Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification and crystallization of the respiratory complex formate dehydrogenase-N from *Escherichia coli*

Mika Jormakka,[a] Susanna Törnroth,[b] Jeff Abramson,[c] Bernadette Byrne[c] and So Iwata[a,b,c]*

[a]Division of Biomedical Sciences, Imperial College, London SW7 2AZ, England, [b]Department of Biochemistry, Uppsala University, BMC BOX 576, S-75123 Uppsala, Sweden, and [c]Department of Biological Sciences, Imperial College, London SW7 2AZ, England

Correspondence e-mail: s.iwata@ic.ac.uk

Received 16 September 2001
Accepted 18 October 2001

A membrane-protein complex, formate dehydrogenase-N from *Escherichia coli*, has been purified and crystallized. This molybdenum-containing enzyme, composed of α, β and γ subunits, is the major electron donor to the nitrate respiratory chain of *E. coli*. The formate dehydrogenase-N crystals belong to the cubic space group $P2_13$, with unit-cell parameters $a = b = c = 203$ Å. An asymmetric unit of the crystals is assumed to contain one formate dehydrogenase-N monomer (MW 170 kDa). One data set to 1.6 Å resolution, with 342 711 independent observations (94.4% complete) and an $R_{\rm merge}$ of 0.08, has been collected from a single crystal. This is the highest resolution data set reported for a membrane-protein complex to date.

1. Introduction

In *E. coli*, the respiration of nitrate constitutes a major respiratory pathway in anaerobic environments (Berg & Stewart, 1990). This pathway utilizes the two-electron oxidation of formate as an electron donor, leading to the reduction of nitrate to nitrite. The two major energy-yielding enzymes involved in this respiratory pathway are the integral membrane proteins formate dehydrogenase-N (Fdh-N) and dissimilatory nitrate reductase (Nar), which belong to the prokaryotic molybdopterin-containing oxidoreductase family. These are members of the subgroup of molybdo-enzymes binding the bis-molybdopterin guanine dinucleotide (bis-MGD) form of the molybdopterin cofactor (Berks *et al.*, 1995). Both Fdh-N and Nar are three-subunit proteins, consisting of two membrane-associated domains and an integral membrane domain. All three subunits of Fdh-N are coded for by the *FdnGHI* operon. The α-subunit (FdnG) is a membrane-associated subunit, incorporating a selenocysteine (SeCys) residue and a [4Fe–4S] cluster in addition to two bis-MGD cofactors (Berg *et al.*, 1991). Fdh-N and the two formate dehydrogenase isozymes, formate dehydrogenase-O (Fdh-O) and formate dehydrogenase-H (Fdh-H), are the only proteins within *E. coli* to incorporate SeCys (Abaibou *et al.*, 1995; Zinoni *et al.*, 1986). The β-subunit (FdnH) is another membrane-associated subunit which contains four [4Fe–4S] clusters and is postulated to contain one transmembrane helix. Currently, it is unclear on which side of the membrane the two membrane-associated Fdh-N subunits are localized. The γ-subunit (FdnI) is the integral membrane subunit which anchors the other two subunits to the membrane. It also incorporates two haem *b* groups and a menaquinone binding site. Similarly, the *NarGHI* operon codes for the three subunits of Nar. *NarG* and *NarH* code for the membrane-associated subunits, which bind bis-MGD cofactors and four iron–sulfur clusters, respectively. The product of *NarI* is the integral membrane subunit, which contains two haem *b* groups and a menaquinol oxidation site. In these two enzymes, the presence of the iron–sulfur clusters and membrane-bound subunits allows the redox reaction at bis-MGD cofactors to be coupled to electron transfer into or out of the menaquinone Q/QH_2 pool in the membrane. Fdh-N and Nar are believed to translocate protons across the membrane by a redox loop mechanism using this Q/QH_2 pool. The generated proton gradient is then used by ATPase and other enzymes.

X-ray structures are available for a number of bis-MGD enzymes, including Fdh-H, a component of the anaerobic formate hydrogen lyase complex of *E. coli* (Boyington *et al.*, 1997). The structure of this soluble formate dehydrogenase has been solved (reduced form at 2.3 Å, oxidized form at 2.8 Å and NO^{2-} complex at 2.9 Å) and revealed that the molybdenum is directly coordinated to the selenium and both bis-MGD cofactors. The structure of a soluble nitrate reductase from *Desulfovibrio desulfuricans* has been also solved at 1.9 Å resolution (Dias *et al.*, 1999) and shown to be very similar to that of Fdh-H,

except for the lack of the SeCys residue. However, there is no structural information available for the iron–sulfur (NarH and FdnH) and transmembrane (NarI and FdnI) subunits for the Fdh-N and Nar enzymes.

