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Crystallization and preliminary X-ray analysis of the recombinant dihaem cytochrome *c* (NapB) from *Haemophilus influenzae*

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The *napB* gene of the pathogenic bacterium *Haemophilus influenzae* encodes a dihaem cytochrome *c*, the small subunit of a heterodimeric periplasmic nitrate reductase (Nap). Recombinant NapB was overproduced in *Escherichia coli*, purified to near-homogeneity and crystallized using the hanging-drop method. Thin quadrilateral plates were grown under various conditions but proved to be unsuitable for X-ray analysis. However, a single crystal was grown using 1.75 *M* ammonium sulfate in 0.1 *M* sodium acetate pH 5.5, from which a native data set could be collected to 1.8 Å resolution using synchrotron radiation. Using the same conditions, further crystals were obtained by microseeding. The space group was determined to be $P42_12$, with unit-cell parameters $a = 77.55$, $b = 77.55$, $c = 28.64$ Å and an unusually low solvent content of 16.5%, assuming there to be one molecule of NapB in the asymmetric unit. Analysis of the dissolved crystals indicated that partial proteolysis of the protein had occurred. Taking the molecular mass of the crystallized form (~8500 Da) into account, the solvent content was estimated to be 53%, with a V_M value of 2.64 Å^3 Da^{-1}.

1. Introduction

In bacteria, three different types of nitrate-reducing systems have been described (Moreno-Vivián & Ferguson, 1998; reviewed in Berks, Ferguson *et al.*, 1995): cytoplasmic assimilatory nitrate reductases (Nas), membrane-bound respiratory nitrate reductases (Nar) and periplasmic nitrate reductases (Nap). The latter are terminal oxidoreductases in an electron-transport chain originating from the membrane-bound quinol pool, but do not seem to be involved in the generation of a proton motive force across the membrane. The Nap enzyme is heterodimeric, consisting of a large subunit (NapA) and a small subunit (NapB), which most probably interact in a hydrophobic manner (Richardson *et al.*, 1990). Dissociation of the subunits during purification can occur. NapA (90 kDa) contains a molybdopterine guanine dinucleotide cofactor that is the catalytic site of nitrate reduction and one [4Fe–4S] centre, whereas NapB (13–19 kDa) is a *c*-type cytochrome with two covalently bound haem groups. Both subunits are synthesized as precursors with N-terminal signal sequences and are thus translocated across the cytoplasmic membrane into the periplasmic space. They are encoded by the *napA* and *napB* genes, respectively, which are part of a *nap* operon consisting of at least four genes (*napDABC*) (Siddiqui *et al.*, 1993; Bedzyk *et al.*, 1999). NapC is a membrane-bound tetrahaem cytochrome *c* that most likely donates electrons to the NapAB complex, whereas NapD is suggested to be involved in NapA maturation (Berks, Richardson *et al.*, 1995; Reyes *et al.*, 1998). In the *nap* operons that have been characterized so far, five other *nap* genes can be found in different combinations with *napDABC*: *napF*, *napG* and *napH* encode iron–sulfur redox proteins and *napE* encodes an integral membrane protein with unknown function, while *napK* has only been found in *Rhodobacter sphaeroides* (Reyes *et al.*, 1996, 1998).

Nap systems have been identified at the protein and/or genetic levels in a still increasing number of bacterial species including enterobacteria, aerobic denitrifiers and non-sulfur photosynthetic bacteria. These Nap systems are not only found throughout a phylogenetically diverse range of bacteria, but are also expressed under different conditions. This indicates their importance and physiological versatility. In particular, periplasmic nitrate reduction has been proposed to play a role in aerobic denitrification, a process with agricultural and environmental implications (Bell *et al.*, 1990; Carter *et al.*, 1995; Sabaty *et al.*, 1994), in the adaptation to anaerobic growth (Siddiqui *et al.*, 1993), in redox balancing using nitrate as an electron sink in order to dissipate excess reductant (Richardson *et al.*, 1990; Roldán *et al.*, 1998; Richardson & Ferguson, 1992) or in the scavenging of nitrate

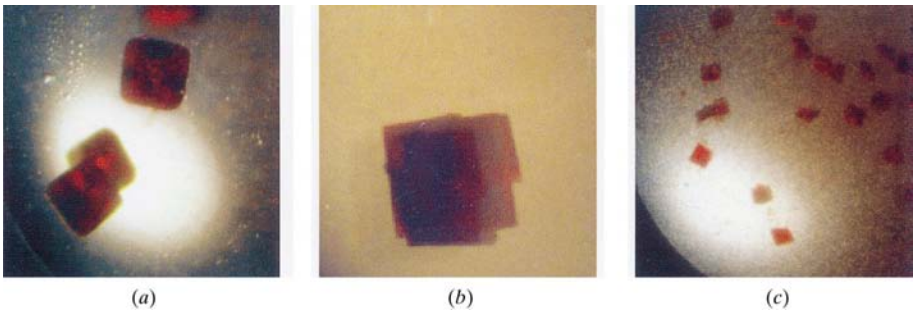

Figure 1
Different crystal forms of the NapB protein. (*a*) Small composite quadrilateral plates; (*b*) clustered plates; (*c*) crystals obtained by streak-seeding.

