NADH Oxidation by the Na\textsuperscript{+}-translocating NADH:Quinone Oxidoreductase from Vibrio cholerae

FUNCTIONAL ROLE OF THE NqrF SUBUNIT

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The Na\textsuperscript{+}-translocating NADH:quinone oxidoreductase from Vibrio cholerae is a six subunit enzyme containing four flavins and a single motif for the binding of a Fe-S cluster on its NqrF subunit. This study reports the production of a soluble variant of NqrF (NqrF\textsuperscript{S}) and its individual flavin and Fe-S-carrying domains using V. cholerae or Escherichia coli as expression hosts. NqrF\textsuperscript{S} and the flavin domain each contain 1 mol of FAD/mol of enzyme and exhibit high NADH oxidation activity (20,000 \textmu mol min\textsuperscript{-1} mg\textsuperscript{-1}). EPR, visible absorption, and circular dichroism spectroscopy indicate that the Fe-S cluster in NqrF\textsuperscript{S} and its Fe-S domain is related to 2Fe 2S in the vertebrate-type. The addition of NADH to NqrF\textsuperscript{S} results in the formation of a neutral flavosemiquinone and a partial reduction of the Fe-S cluster. The NqrF subunit harbors the active site of NADH oxidation and acts as a converter between the cluster and its Fe-S domain is related to 2Fe 2S of the vertebrate-type. The observed electron transfer NADH → FAD → [2Fe-2S] in NqrF requires positioning of the FAD and the Fe-S cluster in close proximity in accordance with a structural model of the subunit.

The ability to diminish the intracellular Na\textsuperscript{+} concentration by specific transporters is common to many organisms. The uphill transport of Na\textsuperscript{+} against an electrochemical potential catalyzed by Na\textsuperscript{+}/H\textsuperscript{+} antiporters is driven by the proton motive force (1). In addition, some bacteria and archaea possess Na\textsuperscript{+} pumps that directly couple an exergonic reaction to the endergonic transport of Na\textsuperscript{+} across the membrane (2). For example, the oxidation of NADH with quinone catalyzed by membrane-bound NADH dehydrogenases generates an electrochemical Na\textsuperscript{+} gradient that can be used to drive the uptake of nutrients. Two distinct classes of bacterial Na\textsuperscript{+}-translocating NADH dehydrogenases are known. Enterobacteria like Escherichia coli possess a Na\textsuperscript{+}-dependent NADH dehydrogenase that is homologous to complex I of the mitochondrial respiratory chain (3–5). Another type of NADH-driven redox pump (called Na\textsuperscript{+}-NQR)\textsuperscript{1} is found in marine bacteria like Vibrio alginolyticus or the human pathogen V. cholerae (6–11). The Na\textsuperscript{+}-NQR is encoded by the nqr operon that comprises the structural genes nqrA-nqrF (12, 13) encoding for six different subunits present in the complex (9). NqrF is the only subunit that contains four closely spaced cysteine residues (14) that are important for the formation of the Fe-S cluster (Cys111-Cys114). The enzyme is efficient in NADH oxidation and acts as a converter between the cluster and its Fe-S domain is related to 2Fe 2S of the vertebrate-type. The observed electron transfer NADH → FAD → [2Fe-2S] in NqrF requires positioning of the FAD and the Fe-S cluster in close proximity in accordance with a structural model of the subunit.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—V. cholerae 0395 N1 (20) served as a source of genomic DNA for PCR cloning of nqrF constructs. Cloning was carried out in E. coli DH5\textalpha using standard techniques (21). Gene sequences encoding the shortened NqrF subunit, its Fe-S domain, or flavin domain were amplified from chromosomal DNA by PCR. The forward primers for amplification of sequences encoding a truncated NqrF subunit or its Fe-S domain were designed to excise bp 7–75 of the nqrF gene. Hereby, amino acids Thr\textsuperscript{3}–Ala\textsuperscript{25} including the predicted single N-terminal transmembrane helix (Val\textsuperscript{11}–Ala\textsuperscript{25}) of the NqrF subunit were removed, and soluble variants of NqrF (NqrF\textsuperscript{S}) and its Fe-S domain were produced (see Fig. 1). Primer sequences and restriction sites are given in Table I. The 3’-ends of the reverse primers for NqrF\textsuperscript{S} and the flavin domain construct were homologous to sequences downstream of the stop codon of nqrF. PCR amplification by Pfu polymerase (Stratagene) was carried out as described by the supplier with an annealing temperature of 56 °C and an amplification time of 2 min and 40 s. PCR products encoding for NqrF\textsuperscript{S} or the flavin domain were digested with NdeI and EcoRI and ligated into the arabinose-inducible expression vector pEC422 (22), yielding pNF3 and pFNF8. The PCR fragment encoding the Fe-S domain was digested with NdeI and XhoI and inserted into pET-16b (Novagen) to give pFS224. The translation product of pFS224 has a C-terminal extension of 21 amino acids, and the stop codon is conferred by the vector. All three constructs add N-terminal