We have recently succeeded in purifying and crystallizing Fdh-N from *E. coli* and in this paper we report our preliminary crystallographic studies. Unusually for membrane proteins, the crystals diffracted X-rays to 1.6 Å resolution. The structure of Fdh-N at this resolution will certainly reveal the molecular details of this large membrane-protein complex. Moreover, since the structures of formate Fdh-N and Nar are closely related, the high-resolution structure of Fdh-N will provide important information for the understanding of the complete proton-translocation system in which both enzymes are involved.

2. Materials and methods

2.1. Expression and purification

Native Fdh-N was purified from GL101 cells (Lemieux *et al.*, 1992) grown under anaerobic conditions. A single colony of GL101 cells was used to inoculate 10 ml LB with 100 mg ml^{-1} kanamycin and the culture was grown under aerobic conditions at 310 K overnight. This overnight culture was then used to inoculate 100 ml LB with 100 mg ml^{-1} kanamycin and incubated at 310 K to an OD_{600} reading of 1.0. This culture was then added to a 12 l carboy with LB medium containing 0.4%(*w*/*v*) glucose, 40 m*M* sodium nitrate, 2 µ*M* sodium selenite, 2 µ*M* ammonium molybdate and 100 mg ml^{-1} kanamycin. The carboy was filled to the neck with medium and sealed to prevent aeration. The culture was grown under anaerobic conditions at 310 K under slow stirring until OD_{600} reached 1.5 (∼12 h).

Cells were harvested by centrifugation (277 K, 20 min) and then resuspended in lysozyme treatment buffer (200 m*M* Tris–HCl pH 8.8, 20 m*M* EDTA pH 8.0, 500 mM sucrose) to a final volume of 1 l. All subsequent steps were performed at 277 K. Membranes were purified as previously described (Abramson *et al.*, 2000). Purified membranes were resuspended in a minimal volume of membrane resuspension buffer (20 ml Tris–HCl pH 8.5) with one tablet of Complete EDTA-free protease-inhibitor cocktail (Roche Molecular Biochemicals, IN, USA); proteins were then solubilized by the addition of 1% *n*-dodecyl-β-D-maltoside (DDM; Anatrace, IL, USA) under slow stirring. Lipid and debris were removed by centrifugation at 100 000*g* for 30 min and the supernatant was then directly applied to a Q Sepharose FF column (Amersham Pharmacia Biotech, Sweden) equilibrated with buffer *A* (20 m*M* Tris–HCl pH 8.5 plus 0.03% DDM). The column was subsequently washed with four column volumes of buffer *A* before protein was eluted with a linear gradient of 0–100% buffer *B* (20 m*M* Tris–HCl pH 8.5, 1 *M* NaCl, 0.03% DDM). All fractions absorbing at 416 nm was pooled and then concentrated to minimal volume using an Amicon cell (Millipore, CA, USA) with a 100 kDa cutoff filter.

The concentrated protein was applied to a Highprep Sephacryl S-300 gel-filtration column (Amersham Pharmacia Biotech, Sweden) equilibrated with two column volumes of buffer (0.1 *M* Tris–HCl pH 8.5, 1 *M* NaCl, 0.03% DDM) and thereafter eluted using the same buffer. Fractions absorbing at 416 nm were pooled and concentrated in an Amicon cell using a 100 kDa cutoff filter and then applied to an anion-exchange column (MonoQ 5/5; Amersham Pharmacia Biotech, Sweden) pre-equilibrated with monoQ buffer *A* (20 m*M* Tris–HCl pH 8.5, 1% *n*-octyl-β-D-glucopyranoside (OG; Anatrace, IL, USA). The column was washed with monoQ buffer *A* before protein was eluted with a linear gradient of 0–100% monoQ buffer *B* (20 m*M* Tris–HCl pH 8.5, 1 *M* NaCl, 1% OG). Fractions corresponding to Fdh-N were pooled and concentrated using a Centricon YM-100. The buffer was then exchanged to 20 m*M* Tris–HCl pH 7.5 with 1% OG in a Centricon YM-100. The protein was finally concentrated to 20 mg ml^{-1} and used for crystallization trials.

