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# $Na^+$-translocating NADH:Quinone Oxidoreductases from *Vibrio cholerae* and *Yarrowia lipolytica*

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PO-CHI LIN

Dipl. Natw. ETH Zürich

born May 7, 1978

citizen of Switzerland

accepted on the recommendation of

Prof. Dr. M. Aebi, examiner

Prof. Dr. H. Hilbi, co-examiner

Prof. Dr. J. Fritz-Steuber, co-examiner

Prof. Dr. P. Dimroth, co-examiner

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*For mom and dad*

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## Abbreviations

| | |
|---|---|
| ACMA | 9-amino-6-chloro-2-methoxyacridin |
| ATP | adenosine triphosphate |
| CCCP | carbonyl cyanide *m*-chlorophenylhydrazone |
| DCCD | N,N’-di-cyclohexylcarbodiimide |
| EPR | electron paramagnetic resonance |
| $E_m$ | midpoint redox potential |
| FAD | flavin adenine dinucleotid |
| FCCP | carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone |
| Fd | ferredoxin |
| FMN | flavin mononucleotide |
| HQNO | 2-heptyl-4-hydroxyquinoline N-oxide |
| ISC | iron-sulfur cluster |
| MCD | magnetic circular dichroism |
| $Na^+$-NQR | $Na^+$-translocating NADH: quinone oxidoreductase |
| NDH-1 | NADH dehydrogenase I, the bacterial homologue of eukaryotic complex I |
| NDH-2 | an alternative, non-electrogenic NADH dehydrogenase |
| PDB | Protein Data Bank |
| $P_i$ | inorganic phosphate |
| Q/ QH2 | ubiquinone / ubiquinol |
| Q1 | ubiquinone-1 (2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone) |
| SMPs | submitochondrial particles |
| T | tesla |
| VTMCD | variable temperature magnetic circular dichroism |
| $\Delta\Psi$ | membrane potential |
| ΔpH | transmembrane pH difference |
| ΔpK | transmembrane potassium difference |
| ΔpNa | transmembrane sodium difference |
| $\Delta\tilde{\mu}H^+$ | proton electrochemical potential (proton motive force) |

# Zusammenfassung

NqrF, die NADH-oxidierende Untereinheit der $Na^+$-transportierenden NADH:Q Oxidoreduktase ($Na^+$-NQR) von *Vibrio cholerae*, enthält einen [2Fe-2S] Cluster. In dieser Studie wurde eine gekürzte Form (Fe-S Domäne) von NqrF verwendet, um die elektronischen Eigenschaften des Fe-S Zentrums zu untersuchen. Ein Vergleich der Aminosäurensequenz zeigte, dass die Sequenz des Fe-S Cluster-bindenden Motivs von der Fe-S Domäne konserviert und mit derjenigen von Ferredoxinen der Wirbeltierfamilie verwandt ist. Das binukleare Fe-S Cluster der Fe-S Domäne wurde von folgenden Cysteinen koordiniert: $Cys^{70}$, $Cys^{75}$, $Cys^{79}$ und $Cys^{111}$. Das Null-Feld Mössbauer Spektrum der oxidierten Fe-S Domäne wurde bei 80 K aufgenommen und zeigte ein asymmetrisches Doublet mit einer Quadrupolspaltung $\Delta EQ = 0.613$ mm/s und einer Isomerverlagerung $\delta = 0.283$ mm/s, die dem Fe(III)-Fe(III) Paar des binuklearen $[2Fe\text{-}2S]^{2+}$ Clusters zugeordnet wurden. Dieses Resultat wurde weiterhin durch den Fe- und $S^{2-}$-Gehalt der Fe-S Domäne (jeweils $1.61 \pm 0.28$ und $1.31 \pm 0.11$ mol/mol Protein) unterstützt. Das temperaturabhängige magnetische Circulardichroismus-Spektrum von der photochemisch reduzierten Fe-S Domäne zeigte ein negatives Maximum bei 315 nm, das den Ladungsübergängen der Fe(II)-S Bindung zugeordnet wurde, und positive Maxima bei 358, 409, 554, 629 und 694 nm, die den Ladungsübergängen der Fe(III)-S Bbindung zugeordnet wurden. Dieses Muster aus positiven und negativen Maxima ähnelte demjenigen des [2Fe-2S] Clusters von ISC-Typ Ferredoxinen, die in der Assemblierung von Fe-S Zentren eine Rolle spielen.

Die $Na^+$-NQR von *V. cholerae* produziert extrazellulär reaktive Sauerstoffspezies (Superoxid und Wasserstoffperoxid), die zu der Pathogenität von *V. cholerae* durch oxidative Beschädigung der infizierten Wirtszellen beitragen könnten. In nativen, mit NADH reduzierten Membranen wurde ein organisches Radikal mit Hilfe der elektronenparamagnetischen Resonanzspektroskopie beobachtet und einem durch die $Na^+$-NQR erzeugten Ubisemichinon zugeordnet. Die Ubisemichinonbildung wurde von $Na^+$ stimuliert; die Radikalkonzentration nahm von 0.2 mM bei 0.08 mM $Na^+$ auf 0.4 mM bei 14.7 mM $Na^+$ zu. Während der Atmung produzierte *V. cholerae* extrazelluläres Superoxid mit einer spezifischen Aktivität von 10.2 nmol $min^{-1}$ $mg^{-1}$, während ein *V. cholerae*-Stamm ohne eine $Na^+$-NQR nur eine Aktivität von 3.1 nmol $min^{-1}$ $mg^{-1}$ aufwies. Wurde die Natriumkonzentration von 0.1 mM auf 5 mM erhöht, stieg die Rate der Superoxidbildung in *V. cholerae* um 70 % an. Diese Resultate zeigten, dass die extrazelluläre Natriumkonzentration

die Ubisemichinonbildung der $Na^+$-NQR stimulieren und hiermit die Produktion der reaktiven Sauerstoffspezies aus der Autooxidation des Chinols fördern könnte.

Ein anderes Familienmitglied der membrangebundenen NADH:Q Oxidoreduktasen ist der Komplex I der Atmungskette. Obwohl keine Sequenz- oder Strukturhomologie besteht, setzen $Na^+$-NQR und Komplex I beide die aus Redoxreaktion gewonnene Energie in Kationentransport um. Unsere Studie zeigte, dass die NADH-Oxidation von submitochondrialen Partikeln aus der Hefe *Yarrowia lipolytica* mit der protonophor-resistenten Natriumaufnahme (0.12 µmol $min^{-1}$ $mg^{-1}$) gekoppelt wurde, was die Präsenz einer redox-getriebenen funktionsfähigen Primärnatriumpumpe in der inneren mitochondrialen Membran andeutet. Durch Reinigung und Rekonstitution in Proteoliposomen wurde eine NADH Dehydrogenase identifiziert, die NADH-abhängige Reduktion des Ubichinons (1.4 µmol $min^{-1}$ $mg^{-1}$) mit Natriumtransport (2.0 µmol $min^{-1}$ $mg^{-1}$) koppelte. Der NADH-getriebene Natriumtransport konnte durch Rotenon gehemmt werden. Rotenon ist ein spezifischer Komplex I Inhibitor, und daraus folgern wir, dass Mitochondrien von *Y. lipolytica* eine NADH-getriebene Natriumpumpe enthalten. Wir schlagen vor, dass sie dem Komplex I der Atmungskette entspricht. Unsere Studie zeigt, dass die Energienutzung der Mitochondrien nicht nur auf dem Protonengradienten („proton motive force“) beruht, sondern dass ein durch Komplex I aufgebauter elektrochemischer Natriumgradient von Nutzen sein könnte.

# Summary

NqrF, the NADH oxidizing subunit of the $Na^+$-translocating NADH:Q oxidoreductase ($Na^+$-NQR) from *Vibrio cholerae*, contains a [2Fe-2S] cluster. Here we used a truncated version (Fe-S domain) of NqrF to study the electronic properties of the Fe-S center. A comparison of the amino acid sequence of the Fe-S domain showed that the sequence of Fe-S cluster binding motif is conserved and related to ferredoxins of the vertebrate-type family. The binuclear Fe-S cluster is coordinated by the cysteine residues $Cys^{70}$, $Cys^{76}$, $Cys^{79}$ and $Cys^{111}$. The zero-field Mössbauer spectrum of the oxidized Fe-S domain recorded at 80K showed an asymmetric doublet with a quadrupole splitting $\Delta EQ = 0.613$ mm/s and an isomer shift $\delta = 0.283$ mm/s which were assigned to the Fe(III)-Fe(III) pair of the binuclear [2Fe-2S] cluster. This result was further supported by the Fe and $S^{2-}$ content of the Fe-S domain (1.61±0.28 and 1.31±0.11 mol/mol protein, respectively). The variable temperature magnetic circular dichroism (VTMCD) spectrum of the photochemically reduced Fe-S domain revealed a negative band at 315 nm assigned to Fe(II)-S charge transitions and positive bands at 358, 409, 554, 629 and 694 nm assigned to Fe(III)-S charge transitions. This pattern of positive and negative bands resembles those of the [2Fe-2S] clusters found in the ISC-type ferredoxins. We conclude that the [2Fe-2S] cluster in the isolated Fe-S domain of NqrF is very similar to the 2Fe-clusters from vertebrate-type ferredoxins and among these is mostly related to the [2Fe-2S] cluster present in the ISC-type ferredoxins.

$Na^+$-NQR from *V. cholerae* generates extracellular reactive oxygen species, such as superoxide and hydrogen peroxide, which may contribute to the pathogenicity of *V. cholerae* by causing oxidative damages to the infected host cells. Upon reduction with NADH, an organic radical was detected in native membranes by electron paramagnetic resonance spectroscopy which was assigned to ubisemiquinone generated by the $Na^+$-NQR. The formation of ubisemiquinone was stimulated by $Na^+$; the concentration of radical increased from 0.2 mM at 0.08 mM $Na^+$ to 0.4 mM at 14.7 mM $Na^+$. During respiration *V. cholerae* produced extracellular superoxide with a specific activity of 10.2 nmol $min^{-1}$ $mg^{-1}$ in the wild-type strain, whereas the $Na^+$-NQR-depleted strain had only a specific activity of 3.1 nmol $min^{-1}$ $mg^{-1}$. Raising the $Na^+$ concentration from 0.1 mM to 5 mM increased the rate of superoxide formation in the wild-type strain by at least 70 %. These results showed that extracellular $Na^+$ concentration could stimulate ubisemiquinone formation by the $Na^+$-NQR

and hereby enhance the production of reactive oxygen species formed during the autooxidation of reduced quinone.

Another family member of membrane-bound NADH:Q oxidoreductases is the respiratory complex I. Although sharing no sequencial or structural homology both $Na^+$-NQR and complex I convey the energy gained from the redox reaction to translocate cation across the membrane. Our study showed that oxidation of NADH by submitochondrial particles from the yeast *Yarrowia lipolytica* is coupled to protonophore-resistent $Na^+$ uptake (0.12 μmol $min^{-1}$ $mg^{-1}$), indicating that a redox-driven primary $Na^+$ pump is operative in the inner mitochondrial membrane. By purifying and reconstitution into proteoliposomes, a respiratory NADH dehydrogenase was identified which coupled NADH-dependent reduction of ubiquinone (1.4 μmol $min^{-1}$ $mg^{-1}$) to $Na^+$ translocation (2.0 μmol $min^{-1}$ $mg^{-1}$). NADH-driven $Na^+$ transport was sensitive towards rotenone, a specific inhibitor of complex I. We conclude that mitochondria from *Y. lipolytica* contain a NADH-driven $Na^+$ pump and propose that it represents the complex I of the respiratory chain. Our study indicates that energy conversion by mitochondria does not exclusively rely on the proton motive force but may benefit from the electrochemical $Na^+$ gradient established by complex I.

# 1 Introduction

## 1.1 Electron Transfer Chain

Oxidative phosphorylation is one of the fundamental metabolic reactions in the cell. This process transfers the energy gained from oxidizing nutrition molecules to form ATP for use in all energy-consuming activities of the cell. Oxidative phosphorylation is catalyzed by the electron transfer chain (ETC), which is found in the mitochondria and chloroplasts of eukaryotes and in the plasma membrane of prokaryotes. The ETC is composed of numerous polypeptides that are grouped together into different enzymatic complexes (Figure 1.1). Through this chain of polypeptide complexes the transfer of reducing equivalents from NADH or $FADH_2$ to molecular oxygen is coupled to the pumping of protons (sodium ions in some organisms) across the membrane. This transfer of ions generates an electrochemical gradient, which drives the ATP synthase to phosphorylate ADP (Mitchell 1961).

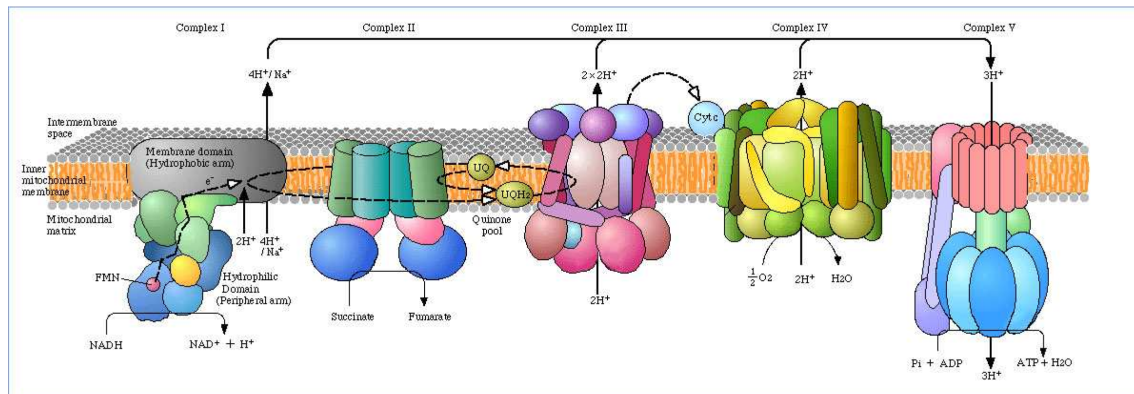
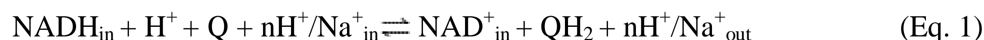



**Figure 1.1 Electron transfer chain in mitochondria**
Complex I (NADH:quinone oxidoreductase), complex II (succinate dehydrogenase), complex III (ubiquinol:cytochrome *c* oxidoreductase, also called $bc_1$ complex) and complex IV (cytochrome *c* oxidase) generate an electrochemical gradient across the inner mitochondrial membrane using the energy gained from redox reactions. This electrochemical gradient, in turn, drives complex V (ATP synthase) to phosphorylate ADP to ATP (Mitchell 1961). This diagram is adapted from http://www.genome.ad.jp/kegg/pathway.html.

Complex I (NADH:quinone oxidoreductase, EC 1.6.5.3.) is the first enzymatic complex of the ETC and is the entry site for the reducing equivalents into ETC. Complex I couples the energy of NADH oxidation and quinone reduction to transfer of ions across membrane (Eq. 1).



$$NADH_{in} + H^+ + Q + nH^+/Na^+_{in} \rightleftharpoons NAD^+_{in} + QH_2 + nH^+/Na^+_{out} \quad \text{(Eq. 1)}$$

In bacteria, membrane-bound NADH:Q oxidoreductases can be divided into three groups: the proton- (or $Na^+$-) translocating NADH:quinone oxidoreductase (NDH-1, the homolog of complex I), the NADH:quinone oxidoreductase (NDH-2) that lacks the ability to transfer ions across the membrane and the $Na^+$-translocating NADH:quinone oxidoreductase ($Na^+$-NQR). The NDH-1 is composed of 14 different subunits. It has a non-covalently bound FMN and several iron-sulfur clusters as prosthetic groups (Yagi and Matsuno-Yagi 2003). The NDH-2 is a single polypeptide and bears no coupling site for ion transport. It contains a non-covalently bound FAD and no iron-sulfur clusters (Yagi 1991). The $Na^+$-NQR is composed of 6 subunits and contains a non-covalently bound FAD (Hayashi and Unemoto 1987; Pfenninger-Li, Albracht et al. 1996), two covalently bound FMNs (Zhou, Bertsova et al. 1999) and one iron-sulfur cluster as prosthetic groups (Pfenninger-Li, Albracht et al. 1996). The presence of one additional riboflavin is discussed controversially (Zhou, Bertsova et al. 1999; Barquera, Zhou et al. 2002).

The eukaryotic complex I is composed of up to 46 subunits (Carroll, Shannon et al. 2002) and has a molecular weight of approximately 1’000 kDa. Among the subunits of the eukaryotic complex I homologues of the 14 subunits from the bacterial complex I (NDH-1) have been identified (Dupuis, Chevallet et al. 1998; Friedrich 1998; Yagi, Yano et al. 1998) and these 14 subunits are referred to as the central subunits. They are likely to form the core of complex I. Subunits of the eukaryotic complex I, which have no homologues in bacteria are called accessory subunits. Studies have shown that these subunits are necessary, not only for the assembly and stability of the complex I (Videira 1998; Videira and Duarte 2001), but also for the regulation of the complex I (Au, Seo et al. 1999; Yadava, Potluri et al. 2002; Grivennikova, Serebryanaya et al. 2003) and possibly in other metabolic processes (Mikolajczyk and Brody 1990; Fearnley, Carroll et al. 2001).

## 1.2 Complex I

### 1.2.1. Structure of complex I

By electron microscopic analysis, Leonard *et al*. reported that complex I of *Neurosopora crassa* has a L-shaped structure (Hofhaus, Weiss et al. 1991). Later on this L-shaped structure

has been also observed in the complex I from *Escherichia coli* (Guenebaut, Schlitt et al. 1998), from bovine heart mitochondria (Grigorieff 1998) and from the yeast *Yarrowia lipolytica* (Djafarzadeh, Kerscher et al. 2000). Of this L-shaped structure, one arm of the complex I is hydrophobic and is embedded in the inner mitochondrial membrane (or cytoplasmic membrane in bacteria), whereas the other, often termed as peripheral arm, protrudes into the matrix of the mitochondria (or cytoplasm in bacteria) (Figure 1.2). Seven out of the fourteen central subunits in the eukaryotic complex I are encoded in the nuclear genome, they are hydrophilic and all located in the peripheral arm; whereas the other seven subunits are encoded in the mitochondrial genome. They are hydrophobic and located in the membrane arm.

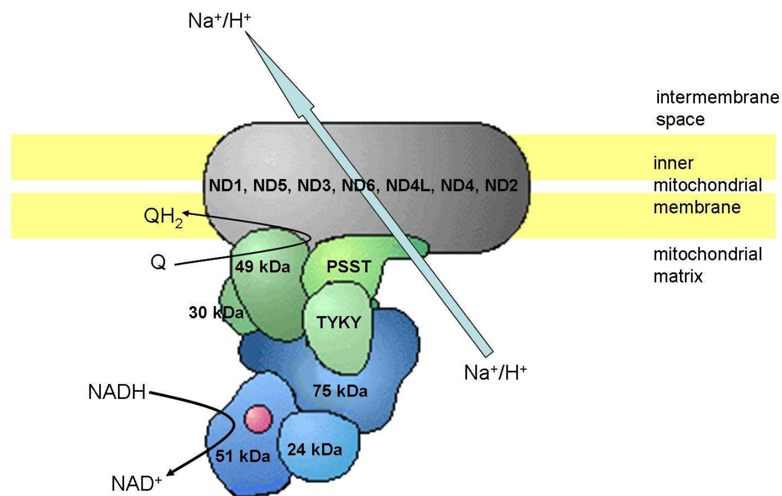



**Figure 1.2 A simplified model of *Yarrowia lipolytica* complex I**
In this model, only the central subunits of complex I are shown. The subunits 51kDa, 24kDa and 75kDa form the peripheral NADH dehydrogenase fragment (FP, shown in blue). The cofactor FMN, shown in magenta, resides in the 51 kDa subunit (Leif, Sled et al. 1995). The connecting fragment (IP), shown in green, consists of the subunits 30kDa, 49kDa, TYKY and PSST. It is proposed that the subunits 49kDa and PSST form a quinone binding site, at which quinone is reduced (Sazanov and Hinchliffe 2006). The subunits ND1-6 and ND4L form the membrane fragment (HP), shown in grey. The organization of the subunits in the membrane fragment is still under investigation (Holt, Morgan et al. 2003; Baranova, Morgan et al.). This model is adapted from http://www.genome.ad.jp/kegg/pathway.html and (Sazanov and Hinchliffe 2006).

Complex I can be divided into 3 functional distinct fragments: the peripheral NADH dehydrogenase fragment (FP), the connecting fragment (IP) and the membrane fragment (HP). From *E. coli* complex I, these fragments can be separated by increasing the pH of the buffer to

pH 7.5 and using chaotropic agents (Leif, Sled et al. 1995). The HP fragment contains seven membrane embedded subunits, ND1-6 and ND4L. ND2, ND4 and ND5 are homologous to each other and belong to a family that shares a common ancestor with $Na^+/H^+$ antiporters (Finel, Skehel et al. 1992; Mathiesen and Hägerhäll 2002). It is proposed that these subunits play an important role in the vectorial ion transport of complex I (Gemperli, Schaffitzel et al. 2007). Furthermore homologues of the subunit ND5 are found to be the transporter subunit of the type 3 hydrogenases (Fearnley and Walker 1992).

The connecting fragment (IP) consists of the subunits 49kDa, 30kDa, PSST and TYKY. This fragment holds three tetranuclear iron-sulfur clusters, termed N2, N6a and N6b (Walker 1992; Böttcher, Scheide et al. 2002). The peripheral fragment (FP) is composed of the subunits 75kDa, 51kDa and 24kDa and it contains two binuclear (N1a and N1b) and three tetranuclear iron-sulfur clusters (N3, N4 and N5) (Leif, Sled et al. 1995). Besides iron-sulfur clusters, complex I also contains a FMN, which is non-covalently bound to the subunit 51kDa (Leif, Sled et al. 1995).

### 1.2.2. The redox factors of complex I

In 2006 Sazanov *et al*. solved the crystal structure of the peripheral arm of complex I from *Thermus thermophilus* (Figure 1.3) (Sazanov and Hinchliffe 2006). Their work provides a very useful framework in regard of the coordination of the cofactors of complex I. Three different types of prosthetic groups are found in complex I: FMN, iron-sulfur clusters and protein-bound quinones. FMN functions both as one- and two-electron acceptor and feeds single electrons to a chain of iron-sulfur clusters (Hinchliffe and Sazanov 2005). The crystal structure of the *Thermus thermophilus* complex I showed that FMN is bound at the deep end of a solvent-exposed cavity and is hold in place by a network of hydrogen bounds. It interacts not only with the conserved residues that are previously predicted to form a FMN-binding domain, but also with those that are suggested to bind NADH (Walker 1992). The crystal structure also showed that the FMN-binding cavity can easily accommodate one NADH molecule (Sazanov and Hinchliffe 2006) (Figure 1.3).

Previous studies showed that all iron-sulfur clusters reside in the peripheral arm of complex I (Hirst 2005). Two [2Fe-2S] clusters and six [4Fe-4S] clusters have been observed by EPR spectroscopy (Yagi and Matsuno-Yagi 2003). Besides FMN, the 51kDa subunit also binds a tetranuclear iron-sulfur cluster which was assigned experimentally as N3 (Fecke, Sled et al.

1994; Yano, Sled et al. 1996). The binuclear cluster N1a was assigned to the 24kDa subunit (Yano, Sled et al. 1996), however this cluster is not detectable by EPR spectroscopy in fungal complex I (Wang, Meinhardt et al. 1991; Djafarzadeh, Kerscher et al. 2000). The tetranuclear cluster N4 and N5 are assigned to the 75kDa subunit (Ohnishi 1998). In some bacteria, the 75kDa subunit binds a third tetranuclear iron-sulfur cluster, N7 (Melo, Lobo et al. 2005; Nakamaru-Ogiso, Yano et al. 2005; Uhlmann and Friedrich 2005). TYKY subunit binds two tetranuclear clusters, N6a and N6b (Rasmussen 2001). The tetranuclear cluster N2 is found in PSST subunit (Ahlers, Zwicker et al. 2000; Duarte, Pópulo et al. 2002; Flemming, Schlitt et al. 2003; Garofano, Zwicker et al. 2003).

