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Crystallization and preliminary X-ray analysis of a nitrate reductase from *Desulfovibrio desulfuricans* ATCC 27774

João M. Dias,[a,b] Sergey Bursakov,[c]† Carla Carneiro,[c] José J. G. Moura,[c] Isabel Moura[c] and Maria João Romão[a]*

[a]Departamento de Quimica, CQFB, FCT, Universidade Nova de Lisboa, 2825-114 Caparica, Portugal, [b]ITQB, Apt. 127, 2780 Oeiras, Portugal, and [c]Departamento de Química, CQFB, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2825 Monte de Caparica, Portugal

† On leave of absence from A. N. Bach Institute of Biochemistry RAS, 117071 Moscow, Russia.

Correspondence e-mail: mromao@dq.fct.unl.pt

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Periplasmic nitrate reductase from the sulfate-reducing bacterium *Desulfovibrio desulfuricans* ATCC 27774 contains two molybdopterin guanine dinucleotide cofactors and one [4Fe–4S] cluster as prosthetic groups and catalyzes the conversion of nitrate to nitrite. Crystals of the oxidized form of this enzyme were obtained using PEG as precipitant and belong to space group $P3_121$ or $P3_221$, with unit-cell dimensions $a = b = 106.3$, $c = 135.1$ Å. There is one monomer of 80 kDa in the asymmetric unit, which corresponds to a Matthews ratio of 2.75 $Å^3$ Da^{-1}. Using cryo-cooling procedures and X-rays from a rotating-anode generator, diffraction was observed to beyond 3.0 Å resolution.

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1. Introduction

The ability to reduce nitrate is widely distributed among prokaryotes and eukaryotes. Owing to its physiological importance, the two-electron reduction step of nitrate to nitrite and the corresponding enzymes have been extensively studied. However, the capacity to use nitrate has been discovered only in a few sulfate-reducing bacteria including *Desulfovibrio* (Senez & Pichinoty, 1958; Liu & Peck, 1981; McCready *et al.*, 1983; Seitz & Cypionka, 1986; Keith & Herbert, 1983; Mitchell *et al.*, 1986), *Desulfobulbus* (Widdel & Pfenning, 1982), *Desulfomonas* (Widdel & Pfenning, 1984) and *Desulfobacterium* (Szewzyk & Pfenning, 1987). Nitrate is reduced to ammonia in a respiratory process coupled to electron-transport phosphorylation (Seitz & Cypionka, 1986). The bacterial nitrate reductases are diverse in terms of subunit structure, active-site composition, cellular localization and physiological function. However, all nitrate reductases described so far are molybdenum-containing enzymes with molybdopterin cofactors at their active sites.

The first nitrate reductase purified and characterized from a sulfate-reducing bacterium was the periplasmic enzyme from *D. desulfuricans* ATCC 27774 (Bursakov *et al.*, 1995), induced by the presence of nitrate in the media (Moura *et al.*, 1997). This nitrate reductase (NAP) was shown to be monomeric (molecular mass 80 kDa). This enzyme presents peculiar catalytic properties with respect to ionic strength and cation/anion activity responses (Bursakov *et al.*, 1997). It was shown that monovalent cations (potassium and sodium) stimulate NAP activity and divalent (magnesium and calcium) inhibited it.

The enzyme has an Mo atom coordinated to two molybdopterin guanine dinucleotide (MGD) cofactors, and EPR signals from one [4Fe–4S] centre and from Mo(V) were identified in the reduced state of the enzyme and in the presence of nitrate (Bursakov *et al.*, 1995). Molybdopterin-containing enzymes have been grouped into three large classes according to their different spectroscopic features, amino-acid homology and active-site composition: (i) the xanthine oxidase family, (ii) the DMSO reductase family and (iii) the sulfite oxidase family. NAP is a member of the DMSO reductase family, which also includes dissimilatory nitrate reductases and formate dehydrogenases (Romão *et al.*, 1997). In the absence of any three-dimensional structure of such a nitrate reductase, efforts were made towards the crystallization and X-ray analysis of NAP from *D. desulfuricans* ATCC 27774 to contribute to our knowledge of molybdenum-containing hydroxylases.