2.2. Crystallization and X-ray data collection

Crystals of Fdh-N were grown using the hanging-drop vapour-diffusion technique. The protein solution contained 20 m*M* Tris–HCl pH 7.5, 1% OG and 15–20 mg ml^{-1} protein. The reservoir solution [100 m*M* HEPES–NaOH pH 7.5–8.2, 6–12%(*w*/*v*) PEG 1500, 100 m*M* NaCl and 5% EtOH] and protein solution was mixed in a 1:1 ratio and left to equilibrate at 277 K. Crystals appeared after 5 d and reached maximum dimensions of 0.1 × 0.1 × 0.05 mm within two weeks. Prior to data collection, crystals were soaked in cryosolutions with increasing concentrations of PEG 1500 (9–15%) and glycerol (5–20%) and frozen in liquid

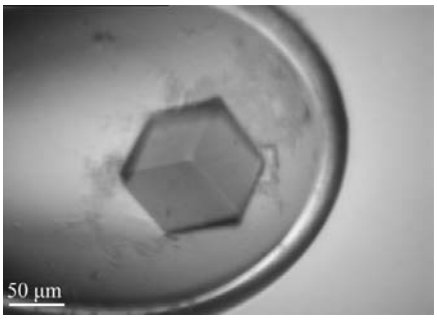

Figure 1
Crystal of formate dehydrogenase N (Fdh-N) from *E. coli*. The crystal has approximate dimensions of 0.1 × 0.1 × 0.05 mm.

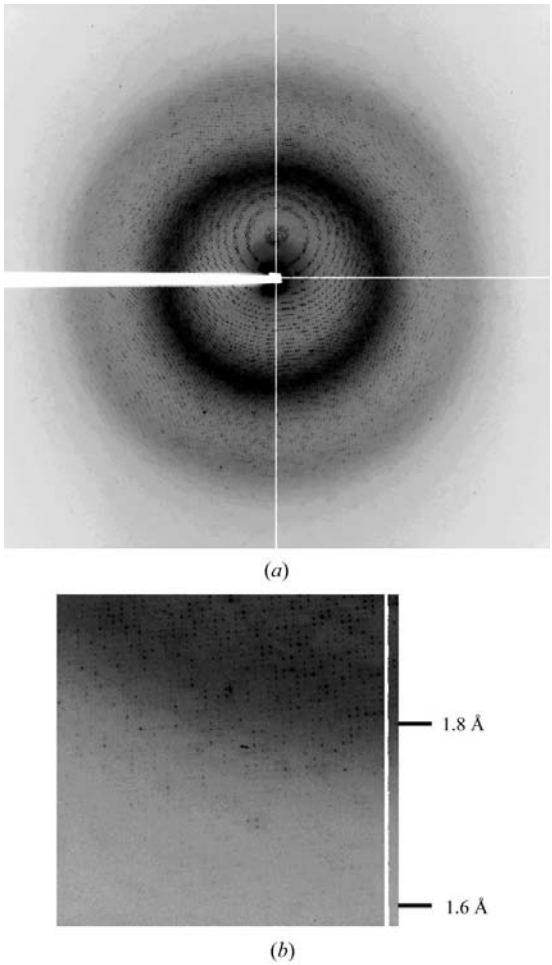

Figure 2
(*a*) X-ray diffraction pattern from the Fdh-N crystal collected at ID14/EH2, ESRF. Beam size, 50 µm; exposure time, 20 s; distance, 140 mm; oscillation angle, 0.3°. An ADSC Quantum 4 CCD detector was used to record the image. (*b*) A magnified view of the lower area of (*a*). Diffraction spots extend to 1.6 Å.

nitrogen. Data collections from frozen crystals were performed at 100 K. Diffraction data to 1.6 Å were collected at beamline ID14/EH3 of the European Synchrotron Radiation Facility (ESRF) using an ADSC Quantum 4 CCD detector. The beam size, oscillation range, exposure time and crystal-to-film distance were 50 × 50 µm, 0.3°, 20 s per frame and 140 mm, respectively. Image data were processed using the program packages *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). All data better than $-3.0\sigma(I)$ were used for scaling.

3. Results and discussion

It proved to be difficult to obtain a pure Fdh-N sample owing to problems in separating it from Nar. However, purity does not seem to be a critical factor for the crystallization of Fdh-N. It was possible to obtain well diffracting crystals even from samples with up to 50% contamination of Nar. We have examined the crystals by SDS–PAGE and confirmed that they contain only the three subunits of Fdh-N. Thus, crystallization is working as a purification step for this enzyme.

A crystallization screen based on previously crystallized membrane proteins (Abramson & Iwata, 1999) was used for initial screening. During further optimization trials, crystals were obtained under different conditions (using DDM instead of OG or using PEG 4000 instead of PEG 1500), although the initial condition yielded the best quality crystals.

