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# Crystallization of the $Na^+$-translocating NADH:quinone oxidoreductase from *Vibrio cholerae*

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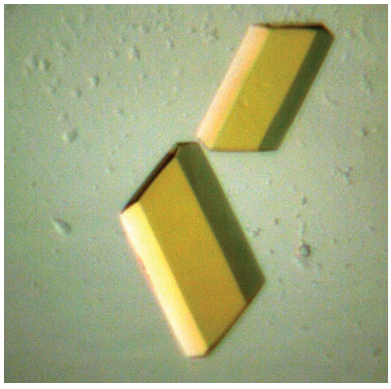
The $Na^+$-translocating NADH:quinone oxidoreductase ($Na^+$-NQR) from the human pathogen *Vibrio cholerae* couples the exergonic oxidation of NADH by membrane-bound quinone to $Na^+$ translocation across the membrane. $Na^+$-NQR consists of six different subunits (NqrA–NqrF) and contains a [2Fe–2S] cluster, a noncovalently bound FAD, a noncovalently bound riboflavin, two covalently bound FMNs and potentially $Q_8$ as cofactors. Initial crystallization of the entire $Na^+$-NQR complex was achieved by the sitting-drop method using a nanolitre dispenser. Optimization of the crystallization conditions yielded flat yellow-coloured crystals with dimensions of up to 200 × 80 × 20 µm. The crystals diffracted to 4.0 Å resolution and belonged to space group $P2_1$, with unit-cell parameters $a$ = 94, $b$ = 146, $c$ = 105 Å, $\alpha = \gamma = 90$, $\beta = 111^\circ$.

## 1. Introduction

*Vibrio cholerae* is a Gram-negative bacterium that lives in brackish or sweet water environments. Some strains of *V. cholerae* infect the human gut and cause cholera. *V. cholerae* belongs to a group of pathogenic bacteria, including, for example, *Yersinia pestis* (black death), *Klebsiella pneumoniae* (pneumonia) and *Neisseria meningitis* (meningitis), which may generate an $Na^+$ gradient across the cytoplasmic membrane. This $Na^+$ motive force is beneficial for supporting processes such as substrate uptake, motility and efflux of antibiotics. It is assumed that this special energy metabolism increases pathogenicity (Häse *et al.*, 2001). The $Na^+$ motive force is generated by a respiratory NADH:ubiquinone oxidoreductase, which couples the exergonic oxidation of NADH to the uphill transport of $Na^+$ across the cytosolic membrane. The sodium-translocating NADH:quinone oxidoreductase ($Na^+$-NQR) is a heterohexameric complex consisting of six different protein subunits (NqrA–NqrF) and contains a [2Fe–2S] cluster, a noncovalently bound FAD, a noncovalently bound riboflavin, two covalently bound FMNs and potentially $Q_8$ as cofactors (Casutt *et al.*, 2010; Tao *et al.*, 2008; Türk *et al.*, 2004; Juárez *et al.*, 2008, 2009; Bogachev *et al.*, 2001). The entire complex has a size of 213 kDa. High-resolution structures of $Na^+$-NQR are only available to date for the FAD-binding domains of the NqrF subunits from *V. cholerae* (Tao *et al.*, 2006) and *Porphyromonas gingivalis* (PDB code 2r6h; Y. Kim, R. Mulligan, S. Moy & A. Joachimiak, unpublished work), which comprise the catalytic binding site for NADH and represent the electron-entry site of the complex (Türk *et al.*, 2004). However, high-resolution structures are not available for the other subunits or for the entire $Na^+$-NQR. Detailed structural information as provided by X-ray crystallography will be an important step towards understanding the still unresolved coupling between electron transfer and transmembrane ion transport. Here, we report the crystallization and preliminary X-ray analysis of the entire $Na^+$-NQR complex from *V. cholerae*.





## 2. Materials and methods

### 2.1. Protein expression and purification

$Na^+$-NQR was expressed and purified as described previously (Tao *et al.*, 2008) with minor modifications. Briefly, hexahistidine-tagged $Na^+$-NQR was expressed in *V. cholerae* strain Δ*nqr* lacking

expression of the NQR complex. Membranes were isolated and solubilized with *n*-dodecyl-$\beta$-D-maltoside (DDM; Glycon). $Na^+$-NQR was purified *via* Ni–NTA agarose (Qiagen) utilizing the N-terminal hexahistidine tag fused to the NqrA subunit. Purified $Na^+$-NQR was dialyzed against 50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 5% glycerol. The $Na^+$-NQR was concentrated by ultrafiltration (Amicon, 100 kDa cutoff) and further purified by size-exclusion chromatography using a Superdex 200 (16/60) column (GE Healthcare) equilibrated in 10 m*M* HEPES–NaOH pH 8.5, 5% glycerol, 300 m*M* NaCl and 0.05% DDM. If required, the hexahistidine tag was removed from NqrA by thrombin proteolysis prior to size-exclusion chromatography. For proteolysis, the protein concentration was adjusted to approximately 1 mg $ml^{-1}$ by ultrafiltration (Amicon, 100 kDa cutoff) and $CaCl_2$ was added to a final concentration of 2.5 m*M*. 10 U thrombin (SERVA) per milligram of protein was allowed to react with the protein for 1 h at 295 K. The digest was supplemented with 0.25 $\mu M$ phenylmethylsulfonyl fluoride and again passed over Ni–NTA agarose in order to remove undigested protein as well as the cleaved hexahistidine tag.

