SHORT COMMUNICATIONS

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Crystallization of *Escherichia coli* enoyl reductase and its complex with diazaborine

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Abstract

Recent work has shown that the NADH-dependent enoyl acyl carrier protein reductase from *Escherichia coli* is the target for diazaborine, an antibacterial agent. This enzyme has been crystallized by the hanging-drop method of vapour diffusion complexed with NAD^+ and in the presence and absence of a thieno diazaborine. The crystals grown in the absence of diazaborine (form *A*) are in the space group $P2_1$ with unit-cell dimensions $a = 74.0$, $b = 81.2$, $c = 79.0$ Å and $\beta = 92.9°$, and with a tetramer in the asymmetric unit, whilst those grown in the presence of diazaborine (form *B*) are in the space group $P6_122$ (or $P6_522$) with unit-cell dimensions $a = b = 80.9$ and $c = 328.3$ Å, and with a dimer in the asymmetric unit. The structure determination of this enzyme in the presence of diazaborine will provide information on the nature of the drug binding site and contribute to a programme of rational drug design.

1. Introduction

With the increasing emergence of drug-resistant strains of bacteria, recent attention has focused on the identification of new targets for drug development and the use of structural data in strategies for the rational design of new pharmaceuticals (von Itzstein *et al.*, 1993). Lipids are synthesized by the multi-functional enzyme complex fatty acid synthase (FAS) by the extension of the acetyl group attached to an acyl carrier protein (ACP), by two carbon units derived from malonyl-CoA in a stepwise reaction (Wakil, 1989). There are two distinct classes of FAS: type I found in eukaryotes and yeast, where all the catalytic domains are found on one or two polypeptides, and type II found in plants and most prokaryotes, where the enzymes that catalyse the individual steps are found on separate polypeptides (McCarthy & Hardie, 1984). The differences between the molecular structures of the two types of FAS makes this biosynthetic pathway an attractive target for the design of new drugs which might inhibit bacterial fatty acid synthesis, but not affect the mammalian enzyme.

Enoyl reductase (ENR) is a homo-tetramer of subunit molecular weight of approximately 28 kDa, which catalyses the final reaction of FAS, the reduction of a double bond in an enoyl-ACP. Previous work has led to the identification of this enzyme as the target for a number of therapeutic agents against *E. coli* (Bergler *et al.*, 1994) and *Mycobacterium tuberculosis* (Banerjee *et al.*, 1994). The antibacterial agent diazaborine, a heterocyclic boron-containing compound, is thought to kill *E. coli* by inhibiting ENR and preventing lipopolysaccharide synthesis (Hogenauer & Woisetschlager, 1981). Biochemical studies have shown that NAD^+ is required for diazaborine binding and this has led to the suggestion that the drug either binds to ENR in association with NAD^+ or that NAD^+ converts the drug to an active form (Kater, Koningstein, Nijkamp & Stuitje, 1994).

Unlike *E. coli* ENR, which is directly inhibited by diazaborine, the inhibition of the mycobacterial enzyme by isoniazid is due to a hitherto uncharacterized metabolite of this compound and thus, as yet, details of the enzyme–inhibitor complex are not available. The emergence of drug-resistant strains of *M. tuberculosis* associated with ENR mutations has led to problems in therapy with isoniazid, the drug used in the front-line treatment of this disease. The crystal structure of ENR from *M. tuberculosis* has indicated that the mutation S94A in the enzyme from the isoniazid-resistant strain generates drug resistance through the disruption of a hydrogen-bonding network that stabilizes NADH binding (Dessen, Quemard, Blanchard, Jacobs & Sacchettini, 1995). Interestingly, a Gly93 to Ser93 mutation in *E. coli* ENR, which is thought to be located in the same region of the structure as Ser94 in the mycobacterial enzyme, leads to diazaborine resistance (Turnowsky, Fuchs, Jeschek & Hogenauer, 1989). This suggests that both compounds bind to a similar region of their respective targets and that there may be a similarity in the mechanisms of drug resistance, therefore.

In previous work we have determined the structure of ENR from *Brassica napus* at 1.9 Å (Rafferty *et al.*, 1995). This enzyme is not inhibited by diazaborine compounds, despite a 33% sequence identity between the bacterial and plant enzymes. Therefore, we have initiated a structural study of ENR from *E. coli* in order to compare the structure with that of the *B. napus* enzyme and to examine the molecular details of drug binding with the aim of initiating a programme of rational drug design.

