The Na+-translocating NADH:quinone oxidoreductase (Na+-NQR) from Vibrio cholerae enhances insertion of FeS in overproduced NqrF subunit

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Abstract

The Na⁺-translocating NADH:quinone oxidoreductase (Na⁺-NQR) from Vibrio cholerae is a membrane-bound, respiratory Na⁺ pump. Its NqrF subunit contains one FAD and a [2Fe-2S] cluster and catalyzes the initial oxidation of NADH. A soluble variant of NqrF lacking its hydrophobic, N-terminal helix (NqrF′) was produced in V. cholerae wild type and nqr deletion strain. Under identical conditions of growth and induction, the yield of NqrF′ increased by 30% in the presence of the Na⁺-NQR. FAD-containing NqrF′ species with or without the FeS cluster were observed, indicating that assembly of the FeS center, but not insertion of the flavin cofactor, was limited during overproduction in V. cholerae. A comparison of these distinct NqrF′ species with regard to specific NADH dehydrogenase activity, pH dependence of activity and thermal inactivation showed that NqrF′ lacking the [2Fe-2S] cluster was less stable, partially unfolded, and therefore prone to proteolytic degradation in V. cholerae. We conclude that the overall yield of NqrF′ critically depends on the amount of fully assembled, FeS-containing NqrF′ in the V. cholerae host cells. The Na⁺-NQR is proposed to increase the stability of NqrF′ by stimulating the maturation of FeS centers.
The Na\textsuperscript{+} -translocating NADH:quinone oxidoreductase (Na\textsuperscript{+} -NQR) from

\textit{Vibrio cholerae} enhances insertion of FeS in overproduced NqrF subunit.

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Abstract
The Na\textsuperscript{+}-translocating NADH:quinone oxidoreductase (Na\textsuperscript{+}-NQR) from \textit{Vibrio cholerae} is a membrane-bound, respiratory Na\textsuperscript{+} pump. Its NqrF subunit contains one FAD and a [2Fe-2S] cluster and catalyzes the initial oxidation of NADH. A soluble variant of NqrF lacking its hydrophobic, N-terminal helix (NqrF\textsuperscript{´}) was produced in \textit{V. cholerae} wild type and \textit{nqr} deletion strain. Under identical conditions of growth and induction, the yield of NqrF\textsuperscript{´} increased by 30\% in the presence of the Na\textsuperscript{+}-NQR. FAD-containing NqrF\textsuperscript{´} species with or without the FeS cluster were observed, indicating that assembly of the FeS center, but not insertion of the flavin cofactor, was limited during overproduction in \textit{V. cholerae}. A comparison of these distinct NqrF\textsuperscript{´} species with regard to specific NADH dehydrogenase activity, pH dependence of activity and thermal inactivation showed that NqrF\textsuperscript{´} lacking the [2Fe-2S] cluster was less stable, partially unfolded, and therefore prone to proteolytic degradation in \textit{V. cholerae}. We conclude that the overall yield of NqrF\textsuperscript{´} critically depends on the amount of fully assembled, FeS-containing NqrF\textsuperscript{´} in the \textit{V. cholerae} host cells. The Na\textsuperscript{+}-NQR is proposed to increase the stability of NqrF\textsuperscript{´} by stimulating the maturation of FeS centers.

1. Introduction

\textit{Vibrio cholerae} is a pathogenic bacterium which may cause the severe diarrheal disease cholera [1]. As an adaptation for growth at high NaCl concentrations, \textit{V. cholerae} expels sodium ions from the cytoplasm during respiration and establishes a sodium motive force across its inner membrane [2]. This respiratory Na\textsuperscript{+} transport is catalyzed by the Na\textsuperscript{+}-
translocating NADH:quinone oxidoreductase (Na⁺-NQR) which consists of six subunits, NqrA to -F, and contains one Fe-S centre, two covalently bound FMNs, one non-covalently bound FAD and ubiquinone-8 as prosthetic groups [3]. The presence of an additional riboflavin cofactor in the Na⁺-NQR is discussed controversially [4, 5]. The electron transfer pathway in the Na⁺-NQR starts with a hydride transfer from the substrate NADH to the non-covalently bound FAD on the flavin domain of the NqrF subunit [6], followed by one-electron transfer to the vertebrate-type [2Fe-2S] cluster in the N-terminal domain of NqrF [7].

