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Review

Na^+ translocation by bacterial NADH:quinone oxidoreductases: an extension to the complex-I family of primary redox pumps[1]

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Abstract

The current knowledge on the Na^+-translocating NADH:ubiquinone oxidoreductase of the Na^+-NQR type from *Vibrio alginolyticus*, and on Na^+ transport by the electrogenic NADH:Q oxidoreductases from *Escherichia coli* and *Klebsiella pneumoniae* (complex I, or NDH-I) is summarized. A general mode of redox-linked Na^+ transport by NADH:Q oxidoreductases is proposed that is based on the electrostatic attraction of a positively charged Na^+ towards a negatively charged, enzyme-bound ubisemiquinone anion in a medium of low dielectricity. A structural model of the [2Fe–2S]- and FAD-carrying NqrF subunit of the Na^+-NQR from *V. alginolyticus* based on ferredoxin and ferredoxin:$NADP^+$ oxidoreductase suggests that a direct participation of the Fe/S center in Na^+ transport is rather unlikely. A ubisemiquinone-dependent mechanism of Na^+ translocation is proposed that results in the transport of two Na^+ ions per two electrons transferred. Whereas this stoichiometry of the pump is in accordance with in vivo determinations of Na^+ transport by the respiratory chain of *V. alginolyticus*, higher (Na^+ or H^+) transport stoichiometries are expected for complex I, suggesting the presence of a second coupling site.

Keywords: Complex I; Na^+ transport; NADH; Ubiquinone; Na^+/H^+ antiporter; *Vibrio alginolyticus*; *Klebsiella pneumoniae*

1. Introduction

Na^+-translocating NADH:ubiquinone oxidoreductases are redox-driven pumps that couple the exergonic oxidation of NADH ($E^{\circ\prime}_{NAD/NADH} = -320$ mV) with ubiquinone ($E_{0'Q/QH^2} = +100$ mV) to the transport of Na^+ ions from the inside of a bacterial cell to the periplasmic space according to:

$$NADH_{in} + H^+ + Q + nNa^+_{in} \rightleftharpoons NAD^+_{in} + QH_2 + nNa^+_{out} \quad (1)$$

This reaction diminishes the internal Na^+ concentration and contributes to the generation of an electrochemical Na^+ gradient (outside positive). The prototype of this sodium pump is the Na^+-translocating NADH:Q oxidoreductase (Na^+-NQR) from the marine, aerobic bacterium *Vibrio alginolyticus* [1–3] described in the first part of this review. Although the overall reaction catalyzed by the Na^+-NQR resembles that of complex I, the H^+-translocating NADH:Q oxidoreductase from mitochondria or bacteria, there is no sequence similarity between these

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[1] Dedicated to Peter Dimroth on the occasion of his 60th birthday.

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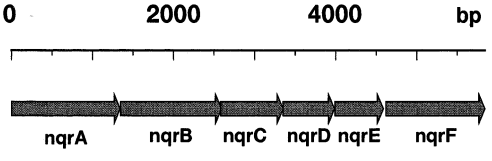
redox pumps. The electrogenic NADH:Q oxidoreductase (complex I, or NDH-I) from *Escherichia coli* is homologous to the eukaryotic complex I and like the latter is assumed to transport protons [4]. However, there is recent evidence indicating that complex I from *E. coli* and the related bacterium *Klebsiella pneumoniae* catalyzes net Na^+ transport according to Eq. 1. These results will be summarized in the second part of the review, and the implications for the mechanism of cation transport by complex I in general will be discussed.

2. The Na^+-NQR from *V. alginolyticus*

The Na^+-NQR of *V. alginolyticus* and other marine bacteria [5] generates an electrochemical sodium gradient which is utilized for various cellular processes such as nutrient uptake and flagellar rotation [2]. The enzyme was discovered by Tokuda and Unemoto who showed that protonophore-resistant Na^+ transport in *V. alginolyticus* is confined to the NADH:Q segment of the respiratory chain [6,7].

2.1. Subunits and redox cofactors of the Na^+-NQR

The Na^+-NQR from *V. alginolyticus* is currently regarded as a complex composed of six subunits encoded by the *nqr* operon with an approximate mass of 200 kDa (Fig. 1). Except for the NqrA subunit, all subunits are anchored to the membrane via transmembrane helices (Fig. 2). Note that so far, there are no experimental data confirming the cytoplasmic localization of the peripheral NqrC domain, or the



Molecular mass [kDa]	48.6	45.2	27.5	22.5	21.5	45.3
Apparent mass [kDa]	50	30 – 35	32	20	19	46
Subunit	α	γ2	γ			β

Fig. 1. Gene cluster and subunits of the Na^+-NQR from *V. alginolyticus*. The properties of the Na^+-NQR from *V. alginolyticus* (accession number AB008030) are compiled from [3,12,14].

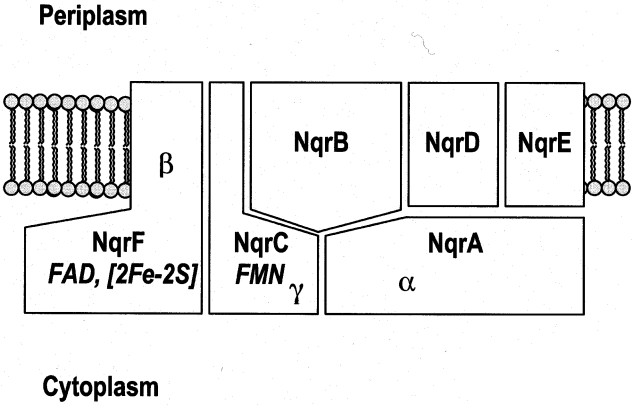


Fig. 2. Putative subunit topology and localization of cofactors in the Na^+-NQR from *V. alginolyticus*. The membranous or peripheral localizations of subunits NqrA–E are derived from a hydropathy analysis of the Nqr subunits using the program Toppred2 [54], with 0 (NqrA), 7 (NqrB), 1 (NqrC), 5 (NqrD), and 6 (NqrE) 'certain' transmembrane helices. For the NqrF subunit, two 'certain' N-terminal transmembrane helices are predicted, which is in conflict with the structural model as discussed in the text.

NqrA subunit. The Na^+-NQR contains FAD, FMN, a [2Fe–2S] cluster and ubiquinone-8 as redox prosthetic groups. Since the stoichiometry and localization of the subunits and the cofactors, especially the FMN prosthetic group, are still a matter of debate, the properties of (sub)complexes of the Na^+-NQR characterized so far will be described in detail.

