NADH_{ox}}$	
subunit	enzyme species	Na^+	K^+	Na^+	K^+	Na^+	K^+
	wild type	1.31	1.18	7.84	1.02	0.58	0.91
B	E28A	1.27	1.32	1.71	0.90	1.21	1.37
	E144L	1.30	1.32	2.66	0.96	1.00	1.37
	D224A	1.88	1.33	4.21	1.09	0.96	1.37
	E274A	1.78	1.42	5.2	0.85	0.84	1.53
	D346A	1.21	1.29	1.21	1.04	1.21	1.31
	E380A	1.47	1.26	3.8	0.87	0.79	1.32
	D397A	1.09	1.38	1.05	1.09	1.13	1.38
D	D17A	1.96	1.40	8.75	1.11	0.93	1.44
	E40L	1.55	1.41	5.72	1.00	0.83	1.48
	D88L	1.09	1.15	1.54	0.94	1.04	1.18
	E119A	1.86	1.61	6.18	1.02	1.18	1.68
	D133A	1.25	1.42	1.89	0.95	1.18	1.48
	E153A	1.56	1.36	7.82	1.16	0.78	1.39
E	E15A	1.98	1.37	7.57	1.10	1.19	1.41
	E95A	1.41	1.27	1.50	1.32	1.40	1.26
	E138A	1.75	1.31	8.26	1.08	0.58	1.35
	E162G	1.78	1.38	8.30	0.88	0.88	1.44

[a] Activities were measured as described in Experimental Procedures in the absence of cations and in the presence of NaCl (100 mM) or KCl (100 mM).

Table 5: Formation of $\Delta\Psi$[a] in Inverted Vesicles and Wild-Type and Mutant Enzymes Reconstituted in Proteoliposomes

		Na^+ stimulation		
subunit	enzyme species	purified enzyme	reconstituted enzyme	$\Delta\Psi$ (mV)
	wild type	7.74	9.6	−165
B	E28A	1.87	2.6	−35
	E144L	2.48	3.5	−65
	D224A	3.87		−90
	E274A	4.50		
	D346A	1.30	1.12	[b]
	E380A	3.92		
	D397A	0.90	1.1	[b]
D	D17A	5.22		
	E40L	4.05		
	D88L	1.87	2.3	−25
	E119A	5.76		
	D133A	1.67	1.8	−10
	E153A	4.46		
E	E15A	4.96		
	E95A	1.30	1.6	[b]
	E138A	5.96		
	E162G	4.64		

[a] $\Delta\Psi$ formation was assessed in preparations of purified enzyme reconstituted into proteoliposomes, using Oxonol IV, as indicated in Experimental Procedures. The spectrophotometric signal of Oxonol IV was calibrated by changing the diffusion potential of potassium. [b] Undetectable (less than −5 mV).

that these mutants exert their major effect by perturbing sodium transport in the enzyme.

We also studied the ion selectivity, comparing the effects of sodium and potassium on the activity of wild-type and mutant Na^+-NQR enzymes (Table 4). The results with the wild-type enzyme confirm earlier findings by Hayashi and Unemoto (*26*) using the enzyme from *Vibrio alginolyticus* (see also refs *6*, *27*, and *29*). The effect of sodium on the Q_{red} activity is approximately 7 times larger than the effect of potassium, indicating that the dominant effect is not due to ionic strength. However, the apparent stimulation of the $\mathrm{NADH_{DH}}$ and $\mathrm{NADH_{ox}}$ activities may be due to ionic strength, since the effects of sodium and potassium are similar.

The same pattern is generally observed in the mutants. In a few cases where there is very little activation by sodium, the difference between sodium and potassium activation is not large. However, in no case does the mutation result in a notable increase in the level of activation of Q_{red} activity by potassium.

