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Crystallization and preliminary X-ray analysis of the periplasmic nitrate reductase (NapA–NapB complex) from *Rhodobacter sphaeroides* f. sp. *denitrificans*

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The periplasmic nitrate reductase of *Rhodobacter sphaeroides* f. sp. *denitrificans* is a heterodimer responsible for the first step of reduction in the denitrification process by the conversion of nitrate to nitrite. It consists of a 91 kDa molybdenum-containing catalytic subunit (NapA) and a 17 kDa dihaem cytochrome *c* (NapB). Crystals of the NapA–NapB complex were obtained by the vapour-diffusion method using ammonium sulfate as precipitant. They belong to the $P6_122$ space group, with unit-cell parameters $a = b = 151.9$, $c = 255.8$ Å, and contain a single complex in the asymmetric unit. A complete native data set was collected at a synchrotron source to 3.1 Å resolution.

1. Introduction

Three different types of nitrate-reducing system have been described in bacteria: cytoplasmic assimilatory nitrate reductase (Nas), membrane-bound respiratory nitrate reductase (Nar) and periplasmic nitrate reductase (Nap) (Berks *et al.*, 1995). Nas is associated with nitrate assimilation, whereas Nar and Nap are involved in respiratory pathways. Nar catalyses nitrate respiration under anaerobic conditions, whereas Nap probably participates in redox balance and aerobic nitrate respiration (Phillipot & Hojberg, 1999). However, in *R. sphaeroides* Nap is responsible for the reduction of nitrate even under anaerobic conditions (Sabaty *et al.*, 1999). Dissimilatory and assimilatory nitrate reductases are different proteins in terms of subunit structure and cell localization, but they are all molybdoenzymes which catalyse the two-electron reduction step of nitrate to nitrite using a molybdopterin cofactor at their active site.

Periplasmic respiratory nitrate reductases have been studied at the biochemical and genetic level in several Gram-negative bacteria. They are heterodimers composed of a catalytic 90 kDa NapA subunit that contains a molybdopterine guanine dinucleotide cofactor, a (4Fe–4S) centre and a 13–19 kDa bihaem *c*-type cytochrome NapB subunit. The interaction between the two subunits is probably hydrophobic (Richardson *et al.*, 1990). They are encoded respectively by the *napA* and *napB* genes of the (*nap*) operon, which consists of at least four genes (Reyes *et al.*, 1998). NapC is a membrane-bound tetrahaem cytochrome that transfers electrons from the quinol pool to NapB; NapD is a cytoplasmic protein probably involved in NapA maturation.

The crystal structure of the monomeric nitrate reductase from *Desulfovibrio desulfuricans* (NapA) has been reported previously (Dias *et al.*, 1999). The polypeptide folding and the arrangement of the cofactors are related to that of formate dehydrogenase (Boyington *et al.*, 1997): the enzyme is folded into four domains with α/β-type topology and the catalytic molybdenum site, positioned 12 Å from the (4Fe–4S) cluster, is coordinated to two molybdopterin guanidine dinucleotide cofactors. On the basis of the structure, a putative reaction mechanism has been proposed.

No structural information on the NapB subunit or NapA–NapB association is available, although the crystallization of a 8 kDa proteolytic fragment of the NapB subunit from *Haemophilus influenzae* has been reported recently (Brigé *et al.*, 2001). Here, we report the crystallization and preliminary X-ray analysis of the 108 kDa NapA–NapB complex from *R. sphaeroides* f. sp. *denitrificans*. This is a first step towards the description of the complete structural organization of the Nap system.

2. Bacterial overexpression and purification

The NapA–NapB complex was overexpressed in *R. sphaeroides*. A plasmid containing the *napA* and *napB* genes modified by the addition of six codons for histidine to the 3′ end was introduced into MS523, a *R. sphaeroides* mutant which does not synthesize Nap (Sabaty *et al.*, 2001). Cells were grown semi-aerobically in Hutner medium (Clayton, 1960) for 24 h at 303 K. 5 l of NapA–NapB overexpressing cells were harvested by centrifugation at 5000g for 10 min and resuspended in 300 ml buffer *A* [20

m*M* phosphate buffer pH 8.0, 20%(*w*/*v*) sucrose]. 10 ml 0.5 *M* EDTA was added and after 10 min incubation the suspension was centrifuged at 5000*g* for 10 min. The pellet was washed in 150 ml cold water and centrifuged at 5000*g* for 20 min. The periplasmic fraction was prepared by resuspension of the pellet in 150 ml buffer *A* containing 1 mg ml^{-1} lysozyme. After 1 h incubation, the suspension was centrifuged for 20 min at 5000*g*. The supernatant was centrifuged at 200 000*g* to remove cell-wall debris. NaCl was added to the solution to a final concentration of 250 m*M*. The periplasmic fraction was loaded onto a Ni-charged column containing about 2 ml of resin. The column was thoroughly washed with 20 m*M* phosphate buffer pH 8.0, 250 m*M* NaCl, 15 m*M* imidazole to remove the contaminating proteins. The bound protein was then eluted with 20 m*M* phosphate pH 8.0, 250 m*M* NaCl, 100 m*M* imidazole. The nitrate-reductase activity was determined at each step of the purification process (Sabaty *et al.*, 1999). The typical yield of NapA–NapB complex was about 1 mg from 1 l of cell culture. Silver staining following SDS gel electrophoresis revealed that the purity of preparation was close to 100% and that no dissociation of the complex occurs in the course of the purification. No attempt was made to remove the His_6 tag and the preparation was directly concentrated prior to crystallization trials.