The initial purification of the Na^+-NQR from *V. alginolyticus* by Hayashi and Unemoto [8] yielded two enzyme preparations. The so-called NADH dehydrogenase consisted of a single subunit with an apparent mass of 46 kDa that catalyzed only one-electron transfer to quinone derivatives and was not activated by Na^+ ions. This NADH dehydrogenase fragment was later identified as the β subunit, or NqrF (Fig. 3), and is regarded as the electron input device of the complex (see Section 2.2). In addition, a so-called Q reductase was enriched which catalyzed Na^+-stimulated ubiquinone-1 reduction to QH_2 with NADH as the electron donor. This Q reductase contained the β (NqrF) subunit and additional polypeptides. Further purification of the Q reductase led to the identification of the α (50 kDa) and the γ (30–35 kDa) fractions, and the Na^+-NQR was proposed to be a complex of α, β, and γ subunits containing FAD and FMN as prosthetic groups. FAD was assigned

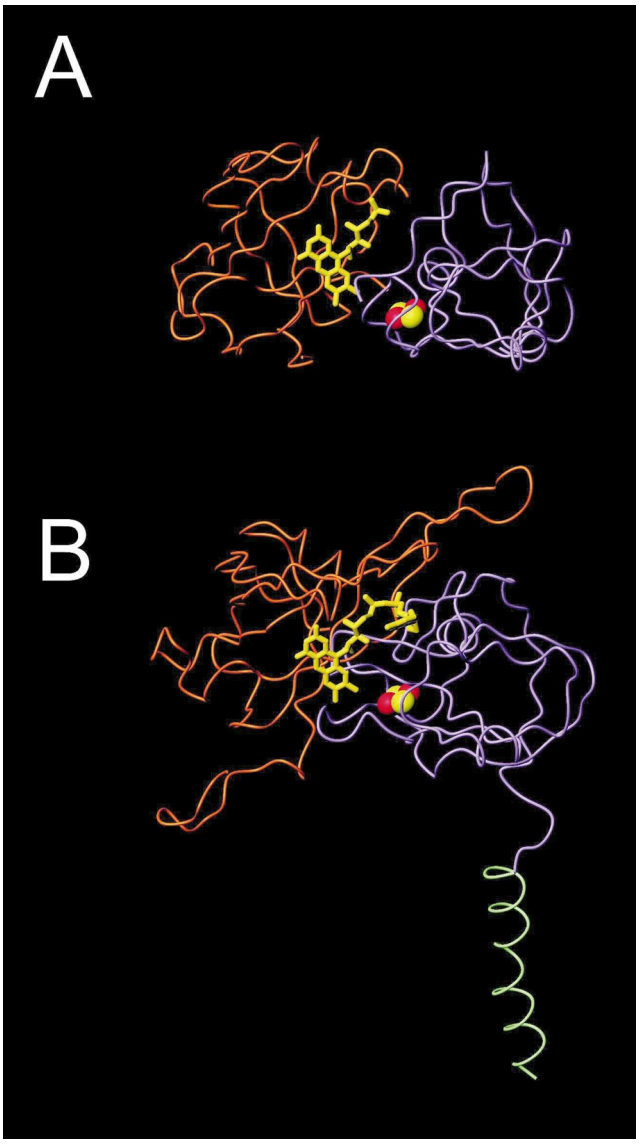


Fig. 3. Structural model of the NqrF subunit of the Na^+-NQR from *V. alginolyticus*. The sequence of the NqrF subunit from *V. alginolyticus* (Lys25–Gly407) was aligned with the sequences of ferredoxin and ferredoxin:$NADP^+$ oxidoreductase (FNR) from *Anabaena* PC7719 based on conserved residues characterizing the extended family of flavoprotein reductases [20] and using the program ClustalX [55]. This alignment served to build the model using the program Swiss PDB viewer [56], based on high-resolution structures of ferredoxin (PDB accession number 1CZP) [21] and ferredoxin:$NADP^+$ oxidoreductase (FNR; PDB accession number 1QGO) [19] from *Anabaena* PC7719, and phthalate dioxygenase reductase from *Burkholderia cepacia* (formerly known as *Pseudomonas cepacia*, PDB accession number 2PIA) [22]. A close contact between the Fe/S cluster and the FMN in the phthalate dioxygenase reductase is established by the ferredoxin domain (violet) and the FNR domain (gold) (A). The model of the NqrF subunit supports a similar arrangement of the FAD-containing FNR domain (gold) and the [2Fe–2S] domain (violet) (B). In addition, a hydrophobic N-terminal extension (Asp2–Ala24) is assumed to anchor the NqrF subunit to the membrane (green). A second hydrophobic stretch in the N-terminal part of the NqrF subunit comprises the cysteine residues 69, 75 and 78 that are required for the coordination of the [2Fe–2S] cluster (C). The figure was prepared with the program MOLMOL [57].

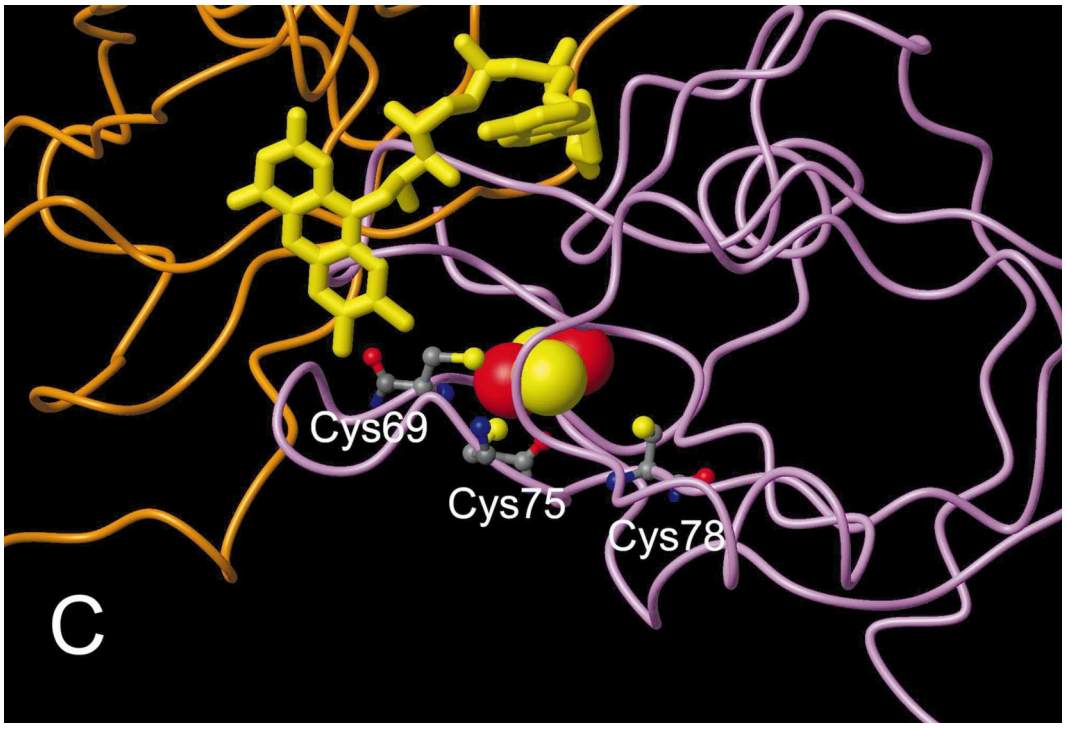


Fig. 3 (*continued*).