Sodium Pumping Activities. Sodium pumping activity was measured spectrophotometrically by means of the $\Delta\Psi$-sensitive probe Oxonol VI, which partitions across the membrane in a voltage-sensitive manner (*23*, *24*) (Table 5). To have a simple system that would allow us to address the activity of Na^+-NQR directly, these measurements were made on the isolated enzyme reconstituted into phospholipid vesicles. To obtain a quantitative measurement, the spectrophotometric signal of Oxonol VI was calibrated by means of diffusion potential created with potassium and valinomycin. Figure 2A shows that the reconstituted wild-type enzyme is able to sustain a $\Delta\Psi$ of approximately −160 mV, which is insensitive to the protonophore CCCP and is collapsed by the addition of the sodium ionophore ETH-157, indicating that the $\Delta\Psi$ is the result of electrogenic sodium translocation. The results in Figure 2B show that the mutants NqrB-D346A, NqrB-D397A, and NqrE-E95A, in which Q_{red} activity is insensitive to sodium, were unable to form a sodium gradient. The mutants NqrB-E28A, NqrB-E144L, NqrD-D133A, and NqrD-D88L, in which sodium stimulation of Q_{red} activity was lowered significantly but not eliminated, produced diminished levels of $\Delta\Psi$.

Kinetic Properties of Wild-Type Na^+-NQR and Its Acid Mutants. To gain a deeper insight into the effect of the mutations on the interaction of sodium with the enzyme, we studied the steady-state turnover over a range of sodium concentrations (Table 6). These experiments were performed with near-saturating concentrations of NADH (150 μM) and Q-1 (50 μM), to minimize rate limitation by substrates other than sodium. A $K\mathrm{m_{app}}^{Na+}$ for sodium of ∼1 mM was obtained for the wild-type enzyme, close to the value reported for other preparations (*29*). It is worth noting that the $K\mathrm{m_{app}}^{Na+}$ for sodium obtained by steady-state measurements is similar to the $K\mathrm{m_{app}}^{Na+}$ for sodium of the rate-limiting step in the reduction of Na^+-NQR by NADH (*29*), indicating that this value could be close to the actual dissociation constant for sodium.

Three mutants (NqrB-D397A, NqrD-D133A, and NqrE-E95A), which are located in the part of the transmembrane helices facing the cytosol, showed an increase of ≥9-fold in the $K\mathrm{m_{app}}^{Na+}$ for sodium, suggesting that the mutations result in a change in the Na^+ affinity of the enzyme (Table 6 and Figure 3). However, in the case of NqrB-D397A, the turnover rate increased linearly all the way to the highest concentration of sodium tested (300 mM). This could be consistent with either (1) a very large change in the sodium affinity of the enzyme or (2) conditions under which sodium uptake is so severely restricted that the bimolecular process (collision of sodium with the enzyme) is always rate-limiting. The NqrB-E144L mutant could be considered part of this group, because it produced a 4-fold decrease in the $K\mathrm{m_{app}}^{Na+}$ for sodium. However, the change is small compared with those of the other mutants, and in contrast with many of them, this mutation does not abolish the capacity for ion pumping (Table 5).