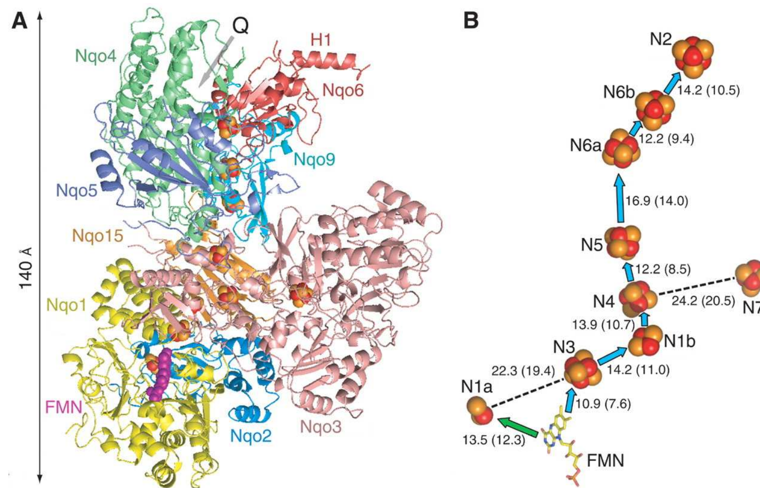



**Figure 1.3 The structure of the hydrophilic domain of the *Thermus thermophilus* complex I** (Sazanov and Hinchliffe 2006)
(A) Each subunit of the hydrophilic domain is colored differently. FMN is shown in magenta and a possible Q binding site is indicated by the arrow. The iron-sulfur clusters are shown in red spheres (iron) and orange spheres (sulfur). (B) The electron transfer pathway within the complex is displayed here separately. The blue arrows indicate the main pathway, whereas the green arrow shows a diversion of the pathway to the cluster N1a. The distances between the clusters are calculated both center-to-center and edge-to-edge (shown in parenthesis). The distance between the cluster N7 and its closest cluster is too far for an electron transfer, therefore it is suggested that the cluster N7 may contribute to the structural stability of the *Thermus thermophilus* complex I (Hinchliffe and Sazanov 2005).

Having obtained the crystal structure, Sazanov *et al.* proposed the following electron transfer pathway within complex I: NADH-FMN-N3-N1b-N4-N5-N6a-N6b-N2-quinone (Figure 1.3 (B)). The cluster N1a does not participate in the electron transfer pathway, but it lies within the range to accept electrons from FMN. Therefore, it is proposed that a pair of electrons may

be transferred simultaneously from FMN to the cluster N3 and N1a. As soon as the cluster N3 is re-oxidized, the electron would be passed on from the cluster N1a via FMN to the cluster N3. The cluster N1a is shielded from solvents, whereas FMN is exposed to exterior environment. Both reduced FMN and flavinsemiquinone can produce reactive oxygen species (ROS) (Kudin, Bimpong-Buta et al. 2004) that damage for the cell, therefore the cluster N1a is likely to serve as a temporary electron storage to minimize the formation of ROS during the turnover of complex I (Sazanov and Hinchliffe 2006). The additional cluster N7 is found only in *Thermus thermophilus* and several other bacteria. Its position lies too far away to be involved in the electron pathway, but it is suggested that the cluster N7 may contribute to the structural stability of complex I (Hinchliffe and Sazanov 2005).

EPR spectroscopy studies showed that there is a semiquinone stabilized in complex I at a distance of 12 Å from the iron-sulfur cluster N2 (Yano, Dunham et al. 2005). The crystal structure of *Thermus thermophilus* complex I showed that in the adjacency of N2 there is a cavity formed by the subunit Nqo4 and Nqo6 (homologues of subunit 49kDa and PSST, respectively). This cavity is most likely to be the quinone binding site where the electrons are passed on from N2 to quinone (Sazanov and Hinchliffe 2006).

### 1.2.3 Accessory subunits

Unlike the central subunits, the accessory subunits found in complex I of higher organisms are not strictly conserved. Proteomics analyses showed that some accessory subunits are only found in fungi and some only in plants (Brandt 2006). The functions of these subunits have not been assigned yet, but in general it is suggested they are involved in the assembly, regulation or stability of complex I. Some subunits may be also involved in other metabolic processes than NADH oxidation.

Subunit MWFE is located in the membrane arm of complex I (Carroll, Fearnley et al. 2003) and is composed of 70 amino acids. The first 25 amino acids at the N-termius are likely to form a single transmembrane domain, whereas the remaining part forms a hydrophilic domain which probably extrudes into the intermembrane space (Yadava and Scheffler 2004). Studies showed that absence of MWFE or hampered import of MWFE into mitochondria results in incompletely assembled complex I (Yadava, Potluri et al. 2002; Marques, Duarte et al. 2003). Also mutations have been found in the subunit NDUFA1, the human homologue of MWFE, in the patients with Leigh’s syndrome which leads to decreased level of intact complex I

(Fernandez-Moreira, Ugalde et al. 2007). This suggests a crucial role of MWFE in complex I assembly.

The subunit 18kDa is composed of 133 amino acids and is highly conserved among mammals (Walker 1992; Heuvel, Ruitenbeek et al. 1998). At position 121-131 it has a consensus site for cAMP-dependent phosphorylation (Papa, Sardanelli et al. 1999). Other studies showed that cAMP promotes the phosphorylation of subunit 18kDa which in turn activates complex I (Papa, Sardanelli et al. 1999; Scacco, Vergari et al. 2000; Papa, Scacco et al. 2001; Technikova-Dobrova, Sardanelli et al. 2001). Subunit 18kDa is probably located at the junction of the peripheral and membrane arm of complex I (Walker 1992). The absence of subunit 18kDa completely abolishes the assembly of a functional complex I (Petruzzella, Vergari et al. 2001). These studies showed that the subunit 18kDa is not only involved in the regulation of complex I, but also plays an important role in its assembly.

The subunit B16.6 is identical to protein GRIM19 (Gene associated with retinoid-interferone induced mortality) in human, which is an apoptosis inducing factor (Angell, Lindner et al. 2000; Fearnley, Carroll et al. 2001; Lufei, Ma et al. 2003). In the mouse model, knockout of gene *grim19* results in disruption of complex I assembly (Huang, Lu et al. 2004), but it is still not known whether this subunit of complex I participates directly in the apoptotic process.

### 1.2.4 Complex I deficiency

Complex I deficiency has been found in several progressive neurodegenerative diseases and is often part of a combined deficiency. Both mutations in nuclear and mitochondrial encoded DNA have been identified to be the cause (Thyagarajan and Byrne 2002). The syndromes of complex I deficiency are often tissue-specific (Kirby, Crawford et al. 1999). The most characteristic syndrome of complex I deficiency in muscles is mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) (Loeffen, Smeitink et al. 2000), and the phenotypes of complex I deficiency in neurons are Leigh-type encephalomyopathy, Leber's hereditary optic neuropathy (LHON), or a leucodystrophy with myoclonus epilepsy (Heuvel, Ruitenbeek et al. 1998; Brown 1999; Schuelke, Smeitink et al. 1999; Loeffen, Smeitink et al. 2000; Carelli, Rugolo et al. 2004). In some patients with Parkinson disease (PD), complex I deficiency has been observed in muscle, brain, and platelets (Janetzky, Hauck et al. 1994; Schapira, Gu et al. 1998; Tretter, Sipos et al. 2004) (Table 1.1).

Leigh syndrome is a severe progressive neurodegenerative disease that is caused by mutations in the genes for biotindase (Baumgartner, Suormala et al. 1989), pyruvate dehydrogenase and the complexes I, III and IV of the respiratory chain (Willems, Monnens et al. 1977; Bourgeron, Rustin et al. 1995; Triepels, van den Heuvel et al. 1999; Loeffen, Smeitink et al. 2000; Bénit, Slama et al. 2004). The complex I mutations found in the patients with Leigh syndrome, V122M in NDFS (PSST), P76L and R102M in NDUFS8 (TYKY), P22Q and several other mutations in NDUFS2 (49 kDa) were successfully reconstructed into the corresponding positions of *Yarrowia lipolytica* complex I (Ahlers, Garofano et al. 2000; Ahlers, Zwicker et al. 2000; Kerscher, Grgic et al. 2004). These complex I variants exhibited ≤ 50% NADH:quinone oxidoreductase activity and showed altered affinities for the substrate quinone and for quinone-like inhibitors. Theses studies provided possible clues toward a molecular understanding of Leigh disease.

**Table 1.1 Subunits of human complex I with pathological mutations and their associated diseases**

| Subunit | Phenotype | Reference |
|---|---|---|
| NDUFS1 (75kDa) | Leigh syndrome | (Martin, Blazquez et al. 2005) |
| NDUFS2 (49kDa) | Leigh syndrome | (Ahlers, Garofano et al. 2000) |
| NDUFS3 (30kDa) | Leigh syndrome | (Bénit, Slama et al. 2004) |
| NDUFS4 (AQDQ) | fatal multisystem disorder | (Heuvel, Ruitenbeek et al. 1998) |
| NDUFV1 (51kDa) | leucodystrophy, myoclonic epilepsy | (Schuelke, Smeitink et al. 1999; Martin, Blazquez et al. 2005) |
| NDUFV2 (24kDa) | hypertrophic cardiomyopathy and encephalopathy | (Bénit, Beugnot et al. 2003; Hinttala, Uusimma et al. 2005) |
| NDUFS8 (TYKY) | Leigh syndrome | (Loeffen, Smeitink et al. 1998; Martin, Blazquez et al. 2005) |
| NDUFS7 (PSST) | Leigh syndrome | (Triepels, van den Heuvel et al. 1999; Martin, Blazquez et al. 2005) |
| ND1 | MELAS, NIDDM, LHON, ADPD, dystonia, CPEO | (Wallace, Singh et al. 1988; Hirai, Suzuki et al. 1996; Schapira, Gu et al. 1998; Kirby, McFarland et al. 2004; Valentino, Barboni et al. 2004; Malfatti, Bugiani et al. 2007) |
| ND2 | LHON, ADPD | (Schapira, Cooper et al. |

| | | |
|---|---|---|
| | | 1990; Brown, Voljavec et al. 1992) |
| ND3 | Leigh syndrome, ESOC | (Taylor, Singh-Kler et al. 2001; Crimi, Papadimitriou et al. 2004) |
| ND4 | MELAS, LHON, Leigh syndrome, CPEO, DM, thyroid cancer line | (Schapira, Cooper et al. 1990; Chen, Li et al. 1998) |
| ND4L | LHON | (Schapira, Cooper et al. 1990) |
| ND5 | MELAS, LHON, Leigh syndrome, thyroid cancer line | (Brown, Voljavec et al. 1992; Santorelli, Tanji et al. 1997; Blok, Spruijt et al. 2007) |
| ND6 | MELAS, LHON, dystonia, thyroid cancer cell line, SNHL, LDYT | (Schapira, Warner et al. 1997; Valentino, Avoni et al. 2002; Raspall-Chaure, Solano et al. 2004) |

The nomenclature of the corresponding homologues in *Yarrowia lipolytica* complex I is given in parenthesis. ADPD: Alzheimer's disease and Parkinson's disease; CPEO: chronic progressive external ophthalmoplegia; DM: diabetes mellitus; ESOC: epilepsy, stroke-like episodes, optic atrophy and cognitive decline; LDYT: Leber's hereditary optic neuropathy and dystonia ; LHON: Leber's hereditary optic neuropathy; MELAS: mitochondrial encephalomypathy, lactic acidosis, and stroke-like episodes; NIDDM: non-insulin dependent diabetes mellitus; SNHL: sensorineural hearing loss.

### 1.2.5 *Yarrowia lipolytica*

The hemiascomyceteous yeast *Yarrowia lipolytica*, formerly classified as *Candida lipolytica,* later as *Saccharomyces lipolytica* (Wickerham, Kurtzman et al. 1990) is an obligate aerobe and is heterthallic and dimorphic, e.g. being able to form either yeast cells or hyphae and pseudohyphae depending on the growth conditions. This yeast is of great industrial interest due to its efficient capacity of secretion (Ogrydziak and Scharf 1982; Barth and Gaillardin 1997) and due to its ability to grow on cheap carbon sources, such as fatty acids, alkanes, oils and *n*-paraffins (Barth and Gaillardin 1997). Therefore, *Y. lipolytica* is used in the production of organic acids like citric acid, α-ketoglutaric acid and a wide variety of proteins from viruses, bacteria to plants and higher eukaryotes (Madzak, Gaillardin et al. 2004). Other industrial applications of *Y. lipolytica* include processing of diary products (Guerzoni, Lanciotti et al. 2001; Suzzi, Lanorte et al. 2001). *Y. lipolytica* is a non-pathogenic organism and several industrial processes based on it are classified by the Food and Drug Administration (FDA) as GRAS (generally regarded as safe). As a reliable and versatile host for heterologous protein production with easy-to-handle vectors (reviewed in (Madzak, Gaillardin et al. 2004)) and possibilities of large-scale applications, *Y. lipolytica* is a very

attractive candidate for industrial processes. *Y. lipolytica* has been studied as model organism for the metabolism of alkanes and fatty acids (Wang, Le Dall et al. 1999; Mauersberger, Wang et al. 2001), for the control of the dimorphic transition (Dominguez, Ferminan et al. 2000; Pérez-Campo and Domínguez 2001), for the general secretion pathway (Beckerich, Boisrame-Baudevin et al. 1998) and for the peroxisome biogenesis (Titorenko, Smith et al. 2000; Boukh-Viner and Titorenko 2006). In this thesis, the Po1t strain of *Y. lipolytica* was used. This strain is derived from the Po1g strain through the transformation of the plasmid pINA 1269 (Madzak, Treton et al. 2000). In this strain, both alkaline and acid extracellular proteases are deleted with facilitates the purification of complex I by minimizing the proteolytic processes.

Unlike fermentative yeasts like *S. cerevisiae, Y. lipolytica* posseses complex I, which is essential for the survival of the yeast. Previous studies have shown that complex I of *Yarrowia lipolytica* is stable enough to be purified from the mitochondrial membranes (Djafarzadeh, Kerscher et al. 2000; Kashani-Poor, Kerscher et al. 2001). EPR spectroscopy studies revealed that *Yarrowia* complex I has similar characteristics to those of *Neurospora crassa* and bovine heart complex I (Wang, Meinhardt et al. 1991; Ohnishi 1998; Kerscher, Dröse et al. 2002). Since a variety of genetic tools are available for *Yarrowia* (Madzak, Gaillardin et al. 2004) and its genome, including the mitochondrial genome, is sequenced (Casaregola, Neuveglise et al. 2000; Kerscher, Durstewitz et al. 2001), *Y. lipolytica* is an attractive model organism for the mutagenesis studies of eukaryotic complex I (Kerscher, Dröse et al. 2002).

## 1.3 $Na^+$-NQR of *Vibrio cholerae*

### 1.3.1 *Vibrio cholerae*

*Vibrio cholerae* is a motile, Gram-negative, rod-shaped bacterium with a polar flagellum. It is the causative agent of Asiatic cholera. About 200 serogroups are recognized as O, but only the O1 and O139 serogroup are responsible for the pandemic cholera. *Vibrio* species are chemoorganotrophs and facultative anaerobes. They are capable to catabolise a wide variety of sugars, organic acids and some amino acids (Baumann, Furniss et al. 1984). In contrast to other *Vibrio sp., V. cholerae* is capable to ferment glucose and tolerate low sodium ion concentrations during growth. *V. cholerae* growths at temperatures from 20-42°C and at a pH

range from 7.0-9.6 (Makukutu and Guthrie 1986; Hood and Winter 1997; Vital, Fuchslin et al. 2007).

The life cycle of *V. cholerae* consists of a free-swimming phase in marine and estuarine environments and a pathogenic phase inside the human host (Peterson 2002). Lipase, amylase, gelatinase and chitinase activities (Connell, Metzger et al. 1998) have been described in *V. cholerae* indicating the capability of *V. cholerae* to utilize polymers as carbon source during the free-living phase. Outside the host *V. cholerae* survives on various surfaces, such as on crustaceans, insects, filamentous algae, zooplankton or water plants (Huq, Huq et al. 1986; Tamplin, Gauzens et al. 1990; Shukla, Singh et al. 1995; Watnick and Kolter 1999). Once inside the host, *V. cholerae* penetrates the mucus layer of the intestinal villi, adheres to and colonizes the epithelial surface. After entering a non-motile phase, *V. cholerae* replicates and secretes numerous toxins and exoproteins causing massive watery diarrhoeal disease characteristic of cholera. Through diarrhea *V. cholerae* disseminates back into the watery environment, thus completing its life cycle.

Two biotypes of *V. cholerae* serogroup O1 have been found, which are associated with at least three of eight worldwide cholera pandemics in the recorded history. The “classical” biotype is characterized by a capsule and has two chromosomal copies of CTXΦ, a *V. cholerae*-specific filamentous bacteriophage encoding cholera toxin (CT) and other structural proteins (Waldor and Mekalanos 1996; Lencer and Tsai 2003; McLeod, Kimsey et al. 2005). The biotype “El Tor” contains only one chromosomal copy of CTXΦ and produces hemolysins (Kalsow and F.S. 1968; Mekalanos 1985; Butterton, Beattie et al. 1995). The CT encoding genes (*ctx*) along with other genes belonging to CTXΦ reside on the larger of the two *V. cholerae* chromosomes (Chromosome I) (Waldor and Mekalanos 1994; Sharma, Maiti et al. 1997; Davis, Moyer et al. 2000; Garg, Nandy et al. 2000), whereas genes encoding other virulence factors such as toxin-coregulated pilus TCP (*tcp*), accessory colonization factors ACFs (*acf*) and the regulatory protein ToxT (*toxT*) are located on a ~40-kb locus termed *Vibrio* pathogenicity island (VPI) (Karaolis, Johnson et al. 1998). To adapt differently to environments in its life cycle, *V. cholerae* regulates virulence genes both temporally and spatially. Once *V. cholerae* reaches its destination, i.e. is attached to the villi of the epithelial cells in the intestine, certain environmental conditions which have not yet been identified are fulfilled, and two transcription activators AphA and AphB initiate the expression of TcpP/H (Kovacikova and Skorupski 1999; Skorupski and Taylor 1999). Together with other activators,

ToxR/S, TcpP/H activate the transcription of *toxT* (Higgins and DiRita 1994; Häse and Mekalanos 1998). ToxT in turn activates the transcription of *tcp* and *ctx* genes, as well as additional genes such as *acf* genes (DiRita, Parsot et al. 1991; Higgins, Nazareno et al. 1992). Interestingly, elevated *toxT* expression has been observed in non-motile mutants or when the $Na^+$-NQR is inactivated (Gardel and Mekalanos 1996). $Na^+$-NQR is the main contributor of the generation of sodium motive force which drives the flagellar motor of *V. cholerae*, but seems also to play an important role in the antagonistic regulation of motility and virulence (Häse and Mekalanos 1999).

### 1.3.2 $Na^+$ -NQR of *Vibrio cholerae*

The $Na^+$-translocating NADH:quinone oxidoreductase ($Na^+$-NQR) is an integral membrane enzyme of 210 kDa and the first complex of the respiratory chain of many marine and pathogenic bacteria (Zhou, Bertsova et al. 1999). The complex consists of 6 subunits, termed NqrA-F, and oxidizes NADH under reduction of quinone. The free energy released in this redox reaction is used to generate a sodium motive force (Eq. 2) that in turn is used for several metabolic processes, such as $Na^+$/substrate symport, $Na^+$/drug antiport, and motility (Kojima, Yamamoto et al. 1999; Zhou, Bertsova et al. 1999; Hayashi, Nakayama et al. 2001; Ito, Guffanti et al. 2001; Steuber 2001; Barquera, Hellwig et al. 2002; Barquera, Zhou et al. 2002; Bertsova and Bogachev 2004; Bogachev and Verkhovsky 2005).

$$NADH + H^+ + Q + nNa^+_{in} \rightleftharpoons NAD^+ + QH_2 + nNa^+_{out} \qquad \text{(Eq. 2)}$$

So far only limited information about the structure of the complex is available. Using topology models, nine transmembrane helices have been predicted in NqrB, two in NqrC, one in NqrF and six in NqrD and E, respectively. NqrA is the only subunit that does not contain hydrophobic stretches predicted to insert into the membrane. The $Na^+$-NQR complex contains a number of cofactors including a [2Fe-2S] cluster and several flavins (riboflavin, FMN and FAD) (Barquera, Hellwig et al. 2002; Barquera, Nilges et al. 2004; Bogachev and Verkhovsky 2005). Two FMNs are covalently bound to NqrB and C at the residues T236 and T225, respectively, and a putative quinone binding site in NqrB at G141 has been identified on the basis of inhibitor studies (Zhou, Bertsova et al. 1999; Hayashi, Nakayama et al. 2001; Duffy and Barquera 2006). Using reporter group gene-fusion methods, it has been determined that the N- and C-termini of NqrD reside in the cytoplasm, whereas the N- and C- termini of NqrE are in the periplasm (Duffy and Barquera 2006). The topological homologues of NqrD

and E in *E. coli*, YdgQ and L, respectively, show the same orientation (Saaf, Johansson et al. 1999).

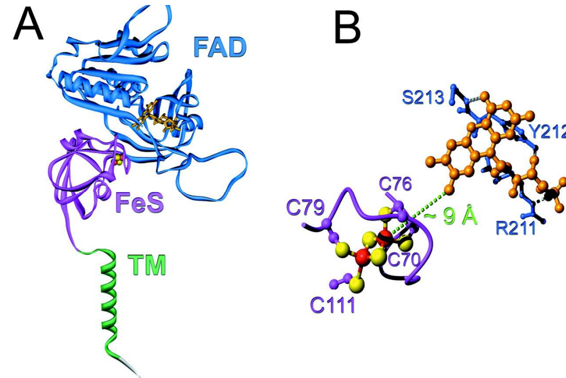



**Figure 1.4 A structural model of NqrF** (Türk, Puhar et al. 2004)
Using the structure of benzoate 1, 2- dioxygenase reductase from *Acinetobacter spp.,* which shows 23 % overall homology to NqrF, this structural model for NqrF is constructed. (A) NqrF is anchored to the membrane by a transmembrane helix, shown in green, and it has two prosthetic groups: a [2Fe-2S] cluster, shown in yellow (S) and red (Fe) and a non-covalently bound FAD, shown in orange. The polypeptides that bind the iron-sulfur cluster are shown in violet and the domain that holds FAD is shown in blue. (B) In this model, the coordinating residues that bind the iron-sulfur cluster and the FAD are indicated explicitly. The calculated distance between the iron-sulfur cluster and the FAD is small enough to allow electron transfer.

NqrF catalyses the initial electron transfer reaction in the $Na^+$-NQR complex with a [2Fe-2S] cluster binding motif at the N-terminus and a FAD and NADH binding motif at the C-terminus. Based on the high-resolution structure of the benzoate 1,2-dioxygenase reductase (BenC) from *Acinetobacter sp* strain ADP1 (Karlsson, Beharry et al. 2002) and related proteins (Lu, Lindqvist et al. 1995; Müller, Müller et al. 1998) a structural model for NqrF was created (Türk, Puhar et al. 2004) (Figure 1.4). Like NqrF, BenC also contains a [2Fe-2S] cluster and FAD as prosthetic group and exhibits 23% overall sequence identity to NqrF. A soluble variant of NqrF devoid of the putative α-helix at the N-terminus, termed NqrF’, the FAD- and the iron-sulfur cluster-binding domain of NqrF have been successfully overexpressed, purified and characterized separately (Türk, Puhar et al. 2004). Both NqrF’ and the flavin domain contain 1 mol of FAD per mol enzyme. The FAD of the flavin domain exhibits a midpoint redox potential $E_m(FAD/FADH_2)$ = -286 mV. The [2Fe-2S] cluster of NqrF showed an axial spectrum in EPR and Mössbauer spectroscopic properties reminiscent of the vertebrate-type 2Fe-ferredoxins (Lin, Puhar et al. 2005). The model of NqrF suggests

that the two cofactors are in close proximity of 9 Å, which allows electron transfer from the FAD to the 2Fe-2S cluster (Türk, Puhar et al. 2004).

## 1.4 Aim of This Work

My goal was to elucidate the properties of $Na^+$-translocating NADH:quinone oxidoreductase ($Na^+$-NQR) from *Vibrio cholerae* with respect to its cofactors and enzymatic functions. In the first part of this thesis, the iron-sulfur cluster of the subunit NqrF from $Na^+$-NQR is characterized in detail using various spectroscopic methods, such as electron paramagnetic resonance (EPR) spectroscopy, Mössbauer spectroscopy and magnetic circular dichroism (MCD) spectroscopy. The oxidation of the reduced cofactor in redox enzymes with $O_2$ can produce reactive oxygen species. In the second part of this thesis, novel functions of $Na^+$-NQR with respect to formation of organic radicals and reactive oxygen species were investigated. The observation that a bacterial complex I translocates $Na^+$ rather than $H^+$ raised the question whether a eukaryotic complex I may also have the capacity to translocate cations other than protons. In the third part of this thesis, the $Na^+$-transport activity of the complex I from the yeast *Yarrowia lipolytica* was investigated in isolated submitochondrial particles (SMPs) or proteoliposomes with purified and reconstituted complex I. The results of this study shall provide clues to the question whether the ability of complex I to translocate $Na^+$ originates farther back in the evolution, thus observed both in bacteria and eukaryotes, or whether it represents a newly-developed feature to adapt to new environments.