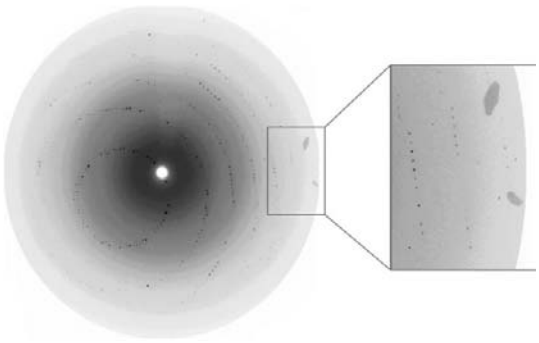

Figure 2
Diffraction pattern of the NapB crystal. The detector edge corresponds to 1.8 Å resolution (see detail).

in nitrate-limited environments, such as the human body (Potter *et al.*, 1999).

Genome-sequencing projects revealed the presence of *nap* genes in a number of pathogenic bacteria, including *H. influenzae* (Fleischmann *et al.*, 1995) and more recently *Salmonella typhimurium*, *Vibrio cholerae*, *Yersinia pestis* and *Campylobacter jejuni* (http://www.tigr.org). This suggests that Nap might play an important role in pathogenic bacteria.

Except for the NapA protein of *Desulfovibrio desulfuricans* (Dias *et al.*, 1999), no structural information on Nap proteins is available. Here, we report the crystallization and preliminary X-ray analysis of the dihaem cytochrome *c* (NapB) of *H. influenzae*, a pathogenic organism that has only been found in human hosts.

2. Bacterial overexpression and purification

The overexpression and purification of NapB will be described in detail elsewhere. Briefly, the gene sequence encoding mature NapB was cloned in the pLPPsompArPDI expression vector (De Sutter *et al.*, 1994), allowing periplasmic expression. NapB was co-expressed with the *ccm* (cytochrome *c* maturation) genes in a DegP protease-deficient *E. coli* strain (Arslan *et al.*, 1998).

Recombinant NapB was purified from the periplasmic protein fraction in five steps, including ammonium sulfate precipitation, hydrophobic interaction and ion-exchange chromatography, and gel filtration. Silver staining following SDS gel electrophoresis indicated that the protein was purified to near-homogeneity. Using electrospray mass spectrometry, the molecular mass of recombinant NapB was determined to be 14 748.68 Da, a value that corresponds perfectly to the calculated value of 14 748.60 Da. The purified protein was concentrated to $\sim$20 mg ml^{-1}.

3. Crystallization

Initial crystallization trials were set up using the sparse-matrix Structure Screen I (Stratech Ltd, Bedfordshire, UK) and the hanging-drop method. In these and all further trials, hanging drops consisted of 1 µl of the concentrated protein solution and 1 µl of the reservoir solution; trials were stored at 277 K. Within two weeks, red quadrilateral plates (Fig. 1*a*), which were small and composite, grew from condition numbers 15 and 26 of the screen. The reservoirs consisted of 20% PEG 8000, 0.2 *M* magnesium acetate tetrahydrate, 0.1 *M* sodium cacodylate pH 6.5 and 0.8 *M* potassium/sodium tartrate tetrahydrate, 0.1 *M* Na HEPES pH 7.5, respectively. After two months, the same type of crystal was grown from condition 27, *i.e.* 1.5 *M* lithium sulfate monohydrate, 0.1 *M* Na HEPES pH 7.5. In an attempt to grow larger and single crystals, these conditions were further explored by varying the pH and the salt concentrations. Because all attempts gave composite crystals, further crystallization trials were initiated using Structure Screen II (Stratech Ltd, Bedfordshire, UK) and PEG/Ion Screen (Hampton Research, Laguna Hills, CA 92653, USA), in addition to trials with ammonium sulfate as precipitant. Composite microcrystals were grown from condition 23 [1.6 *M* ammonium sulfate, 0.1 *M* MES pH 6.5, 10%(*v*/*v*) dioxane] of Screen II. Further exploration of this condition resulted in the same composite small crystals. Larger plate-like crystals (Fig. 1*b*) were grown in the trials with ammonium sulfate as precipitant, but these crystals were clustered, cracked upon detachment and were too small for data collection using a rotating-anode generator. However, in a condition with 0.1 *M* sodium acetate pH 5.5, 1.75 *M* ammonium sulfate, a single crystal was grown that proved to be of good X-ray quality. Growth of crystals using this condition could only be reproduced by microseeding (streak-seeding; Fig. 1*c*).