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¶ Dedicated to Peter M. H. Kroneck on the occasion of his 60th birthday.

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1 The abbreviations used are: Na\textsuperscript{+}-NQR, Na\textsuperscript{+}-translocating NADH:quinone oxidoreductase; FNR, ferredoxin:NADP\textsuperscript{+} reductase; BenC, benzoate 1,2-dioxigenase reductase; LB, Luria-Bertani; Ni-NTA, nickel-nitrilotriacetic acid; E\textsubscript{m}, midpoint redox potential; W, watt.
His tags to the target proteins consisting of six histidine residues in the case of the NqrF and its flavin domain and ten histidine residues in the case of the Fe-S domain. The expression vectors pNF3 or pFN8 were transformed into V. cholerae as described in Ref. 23. E. coli BL21 (DE3) (Stratagene) was transformed with pFS224. The cloned gene fragments were sequenced, and in the case of the fragments encoding the NqrF or the flavin domain the fragments were found to be identical to the restriction sites for NdeI and EcoRI (NqrF (1-ml bed volume, 1.4-cm diameter, Merck). Concentrated NqrF was diluted 10-fold in 50 mM NaCl and the Flavins domain was eluted with 400 mM imidazole.

Fe-S domain was purified from the reaction mixture by affinity chromatography on the Ni-NTA-agarose column. The soluble fraction containing the Fe-S domain was loaded onto a Ni-NTA-agarse column (Qiagen) equilibrated with buffer A (20 mM Tris/HCl, pH 8.0, 0.5 mM isopropyl-1-thio-D-galactopyranoside, growth was continued by dialyzing against 30 mM imidazole in buffer A, the eluate was collected under a stream of N2. Unbound contaminants identified in NqrF were sequenced, and in the case of the fragments encoding the NqrF and the flavin domain the fragments were found to be identical to the predicted polypeptides derived from pNF3 from V. cholerae El Tor N19681 (Gen-Bank accession number AAP96434) (54). The cloned gene fragments encoding the Fe-S domain contained a single G→A mutation at bp 273 (nqrF numbering). The identity of the Fe-S and the flavin domains with the predicted polypeptides derived from pNF3 from V. cholerae El Tor N19681 was further supported by matrix-assisted laser desorption ionization-mass spectrometry. The observed mass was 17895 Da (calculated, 17873 Da) for the Fe-S domain and 33983 Da for the flavin domain (calculated, 34047 Da). NqrF, the FAD, and the Fe-S domain showed the expected N-terminal sequences.

Cultivation of Bacteria—All strains were cultivated aerobically in Luria-Bertani (LB) medium (21). V. cholerae O395 N1 was cultivated in LB medium supplemented with 10 mM glucose and 50 mM potassium phosphate buffer, pH 7.3, in the presence of 50 μg ml⁻¹ streptomycin and 200 μg ml⁻¹ ampicillin. For the production of NqrF or the flavin domain, 10 liters of medium were inoculated with 250 ml of 10, 4, and 5 mg of Fe-S domain, flavin domain, and NqrF (25) under exclusion of oxygen in cuvettes sealed with a rubber stopper. NADH or sodium dithionite were added in the glove box or with gas-tight syringes. The standard reduction potential of the FAD of the flavin domain was determined by the xanthine/xanthine oxidase method (26) in 100 mM Tris/HCl, pH 7.5, 13.7 mM ubiquinone-1 (ubiquinone-1), ubiquinone-1 (ubiquinone-1), and methyl viologen (Em = −400 mV, pH 7.5, n = 2) were used as redox mediators (27) at final concentrations of 1.8 μM each. The redox state of flavin or NqrF was monitored at 404 or 518 nm, respectively. The Nernst coefficient was determined from the slope of the line ln(FAD+/FAD−) versus ln(PS+/PS−). The difference between the Em of FAD in the flavin domain and the Em of NqrF was calculated from the vertical intercept of the line.