The nqrABCDEF-genes are related to the rnfBDGAEC-genes, respectively, which encode for the membrane-bound Rnf complex first identified as a complex required for nitrogen fixation by the photoautotrophic bacterium Rhodobacter capsulatus. Deletion of the rnf genes did not affect the nitrogenase system from R. capsulatus itself, but revealed a failure in the electron transport to nitrogenase [8]. Another N₂-fixing bacterium, Azotobacter vinelandii, contains two sets of rnf-gene clusters. The expression of rnf1 is regulated together with other nif-genes implicated in nitrogen fixation, whereas that of rnf2 is independent of the nitrogen source. The rnf-gene products are essential for the rapid accumulation of the matured, [4Fe-4S]-containing dinitrogenase reductase [9], but the redox partner(s) of Rnf, and its precise role in FeS maturation, are unknown.

Ferredoxins were proposed to act as electron donors for Rnf complexes from Clostridia [10, 11] and the archaeon Methanosarcina acetivorans [10]. In Escherichia coli, the Rnf complex is called Rsx. During aerobic growth, this complex keeps the [2Fe-2S] cluster of the SoxR regulatory protein in the reduced inactive state. Under conditions of oxygen stress, the [2Fe-2S] cluster becomes oxidised and triggers transcription of soxS whose
product induces other genes to protect against oxidative stress [11]. In summary, Na\textsuperscript{+} -NQR and Rnf represent redox-driven, cation-translocating complexes which react with different substrates or redox carriers like NADH, quinones or [2Fe-2S] ferredoxins. We previously reported the cloning, expression and partial characterization of NqrF\textprime, a soluble variant of the NADH-oxidizing NqrF subunit devoid of its N-terminal, hydrophobic helix, and its individual FAD- and FeS-carrying domains [6]. Here we demonstrate that the Na\textsuperscript{+}-NQR complex stimulates overproduction of NqrF\textprime in V. cholerae, and show that the FeS cluster is essential for NqrF\textprime stability. We propose that the Na\textsuperscript{+}-NQR from V. cholerae, like the related Rnf complex from A. vinelandii, is involved in the maturation of specific iron-sulfur proteins.