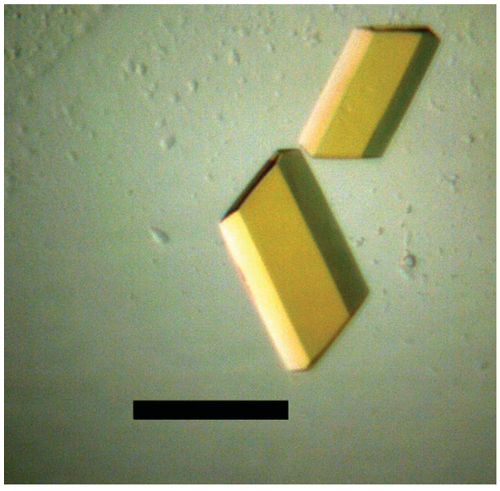


**Figure 1**
Crystals of the $Na^+$-NQR complex from *V. cholerae* grown under refined conditions. The bar represents a length of 100 µm.

### 2.2. Protein crystallization

Prior to crystallization, $Na^+$-NQR was concentrated to 7 mg $ml^{-1}$ by ultrafiltration (Amicon, 100 kDa cutoff). Crystallization conditions for the hexahistidine-tagged $Na^+$-NQR from *V. cholerae* were initially tested by the sitting-drop vapour-diffusion method on the nanolitre scale using a Honeybee 963 dispenser (Digilab Genomic Solutions). Each drop was prepared by mixing 50–200 nl protein solution with crystallization solution in a ratio of between 3:1 and 1:3 and was equilibrated by vapour diffusion against 100 µl crystallization solution. Crystallization trials were performed at 295 and 277 K. Manual crystallization screens were set up using the sitting-drop vapour-diffusion method in Cryschem 24-1 SBS microplates (Hampton Research). Drops were set up by mixing 2 µl protein solution and 2 µl crystallization solution (40 m*M* KSCN, 21.0% PEG 2000 MME, 100 m*M* Tris acetate pH 8.5 and 8% 1-propanol) and were equilibrated by vapour diffusion against 100 µl crystallization solution at 277 K. Cofactors such as FAD, riboflavin or NADH were not added during crystallization. For cryoprotection, PEG 550 MME was added to the crystallization solution to final concentrations of 0, 5, 10, 15 and 20% and the crystals were soaked in each solution for 5–10 s and flash-frozen immediately in liquid nitrogen. X-ray data were collected using monochromatic synchrotron radiation ($\lambda$ = 1.0 Å) on beamlines X06SA and X06DA at the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland. The diffraction data were processed with the *XDS* program package (Kabsch, 1988, 2010).

## 3. Results and discussion

Initial crystallization conditions for hexahistidine-tagged $Na^+$-NQR from *V. cholerae* were found by the sitting-drop vapour-diffusion method. Initial crystals only grew at 277 K from a 1:1 mixture of protein solution and crystallization solution consisting of 25% PEG 2000 MME, 200 m*M* KSCN and 100 m*M* Tris acetate pH 8.5. These yellow-coloured crystals had a maximum dimension of 25 µm and diffracted to 16 Å resolution. The crystallization conditions were

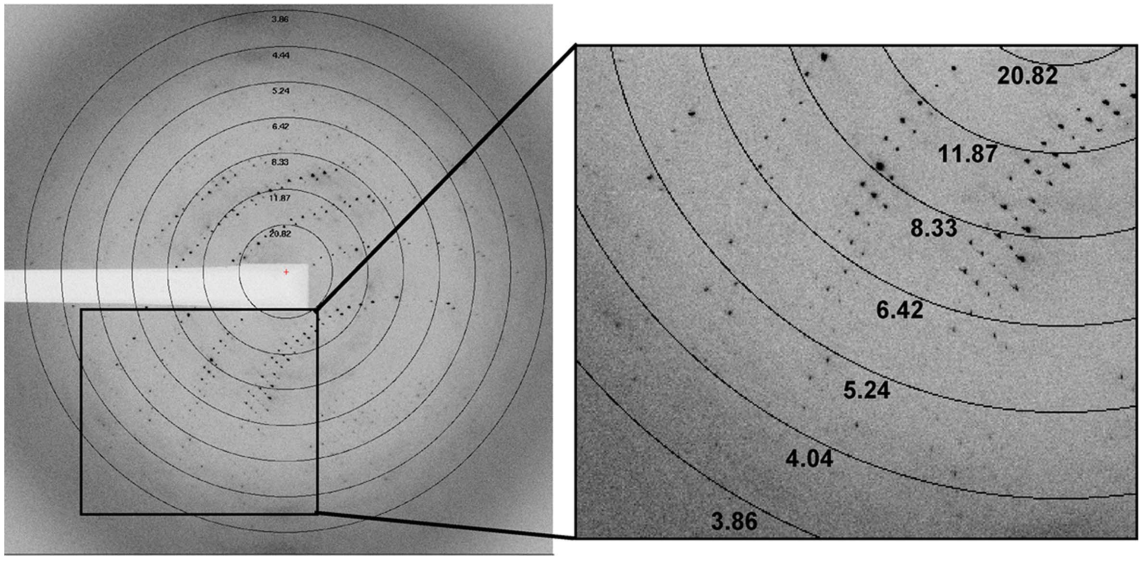



**Figure 2**
Typical diffraction pattern from an $Na^+$-NQR crystal. The resolution at the image border is 3.7 Å. A detail of the diffraction pattern showing spots to a resolution of 4 Å is shown on the right.