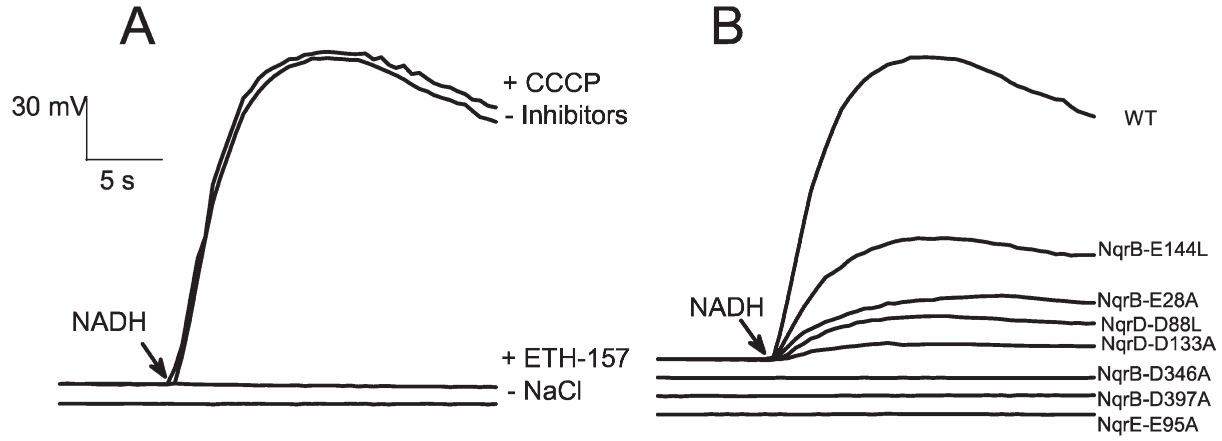

FIGURE 2: Formation of $\Delta\Psi$ by wild-type Na^+-NQR and acidic mutants reconstituted into proteoliposomes. (A) Formation of $\Delta\Psi$ by wild-type Na^+-NQR showing the effects of CCCP (5 μM), ETH-157 (5 μM), and the omission of NaCl from the medium. (B) Formation of $\Delta\Psi$ by wild-type Na^+-NQR and acidic mutants. The assay buffer contained 100 μM Q-1, 100 mM HEPES, 100 mM NaCl (except where noted), 150 mM KCl, and 1 mM EDTA (pH 7.5). The reaction was started by the addition of NADH (200 μM).

Table 6: Apparent Kinetic Parameters[a] of Wild-Type Na^+-NQR and Its Acid Mutants

subunit	enzyme species	orientation[b]	Km_{app}^{Na+} (mM)	$V_{max}^{Na+}/V_{max}^{no\ Na+}$
	wild type		1.1	6.2
B	E28A	P	2.1	3.5
	E144L	N	4.2	4.8
	D346A	P	0.9	1.4
	D397A	N	> 100	–
D	D88L	P	1.4	3.1
	D133A	N	9.3	2.8
E	E95A	N	13.1	1.3

[a] The Q_{red} activity of the mutants was measured with different concentrations of NaCl using near-saturating amounts of NADH (150 μM) and Q-1 (50 μM). The kinetic parameters were calculated from the saturation curve using the Michaelis–Menten equation. The orientation was obtained from the topology studies published previously (*15*). [b] N, negative side of the membrane (facing the cytosol); P, positive side of the membrane (facing the periplasmic space).

NqrB-E28A, NqrB-D346A, and NqrD-D88L mutants caused little or no change in the Km_{app}^{Na+} for sodium, in spite of the fact that the overall sensitivity of the turnover rate to sodium was significantly diminished as compared to that of the wild-type enzyme. This suggests that in these mutants, the sodium binding affinity of the enzyme has not changed and that the mutations exert their effects at some other point in the reaction cycle. This would be consistent with a mechanism limited by restricted Na^+ ejection, rather than uptake. It is interesting to note that all of the residues in the latter group are located near the positive side of the membrane facing the periplasm where sodium release occurs during turnover. Residues in the former group (NqrB-D397A, NqrD-D133A, and NqrE-E95A) are all located near the negative side of the membrane, facing the cytoplasm, where sodium uptake occurs (Table 6). It is important to stress that the *K*m or the Km_{app}^{Na+} of an enzyme does not directly reflect the dissociation constant for the substrate, because in both cases they are complex parameters that are composed of many rate constants, which vary depending on the mechanism of the enzyme. However, if we consider that these mutations had a selective effect and that they did not modify the binding of other substrates, or the stability of the protein, the Km_{app}^{Na+} values could be used as a rough indication of the affinity for substrate.