to the β fraction, FMN to the α fraction of the Na^+-NQR [9].

The sequencing of the *nqr* operon from *V. alginolyticus* (accession number AB008030) [10,11] led to the identification of six putative *nqr* genes. The α, β, and γ subunits were assigned to the *nqrA*, *nqrF* and *nqrC* genes, respectively. The presence of non-covalently bound FMN in the α (NqrA) subunit was questioned when a complex consisting of the NqrABCF subunits was purified containing non-covalently attached FAD, a [2Fe–2S] cluster, and ubiquinone-8 [3]. The FAD and [2Fe–2S] cluster are most likely bound to the NqrF subunit, which exhibits sequence similarities to ferredoxins and FNR, the ferredoxin:$NADP^+$ oxidoreductase [12] (Fig. 3). Ag^+ acts as an inhibitor of the Na^+-NQR by displacing FAD from the NqrF subunit [13]. A re-evaluation by Unemoto and coworkers showed that the so-called α fraction containing FMN [9] comprises the subunits NqrA, NqrD, NqrE, and a subunit with an approximate mass of 30 kDa. Since this polypeptide was not unequivocally identified by N-terminal sequencing, it could be either the NqrB or NqrC subunit that may comigrate on SDS–PAGE [14]. Recently, a covalently bound FMN was discovered on the NqrC subunit of the Na^+-NQR from *Vibrio harveyi* and *V. alginolyticus* [15]. In a subsequent study, Nakayama et al. identified a covalently bound flavin on the NqrB subunit in addition to the FMN-containing NqrC subunit [16]. The participation of the covalently bound flavins on the hydrophobic NqrB and C subunits in electron transfer or Na^+ transport remains to be investigated.

The interactions of subunits and localizations of cofactors of the Na^+-NQR from *V. alginolyticus* as depicted in Fig. 2 are based on the properties of the different subcomplexes of the Na^+-NQR that have been isolated. From the high sequence similarity of the *nqr* genes from *V. alginolyticus* with the corresponding genes from *V. harveyi* (accession number AF165980) [15] and *Vibrio cholerae* (accession number AF117331) [17], it is reasonable to assume that the Na^+-NQRs from these *Vibrio* sp. exhibit very similar properties. The occurrence of Na^+-NQRs in *V. cholerae* and other pathogenic bacteria, and a possible role for the sodium motive force generated by the Na^+-NQR in pathogenicity, are outlined in the article by Häse and Barquera in this issue.

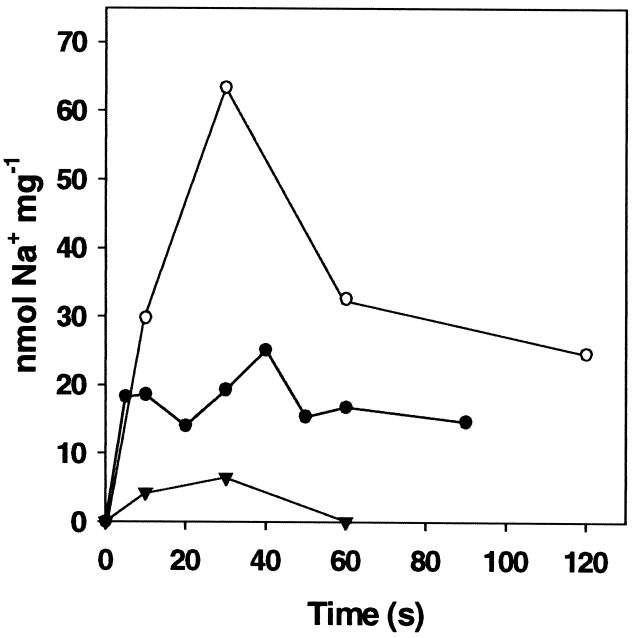


Fig. 4. Respiratory Na^+ transport catalyzed by complex I. The oxidation of deaminoNADH by inverted membrane vesicles of *K. pneumoniae* with oxygen as electron acceptor drives the transport of Na^+ ions into the vesicles (●). The uptake is stimulated by the protonophore CCCP (○), but inhibited by the complex I-specific inhibitor rotenone (▼).

2.2. The electron input subunit

The initial oxidation of NADH by the Na^+-NQR is catalyzed by a flavin prosthetic group, therefore the site of electron entry is located either on the FAD-carrying NqrF subunit or on the FMN-containing NqrC subunit. There are several indications that point to the NqrF subunit being the electron input subunit of the Na^+-NQR. Whereas the isolated NqrF subunit oxidizes NADH with artificial electron acceptors, this reaction is not catalyzed by subcomplexes of the Na^+-NQR comprising the NqrC subunit that are devoid of the NqrF subunit. Furthermore, the NqrF harbors a [2Fe–2S] cluster besides the FAD which is well suited to convert the two-electron process of NADH oxidation into one-electron transfer reaction steps. The one-electron reduction of an internal redox cofactor in a low dielectric environment is a central feature of the mechanisms of redox-linked Na^+ transport by the Na^+-NQR that have been put forward. According to the proposal of Rich et al. [12], the positively charged Na^+ is counterbalanced by the reduced Fe/S center, whereas Dimroth suggested that the Na^+ ion is attracted by an internal, negatively charged ubisemiquinone anion stabilized in the Na^+-NQR [18] (Fig. 5).