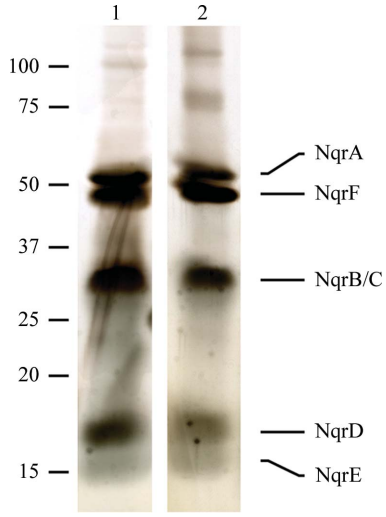



**Figure 3**
SDS–PAGE analysis of crystals of $Na^+$-NQR. Lane 1, $Na^+$-NQR (30 µg) from size-exclusion chromatography; lane 2, $Na^+$-NQR (14 µg) from crystals dissolved in SDS sample buffer. Protein was stained with silver. Nqr subunits are labelled and molecular-mass standards are indicated in kDa. Subunits NqrB and NqrC co-migrated in a single band running between 25 and 37 kDa.

refined by varying the PEG and KSCN concentrations and the pH and adding additives such as 1-propanol. Using these conditions, crystals grew to dimensions of up to 200 × 80 × 20 µm (Fig. 1). These crystals had a yellow–brownish colour, indicating the presence of Fe–S and flavin cofactors. However, these crystals did not diffract beyond 7 Å resolution. Searches for further crystallization conditions for $Na^+$-NQR by broad screening were not successful. In order to improve the crystals, we considered removal of the hexahistidine tag. The tag has a total length of 20 residues and is likely to be rather flexible, thereby masking some potential crystal contacts. Indeed, crystals formed from $Na^+$-NQR after proteolytic removal of the hexahistidine tag showed improved diffraction and slightly smaller unit-cell axes. Using synchrotron radiation on beamline X06SA of the Swiss Light Source, these crystals diffracted to 4.0 Å resolution and a complete data set was recorded to 4.3 Å resolution (Fig. 2). Data statistics are given in Table 1. The space group was determined to be $P2_1$, with unit-cell parameters $a$ = 94, $b$ = 146, $c$ = 105 Å, $\alpha = \gamma = 90$, $\beta = 111^\circ$. In order to check for the presence of all six subunits in the crystal, we dissolved 190 protein crystals (14 µg) for analysis by SDS–PAGE and compared it with that of holo NQR from size-exclusion chromatography. The same pattern of subunits was observed (Fig. 3). Assuming the presence of one $Na^+$-NQR complex (molecular weight of 213 kDa) in the asymmetric unit, the Matthews coefficient ($V_M$) of the crystal (Matthews, 1968) was calculated to be 3.01 $Å^3$ $Da^{-1}$, corresponding to an estimated solvent content of 59.2%. Structure determination of the $Na^+$-NQR complex by heavy-atom phasing is in progress.

**Table 1**
Data-processing statistics.

| | |
|---|---|
| Wavelength (Å) | 1.00 |
| Space group | $P2_1$ |
| Unit-cell parameters (Å, °) | $a$ = 94.15, $b$ = 145.53, $c$ = 104.76, $\alpha = \gamma = 90$, $\beta = 110.9$ |
| Resolution (Å) | 50–4.3 (4.7–4.3) |
| Completeness (%) | 98.2 (98.9) |
| No. of unique reflections | 17536 (4134) |
| Redundancy | 3.4 (3.5) |
| $R_{merge}$† (%) | 11.6 (70.6) |
| $R_{meas}$‡ (%) | 13.8 (83.4) |
| $R_{mrgd\text{-}F}$§ (%) | 32.4 (100.7) |
| $\langle I/\sigma(I)\rangle$ | 6.8 (2.1) |
| No. of molecules per asymmetric unit | 1 $Na^+$-NQR complex |
| Matthew coefficient ($Å^3$ $Da^{-1}$) | 3.01 |
| Solvent content (%) | 59.2 |

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ $R_{meas} = \sum_{hkl}[N/(N-1)]^{1/2} \times \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. § $R_{mrgd\text{-}F} = \sum_{hkl}[N/(N-1)]^{1/2}\sum_i |F_i(hkl) - \langle F(hkl)\rangle| / \sum_{hkl}\sum_i F_i(hkl)$.

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