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Crystallization and preliminary diffraction studies of pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2

Peter C. E. Moody,[a]* Nita Shikotra,[a] Christopher E. French,[b] Neil C. Bruce[b] and Nigel S. Scrutton[a] at [a]*Department of Biochemistry, University of Leicester, Adrian Building, University Road, Leicester LE1 7RH, England, and* [b]*Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, England. E-mail: pcem1@le.ac.uk*

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Abstract

Pentaerythritol tetranitrate (PETN) reductase of *Enterobacter cloacae* PB2, a flavoprotein involved in the biodegradation of the explosive PETN, ethylene glycol dinitrate (EGDN) and glycerol trinitrate (GTN), was purified from an overexpressing strain of *E. coli* and crystallized at 293 K using the sitting-drop vapour-diffusion method. Diffraction data can be seen at 1.8 Å. The primitive orthorhombic cell has a monomer in the asymmetric unit. Preliminary molecular-replacement calculations have been performed using a search model based on Old Yellow enzyme.

1. Introduction

Pentaerythritol tetranitrate [2,2-bis[(nitroxy)methyl]-1,3-propanediol dinitrate (ester)] is a nitrate ester produced by nitration of pentaerythritol and is widely used both as an explosive and as a vasodilator. A strain of *Enterobacter cloacae* was previously isolated from explosive contaminated land on the basis of its ability to use nitrate esters such as pentaerythritol tetranitrate (PETN), ethylene glycol dinitrate (EGDN) and glycerol trinitrate (GTN) as nitrogen sources (Binks *et al.*, 1996). *E. cloacae* strain PB2 possesses a soluble PETN reductase capable of reductively liberating nitrite from nitrate esters with oxidation of NADPH (Fig. 1). The enzyme is a flavoprotein containing 1 mol equivalent of flavin mononucleotide (FMN) per subunit (French *et al.*, 1996). Nitrate esters, although produced in large quantities for use as explosives and vasodilators (Rosenblatt *et al.*, 1991), are rare in nature (Hall *et al.*, 1992; Roberts, 1990) and multiply substituted nitrate esters are not known to occur naturally. For this reason and because of the potential applications of enzymes that act on multiply substituted nitrate esters for bioremediation purposes, we have embarked on a structural study of PETN reductase from *E. cloacae* strain PB2.

The gene sequence of PETN reductase reveals that the enzyme is closely related to the Old Yellow enzyme family of flavoproteins, which includes three isoforms of Old Yellow enzyme (Stott *et al.*, 1993), bacterial morphinone reductase (French & Bruce, 1995), various NADH oxidases and enzymes involved in the transformation of bile acids (Liu & Scopes, 1993; Franklund *et al.*, 1993; Mallonee *et al.*, 1990), bacterial tri- and dimethylamine dehydrogenases (Boyd *et al.*, 1992; Yang *et al.*, 1995) and several less well characterized flavoproteins (see Scrutton, 1994; French & Bruce, 1995 and references therein). The sequence identity of PETN reductase with that of Old Yellow enzyme is approximately 39%. Crystal structures for Old Yellow enzyme (Fox & Karplus, 1994) and bacterial trimethylamine dehydrogenase (Lim *et al.*, 1986) exist, and a preliminary diffraction study for morphinone reductase has been reported (Moody *et al.*, 1997). The ability of this family of enzymes to accept a variety of widely differing substrate molecules and to display variable chemistries makes a struc-