Analytical Methods—Protein was determined by the bicinchoninic acid method using the reagent from Pierce (28) or by the microburet method (29) preceded by trichloroacetic acid precipitation. Bovine serum albumin served as the standard. The concentration of active NqrF and the flavin domain was estimated from the content of oxygen using a MonoQ HR5/5 anionic exchange column connected to an Akta FPLC station (Amersham Biosciences). The flavin domain was diluted 10-fold in 50 mM Hepes/NaOH, pH 7.0, and loaded onto the MonoQ column. A linear gradient of 15 volumes from 0.0–0.4 M NaCl led to the elution of the flavin domain at 0.32 mM NaCl.

| Product      | PCR primers (5'-3')                          | Vector expression host |
|--------------|---------------------------------------------|------------------------|
| NqrF Forward | CGATATGACATCAGCTGTTAACATTAACGGAATGACCGAGG  | pNF3 V. cholerae O395 N1|
| NqrF Reverse | CGTAGAGAATTCCTGAGGAAACATTGCCGCCGGCC      |                        |
| FAD domain   | GCATATGACATGCGCTGAAGATGGGTGAAATGC            | pFN8 V. cholerae O395 N1|
| FAD domain   | GCATGAGAATTCGAGGAGCCAGGCAAT                 |                        |
| Fe-S domain  | GCATATGCTATC7GCTAACATTCAACGTAATGGCGACGAGG  | pFS224 E. coli BL21 (DE3) |
| Fe-S domain  | CATACTCAGAGCTTGTACAGAATTGAAAGGCGGTTCG      |                        |

Iron was determined colorimetrically by the 3-(2-pyridyl)-5,6-bis(2-
NqrF Subunit of the Sodium Pump from Vibrio cholerae

RESULTS

Cofactor Content and Activity of NqrF-derived Polypeptides—The N-terminal half of NqrF with its binding motif for an Fe-S cluster is related to ferredoxins, whereas the C-terminal half comprises motifs for the binding of flavin and NADH (Fig. 1). Three NqrF-derived polypeptides were produced, the NqrF subunit devoid of a putative N-terminal α-helix with a molecular mass of 44,596 Da (NqrF*) and its Fe-S and flavin-containing domains with molecular masses of 17,873 and 34,047 Da, respectively (Fig. 2). NqrF* contained 0.94 ± 0.06 mol of FAD/mol (21.1 ± 1.3 nmol of non-covalently bound FAD/mg). In addition, less than 3.2 nmol/mg FMN and 0.3 nmol of riboflavin/mg were present in NqrF*. The FAD domain contained 0.95 ± 0.06 mol of non-covalently bound FAD/mol (29.6 ± 1.9 nmol of FAD/mg). Again, small amounts of FMN (<2.8 nmol/mg) and riboflavin (<0.2 nmol/mg) were also detected. These results demonstrate that the non-covalently bound FAD present in the Na⁺-NQR complex resides in the NqrF subunit. Both NqrF* and the flavin domain exhibited high NADH dehydrogenase activities with ubiquinone-1 as an artificial electron acceptor (up to 20,000 μmol min⁻¹ mg⁻¹), depending on the FAD content of the enzyme specimen. The increase of enzymatic activity with increasing concentrations of NADH was very similar for NqrF* and the flavin domain, with half-maximal activities observed in the presence of 2–4 μM NADH. A characteristic property of Na⁺-NQR is its inhibition by silver ions (44). Half-maximal inhibition of the flavin domain by Ag⁺ was observed in the presence of 670 nM Ag⁺. NqrF* produced in V. cholerae contained 0.35 ± 0.02 mol of Fe and 0.38 ± 0.12 mol of acid-labile sulfide/mol of NqrF*. Although FAD was efficiently inserted during overproduction of NqrF*, the assembly of the Fe-S cluster was clearly substoichiometric. Assuming that the Fe-S cluster on NqrF* is of the 2Fe-2S type (see below), our results indicate that 18% of NqrF* overproduced in V. cholerae contained an Fe-S cluster. Treatment with the cysteine desulfurase NifS did not increase the amounts of iron and acid-labile sulfide in NqrF* and did not result in higher spin concentrations monitored by EPR. Attempts to reconstitute the Fe-S cluster under slightly denaturing conditions resulted in the loss of the FAD. The situation was different in the isolated Fe-S domain of the NqrF subunit. Here, the amount of Fe-S cluster inserted in vivo (0.40 ± 0.08 mol of Fe and 0.40 ± 0.10 mol of acid-labile sulfide/mol) could be increased by subsequent in vitro assembly with the help of NiFS (1.61 ± 0.28 mol of Fe and 1.31 ± 0.11 mol of acid-labile sulfide/mol). The NiFS-catalyzed insertion of the Fe-S cluster was accompanied by an