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# 2 A Vertebrate-type Ferredoxin Domain in the $Na^+$-translocating NADH Dehydrogenase from *Vibrio cholerae*

Po-Chi Lin[‡], Andrea Puhar[‡], Karin Türk[‡], Stergios Piligkos[||], Eckhard Bill[||], Frank Neese[||], and Julia Steuber[‡]

From the [‡]Mikrobiologisches Institut der Eidgenössischen Technischen Hochschule, ETH-Hönggerberg, CH-8093 Zürich, Switzerland and the [||]Max-Planck-Institut für Bioanorganische Chemie, D-45470 Mülheim an der Ruhr, Germany

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## 2.1 Abstract

The $Na^+$-translocating NADH:quinone oxidoreductase from *Vibrio cholerae* contains a single Fe-S cluster localized in subunit NqrF. Here we study the electronic properties of the Fe-S center in a truncated version of the NqrF subunit comprising only its ferredoxin-like Fe-S domain. Mössbauer spectroscopy of the Fe-S domain in the oxidized state is consistent with a binuclear Fe-S cluster with tetrahedral sulfur coordination by the cysteine residues $Cys^{70}$, $Cys^{76}$, $Cys^{79}$, and $Cys^{111}$. Important sequence motifs surrounding these cysteines are conserved in the Fe-S domain and in vertebrate-type ferredoxins. The magnetic circular dichroism spectra of the photochemically reduced Fe-S domain exhibit a striking similarity to the magnetic circular dichroism spectra of vertebrate-type ferredoxins required for the in vivo assembly of iron-sulfur clusters. This study reveals a novel function for vertebrate-type [2Fe-2S] clusters as redox cofactors in respiratory dehydrogenases.

## 2.2 Introduction

Iron-sulfur proteins are present in all domains of living organisms where they exhibit diverse functions like electron transport, catalysis, and sensing in regulatory processes (Beinert, Holm et al. 1997). In some NADH-oxidizing, respiratory complexes, Fe-S centers accept electrons from flavin cofactors in an overall exergonic reaction that results in the reduction of quinone. This electron transfer reaction drives the uphill transport of protons or $Na^+$ across the inner membrane of mitochondria or bacteria. The NADH:quinone oxidoreductase ($Na^+$-NQR) from the human pathogen *Vibrio cholerae* maintains an electrochemical $Na^+$ gradient across the inner bacterial membrane, which strongly influences the production of virulence factors (Häse and Mekalanos 1999). The $Na^+$-NQR consists of six subunits, NqrA-F, and contains one Fe-S center, two covalently bound FMNs, one non-covalently bound FAD, one riboflavin, and ubiquinone-8 as prosthetic groups (Pfenninger-Li, Albracht et al. 1996; Hayashi, Nakayama et al. 2001; Barquera, Zhou et al. 2002; Bogachev, Bertsova et al. 2002; Türk, Puhar et al. 2004). The NqrF subunit of the $Na^+$-NQR complex is anchored to the inner membrane and displays a clearly defined domain structure. The N-terminal Fe-S domain harbors the [2Fe-2S] cluster, while the binding sites for the non-covalently bound FAD and NADH are located in the C-terminal domain of NqrF. The initial oxidation of NADH by the NqrF subunit results in the two-electron reduction of the FAD followed by one-electron transfer steps to the [2Fe-2S]

cluster in the Fe-S domain (Türk, Puhar et al. 2004). Here we study the electronic properties of the [2Fe-2S] cluster in the isolated Fe-S domain of NqrF. A comparison of its amino acid sequence with sequences of [2Fe-2S] ferredoxins from vertebrates and plants reveals that the Fe-S domain is related to ferredoxins of the vertebrate-type family. Vertebrate-type ferredoxins are soluble redox carriers that accept electrons from specific NADH:ferredoxin reductases and deliver them to enzymatic systems catalyzing the hydroxylation of various compounds like steroids or camphor (Grinberg, Hannemann et al. 2000). The Fe-S domain exhibits highest sequence similarity to vertebrate-type ferredoxins required for the in vivo assembly of iron-sulfur clusters (ISC-type ferredoxins) (Johnson, Dean et al. 2005). This is further supported by Mössbauer and magnetic circular dichroism (MCD) spectra of the Fe-S domain, which are reminiscent to ISC-type ferredoxins. Our finding that a vertebrate-type [2Fe-2S] cluster is an intrinsic redox cofactor of the $Na^+$-translocating NADH dehydrogenase adds a novel function in respiration to this class of Fe-S centers.

## 2.3 Material and Methods

### 2.3.1 Preparation of the Fe-S domain

The Fe-S domain of the $Na^+$-NQR comprises the amino acids $Met^1$-$Phe^{146}$ of subunit NqrF devoid of the hydrophobic residues $Val^8$–$Ala^{25}$, which are likely to anchor the NqrF subunit to the membrane. The molecular mass of the Fe-S domain including its N-terminal polyhistidine tag is 17,873 Da. Following NifS-mediated in vitro reconstitution of the Fe-S cluster (Zheng 1993), the Fe-S domain was purified by nickel-nitrilotriacetic acid affinity chromatography to remove precipitated iron sulfides and the cysteine desulfurase, NifS (Türk, Puhar et al. 2004).

### 2.3.2 Spectroscopy

For Mössbauer spectroscopy, the NifS-mediated reconstitution of the Fe-S cluster of the Fe-S domain (Türk, Puhar et al. 2004) was carried out with the $^{57}$Fe-enriched Mohr's salt, $(NH4)_2{}^{57}Fe(SO_4)_2{\cdot}6H_2O$, as source of redox-stable ferrous ions. The salt was obtained from metallic iron foil (95% $^{57}$Fe) by adding a stoichiometric amount of 1 M $H_2SO_4$ to 1.88 x $10^{-4}$ mol of metallic $^{57}$Fe. The solution was heated until the iron foil was completely dissolved. The volume was kept constant by adding distilled water to compensate for evaporation. Subsequently, the solution was concentrated at 80 °C until a thin crystal film appeared on its surface. Likewise, 1.88 x $10^{-4}$ mol of $(NH_4)_2SO_4$ was dissolved in a few drops of distilled

water and concentrated at 80 °C until crystallization commenced. The two hot solutions were mixed and crystals of $(NH_4)_2{}^{57}Fe(SO_4)_2 \cdot 6H_2O$ formed overnight at room temperature. The mother liquor was removed and the dried crystals were stored in the anaerobic chamber until use. Mössbauer data were recorded with a spectrometer of the alternating constant-acceleration type equipped with a Variox Cryostat (Oxford Instruments). The minimum experimental line width was 0.24 mm $s^{-1}$ (full width at half-height). The $^{57}Co$/Rh source (1.8 GBq) was positioned at room temperature inside the gap of the magnet system at a zero-field position. Isomer shifts are quoted relative to iron metal at 300 K. For MCD spectroscopy, the Fe-S domain in 50 mM Tris/HCl, pH 7.8, 40 mM oxalate, 4 µM 5-deazaflavin, and 50% (v/v) glycerol was irradiated with white light for 10 min in the MCD cell immediately prior to freezing in liquid nitrogen. The photochemical 5-deazaflavin/oxalate system generates electrons at very low potentials of –650 mV (Massey, Stankovich et al. 1978) required for the reduction of the Fe-S domain. MCD spectra were obtained at liquid He temperatures (1.8–50.4 K) on a Jasco J-715 (200–1060 nm) with an extended S-20 and S-1 photomultiplier tube (Hammamatsu). The J-500C spectrometer was equipped with an Oxford Instruments SM4-11 T superconducting magnet/cryostat capable of fields up to 11 T and temperatures down to 1.5 K.

### 2.3.3 Analytical methods

Protein was determined by the microbiuret method (Goa 1953) preceded by trichloroacetic acid precipitation. Bovine serum albumin served as standard. The concentration of the Fe-S domain was determined by the microbiuret method standardized by UV spectroscopy using the theoretical extinction coefficient at 280 nm, 6290 $M^{-1}$ $cm^{-1}$, of the colorless Fe-S domain devoid of its Fe-S cluster. Iron was determined colorimetrically by the 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazinedisodium salt trihydrate (ferene) complex (Beinert 1978). For the determination of acid-labile sulfur the methylene blue method (Beinert 1983) was applied.

## 2.4 Results and Discussion

Visible absorption and EPR spectra of the Fe-S domain of the NqrF subunit are reminiscent of [2Fe-2S] ferredoxins of the vertebrate type, with a maximum around 540 nm in the visible spectrum and a nearly axial EPR signal with $g_{\|} \approx 2.020$ and $g_{\|} \approx 1.938$ in the reduced state. The

EPR spectrum of the Fe-S domain was indistinguishable from the spectrum of the NqrF subunit, indicating a very similar structural environment of the cluster in the two proteins (Türk, Puhar et al. 2004). Axial $S = 1/2$ EPR signals are typical of $[2Fe\text{-}2S]^+$ clusters in vertebrate-type ferredoxins (Ta and Vickery 1992), while $[2Fe\text{-}2S]^+$ centers of plant-type ferredoxins exhibit rhombic $S = 1/2$ resonances. Sequence comparisons were performed with the aim to assign the Fe-S domain to known classes of ferredoxins. The NqrF subunit is highly conserved among $Na^+$-NQRs of different organisms, especially among *Vibrio sp.* (identity >90%). Including NqrF of *Chlamydia sp.*, which form a group apart from other identified NqrF sequences, reduces the consensus to 36% identity (Figure 2.1). The [2Fe-2S] cluster in NqrF is coordinated by the conserved residues $Cys^{70}$, $Cys^{76}$, $Cys^{79}$, and $Cys^{111}$ (Barquera, Nilges et al. 2004). The arrangement of these cysteine residues matches the C-X(5)-C-X(2)-C-x(30 $< n <$ 39)-C cluster binding motif characteristic of vertebrate-type [2Fe-2S] ferredoxins with the conserved residues RLXCQ surrounding the fourth cysteine ligand ($C^{111}$ in *V. cholerae* NqrF). In addition to the cluster binding motif, an important region conserved between NqrF and vertebrate-type ferredoxins is the $\beta$ strand from $M^{119}$ to $L^{123}$, corresponding to $Met^{103}$–$Val^{107}$ in adrenodoxin, followed by a conserved proline ($P^{124}$ in NqrF) (Grinberg, Hannemann et al. 2000). A BLAST search (Altschul, Gish et al. 1990) was performed using only the Fe-S domain of NqrF from *V. cholerae* as the input sequence. The highest similarity of the Fe-S domain to a biochemically characterized 2Fe ferredoxin was found for the ISC-related ferredoxin 5 from *Aquifex aeolicus* (21% identity and 34% similarity) (Mitou, Higgins et al. 2003), followed by the ISC-type ferredoxin Yah1p from *Saccharomyces cerevisiae* (Lange, Kaut et al. 2000; Mühlenhoff, Gerber et al. 2003) and ferredoxin IV from *Azotobacter vinelandii* (Zheng, Cash et al. 1998; Jung, Gao-Sheridan et al. 1999) (Figure 2.1). Yah1p and FdIV are essential components of the ISC assembly machinery, a biochemical pathway for the formation of iron-sulfur proteins in eukaryotes and bacteria (Johnson, Dean et al. 2005; Lill and Mühlenhoff 2005).

Mössbauer spectroscopy is a powerful tool to determine the number of irons per cluster, its oxidation state, and the type of ligands to the Fe atoms (Que and Münck 2000). The zero-field Mössbauer spectrum of the oxidized Fe-S domain of $Na^+$-NQR recorded at 80 K shows an asymmetric doublet as depicted in Figure 2.2. The spectrum could be deconvoluted into three symmetric quadrupole doublets with Lorentzian line shape. The major component, with 80% relative intensity, has small quadrupole splitting $\Delta EQ = 0.61$ mm/s and characteristic low isomer shift $\delta = 0.283$ mm/s, which is typical of ferric iron with tetrahedral sulfur

coordination (Münck, Debrunner et al. 1972; Huynh and Kent 1984; Mitou, Higgins et al. 2003).

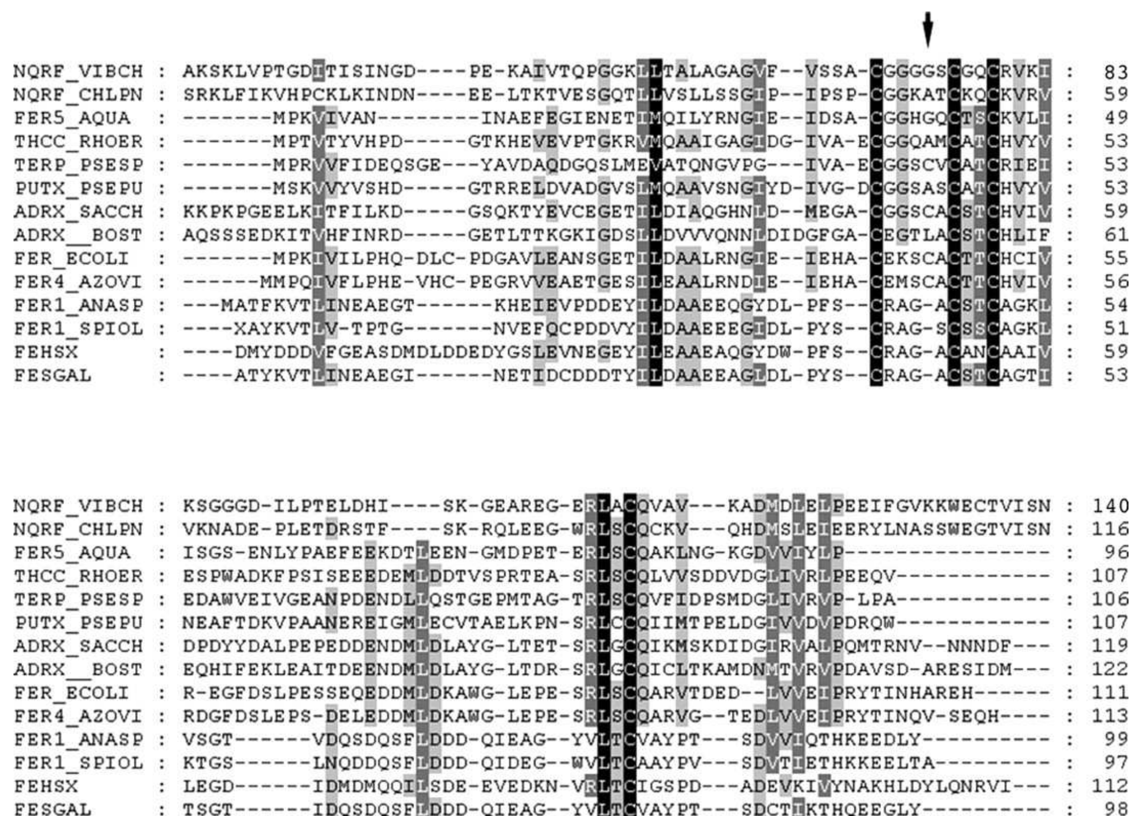


**Figure 2.1. Sequence alignment of the Fe-S domain of $Na^+$-NQR with [2Fe-2S] ferredoxins.**
The Fe-S domain ($Ala^{25}$–$Asn^{140}$) of the NqrF subunit from *V. cholerae* (NqrF_VIBCH, Q9X4Q8) was aligned with the putative NqrF subunit from *Chlamydophila pneumoniae* (NqrF_CHLPN, 15214181) and with representatives of vertebrate- and plant-type ferredoxins with special focus on the arrangement of the cysteine residues $Cys^{70}$, $Cys^{76}$, $Cys^{79}$, and $Cys^{111}$, which ligate the Fe-S cluster (Barquera, Nilges et al. 2004). The alignment was created with Clustal W and was adjusted considering structural elements. FER5_AQUAE, *A. aeolicus* Fd5 (P59799); THCC_RHOER, *Rhodococcus erythropolis* rhodocoxin (P43493); TERP_PSESP, *Pseudomonas sp.* terpredoxin (P33007; PDB entry 1B9R); PUTX_PSEPU, *Pseudomonas putida* putidaredoxin (PXPSEP; PDB entry 1PUT); ADRX_SACCE, *S. cerevisiae* Yah1p (S0006173); ADRX_BOST, *Bos taurus* adrenodoxin (BAA00363 PDB entry 1AYF); FER_ECOLI, *Escherichia coli* ISC-Fd (P25528, PDB entry 1I7H); FER4_AZOVI, *A. vinelandii* ISC-Fd (FdIV) (T44286); FER1_ANASP, *Anabaena* strain PCC 7120 Fd (P06543, PDB entry 1FXA); FER1_SPIOL, *Spinacia oleracea* FdI; FEHSX, *Haloarcula marismortui* (PDB entry 1DOI); FESGAL, *Spirulina platensis* Fd (PDB entry 4FXC). The arrow indicates the fourth amino acid residue between the first and the second cysteine ligand of the Fe-S cluster, which is lacking in plant-type ferredoxins. Consensus level of amino acid residues: fully conserved (black), intermediate (≥80%) (dark gray), low (≥60%) (light gray).

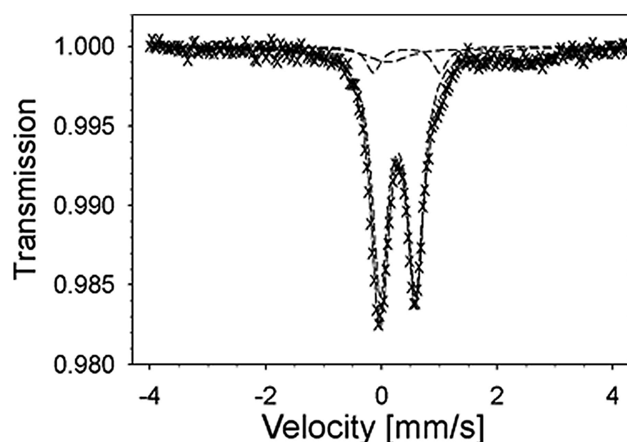


**Figure 2.2. Zero-field Mössbauer spectrum of the Fe-S domain of the Na$^+$-translocating NADH:quinone oxidoreductase.**
The Mössbauer spectrum of the Fe-S domain (0.4 mM) was recorded at zero magnetic field at 80 K. x marks indicate the experimental values. The solid line is a theoretical spectrum that represents the sum of the simulated spectra of three components. The major component (80% weight) is assigned to a diamagnetic $[2Fe\text{-}2S]^{2+}$ cluster with $\Delta E_Q = 0.613$ mm/s and $\delta = 0.283$.

Since the subspectrum does not show any indications of paramagnetic broadening, and moreover, the Fe-S domain in the as isolated state is EPR-silent, the major contribution of the Mössbauer subspectrum can be assigned to a diamagnetic Fe(III)-Fe(III) pair due to the presence of binuclear $[2Fe\text{-}2S]^{2+}$ clusters. This is further supported by the content of Fe and $S^{2-}$ (1.61 ± 0.28 and 1.31 ± 0.11 mol/mol of Fe-S domain, respectively). Two further subcomponents are found in the Mössbauer spectrum with the following simulation parameters: $\Delta EQ = 1.142$ mm/s and $\delta = 0.436$ mm/s, weight 8% (component 2) and $\Delta EQ = 2.469$ mm/s and $\delta = 1.342$ mm/s, weight 12% (component 3). The distinct high isomer shift of component 3 is typical of hexa-coordinated Fe(II) with "hard" donor ligands. We assign it to remaining Fe(II) ions in the solution (hex-aquo complex or non-specifically bound iron), originating from $(NH_4)_2{}^{57}Fe(SO_4)_2 \cdot 6\ H_2O$, the iron source in the reconstitution assay. The Mössbauer parameters of the minor component 2 resemble those of the delocalized mixed valence Fe(2.5)/Fe(2.5) pair of a cubane 4Fe-4S cluster (Beinert, Holm et al. 1997). However, the component cannot be clearly discriminated from high spin Fe(III) precipitates due to the presence of some non-specifically bound iron oxide or hydroxide.

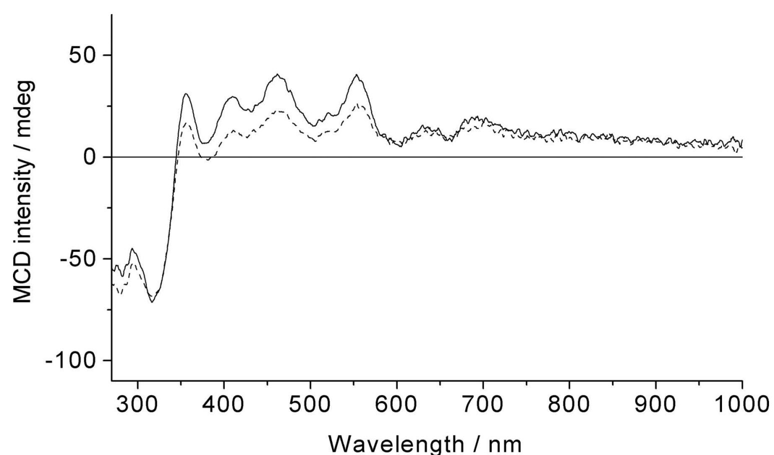


**Figure 2.3. Magnetic circular dichroism spectra of the [2Fe-2S]$^{+}$ cluster in the photochemically reduced Fe-S domain of the Na$^{+}$-NQR**
The spectra were recorded at 5.2 K (dashed line) and 1.6 K (solid line) with an applied field of 5 T. All bands increase in intensity with decreasing temperature indicating C-term behavior. The concentration of the Fe-S domain was 1.1 mM.

To further compare the [2Fe-2S] cluster in the Fe-S domain with other ferredoxins, we applied variable temperature magnetic circular dichroism (VTMCD) spectroscopy, a complementary approach to EPR for investigating the electronic properties of paramagnetic iron-sulfur clusters. In particular, VTMCD spectroscopy allows to distinguish between vertebrate and plant-type [2Fe-2S]$^{+}$ centers and provides a sensitive probe of the electronic structure of clusters with paramagnetic ground states (Johnson, Robinson et al. 1982). Each electronic transition from a spin degenerate ground state gives rise to positive or negative absorption-shaped MCD bands that increase in intensity with decreasing temperature. These C-term features commonly dominate the MCD spectra of paramagnetic species. The VTMCD spectrum of the photochemically reduced Fe-S domain (Figure 2.3) reveals a negative band at 315 nm assigned to Fe(II)-S charge transitions and positive bands at 358, 409, 461, 554, 629, and 694 nm assigned to Fe(III)-S charge transitions. This pattern of negative and positive bands is strikingly similar to the MCD spectrum of the [2Fe-2S] cluster found in the ISC-type Fd IV (Mitou, Higgins et al. 2003) or in the central domain of the ISC scaffold protein NifU (Fu, Jack et al. 1994). The spectrum of the Fe-S domain is clearly distinct from the MCD spectra of plant-type ferredoxins, which exhibit a prominent positive band at 515 nm (Fu, Drozdzewski et al. 1992). A comparison of the MCD spectrum of the Fe-S domain with the spectrum of other vertebrate-type Fds like putidaredoxin (Fu, Drozdzewski et al. 1992) shows that the latter displays an additional band in the range between 409 and 461 nm, which is not present in the Fe-S domain. We conclude that the [2Fe-2S] cluster in the isolated Fe-S domain

of the $Na^+$ redox pump is very similar to the 2Fe-cluster from vertebrate-type ferredoxins and among these is mostly related to the [2Fe-2S] cluster present in ISC-type ferredoxins. ISC-type Fds catalyze an essential step during the biogenesis of iron-sulfur clusters (Johnson, Dean et al. 2005). The *in vitro* formation of Fe-S clusters with nuclearity >2 requires a reductive step (Beinert, Holm et al. 1997). *In vivo*, this redox reaction is likely to be catalyzed by ISC-type ferredoxins and specific dehydrogenases, which act as electron carriers during ISC assembly (Mühlenhoff, Gerber et al. 2003; Mansy and Cowan 2004; Johnson, Dean et al. 2005; Lill and Mühlenhoff 2005). The NqrF subunit of the $Na^+$-translocating NADH:quinone oxidoreductase represents a model for studying electron transfer from NADH to an iron-sulfur cluster that is very similar to the [2Fe-2S] center found in ISC-type ferredoxins.