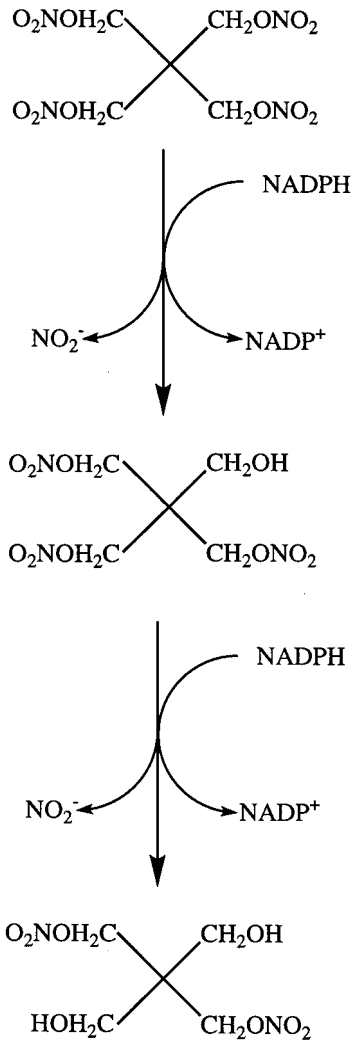

Fig. 1. The reactions catalyzed by pentaerythritol tetranitrate reductase. Pentaerythritol tetranitrate is reduced to form pentaerythritol trinitrate which is, in turn, reduced to pentaerythritol dinitrate. Each reduction is achieved with the oxidation of NADPH and the liberation of nitrate. The elimination of two nitrate groups occurs rapidly to form pentaerythritol dinitrate but no further elimination is seen over a period of 2 h.

tural study of its individual members an attractive proposition. PETN reductase itself is able to act on three nitrate esters PETN, EGDN and GTN and, like morphinone reductase and Old Yellow enzyme, is inhibited by steroids (French *et al.*, 1996).

2. Materials and methods

PETN reductase was purified from a recombinant strain of *Escherichia coli* by affinity chromatography as described previously (French *et al.*, 1996). The phosphate buffer used for storage of the enzyme was exchanged for filtered deionized water and the protein concentrated to *ca* 10 mg ml^{-1} using a centricon-30. Screening of crystallization conditions was using the sparse-matrix factorial search method of Jancarick & Kim (1991) as made available commercially in kits (Hampton Research). Sitting drops made of 2 µl protein solution and 2 µl of precipitant solution placed in Crystal Clear strips (Douglas Instruments Ltd) and allowed to equilibrate with 100 µl of the precipitant solution gave well formed crystals from 0.2 *M* sodium acetate, 0.1 *M* sodium cacodylate, 30% PEG 8000, pH 6.5. Crystals grew to 0.2 mm in cross section in 5 d. Smaller crystals were also seen in drops equilibrated with 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5, and 30% PEG 4000, and very small crystals seen growing from 0.2 *M* zinc acetate, 0.1 *M* sodium cacodylate pH 6.5 and 18% PEG 8000.

3. Results and conclusions

Data were collected at room temperature from a crystal mounted directly from the screening plate into a thin-walled glass capillary. Diffraction data were collected using a Rigaku R-axis IV image-plate system mounted on a Rigaku RU200HB rotating-anode generator with a copper anode, a nominal focus of 0.3 × 3.0 mm and powered at 50 kV, 100 mA. MSC/Yale mirrors and nickel foil were used to focus and select Cu $K\alpha$ radiation. Initial exposures showed good diffraction and 92° of data were collected and measured to 1.9 Å using 20 min exposures of 1° oscillation and a crystal-to-detector distance of 150 mm. The first image (shown in Fig. 2) was subjected to the auto-indexing routines in *DENZO* (Otwinowski, 1993) and the *REFIX* (Kabsch, 1993) autoindexing routines in *MOSFLM* (V5.41, A. G. W. Leslie, LMB, Cambridge, UK). Both gave solutions consistent with a primitive orthorhombic cell that refines to a = 58.2 b = 70.9 c = 89.3 Å. Measurement and reduction [using *MOSFLM* and the *CCP*4 (V3.2) suite of programs (Collaborative Computational Project, Number 4, 1994)] of the 92° of data collected allowed examination of the distribution of intensities along the principal axes. For these axis the average $I/\sigma(I)$ is 12.0 for the 31 expected reflections in $P2_12_12_1$ (h00, 0k0, 00l where h, k, or $l = 2n$). The 31 expected absences (h00, 0k0, 00l where h, k, or $l \neq 2n$) had an $I/\sigma(I)$ of 1.7, this value is inflated by an apparently strong reflection measured with indices 0,0,9 because of Cu $K\beta$ contamination from the very strong 0,0,10 reflection. The $R_{\rm merge}$ for 97 281 observations of 28 888 reflections to 1.9 Å was 7.4% with an average $I/\sigma(I)$ of 6.8 and 97.1% completeness (the data is greater than 99% complete to 2.45 Å). In the outer resolution shell (2.12 to 1.9 Å), 54.9% of reflections have $I/\sigma(I)$ greater than 3 and the data is 91.8% complete. A monomer in the asymmetric unit would correspond to a V_m of 2.3 and a solvent content of approximately 38% (Matthews, 1968).