2. Materials and methods

2.1. Preparation of NqrF\textprime

The soluble variant of NqrF, NqrF\textprime, comprises an N-terminal polyhistidine tag and lacks the amino acid residues Thr\textsuperscript{3}-Ala\textsuperscript{25} required for the attachment of NqrF to the membrane [6]. V. cholerae O395N1 [12] or V. cholerae O395N1\textDelta nqr lacking the Na\textsuperscript{+}-NQR [13] were transformed with plasmid pNF3 encoding NqrF\textprime [6]. Cells (1 l) were grown aerobically at 37 °C in 5 liter Erlenmeyer flasks in the presence of 171 mM NaCl. The medium contained 10 g l\textsuperscript{-1} tryptone, 5 g l\textsuperscript{-1} yeast extract, 50 mM potassium phosphate (pH 7.3), 10 mM glucose, 50 µg ml\textsuperscript{-1} streptomycin and 200 µg ml\textsuperscript{-1} ampicillin. Expression of NqrF\textprime was induced by adding 10 mM L-arabinose after an optical density $A_{600\text{ nm}}$ of 1 was reached, and growth was continued to the beginning of the stationary phase. Cells were
harvested, washed with buffer (300 mM NaCl in 10 mM Tris/HCl, pH 7.4), shock-frozen in liquid N\textsubscript{2} and stored at -80°C. Expression and affinity purification of NqrF’ was performed in parallel using \textit{V. cholerae} O395N1 and \textit{V. cholerae} O395N1Δnqr as hosts to compare the yields and cofactor content of NqrF’ produced in the presence and absence of the Na\textsuperscript{+}-NQR. Ten g cells (wet weight) were resuspended in 30 ml buffer containing 1 mM phenylmethylsulfonyl fluoride, 0.1 mM diisopropylfluorophosphate, 1 mM dithiothreitol, 5 mM MgCl\textsubscript{2}, and traces of DNase I (Roche Diagnostics). The suspension was passed once through a French pressure cell at 83 MPa, and the eluate was collected under a stream of N\textsubscript{2}. Unbroken cells and debris were removed by centrifugation at 35,000 x g for 20 min. All subsequent manipulations were performed under exclusion of O\textsubscript{2} using an anaerobe chamber (90% N\textsubscript{2}, 10 % H\textsubscript{2} in the gas phase, Coy). Soluble proteins were separated from the membrane fraction by ultracentrifugation (150,000 x g, 1 h, 4° C) and loaded on a Ni-NTA superflow column (3-ml bed volume, 1.4-cm diameter, Qiagen) equilibrated with 20 mM Tris/HCl pH 8.0, 0.5 M NaCl. After a washing step with 30 mM imidazole, NqrF’ was eluted with 400 mM imidazole, immediately diluted 10-fold with 50 mM Tris/HCl, pH 7.5 and loaded on a Fractogel TSK DEAE 650 column (1-ml bed volume, 1.4-cm diameter, Merck). NqrF’ was eluted with 300 mM NaCl and its concentration was adjusted to approximately 1 mg ml\textsuperscript{-1} in Tris buffer containing 150 mM NaCl using Ultra-15 centrifugation filters (10 kDa cut off, Amicon). To remove the N-terminal polyhistidine tag of NqrF’, 5 Units thrombin (Serva) per mg protein and 2.5 mM CaCl\textsubscript{2} were added. After 1 h at 25° C, the mix was loaded on the Ni-NTA column to obtain NqrF’ devoid of the affinity tag in the flow-through. The protein was diluted 10-fold in 50 mM Hepes/NaOH, pH 7.0, and loaded on a MonoQ
HR10/10 anionic exchange column connected to an Äkta station (Amersham Biosciences). By applying a linear gradient of 20 volumes from 0-1 M NaCl, NqrF’ eluted in two fractions at 0.5 M and 0.6 M NaCl, respectively. If not indicated otherwise, NqrF’ was produced using wild-type \textit{V. cholerae} as expression host.

2.2. Analytical Methods

Protein was determined by the bicinchoninic acid method [14] using the reagent from Pierce. Bovine serum albumin served as the standard. The content of non-covalently bound FAD in NqrF’ was determined according to Macheroux [15]. Acid-labile sulfur was determined by the method of Beinert [16]. Iron was determined by atomic absorption spectroscopy (AA240FS spectrometer, Varian) using Fe standards from 0.1 – 10 mg l$^{-1}$ (Fluka). SDS-PAGE was performed with 10\% polyacrylamide according to Lämmli [17]. To prevent dimerization by disulfide bond formation, NqrF’ was boiled for 5 min in sample buffer containing 50 mM dithiothreitol. Tryptic in-gel digestion of proteins separated by SDS-PAGE was performed according to [18]. The digests were analyzed on a QTOF Ultima API mass spectrometer (Waters), and peptides were identified using the ProteinLynx Global Server software (Waters).

