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*Acta Cryst.* (1997). D53, 619–621

# Crystallization and preliminary diffraction studies of morphinone reductase, a flavoprotein involved in the degradation of morphine alkaloids

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(*Received 3 December 1996; accepted 13 March 1997*)

**Abstract**

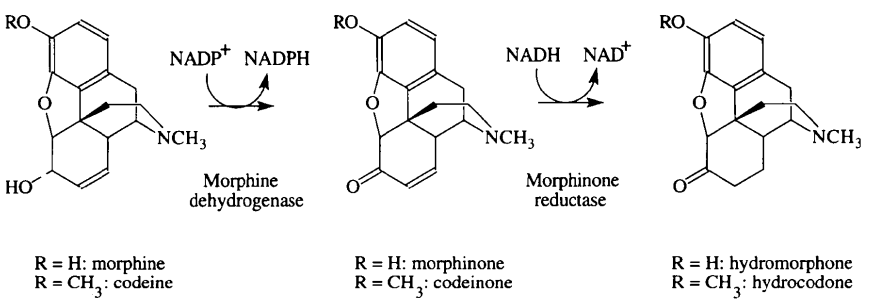
Morphinone reductase from *Pseudomonas putida* M10, a flavoprotein involved in the degradation of morphine alkaloids, was purified from an overexpressing strain of *Escherichia coli* and crystallized using the hanging-drop vapour-diffusion method. Diffraction data were collected to 2.5 Å. The *I*-centred orthorhombic cell has a monomer in the asymmetric unit. Preliminary molecular replacement calculations have been performed using Old Yellow Enzyme as the search model.

## 1. Introduction

A search for enzymes able to degrade morphine has resulted in the identification of two enzymes, morphine dehydrogenase and morphinone reductase from *Pseudomonas putida* strain M10 (Bruce, French, Hailes, Long & Rathbone, 1990). These enzymes catalyse the first steps in the degradation of morphine and codeine. Morphine dehydrogenase catalyses the oxidation of morphine to morphinone, and morphinone reductase reduces morphinone to hydromorphone (Fig. 1). Both enzymes are potentially useful for the production of semisynthetic opiate drugs (Bruce *et al.*, 1995). The synthesis of hydromorphone by existing methods is problematic because of the difficulty associated with the specific oxidation of the C6 hydroxy group. Given the widespread use of hydromorphone as a powerful analgesic, the use of specific biocatalysts to produce hydromorphone is an attractive alternative to the traditional methods of synthesis and is worthy of investigation (Long, Hailes, Kirby & Bruce, 1995; Bruce *et al.*, 1995).

Morphinone reductase is readily purified from cell extracts of *P. putida* M10 (French & Bruce, 1994), and the enzyme was shown to be a homodimer of subunit mass 41.1 kDa. Each subunit contains one molecule of flavin mononucleotide (FMN) bound non-covalently to the enzyme. The enzyme is specific for morphinone and the related compounds codeinone, neopinone and 2-cyclohexen-1-one, but displays no activity towards morphine, codeine or isocodeine. The reaction catalysed is essentially irreversible. As part of a program of work aimed at the synthesis of hydromorphone and hydrocodone using recombinant strains of bacteria (French *et al.*, 1995), the genes encoding morphine dehydrogenase and morphinone reductase have been cloned and expressed (Willey, Caswell, Lowe & Bruce, 1993; French & Bruce, 1995). Sequence analysis of the morphinone reductase gene (*mor*B) has revealed that the enzyme is related to the flavoprotein $\beta/\alpha$ barrel oxidoreductase family (Scrutton, 1994; French & Bruce, 1995) and in particular the enzyme is closely related to the isoforms of Old Yellow Enzyme from *Saccharomyces* spp. (Stott, Saito, Thiele & Massey, 1993). Members of this family of enzymes display various chemistries. For example, following reduction of the protein-bound FMN, the more complex members catalyse long-range electron transfer to other redox centres held in the protein scaffold. Bacterial trimethylamine dehydrogenase (Boyd, Mathews, Packman & Scrutton, 1992) serves as the structural parent for these more complex proteins (Lim *et al.*, 1986) and the crystallographic structure of the enzyme reveals that domains fused to the N-terminal FMN-barrel domain contain additional redox centres that participate in long-range electron transfer. Other complex members *e.g.* the NADH oxidase of *Thermoanaerobium brockii*, the bile-acid induced proteins H (an NADH oxidase; Franklund, Baron & Hylemon, 1993) and C (believed to be involved in the 7-dehydroxylation of steroids; Mallonee, White & Hylemon, 1990) of *Eubacterium* sp. VPI 12708 and dimethylamine dehydrogenase of *Hyphomicrobium X* (Yang, Packman & Scrutton, 1995; Raine *et al.*, 1995) may be similarly constructed.