![Fig. 1. Design of functional domains from the Fe-S and flavin-carrying NqrF subunit of the Na⁺-translocating NADH:quinone oxido-reductase (Na⁺-NQR).](image1)

![Fig. 2. SDS-PAGE of the N terminally truncated NqrF subunit and its flavin and Fe-S domains.](image2)
increase in absorption with typical maxima at 340, 410, 451, and 560 nm. The content of iron and acid-labile sulfide indicated that the reconstituted Fe-S domain harbors a 2Fe-2S cluster in accordance with its spectroscopic properties (see below). We observed a rapid degradation of the Fe-S cluster in NqrF\(^{11032}\) and the Fe-S domain during few minutes after exposure to air. The NqrF\(^{11032}\) domain lost its brown-yellow color typical for Fe-S-containing flavoproteins and turned yellow, whereas the Fe-S domain bleached completely. A loss of the Fe-S center during purification in the presence of oxygen was also observed with the Na\(^{-}\)/H\(^{+}\)-NQR complex from V. alginolyticus (10).

Midpoint Redox Potential of the FAD in the Flavin Domain

The flavin domain exhibited an optical spectrum typical for oxidized flavoproteins with maxima at 396 and 454 nm and a shoulder at 480 nm (Fig. 3). Upon addition of substoichiometric amounts of NADH (10 \(\mu\)M) to the flavin domain (14 \(\mu\)M), the transient formation of a blue neutral flavosemiquinone with a characteristic absorbance in the range from 520 to 660 nm was observed (Fig. 3A). The flavosemiquinone was stable for 20–30 min. From the difference in absorbance at 580 nm and the absorption coefficient of the neutral flavosemiquinone in flavodoxin (\(\epsilon_{580} = 4.5 \text{ mm}^{-1} \text{ cm}^{-1}\) (45), a flavosemiquinone concentration of 4 \(\mu\)M was calculated indicating that \(\sim\)30% of the FAD cofactor in the flavin domain was in the one-electron-reduced state under these conditions. The formation of the one-electron-reduced flavin from the obligate two-electron donor NADH is unexpected and might be the result of a compartmentation reaction between two FAD domains in the fully oxidized and the fully reduced state, respectively (46). Addition of excess NADH (73 \(\mu\)M) to the FAD domain (30 \(\mu\)M) resulted in the complete reduction of the FAD (Fig. 3B). The Na\(^{-}\)-NQR complex purified in the presence of oxygen stabilized a neutral flavosemiquinone in the as isolated state, whereas an anionic flavosemiquinone was observed in the Na\(^{-}\)-NQR after addition of excess dithionite (7, 47). We did not detect radicals in the flavin domain as isolated or in the flavin domain treated with an excess of NADH by EPR spectroscopy. The midpoint redox potential of the FAD in the flavin domain was determined by comparison with a suitable redox indicator of known midpoint potential. Using the two-electron donor phenosafranin, a theoretical Nernst coefficient of 1.0 is expected if the FAD in the flavin domain undergoes a two-electron reduction. A Nernst coefficient of 1.33 was obtained for the FAD in the flavin domain indicating that the FAD acted as two-electron acceptor during the redox titration. The FAD in the flavin domain of the NqrF subunit exhibited an overall redox midpoint potential of...
The Na⁺-NQR contains four flavins, one Fe-S center, and ubiquinone-8 as cofactors. These redox centers are likely to participate in the electron transfer from NADH to membrane-bound quinones coupled to the transport of Na⁺ against an electrochemical potential. The description of the individual redox centers is a prerequisite to elucidate the transport mechanism of Na⁺-NQR. A role for flavosemiquinones during redox-driven Na⁺ transport has been proposed for the Na⁺-NQR complexes from *V. cholerae* (47) and *V. harveyi* (7). In both studies, a neutral flavosemiquinone was observed in the Na⁺-NQR purified in the presence of oxygen. Upon the addition of a...
reductant, this neutral flavosemiquinone disappeared, and an anionic flavosemiquinone was detected. Here we demonstrate that the NqrF subunit represents the site of electron entry into the Na⁺-NQR complex. The FAD in NqrF acts as a converter between the hydride donor, NADH, and the one-electron acceptor, the 2Fe-2S cluster, under transient formation of a neutral flavosemiquinone. The anionic flavosemiquinone identified in the Na⁺-NQR (7, 47) must therefore arise from one of the covalently bound FMNs on NqrB and NqrC, or from the riboflavin that is also present in the complex (18).