2.3. Enzymatic tests and thermal inactivation

Oxidation of 0.1 mM NADH (\(\varepsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}\)) by NqrF’ with 0.1 mM ubiquinone-1 as electron acceptor was followed on an 8452A diode array spectrophotometer (Hewlett Packard) in the presence of 50 mg ml$^{-1}$ bovine serum albumin and 50 mM Na$_2$SO$_4$ at pH 7.0 as described previously [6]. The pH dependence of activity was followed in 20 mM
MES/NaOH (pH 5.5 - 6.5) or 20 mM TRIZMA/HCl (pH 7.0 - 9.5). Thermal inactivation was followed with NqrF′ (0.9 mg ml\(^{-1}\) in 50 mM Hepes/NaOH, pH 7.0, 500 mM NaCl) incubated at 37°C or 57°C. Aliquots were removed at intervals and immediately analyzed for NADH dehydrogenase activity as described above. To monitor the dissociation of FAD during heat denaturation, NqrF′ (0.4 mg ml\(^{-1}\)) was diluted tenfold in pre-warmed 50 mM Hepes/NaOH, pH 7.0, 500 mM NaCl in a temperature-controlled cuvette (55 °C) at \(t = 0\) s. At indicated times, the intensity of fluorescence emission at 526 nm of the FAD (\(\lambda\) excitation, 450 nm) was determined with a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon Inc.). To confirm that the observed increase in fluorescence intensity resulted from the dissociation of FAD, but not from decreased fluorescence quenching of FAD bound to partially unfolded protein, NqrF′ with or without the FeS cluster was incubated at 55°C for 4 min and concentrated with Amicon Ultrafree-MC centrifugal filters (molecular weight cutoff, 5 kDa). The filtrates contained 83% (NqrF′ with FeS) or 85% (NqrF′ without FeS) of the FAD bound to the protein prior to thermal inactivation.

2.4. Quantification of Na\(^+\)-NQR in V. cholerae O395N1

Ag\(^+\) is a specific inhibitor of the Na\(^+\)-NQR which promotes the dissociation of the non-covalently bound FAD from the NqrF subunit and thereby prevents the initial oxidation of NADH [19]. In the presence of 1 μM Ag\(^+\), rates of NADH oxidation by membranes from V. cholerae O395N1 grown under the specified condition decreased to 8% of the activity observed in the absence of the inhibitor. In contrast, no inhibition of NADH oxidation by Ag\(^+\) was observed with membranes from the \(nqr\) deletion strain. The inhibition by Ag\(^+\) indicated that at least 92% of the NADH dehydrogenase activity of
membranes from the *V. cholerae* wild-type strain was catalyzed by the Na\(^+\)-NQR, corresponding to 0.77 µmol NADH min\(^{-1}\) mg\(^{-1}\). We calculated the content of Na\(^+\)-NQR in the membranes by dividing the total Na\(^+\)-NQR activity in 1 mg membrane protein (0.77 µmol NADH min\(^{-1}\)) with the turnover number determined for purified Na\(^+\)-NQR (16,000 µmol NADH µmol\(^{-1}\) Na\(^+\)-NQR min\(^{-1}\)) [13], and obtained a value of 0.05 nmol Na\(^+\)-NQR mg\(^{-1}\) protein.

### 3. Results

#### 3.1. Increased production of NqrF´ in the presence of the NQR complex

We asked whether the presence of the Na\(^+\)-NQR had an effect on the overproduction of its FeS-carrying NqrF subunit and compared the yield of NqrF´ after affinity and TSK DEAE chromatography using the *V. cholerae* wild-type and the *nqr* deletion strain as hosts for protein production. The growth behavior of both strains before and after induction was very similar, and the final cell yields were essentially identical (Fig.1). Under the specified growth conditions, energy conversion in *V. cholerae* was not dependent on the Na\(^+\)-NQR. Expression of *nqrF´* in the presence of the NQR complex raised the yield of NqrF´ per liter culture volume to 4.6 ± 0.4 mg l\(^{-1}\), compared to 3.6 ± 0.2 mg l\(^{-1}\) in the absence of the Na\(^+\)-NQR, determined in triplicates and based on protein determination by the bicinchoninic acid method. The FAD content of NqrF´ was not influenced by the genetic background of the expression host. Plasmid pNF3 used for NqrF´ production is a multicopy expression vector which is under control of the vector-encoded AraC regulator protein. It has been shown that protein production in these
expression vectors is not limited by mRNA synthesis [20]. Therefore, transcription and translation of the plasmid-encoded NqrF’ is not determined by the genetic background and will result in the same amount of apo-NqrF’ in the V. cholerae mutant and wild-type strains. We conclude that the higher yield of NqrF’ observed in the presence of the Na\textsuperscript{+}-NQR is caused by increased stability and/or decreased degradation of holo-NqrF’. To address this hypothesis, we purified NqrF’ and studied its cofactor composition, activity and stability.

