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Crystallization and preliminary X-ray analysis of the membrane-bound cytochrome *c* nitrite reductase complex (NrfHA) from *Wolinella succinogenes*

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Crystals of the complex between the enzyme cytochrome *c* nitrite reductase (NrfA) and the membrane-bound quinol oxidase and electron carrier NrfH were grown by vapour diffusion using ammonium sulfate as a precipitant. In the ε-proteobacterium *Wolinella succinogenes*, NrfA and NrfH form a functional membrane-bound complex which catalyzes the last step in the metabolic pathway of nitrate dissimilation. NrfH represents a prototype of a large family of putative bacterial quinol oxidases, the NapC/NirT family, which have been proposed to serve as electron donors for a variety of reductases. Crystal growth of the NrfHA complex was strongly dependent on the presence of detergent; the crystals grown belonged to space group *I*422.

1. Introduction

Cytochrome *c* nitrite reductase is the terminal enzyme in the metabolic pathway of dissimilatory nitrate reduction to ammonia. It catalyzes the six-electron reduction of nitrite to ammonia, obtaining electrons from formate or hydrogen through the membraneous quinone pool (Berks *et al.*, 1995; Einsle *et al.*, 1999). In all sequences from δ- and ε-proteobacteria known so far, the nitrite reductase system consists of two proteins, NrfH and NrfA, which form a membrane-bound respiratory complex. The purified NrfHA complex could be reconstituted into liposomes together with the membraneous formate dehydrogenase and this system was able to couple electron transfer from formate to nitrite to the generation of an electrochemical proton potential (Simon *et al.*, 2000). The enzymatic activity of nitrite reduction is located on the NrfA protein, which has been shown to be soluble and active as a monomer (Schumacher *et al.*, 1994). However, the X-ray structures of NrfA from *Sulfurospirillum deleyianum* (Einsle *et al.*, 1999) and *W. succinogenes* (Einsle *et al.*, 2000) suggest that NrfA is a functional dimer.

According to sequence alignments, the second component of the nitrite reductase complex, NrfH, belongs to the NapC/NirT family of multihaem cytochromes. This family of proteins is involved in a large number of periplasmic reductase and oxidoreductase systems in bacteria, including nitrate reductase (NapC; Roldán *et al.*, 1998), cytochrome cd_1 nitrite reductase (NirT; Jüngst *et al.*, 1991) and FccA methacrylate reductase (FccC; Simon *et al.*, 1998). These peripheral membrane proteins have a single N-terminal transmembrane helix and are proposed to oxidize quinols in the membrane in order to provide downstream elements of the respective reductase systems with electrons. In most cases, but not in the NrfHA system, the actual reductase is coupled to these quinol oxidases through another soluble electron carrier, usually a *c*-type cytochrome. Thus, the stable NrfHA complex in *W. succinogenes* represents an ideal system to investigate this novel class of quinol oxidases and its high-resolution X-ray structure will allow a detailed picture of a respiratory electron-transfer complex to emerge.

2. Materials and methods

2.1. Protein preparation

Growth of cells and purification of the cytochrome *c* nitrite reductase complex NrfHA from *W. succinogenes* were carried out as described previously (Schumacher *et al.*, 1994; Simon *et al.*, 2000).

2.2. Detergent removal

While the presence of detergent was essential for solubilization of the protein from the membrane as well as for crystallization, the complex itself was stable in solution without detergent. Thus, it was possible to prepare detergent-free protein solution to be used for crystallization experiments with a wide range of different detergents. For detergent removal, the protein buffer (10 m*M* HEPES–NaOH pH 7.0) was exchanged for water using a NAP10 desalting column (Pharmacia) and the salt-free

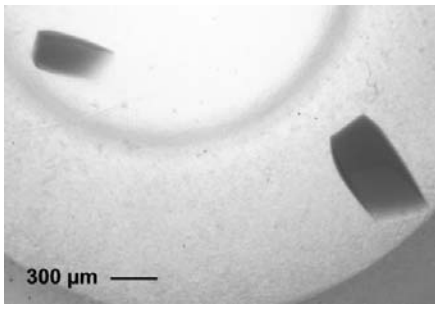

Figure 1
Crystals of the NrfHA complex obtained by sitting-drop vapour diffusion.

sample was then applied to a DetergentOUT DTG-100X spin column (Geno Technology). Protein was stored at 273 K at concentrations greater than 15 mg ml^{-1}.