Productive electron transfer from the FAD to the 2Fe-2S cluster in NqrF requires a spacing of 14 Å or less between these redox centers (52). The structural arrangement of the FAD and the 2Fe-2S cluster can be predicted from a model of NqrF (Fig. 8) derived from high-resolution structures of related enzymes. Sequence comparisons indicate that the NqrF subunit comprises two functional units. The N-terminal part contains the cysteine motif required for the binding of the Fe-S cluster, whereas the C-terminal part is related to NADP⁺-ferredoxin reductases (FNRs) (53) and contains the NADH- and FAD-binding domains. NADP⁺-ferredoxin reductases accept electrons from ferredoxin and reduce NADP⁺ to NADPH. The NqrF subunit can be viewed as a fusion of a 2Fe ferredoxin with its reduct partner, FNR. The structural prototype of a 2Fe ferredoxin and a NADH-oxidizing FAD domain arranged in a single enzyme is represented by BenC. Like NqrF, BenC contains a 2Fe-2S cluster and FAD as redox cofactors and stabilizes a neutral flavosemiquinone upon reduction by NADH (54). A comparison of BenC with the related enzymes phthalate dioxygenase reductase and FNR in complex with ferredoxin revealed that the flavin and 2Fe-2S cluster can be superimposed in all three structures (39) indicating that the arrangement of the redox cofactors is highly conserved in this family of NAD(P)H dehydrogenases. We used the high-resolution structures of BenC (39) and related proteins (40, 42) to model the structure of the NqrF subunit of the Na⁺-NQR from V. cholerae. Important amino acid residues that act as ligands or form hydrogen bonds to the cofactors in BenC are also present in NqrF. For example, residues Arg156, Tyr158, and Ser159, which form hydrogen bonds to the phosphate, the ribityl and the isoalloxazine moieties of the FAD in BenC are fully conserved in NqrF (Arg210, Tyr212, and Ser213). A stacking interaction of the isoalloxazine ring with Phe335 is observed in BenC suggesting a similar arrangement of Phe106 in NqrF. The ferredoxin domain of NqrF contains the four conserved cysteine Cys70, Cys76, Cys79, and Cys111, which act as ligands to the 2Fe-2S cluster. The Fe-S cluster is probably held in position by Gly77 and Gin112 (NqrF numbering), which in BenC are hydrogen bonded to the acid-labile sulfide S-2 of the 2Fe-2S cluster and to the isoalloxazine moiety of the FAD. The adenosine moiety of the FAD was omitted for clarity. The figure was prepared with the program DS ViewerPro 5.0 (accelrys.com).
the sulfur atom of the fourth cysteine, respectively. In summary, the FAD and the 2Fe-2S cluster are likely to adopt very similar positions in NqrF and BenC. The model of NqrF suggests a distance from the C(8) methyl group of the isoaloxazine ring of FAD to the closest iron atom of the 2Fe-2S cluster of ∼9 Å (Fig. 8). This spacing would allow efficient electron transfer between the two redox cofactors, which is in agreement with the experimental results. The redox chain NADH → FAD → 2Fe-2S on the NqrF subunit represents the initial electron transfer pathway in the Na+-NQR complex. From the Fe-S center, electron transfer could proceed to one of the flavins or to ubiquinone-8 present in the complex. Subunits NqrC and NqrF were copurified as a fragment of the Na+-NQR from V. alginolyticus (9). It therefore seems likely that the covalently attached FMN on NqrC is located in close proximity to NqrF and acts as an electron acceptor for the Fe-S center. It will now be important to investigate whether the oxidation of the Fe-S center precedes the formation of the anionic flavosequinone in the Na+-NQR complex and whether this anionic flavosequinone is located on the NqrC subunit.

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