2. Material and methods

2.1. Protein purification

The *D. desulfuricans* ATCC 27774 cells were grown as previously described (Bursakov *et al.*, 1995, 1997), harvested in 10 m*M* Tris–HCl (pH 7.6) and passed through a Manton–Gaulin press at 6.205 × 10^6 Pa. The extract was centrifuged at 10000*g* for 1 h and then at 180000*g* for 1 h to eliminate the membrane fraction. The purification procedure was adapted from one published previously (Bursakov *et al.*, 1995) with modifications (Bursakov *et al.*, 1997). The activity was determined at each step of the purification process (Lowe & Evans, 1964; Garrett &

Nason, 1969) and the purity of the final preparation was confirmed by polyacrylamide gel electrophoresis. The active nitrate reductase was distributed in small aliquots of 50 µl with a protein concentration of 10 mg ml^{-1} in 10 m*M* Tris–HCl buffer pH 7.5 and was stored at 203 K, ready for initial crystallization trials.

2.2. Crystallization

Initial crystallization conditions were screened using an in-house modified version of the sparse-matrix method of Jancarik & Kim (1991), in combination with the commercial Hampton Research (California, USA) Crystal Screen and Crystal Screen 2. Crystallization trials were carried out at constant temperature (277 and 294 K), using the hanging-drop and sitting-drop vapour-diffusion methods. The first experiments yielded multiple small crystals when employing 20% PEG 20K as precipitant and 0.1 *M* MES buffer pH 6.5. The crystal quality was first improved by lowering the PEG concentration and changing to PEG 8K. Different buffers and additives did not improve the crystallization conditions and salts induced solubilization of the protein. Seeding techniques were not successful, as microseeding repeatedly produced microcrystals, which also did not improve with macroseeding.

The optimized experimental conditions were reached after increasing the initial concentration of the protein to 40 mg ml^{-1} in 10 m*M* Tris–HCl buffer pH 7.5, using a Centricon 30 concentrator (Amicon, USA). The largest crystals were obtained using 2 µl of this 40 mg ml^{-1} protein solution and 2 µl of a reservoir solution containing 6% PEG 8K with 0.1 *M* MES buffer pH 6.5. At room temperature, the crystals grew overnight to 0.2 × 0.2 × 0.2 mm and within 5 d they reached a maximum size of 0.8 × 0.3 × 0.2 mm (Fig. 1).

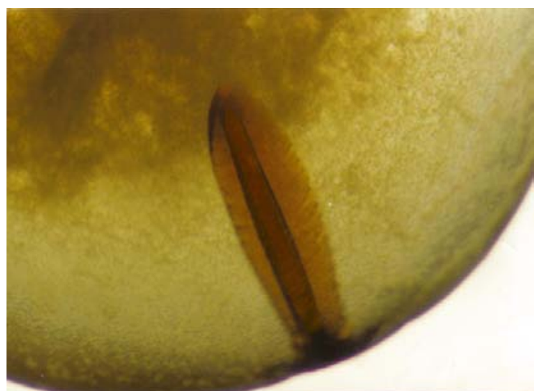

Figure 1
Crystal of periplasmic nitrate reductase (NAP) from *Desulfovibrio desulfuricans* ATCC 27774 grown at 293 K. The crystal is approximately 0.8 × 0.3 × 0.2 mm.

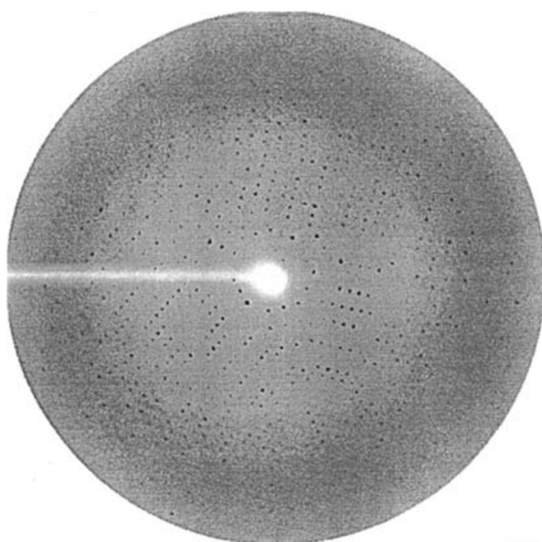

Figure 2
A 1° oscillation image of a native NAP crystal. The image was taken with a MAR Research imaging-plate detector at a crystal-to-detector distance of 160 mm using a copper rotating-anode source. The exposure time was 30 min. The edge of the image corresponds to 3.0 Å, but a complete data set with significant intensities goes only as far as 3.5 Å.