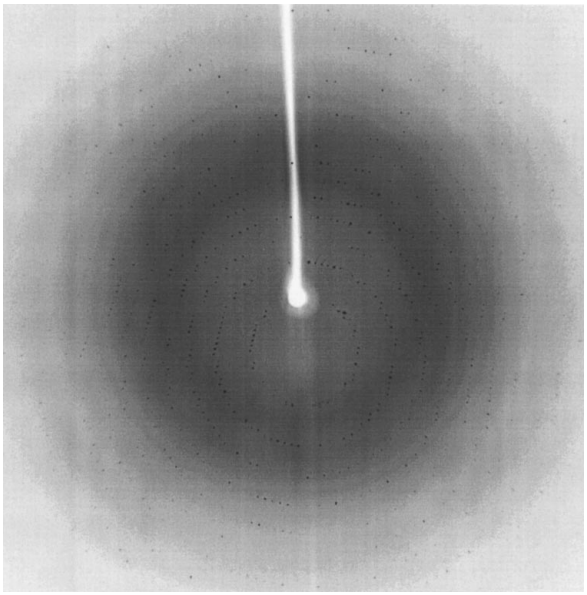

Fig. 2. 20 min exposure with 1° oscillation from a crystal of PETN reductase, the crystal-to-detector distance is 150 mm and the plate is 300 mm wide. The edge of the detector is at 2.0 Å.

Preliminary molecular-replacement calculations were carried out using Old Yellow enzyme (pdb code 1OYA) (Fox & Karplus, 1994) as a search model in the *CCP*4 (Collaborative Computational Project, Number 4, 1994) implementation of *AMoRe* (Navaza, 1994). Old Yellow enzyme shares approximately 39% sequence identity with that of PETN reductase. The rotation function (using data in the range 20.0–4.0 Å) gave a clear peak at 7.73 times r.m.s. deviation from the mean. The next highest peaks all had values of 3.9 times the r.m.s. or less. The translation function was tried for the top eight solutions (using data in the range 8.0–3.5 Å) and the best solution gave a correlation coefficient of 27.1 (three times the height of the next peak). This improved to 39.6 with rigid-body refinement (R = 48.4%). Further search models were derived from the Old Yellow enzyme (1OYA) coordinates and the sequence alignment of French *et al.* (1996). One of these was made by deleting all insertions in the 1OYA sequence and truncating all non-identical side chains to alanine or glycine (consensus model), this comprises approximately 75% of the non-H atoms in PETN reductase. Another model was made by deleting the same insertions and truncating all side chains to alanine or glycine (poly-Ala model), this is approximately 63% of the non-H atoms. Molecular-replacement calculations with all models used data in the same ranges and provided the same solution. In the case of the consensus model, the solution to the rotation function was the highest peak with a correlation coefficient of 14.7 (7.25 times the r.m.s. of the map) and the next peak was at 4.4 times the r.m.s. The translation function for this solution gave a coefficient of 28.0 and this refined to 51.8 (R = 46.9%). The correct solution for rotation function with the poly-Ala model was still the highest peak at 12.0 (5.5 times r.m.s.), but the next highest was very close at 5 times r.m.s. However, applying the translation function to this gave a coefficient of 20.0 (2.7 times the best solution for the second highest solution to the rotation function) and this refined to 53.8 (R = 48.4%). If this observation can be applied to other examples, then for more difficult molecular-replacement

problems, it may be worth using as full a model as possible for the rotation searches, and using a truncated model for the translation searches.

Examination of the packing of these solutions in the unit cell shows no significant parts of the molecule form unfavorable contacts.

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