2. Materials and methods

Enoyl reductase was purified from an overexpressing *E. coli* strain BL21 (DE3) harbouring the pENVM5 plasmid, which encodes the gene for *E. coli* ENR (Bergler *et al.*, 1994). Cells were pelleted, washed and finally resuspended in 20 m*M* $NaPO_4$ buffer, pH 6.1, 1 m*M* DTT, 1 m*M* benzamidine–HCl and frozen in liquid nitrogen as droplets. For the purification, cells from a 500 ml culture were defrosted and broken using a French Press

at 20 000 p.s.i. Debris was removed by centrifugation at 18 000 rev min^{-1}, in a JA-20 rotor (J-21 centrifuge, Beckman) for 10 min and the pH adjusted by the addition of 0.2 *M* Tris–HCl to pH 8.5. The crude extract was then loaded onto a column containing 20 ml of DEAE-Sepharose Fast Flow (Pharmacia) and enoyl reductase was eluted from the column using a 400 ml gradient over the concentration range 0.1–0.25 *M* NaCl in buffer containing 50 m*M* Tris–HCl, pH 8.5, 2 m*M* EDTA and 2 m*M* 2-mercaptoethanol. The enzyme was found in fractions with a NaCl concentration of ~ 0.15 *M*. The final preparation was 95% pure as estimated by SDS PAGE.

For crystallization the protein was concentrated to ~ 10 mg ml^{-1} in 10 m*M* $NaPO_4$ buffer, pH 7.0, containing 1 m*M* DTT and NAD^+ added to a concentration of 10 m*M*. In addition, trials were also conducted with the inclusion of 5 m*M* 1,2-dihydro-1-hydroxy-2-(prop-1-ylsulfonyl)(5-methylthieno)-[3,2-*e*][1,2,3]diazaborine to the above mixture. Crystallization trials were conducted using the hanging-drop method of vapour diffusion with LINBRO crystallization plates and 10 µl samples of protein mixed with an equal volume of a range of precipitants. Crystals of form *A* (no diazaborine) were obtained after 1 week with 12% PEG 400, 100 m*M* acetate (pH 5.0) as the precipitant, and had the morphology of rhomboids with maximum dimensions 1.2 × 0.3 × 0.3 mm. Crystals of form *B* (plus diazaborine) were obtained after 2 weeks with 15% PEG 400, 100 m*M* acetate, pH 5.0, as the precipitant and had the morphology of hexagonal bipyramids with maximum dimensions 0.6 × 0.3 × 0.3 mm.

3. Results and discussion

A preliminary data set was collected on the form *A* crystal on a twin San Diego multiwire systems (SDMS) area detector (Hamlin, 1985; Xuong, Nielsen, Hamlin & Anderson, 1985) with a Rigaku AFC-6 goniostat system mounted on a Rigaku RU200 rotating Cu anode X-ray generator, to a resolution of 2.5 Å. A total of 114 615 measurements were made of 27 520 independent reflections and the data were merged to an *R* factor of 7.1% with 78% completeness of the data from 20 to 2.5 Å resolution. Analysis of the data using the autoindexing algorithm supplied with the SDMS detector system (Howard, Nielson & Xuong, 1985) showed that the crystals either belong to the space group $P2$ or $P2_1$ with unit-cell dimensions $a = 74.0$, $b = 81.2$, $c = 79.0$ Å and $\beta = 92.9$ and a cell volume of 4.74×10^5 Å^3. Given the subunit molecular weight of 28 000 Da, the V_M value for a tetramer in the asymmetric unit is 2.11 Å^3 Da^{-1} and corresponds to a solvent content of 41.7%, which is at the lower end of the range of values observed for crystals of globular proteins (Matthews, 1977). Use of the program *HKLVIEW* (Collaborative Computational Project, Number 4, 1994) to examine the diffraction pattern in the *hk*0 zone showed that reflections with *k* odd appear to be systematically absent. A self-rotation function was calculated on data from 8 to 6 Å resolution with a 20 Å radius of integration using the program *POLARRFN* (W. Kabsch, unpublished work; Collaborative Computational Project, Number 4, 1994) and gave two peaks corresponding to the non-crystallographic axes. These lie at $\omega = 107$, $\varphi = 0$, $\kappa = 180$ and $\omega = 163$, $\varphi = 180$, $\kappa = 180$, and form an orthogonal set with the crystallographic twofold axis. Furthermore, the calculation of a self-Patterson using the native data from 10 to 6 Å resolution indicated a strong peak equivalent to 18% of the origin at position $\frac{1}{2},\frac{1}{2},\frac{1}{2}$. Given that the systematic absences along the *k* axis may arise from pseudo-symmetry, three possible packing arrangements of the molecule can be envisaged. These are as follows.