3.2. Separation of NqrF’ with varying FeS content

A previously reported purification protocol for NqrF’ yielded a partially purified preparation after Ni-NTA affinity chromatography [6]. We performed an additional MonoQ anionic exchange chromatographic step after proteolytic cleavage of the polyhistidine tag (Table 1). NqrF’ eluted in two fractions, A and B (Fig. 2), as confirmed by SDS-PAGE and mass spectroscopic analysis of the polypeptides after in-gel digestion (not shown). The masses of NqrF’ before (44621 Da) and after removal of the polyhistidine tag (42856 Da) were close to the calculated values of 44596 Da and 42827 Da, indicating that treatment with thrombin did not result in unspecific proteolysis of NqrF’. The specific NADH dehydrogenase activity of NqrF’ in fraction A increased 7-fold after MonoQ chromatography compared to the eluate from the first affinity purification step (Table 1). Inspection by UV-Vis spectroscopy revealed that NqrF’ eluting at 0.55 M NaCl (fraction A) exhibited typical maxima of the flavin cofactor at 396 nm and 456 nm, and absorbances around 350 nm and 560 nm assigned to the [2Fe-2S] cluster of NqrF [7]. In contrast, NqrF’ eluting at 0.75 M NaCl (fraction B) exhibited
maxima characteristic for flavin but lacked the prominent shoulder at 560 nm which would indicate the presence of the [2Fe-2S] cluster (Fig. 3). NqrF− in fraction A contained 0.54 ± 0.02 mg Fe mg\(^{-1}\) protein, or 0.41 ± 0.02 mol Fe per mol NqrF−, compared to 1.04 ± 0.04 mol mol\(^{-1}\) FAD (Table 1). We found no evidence for protein-bound Fe in NqrF− in fraction B which only contained residual iron from the elution buffer (0.04 mg Fe l\(^{-1}\)). None of the proteins eluting from the MonoQ column represented NqrF− containing the [2Fe-2S] cluster but lacking the FAD. The prominent peak at 0.25 M NaCl represented free flavin. It is concluded that NqrF− readily binds FAD, while insertion of the FeS cluster is limited during overproduction in \textit{V. cholerae}, yielding two distinct NqrF− species with or without the FeS center, respectively. The yield of the two NqrF− species after MonoQ chromatography was influenced by the \textit{V. cholerae} expression host. Using the \textit{nqr} deletion strain as production host, the amount of NqrF− containing the [2Fe-2S] cluster was approximately twofold higher than the amount of NqrF− devoid of FeS center. In the \textit{V. cholerae} wild type strain, the yield of NqrF− with FeS was fourfold higher than the yield of NqrF− containing only the FAD cofactor.