The central role of the NqrF subunit in NADH oxidation is also supported by the sequence similarity of its C-terminal part to ferredoxin:$NADP^+$ oxidoreductases (FNR) from plants, algae or photosynthetic bacteria. FNRs are FAD-containing enzymes that accept electrons from the one-electron donor ferredoxin and subsequently reduce $NADP^+$ to NADPH in a two-electron transfer reaction [19]. Since its N-terminal part is related to [2Fe–2S] ferredoxins, the NqrF subunit resembles a fused ferredoxin–FNR complex, as pointed out by Rich et al. [12]. The structural prototype of a [2Fe–2S] cluster and a NADH-oxidizing FMN moiety arranged in a

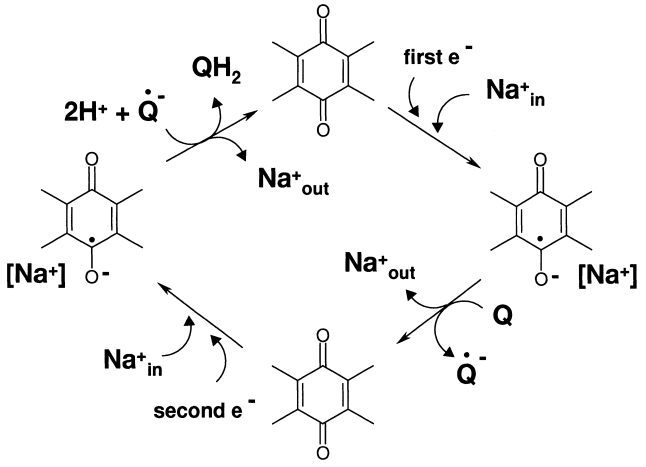


Fig. 5. A putative mechanism of Na^+ translocation by a NADH:quinone oxidoreductase redox pump. The central redox cofactor of the pump is a bound quinoid compound (symbolized by the quinoid ring) that shuttles between the oxidized and one-electron reduced, deprotonated state (semiquinone anion). Q, $Q^{\bullet-}$, and QH_2 indicate the oxidized, one-electron, and two-electron reduced states of external quinone acting as substrate. Upon reduction by the first electron, the bound quinone is converted into the semiquinone anion and Na^+ is taken up from the negative side of the membrane to form the ion pair ($Q^{\bullet-}$ [Na^+]). Upon reoxidation of the semiquinone anion by external Q, Na^+ escapes to the positive side of the membrane. This one-electron transfer reaction converts the external Q into the semiquinone state, in which it probably remains enzyme-bound until it becomes fully reduced to the quinol in the second cycle. The second cycle of Na^+ uptake and extrusion is initiated by the incoming second electron. Per NADH oxidized, two electrons are transferred to quinone, and two Na^+ are translocated.

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 NaH_BacC125 MTLLHWATVSPFLLAIFIPILYKYTRS-----IHTGWFVLVLPLVLFGYFLSLLPITSNGGVIHETIPWVPSLGINFTVY    75
  MrpA_Bacsub ------------------------------MHTGWFVLILPVLLFIYFLPMIRMTQSGETLRSVLEWIPSLGINFTVY    48
MnhA_Staphau MSLLHIAVILPLIFALIIPILYRFFKR-----IHLGWFVLSVPIVIFIYMLTLIKTTMSGNTVMKTLNWMPHFGMNFDLY    75
  nuoL_Ecoli MNMLALTIILPLIGFVLLAFSRGRWSENVSAIVGVGSVGLAALVTAFIGVDFFANGEQTYSQPLWTWMSVGDFNIGFNLV    80

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 NaH_BacC125 VDGLSLLFALLITGIGTLVALYSIYYLSKKTEKLNNFYVYLLMFMGAMLGVVLSDNLIVLYVFWELTSLASSLLISYWFH   155
  MrpA_Bacsub IDGLGLLFALLITGIGSLVTLYSIFYLSKEKEQLGPFYVYLLMFMGAMLGVVLVDNVMVLYMFWELTSLSSFLLIGYWYK   128
MnhA_Staphau LDGLGLLFSLLISGIGSLVVLYSIGYLSK-SEQLGNFYCYLLLFMGAMLGVVLSDNVIILYLFWELTSFSSFLLISFWRE   154
  nuoL_Ecoli LDGLSLTMLSVVTGVGFLIHMYASWYMRG-EEGYSRFFAYTNLFIASMVVLVLADNLLLMYLGWEGVGLCSYLLIGFYYT   159

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 NaH_BacC125 REKSTYGAQKSMLITVFGGFAMLGGFSLLYVITGTFSIREIIEQADAVLA--STLFLPAMLLVLLGAFTKSAQFPFHIWL   233
  MrpA_Bacsub REKSRYGAAKSLLITVSGGLCMLGGFILLYLITDSFSIREMVHQVQLIAG--HELFIPAMILILLGAFTKSAQFPFYIWL   206
MnhA_Staphau RQASIYGAQKSLIITVFGGLSLLGGIILLAIPTQSFSIQYMIQHASEIQN--SPFFIFAMILIMIGAFTKSAQFPFYIWL   232
  nuoL_Ecoli DPKNGAAAMKAFVVTRVGDVFLAFALFILYNELGTLNFREMVELAPAHFADGNNMLMWATLMLLGGAVGKSAQLPLQTWL   239

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 NaH_BacC125 PDAMEAPTPVSAYLHSATMVKAGLYLVARLTPVFGGTPEWFWLLAGFGIITLCWGSISAVRQKDLKSILAFSTISQLGLI   313
  MrpA_Bacsub PDAMEAPTPVSAYLHSATMVKAGIYVIARFSPIFAFSAQWFWIVSLVGLFTMVWGSFHAVKQTDLKSILAFSTVSQLGMI   286
MnhA_Staphau PDAMEAPTPVSAYLHSATMVKAGLYLIARMTPIFAASQGWVWTVTLVGLITLFWASLNATKQQDLKGILAFSTVSQLGMI   312
  nuoL_Ecoli ADAMAGPTPVSALIHAATMVTAGVYLIARTHGLFLMTPEVLHLVGIVGAVTLLLAGFAALVQTDIKRVLAYSTMSQIGYM   319

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 NaH_BacC125 MCLFGLGSATLHFDPTDSMIKFYATATLAAVFHLINHATFKGSLFMTVG-IIDHETGTRDIRKLGGLMAIMPVTFTVSLI   392
  MrpA_Bacsub ISMLGVSAAALHYGHT----EYYTVAAMAAIFHLINHATFKGSLFMAVG-IIDHETGTRDIRKLGGLMAIMPITFTISLI   361
MnhA_Staphau MAMLGIGAISYHYQ--GDDSKIYAAAFTAAIFHLINHATFKGALFMITG-AVDHSTGTRDVKKLGGLLTIMPISFTITVI   389
  nuoL_Ecoli FLALGV------------Q------AWDAAIFHLMTHAFFKALLFLASGSVILACHHEQNIFKMGGLRKSIPLVYLCFLV   381