Bacterial morphinone reductase is more closely related in sequence to the simpler members of the class I flavin oxidoreductase family, and this sub-category comprises ten proteins. Curiously, the other members of this sub-group are eukaryotic proteins and include the Old Yellow Enzymes of *Saccharomyces* spp. (Stott *et al.*, 1993), the oestrogen-binding protein of *Candida albicans* (Madani, Malloy, Rodriguez-Pombo, Krishnan & Feldman, 1994) and several expressed sequence tags from the plants *Arabidopsis thaliana*, *Brassica*




R = H: morphine
R = $CH_3$: codeine

R = H: morphinone
R = $CH_3$: codeinone

R = H: hydromorphone
R = $CH_3$: hydrocodone

Fig. 1. The reactions catalysed by morphine dehydrogenase and morphinone reductase. Morphine dehydrogenase catalyses the oxidation of the C6 hydroxy group of morphine to form morphinone and morphinone reductase reduces the 7,8-olefinic bond of morphinone to yield hydromorphone.

*campestris* and *Oryza sativa* (see French & Bruce, 1995, and references therein). The crystal structure of Old Yellow Enzyme shows that each subunit of the homodimer forms an eightfold $\beta/\alpha$-barrel similar to the N-terminal domain of trimethylamine dehydrogenase (Fox & Karplus, 1994) and the high level of sequence identity (approximately 39%) between morphinone reductase and Old Yellow Enzyme makes it certain that their overall structures will be similar. In this regard, it is relevant that Old Yellow Enzyme, oestrogen-binding protein and morphinone reductase all have affinity for steroids and each can use 2-cyclohexen-1-one as a substrate. In the case of Old Yellow Enzyme and oestrogen-binding protein, steroids are probably the physiological substrates, whereas for morphinone reductase progesterone and cortisone are known to act as competitive inhibitors (French & Bruce, 1994). The recognition of steroid and alkaloid substrates within this family of enzymes is therefore intriguing, and with a view to understanding in more detail the structures of the enzyme–substrate and enzyme–inhibitor complexes we have begun a crystallographic study of morphinone reductase.

## 2. Methods and results

Morphinone reductase was purified from a recombinant strain of *Escherichia coli* by affinity chromatography as described previously (French & Bruce, 1994, 1995). The phosphate buffer used for storage of the enzyme was exchanged for 10 m*M* Tris–HCl, 0.1 m*M* EDTA pH 8.0 (TE buffer), 5 m*M* dithiothreitol (DTT) and the protein concentrated to *ca* 10 mg ml$^{-1}$ using a centricon-30. Initial screening of crystallization conditions was used the sparse-matrix factorial search method of Jancarik & Kim (1991) as made available commercially in kit form (Hampton Research). 2 + 2 µl sitting drops in CrystalClear Strips (Douglas Instruments Ltd) only gave small crystals from 0.2 *M* magnesium acetate, 0.1 *M* sodium cacodyate, 20% PEG 8000 pH 6.5 that could not be improved upon by refining the conditions or by using hanging drops in Linbro plates. However, if the protein was concentrated into filtered deionized water rather than TE buffer, the same screen conditions showed that crystals could be grown in 2 d from 28% PEG 400, 0.1 *M* HEPES pH 7.5, 0.2 *M* $CaCl_2$ and 30% PEG 400, 0.1 *M* HEPES pH 7.5, 0.2 *M* $MgCl_2$. Refinement of these conditions showed that X-ray quality crystals could be grown reproducibly from hanging drops of 4 µl 4–6 mg ml$^{-1}$ protein with an equal volume of 30–35% PEG 400, 0.1 *M* HEPES, pH 7.0–7.5, 0.1 *M* $MgCl_2$ suspended over 1 ml of the latter mixture from glass cover slips in 24-well (Linbro) plates. Crystals grew to 0.4 mm in cross section in 2 d.

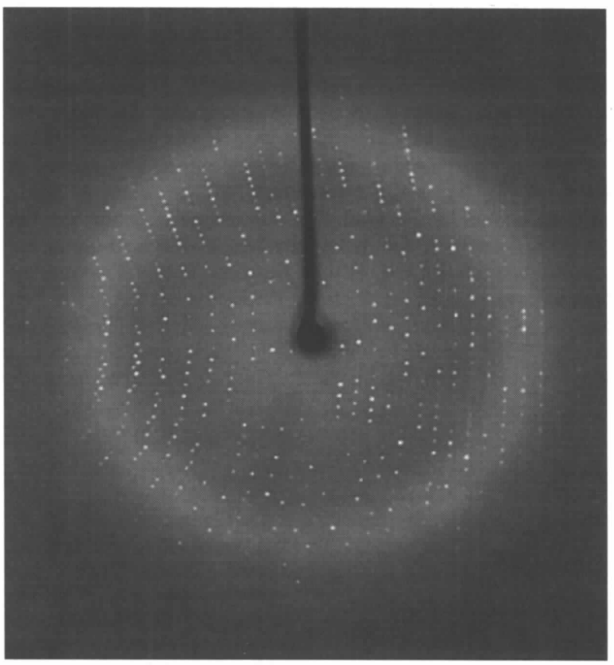


Fig. 2. 10 min exposure with 1° oscillation from a crystal of mophinone reductase, the crystal-to-detector distance is 130 mm and the plate is 200 mm wide. The edge of the detector is at 2.45 Å.