3.3. Enzymatic properties of NqrF−

The two NqrF− species separated by MonoQ chromatography both contained stoichiometric amounts of FAD, yet NqrF− devoid of FeS exhibited only 24% of the specific NADH oxidation activity of NqrF− containing the [2Fe-2S] cluster (Table 1). We previously showed that the specific NADH oxidation activity, and the affinity towards NADH, is essentially identical in NqrF− and its isolated FAD domain. The FAD domain is obtained by truncation of the N-terminal, FeS-binding part of NqrF− [6]. This
demonstrates that in NqrF’, the [2Fe-2S] cluster is not required for electron transfer from NADH to the substrate Q1 used as artificial electron acceptor in the enzymatic assays. Besides the difference in specific NADH dehydrogenase activity, the two NqrF’ species differed with respect to the pH optimum of activity (Fig. 4). NqrF’ containing the [2Fe-2S] cluster showed a prominent maximum of activity at pH 7.0, while the NADH oxidation activity of NqrF’ devoid of FeS was not dependent on pH in the range from 5.5 to 8.0 (Fig. 4). The results suggested that the lack of the FeS center in NqrF’ was accompanied with structural rearrangement(s) of amino acid residue(s) close to the active site caused by partial unfolding of the protein. To address this question, we compared the thermal stability of the two NqrF’ species.

3.4. Thermal inactivation

The enzymatic activity of NqrF’ is a measure for the structural integrity of the protein. We followed the time course of inactivation of NqrF’ with or without FeS center at 37°C or 57°C to monitor denaturation (Fig. 5). There was a slight decrease of activity after 5 min at 37°C which was more pronounced in NqrF’ devoid of FeS. The increased thermal inactivation of NqrF’ lacking the FeS center was more evident at 57°C, where activity dropped to 25% after 1 min and was completely lost after 2 min. In contrast, NqrF’ containing the [2Fe-2S] cluster retained 85% or 22% of its initial activity after 1 min or 2 min, respectively (Fig. 5). The results suggested that NqrF’ lacking the FeS center was partially unfolded which promoted the denaturation of the protein by heat. The rapid thermal inactivation of NqrF’ devoid of FeS could also be due to a diminished binding affinity of the FAD cofactor. To test this hypothesis, we followed the increase in
fluorescence emission intensity of FAD during heat denaturation which reflects the rise of free FAD concomitant with the unfolding of NqrF’. The time course of FAD release from NqrF’ with or without the FeS center did not differ significantly (Fig. 6). Assuming complete release of FAD after 4 min (Fig. 6), we estimate that during the first minute of thermal inactivation, the FAD cofactor was retained in at least 70% of the NqrF’ molecules. We conclude that local structural rearrangements of the substrate binding site(s), rather than a lower affinity towards FAD, are the cause for the faster denaturation observed with NqrF’ lacking the FeS center. The results demonstrate that the [2Fe-2S] cluster represents a structural element of NqrF’ which impedes unfolding of the protein. A likely explanation for the diminished activity, altered pH profile and increased thermal inactivation of NqrF’ devoid of FeS is the incorrect folding of the polypeptide in the proximity of the active site(s).

4. Discussion

With six subunits, three (or perhaps four) flavins, and one [2Fe-2S] cluster, the Na+-translocating NADH:quinone oxidoreductase represents a sophisticated, membrane-embedded redox pump. Its biosynthesis requires a specific pathway for the folding of the polypeptides to subunits, insertion of cofactors and assembly of the complex. For example, only the apo-form of subunit NqrC was produced in Escherichia coli which lacks the Na+-NQR [21]. In V. cholerae, NqrC contains covalently attached FMN, and the authors speculated that attachment of the flavin to a threonine in NqrC requires additional factors that are not present in the E. coli expression host [21]. Quite
unexpected, neither holo- nor apo-NqrF' was produced in *E. coli* [6], although the organism is routinely used as host for the heterologous production of bacterial FeS proteins containing non-covalently bound flavins [22, 23]. Our observation that the Na\(^+\) -NQR enhances insertion of FeS into overproduced NqrF' further supports the notion of a specific system for the assembly of the Na\(^+\) -NQR in *V. cholerae*. It is noteworthy that the lack of the Na\(^+\) -NQR which resulted in diminished production of NqrF' had no effect on the content of succinate dehydrogenase, a respiratory complex containing covalently attached FAD and three FeS clusters [24].