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 NaH_BacC125 GLAAMAGLPPFNG-FLSKEMFFSGVVTATTLGIFNMETWGFLFPVLAWVASVFTFVYCAIMFFRTFTGEFKKENYDVHVH   471
  MrpA_Bacsub GTFSMAGLPPFNG-FLSKEMFFTSMLRVTHFDLFNVQTWGVLFPLFAWIGSVFTFIYSMKLLFKTFRGNYQPEQLEKQAH   440
MnhA_Staphau TALSMAGVPPFNG-FLSKESFLETTFTASQANLFSVDTLGYLFPIIGIVGSVFTFVYSIKFIMHIFFGQYKPEQLPKKAH   468
  nuoL_Ecoli GGAALSALPLVTAGFFSKDEILAGAMANGHINLM--------V--AGLVGAFMTSLYTFRMIFIVFHGKEQIHAHAVKG-   450

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 NaH_BacC125 EAPMGMLISPVILGSLVVIFGLFPNLLTYTIIEPAVTAILGADVFGGGE---ANIYLWHGFNPELFMTIAVVAFGTLIFM   548
  MrpA_Bacsub EAPVGMLVPPVILVALAVSLFFFPNILSYSLIEPAMNSIY-PTLLDGHEKFHVHISQWHGVTTELLMTAGIVVIGTIGYL   519
MnhA_Staphau EVSILMLLSPAILATLVIVFGLFPGILTNSIIEPATSSIN-HTVIDD-----VEFHMFHGLTPAFLSTLVIYILGILLIV   542
  nuoL_Ecoli ----------VTHSLPLIVLLILSTFVGALIVPPLQGVLPQTTELAHG------SMLTLEITSGVVAVVGILLAAWLWLG   514

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 NaH_BacC125 NMKKWQQTAFYLRERDPLNRVYDSS-LDWLIKGSQGITRVQMTGML-RDYFAYMCVFMILLFGYTMVRYNAFAIDTVNVS   626
  MrpA_Bacsub SLNKWKGIYKLFPSKLTLNRLYDKL-LTMMEKGSYRVTKQYMTGFL-RDYLLYIFAGFIILIGGAFAIKGGFSFKTEGMA   597
MnhA_Staphau TFSYWVKLLQRQPGKLTFNYWYNRS-ANVIPNYSEKMTNSYVTDYS-RNNLVIIFGALILLT-FVTIFSVPFNININFKDVS   619
  nuoL_Ecoli KRTLVTSIANSAPGRLLGTWWYNAWGFDWLYDKVFVKPFLGIAWLLKRDPLNSMMNIPAVLS-----RFAGKGLLLSENG   589

             :  :   : :
 NaH_BacC125 VISPYMWVLTLVFIGSTLAIPFINHRITAIIVVGVIGFLLALFFVIFRAPDLALTQLLVETVTVVLFMLAFYHLPELRKE   706
  MrpA_Bacsub KIGVYEIILTLVMISATVATVFARSRLTAIIALGVVGYTLALFFVIFRAPDLALTQLVIETISVALFLLCFYHLPKLRLK   677
MnhA_Staphau PIRIFEVCIVILLLSAAFLILFAKSRLFSIIMLSAVGYAVSVLFIFFKAPDLALTQFVVESISTALFLLCFYHLPNLNRY   699
  nuoL_Ecoli YLRWYVASMSI-------------------------------------------------------------------   600


 NaH_BacC125 KFTPRFNLVNLFISIGVGGLVTLIALSSFALGTEAGLGTISEYFIENSKELAGGYNMVNVILVDFRGLDTLLEVLVLGIA   786
  MrpA_Bacsub TKTRTFRMTNFIISLGVGVIVTLLGIASSSQRTKD---SIASFFVKHSHDLGGGDNVVNVILVDFRGFDTMFEITVLTIA   754
MnhA_Staphau NEKRSFQLTNALIAGGVGLSVIIIGLIAYGN---RHFESISKFYQEHVYDLAHGKNMVNVILVDFRGMDTLFESSVLGIA   776
  nuoL_Ecoli ----------------------------------------------------------------------------   600


 NaH_BacC125 ALGVIAMIKLR---MTGREDV----   804
  MrpA_Bacsub ALGIYSMIKTKVK-EEGKSGE----   774
MnhA_Staphau GLAVYTMIKLRKKRQTQGNEVKNHE   801
  nuoL_Ecoli -------------------------   600
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Fig. 6. Conserved domains in secondary Na^+/H^+ antiporters and the NuoL subunit from *E. coli* complex I. The sequences of Na^+/H^+ antiporters from *Bacillus* strain C-125 (accession number BAA06609) [47], *Bacillus subtilis* (YufT, designated MrpA, accession number Z93937) [50], and *Staphylococcus aureus* (MnhA, accession number BAA35095) [48] were aligned with the NuoL subunit from *E. coli* (accession number AE000317) [58] using the program ClustalX [55]. Conserved residues are indicated by asterisks.
←

single enzyme is represented by phthalate dioxygenase reductase [20] (Fig. 3A).

Several spectroscopic features of the NqrF subunit underline its similarity to FNR. Like in FNR, a blue neutral flavosemiquinone is transiently observed in the Na^+-NQR from *V. alginolyticus* under low oxygen tension. During a reductive titration and subsequent reoxidation of the Na^+-NQR with ubiquinone-1 under exclusion of oxygen, no flavosemiquinone is stabilized, but a transient increase in absorbance around 800 nm is observed that is tentatively assigned as a charge transfer complex between reduced flavin and NAD^+ [13]. Again, similar features are displayed by FNR from *Anabaena* PCC7119, where the stacking of the nicotinamide moiety to the isoalloxazine ring was confirmed in the high-resolution structure of a mutant FNR [19]. The spectroscopic properties of the NqrF subunit, together with the conserved domains required for the binding of FAD and the [2Fe–2S] cluster, make it possible to propose a structural model based on the high-resolution structures of ferredoxin from *Anabaena* PCC7119 [21] (N-terminal domain of NqrF, Val8–Glu133) and FNR from the same organism [19] (C-terminal domain of NqrF, Cys134–Gly407) (Fig. 3B). The two domains were modeled separately and combined in accordance with the relative orientation of the flavin and Fe/S domains found in the phthalate dioxygenase reductase (Fig. 3A) [22]. Since FNR, ferredoxin, and phthalate dioxygenase reductase are soluble proteins, no reasonable prediction for the relative orientation of the N-terminal anchor of the NqrF subunit can be made. Hydropathy analysis programs predict that the NqrF subunit possesses two N-terminal, transmembrane helices, but the structural model clearly illustrates that only the first hydrophobic region (Ile4–Ala24) could insert into the membrane (Fig. 3B). The second hydrophobic stretch (Gly51–Ser74) contributes to the Cys69-$(X)_5$-Cys75-$(X)_2$-Cys78 motif. This characteristic arrangement of cysteine residues allows the ligation of Fe atoms in a conserved loop in the N-terminal ferredoxin domain of NqrF that cannot be reconciled with an α-helical structural element (Fig. 3C). The