The crystals of Fdh-N belonged to the cubic space group $P2_13$, with unit-cell parameters $a = b = c = 203.0$ Å. We have completed one data set from the largest crystal obtained (Fig. 1). The initial images clearly show diffraction to 1.6 Å (Fig. 2), although later images showed some radiation damage. The data were integrated and scaled to 1.6 Å resolution. We have obtained a data set with 909 888 total and 342 711 independent observations in the resolution range 40–1.6 Å, with an overall completeness of 94.4% (93.3% for the last shell) and an $R_{\rm merge}$ of 8%. The overall $I/\sigma(I)$ is 15.0. $R_{\rm merge}$ for the last shell (1.66–1.6 Å) is as high as 72% owing to the radiation damage for the last frames. $R_{\rm merge}$ for the 1.80–1.72 Å shell is 38% and this indicates that the current data set is of reasonable quality up to this resolution range. Assuming one Fdh-N monomer in the asymmetric unit, the calculated $V_{\rm M}$ (Matthews, 1968) of the crystals is 4.2 Å^3 Da^{-1}, corresponding to a solvent content of 66%. The high $V_{\rm M}$ and solvent content are common for membrane-protein complexes owing to the detergent micelle surrounding the proteins (Ostermeier *et al.*, 1995; Iwata *et al.*, 1998).

The crystals obviously have the best quality reported for large membrane-protein complexes to date. The bacterial reaction centre at 2.0 Å (Lancaster *et al.*, 2000) is the highest resolution structure so far reported for a membrane-protein complex. The 1.6 Å resolution obtained for Fdh-N is the second highest for any membrane protein, next only to the 1.55 Å bacteriorhodopsin structure (Luecke *et al.*, 1999). Structure determination by the multiwavelength anomalous dispersion (MAD) technique using the intrinsic Fe atoms (22 Fe atoms per 170 kDa) is in progress. The structure of Fdh-N at this resolution could answer many questions about the molecular mechanisms of this enzyme and the Fdh-N/Nar system. In addition, the high-resolution structure should provide essential structural information about the architecture of membrane-protein complexes, including helix–helix, protein–lipid/detergent and protein–solvent interactions.

This research was supported by the Biotechnology and Biological Sciences Research Council of the UK and the Structural Biology Network of Sweden. We would like to thank Edward Mitchell at ESRF, Grenoble for his technical support in the data collection.

References

Abaibou, H., Pommier, J., Benoit, S., Giordano, G. & Mandrand-Berthelot, M. A. (1995). *J. Bacteriol.* **177**, 7141–7149.
Abramson, J. & Iwata, S. (1999). *Protein Crystallization*, edited by T. M. Bergfors, pp. 199–210. La Jolla, CA, USA: International University Line.
Abramson, J., Larsson, G., Byrne, B., Puustinen, A., Garcia-Horsman, A. & Iwata, S. (2000). *Acta Cryst.* D**56**, 1076–1078.
Berg, B. L., Li, J., Heider, J. & Stewart, V. (1991). *J. Biol. Chem.* **266**, 22380–22385.
Berg, B. L. & Stewart, V. (1990). *Genetics*, **125**, 691–702.
Berks, B. C., Page, M. D., Richardson, D. J., Reilly, A., Cavill, A., Outen, F. & Ferguson, S. J. (1995). *Mol. Microbiol.* **15**, 319–331.
Boyington, J. C., Gladyshev, V. N., Khangulov, S. V., Stadtman, T. C. & Sun, P. D. (1997). *Science*, **275**, 1305–1308.
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., Moura, I. & Romao, M. J. (1999). *Structure Fold. Des.* **7**, 65–79.
Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Iwata, M., Rasmussen, B., Link, T. A., Ramaswamy, S. & Jap, B. (1998). *Science*, **281**, 64–71.
Lancaster, C. R., Bibikova, M. V., Sabatino, P., Oesterhelt, D. & Michel, H. (2000). *J. Biol. Chem.* **275**, 39364–39368.
Lemieux, L. J., Calhoun, M. W., Thomas, J. W., Ingledew, J. & Gennis, R. B. (1992). *J. Biol. Chem.* **267**, 2105–2113.
Luecke, H., Schobert, B., Richter, H. T., Cartailler, J. P. & Lanyi, J. K. (1999). *J. Mol. Biol.* **291**, 899–911.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Ostermeier, C., Iwata, S., Ludwig, B. & Michel, H. (1995). *Nature Struct. Biol.* **2**, 842–846.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Zinoni, F., Birkmann, A., Stadtman, T. C. & Bock, A. (1986). *Proc. Natl Acad. Sci. USA*, **83**, 4650–4654.