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# 3 Quinone Reduction by the Na$^{+}$-translocating NADH Dehydrogenase Promotes Extracellular Superoxide Production in *Vibrio cholerae*

**Po-Chi Lin,[1] Karin Türk,[2] Claudia C. Häse,[3] Günter Fritz,[4] and Julia Steuber[1]**

Biochemisches Institut, Universität Zürich, CH-8057 Zürich, Switzerland,[1] School of Engineering and Science, International University Bremen, D-28759 Bremen, Germany,[2] Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331,[3] Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany[4]

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## 3.1 Abstract

The pathogenicity of *Vibrio cholerae* is influenced by sodium ions which are actively extruded from the cell by the $Na^+$-translocating NADH:quinone oxidoreductase ($Na^+$-NQR). To study the function of the $Na^+$-NQR in the respiratory chain of *V. cholerae*, we examined the formation of organic radicals and superoxide in a wild-type strain and a mutant strain lacking the $Na^+$-NQR. Upon reduction with NADH, an organic radical was detected in native membranes by electron paramagnetic resonance spectroscopy which was assigned to ubisemiquinones generated by the $Na^+$-NQR. The radical concentration increased from 0.2 mM at 0.08 mM $Na^+$ to 0.4 mM at 14.7 mM $Na^+$, indicating that the concentration of the coupling cation influences the redox state of the quinone pool in *V. cholerae* membranes. During respiration, *V. cholerae* cells produced extracellular superoxide with a specific activity of 10.2 nmol $min^{-1}$ $mg^{-1}$ in the wild type compared to 3.1 nmol $min^{-1}$ $mg^{-1}$ in the NQR deletion strain. Raising the $Na^+$ concentration from 0.1 to 5 mM increased the rate of superoxide formation in the wild-type *V. cholerae* strain by at least 70%. Rates of respiratory $H_2O_2$ formation by wild-type *V. cholerae* cells (30.9 nmol $min^{-1}$ $mg^{-1}$) were threefold higher than rates observed with the mutant strain lacking the $Na^+$-NQR (9.7 nmol $min^{-1}$ $mg^{-1}$). Our study shows that environmental $Na^+$ could stimulate ubisemiquinone formation by the $Na^+$-NQR and hereby enhance the production of reactive oxygen species formed during the autoxidation of reduced quinones.

## 3.2 Introduction

The gram-negative bacterium *Vibrio cholerae* naturally inhabits aquatic ecosystems, but some strains are able to colonize the human intestine, where they can cause the severe diarrheal disease cholera (Guerrant, Carneiro-Filho et al. 2003). As an adaptation for growth at high NaCl concentrations, *V. cholerae* expels sodium ions from the cytoplasm during respiration and establishes a sodium motive force across its inner membrane (Häse and Barquera 2001). This respiratory $Na^+$ transport is catalyzed by the $Na^+$-translocating NADH:quinone oxidoreductase ($Na^+$-NQR), which consists of six subunits, NqrA to -F, and contains one Fe-S center, two covalently bound flavin mononucleotides, one non-covalently bound flavin adenine dinucleotide (FAD), one riboflavin, and ubiquinone-8 as prosthetic groups (Hayashi

and Unemoto 2004; Türk, Puhar et al. 2004; Bogachev and Verkhovsky 2005). Genome comparisons reveal that a $Na^+$-NQR is present in many pathogenic bacteria, indicating that pathogens may benefit from a sodium cycle for nutrient uptake or motility (Häse and Barquera 2001). The sodium motive force which is maintained by the $Na^+$-NQR strongly influences the production of virulence factors in *Vibrio cholerae* (Häse and Mekalanos 1999), and environmental $Na^+$ is likely to be an important parameter during infection both as stimulus and as respiratory coupling ion (Häse and Barquera 2001). Loss of the $Na^+$-NQR, either by mutation or by chemical inhibition, results in altered virulence gene regulation in *V. cholerae* (Häse and Mekalanos 1999), but the putative link between sodium membrane energetics and virulence has not been identified yet.

Superoxide ($O_2^-$) is an anionic free radical produced by the oxidation of reduced cofactors of redox enzymes with $O_2$ (Imlay 2003). As a charged species, it cannot cross membranes at physiological pH and therefore is constrained to the compartment where it originated, e.g., the cytoplasm or periplasm of a bacterial cell (Korshunov and Imlay 2002). Respiratory NADH dehydrogenases may directly produce superoxide from the reaction of their reduced flavin cofactors with $O_2$ (Massey 1994; Kussmaul and Hirst 2006), or they may indirectly contribute to superoxide formation in a bacterial cell by producing reduced quinones which are reoxidized by $O_2$ (Korshunov and Imlay 2006). Here we investigate the function of the $Na^+$-NQR in *V. cholerae* using native membranes and whole cells. It is shown that the $Na^+$-NQR represents a major source for extracellular superoxide produced by respiring *V. cholerae* cells.

## 3.3 Materials and Methods

### 3.3.1 Growth of *Vibrio cholerae*

The parent strain *V. cholerae* O395 N1 (Mekalanos, Swartz et al. 1983) and *V. cholerae* O395 N1 (*nqrC*::Tn*bla*) (Häse and Mekalanos 1999) with a Tn*5* transposon in the *nqrC* gene were cultivated aerobically in Luria-Bertani medium supplemented with 10 mM glucose in the presence of 50 µg $ml^{-1}$ streptomycin at 37°C. For maintenance of the Tn*bla* cassette insertion in *nqrC* in *V. cholerae* O395 N1 (*nqrC*::Tn*bla*) 100 µg $ml^{-1}$ ampicillin was added to the medium. *V. cholerae* cells were grown for 6 to 8 h to the late exponential phase and harvested by centrifugation. The cells were resuspended in 10 mM HEPES-KOH, pH 7.5, 0.2 M $K_2SO_4$, 10% glycerol; frozen in liquid nitrogen; and stored at –80°C until use.

### 3.3.2 Preparation of membranes

Cells were disrupted under exclusion of $O_2$. Prior to use all solutions were degassed and purged with $N_2$ followed by equilibration in a Coy glove box (95% $N_2$, 5% $H_2$) at least overnight ($O_2$ concentration of <0.3 µM in buffers). Cells (8 to 11 g, wet weight) were resuspended in 25 ml buffer (10 mM HEPES-KOH, pH 7.5, 0.2 M $K_2SO_4$) containing 5 mM $MgCl_2$ and traces of DNase I (Roche Diagnostics). The cell suspension was passed once through a French pressure cell at 83 MPa, and the eluate was collected under a stream of $N_2$. Unbroken cells and large debris were removed by centrifugation at 35,000 x *g* for 20 min. To the supernatant containing the membrane vesicles, 50 mM $K_2$-EDTA was added as a chelator for $Mn^{2+}$ and $Cu^{2+}$, which perturb electron paramagnetic resonance (EPR) spectra. If not indicated otherwise, all subsequent manipulations were performed in the glove box at room temperature. The reddish-brown membranes were collected by ultracentrifugation (150,000 x *g*, 1 h, 4 °C) and were washed once with 60 ml buffer containing 10 mM $K_2$EDTA. Two additional washing steps in 60 ml buffer without $K_2$EDTA were performed to further decrease the concentrations of $Na^+$, $Mn^{2+}$, and $Cu^{2+}$. Membranes were thoroughly mixed with buffer to yield suspensions containing 40 ± 10 mg protein $ml^{-1}$ and <0.1 mM $Na^+$.

### 3.3.3 EPR spectroscopy.

EPR samples were prepared in the anaerobe chamber. Membranes (0.27 ml in a reaction tube) were mixed with 0.03 ml substrate (NADH or succinate) in the presence of $Na^+$ or inhibitors as indicated in the legends to the figures. The viscous suspension was transferred to an EPR quartz tube using plastic tubing fitted to a syringe. The time between mixing with substrate and complete freezing of the membranes in liquid $N_2$ was 5 to 8 min. The EPR spectra were recorded as described elsewhere (Steuber, Rufibach et al. 2002) and simulated using the program EPR (Neese 1995). The concentration of a paramagnetic species was calculated by comparing the total intensity of its simulated EPR spectrum with the intensity of the EPR spectrum of a $CuSO_4$ standard (Neese, Zumft et al. 1996) (see also Figure. S1 in the supplemental material).

### 3.3.4 Analytical methods.

Oxidation of NADH (0.1 mM) by membrane vesicles was followed in 20 mM Tris-$H_2SO_4$, pH 7.5, containing 50 mM $Na_2SO_4$, 50 µg/ml bovine serum albumin, and 0.1 mM ubiquinone-1 as an electron acceptor (Türk, Puhar et al. 2004). Protein was determined by the microbiuret

method (Goa 1953) using bovine serum albumin as standard. $Na^+$ was determined by atomic absorption spectroscopy with a Shimadzu AA-646 spectrometer.

The formation of superoxide was followed according to the procedure in reference (Huycke, Joyce et al. 1996), modified as follows. To prepare cell suspensions, anoxic buffers with $K^+$ salts replacing the corresponding $Na^+$ salts were used, and cells were kept in the anaerobe chamber until the reactions were started. The residual $Na^+$ concentration in cell suspensions was <0.1 mM. Fifty milliliters of *V. cholerae* O395 N1 or *V. cholerae* O395 N1 (*nqrC*::Tn*bla*) was grown as described above and harvested at an approximate optical density at 550 nm of 1.2 to 1.4. Cells were washed twice with phosphate-buffered saline (4.3 mM $K_2HPO_4$, 1.4 mM $KH_2PO_4$, 140 mM KCl, pH 7.5) and resuspended in ice-cold Hanks balanced salt solution (5.6 mM glucose, 142 mM KCl, 0.3 mM $K_2HPO_4$, 0.4 mM $KH_2PO_4$, 4.2 mM $KHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, pH 7.5) to an optical density at 550 nm of 0.2. The reaction was started by adding 0.99 ml anoxic, prewarmed cell suspension (25°C) to 10 µl cytochrome *c* (from beef heart; Sigma; final concentration, 20 µM) in a cuvette under magnetic stirring to aerate the cells. The reduction of cytochrome *c* by superoxide was followed with a UV-visible-light spectrometer (HP 8452A) at 550 nm. If indicated, 0.5 mg superoxide dismutase (SOD; Fluka; 3,231 U $mg^{-1}$) or NADH (0.7 mM) was added. Rates of cytochrome *c* reduction were calculated using an extinction coefficient of 21.5 $mM^{-1}$ $cm^{-1}$ (Massey 1959).

Hydrogen peroxide was determined using horseradish peroxidase according to the method in reference (Green and Hill 1984). The reaction was started by adding 0.97 ml anoxic, prewarmed cell suspension to 30 µl Hanks solution containing 4-aminoantipyrine (0.49 mM), phenol (1.06 mM), and 4 U horseradish peroxidase from Sigma (final concentrations in the assay). The $H_2O_2$-dependent formation of the quinoneimine dye (Saito, Mifune et al. 1987) was followed at 550 nm in a cuvette under magnetic stirring to aerate the cells. The extinction coefficient ($\varepsilon_{550} = 4.3$ $mM^{-1}$ $cm^{-1}$) was determined from standards of 44 to 440 µM hydrogen peroxide in 970 µl Hanks solution in the absence of cells. Rates of $H_2O_2$ formation were corrected by subtracting the residual activity (0.28 to 0.30 nmol $min^{-1}$) of the cell-free supernatant obtained by centrifugation of the cell suspension. Superoxide and $H_2O_2$ formation activities from three to four experiments are presented. The protein content of *V. cholerae* cell suspensions was estimated from the optical density, assuming that 1 unit of absorbance at 550 nm corresponds to 0.33 g total dry weight $liter^{-1}$ (Underwood, Buszko et al. 2004) and that 55% of the total dry weight represents protein (Neidhardt, Ingraham et al. 1996).

### 3.3.5 Inhibition studies.

Inhibitors were added in the anaerobe chamber 10 min prior to the start of the reaction. 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) was added from a stock solution in ethanol to *V. cholerae* cells or membranes. KCN was added from a buffered stock solution to membranes (final concentration, 29 mM). Note that cyanide interferes with the detection of extracellular superoxide since it acts as a ligand to the heme iron of cytochrome *c* (Schejter, Ryan et al. 2006).

## 3.4 Results

### 3.4.1 Respiratory chain complexes in wild-type *V. cholerae* and a mutant devoid of a functional Na$^+$-NQR

We compared the EPR spectra of membranes from wild-type *V. cholerae* and a mutant carrying an insertion in the *nqrC* gene with the aim of assigning organic radicals to the Na$^+$-NQR in its membrane-bound state. The NqrF subunit of the Na$^+$-NQR complex harbors a [2Fe-2S] cluster which accepts electrons from the FAD cofactor located in close proximity. Upon addition of NADH, a resonance at $g = 1.94$ appeared in the EPR spectrum of membranes from the wild-type *V. cholerae* strain but not in membranes from the mutant carrying a transposon in the *nqrC* gene (Figure 3.1). In a previous study using the isolated NqrF subunit, the $g = 1.94$ resonance was assigned to the $g_{x,y}$ component of a nearly axial signal of the one-electron reduced [2Fe-2S] cluster in NqrF (Türk, Puhar et al. 2004). Thus, the disruption of the *nqrC* gene encoding the membrane-bound NqrC subunit prevented the synthesis or assembly of the NqrF subunit. We could not detect the $g_z$ component of the [2Fe-2S] from NqrF in membranes from the parent strain since the $g > 2$ region of the EPR spectrum was dominated by unassigned resonances (Figure 3.1).

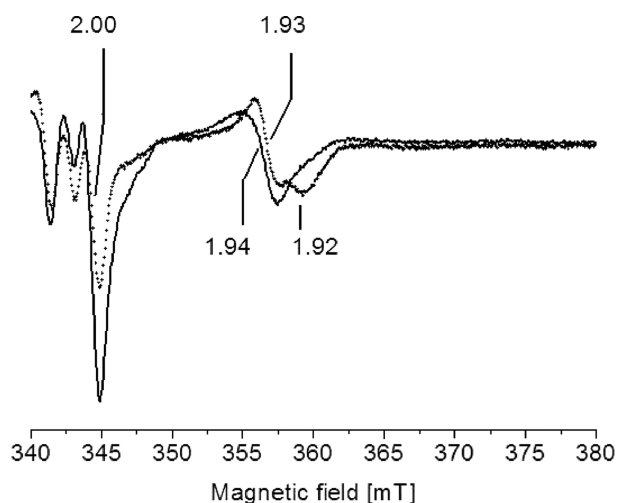


**Figure. 3.1. Detection of the [2Fe-2S] cluster localized in subunit NqrF of the membrane-bound $Na^+$-NQR by EPR spectroscopy.**
Membranes from the wild-type *V. cholerae* strain containing the $Na^+$-NQR (solid line) and from the *nqrC* insertion mutant (dotted line) were mixed with 7.3 mM $Na_2$-NADH prior to freezing. EPR conditions: microwave frequency, 9.652 GHz; modulation amplitude, 0.5 mT; microwave power, 2 mW; temperature, 40 K. Characteristic *g* values are indicated.

The NADH dehydrogenase activities of membranes (0.4 to 0.5 µmol $min^{-1}$ $mg^{-1}$) did not differ significantly in the mutant and the wild-type strains, suggesting that the lack of a functional $Na^+$-NQR complex in the mutant was compensated for by a nonelectrogenic NADH dehydrogenase encoded on the genome of *V. cholerae*. On the two chromosomes of *V. cholerae* El Tor, four open reading frames encoding NADH dehydrogenases of the membrane-bound, nonelectrogenic type were identified: VC1581, VCA0155, VCA0157, and VC1890 (only the last is annotated *ndh*) (Heidelberg, Eisen et al. 2000). We also considered the possibility that NADH was oxidized by a subcomplex of $Na^+$-NQR assembled in the mutant strain even in the absence of the NqrC subunit. $Ag^+$ 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 (Steuber, Krebs et al. 1997). In the presence of $Ag^+$, rates of NADH oxidation by membranes from the parent *V. cholerae* strain decreased to 50% (0.1 µM $Ag^+$) or 14% (1 µM $Ag^+$) 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 mutant strain under identical conditions (data not shown). These results further corroborate our assumption that the mutant strain lacks a functional $Na^+$-NQR and oxidizes NADH via an alternative NADH dehydrogenase.

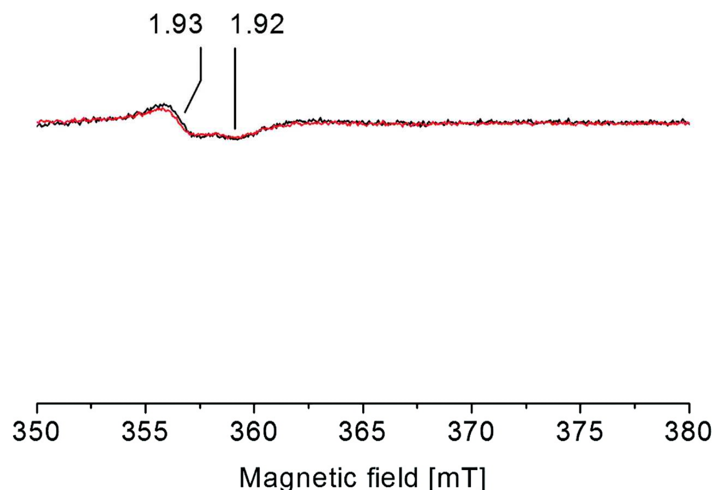


**Figure. 3.2. Detection of the Fe-S center I from succinate dehydrogenase/fumarate reductase in *V. cholerae* membranes by EPR spectroscopy.**
Membranes from the wild-type *V. cholerae* strain containing the Na$^+$-NQR (black trace) and from the *nqrC* insertion mutant (red trace) were mixed with 36.4 mM $Na_2$-succinate prior to freezing. Characteristic *g* values are indicated. For EPR conditions, see the legend to Figure. 3.1.

The wild-type and the mutant strains contained similar amounts of succinate dehydrogenase or fumarate reductase as judged from the intensities of the succinate-induced EPR signal at $g_{x,y}$ = 1.93 and 1.92 (Figure 3.2). These resonances arise from the one-electron reduced [2Fe-2S] center I of succinate dehydrogenase-fumarate reductase (Ackrell, Johnson et al. 1992). Center I was also observed in NADH-treated membranes from the mutant strain, demonstrating that electrons were delivered from NADH to the quinone pool even in the absence of a functional Na$^+$-NQR (Figure 3.1). We conclude that the disruption of the *nqrC* gene had no significant effect on the respiratory chain complexes located downstream of the succinate:quinone segment. By comparing the EPR spectra of membranes from the wild-type and the mutant *V. cholerae* strains, we could assign NADH-induced organic radicals to the Na$^+$-NQR in its membrane-bound state, as described below.

### 3.4.2 Na$^+$ stimulates radical formation by the Na$^+$-NQR

The NqrF subunit acts as a converter between the two-electron donor NADH and subsequent one-electron transfer steps in the Na$^+$-NQR complex, which could result in the formation of organic radicals like flavo- or ubisemiquinones. Membranes from the wild type and the mutant

strain devoid of a functional $Na^+$-NQR complex were inspected by EPR under conditions, which are optimal for the detection of organic radicals. Essentially identical amounts of organic radicals centered at $g = 2.00$ were detected in the membranes after incubation with 36.4 mM succinate for at least 6 min in the absence of $O_2$ (Figure 3.3). We conclude that the two *V. cholerae* strains contained very similar amounts of organic radicals in the succinate:$O_2$ segment of the respiratory chain. Using NADH (7.3 mM) as an electron donor, approximately twice the amount of organic radicals detected in the mutant strain was detected in membranes from the parent strain (Figure 3.3), suggesting that the $Na^+$-NQR promoted the one-electron reduction of organic redox carriers in *V. cholerae* membrane vesicles. These vesicles obtained by French press cell rupture were presumed to be predominantly oriented inside-out (Reenstra, Patel et al. 1980), hereby exposing the cytoplasmic aspect of the inner membrane and the NADH-oxidizing domain of the $Na^+$-NQR complex. We asked whether NADH-induced radical formation by the $Na^+$-NQR was influenced by the coupling cation and added $Na^+$ to the external buffer which represents the cytoplasmic aspect of the membrane vesicles. Increasing the $Na^+$ concentration from 0.08 to 14.7 mM led to a significant increase of NADH-induced organic radicals in membranes from the wild-type strain compared to the mutant lacking a functional $Na^+$-NQR (Figure. 3.4). Spin quantification of the radical signal at low $Na^+$ concentrations (0.08 mM) gave an approximate radical concentration of 200 µM in 0.3 ml membrane suspension, or 5 nmol $mg^{-1}$ protein. In the presence of 14.7 mM $Na^+$, the radical concentration increased to approximately 400 µM, or 10 nmol $mg^{-1}$ protein (see Figure. S1 in the supplemental material). This twofold increase in radical concentration can be assigned to a $Na^+$-dependent redox reaction catalyzed by the $Na^+$-NQR if one takes into account that increasing the $Na^+$ concentration from below 0.1 mM to 25 mM resulted in a threefold stimulation of NADH:quinone oxidoreduction activity of the purified enzyme (Barquera, Hellwig et al. 2002) and that the organic radicals detected in NADH-reduced membranes from wild-type *V. cholerae* did not exclusively arise from the $Na^+$-NQR. Organic radicals associated with the succinate dehydrogenase/fumarate reductase (Figure. 3.2) or with other respiratory complexes in the quinol:$O_2$ segment of the respiratory chain will contribute to the EPR spectrum of NADH-reduced membranes but will not be affected by a rise in the $Na^+$ concentration.

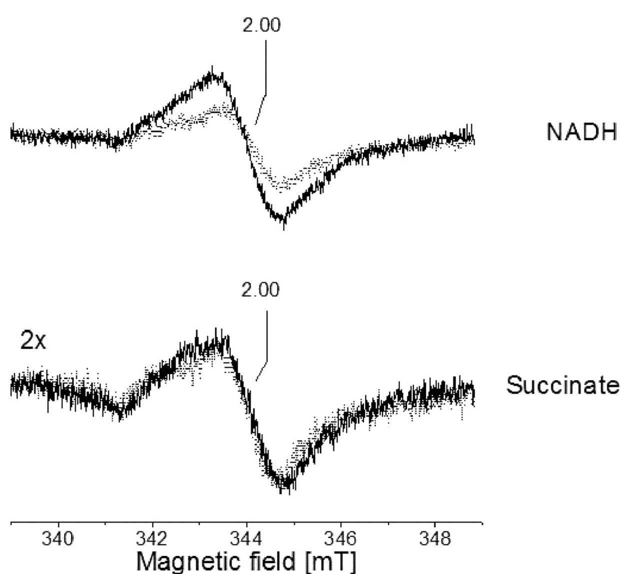


**Figure. 3. 3. Respiration-induced organic radicals in *V. cholerae* membranes in the presence and absence of a functional Na$^+$-NQR.** Membranes from the wild-type *V. cholerae* strain (black trace) and from the *nqrC* insertion mutant (gray trace) were mixed with 7.3 mM $Na_2$-NADH (top) or 36.4 mM $Na_2$-succinate (bottom) prior to freezing. EPR conditions: microwave frequency, 9.652 GHz; modulation amplitude, 0.5 mT; microwave power, 4 µW; temperature, 70 K.

The inhibition by $Ag^+$ indicated that at least 86% of the NADH dehydrogenase activity of membranes from the parent *V. cholerae* strain was catalyzed by the Na$^+$-NQR, corresponding to 0.43 µmol NADH min$^{-1}$ mg$^{-1}$. We estimated the content of Na$^+$-NQR in the membranes by dividing the total Na$^+$-NQR activity in 1 mg membrane protein (0.43 µmol NADH min$^{-1}$) by the turnover number determined for purified Na$^+$-NQR (16,000 µmol NADH µmol$^{-1}$ Na$^+$-NQR min$^{-1}$) (Barquera, Hellwig et al. 2002). Comparing the content of Na$^+$-NQR in the membranes (0.03 nmol mg$^{-1}$ protein) with the amount of NADH-induced organic radicals (5 to 10 nmol mg$^{-1}$ protein) revealed that radical formation by native membrane vesicles from *V. cholerae* was clearly overstoichiometric compared to the Na$^+$-NQR, with a [radical]/[Na$^+$-NQR] ratio of 170:1 at 0.08 mM Na$^+$ or 340:1 at 14.7 mM Na$^+$, respectively. The NADH-induced organic radicals cannot exclusively result from the flavin cofactors of the Na$^+$-NQR, and we conclude that a significant fraction of membrane-bound quinones in *V. cholerae* is converted to the one-electron reduced state during oxidation of NADH by the Na$^+$-NQR. The isolated Na$^+$-NQR from *Vibrio alginolyticus* produced ubisemiquinone radicals which were reoxidized by $O_2$ under formation of superoxide (Hayashi and Unemoto 1984; Pfenninger-Li, Albracht et al. 1996; Steuber, Rufibach et al. 2002). We asked whether the Na$^+$-NQR could also contribute to superoxide formation in vivo and studied the formation of reactive oxygen species by intact *V. cholerae* cells.