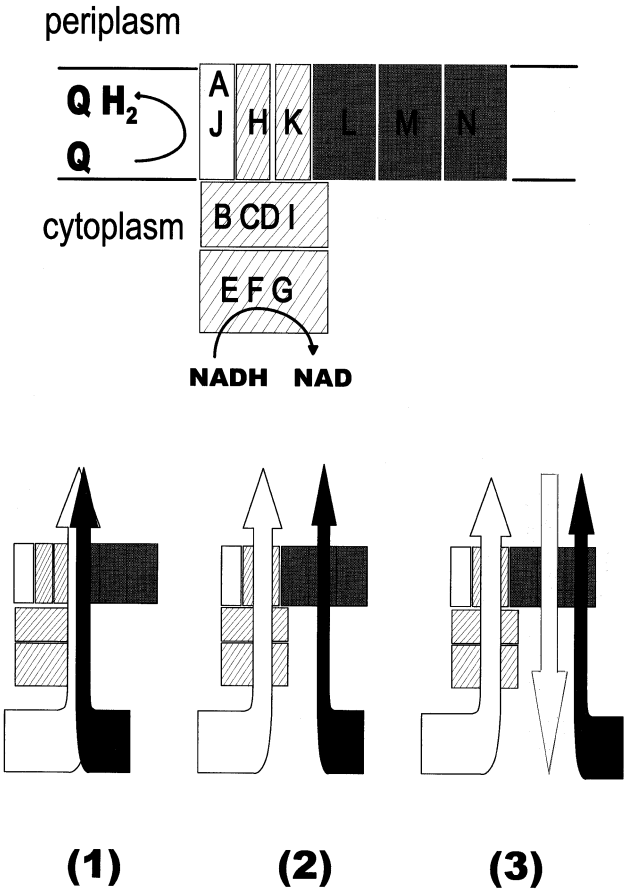

Fig. 7. Three putative mechanisms of H^+ and Na^+ translocation by complex I. The oxidation of NADH is coupled to the translocation of H^+ or Na^+ from the cytoplasmic to the periplasmic space. The electron transfer proceeds from NADH via FMN and Fe/S clusters to external quinone in the membrane. Nuo (NADH:ubiquinone oxidoreductase) subunits exhibiting sequence similarity to hydrogenases are hatched, whereas the Na^+/H^+ antiporter-related NuoL, NuoM and NuoN subunits are given in gray. Note that the NuoL [45] or NuoL and M subunits [25] are also thought to be evolutionarily related to subunits of membrane-bound hydrogenases. White arrows indicate movements of protons, black arrows indicate movement of Na^+ across the membrane. (1) A single cation-translocating site acting either on H^+ or on Na^+. (2) Two distinct coupling sites for active H^+ or Na^+ extrusion. (3) A primary H^+ pump fused to a Na^+/H^+ antiporter.

[2Fe–2S] center is supposed to be located at the cytoplasmic aspect of the membrane in a high dielectric environment, where the uptake of an electron could be compensated by proton uptake from the aqueous phase (Fig. 3B). Therefore, the direct participation of the Fe/S center in Na^+ transport suggested by Rich et al. [12] seems rather unlikely. Moreover, NADH oxidation catalyzed by the isolated NqrF subunit is not activated by sodium ions [8], as would be expected for a redox reaction that is coupled to Na^+ transport.

There are several open questions regarding the redox properties of the FAD and the [2Fe–2S] cluster on the NqrF subunit. An optical titration of a flavin prosthetic group of the Na^+-NQR from *V. alginolyticus* under aerobic conditions revealed a single $n=2$ component with a midpoint redox potential of −295 mV that could not be assigned to either FAD or FMN [23]. In contrast, a flavosemiquinone was observed in titrations performed with the Na^+-NQR and the NADH dehydrogenase subcomplex, indicating the formation of a one-electron reduced FAD on the NqrF subunit [13]. Knowledge of the redox potentials of the flavin and Fe/S cofactors will be a prerequisite to understand the electron transfer reactions in the Na^+-NQR. Determination of the flavin redox potentials probably requires the dissection of the complex, whereas redox titrations of the [2Fe–2S] cluster followed by ESR have been hampered by unspecific radical signals, probably due to superoxide damage [3].

3. Na^+ translocation by complex I (NADH:quinone oxidoreductase) from *K. pneumoniae* and *E. coli*

3.1. Architecture and cofactors of bacterial complex I

Complex I from bacteria is composed of 14 subunits encoded by the *nuo* operon. In *E. coli*, the *nuoC* and *nuoD* genes are fused, resulting in a NuoCD subunit [4]. The bacterial complex I, also termed NDH-I, has been studied with respect to primary sequence, cofactor content and regulation in various microorganisms [4,24–27]. Since every subunit present in the bacterial complex I has a homologue in the mitochondrial enzyme, the smaller bacterial complex I is regarded as a model for the analysis of the structure and function of its larger counterpart. The sequence of complex I from *K. pneumoniae* is being determined in a genome sequencing project [28] (http://genome.wustl.edu/gsc/Projects/bacteria.shtml). Due to its high degree of sequence identity with complex I from *E. coli* (84%), it is reasonable to assume that both enzymes exhibit similar biochemical and physicochemical properties. Complex I from *E. coli* is an L-shaped multisubunit complex with an approximate mass of 530 kDa [29]. Upon purification, the complex tends to disrupt into a peripheral and a so-called connecting fragment harboring the FMN and Fe/S clusters (subunits NuoB, CD, E, F, G, I), and a membranous fragment composed of the subunits NuoA, H, J, K, L, M, N [4] (Fig. 7). For a detailed description of the redox properties and localizations of the different Fe/S clusters, the reader is referred to [30,31]. The oxidation of NADH is catalyzed by the peripheral NuoF subunit harboring FMN and a [4Fe–4S] cluster. Subsequently, electrons are delivered via additional Fe/S centers to the cluster(s) with the highest midpoint redox potential, termed N2 (or 2). It seems to be generally accepted that cluster N2 donates electrons to an as yet unidentified, enzyme-bound quinone or quinoid cofactor. Re-oxidation of this cofactor by external Q acting as a substrate is thought to be linked to H^+ translocation [32,33]. Inhibitors acting on the Q site(s) of complex I were used to identify subunits that might participate in Q binding. Biochemical data point to the NuoB subunit [34], whereas mutational analysis indicates the participation of the NuoD subunit [35]. As both subunits belong to the connecting fragment of complex I, these putative Q sites are difficult to envisage as a component of a H^+-pumping, membrane-embedded redox unit.