(*a*) The crystals belong to space group $P2$ with a molecular twofold axis approximately parallel to, but not coincident with, the crystallographic axis, with a tetramer in the asymmetric unit.

(*b*) The crystals belong to space group $P2$ with a molecular twofold axis coincident with the crystallographic axis, with two independent dimers in the asymmetric unit.

(*c*) The crystals belong to the space group $P2_1$ with a molecular twofold axis parallel to, but not coincident with, the crystallographic axis, with a tetramer in the asymmetric unit.

We can rule out case (*a*) since we know from previous structural work that the approximate diameter of ENR is 50 Å and, therefore, packing considerations suggest that there is insufficient space in the cell to accommodate two tetramers related by a crystallographic twofold axis in the *ac* plane. Case (*b*) is a possibility if the unit cell contains two tetramers with the asymmetric unit being constructed from two independent dimers related by an approximate translation of $\frac{1}{2},\frac{1}{2},\frac{1}{2}$. This packing arrangement would give rise to a pseudo-twofold screw axis along *b*. Case (*c*) is also clearly consistent with the self-rotation function and the self-Patterson. Therefore, in this situation, both cases (*b*) and (*c*) remain a distinct possibility.

A cross rotation function was calculated using *POLARRFN* on data from 8 to 3.4 Å resolution with a 20 Å radius of integration with a tetrameric model of *B. napus* ENR as a search model, in which the three molecular twofold axes were aligned coincidentally with the orthogonal coordinate system defined

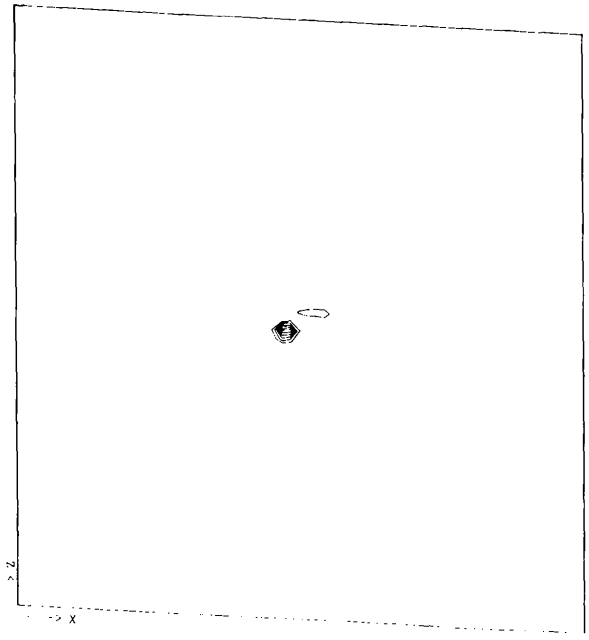

Fig. 1. A section through the translation-function solution for ENR from *E. coli* crystal form A in space group $P2_1$ calculated using *TFFC* (Collaborative Computational Project, Number 4, 1994) and a tetramer of ENR from *B. napus* as the search model. This section is at $y = 0$ and covers the range from $x = 0$–$\frac{1}{2}$ and $z = 0$–$\frac{1}{2}$ and is contoured from 1.2 to 7 by 1.2.

within the rotation function. A clear solution was obtained at $\omega = 90$, $\varphi = 90$, $\kappa = 108°$, which is consistent with either one of the molecular twofold axes lying coincident with the crystallographic b axis (as required by the space group $P2$) or lying very close to this axis (as allowed by space group $P2_1$), such that the peaks from two symmetry-related tetramers overlap. Using a model rotated by the above solution, a translation function calculated with the program *TFFC* (Collaborative Computational Project, Number 4, 1994) in space group $P2_1$ gave a peak height of 4σ. However, systematic small adjustments to the rotation angles, prior to the calculation of the translation function, increased the peak height to 16σ (Fig. 1). The small alterations to the rotation angles correspond to a rotation of the molecular twofold approximately 5° away from the crystallographic twofold, consistent with the identification of the space group as $P2_1$.