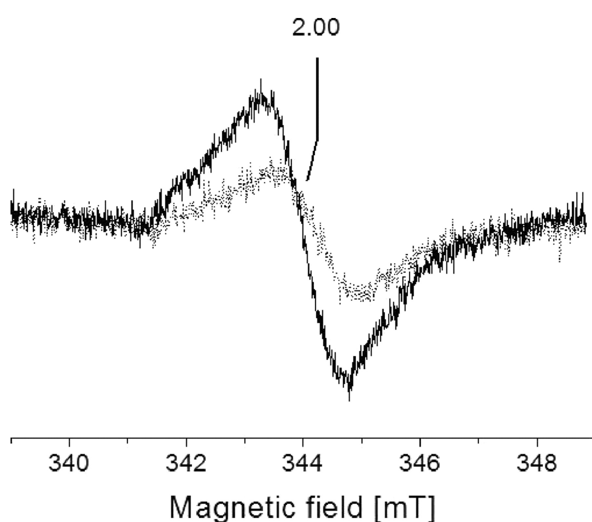


**Figure 3.4. Effect of Na$^{+}$ on the NADH-induced formation of organic radicals by the membrane-bound Na$^{+}$-NQR.** Membranes from the wild-type *V. cholerae* strain were reduced with 7.3 mM NADH in the presence of 0.08 mM Na$^{+}$ (gray trace) or 14.7 mM Na$^{+}$ (black trace). For EPR conditions, see the legend to Figure. 3.3.

### 3.4.3 Extracellular superoxide production by *V. cholerae*

With a standard redox potential $O_2/O_2^-$ of –0.16 V (Imlay 2003), the superoxide anion acts as a one-electron donor for ferricytochrome *c* with $E_0'$ = +0.24 mV. As a 12-kDa protein, cytochrome *c* does not diffuse across intact cellular membranes and can therefore be used to monitor extracellular superoxide production by *V. cholerae* cells. Using late-exponential *V. cholerae* cells and glucose as substrate, superoxide formation was initiated by rapid mixing of the cells with air (Figure. 3.5). Cytochrome *c* reduction rates were highest in the wild-type strain containing the Na$^{+}$-NQR (15.7 nmol min$^{-1}$ mg$^{-1}$) compared to 8.9 nmol min$^{-1}$ mg$^{-1}$ in the *nqrC* deletion mutant, indicating that the Na$^{+}$-NQR represents a major source for superoxide in respiring *V. cholerae* cells (Table 3.1). Raising the Na$^{+}$ concentration from 0.1 to 5 mM increased the cytochrome *c* reduction rates in the wild-type *V. cholerae* by at least 70% but showed no significant effect in the mutant strain (data not shown). In both strains, rates of cytochrome *c* reduction decreased to 5 to 6 nmol min$^{-1}$ mg$^{-1}$ upon addition of SOD, indicating that $O_2^-$ produced in the periplasm of *V. cholerae* represented the major electron donor for cytochrome *c* reduction (Table 3.1; Figure. 3.5).

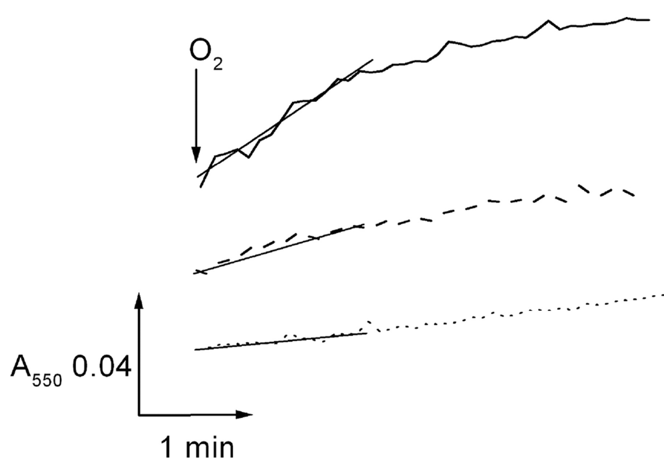


**Figure 3.5. Superoxide production by respiring *V. cholerae* cells in the presence and absence of a functional Na$^+$-NQR.**
The reaction was started by mixing the cell suspension with cytochrome *c* in air-saturated buffer. The superoxide produced was reoxidized by cytochrome *c* in a nonenzymatic reaction. The reduction of cytochrome *c* was followed at 550 nm. Solid trace, with Na$^+$-NQR; dashed trace, without Na$^+$-NQR; dotted trace, with Na$^+$-NQR and 1,615 U SOD.

The residual activity observed in the presence of SOD could result from the reduction of cytochrome *c* by an unknown low-molecular-weight compound excreted by *V. cholerae*, as proposed for *Escherichia coli* cells which also exhibited SOD-insensitive cytochrome *c* reduction activity (Korshunov and Imlay 2002). By subtracting the SOD-insensitive rate, superoxide formation activities of 10.2 nmol min$^{-1}$ mg$^{-1}$ for the wild-type *V. cholerae* and 3.1 nmol min$^{-1}$ mg$^{-1}$ for the NQR deletion strain were calculated (Table 3.1). To exclude the possibility that superoxide was produced by lysed cells, we followed the reduction of cytochrome *c* in the presence of NADH (Hassan and Fridovich 1979). Like the superoxide anion (Korshunov and Imlay 2002), NADH does not readily permeate through the inner bacterial membrane of intact cells but will react exclusively with cytoplasmic redox enzymes from broken cells, thereby increasing the overall amount of superoxide formed. Rates of superoxide formation were essentially identical with or without added NADH (Table 3.1), indicating that the *V. cholerae* cells were intact. In *E. coli*, superoxide is disproportionated to $H_2O_2$ and $O_2$ by SODs found in the cytoplasm and periplasm (Korshunov and Imlay 2006). *V. cholerae* possesses a periplasmic SOD (Gabbianelli, Signoretti et al. 2004), which could contribute to the overall formation of $H_2O_2$ by converting the superoxide generated in the periplasm. We followed $H_2O_2$ formation by respiring *V. cholerae* cells and observed approximately threefold-higher rates in the wild-type strain (30.9 nmol $H_2O_2$ min$^{-1}$ mg$^{-1}$) than in the mutant lacking the Na$^+$-NQR (9.7 $H_2O_2$ nmol min$^{-1}$ mg$^{-1}$), indicating that the Na$^+$-NQR represents a major source for reactive oxygen species in *V. cholerae*. Since $H_2O_2$ in contrast to the superoxide anion is membrane permeable, these rates reflect both cytoplasmic and periplasmic $H_2O_2$ production activities and therefore are expected to be higher than the rates

of extracellular superoxide formation. The source for intracellular $H_2O_2$ in *V. cholerae* remains to be identified.

**Table 3.1. Reduction of cytochrome *c* by respiring *V. cholerae* cells**

| Strain | Sp act[a] (nmol $min^{-1}$ $mg^{-1}$) | | | |
|---|---|---|---|---|
| | No addition | With 1,615 U SOD | With 0.7 mM NADH | Superoxide formation[b] |
| *V. cholerae* O395 N1 | 15.7 ± 1.9 | 5.5 ± 0.4 | 14.7 ± 3.8 | 10.2 |
| *V. cholerae* O395 N1 (*nqrC*::Tn*bla*) | 8.9 ± 2.3 | 5.8 ± 0.5 | 8.0 ± 0.3 | 3.1 |

[a] Mean values from three experiments.
[b] The superoxide formation activity was calculated by subtracting the cytochrome c reduction rate observed in the presence of SOD from the rate observed without addition.

### 3.4.4 Effect of respiratory chain inhibitors on radical formation and superoxide production.

The $Na^+$-NQR contains several redox-active flavins which participate in electron transfer from NADH to quinone (Türk, Puhar et al. 2004; Barquera, Ramirez-Silva et al. 2006). Hence, reduced flavin(s) in the $Na^+$-NQR rather than ubisemiquinones formed by the $Na^+$-NQR could act as an electron donor for extracellular superoxide formation in *V. cholerae*. We addressed this question by studying the production of superoxide and the formation of radicals in the presence of HQNO. HQNO interacts with quinone-binding sites of respiratory complexes (Rich 1984) and also effectively inhibits the $Na^+$-NQR (Pfenninger-Li, Albracht et al. 1996; Barquera, Hellwig et al. 2002). HQNO (0.3 mM) inhibited extracellular superoxide formation of wild-type *V. cholerae* cells by 60 to 80% (data not shown). At the same time, the NADH-induced radical signal in wild-type *V. cholerae* membranes was decreased by approximately 50% in the presence of 0.5 mM HQNO (Figure. 3.6). The genome of *V. cholerae* encodes heme-containing respiratory complexes like the $bc_1$ complex (VC0573 to VC0575) and the quinol oxidase (VC1570 and VC1571) which could accept electrons from reduced quinones formed during NADH oxidation (Heidelberg, Eisen et al. 2000). Cyanide inhibits electron transfer to these complexes, and as a consequence, the ratio of reduced to oxidized quinones in the Q pool will increase. The signal intensity of the organic radical detected in NADH-reduced membranes from wild-type *V. cholerae* increased by approximately 60% upon addition of KCN (Figure. 3.6).

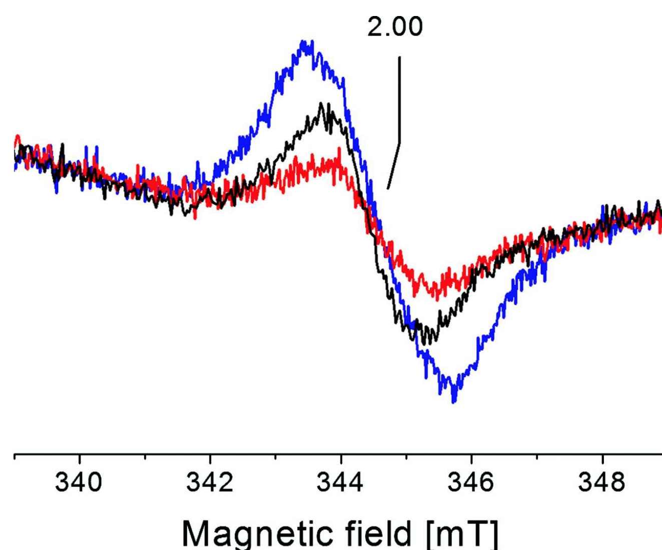


**Figure 3.6. Effect of respiratory chain inhibitors on the NADH-induced formation of organic radicals by the membrane-bound Na$^+$-NQR.**
Membranes from the wild-type *V. cholerae* strain were mixed with 29 mM KCN (blue trace) or 0.5 mM HQNO (red trace). After 10 min, 9 mM $Na_2$NADH was added, and the samples were frozen in liquid $N_2$. Black trace, membranes reduced with NADH in the absence of inhibitor. EPR conditions: microwave frequency, 9.674 GHz; modulation amplitude, 0.5 mT; microwave power, 1 mW; temperature, 70 K.

These results further corroborate our notion that the organic radicals produced during NADH oxidation by the Na$^+$-NQR represent ubisemiquinones which act as electron donors for superoxide formation.

## 3.5 Discussion

The rates of extracellular superoxide production of the gram-negative *V. cholerae* (10 nmol min$^{-1}$ mg$^{-1}$ protein) were lower than activities observed with the gram-positive *Enterococcus faecalis* (26 nmol min$^{-1}$ mg$^{-1}$ protein) (Huycke, Joyce et al. 1996) but significantly exceeded the rates observed with the gram-negative *E. coli* (1 nmol min$^{-1}$ mg$^{-1}$ protein) (Korshunov and Imlay 2006). Like *E. coli*, *V. cholerae* possesses a periplasmic SOD (Gabbianelli, Signoretti et al. 2004) which should scavenge superoxide formed in the periplasm. A possible explanation for the increased production of superoxide in *V. cholerae* compared to *E. coli* is that disproportionation to $H_2O_2$ is less efficient in *V. cholerae* or that *V. cholerae* produces significantly higher amounts of superoxide than does *E. coli*. In support of the latter hypothesis, our study identifies the Na$^+$-NQR which is not present in *E. coli* as a major source for extracellular superoxide.

The electron transfer pathway in the Na$^+$-NQR starts by a hydride transfer from the substrate NADH to the non-covalently bound FAD on the flavin domain of the NqrF subunit (Türk, Puhar et al. 2004), followed by one-electron transfer to the vertebrate-type [2Fe-2S] cluster in the N-terminal domain of NqrF (Lin, Puhar et al. 2005). How electron transport from this FeS center to other flavin and ubiquinone cofactors in the complex and to the substrate quinone proceeds and how this overall exergonic reaction is coupled to Na$^+$ transport are still enigmatic. Compared to other respiratory NADH dehydrogenases, the Na$^+$-NQR is unique as it stabilizes the one-electron reduced state of flavin cofactors which are covalently attached to the membrane-bound NqrB and NqrC subunits, respectively (Barquera, Ramirez-Silva et al. 2006). These flavins in their one- or two-electron reduced state could reduce $O_2$ (Massey 1994; Kussmaul and Hirst 2006). Alternatively, superoxide could be formed during the autoxidation of reduced ubiquinones generated by the Na$^+$-NQR. In *E. faecalis* and *E. coli*, extracellular superoxide was proposed to result from the reaction of molecular oxygen with reduced quinones produced by the respiratory chain. Both two-electron reduced quinones (quinols) and one-electron reduced quinones (semiquinones) were considered as possible electron donors (Huycke, Moore et al. 2001; Korshunov and Imlay 2006). Similarly, quinones reduced by the Na$^+$-NQR could act as electron donors for $O_2$ reduction in *V. cholerae*.

We favor ubisemiquinones generated by the Na$^+$-NQR as a source for in vivo superoxide formation, since this reaction was also observed with the isolated Na$^+$-NQR (Hayashi and Unemoto 1984; Pfenninger-Li, Albracht et al. 1996). Moreover, Na$^+$ which stimulated quinone reduction by the isolated Na$^+$-NQR (Barquera, Hellwig et al. 2002) also promoted ubisemiquinone formation and extracellular superoxide production by the Na$^+$-NQR in its native membrane environment. Further support comes from the finding that superoxide formation by *V. cholerae* cells was greatly diminished in the presence of the quinone-type inhibitor HQNO. Reduced flavins are unlikely electron donors for extracellular superoxide production since all flavin cofactors of the Na$^+$-NQR with known locations are bound to cytoplasmic domains of the complex (Duffy and Barquera 2006).

The question arises whether superoxide formation by the Na$^+$-NQR takes place in the general membrane milieu or whether it occurs at a quinone-binding site of the enzyme. EPR studies of ubisemiquinones associated with the proton-pumping mitochondrial NADH dehydrogenase (complex I) revealed two distinct ubiquinone-binding sites within the membrane-embedded part of the complex (Magnitsky, Toulokhonova et al. 2002). One of these ubisemiquinones is stabilized by applying an electrochemical proton potential and is proposed to play a central

role in the coupling of electron transfer and proton transport by complex I (Ohnishi and Salerno 2005). At least 1 mole of ubiquinone-8 per mole is found in the Na$^+$-NQR from *V. cholerae* (Barquera, Hellwig et al. 2002), suggesting that like complex I, the Na$^+$-NQR comprises one or several binding sites which may accommodate quinone(s) in its different redox states. Although we cannot exclude the possibility that $O_2$ reduction by a protein-bound ubisemiquinone contributes to NADH-dependent superoxide formation by the Na$^+$-NQR, the overstoichiometric amount of ubisemiquinones in membranes compared to the Na$^+$-NQR suggests that ubisemiquinones in the lipid bilayer represent the major reductant for superoxide formation by *V. cholerae* cells.

The superoxide anion and secondary reactive oxygen species like $H_2O_2$ and the hydroxyl radical are highly toxic compounds which may severely damage proteins, lipids, and DNA (Imlay 2003). In *E. faecalis*, extracellular $O_2^-$ formation was associated with invasiveness, and superoxide was considered to be a virulence factor (Huycke, Joyce et al. 1996). The Na$^+$ concentration in the small intestine and in stool from cholera patients is in the range from 90 to 150 mM (Bennish 1994; Guyton and Hall 2000). In the human host, respiratory electron transfer by the Na$^+$-NQR in *V. cholerae*, therefore, is not expected to be limited by Na$^+$. Our study opens the possibility that the NQR-dependent production of superoxide might augment the pathogenicity of *V. cholerae*.

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## 3.7 Supplemental Material

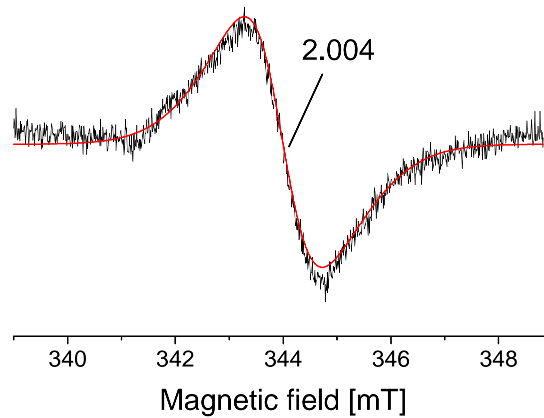


**Figure S 3.1: Simulation of the NADH-induced EPR signal in *V. cholerae* membranes**
Membranes from the wild-type *V. cholerae* strain (black trace) were mixed with 7.3 mM $Na_2$-NADH prior to freezing. EPR conditions: microwave frequency, 9.652 GHz; modulation amplitude, 0.5 mT; microwave power, 4 µW; temperature, 70 K. The red trace is a simulation of the spectrum using the program EPR (Neese 1995), assuming a spin = 0.5 system with $g_x$ = 2.0048, $g_y$ = 2.0044, $g_z$ = 2.0044 and line widths $W^x$ = 0.5 mT, $W_y$ = 1.6 mT, $W_z$ = 1.2 mT. To determine the spin concentration in the membranes, the total intensity of the simulated spectrum was compared with the intensity of the EPR spectrum of a $CuSO_4$ standard (Neese, Zumft et al. 1996).

# 4 NADH Oxidation Drives Respiratory $Na^+$ Transport in Yeast Mitochondria

Po-Chi Lin, Andrea Puhar[‡], and Julia Steuber*

Biochemisches Institut, Universität Zürich, CH-8057, Switzerland

*corresponding author

Biochemisches Institut, Universität Zürich, CH-8057, Switzerland, Tel. ++41 44 635 55 67, Fax ++41 44 635 57 12, E-mail: steuber@bioc.uzh.ch

[‡]present adress

Dipartimento di Scienze Biomediche Sperimentali, Universita di Padova, Viale G. Colombo, 3, I-35121 Padova, Italy

submitted

### 4.1 Summary

It is generally assumed that respiratory complexes exclusively use protons to energize the inner mitochondrial membrane. Here we show that oxidation of NADH by submitochondrial particles from the yeast *Yarrowia lipolytica* is coupled to protonophore-resistant $Na^+$ uptake, indicating that a redox-driven, primary $Na^+$ pump is operative in the inner mitochondrial membrane. By purifying and reconstitution into proteoliposomes, a respiratory NADH dehydrogenase was identified which coupled NADH-dependent reduction of ubiquinone (1.4 μmol $min^{-1}$ $mg^{-1}$) to $Na^+$ translocation (2.0 μmol $min^{-1}$ $mg^{-1}$). NADH-driven $Na^+$ transport was sensitive towards rotenone, a specific inhibitor of complex I. We conclude that mitochondria from *Y. lipolytica* contain a NADH-driven $Na^+$ pump and propose that it represents the complex I of the respiratory chain. Our study indicates that energy conversion by mitochondria does not exclusively rely on the proton motive force but may benefit from the electrochemical $Na^+$ gradient established by complex I.

### 4.2 Introduction

Mitochondria are vital for the synthesis of ATP, the regulation of programmed cell death, and the modulation of intracellular $Ca^{2+}$ concentration (McBride, Neuspiel et al. 2006). These processes are governed by mitochondrial respiration which creates a proton motive force ($\Delta p$) consisting of $\Delta\Psi$, the transmembrane voltage, and $\Delta pH$, the proton concentration gradient (Mitchell 1961). Respiring mitochondria maintain a $\Delta p$ of 170mV which essentially consists of $\Delta\Psi$ as $\Delta pH$ contributes less than 10mV (Murphy and Brand 1987). Three electron transfer reactions from NADH to quinone, quinole to ferricytochrome *c*, and ferrocytochrome *c* to $O_2$ provide the driving force for the generation of $\Delta p$ by the respiratory complexes I, III, and IV, respectively. The stoichiometries of translocated protons per transferred electrons in each of these respiratory segments were determined with intact mitochondria and inside-out vesicles of mitochondrial membranes, or submitochondrial particles (SMPs). $H^+/e^+$ stoichiometries were also studied with the purified complexes reconstituted into artificial membrane systems. By this approach, consistent ratios for the mitochondrial complex III (the $bc_1$ complex) and IV (the cytochrome *c* oxidase) were found in many different laboratories (for review, see (Trumpower and Gennis 1994; Ruitenberg, Kannt et al. 2002; Hunte, Palsdottir et al. 2003; Wikström 2004; Osyczka, Moser et al. 2005) and references therein). Studies with eukaryotic

(Hirst 2005; Brandt 2006) or bacterial model organisms (Yagi and Matsuno-Yagi 2003; Sazanov and Hinchliffe 2006) revealed that complex I is L-shaped, with a NADH-oxidizing arm that protrudes in the mitochondrial matrix or the bacterial cytoplasm, and a cation-translocating arm embedded in the membrane. Compared to complexes III and IV, very little is known about the mechanism of redox-driven proton transport by complex I.

It is generally assumed that the transmembrane voltage established during mitochondrial respiration exclusively results from the electrogenic transport of protons. In contrast, bacterial respiratory chains may contain smaller NADH:quinone reductases not related to complex I (Hayashi and Unemoto 2004; Türk, Puhar et al. 2004) or terminal oxidases (Kim, Stark et al. 2005) which catalyze redox-driven $Na^+$ transport. In 1999 we showed that the enterobacterium *Klebsiella pneumoniae* contains a $Na^+$-translocating NADH dehydrogenase which belongs to the complex I family of respiratory enzymes (Krebs, Steuber et al. 1999). We subsequently determined the cofactor composition and $Na^+$/electron transport stoichiometry of complex I (Gemperli, Dimroth et al. 2002) and showed that NADH-driven $Na^+$ transport is electrogenic (Gemperli, Dimroth et al. 2003). Our views have been challenged by Bertsova and Bogachev who claimed that the *K. pneumoniae* complex I exclusively acts as a proton pump (Bertsova and Bogachev 2004). In a recent study, we corroborated our previous notion of $Na^+$ transport by complex I from *K. pneumoniae* and identified a functional $Na^+$ binding site in its membrane-embedded part (Vgenopoulou, Gemperli et al. 2006).

The coupling ion specificity ($Na^+$ versus $H^+$) of complex I has important implications for its transport mechanism. Our finding that the smaller complex I from some enterobacteria transports $Na^+$ rather than $H^+$ raised the question whether the larger mitochondrial complex I also has the capacity to translocate $Na^+$. Primary $Na^+$ pumps couple an exergonic reaction to the translocation of $Na^+$, whereas $Na^+$ translocation by secondary $Na^+/H^+$ antiporters depends on the proton motive force generated by respiratory chain complexes. Here we show that oxidation of NADH by SMPs from the yeast *Yarrowia lipolytica* is coupled to protonophore-resistant $Na^+$ uptake, indicating that a redox-driven $Na^+$ pump is operative in the inner mitochondrial membrane. This is the first report on the use of $Na^+$ as respiratory coupling ion mitochondria. We unequivocally assign the redox-driven $Na^+$ translocation activity to the NAHD:quinone segment of the respiratory chain, and propose that $Na^+$ transport is an intrinsic catalytic property of complex I from *Y. lipolytica*.

## 4.3 Materials and Methods

### 4.3.1 Growth of *Yarrowia lipolytica*

*Yarrowia lipolytica* strain po1t (Madzak, Treton et al. 2000) was grown at 30 °C in 1 L YPD media (1 % Bacto-yeast extract, 2 % Bacto-peptone and 2 % glucose) in a 5 L baffled Erlenmeyer flask for 18-22 h to the late exponential phase. The cells were harvested by centrifugation and washed with 100 mL deionised water. The cell suspension was frozen in liquid nitrogen and stored at -80 °C.

### 4.3.2 Isolation of mitochondria

All steps were carried out at 4 °C. *Y. lipolytica* cells (0.5 g $mL^{-1}$) in 20 mM HEPES/KOH, pH 7.4, 0.6 M D-mannitol, 1 mM DL-dithiotreitol, 2 mM phenylmethanesulfonyl fluoride (PMSF), and 3 mM benzamidine (Yaffe 1991) were mixed with an equal volume of acid-washed glass beads (425-600 microns) and broken in a mix mill (TissueLyser, Qiagen) at 30 Hz for 15 min. The supernatant was combined with 15 mL buffer used to rinse the glass beads, and PMSF was added to a final concentration of 4 mM. Non-broken cells were removed by centrifugation (4300 x g, 20 min), and the crude extract was centrifuged at 36000 x g for 30 min. The supernatant was centrifuged at 150000 x g for 90 min to collect the mitochondria which were resuspended to a concentration of 30 mg protein $mL^{-1}$ and stored in liquid $N_2$.