3.2. Experimental evidence for Na^+ transport by complex I

The conclusion that complex I from *K. pneumoniae* or *E. coli* catalyzes primary Na^+ transport is based on four lines of evidence, namely measurements of respiratory Na^+ transport by native membrane vesicles, identification of subunits from complex I in Na^+-stimulated NADH:Q oxidoreductase fractions, growth studies and determination of Na^+ transport in a Na^+/H^+ antiporter-deficient *E. coli*

strain, and controls performed with an *E. coli* mutant devoid of complex I.

The respiratory Na^+ transport into native membrane vesicles from *K. pneumoniae* or *E. coli* during the oxidation of NADH or the complex I-specific substrate deaminoNADH with oxygen did not collapse in the presence of a protonophore, indicating that the transport was accomplished by a primary Na^+ pump and not by the combined action of a proton pump and the Na^+/H^+ antiporter(s). In the case of *K. pneumoniae*, the rates increased even threefold in the presence of protonophore, resulting in an internal Na^+ concentration that was three times higher than the external Na^+ concentration (Fig. 4). With both *E. coli* and *K. pneumoniae* vesicles, the Na^+ transport was severely inhibited by the complex I-specific inhibitor rotenone [36,37] (Fig. 4). An enrichment of *K. pneumoniae* complex I was achieved following the Na^+-stimulated Q reductase activity during purification. This increase in enzymatic activity of complex I in the presence of its coupling ion is reminiscent of other primary Na^+ pumps. N-terminal sequencing of two polypeptides present in the Na^+-stimulated NADH:Q oxidoreductase confirmed the identity with the corresponding complex I subunits [36]. Further support for the hypothesis that complex I from *E. coli* acts as a Na^+ pump came from studies with mutant strains devoid of either secondary Na^+/H^+ antiporters or complex I. The antiporter-deficient strain did not tolerate high concentrations of NaCl in the growth medium under conditions where complex I was repressed, but was not affected by Na^+ ions if grown under complex I-inducing conditions. On the other hand, with membrane vesicles from the *E. coli* mutant devoid of complex I, the rate of respiratory Na^+ transport was severely reduced [37]. Taken together, these data clearly point to Na^+ transport by complex I from *K. pneumoniae* and *E. coli*.

3.3. Coupling of Na^+ transport to electron transfer

The finding that the electrogenic NADH:Q oxidoreductases (NDH-I) from *K. pneumoniae* and *E. coli* are capable of Na^+ translocation has important implications for the mechanism of cation translocation by complex I in general. Detailed descriptions of putative mechanisms of H^+ translocation by complex I are given in the reviews of Brandt [32] and Dutton and colleagues [33]. Since only H^+, but not Na^+, can be transported across the membrane by diffusion of the proton carrier QH_2, a mechanism of cation translocation by complex I from *K. pneumoniae* and *E. coli* that exclusively relies on a Q-cycle type of mechanism [38] can be excluded. As a consequence, a Na^+-translocating redox pump is likely to operate. A general reaction mechanism of redox-linked Na^+ transport is described that might function in the Na^+-NQR from *Vibrio* sp. and the Na^+ translocating complex I from *E. coli* and *K. pneumoniae*. It is proposed that the central unit of a Na^+-translocating NADH:quinone oxidoreductase is a redox cofactor that accepts a Na^+ from the negative side of the membrane upon reduction. During reoxidation of the cofactor, the Na^+ is pushed to the positive side of the membrane, thereby generating an electrochemical potential. This concept is based on the electroneutrality principle that states that a negative charge generated in an environment of low dielectric strength is compensated by the uptake of a positive charge [12]. The central redox cofactor of the Na^+-translocating NADH:quinone oxidoreductase is thought to be a bound quinoid group that shuttles between the fully oxidized and the one-electron reduced, anionic state (Fig. 5). The quinoid cofactor is most likely reduced by a Fe/S cluster, namely the [2Fe–2S] cluster in the case of the Na^+-NQR, or the high-potential cluster N2 in the case of complex I. By this one-electron transfer, an enzyme-bound semiquinone is generated that is deprotonated at physiological pH (pK_a $QH^\bullet/Q^{\bullet -} = 4.9$) [39]. Upon formation of the bound semiquinone anion, Na^+ is taken up from the negative side of the membrane, forming an ion pair with the negatively charged semiquinone. The one-electron oxidation of the semiquinone by external Q (substrate) is coupled to the extrusion of Na^+ against the electrochemical potential to the positive side of the membrane. A second cycle of Na^+ uptake and extrusion is initiated by the incoming second electron that again generates a semiquinone anion. This time, the bound semiquinone anion is oxidized by the substrate semiquinone remaining bound to the enzyme, and the two-electron reduced product quinol is formed (Fig. 5). The formation of quinol from semiquinone is an exergonic reaction ($E^{\circ\prime}$ $QH_2/QH^\bullet + H^+ = +190$ mV) that

drives the unfavorable reduction of quinone to semiquinone ($E^{\circ\prime}$ $Q^{\bullet-}/Q = -240$ mV) [39] in the preceding reaction steps. In one complete turnover of the Na^+ translocating NQR, two electrons from NADH are transferred to quinone, and $2Na^+/2e^-$ are translocated.

This model is in accordance with a Na^+/e^- stoichiometry equal to 0.71 of the Na^+-NQR determined with whole cells of *V. alginolyticus* [40]. With the purified Na^+-NQR reconstituted into proteoliposomes, the value decreased to 0.5 Na^+ ions transported per two electrons transferred [3]. This low ratio probably reflects a minimum value since reconstituted proteoliposomes are usually less perfectly coupled than ion pumps working under in vivo conditions. There is no experimental evidence for H^+ transport catalyzed by the Na^+-NQR. The transport stoichiometry of complex I has not been determined yet, since neither mitochondrial nor bacterial complex I has been purified in an active state that allows reconstitution into proteoliposomes. From studies with submitochondrial particles or whole *E. coli* cells, the ratio of protons translocated to electrons transferred by complex I is considered to be $4H^+/2e^-$ [32,41,42] or $3H^+/2e^-$ [43]. Higher stoichiometries of up to $6H^+/2e^-$ are also discussed, which are in line with the large ΔG° of the substrates of complex I [33]. The determination of transport stoichiometries of reconstituted, Na^+-translocating complex I is in progress. If the ratio of cations transported to electrons transferred proves to be greater than one, a second coupling site for complex I has to be assumed in addition to the Na^+ redox pump described above.