A preliminary data set was collected at the DRAL Synchrotron on station 9.6 on a form *B* crystal to 2.2 Å resolution using the rotation method with rotations of 1.5° per frame. Observations were recorded on a large MAR research image plate at a wavelength of 0.87 Å (Fig. 2). A total of 76 176 measurements were made of 31 179 independent reflections and the data were merged to an R factor of 5.8% with 95% completeness of the data from 20 to 2.2 Å resolution. The diffraction images were indexed using the *REFIX* program (Kabsch, 1988) in a primitive hexagonal cell dimensions of $a = b = 80.9$ Å and $c = 328.3$ Å and a cell volume of 1.86×10^6 Å^3. Considerations of the cell volume suggest that these crystals contain a dimer in the asymmetric unit with a V_M of 2.773 Å^3 Da^{-1}, corresponding to a solvent content of 55.6% (Matthews, 1977). Use of the program *HKLVIEW* to look at the diffraction pattern indicated strong 622 symmetry in all layers. Given the length of the c axis and the known radius of the molecule, packing considerations suggest that the space group must be one of the two enantiomorphs $P6_122$ or $P6_522$. This would appear to suggest that these crystals are related to those of the free enzyme reported previously by Wagner and co-workers (Wagner *et al.*, 1994).

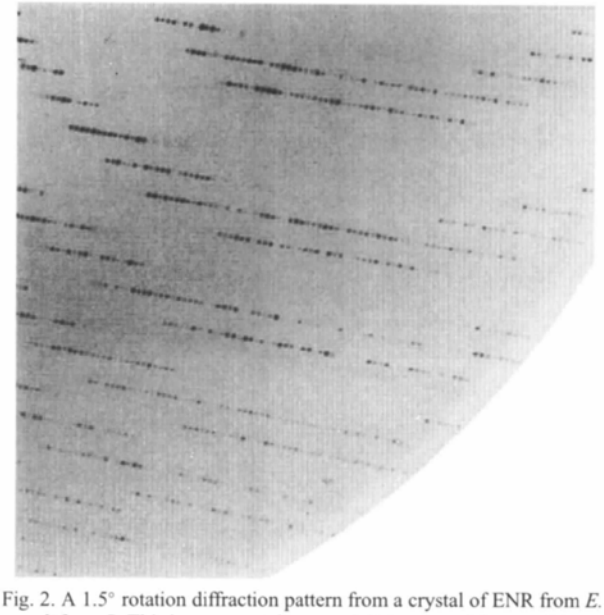

Fig. 2. A 1.5° rotation diffraction pattern from a crystal of ENR from *E. coli* form *B*. This image was taken on a MAR research image plate on station 9.6 at DRAL Synchrotron. The image has been magnified to show the edge of the plate, which is at a resolution of 2.2 Å.

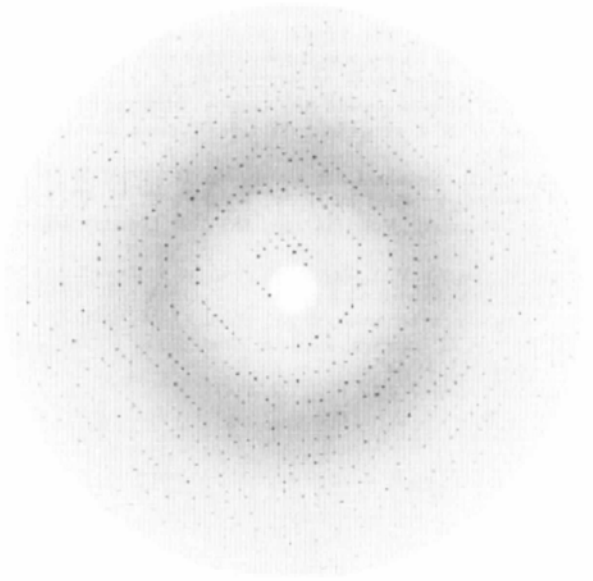

Fig. 3. A 1.2° rotation diffraction pattern from a crystal of ENR from *E. coli* form *A*. This image was taken on a MAR research image plate on station 9.5 at DRAL Synchrotron and is at a resolution of 1.8 Å at the edge of the plate.

The cell dimensions of form *A* crystals are more immediately convenient for structural analysis. Furthermore, X-ray patterns recorded from form *A* crystals at the DRAL Synchrotron on station 9.5 show that the diffraction extends to beyond 1.8 Å (Fig. 3). Hence, our strategy for solving the structure and for examining the nature of the drug binding site will be to solve form *A* by molecular replacement and then to use that refined structure to determine the structure of form *B*.

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