Quantification of the amount of FeS proteins produced under different expression conditions is an important biochemical approach to identify FeS maturation factors. For example, coexpression of an *E. coli* DNA fragment containing the iscS-iscU-iscA-hscB-hscA-fdx genes resulted in a 1.3 -fold increase in the production yield of the ferredoxin from *Bacillus thermoproteolyticus* [25]. In *V. cholerae*, the yield of NqrF' production increased by approximately 30% in the wild-type compared to the nqr deletion strain under identical conditions of growth and nqrF’ expression. In *Azotobacter vinelandii*, deletion of the Rnf complex which exhibits 20-40% sequence identity with the *V. cholerae* NQR diminished the rates of FeS insertion into the nitrogenase Fe protein but not the amount of apo-protein formed [26].

Insertion of FeS into NqrF' by *V. cholerae* was limited, and NqrF' containing the [2Fe-2S] cluster exhibited increased thermal stability compared to NqrF' devoid of FeS. The thermal stability of proteins is known to be associated with resistance to proteolysis [27]. Overproduction of NqrF' will therefore critically depend on the amount of mature NqrF' which is less prone to proteolytic degradation in *V. cholerae*. A similar stabilizing effect
of an iron-sulfur cluster which led to increased protein yields was reported for subunit
NuoG from the NADH:quinone oxidoreductase (complex I) from *Escherichia coli* [28].  
Note that the loss of FeS does not necessarily result in diminished stability of the apo-
protein, as exemplified by the cytoplasmic aconitase which, upon iron depletion, is
converted to an iron-responsive element (IRE) binding protein [29].  
The Na\(^+\) -NQR appears to be involved in the maturation of Fe/S clusters, hereby
enhancing the stability of NqrF\(^-\) and increasing its yield during overproduction in *V.
cholerae*. One could argue that the amount of holo-NqrF\(^-\) produced by *V. cholerae* is not
determined by the formation of the FeS cluster but by its degradation. For example,
reactive oxygen species like superoxide or hydrogen peroxide are known to destroy FeS
clusters [30]. Following this argument, one would propose that *V. cholerae* cells lacking
the Na\(^+\) -NQR produce increased amounts of reactive oxygen species which promote the
degradation of NqrF\(^-\) *in vivo*. On the contrary, we recently showed that the formation of
reactive oxygen species by wild type *V. cholerae* cells is increased two-fold compared to
the *nqr* deletion strain [24], which argues against oxidative destruction of the FeS center
as cause for diminished NqrF\(^-\) production in the *V. cholerae* mutant.  
FeS maturation of NqrF\(^-\) in *V. cholerae* was also observed in the *nqr* deletion strain and
therefore did not obligatorily depend on the Na\(^+\) -NQR. This implies that other systems
for iron-sulfur cluster assembly exist in *V. cholerae* which may become limiting under
conditions of overproduction of NqrF\(^-\). To our knowledge, the pathways of FeS protein
assembly in *V. cholerae* have not been studied yet. Analysis of the genome from the El
Tor strain (http://cmr.tigr.org) indicates that *V. cholerae* possesses homologs of the ISC
(VC_0747) and SUF systems (VC_2720) for the assembly of FeS proteins [31], and a
homolog of the Rnf complex (VC_1017). Our study shows that the Na\textsuperscript{+} -NQR, in addition to its function in respiration and energy conversion, plays a role in the biosynthetic pathway leading to a mature FeS protein. The FeS cluster of NqrF is structurally related to ISC-type ferredoxins [7] which are proposed to act as redox mediators during FeS assembly [31, 32]. It remains to be investigated whether this structural similarity has functional significance, and how electron transfer reactions catalyzed by membrane-bound complexes like Rnf or Na\textsuperscript{+} -NQR might contribute to the maturation of FeS proteins in bacteria.

5. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Na\textsuperscript{+} -NQR</td>
<td>Na\textsuperscript{+} -translocating NADH:quinone oxidoreductase</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>Q1</td>
<td>ubiquinone-1 [2,5-cyclohexadiene-1,4-dione,2,3-dimethoxy-5-methyl-6-(3-methyl-2-buteryl)-]</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>SDS–PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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Acknowledgments

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Freisinger, University of Zürich, for the analysis of Fe by atomic absorption spectroscopy.
References


Table 1: Purification of NqrF´

<table>
<thead>
<tr>
<th>Step Description</th>
<th>Protein [mg]</th>
<th>NADH dehydrogenase activity [µmol min⁻¹ mg⁻¹]</th>
<th>FAD content [mol mol⁻¹ NqrF´]</th>
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<tbody>
<tr>
<td>Soluble fraction</td>
<td>457</td>
<td>n.d.*</td>
<td>n.d.*</td>
</tr>
<tr>
<td>Fractogel TSK DEAE 650 (after first Ni-NTA column)</td>
<td>22.7</td>
<td>291</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>Second Ni-NTA column (after thrombin digestion)</td>
<td>10.9</td>
<td>459</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>MonoQ, fraction A</td>
<td>2.2</td>
<td>2043</td>
<td>1.0 ± 0.04</td>
</tr>
<tr>
<td>MonoQ, fraction B</td>
<td>0.6</td>
<td>493</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

n.d., not determined; *Activity and FAD determinations do not reflect the content of NqrF´ in the soluble fraction due to a high content of endogenous NADH dehydrogenases.
Figure 1: Growth of the *V. cholerae* wild-type and the *nqr*- deletion strains during production of NqrF’. *V. cholerae* O395N1 (square) and *V. cholerae* O395N1 Δnqr (circle) transformed with plasmid pNF3 were grown as described in Materials and Methods. Arrows indicate the addition of 10 mM L-arabinose to induce the expression of *nqrF’,* and the harvesting of cells for subsequent NqrF’ purification.

Figure 2: Separation of NqrF’ by MonoQ anionic exchange chromatography

*V. cholerae* O395N1 Δnqr was used as expression host. Two distinct NqrF’ species eluted in fractions labeled with A and B, respectively. The dotted trace indicates the concentration of NaCl.

Figure 3: UV-Visible absorption spectra of NqrF’ after MonoQ anionic exchange chromatography

NqrF’ species representing fully assembled NqrF’ (upper panel) and NqrF’ lacking the [2Fe-2S] cluster (lower panel) were separated by MonoQ HR10/10 anionic exchange chromatography.

Figure 4: Dependency of NADH dehydrogenase activity of NqrF’ on pH

Specific activities of NqrF’ with (upper panel) or without [2Fe-2S] cluster (lower panel) were determined at different pH values as described in Materials and Methods. 100% activity at pH 7.0 corresponds to 2042 µmol min⁻¹ mg⁻¹ (NqrF’ with FeS) or 493 µmol min⁻¹ mg⁻¹ (NqrF’ without FeS), respectively.
Figure 5: Thermal inactivation of NqrF´

NqrF´ (0.9 mg ml⁻¹) containing the [2Fe-2S] cluster (upper panel) or devoid of FeS (lower panel) in 50 mM Hepes/NaOH, pH 7.0, 500 mM NaCl was incubated at 37° C (squares) or 57° C (triangles). At indicated times, aliquots were immediately analyzed for NADH dehydrogenase activity in triplicates. The data are presented as percentage of activity determined at t = 0 s.

Figure 6: Dissociation of FAD from NqrF´ during heat denaturation

NqrF´ (0.04 mg ml⁻¹) containing the [2Fe-2S] cluster (closed square) or devoid of FeS (open square) in 50 mM Hepes/NaOH, pH 7.0, 500 mM NaCl was incubated at 55° C. The fluorescence emission intensity I was determined at indicated times and divided by the intensity I₀ observed at the start of the reaction. Excitation wavelength, 450 nm; emission wavelength, 526 nm.
Figure 1

[Graph showing OD values over time after induction.]
Figure 2
Figure 3

A

Absorbance (A.U.)

Wavelength (nm)

B

Absorbance (A.U.)

Wavelength (nm)
Figure 4
Figure 6