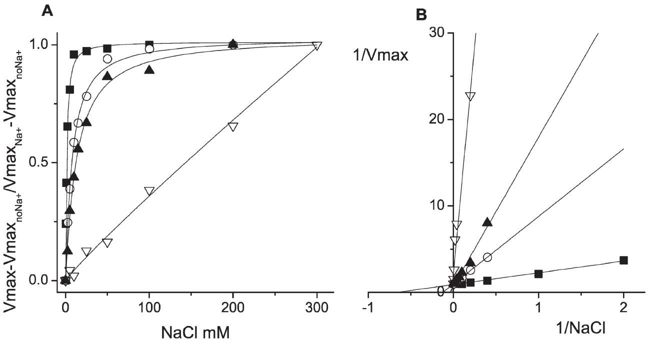

FIGURE 3: (A) Saturation kinetics for sodium of the wild type (■) and mutants NqrB-D397A (▽), NqrD-D133A (○), and NqrE-E95A (▲). For presentation purposes, the $V_{max}^{no\ Na+}$ was subtracted from the individual points of the saturation curves and then the data were normalized by the V_{max} value obtained from the fit of the data of each mutant. In the case of the NqrB-D397A mutant, data were normalized by the V_{max} obtained with 300 mM NaCl, because of the apparently unsaturable behavior. (B) Double-reciprocal plots of saturation kinetics. The analysis of the data was performed using nonlinear curve fitting with a modified Michaelis–Menten equation [$v = V_{max}Na/(Km_{app}^{Na+} + Na) + b$] that contains the V_{max} and Km_{app}^{Na+} values, as well as the activity of the enzyme without sodium (*b*).

It was important to verify that the changes in Km_{app}^{Na+} for sodium measured in these mutants did not arise as secondary effects of inhibition of the redox reaction in the mutants. To this end, we determined the Km_{app}^{Na+} for sodium in the wild-type enzyme at two different concentrations of NADH (Figure 4). The Km_{app}^{Na+} for sodium changes very little with the changing NADH concentration and in fact increases with an increasing turnover rate, the opposite of what is observed in the mutants. We also studied the effect of sodium concentration on the *K*m values for NADH and Q-1 for the wild type and selected mutants (NqrB-D346A and NqrB-D397A). The *K*m values for NADH and Q-1 were essentially unaffected by sodium concentration and were not significantly altered by the mutations (data not shown).

DISCUSSION

To achieve sodium transport, Na^+-NQR must be able to bind the cation with a physiologically relevant affinity and specificity, and sodium must be able to move across the membrane through a pore or passageway. The gating or unidirectionality of sodium

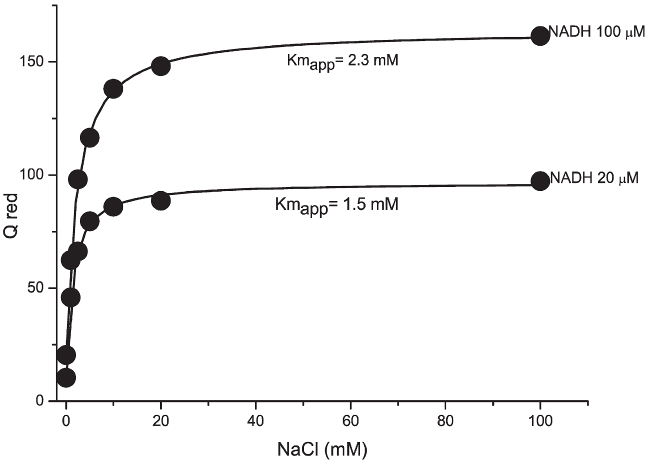

FIGURE 4: Dependence of Q_{red} activity of wild-type Na^+-NQR on sodium at two different concentrations of NADH.

transport could be accomplished by coupling the energy produced in the redox reaction of the enzyme to structural changes in the protein, which would prevent ion back flow. The characterization of the sodium-binding site (or sites) and sodium passageway is a prerequisite for understanding the coupling mechanism in Na^+-NQR.

Negatively charged residues located in transmembrane segments of proteins are ideal candidates for the passageways or binding sites of cations in either pumps or ion channels. In fact, aspartates and glutamates are common components of sodium-binding sites of both soluble and membrane proteins (*30*).

Acid Residues with a Significant Role in Sodium Translocation. Na^+-NQR has a surprisingly large number of aspartates and glutamates in the membrane segments of subunits B, D, and E. This indicates that, although these three subunits are very hydrophobic, they contain hydrophilic sites that could be important for sodium translocation. Our initial goal was to determine which of these amino acids have a significant role in enzyme activity and sodium binding.

There are 17 acid residues in transmembrane helices highly conserved across Na^+-NQR sequences. Of these, 14 are conserved and semiconserved in the related enzyme RNF, indicating that these residues are likely to be of special importance.