3.4. Redox pump versus transporter: putative sites of Na^+ (and H^+) transport in complex I

The observation that Na^+ is transported by complex I from *K. pneumoniae* and *E. coli* raises two fundamental questions: are Na^+ ions transported instead of (or in addition to) protons, and which subunits of complex I contribute to Na^+ translocation? Without a direct in vivo or in vitro assay for cation transport by complex I, no site-directed mutants can be analyzed for their transport capacity. Therefore, the assignment of transport functions to distinct subunits is speculative and based on sequence comparisons. It has been early recognized that the NuoE, F, and G subunits of the peripheral complex I domain are related to NAD-reducing, soluble hydrogenases [44]. Furthermore, membranous subunits of complex I were found to be evolutionarily related to subunits of membrane-bound hydrogenases [25,45]. These multisubunit complexes catalyze the reversible oxidation of ferredoxin or formate and generation of H_2 from H^+. As a consequence, H^+ channel and/or H^+ transport functions were discussed for the membranous NuoK, L, M, and N subunits of complex I [25]. The NuoL, M, and N subunits have very likely arisen from a common ancestor [46] and show sequence similarity to Na^+/H^+ antiporters, which further seemed to support their participation in H^+ translocation [4]. The novel class of multisubunit Na^+/H^+ antiporters related to NuoL and NuoN was discovered by complementation of an alkali-sensitive mutant of *Bacillus* C-125 and restored secondary, $\Delta\mu H^+$-driven Na^+ efflux from whole cells [47]. The authors identified a single open reading frame encoding a hydrophobic protein that exhibited striking sequence similarity to the membranous ND5 subunit (NuoL homologue) of complex I. Especially in the N-terminal region, three well conserved domains were recognized (Fig. 6). However, the expression of this putative Na^+/H^+ antiporter did not increase the NADH dehydrogenase activity in *Bacillus* membranes [47]. A corresponding gene, *mnhA*, was identified in *Staphylococcus aureus* (Fig. 6) and was shown to be part of an operon encoding seven genes (*mnhA–G*), with *mnhA* and *mnhD* encoding hydrophobic polypeptides with high similarities to NuoL and NuoN, respectively [48]. From heterologous complementation studies using Na^+/H^+ antiporter-deficient or alkali-sensitive *E. coli* strains, the authors concluded that all seven gene products of the *mnh* operon were required in order to confer Na^+/H^+ antiport activity. Such a large antiporter with an approximate mass of 200 kDa was unexpected compared to the single-subunit, bacterial antiporters described so far [49]. However, genetic studies with the corresponding *mrp* operon in *Bacillus subtilis* showed that although the *mrpF* and *mrpG* gene products (*mnhF* and *mnhG* in *S. aureus*) were also involved in Na^+ or cholate transport, overexpression of the single *mrpA* gene (NuoL homologue) in a polar *mrpA* mutant of *B. subtilis* producing only low levels of the downstream *mrpB, C, D, E, F, G* gene prod-

ucts raised the Na^+/H^+ antiport activity significantly [50]. In summary, it seems likely that the NuoL homologues (MnhA or MrpA) function as Na^+/H^+ antiporters, but that other gene products of the multisubunit Na^+/H^+ antiporter operons may also contribute to Na^+ extrusion.

Based on these studies, it is proposed that Na^+ extrusion is catalyzed by the membranous NuoL (or NuoM and N) subunits of complex I from *E. coli* and *K. pneumoniae*, whereas H^+ pumping is thought to be mediated by subunits that are homologous to membrane-bound hydrogenases (Fig. 7). Most likely, active Na^+ pumping is only catalyzed by the entire complex, but membranous subcomplexes may have the capacity for passive Na^+ (or H^+) translocation as observed with the F_0 part of the F_1F_0 ATP synthase. Assembly studies performed with complex I showed that distinct parts of the membrane arm are formed that might also represent functional units. In the fungus *Neurospora crassa*, a small intermediate containing the ND5 (NuoL) and ND2 (NuoN) subunits was identified that is thought to combine with a second, membranous fragment containing homologues of the membranous NuoA, H, J, K, M subunits [51].

Three hypothetical mechanisms of cation translocation by complex I should be considered (Fig. 7). Protons and Na^+ ions could follow the same translocation pathway through complex I, with either Na^+ or H^+ being the preferred substrate (Fig. 7, 1). This mode of action is found in the Na^+-translocating ATPase from *Propionigenium modestum*, where protons are translocated only in the absence of Na^+ [52]. Note that this model implies a single coupling site per complex I. If a stoichiometry $\geq 4(H^+$ or $Na^+)/2e^-$ for complex I is assumed, this putative mechanism cannot be reconciled with the general model for electron transfer and Na^+ translocation as depicted in Fig. 5. A second possible mechanism implies two coupling sites converting redox energy into an electrochemical potential, one site for protons and one for Na^+. In the third model, a primary H^+ pump is fused to a Na^+/H^+ antiporter, utilizing the proton motive force established during NADH oxidation (Fig. 7, 3). So far, neither model can be rejected unequivocally, but the existence of two coupling sites in complex I is favored (Fig. 7, 2), with a H^+-pumping site established by subunits that are homologous to hydrogenases, and a Na^+-translocating machinery confined to the NuoL, M or N subunits related to Na^+/H^+ antiporters.

4. Prospects

Compared to the H^+-translocating counterparts, Na^+-dependent NADH:quinone oxidoreductases will offer experimental advantages, such as the determination of Na^+/e^- stoichiometries, the identification of Na^+-dependent steps during catalysis, or the identification of critical subunits and amino acid residues required for Na^+ translocation. The latter problem can be approached by complementation of a Na^+-sensitive *E. coli* mutant that is deficient in Na^+/H^+ antiporters [53]. Detailed knowledge of the mechanism of Na^+ transport by complex I from *E. coli* and *K. pneumoniae* will be of relevance to the H^+-pumping, mitochondrial enzyme, assuming that only the cation selectivity, but not the mechanism of transport, is altered in the Na^+- or H^+-translocating complex I.

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