4. Data collection and processing

The cryoprotected crystal (15% glycerol as cryoprotectant agent) was flash-frozen in liquid nitrogen and mounted in a stream of nitrogen gas. A native data set was collected at the synchrotron beamline X11 at DORIS (HASYLAB, DESY, Hamburg, Germany) to 1.8 Å using a MAR CCD imaging-plate detector (Fig. 2). Intensities were integrated with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1993; Table 1). The crystal is tetragonal, with unit-cell parameters a = 77.55, b = 77.55, c = 28.64 Å. Analysis of the data, including systematic absences, suggested that the space group is $P42_12$. Assuming a molecular weight of 14.748 Da and one molecule in the asymmetric unit, the value of the crystal packing parameter V_M is 1.48 Å^3 Da^{-1} (Matthews, 1968). The solvent content was calculated to be only 16.5%. Putting the data set through Todd Yeates' twinning program (http://www.doe-mbi.ucla.edu/Services/Twinning) revealed no twinning for the crystal. However, following the dissolution of several crystals obtained by microseeding, electrospray ionization mass spectrometry and N-terminal amino-acid sequence analysis clearly indicated that proteolysis had occurred at both the N- and C-terminus of the recombinant protein. The average molecular mass of the crystallized molecule was determined to be $\sim$8500 Da. This information allowed recalculation of the solvent content to a value of 53%. The Matthews coefficient was determined to be 2.64 Å^3 Da^{-1}, a value within the normal

Table 1
Data-collection statistics for the crystal of NapB.

Values in parentheses refer to the highest resolution shell.

Space group	$P42_12$
Unit-cell parameters (Å,°)	$a = b = 77.55$, $c = 28.64$, $\alpha = \beta = \gamma = 90$
Resolution (Å)	40.0–1.8 (1.83–1.80)
No. of unique reflections	15375 (733)
Redundancy	2.54 (2.47)
$R_{\rm merge}$† (%)	2.7 (8.7)
Completeness (%)	94.5 (93.3)
$I/\sigma(I)$	35.49 (12.76)

† $R_{\rm merge}(I) = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i I_{h,i}$.

range for protein crystals. One of the crystals grown by microseeding diffracted to 1.1 Å at ESRF (Grenoble, France) on the microfocus beamline ID13. The crystal structure determination of the truncated form of NapB by MAD is under way.

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References

Arslan, E., Schulz, H., Zufferey, R., Künzler, P. & Thöny-Meyer, L. (1998). *Biochem. Biophys. Res. Commun.* **29**, 744–747.
Bedzyk, L., Wang, T. & Ye, R. W. (1999). *J. Bacteriol.* **181**, 2802–2806.
Bell, L. C., Richardson, D. J. & Ferguson, S. J. (1990). *FEBS Lett.* **265**, 85–87.
Berks, B. C., Ferguson, S. J., Moir, J. W. B. & Richardson, D. J. (1995). *Biochim. Biophys. Acta*, **1232**, 97–173.
Berks, B. C., Richardson, D. J., Reilly, A., Willis, A. C. & Ferguson, S. J. (1995). *Biochem. J.* **309**, 983–992.
Carter, J. P., Richardson, D. J. & Spiro, S. (1995). *Arch. Microbiol.* **163**, 159–166.
De Sutter, K., Hostens, K., Vandekerckhove, J. & Fiers, W. (1994). *Gene*, **141**, 163–170.
Dias, J. M., Than, M. E., Humm, A., Huber, R., Bourenkov, G. P., Bartunik, H. D., Bursakov, S., Calvete, J., Caldeira, J., Carneiro, C., Moura, J. J. & Romão, M. J. (1999). *Structure Fold. Des.* **7**, 65–79.
Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J. D., Scott, J., Shirley, R., Liu, L., Glodek, A., Kelley, J. M., Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghagen, N. S. M., Gnehm, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O. & Venter, J. C (1995). *Science*, **269**, 496–512.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Moreno-Vivián, C. & Ferguson, S. J. (1998). *Mol. Microbiol.* **29**, 664–666.
Otwinowski, Z. & Minor, W. (1993). *DENZO: A Film Processing Program for Macromolecular Crystallography*. Yale University, New Haven, Connecticut, USA.
Potter, L. C., Millington, P., Griffiths, L., Thomas, G. H. & Cole, J. A. (1999). *Biochem. J.* **344**, 77–84.
Reyes, F., Gavira, M., Castillo, F. & Moreno-Vivián, C. (1998). *Biochem. J.* **331**, 897–904.
Reyes, F., Roldán, M. D., Klipp, W., Castillo, F. & Moreno-Vivián, C. (1996). *Mol. Microbiol.* **19**, 1307–1318.
Richardson, D. J. & Ferguson, S. J. (1992). *Arch. Microbiol.* **157**, 535–537.
Richardson, D. J., McEwan, A. G., Page, M. D., Jackson, J. B. & Ferguson, S. J. (1990). *Eur. J. Biochem.* **194**, 263–270.
Roldán, M. D., Sears, H. J., Cheesman, M. R., Ferguson, S. J., Thomson, A. J., Berks, B. C. & Richardson, D. J. (1998). *J. Biol. Chem.* **273**, 28785–28790.
Sabaty, M., Gagnon, J. & Verméglio, A. (1994). *Arch. Microbiol.* **162**, 335–343.
Siddiqui, R. A., Warnecke-Eberz, U., Hengsberger, A., Schneider, B., Kostka, S. & Friedrich, B. (1993). *J. Bacteriol.* **175**, 5867–5876.