NqrB. Mutations of almost all the acid residues in the transmembrane helices of NqrB had a deleterious effect on sodium-dependent enzyme activity or sodium affinity, suggesting that these residues participate in the binding or transportation of sodium. Mutations of NqrB-D346 and NqrB-D397 both led to significant decreases in the rate of enzyme turnover and overall sodium sensitivity. However, in the case of NqrB-D346, which is located near the periplasmic space, there was essentially no change in sodium affinity, while in the case of NqrB-D397, the sodium dependence of enzyme activity produced a straight line (Figure 3) consistent with a very large decrease in the sodium affinity of the enzyme. Mutations of two other residues, NqrB-E380 and NqrB-E274, had only minimal effects on enzyme activity, suggesting that they do not play an important role in the mechanism of sodium pumping. These residues are both located near the ends of transmembrane helices, where they are likely to interact with solvent in the periplasmic space. All of the other residues appear to be buried by at least one turn of the helix (three residues) into the hydrophobic core of the membrane.

Two of these residues, which appear to have a significant role in enzyme activity, NqrB-E28 and NqrB-E144, are highly conserved in Na^+-NQR but not in RNF. This lack of conservation probably reflects differences in the details of the sodium pumping mechanisms of the two complexes.

NqrD. Mutations of two of the six conserved acid residues in NqrD, E133 and D88, resulted in large changes in enzyme activity and sodium sensitivity. In the case of NqrD-D88, the $Km_{app}{}^{Na+}$ for sodium in the mutant is approximately the same as that of the wild type, but in the case of NqrD-D133, the $Km_{app}{}^{Na+}$ for sodium is approximately 10 times higher than that for the wild-type enzyme. It is interesting to note that while NqrD-D88 appears to have an important role in enzyme function, it is predicted to be close to the end of a transmembrane helix and likely to be in contact with solvent in the periplasmic space.

NqrE. Of the four conserved acid residues in NqrE, only NqrE-E95 appears to play a role in enzyme activity. Mutation of this residue, which is located in the hydrophobic part of the membrane, led to a significant decrease in the rate of enzyme turnover (Q_{red}) and sodium sensitivity. Importantly, the apparent *K*m for sodium is ~10-fold higher than in the wild-type enzyme. Mutations of the three remaining acid residues produced very little effect on either enzyme turnover or sodium sensitivity. Two of these residues, NqrE-E138 and NqrE-E162, appear to be exposed to the cytoplasm, which may explain these results. However, NqrE-15 is located in the hydrophobic part of the membrane and thus could have a role in stabilizing the structure.

A Sodium-Binding Site in Na^+-NQR. These results point to three residues (NqrB-D397, NqrD-D133, and NqrE-E95), all facing the cytoplasmic (negative) side of the membrane, which appear to serve an important role in the binding of sodium by Na^+-NQR during turnover. Mutations of these three residues have large effects on the enzyme activity (Q_{red}) and also significantly alter the apparent affinity of the enzyme for sodium (Figure 3). NqrB-D397 is perhaps the most important ligand in this putative Na^+ binding site, since its mutant cannot be saturated with sodium under the experimental conditions tested, which is consistent with a very low affinity for the cation. NqrD-D133 and NqrE-E95 could participate in this putative binding site, since in both cases mutations increased the $Km_{app}{}^{Na+}$ for sodium by approximately 1 order of magnitude (Table 6 and Figure 3).

NqrB-E144 may also have a role in sodium binding. Mutation of this residue (E144A) led to a 4-fold increase in the $Km_{app}{}^{Na+}$ for sodium (4.2 mM vs 1.1 mM for the wild type). This change is smaller than for the other three residues, suggesting a less important role as a ligand. Interestingly, the value of $Km_{app}{}^{Na+}$ in this mutant is similar to that for wild-type *Vibrio harveyi* Na^+-NQR (3.3 mM) (*29*).