2.3. Data collection and reduction

The NAP crystals are extremely sensitive to handling and to radiation, maintaining their shape but no longer diffract. After a few days in the crystallization drop, the NAP crystals undergo degradation, not apparent externally, but revealed by the total absence of diffraction spots. Only very fresh crystal diffracted. When these crystals were analyzed with X-rays at room temperature, their diffraction power rapidly decayed, and after one single exposure of 30 min the crystals did not diffract at all. When diffraction experiments were attempted at 277 K employing a stream of cooled air, the lifetime of the crystals was extended, but only to about 3 h, and radiation damage was still quite severe. Cryoprotection of the NAP crystals was crucial for complete data collection and effectively eliminated radiation damage to the crystals. A number of different cryoprotectant solutions at different concentrations were tried including glycerol and PEG 400. Satisfactory freezing of the NAP crystals was achieved with a cryoprotectant solution containing 25% glycerol, 8% PEG 8K with 0.1 *M* MES buffer pH 6.5. The most efficient protocol was to first transfer the crystal directly from its original mother liquor into this cryoprotectant solution for about 5 min. The protected crystal was then rapidly mounted in a cryoloop followed by flash freezing in a stream of cooled nitrogen gas maintained at 100 K throughout data collection.

X-ray diffraction data were collected on an X-ray imaging-plate system (MAR Research, Hamburg, Germany) using graphite-monochromated Cu $K\alpha$ radiation from a Rigaku rotating-anode generator operated at 5.4 kW. One typical rotation photograph taken from one NAP crystal is shown in Fig. 2. The intensity data were processed with the program packages *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1993).

3. Conclusions

Even after optimization of the cryo-conditions for data collection, we observed that diffraction quality was still very much crystal dependent and many crystals had to be tried. One particularly well diffracting crystal diffracted to beyond 2.7 Å but, owing to technical problems, it was unfortunately not possible to collect a complete data set.

Statistics for this crystal showed an overall completeness of 69.4% between 45.0 and 2.7 Å with an overall $R_{\rm merge}$ of 7.9% and an $R_{\rm merge}$ of 44.1% for the last shell between 2.80 and 2.70 Å. Using a new crystal, a complete data set could be collected with significant intensities up to 3.5 Å resolution, with an $R_{\rm merge}$ for replicate measurements of 20.3% (45.6% in the last shell, 3.62–3.50 Å) and an overall completeness of 98.0% (98.6% in the last shell). Between 3.5 and 3.0 Å the completeness was 98.6%, but the $R_{\rm merge}$ was 62.9%, and this shell was discarded in the final processing.

A summary of the data-collection and final processing statistics is shown in Table 1. The nitrate reductase crystals belong to space group $P3_121$ or to its enantiomorph $P3_221$, with unit-cell dimensions $a = b = 106.3$, $c = 135.1$ Å.

This cell size and symmetry correspond to a Matthews ratio V_M of 2.75 Å^3 Da^{-1}, assuming one monomer of 80 kDa of NAP

Table 1
Data-collection and processing statistics.

Space group	$P3_121$ or $P3_221$	
Unit-cell parameters (Å)	$a = b = 106.3$, $c = 135.1$	
Resolution range (Å)	35.00–3.50	
Number of measured reflections	122438	
Number of unique reflections	11649	
Mosaicity	0.52	
Resolution shell (Å)	35.00–3.50	3.62–3.50
$I/\sigma(I)$	4.2	3.3
R_{merge}† (%)	20.3	45.6
Completeness (%)	98.0	98.6

† $R_{merge}(I) = \sum(|I(k) - \langle I \rangle|)/\sum I(k)$, where $I(k)$ and $\langle I \rangle$ represent diffraction intensity values of individual measurements and the corresponding mean values, respectively. The summation is over all measurements.

per asymmetric unit. This is within the average V_M values found for protein crystals (Matthews, 1968) and indicates a solvent content of approximately 55%.

The major problem of crystal degradation could be overcome only by using cryocrystallography techniques which, however, have not been successful for finding heavy-atom derivatives. Crystals have been soaked in different heavy-atom solutions, but deterioration of the derivatized crystals was significant and no successful results were obtained. The difficulties in obtaining good derivatives and the severe limitations in the availability of well ordered crystals led us to try the multiple anomalous dispersion (MAD) method exploiting the iron absorption edge arising from the presence of the iron–sulfur cluster [4Fe–4S]. The four Fe atoms contribute weak anomalous and dispersive effects of about 5 e^- each (Hendrickson *et al.*, 1988) per molecular weight of 80 kDa, but owing to the presence of the four Fe atoms in a close site with a defined geometry, their positions will be restricted which will simplify the phasing problem. MAD data collection from one single crystal was a successful experiment and analysis of the data is under way.

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