### 4.3.3 Preparation of submitochondrial particles and purification of complex I

All steps were carried out in the anaerobic chamber (COY Laboratory; 95 % $N_2$, 5 % $H_2$). Mitochondria were diluted 5-fold with Tris buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 % glycerol, 1 mM PMSF). The suspension was passed through the French Press Cell (AMICON) flushed with $N_2$. The submitochondrial particles (SMPs) were collected by ultracentrifugation at 150000 x g for 80 min. SMPs for $Na^+$ transport measurements were resuspended in Tris buffer. To purify complex I, SMPs (20 mg protein $mL^{-1}$) in MOPS buffer (20 mM MOPS/KOH, pH 7.0, 50 mM KCl, 1 mM $K_2$EDTA, 1 mM PMSF) were solubilised with 0.5 g dodecylmaltoside (DDM) $g^{-1}$ protein (Djafarzadeh, Kerscher et al. 2000) and centrifuged at 150000 x g for 60 min. The supernatant was loaded on a 15 mL anion exchange chromatography column (DEAE-Sepharose CL-6B, Amersham Biosciences) equilibrated with buffer (25 mM MOPS/KOH, pH 7.0, 1 mM $K_2$EDTA, 0.05% DDM) containing 70 mM KCl. The column was washed with 70 and 100 mM KCl, respectively. A linear gradient of 30 mL from 100 to 500 mM KCl was applied to elute complex I around 300 mM KCl. Fractions

which exhibited rotenone-sensitive quinol formation activity were combined and reconstituted into proteoliposomes.

#### 4.3.4 Preparation of proteoliposomes

Complex I from *Y. lipolytica* or the F1F0 ATPase from *Escherichia coli* were reconstituted by a dilution protocol under exclusion of $O_2$ (Gemperli, Dimroth et al. 2002). To a lipid film of 20 mg L-α-phosphatidylcholine (from soybean, Type II-S, 14-23 % as choline, Sigma), the solubilised proteins were added drop-wise under stirring until the lipid film were fully dispersed. The protein-lipid-detergent mixture was diluted at least 10-fold by adding buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 % glycerol) at 30 drops $min^{-1}$. Below the critical micelle concentration of DDM, proteoliposomes were formed which were collected by ultracentrifugation at 150000 x g over night and resuspended in 1-2 mL buffer. Complex I was also reconstituted with buffer containing 5 mM NaCl. The protein to lipid ratio was 1:40 for complex I, and 1:50 for the F1F0 ATPase purified according to (Ishmukhametov, Galkin et al. 2005). Prior to reconstitution, the ATPase was desalted using a NAP-10 column (GE Healthcare).

#### 4.3.5 Transport measurements

$Na^+$ transport was followed in 0.3 mL 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 10 % glycerol with less than 3 h after the preparation of SMPs or proteoliposomes. To SMPs (1.7 mg protein $mL^{-1}$) or proteoliposomes (0.2 mg complex I $mL^{-1}$), 5 mM NaCl or 5 mM $^{22}NaCl$ and 0.1 mM NADH were added to start the reaction. At indicated times, aliquots (70 µL) were passed through a Dowex cationic exchange column, and the internal $Na^+$ content of the vesicles was determined by atomic absorption spectroscopy (Gemperli, Dimroth et al. 2002) or γ-counting using 5 mM $^{22}NaCl$ (0.37 MBq) (Kaim and Dimroth 1998). Generation of a transmembrane potential ($\Delta\Psi$) by reconstituted complex I was followed using the voltage-sensitive dye oxonol VI. Proteoliposomes were prepared in buffer (100 mM potassium phosphate, pH 7.5, 0.05 mM EDTA, 10 % glycerol, and 0.07 mM $Na^+$) as described above. To 41 µg reconstituted complex I in 1 mL buffer containing 10 µM oxonol VI, 0.1 mM ubiquinone-1 (2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone; Q1), 5 mM NaCl or 10 µM monensin were added as indicated. The reaction mixtures were kept in the anaerobic chamber for 6 min prior to the measurements which were performed under air. To start the reaction, NADH (0.16 mM) was added to the stirred assay mixture, and the formation of $\Delta\Psi$ was estimated from the difference in the absorbance at 625 nm –587 nm on a Shimadzu

UV-3000 dual-wavelength spectrophotometer. Proton transport was followed by fluorescence quenching of 1 μM 9-amino-6-chloro-2-methoxyacridin (ACMA) in 10 mM HEPES-KOH, pH 7.5, 0.1 M KCl, 5 mM $MgCl_2$ (Laubinger and Dimroth 1988). The proton-translocating F1F0 ATPase which was used in control reactions exhibited a specific ATP hydrolysis activity of 0.18 μmol $min^{-1}$ $mg^{-1}$. Upon modification with 0.5 mM N,N'-di-cyclohexylcarbodiimide (DCCD) (Weber and Senior 2003), the ATPase activity decreased to 0.06 μmol $min^{-1}$ $mg^{-1}$, indicating that 66 % of the hydrolytic activity was coupled to proton transport. Assuming a ratio of 4 protons translocated per ATP hydrolyzed (Weber and Senior 2003), the calculated proton transport activity was (0.12 x 4 = 0.48) μmol $min^{-1}$ $mg^{-1}$. During ATP hydrolysis, a transmembrane voltage of at least 30 mV was generated (Gemperli, Dimroth et al. 2003), demonstrating that a proton gradient was maintained across the liposomal membrane.

### 4.3.6 Analytical methods

NADH oxidation and reduction of Q1 were followed simultaneously on a diode array spectrophotometer (Agilent 8452A UV-visible system). The assay buffer (1 mL 50 mM Tris-HCl, pH 7.5, 10 mM KCl) containing 0.1 mM Q1, 0.1 mM NADH and 0.1 mM rotenone (if indicated) was repeatedly degassed under vacuum and flushed with $N_2$ using a quartz cuvette sealed with a rubber stopper. The reaction was started by adding complex I with a gas-tight syringe. Oxidation of NADH was followed at 340 nm ($\varepsilon_{340}$ = 6.22 $mM^{-1}$ $cm^{-1}$). Formation of ubiquinol-1 ($QH_2$) was determined from the difference in absorbance at the wavelength pair 248 and 268 nm ($\Delta\varepsilon_{248-268}$ = 7.8 $mM^{-1}$ $cm^{-1}$). Protein was determined by bicinchoninic acid method (Smith, Krohn et al. 1985) using reagent purchased from Pierce. Bovine serum albumin was used as standard. Acid-labile sulfide was determined according to Beinert (Beinert 1983). For UV-vis spectroscopy, complex I was concentrated with Ultrafree-MC filters (cutoff 30000 Da, Millipore) in the anaerobe chamber. Heme concentration were calculated from the difference in absorbance of dithionite-reduced minus dehydroascorbate-oxidized forms using 10.4 $mM^{-1}$ $cm^{-1}$ at $A_{605}$ (heme *a*) and 28.5 $mM^{-1}$ $cm^{-1}$ at $A_{563}$ (heme *b*) as extinction coefficients (Rieske 1967).

## 4.4 Results

### 4.4.1 A redox-driven $Na^{+}$ pump in the respiratory chain from *Yarrowia lipolytica*

We first studied $Na^{+}$ transport by vesicles obtained from mitochondria (submitochondrial particles or SMPs). In the presence of 50 mM rotenone, the Q reduction activity of SMPs was inhibited by 83 ± 17 % (n = 3), indicating that the vesicles were predominately oriented inside-out, with the NADH-oxidizing part of complex I exposed to the external buffer. In the absence of a respiratory substrate, there was some uptake of $Na^{+}$ uptake by SMPs to a stable plateau of 11 nmol $Na^{+}$ $mg^{-1}$ protein driven by the chemical $Na^{+}$ concentration gradient imposed at the start of the reaction. Addition of NADH significantly increased the amount of $Na^{+}$ entrapped in SMPs during 1 min to 100 – 120 nmol $Na^{+}$ $mg^{-1}$ (Figure 4.1), indicating that respiration stimulated $Na^{+}$ transport. In the presence of the ionophore gramicidin, the $Na^{+}$ content of the SMPs decreased to 6 nmol $mg^{-1}$ protein, demonstrating that $Na^{+}$ did not bind unspecifically but was entrapped by the submitochondrial vesicles. Note that cytochome *c* was not included in the assay, hence electron transfer from NADH to $O_2$ was catalyzed by complex I or the alternative NADH dehydrogenase (Kerscher, Eschemann et al. 2001) and the quinol oxidase (Akimenko, Arinbasarova et al. 2003), which is found in *Y. lipolytica* in addition to the cytochrome *c* oxidases. NADH-driven $Na^{+}$ accumulation was also observed with rat liver SMPs, which exhibited a transport activity of 20-25 nmol $Na^{+}$ $min^{-1}$ $mg^{-1}$ protein (Douglas and Cockrell 1974).

In this study, $Na^{+}$ uptake was completely abolished in the presence of the protonophore carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 4 nmol/4.4 mg mitochondrial protein), and the authors concluded that the observed $Na^{+}$ transport was catalyzed by secondary $Na^{+}/H^{+}$ exchangers (Douglas and Cockrell 1974). With SMPs from *Y. lipolytica*, NADH-driven $Na^{+}$ transport was not inhibited by the protonophore carbonyl-cyanide m-chlorophenylhydrazone (CCCP; Figure 4.1) or FCCP (not shown) at concentration of 30 nmol/0.5 mg protein. We did not find genes encoding for a homolog of the $Na^{+}$-translocating NADH:quinone reductase ($Na^{+}$-NQR) in the genome from *Y. lipolytica* (Dujon, Sherman et al. 2004) (Table 4.1), and NADH oxidation by mitochondrial membranes was not inhibited by 100 µM $Ag^{+}$, a specific inhibitor of the $Na^{+}$-NQR (not shown). These findings prompted us to speculate that NADH-driven $Na^{+}$ transport in SMPs was catalyzed by complex I. To test this hypothesis, we obtained a complex I preparation from *Y. lipolytica* which coupled the

oxidation of NADH to the two-electron reduction of Q, reconstituted the complex into proteoliposmes, and analyzed its coupling cation specificity.

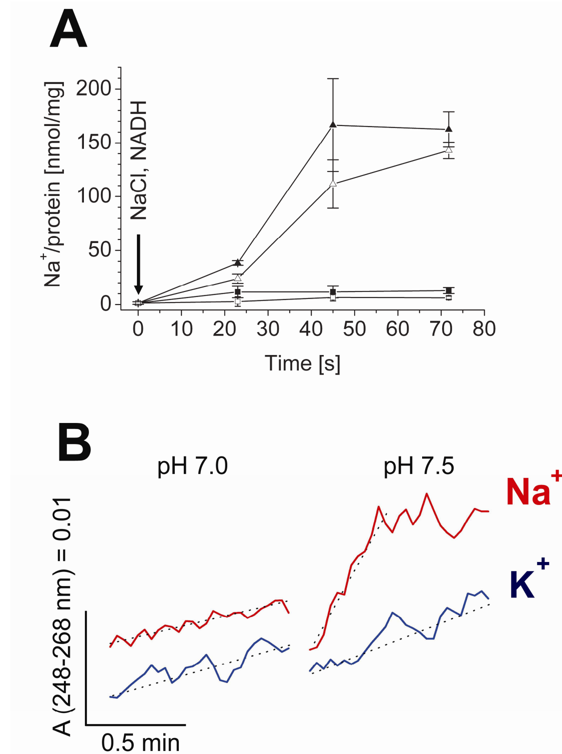


**Figure 4.1 Respiratory NADH:Q oxidoreductase drives $Na^+$ translocation and is stimulated by $Na^+$.**

A, $Na^+$ uptake by submitochondrial particles in the presence of 5 mM NaCl was followed without added NADH (closed square), with 0.1 mM NADH (open triangle), with 0.1 mM NADH and 0.1 mM CCCP (closed triangle), or with 0.1 mM NADH and gramicidin (5 µg $mg^{-1}$ protein; open square). The $Na^+$ content of the particles prior to addition of NaCl was 2 nmol $mg^{-1}$ protein. Mean values from three experiments are presented. B, The reduction of ubiquinone (0.1 mM) with NADH (0.1 mM) by complex I after DEAE chromatography (3 µg) was followed at pH 7.0 or pH 7.5 in the presence of 10 mM NaCl (read) or 10 mM KCl and 0.03 mM $Na^+$ (blue).

### 4.4.2 Properties of complex I

As observed previously with the bacterial complex I (Krebs, Steuber et al. 1999; Gemperli, Dimroth et al. 2002), highest quinol formation activities of complex I from *Y. lipolytica* were achieved by excluding $O_2$ during the purification. Anoxically prepared SMPs exhibited a quinol formation activity of 0.20 µmol $min^{-1}$ $mg^{-1}$ (Table 4. 2), compared to 0.06 µmol $min^{-1}$ $mg^{-1}$ observed with SMPs prepared under air. We used dodecylmaltoside to solubilize the mitochondrial membrane proteins which were separated on a weak anionic exchange column in the anaerobe chamber. NADH oxidation activity was detected in several fractions, but only proteins eluting around 0.3 M KCl exhibited NADH dehydrogenase activity coupled to two-electron reduction of Q1 (Figure 4.2). In these fractions, formation of quinol was completely inhibited by rotenone (50 µM), indicating that quinone reduction was catalyzed by complex I. The purity of our complex I preparation was estimated from the content of heme and FeS clusters determined by VIS spectroscopy and chemical analysis of acid-labile sulfide.

**Table 4.1**: **Search for homologs of subunits of the $Na^+$ -translocating NADH:quinone oxidoreductase ($Na^+$-NQR) in *Yarrowia lipolytica***

| Query | Subject Accession number (function) | Partial amino acid sequence of the *Y. lipolytica* protein (total number of amino acids) |
|---|---|---|
| NqrA | YALI0C21131g (putative nuclear matrix protein) | 559 **KK**P**DLPS**I**GTP**QVVY**I**DKLSELD**KV** 582<br>(724) |
| NqrB | YALI0F16940g (unknown) | 1381 **N**TQV**V**GII**TGSP** 1392<br>1425 **A**FEVKG**NV**VDKNG**NP**QWTVGG**HWH** 1448<br>(1582) |
| NqrC | YALI0E15554g (putative histidine kinase) | 353 **T**PH**AI**PQQPHLQ**TPG**QDAFPGP**PS**AT**D** 379<br>384 **Y**D**ED**TK**P**AR**K**RRDI**G**SAGS**SQHSV**A**G** 409<br>(913) |
| NqrD | YALI0B18502g (unknown) | 110 **WY**N**P**SSST**LLA**Y**SA**AS**L**VAIVPYTLIVM**KP** 139<br>(188) |
| NqrE | YALI0E33583g (putative urea transport protein) | 51 **MV**AN**R**AVGVGLT**A**SAV**F**SSWMWA**N**ETLY**G**A**V** 81<br>84 **YNF**GM**S**GPFW**F**AA**G**LSFHI**A**LMTVV**GI**QV**K**L**K** 115<br>(761) |
| NqrF | YALI0D11330g (putative NADH dehydrogenase) | 81 **L**TK**Y**VTPKGSNVV**RPY**TPVSD**P**DSK**G**EFE**L**V**V** 113<br>118 **G**K**MS**KH**I**HE**LKEGD**TLSFK**GP**IIKYQWQPN 147<br>148 LHK**E**ITLI**G**A**G**T**G**IT**P** 163<br>168 **I**SAIN**K**NPED**K**T**K**VNLF**YG** 186<br>200 **D**AIAK**A**KPQQ**F**NV**H**YF**L** 216<br>219 **PSDNW**K**G**EN**GFI** 230<br>232 **E**EFIKGNSPAA**D**SDNVKVFV**CGPP**PFYKA**I**S**G** 263<br>(291) |

The six subunits (NqrA to NqrF) of the $Na^+$ -NQR from *Vibrio alginolyticus* (accession number BAA22910) were compared with annotated and hypothetical proteins encoded by the genome from *Yarrowia lipolytica* strain CLIB122 (Dujon, Sherman et al. 2004) using the BLASTP program version 2.2.6 (Altschul, Madden et al. 1997) at http://cbi.labri.fr/Genolevures/. Only *Y. lipolytica* proteins exhibiting the highest similarities to Nqr subunits are shown. Bold letters indicate amino acids in the *Y. lipolytica* protein which are also present in the corresponding Nqr subunit used as query sequence in the BLASTP search (*Y. lipolytica* numbering). The total lengths of the *Y. lipolytica* proteins are given in brackets. With the exception of a putative NADH dehydrogenase (YALI0D11330g) which is related to the flavin-binding domain of NqrF, no homologs of Nqr subunits were found in *Y. lipolytica*. We conclude that *Y. lipolytica* does not contain a respiratory complex of the $Na^+$ -NQR-type.

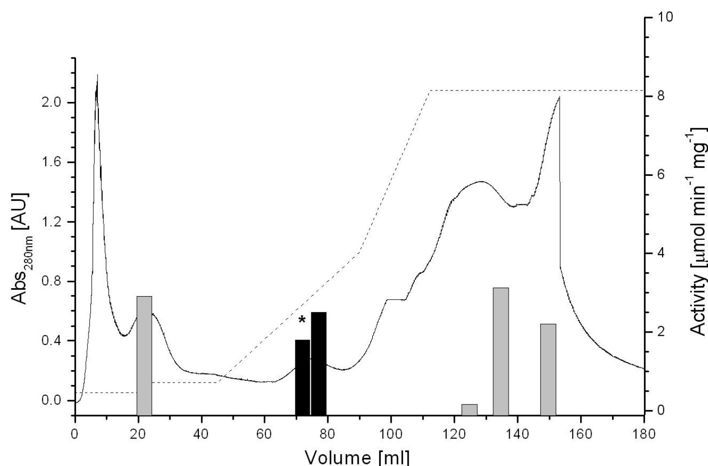


**Figure 4.2: Separation of complex I by anionic exchange chromatography**
Solubilized mitochondrial proteins (black trace) were eluted from the DEAE column with a gradient from 0.07 – 1.0 M KCl (dashed trace) under exclusion of $O_2$. Grey bars indicate proteins that showed NADH dehydrogenase but no significant ubiquinol formation activity. Only fractions with ubiquinol formation activity (black bars) which could be inhibited by rotenone (*) were used for transport studies.

One mg of complex I after anionic exchange chromatography contained 1.2 nmol heme *a*, 0.1 nmol heme *b* (Figure 4.3), and 9.5 nmol acid-labile sulfide $mg^{-1}$ (Table 4.2). Assuming that pure complex I with a molecular weight of 890 kDa containing 28 mol $S^{2-}$ $mol^{-1}$ is analyzed (Brandt 2006), this corresponds to 10.8 mol acid-labile sulfide $mol^{-1}$ complex I and indicates that approximately one third of the total protein in our enzyme preparation represents complex I. Major contaminants were heme *a*-containing complex IV and the heme *b*-containing complex III. Approximately 50% of the heme iron was in the $Fe^{2+}$ state (Figure 4.3), suggesting that complex I purified in the anaerobe chamber also was (partially) reduced.

**Table 4.2**. **Purification of complex I from *Y. lipolytica***

| | Protein | Quinol formation activity | | $S^{2-}$ |
|---|---|---|---|---|
| | [mg] | [µmol min$^{-1}$ mg$^{-1}$] | [µmol min$^{-1}$] | [nmol/mg] |
| Submitochondrial particles | 21.9 | 0.2 | 3.7 | 1.1 |
| Solubilized membranes | 3.8 | 0.4 | 1.5 | 2.4 |
| DEAE column | 0.3 | 1.4 | 0.6 | 9.5 |

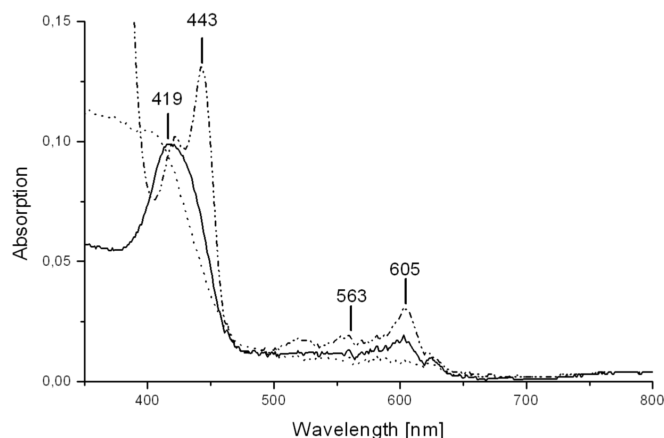


**Figure 4.3 Visible absorption spectra of complex I from DEAE anionic exchange chromatography**

Solid line, complex I (3.2 mg $ml^{-1}$) as isolated; dotted line, with excess dehydroascorbate; dashed line, with excess dithionite.

### 4.4.3 $Na^+$ stimulates Q reduction by complex I

In $Na^+$-translocating NADH dehydrogenases, electron transfer from NADH to quinone is strongly stimulated by $Na^+$ (Hayashi and Unemoto 2004; Türk, Puhar et al. 2004), which suggests binding of $Na^+$ to a specific site relevant for cation pumping. Since the dissociated, NADH-oxidizing part of complex I may contribute to the overall NADH oxidation activity, the coupling ion dependency of electron transfer was studied by following the formation of quinol, which is catalyzed by the membrane-embedded part of complex I. The activation by $Na^+$ is expected to be more prominent under slightly alkaline conditions, since protons may compete with $Na^+$ for the binding to $Na^+$ pumps (Dimroth 1997). At pH 7.0, the rates of NADH-dependent Q1 reduction by complex I from *Y. lipolytica* did not differ significantly in the presence of 10 mM $Na^+$ (0.28 µmol $min^{-1}$ $mg^{-1}$) or 0.03 mM $Na^+$ and 10 mM $K^+$ (0.33 µmol $min^{-1}$ $mg^{-1}$). If the pH was raised to 7.5, the quinol formation activity of complex I increased from 0.33 µmol $min^{-1}$ $mg^{-1}$ at 0.03 mM $Na^+$ and 10 mM $K^+$ to 1.43 µmol $min^{-1}$ $mg^{-1}$ at 10 mM $Na^+$ (Figure 4.1 B). Obviously, binding of $Na^+$ to complex I from *Y. lipolytica* promted electron transfer from NADH to Q1, as reported earlier for the bacterial complex I (Gemperli, Dimroth et al. 2002).

### 4.4.4 $Na^+$ transport

We now asked whether our complex I preparation which was stimulated by $Na^+$ in the electron transfer reduction could also used $Na^+$ as a coupling ion in the transport reaction. The complex was reconstituted into proteoliposomes to follow NADH-driven $Na^+$ uptake. As

observed previously with the bacterial complex I (Gemperli, Dimroth et al. 2002), endogenous quinones from the soy bean lipids used for reconstitution served as electron acceptors. Addition of NADH led to a rapid uptake of 0.5 µmol $Na^+$ $mg^{-1}$, which was inhibited by rotenone (Figure 4.4). Note that uptake was followed in the presence of a $Na^+$ concentration gradient ($Na^+_{out} >> Na^+_{in}$), since the analysis by atomic absorption spectroscopy required low internal $Na^+$ concentration of proteoliposomes at the start of the reaction. We also followed $Na^+$ transport at $Na^+_{in} = Na^+_{out} = 5$ mM and added $^{22}Na^+$ as radioactive tracer to the external lumen. Again, the oxidation of NADH by reconstituted complex I was coupled to the translocation of $^{22}Na^+$ (Figure 4.4). Uptake of $^{22}Na^+$ did not collapse in the presence of the protonophore CCCP, demonstrating that the observed transport did not result from the combined action of a proton-translocating complex I and a $Na^+/H^+$ antiporter (Figure 4.4).

Next we investigated whether the transport of $Na^+$ during NADH:quinone oxidoreduction by the reconstituted NADH dehydrogenase from *Y. lipolycta* resulted in the formation of a transmembrane potential ($\Delta\Psi$) using the voltage-sensitive dye oxonol VI. Generation of $\Delta\Psi$ was dependent on $Na^+$ (5 mM) present in the external lumen of proteoliposomes, and was stimulated by adding the electron acceptor Q1. No $\Delta\Psi$ was established in the presence of 0.07 mM $Na^+$, or 5 mM $Na^+$ and 10 mM monensin, a $Na^+$ ionophore (Figure 4.4C). We conclude that *Y. lipolytica* mitochondria contain an electrogenic NADH dehydrogenase which specifically requires sodium ions for the build-up of a transmembrane potential. The NADH-dependent $Na^+$ transport activity increased significantly upon purification from 0.12 µmol $min^{-1}$ $mg^{-1}$ in SMPs (Figure 4.1) to 2.0 µmol $min^{-1}$ $mg^{-1}$ with reconstituted NADH dehydrogenase (Figure 4.4B), concurrent with an increase in Q reduction activity (Table 4.2). A comparison of the specific quinol formation activity (1.4 µmol $min^{-1}$ $mg^{-1}$, Figure 4.1B) with the rate of $Na^+$ translocation (2.0 µmol $min^{-1}$ $mg^{-1}$) gave a transport ratio of 0.8 $Na^+$/electron transferred.