The side chains of NqrB-D397, NqrD-D133, NqrE-E95, and possibly NqrB-E144 may form a single sodium-binding site. This would follow the pattern found in sodium-binding sites I and II of Na^+/K^+-ATPase, which contain three and four acid residues, respectively (*31*). However, it has been shown that the presence of net negative charges is not absolutely necessary for sodium coordination; in fact, sodium-binding site I in the leucine/Na^+ cotransporter (*30*) and sodium-binding site III of the Na^+/K^+-ATPase (*31*) contain only one net negative charge, while the second cation binding site of the leucine/Na^+ cotransporter does not include any negative charges (*30*). In these cases, the other ligands are parts of serine or threonine residues, the protein

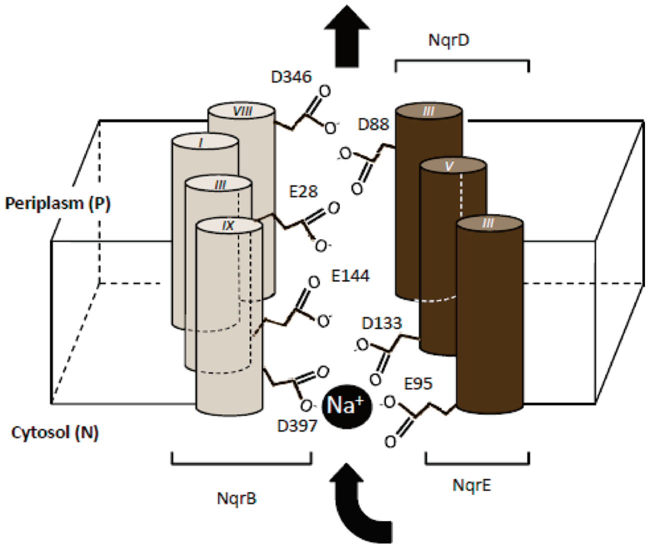

FIGURE 5: Representation of a possible binding site and passageway of sodium in Na$^+$-NQR.

backbone, and water molecules, carrying partial negative charges.

Nayal and Di Cera (*22*) studied ion binding sites in proteins and concluded that the main features that determine the specificity of these sites are the number of ligands, the match between the size of the ion and the binding site, and the spatial distribution of the ligands. They determined that the optimal binding site for sodium consists of six ligands in an octahedral array (*20*). Thus, it is not surprising that the three main residues implicated in sodium binding in Na$^+$-NQR are completely conserved in both Na$^+$-NQR and RNF.

Our results also identified four additional residues (NqrB-E28, NqrB-E144, NqrB-D346, and NqrD-D88), mutants of which alter the enzyme activity (Q_{red}) and its sodium sensitivity, but do not alter the $Km_{\text{app}}{}^{\text{Na+}}$ for sodium. This suggests that these residues may be part of a structure that conducts sodium through the hydrophobic region of the membrane.

Figure 5 shows a possible arrangement of helices in which NqrB-D397, NqrD-D133, and NqrE-E95 take part in a sodium-binding site while NqrB-E144, NqrB-E28, NqrB-D346, and NqrD-D88 participate in a structure that facilitates the passage of sodium through the hydrophobic membrane. The complete mechanism is likely to require additional residues and probably internal water. There are up to 15 conserved polar residues buried in the membrane; three are found in the NqrB subunit (S222, T345, and N390), seven in NqrD (N18, N19, Q22, S55, S81, N111, and C112), and five in NqrE (N16, C26, Q92, N120, and C121). Conservation of these residues cannot be used to directly infer participation in sodium transport, since many of these amino acids play other roles in membrane proteins. For example, serine and threonine are common components of transmembrane helices, accounting for approximately 7% of all amino acid residues (*17*). These residues promote the formation of strong and specific associations between transmembrane helices, mediated by hydrogen bonds; some examples are found in halorhodopsin, Ca^{2+}-ATPase, and cytochrome *c* oxidase (*17*).

All of the mutations studied in this paper involved replacement of acid residues with aliphatic ones, changing both the chemical properties and the size of the side chain. At this point, we cannot rule out the possibility that these mutations may affect the properties of the enzyme indirectly through structural perturbations. To investigate this possibility, we are now constructing new mutations at the most interesting sequence positions, with more conservative changes, for example, D → E, D → N, etc. With these new mutations, we hope to distinguish the steric and chemical effects of the residues. However, this initial survey has succeeded in identifying a subset of the acidic residues within transmembrane helices that are promising candidates for sodium-binding sites in Na$^+$-NQR. This represents an important initial step in the characterization of the coupling mechanism of Na$^+$-NQR and also could be useful for understanding the structural basis of the cation selectivity of the enzyme.

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