3. Crystallization

The purified protein was initially concentrated to 5 mg ml^{-1} as estimated by the Bradford method (Bradford, 1976) in 20 m*M* HEPES pH 7. All the crystallization experiments were carried out at 293 K using the hanging-drop vapour-diffusion technique on Linbro tissue-culture plates. Hanging drops consisted of 2 µl of concentrated protein solution and 2 µl of the reservoir solution. Preliminary crystallization trials were conducted at pH 7.0 using ammonium sulfate or PEG 4000 as precipitant. Thin red needles unsuitable for crystallographic study were obtained within 3 d for many conditions containing ammonium sulfate. In order to identify the critical factors for the crystallization, we resorted to the Incomplete Factorial Approach (INFAC; Carter & Carter, 1979). This procedure permits the sampling of a large number of crystallization conditions using a limited number of experiments. An incomplete factorial experiment using 96 hanging drops was set up to test (i) several pH values (between 5.5 and 7.5), (ii) several ammonium sulfate concentrations, (iii) several protein concentrations (between 5 and 20 mg ml^{-1}) and (iv) various additives such as salts (NaCl, sodium nitrite and sodium nitrate). The resulting optimal crystallization droplets were obtained by mixing 5 µl of NapA–NapB solution (20 mg ml^{-1}) with 5 µl of reservoir solution containing 1 *M* ammonium sulfate, 100 m*M* sodium nitrate, 100 m*M* HEPES pH 7.5 and were equilibrated against 1 ml of reservoir. Red single needle-like crystals with approximate dimensions of 70 × 70 × 300 µm grew in 3 d and reached their final size in about two weeks (Fig. 1). The presence of both NapA and NapB subunits in the crystals was confirmed as follows: crystals were thoroughly washed with the reservoir solution, centrifuged for 2 min at 4000*g* and dissolved in water prior to SDS–PAGE analysis.

Figure 1
Crystals of the NapA–NapB complex (40 × 40 × 200 µm).

4. Data collection and analysis

NapA–NapB complex crystals were cooled to 100 K after soaking for several minutes in a cryobuffer containing 1.2 *M* ammonium sulfate, 50 m*M* HEPES pH 7.5 and 30% glycerol. Diffraction data were collected using synchrotron radiation (ID14-EH1 beamline at the ESRF, France). A MAR CCD detector and X-ray radiation of 0.94 Å wavelength were used. The data sets were processed with *MOSFLM* (Leslie, 1992) and subsequent data reduction was carried out using the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Analysis of symmetry-related reflections and systematic absences indicated that the crystals belong to the $P6_122$ space group or its enantiomorph, with unit-cell parameters $a = b = 151.9$, $c = 255.8$ Å. They diffract to 3.1 Å resolution. One or two NapA–NapB complexes per asymmetric unit give V_M values of 2.0 or 3.9 Å^3 Da^{-1} (Matthews, 1968) and solvent contents of 70 or 30%, respectively. Further details concerning the resulting complete data set are given in Table 1.

Table 1
Data-collection statistics.

Values in parentheses refer to data in the highest resolution shell (3.2–3.1 Å).

Space group	$P6_122$
Unit-cell parameters (Å)	
a, *b*	151.9
c	255.8
Solvent content (%)	70
V_M (Å^3 Da^{-1})	3.9
Resolution (Å)	25–3.1
No. of observations	128073
No. of unique reflections	29337
R_{merge}†	11.9 (23.9)
Completeness	99 (98.7)
Redundancy	4.3
$I/\sigma(I)$	4.8 (2.7)

† $R_{merge} = \sum_{hkl}\sum_i[I_{h(i)} - \langle I_h\rangle]/\sum_{hkl}\sum_i I_{h(i)}$.

Molecular-replacement calculations were performed using the program *AMoRe* (Navaza, 1994). The structure of the nitrate reductase (NapA) from *D. desulfuricans* (PDB code 2nap; Dias *et al.*, 1999) was used as a search model. No significant peak was observed in the space group $P6_522$, whereas a clear peak was found with a correlation coefficient of 23% (18.7% for the next best solution) after translation-function calculations (15–3.5 Å) in the space group $P6_122$. Manual modifications of the model structure are currently in progress.

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