The alternative NADH dehydrogenase (NDH II) of *Y. lipolytica* which is attached to the inner mitochondrial membrane does not act as a cation pump (Kerscher, Eschemann et al. 2001). Kerscher and coworkers also demonstrated that complex I and NDH II are the only respiratory NADH dehydrogenases found in mitochondria from *Y. lipolytica* (Kerscher, Eschemann et al. 2001). We therefore propose that the rotenone-sensitive, $Na^+$-translocating, electrogenic NADH dehydrogenase identified in mitochondria from *Y. lipolytica* represents the complex I of the respiratory chain.

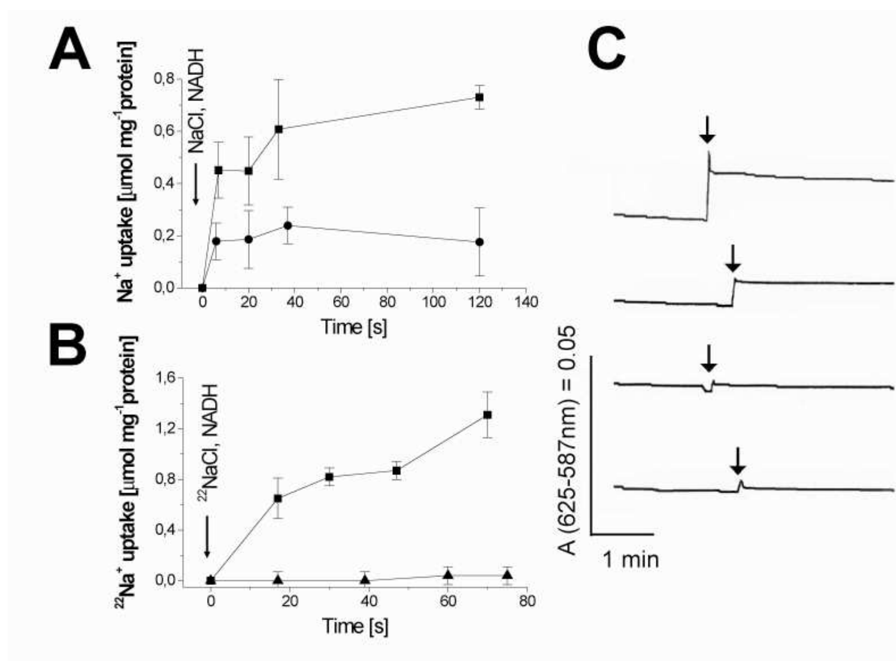


**Figure 4.4 Electrogenic $Na^+$ transport by reconstituted complex I**
$Na^+$ transport was followed by atomic absorption spectroscopy (A) or γ-counting of $^{22}Na^+$ (B) in the presence of 5 mM NaCl. Mean values from three experiments are presented. A, with 0.1 mM NADH in the absence (square) or presence of 0.1 mM rotenone (circle). In A, the data were corrected for the endogenous $Na^+$ content of the proteoliposomes in the absence of substrates (0.42 µmol $mg^{-1}$ protein). B, without (triangle) or with 0.1 mM NADH and 0.1 mM CCCP (square). In B, the internal $Na^+$ concentration at the start of the reaction was 5 mM. In C, the absorption difference of 0.01 mM oxonol VI (625 nm-587 nm) indicated the formation of a transmembrane potential during oxidation of 0.16 mM NADH (arrow) by reconstituted complex I. The assay contained (traces from top to bottom): 5 mM $Na^+$ and 0.1 mM ubiquinone-1 (Q1); 5 mM $Na^+$; 0.07 mM $Na^+$ and 0.1 mM Q1; 5 mM $Na^+$, 0.1 mM Q1 and 10 µM monensin.

### 4.4.5 Proton transport

To investigate whether complex I translocates $H^+$, it was crucial to show that the proteoliposoomes used for $Na^+$ transport experiments were also suitable for the analysis of transmembrane proton movements. Proton transport was followed by ACMA quenching using the $H^+$-translocating F1F0 ATPase as control. Addition of ATP elicited quenching of the fluorescence signal, indicating that a pH gradient (acidic inside) was established by the ATPase. CCCP restored the signal, demonstrating that pH gradient across the liposomal membrane resulted from the vectorial transport of protons from the external to the internal lumen of the proteoliposomes (Figure 4.5, upper trace). In contrast, hardly any quenching was observed upon addition of NADH to proteoliposomes containing complex I. Assuming a

coupling ration of 3.6 $H^{+}$ translocated/2 electron transferred by the *Y. lipolytica* complex I (Galkin, Drose et al. 2006), we would expect a proton transport activity of (3.6 x 0.33 = ) 1.19 µmol $min^{-1}$ $mg^{-1}$ based on the quinol formation activity of our complex I preparation (Figure 4.1B). Considering that the ATPase had an activity of 0.48 µmol $H^{+}$ translocated $min^{-1}$ $mg^{-1}$, proton transport by complex I should have quenched the fluorescence of ACMA to an extent observed with the ATPase in the control reaction. We also tested whether reductive activation of complex I would be required to observed proton transport (Kotlyar and Vinogradov 1990) and added a second aliquot of NADH, but no significant quenching of ACMA fluorescence was observed unless Q1 was also added. However, this Q1-dependent quenching reaction was not accompanied by transmembrane proton movements since it could not be reversed by CCCP (Figure 4.5, middle trace). In fact, Q1 strongly decreased the fluorescence signal even in the absence of NADH, and again, addition of NADH did not significantly quench the remaining fluorescence (Figure 4.5, lower trace). We conclude that there is no evidence for proton transport by reconstituted complex I in this artificial membrane system which allows following NADH-driven $Na^{+}$ transport (Figure 4.4).

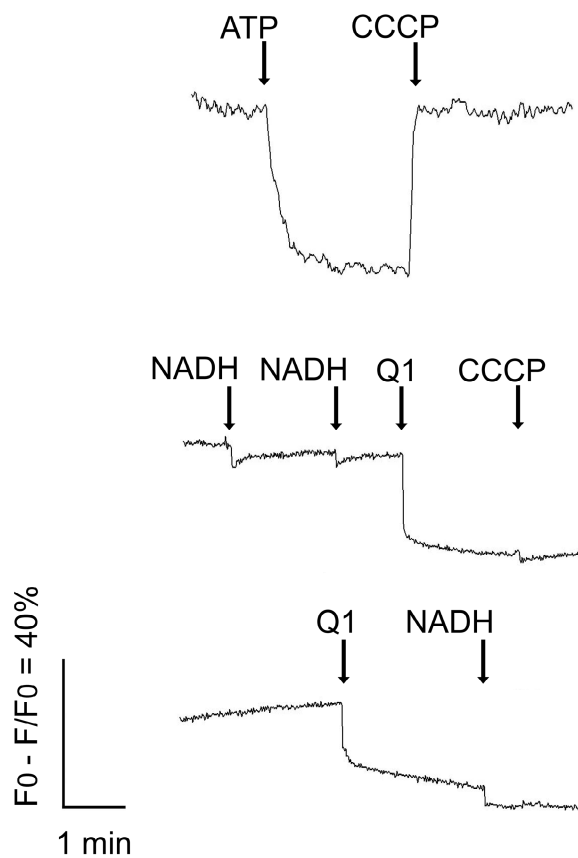


**Figure 4.5 Proton transport followed by quenching of ACMA fluorescence.**

To 48 µg reconstituted ATPase, 2 mM ATP and 10 µM CCCP were added as indicated (upper trace). To 42 µg reconstituted complex I, 0.6 mM NADH (two subsequent additions), 0.1 mM Q1 and 1 µM CCCP (middle trace), or 0.1 mM Q1 and 0.6 mM NADH (lower trace) were added as indicated.

## 4.5 Discussion

With the discovery of $Na^+/H^+$ antiporters in mitochondria (Mitchell and Moyle 1967), it became obvious that a $Na^+$ gradient is maintained across the inner mitochondrial membrane at the expense of the proton motive force. Respiring mitochondria from vertebrates established a $Na^+$ gradient of ~ 8 ($Na^+_{out} >> Na^+_{in}$) which collapsed when the proton motive force was dissipated by protonophores (Douglas and Cockrell 1974; Jung, Apel et al. 1992), suggesting that mitochondrial $Na^+$ transport is exclusively catalyzed by secondary $Na^+$ transporters. In the yeast *Yarrowia lipolytica*, protonophores did not inhibit respiration-driven $Na^+$ transport in mitochondrial membranes, indicating that a redox-driven $Na^+$ pump is operative in this lower eukaryote. Using solubized mitochondrial protein reconstituted into proteoliposomes, we confined the redox-driven $Na^+$ transport activity to the NADH:quinone segment of the respiratory chain from *Y. lipolytica*, and propose that it is an intrinsic catalytic property of complex I. As observed previously with the bacterial complex I (Gemperli, Dimroth et al. 2003), $Na^+$ transport by complex I from *Y. lipolytica* was coupled to the generation of a transmembrane potential. We did not find evidence for proton transport by complex I under our experimental conditions. How can we reconcile our observations with previous investigations? We are aware of three reports on proton transport using solubilized or purified complex I from mitochondria reconstituted into proteoliposomes (Ragan and Hinkle 1975; Dröse, Galkin et al. 2005; Galkin, Drose et al. 2006). In these studies, the possibility that other cations are transported by complex I instead of (or in addition to) protons was not considered (Ragan and Hinkle 1975), or could not be excluded unequivocally (Dröse, Galkin et al. 2005). The electron transfer reaction catalyzed by complex I results in the net consumption of one $H^+$ per NADH oxidized for the formation of ubiquinol ($QH_2$) from ubiquinone (Q). If Q like NADH binds at the matrix side of complex I, electron transfer by reconstituted complex I will be accompanied by an alkalization of the external lumen of the proteoliposomes. Indeed, the reconstituted complex I consumed 1 $H^+$ per NADH oxidized in the presence of a protonophore, demonstrating that proton uptake by complex I is not necessarily coupled to proton transport (Ragan and Hinkle 1975; Galkin, Drose et al. 2006). Brandt and coworkers followed proton uptake by the purified and reconstituted complex I from *Y. lipolytica* using the pH-sensitive fluorescence dye, ACMA. Notably, NADH-dependent quenching of ACMA by reconstituted complex I required short-chain quinones like Q1 (Dröse, Galkin et al. 2005) which very efficiently quenched the fluorescence of ACMA even without added NADH (see Figure 4.5 in this study, and Figure 3 in (Dröse, Galkin et al.

2005)). Hence, it is questionable whether the quenching of ACMA fluorescence during electron transfer from NADH to short-chain quinones described in (Dröse, Galkin et al. 2005) exclusively resulted from vectorial proton transport by reconstituted complex I (Ragan and Hinkle 1975; Dröse, Galkin et al. 2005; Galkin, Drose et al. 2006) was based on a method to determine the proton concentration in the internal lumen of the proteoliposomes during NADH oxidation by complex I. In contrast, the results described here are based on the analysis of $Na^+$ entrapped in SMPs or proteoliposomes. NADH oxidation by reconstituted complex I from *Y. lipolytica* was clearly linked to $Na^+$ transport but did not result in a significant quenching of ACMA fluorescence. Under our experimental conditions, $Na^+$ rather than $H^+$ was the preferred coupling cation of complex I, but proton translocation should be considered nevertheless. Protons may be transported in addition to $Na^+$, or the cation selectivity may switch from $Na^+$ to $H^+$ under conditions of acidic pH and low [$Na^+$], as observed with Na+-translocating F1F0 ATPases (Dimroth 1997).

The use of $Na^+$ as respiratory coupling cation in mitochondria seems to contradict the central tenet of bioenergetics which states that mitochondrial respiration is coupled to ATP synthesis via the proton motive force. On the other hand, bacterial respiratory chain provide many examples where $H^+$-dependent ATP synthases and respiratory proton and $Na^+$ pumps operate in the same membrane (Dimroth 1997). The proton and sodium ion cycles are linked by $Na^+/H^+$ antiporters which exchange $Na^+$ and $H^+$ across the membrane (Padan, Tzubery et al. 2004). They are involved in the homeostasis of $Na^+$ and $H^+$ and are found in membranes and organelles from cells of many different organisms. It has been generally assumed that the $Na^+$ gradient across the inner mitochondrial membrane solely results from the activity of $Na^+/H^+$ antiporters. Our study indicates that the $Na^+$ cycle in mitochondria from *Y. lipolytica* includes the NADH-driven extrusion of $Na^+$ which we propose to be catalyzed by complex I. The cation specificity ($Na^+$ versus $H^+$) of complex I has fundamental implications for our understanding of its mechanism and its role in energy transduction and metabolism. The use of $Na^+$ as respiratory coupling cation might enable *Y. lipolytica* to efficiently adjust the mitochondrial proton and $Na^+$ concentrations in response to cellular demands.

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# 5 General Discussion

## 5.1 ISC-type Iron-Sulfur Cluster in $Na^+$-NQR

Various spectrometric analyses and sequence alignments showed that the Fe-S domain of the subunit NqrF possesses an ISC-type [2Fe-2S] cluster that is related to the ferredoxin of the vertebrate-type family (Lin, Puhar et al. 2005). NqrF is the entry site, where under oxidation the electrons of NADH enter $Na^+$-NQR and are transferred via NqrF's cofactors, FAD and [2Fe-2S] cluster, to other subunits to reduce quinone to quinol (Türk, Puhar et al. 2004). Subsequently, quinol transfers the electrons to the downstream complexes of the respiratory chain. $Na^+$-NQR is an important part of the respiratory chain of *Vibrio cholerae*. However recent studies have shown that $Na^+$-NQR might be involved in processes other than respiration.

NqrF', a soluble derivate of the NqrF subunit, can be overproduced using *V. cholerae* as host, however the insertion of an iron-sulfur cluster seems to be the limiting step of the NqrF' overproduction. NqrF' with fully assembled cofactors FAD and Fe-S cluster shows higher stability than those only with incorporated FAD. Interestingly, the overproduction of NqrF' is better in the wild type *V. cholerae* strain with intact $Na^+$-NQR than in the $Na^+$-NQR-depleted mutant (Tao, Fritz, Steuber, submitted for publication). It seems that the Fe-S cluster of $Na^+$-NQR is not only taking part in the electron transfer (Türk, Puhar et al. 2004), but also promotes the stability and/or assembly of NqrF'.

The genes *nqrABCDEF* are related to the genes *rnfBDGAEC*, respectively, which encode a membrane-bound complex called Rnf that was first described in the $N_2$-fixing bacterium *Rhodobacter capsulatus* for its participation of electron transport to nitrogenase (Jouanneau, Jeong et al. 1998; Jeong and Jouanneau 2000). Another $N_2$-fixing bacterium, *Azotobacter vinelandii,* contains two clusters of *rnf*-genes: *rnf1*, whose expression is regulated together with *nif*-genes; *rnf2*, which is expressed independently of the nitrogen source in the medium (Curatti, Brown et al. 2005). Other than in *V. cholerae*, *rnf*-like genes have been found in *E. coli* (designated *rsxABCDGE*) as well, and they are required to keep the [2Fe-2S] cluster-containing, redox-sensitive transcriptional factor SoxR in its reduced state during normal aerobic growth (Koo, Lee et al. 2003). In *A. vinelandii* it's been shown that Rnf proteins are

required for the rapid accumulation of the matured, [4Fe-4S] cluster-containing form of dinitrogenase reductase (Curatti, Brown et al. 2005). Therefore, it seems that Rnf and Rnf-related complexes like $Na^+$-NQR are important for the maturation of iron-sulfur cluster-containing proteins.

The iron-sulfur cluster assembly machinery in *V. cholerae* is poorly studied, however in the genome of *V. cholerae* (http://cmr.tigr.org and http://www.ncbi.nlm.nih.gov/) homologues of iron-sulfur cluster assembly proteins are found: VC_0748 (NifS/IscS, cysteine desulfurase), VC_0749 (NifU family protein), VC_0747 (HTH-type transcriptional regulator IscR), VC_0752 (chaperone protein HscA), VC_0751 (co-chaperone HscB), VC_0750 (hesB family protein). The *nqr* genes are related to *rnf* genes and the $Na^+$-NQR subunit, NqrF, contains a [2Fe-2S] cluster related to ISC-type ferredoxins. Iron-sulfur cluster assembly machineries like ISC (iron-sulfur-cluster)-, NIF (nitrogen-fixation)- and SUF (sulfur-utilizing-factor)-systems have been intensively studied (reviewed in (Johnson, Dean et al. 2005)). Based on these studies and sequence analysis a putative iron-sulfur cluster assembly machinery in *V. cholerae* including a reductive function for the $Na^+$-NQR can be hypothesized (Figure 5.1).

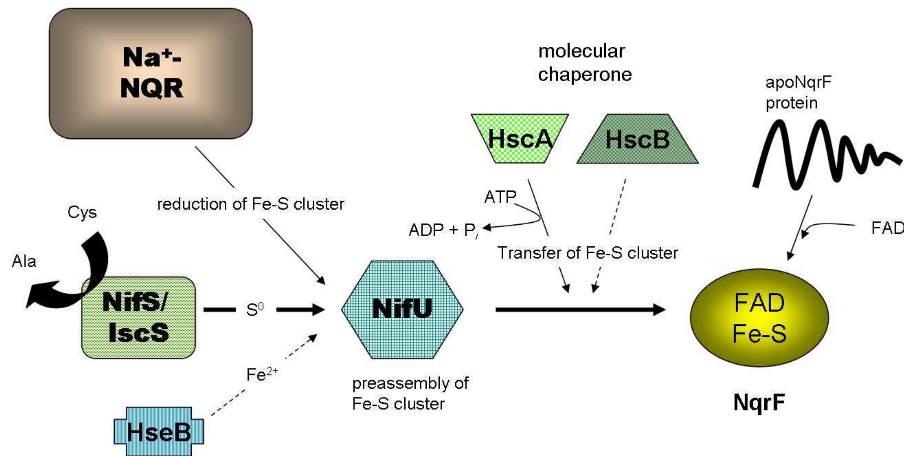


**Figure 5.1 Putative Fe-S cluster assembly machinery in *V. cholerae***
NifS and IscS are both cysteine desulfurases and catalyze the elimination of sulfur from L-cysteine. NifU is a scaffold protein in which the iron-sulfur cluster is preassembled. After the reduction of preassembled iron-sulfur cluster by $Na^+$-NQR, the cluster is transferred to its destined apoprotein (NqrF is displayed here as an example) with help of the chaperones, HscA and HscB under ATP hydrolysis (Takahashi and Nakamura 1999; Chandramouli and Johnson 2006).

IscS is closely related to NifS. They both are pyridoxal phosphate-dependent cysteine desulferases, which catalyse the elimination of sulfur from L-cysteine providing the necessary sulfur for the Fe-S cluster assembly (Jacobson, Cash et al. 1989; Zheng 1993; Zheng, White et al. 1994; Flint 1996; Zheng, Cash et al. 1998). NifU is a homodimeric modular protein that functions as a scaffold protein providing an intermediate site for the assembly of Fe-S cluster, or Fe-S cluster precursors (Smith, Jameson et al. 2005). Both NifS and NifU play crucial roles in the maturation of Fe-S proteins (reviewed in (Johnson, Dean et al. 2005)). HseB is a large protein family to which IscA belongs (Cupp-Vickery, Silberg et al. 2004). IscA is part of the ISC-type assembly system; however its function still remains controversial. It has been proposed that IscA provides iron molecules for the Fe-S cluster assembly (Ding, Clark et al. 2004; Zeng, Zhao et al. 2007). The $Na^+$-NQR subunit NqrF contains a [2Fe-2S] cluster related to ISC-type ferredoxin. The nature of ferredoxin within the iron-sulfur assembly machinery hasnot been fully characterized yet, but ferredoxin is likely to be involved in electron transfer such as to reduce S to $S^{2-}$ (Tokumoto, Nomura et al. 2002), which is essential for the maturation of iron-sulfur cluster. Both HscA and HscB (heat shock cognate proteins) function together as a nucleotide-dependent molecular chaperone system that is specific to IscU (Silberg, Tapley et al. 2004). HscA belongs to the ubiquitous 70 kDa heat shock protein family (Hsp70 or DnaK) that facilitates protein folding, assembly and transport via nucleotide-dependent binding to unfolded, misfolded or unstable polypeptides to prevent unspecific aggregation (Mayer, Brehmer et al. 2001). HscB is a member of J-type cochaperone family (Hsp20 or DnaJ) that escorts the IscU substrate to HscA. Together, HscA and HscB stimulate [2Fe-2S] cluster transfer from IscU to apoferredoxin in a ATP-dependent manner (Chandramouli and Johnson 2006). There are no known chaperones associated with iron-sulfur cluster assembly specific to the nitrogen fixation (NIF) machinery yet (Zheng, Cash et al. 1998; Agar, Zheng et al. 2000). The N-terminal regions of NifU proteins display sequence homology to IscU proteins, and the primary studies in which the valine in the binding-motif sequence (LPPVK) of IscU is replaced by glutamic acid in the binding-motif sequence (LPPEK) of NifU showed no difference in general properties of the interaction with HscA/HscB, dimerization, and iron-sulfur cluster formation, indicating that the HscA/HscB chaperone system may interact with NifU as well (Hoff, Cupp-Vickery et al. 2003).

The facts that $Na^+$-NQR is related to the Rnf complex and contains a [2Fe-2S] cluster related to ISC-type ferredoxin indicate that $Na^+$-NQR might possess properties that promote maturation of iron-sulfur proteins in *V. cholerae*. The beneficial effect of the presence of $Na^+$-

NQR with respect to NqrF' might be due to the activity of $Na^+$-NQR in facilitating insertion of Fe-S cluster into NqrF', hereby promoting the synthesis and the stability of NqrF'.

## 5.2 Reactive Oxygen Species Generated by $Na^+$-NQR

$Na^+$-NQR reduces quinone under oxidation of NADH and uses the gained energy from the redox reaction to translocate sodium ions across the membrane (Steuber 2001). During the reduction of quinone ,semiquinone radicals are formed as intermediate which can be observed using EPR analysis. Semiquinone radicals can react with molecular oxygen forming superoxide (Lin, Türk et al. 2007). *V. cholerae* possesses periplamic superoxide dismutase (Gabbianelli, Signoretti et al. 2004) that catalyzes the dismutation of superoxide into hydrogen peroxide and molecular oxygen (Figure 5.2). Our studies showed that a *V. cholerae* strain with intact $Na^+$-NQR generates significantly higher amounts of extracellular superoxide and hydrogen peroxide than the $Na^+$-NQR-depleted strain, indicating that the generation of reactive oxygen species is linked to $Na^+$-NQR (Lin, Türk et al. 2007). $Na^+$-NQR of *V. cholerae* is situated in the commensurate position within the respiratory chain like complex I as the entry site for electrons, and it is generally known that the respiratory complexes I and III generate small amount of superoxide as a side product of electron transfer during oxidative phosphorylation (Nicholls and Budd 2000; Chen, Vazquez et al. 2003; Turrens 2003). It was observed that complex I generates superoxide when the rate of electron transfer and ATP synthesis is low, and in this state the redox potential of complex I seems to be higher than the NADH/$NAD^+$ couple, resulting a thermodynamically unstable center that leaks electrons to molecular oxygen (Kushnareva, Murphy et al. 2002); whereas complex III generates superoxide during cycling of the electron acceptor ubiquinone when the electron flow is high, and ATP synthesis is fast (Turrens, Alexandre et al. 1985). In both cases, the univalent oxidation of quinol (or univalent reduction of quinone) produces semiquinone radicals that react with molecular oxygen forming superoxide (Barja 1999); however only a small percentage of electrons leaks out of the respiratory chain complexes (Barja 1999). In contrast to mitochondrial respiratory complexes, $Na^+$-NQR produces overstoichiometic amount of reactive oxygen species which are subsequently released into the extracellular environment (Lin, Türk et al. 2007).