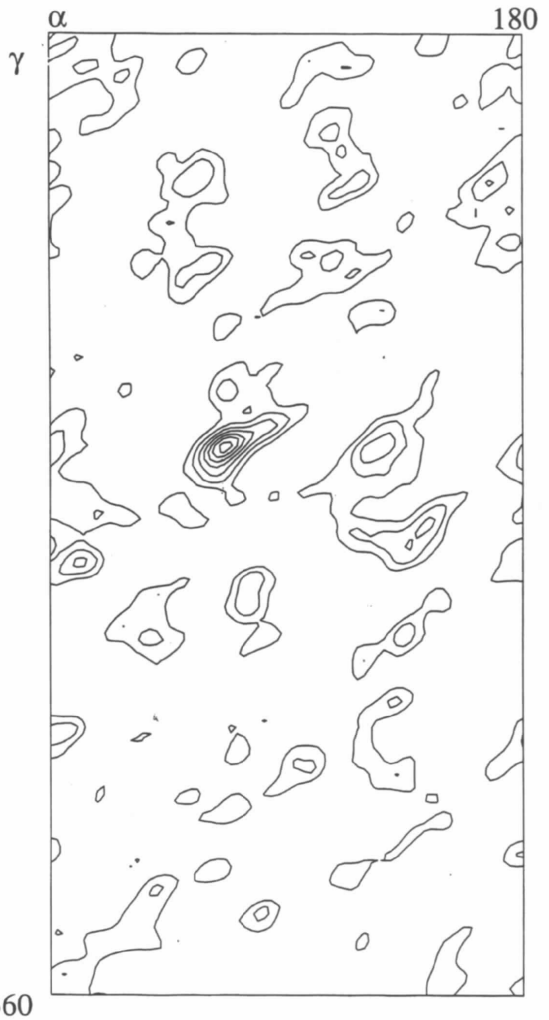



Fig. 3. A section ($\beta = 65°$) of the map of the cross-rotation function calculated within *AMoRe* (Navaza, 1994) using 1OYA as the search model. The peak at Eulerian angles of $\alpha = 65.3$ $\beta = 64.75$ $\gamma = 152.9°$ corresponding to the maximun correlation can be seen. The contours shown are multiples of the r.m.s. of the map.

Data were collected from crystals mounted in a rayon loop and flash-cooled to 100 K in a stream of boiled-off liquid nitrogen. Diffraction data were collected using a R-AXIS IIc image-plate system (Rigaku/Molecular Structure Corporation) mounted on a Rigaku RU-200HB 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 Ni foil were used to focus and select Cu $K\alpha$ (1.5418 Å) radiation. Initial exposures showed good diffraction, and 180° of data were collected to 2.5 Å using 10 min exposures of 1° oscillation and a crystal-to-detector distance of 130 mm (Fig. 2). The first image was subjected to the auto-indexing routines in *DENZO* (Otwinowski, 1993) from which the best refined solution was an *I*-centred orthorhombic cell. The 180° of data allowed the cell to be refined to $a = 49.6$, $b = 121.7$, $c = 178.2$ Å and the distribution of intensities to be examined, confirming the space group to be either $I222$ (23) or $I2_12_12_1$ (24). The systematic absences on the principal axes do not allow these two space groups to be distinguished. A monomer in the asymmetric unit would correspond to a solvent content of about 56% (Matthews, 1968). The data are 99% complete in the range 40–2.5 Å with an $R_{\rm merge}(I)$ of 0.092 for 134 821 observations of 18 992 reflections. The outer shell (2.59–2.5 Å) has 50% of observations with $I/\sigma(I) \geq 5$.

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). The rotation function gave two equivalent solutions with correlation coefficients of 14.8 (Fig. 3), the next highest peaks had values of 9.5. The translation function was tried for the top 20 solutions in both space groups $I222$ and $I2_12_12_1$; the results are clearly better for $I2_12_12_1$. The best solution gave a correlation coefficient of 29.6, which improved to 40.2 with rigid-body refinement. There are, however, some unfavourable contacts resulting from the application of this solution, but these are confined to two small peripheral regions.

This work was funded by the Royal Society (NSS) and the Leicester crystallography facilities are supported by the BBSRC.

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