2.3. X-ray analysis

Diffraction experiments were carried out on beamline BW6 at DESY, Hamburg using synchrotron radiation with a wavelength of 1.05 Å. For multiple-wavelength anomalous dispersion, a fluorescence scan around the iron $K\alpha$ edge was carried out and two further data sets were collected at wavelengths of 1.7378 Å to maximize the anomalous f'' contribution and of 1.7410 Å at the f' inflection. Diffraction data were indexed using *DENZO* (Otwinowski & Minor, 1996).

3. Results and discussion

Crystallization of the NrfHA complex was critically dependent on the use of detergents, presumably because of the need to shield the hydrophobic transmembrane helix of NrfH from the solvent. Although small crystals could be grown with the detergent applied during purification, namely Triton X-100, considerable improvement in crystal quality was achieved after establishing a protocol for detergent exchange. Of 72 detergents screened with the NrfHA complex, Triton X-114 led to the most significant improvement in crystal size and quality (Fig. 1).

The first crystals of *W. succinogenes* NrfHA were obtained by sitting-drop vapour diffusion from Crystal Screen solution 39 (Hampton Research, Laguna Niguel, USA) in the presence of 0.5%(*v*/*v*) Triton X-100. Further refinement of the precipitant concentration and pH value of this buffer and a change of detergent to Triton X-114 led to large single crystals which could be used in diffraction experiments. The final crystallization conditions were 1.9 *M* ammonium sulfate as precipitant with 3% PEG 300 in 0.1 *M* HEPES–NaOH buffer pH 7.0. The presence of both NrfA and NrfH in the crystals was confirmed by SDS–PAGE. For this, crystals were washed three times in protein-free reservoir buffer and subsequently dissolved in water.

In order to collect diffraction data, crystals had to be flash-cooled in a nitrogen stream. While the native crystallization buffer was not suitable for flash-cooling, replacement of ammonium sulfate with chaotropic lithium sulfate was sufficient to prevent formation of ice crystals. The final cryoprotective buffer used contained 2.4 *M* Li_2SO_4, 3% PEG 300 and 0.1 *M* HEPES–NaOH pH 7.0.

A crystal with dimensions of 100 × 100 × 500 Å was used. It diffracted to below 4 Å and could readily be indexed and integrated with *DENZO*. The NrfHA complex was crystallized in space group *I*422, with unit-cell parameters $a = b = 249.0$, $c = 231.1$ Å. Data were 96.6% complete to 4.0 Å (last shell, 97.2%), with an $R_{\rm merge}$ of 0.138 (last shell, 0.435) and an $I/\sigma(I)$ of 6.2 (last shell, 1.6).

The presence of a dimer of NrfA in all crystal forms obtained so far (Einsle *et al.*, 1999, 2000) leads to the assumption that NrfHA forms an $\alpha_2\beta_2$ complex. Two such complexes per asymmetric unit of the complex crystals would result in a Matthews parameter of 2.82 Å^3 Da^{-1} and a solvent content of 56% (Matthews, 1968). However, given the lability of the crystals, the presence of only one complex at a solvent content of 80% cannot be excluded.

Molecular replacement using either the monomer or dimer of *W. succinogenes* NrfA as a search model did not produce a clear solution. Consequently, a three-wavelength MAD experiment at the iron edge was carried out and yielded data to 5 Å. As a next step, we will attempt to use this data to calculate maps of sufficient quality to manually place NrfA and use its additional phasing information to locate and solve the structure of NrfH in the complex.

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