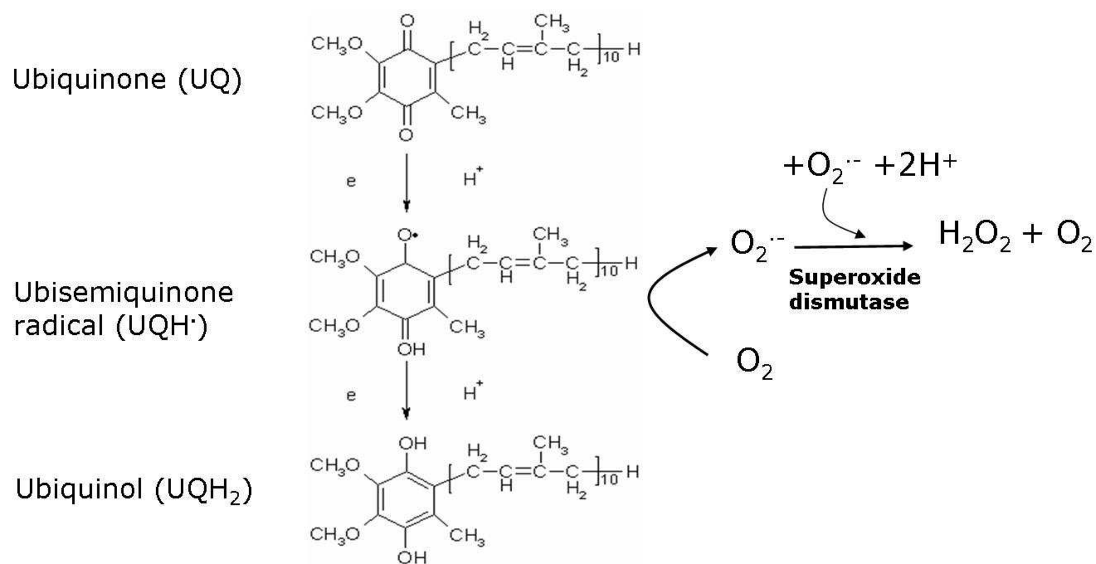


**Figure 5.2 Generation of reactive oxygen species via ubisemiquinone radical**
The reduction of ubiquinone occurs in two steps: firstly, one electron and one proton are accepted and ubisemiquinone radical is formed as an intermediate, subsequently, further electron and proton are accepted and the ubisemiquinone radical is fully reduced to ubiquinol. Ubisemiquinone radical can react with molecular oxygen and form superoxide ($O_2^{\cdot -}$). Superoxide dismutase catalyzes the dismutation of superoxide into hydrogen peroxide and molecular oxygen.

The ability to produce substantial amount of extracellular superoxide is not so uncommon among bacteria; *Enterococcus faecalis* (Huycke, Joyce et al. 1996; Huycke, Moore et al. 2001), *Lactococcus lactis* (Huycke, Joyce et al. 1996; Winters, Schlinke et al. 1998) and *Mycobacterium pneumoniae* (Cohen and Somerson 1967; Cole, Ward et al. 1968) also posses this ability. Our study showed that the production of extracellular superoxide in *V. cholerae* rose from the formation of semiquinone radicals which is contributed mainly by $Na^+$-NQR (Lin, Türk et al. 2007), however there are other quinone-reducing/ quinol-oxidizing enzymes in the respiratory chain of *V. cholerae* that may also be involved in the production of superoxide (Figure 5.3). In our study, it was demonstrated that the succinate dehydrogenase, the respiratory complex II, contributes very little to the formation of semequinone radicals (Lin, Türk et al. 2007); this result agrees with the findings in *E. faecalis* (Huycke, Moore et al. 2001).

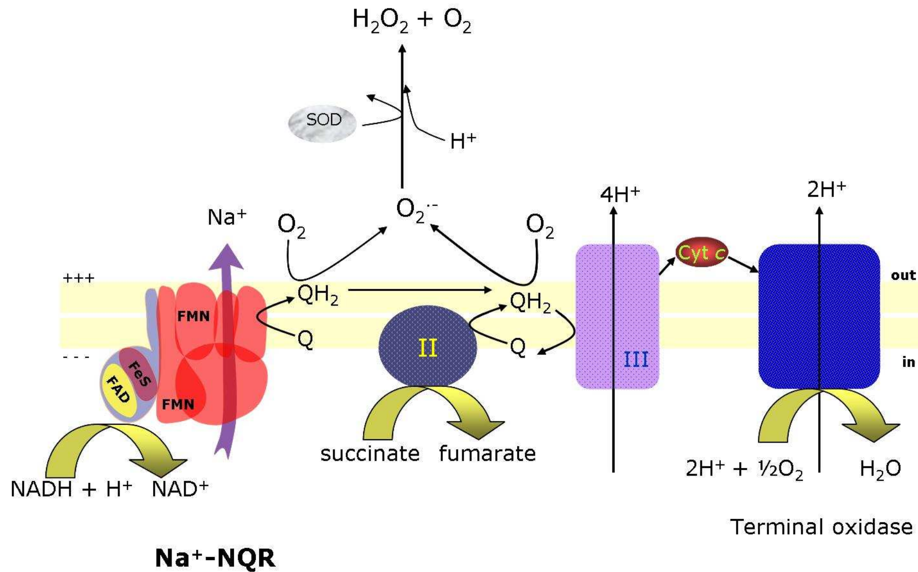


**Figure 5.3 Generation of extracellular reactive oxygen species in the respiratory chain of *Vibrio cholerae***
$Na^+$-NQR produces overstoichiometric amount of extracellular superoxide via formation of semiquinone radicals (Lin, Türk et al. 2007). In the presence of the periplasmic superoxide dismutase (SOD), superoxide is converted into hydrogen peroxide. Succinate dehydrogenase (complex II) also reduces quinone to quinol, but contributes very little to the formation of semiquinone radicals (Huycke, Moore et al. 2001; Lin, Türk et al. 2007), thus it is not considered to participate in the production of extracellular reactive oxygen species. Cyt $bc_1$ complex (complex III) reduces cytochrome *c* under oxidation of quinol, therefore it may contribute to the formation of semiquinone radicals. The terminal oxidase couples the proton transport with the reduction of molecular oxygen under the oxidation of reduced cytochrome *c*.

### 5.2.1 Extracellular reactive oxygen species and pathogenicity

*Vibrio cholerae* produces overstoichiometric amount of extracellular reactive oxygen species, however their physiological function are still unknown, but the studies on *Mycoplasma pneumoniae* infections may provide some indications (Almagor, Yatziv et al. 1983). Like *V. cholerae, M. pneumoniae* is also a pathogen that adheres to the host cell and causes infection. *M. pneumoniae* is known to produce reactive oxygen species such as $H_2O_2$ and $O_2^{\cdot-}$ (Cohen and Somerson 1967; Cole, Ward et al. 1968; Sobeslavsky and Chanock 1968; Lynch and Cole 1980). Studies showed that 70% of the catalase activity in the *M. pneumoniae*-infected host cell was inhibited by superoxides (Almagor, Kahane et al. 1984). And the level of malonyldialdehyde in the host cell was elevated after 2 hours of infection, indicating peroxidation of membrane lipids (Almagor, Kahane et al. 1984). It was suggested that the reactive oxygen species generated by *M. pneumoniae* during the infection inhibit the catalase and CuZn-SOD activity of the host cell (Zimmermann, Flohe et al. 1973; Hodgson and

Fridovich 1975), resulting in accumulation of intracellular reactive oxygen species, which then induce oxidative damages to vital cell constituents such as membrane lipids, DNA or glutathione and finally cell death (Almagor, Kahane et al. 1984; Kahane 1984). The studies on *M. pneumoniae* provided a possible model of how the production of extracellular reactive oxygen species contributes to the pathogenicity of *V. cholerae*, however to what extent the extracellular reactive oxygen species play a role during *V. cholerae* infection still remains to be elucidated.

## 5.3 Complex I and $Na^+$ Ions

### 5.3.1. Ion transport mechanisms of complex I

The finding that the respiratory complex I in the yeast *Yarrowia lipolytica* (Lin 2007 submitted) and in the bacterium *Klebsiella pneumoniae* (Gemperli, Dimroth et al. 2002) is capable of translocating sodium ions instead of proton raises questions not only about the physiological role of complex I but also about its ion transport mechanism. The crystal structure of the hydrophilic arm of complex I suggested that the electrons travel through the complex I by the following pathway of cofactors: FMN-N1b-N3-N4-N5-N6a-N6b-N2 (Friedrich 2001; Sazanov and Hinchliffe 2006). However, without the structure of the hydrophobic arm of complex I, the mechanism of how the reduction of quinone and the transfer of ions are coupled still remains unknown. Two mechanisms have been proposed. In the first model, the ion transport is directly coupled to the redox reaction (Vinogradov 1993; Vinogradov 2001); whereas in the second model, the redox reaction induces conformational changes in complex I which then set off the transport of ions across the membrane (Brandt, Kerscher et al. 2003; Flemming, Stolpe et al. 2005).

#### 5.3.1.1 Redox-linked ligand conduction mechanism

An example for the direct coupling of redox reactions and ion transport is the Q cycle of the cytochrome *bc*$_1$ complex, in which the translocation of protons is linked to the redox-dependent protonation and deprotonation of Q (Xia, Kim et al. 1998; Hirst 2003). In 1998 Dutton *et al* (Dutton, Moser et al. 1998) proposed a reverse Q cycle mechanism for complex I, in which an exogenous Q on the matrix side is reduced to $QH_2$ by the N2 cluster and the endogenous $QH_2$, which forms a semiquinone and releases a proton to the intermembrane space. The exogenous $QH_2$ formed is exchanged with a second Q from the membrane. The

process repeats again, in which the semiquinone is fully oxidized to Q and releases another proton. After both Q and $QH_2$ are replaced, the cycle starts over again. The reverse Q-cycle mechanism requires several quinones and its intermediates (hence ligand conduction) to translocate protons across the membrane. With the discovery of the $Na^+$ transport activity of *K. pneumoniae* complex I (Gemperli, Dimroth et al. 2002), Steuber and coworkers proposed another mechanism for redox-coupled $Na^+$ transport, in which an endogenous quinone acts as a cofactor and is stabilized by the binding to $Na^+$ during the reduction reaction and an exogenous quinone from the Q-pool receives the electrons from the reduced quinone and release $Na^+$ to the other side of the membrane (Gemperli 2002). However, in both proposed mechanisms distinct and different Q binding sites are required, and studies of other groups showed that different complex I inhibitors bind to the same binding pocket (Okun 1999), thus the redox-linked ligand conduction mechanism seems less likely to be the transport mechanism of complex I.

#### 5.3.1.2 Ion transport via conformational energy transfer

With increasing numbers of observations of complex I in different conformations (Belogrudov and Hatefi 1994; Yamaguchi, Belogrudov et al. 1998; Hellwig, Scheide et al. 2000; Mamedova, Holt et al. 2004), the model of ion transport via conformational energy transfer becomes more favorable. It is proposed that the oxidation of the N2 cluster by quinone reduction induces specific conformational changes that are transmitted to the subunits in the membrane arm of complex I, which act as ion pumps (Brandt, Kerscher et al. 2003). Remarkably, studies showed that complex I is sensitive to the amiloride-type inhibitors which are specific for $Na^+/H^+$ antiporters (Kerscher 2001; Nakamaru-Ogiso, Seo et al. 2003; Steuber 2003). Furthermore, the subunits of the membrane arm, ND2, ND4 and ND5, seem to have evolved from a $Na^+/H^+$ antiporter family (Fearnley and Walker 1992; Mathiesen and Hägerhäll 2002). These subunits are considered as the possible candidates responsible for the transport of $Na^+$ or $H^+$. Recently Gemperli *et al* also showed that the NuoL subunit (homologue of ND5) of *E. coli* complex I is capable of translocating $Na^+$ and $K^+$ ions (Gemperli, Schaffitzel et al. 2007). All these results suggest that complex I is more likely to couple the reduction of quinone to the transfer of $Na^+$ (or $H^+$) through $Na^+/H^+$ antiporter-related subunit by conformational changes imposed by the redox reaction.

### 5.3.2 $Na^+$ cycle in mitochondria

The physiological role of $Na^+$ gradient has been intensively studied in bacteria, but not so in mitochondria. Generally a $Na^+$ gradient across the membrane can be generated either by converting ΔpH to ΔpNa through the $Na^+/H^+$ antiporter or by active $Na^+$ pumps such as $Na^+$-translocating NADH:quinone oxidoreductases ($Na^+$-NQR) (Ken-Dror, Preger et al. 1986; Udagawa, Unemoto et al. 1986; Dimroth and Thomer 1989; Tokuda and Kogure 1989; Kostyrko, Semeykina et al. 1991), $Na^+$-decarboxylases (Dimroth 1980; Buckel and Liedtke 1986; Dimroth 1987) or $Na^+$-ATPases (Schwartz and Collins 1982; Skulachev 1988, Dimroth 1987). It was suggested by Skulachev that ΔpNa (and ΔpK) generated by the $Na^+(K^+)/H^+$ antiporter functions as $\Delta\tilde{\mu}H^+$ buffer (Skulachev 1978) and this idea was experimentally proven by other groups (Wagner, Hartmann et al. 1978; Arshavsky, Baryshev et al. 1981; Brown, Galperin et al. 1983; Michels and Bakker 1985). However, the bioenergetic role of $Na^+$ is not limited only in $\Delta\tilde{\mu}H^+$ buffering. In some forms of life, it was shown that $Na^+$-motive force plays a crucial role in energy transductions, transport of metabolites, as well as regulation of pH and volume homeostasis. $Na^+$-NQR and $Na^+$-decarboxylases are mostly found in marine bacteria, whereas $Na^+$-ATPases, enzymes that pump $Na^+$ ions under consumption of ATP, are found in the plasma membrane of eukaryotes (Schwartz and Collins 1982; Skulachev 1988). In bacteria, a different kind of $Na^+$-ATPase is found which utilizes the $Na^+$ motive force to synthesize ATP (Dimroth 1987).

Although a $Na^+$ gradient can be used as the driving force for $Na^+$-metabolites symport, as observed in bacterial and plasma membrane (Skulachev 1989; MacAulay, Hamann et al. 2004), no such $Na^+$-dependent cotransporter has been reported in mitochondria yet. The $Na^+$ gradient in mitochondria is mostly considered to be involved in the release of $Ca^{2+}$ ions into the cytosol (Brierley, Baysal et al. 1994; Nguyen, Dudycha et al. 2007). $Ca^{2+}$ serves as an intracellular second messenger in signal transduction from the ambient medium into the cell (Berridge 1993; Rizzuto, Bastianutto et al. 1994). Mitochondria are known to control free cytosolic calcium ion signals, $[Ca^{2+}]_c$, by uptake and release during cytosolic $Ca^{2+}$ mobilization. Mitochondria operate either as a barrier buffer (Hoth, Fanger et al. 1997; Straub, Giovannucci et al. 2000; Park, Ashby et al. 2001; Camello-Almaraz, Salido et al. 2002; Cancela, Van Coppenolle et al. 2002) or as a facilitating factor in the spreading of calcium signals (Simpson and Russell 1996; Duchen 2000; Simpson 2000).

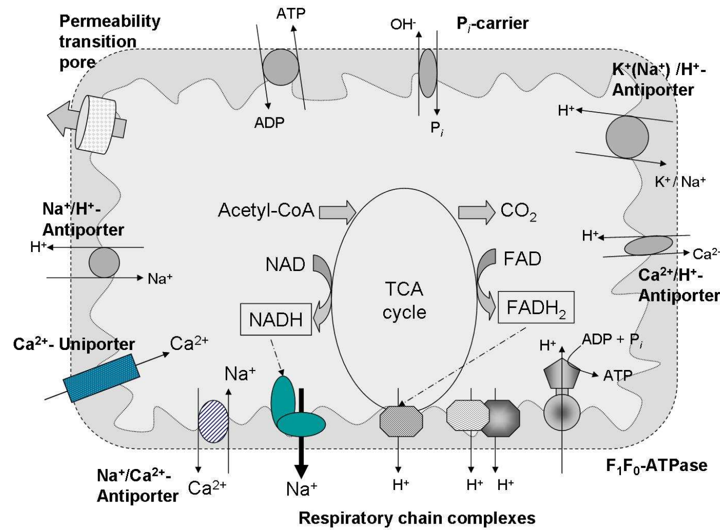


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**Figure 5.4 A schematic diagram of cation gradient in mitochondria from *Yarrowia lipolytica***

The Krebs tricarboxylic acid (TCA) cycle provides the necessary reduction equivalents (NADH and $FADH_2$) for the respiratory chain to build up an electrochemical potential across the membrane; a $Na^+$ gradient in case of complex I and a proton gradient generated by other respiratory complexes. The proton electrochemical gradient is used in many processes such as ATP synthesis, $K^+/H^+$ antiport for pH and volume homeostasis or maintaining a $Na^+$ gradient. The uptake of $Ca^{2+}$ is mediated through an energy-consuming $Ca^{2+}$ uniporter. The release $Ca^{2+}$ into cytosol can be carried out either by $Ca^{2+}/H^+$ antiporter, permeability transition pore or $Na^+/Ca^{2+}$ antiporter. For the latter, a $Na^+$ gradient is most crucial for the efflux of $Ca^{2+}$ (for references see text). This schematic diagram is modified from (Nguyen, Dudycha et al. 2007).

Other studies showed that the relation between mitochondria and the change of free matrix calcium ions, $[Ca^{2+}]_m$, is much more complicated. $Ca^{2+}$-mobilizing signals can become a direct stimulus for the metabolic enzymes in the mitochondrial matrix (pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase) leading to activation of metabolic pathways such as the Krebs tricarboxylic acid cycle and respiratory chain by elevating the level of NADH and $FADH_2$ in the matrix (Denton and McCormack 1980; McCormack and Denton 1981; Brookes, Yoon et al. 2004; Camello-Almaraz, Gomez-Pinilla et al. 2006). In general, increases in $[Ca^{2+}]_c$ stimulate the energy demand of the cell, whereas increases in $[Ca^{2+}]_m$ within a physiological range stimulate the supply for the energy demand (Katz 1991). Recent studies also showed that $Ca^{2+}$ transport is involved in triggering of apoptotic processes (Crompton 1999; Smaili, Hsu et al. 2000; Skulachev 2001; Skulachev 2002; Hajnoczky, Csordas et al. 2006; Saunders, Szymczyk et al. 2007), and stimulation of metabolism during fertilisation (Dumollard, Duchen et al. 2006).

Mitochondrial $Ca^{2+}$ uptake is mediated in an energy-dependent fashion (Vasington and Murphy 1962) via a $Ca^{2+}$ uniporter (Kirichok, Krapivinsky et al. 2004). $Ca^{2+}$ can be released into the cytosol through 3 different pathways: the $Na^+$-dependent pathway via $Na^+/Ca^{2+}$ antiporter in excitable tissues (Crompton, Moser et al. 1978; Nicholls and Scott 1980; Rizzuto, Bernardi et al. 1987; Gunter and Gunter 1994; Hoyt, Stout et al. 1998; Griffiths 1999), the $Na^+$-independent pathway via $Ca^{2+}/H^+$ antiporter in non-excitable tissues (Gunter and Pfeiffer 1990; Gunter and Gunter 1994; Bernardi 1999) and the $Ca^{2+}$-dependent pathway via permeability transition pore (Gunter and Pfeiffer 1990) which can lead to massive and unspecific release of ions and of compounds with a molecular weight less than 15000 kDa, causing $\Delta\Psi$ decay and swelling of mitochondria (Bernardi 1999; Crompton 1999; Skulachev 2001; Skulachev 2002) (Figure 5.4).

In the $Na^+$-dependent $Ca^{2+}$ efflux, the $Na^+$ gradient provides a driving force for the extrusion of $Ca^{2+}$ against a steep electrochemical gradient (Crompton and Heid 1978); the $Na^+$ gradient is in turn maintained via $Na^+/H^+$ antiporter using $\Delta\tilde{\mu}H^+$ generated by the respiratory chain (Gunter and Pfeiffer 1990; Bernardi 1999; Carafoli 2003). There are two antiporters for $Na^+$ and $H^+$ in mitochondria (Nakashima and Garlid 1982). The $K^+/H^+$ antiporter transports all alkali cations and can be inhibited by DCCD (Kakar, Mahdi et al. 1989; Jezek, Mahdi et al. 1990); together with the $P_i$-carrier, they are responsible for the homeostasis of the pH and the volume of mitochondria (Brierley, Baysal et al. 1994; Nguyen, Dudycha et al. 2007).The $Na^+/H^+$ antiporter, on the other hand, is highly selective for $Na^+$ and $Li^+$ (Garlid 1988), sensitive to DCCD (Garlid, Shariat-Madar et al. 1991) and can be inhibited by divalent cations such as $Mn^{2+}$, $Ca^{2+}$ and $Mg^{2+}$ (Douglas and Cockrell 1974; Crompton and Heid 1978; Brierley and Jung 1980; Nath and Garlid 1988).

It's generally assumed that the $Na^+$ gradient in mitochondria is solely generated by $Na^+/H^+$ antiporters, mainly because many experiments of $Na^+$-transport in respiring mitochondria were carried out in the presence of rotenone (Crompton and Heid 1978; Bernardi, Angrilli et al. 1990; Jung, Apel et al. 1992), which is known as a specific complex I inhibitor. It has been assumed that complex I contributes to the generation of a $Na^+$ gradient in mitochondria by generating a proton gradient, which is converted into a $Na^+$ gradient by $Na^+/H^+$ antiporters. The discovery that the mitochondrial complex I of *Y. lipolytica* is also capable of NADH-driven $Na^+$ transport sheds some new lights on how the $Na^+$ gradient can be maintained in mitochondria. In vitro studies showed that $Na^+/H^+$ antiport would lead to alkalinisation of the

matrix in the absence of added $P_i$ (Bernardi, Angrilli et al. 1990). And Kapus and coworkers (Kapus, Ligeti et al. 1989) also observed no $Na^+/H^+$-antiporter activity above pH 7.5 and suggested that the $Na^+/H^+$ antiporter is regulated by matrix pH. Our study showed that the *Y. lipolytica* complex I is still active at this (and higher) pH, indicating that complex I, in addition to $Na^+/H^+$ antiporters, might possibly play a role in maintaining a $Na^+$-gradient at higher pH (Figure 5.4).

## 5.4 Outlook

$Na^+$-NQR and complex I, although sharing no homology in their sequences and structure, both belong to the same enzyme family. They utilize the energy gained from NADH oxidation to transport ions across membranes. Many progresses have been made in the past years, yet there are still many other aspects of these two enzymes remaining to be explored. A high-resolution x-ray structure of the peripheral fragment of complex I has been reported, but the structure of the membrane fragment is still unsolved. And in spite of many efforts, little is known about the structure of $Na^+$-NQR. Obtaining the structural information would be a great help to understand the mechanism of ion transport. Being able to catalyze the same enzymatic reaction and to utilize the free energy to transport ions, complex I and $Na^+$-NQR might share some common principles in the mechanism of ion translocation.

Besides belonging to the same enzymae family, complex I and $Na^+$-NQR also have the same physiological function in the cell: they are the first entry site into the respiratory chain. However, recent studies showed that mitochondrial complex I is playing a crucial role not only in the respiratory chain, but seems to be involved in other metabolic processes as well. And our laboratory also showed that $Na^+$-NQR of *Vibrio cholerae* produces overstoichiometic amounts of extracellular reactive oxygen species, of which the physiological function is still unclear. Therefore, more studies will be necessary in the future to uncover the different physiological functions of complex I and $Na^+$-NQR.

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# Curriculum Vitae

Po-Chi Lin

Born May 7th, 1978, in Taichung, Taiwan, Republic of China

Citizen of Zürich (ZH) Switzerland and Taiwan

| | |
|---|---|
| 1985 – 1991 | Primary school in Taichung, Taiwan |
| 1991 –1998 | Gymnasium in Zug, Switzerland |
| 1998 - 2003 | Studies of Biotechnology, Swiss Federal Institute of Technology Zürich (ETHZ), Switzerland<br>03/2002 – 9/2002<br>Diploma thesis at the Institute of Biotechnology, Swiss Federal Institute of Technology, Zürich (ETHZ), Switzerland<br><br>"Cell-free application of styrene monooxygenase (StyA) coupled to $[Cp^*Rh(bpy)H_2O]^{2+}$ -catalyzed coenzyme regeneration for the production of enantiopure epoxides" |
| 2003 - 2004 | PhD project at the Institute of Microbiology, Swiss Federal Institute of Technology, Zürich (ETHZ) Switzerland |
| 2004 - 2007 | Continuance of the PhD project at the Biochemistry Institute (BCI), University Zürich (UZH), Switzerland |

# Publications

Lin P.-C., Türk K., Hase C.C., Fritz G., Steuber J. (2007) Quinone reduction by the $Na^{+}$-translocating NADH dehydrogenase promotes extracellular superoxide production in *Vibrio cholerae*. J Bacteriol.;189(10):3902-8.

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