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Crystallization of the NADP-dependent β-keto acyl carrier protein reductase from *Escherichia coli*

JOHN B. RAFFERTY,[a] MARTIN FISHER,[a] SARAH J. LANGRIDGE,[a] WAYNE MARTINDALE,[b] NEIL C. THOMAS,[b] J. WILLIAM SIMON,[b] SIÂN BITHELL,[b] ANTONI R. SLABAS[b] AND DAVID W. RICE[a]* *at* [a]*Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, England, and* [b]*Lipid Molecular Biology Group, Department of Biological Sciences, The University of Durham, Durham DH1 3LE, England. E-mail: d.rice@sheffield.ac.uk*

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Abstract

The NADP-dependent β-keto acyl carrier protein reductase (BKR) from *E. coli* has been crystallized by the hanging-drop method of vapour diffusion using poly(ethylene glycol) of average molecular weight 1450. The crystals belong to the hexagonal space group $P6_122$ or $P6_522$ with unit-cell dimensions $a = b = 67.8$, $c = 355.8$ Å. Calculated values for V_m and consideration of the packing suggest that the asymmetric unit contains a dimer. BKR catalyses the first reductive step in the elongation cycle of fatty-acid biosynthesis. It shares extensive sequence homology with the enzyme which catalyzes the second reductive step in the cycle, enoyl acyl carrier protein reductase (ENR), and thus provides an opportunity to study the evolution of enzyme function in a metabolic pathway. The structure determination will permit the analysis of the molecular basis of its catalytic mechanism and substrate specificity.

1. Introduction

Fatty-acid biosynthesis is a critical metabolic function which provides both cellular building materials and energy-storage reserves. It is catalyzed by a complex enzyme system, fatty-acid synthetase (FAS), which has been classified into two types based upon whether the enzymes that catalyse the individual steps in the pathway and the associated acyl carrier protein reside as catalytic sub-domains on one or two multifunctional polypeptides (type I) or are located on separate polypeptide chains (type II) (McCarthy & Hardie, 1984). A type II FAS is found in plants and many bacteria, including *E. coli*, where there are six successive steps in the elongation of a fatty-acid chain (Slabas & Fawcett, 1992). Each cycle involves carboxylation, transacylation, condensation, reduction, dehydration and further reduction of the incoming substrates with the resultant incorporation of a two-carbon unit into the growing chain. The first reductive step of the pathway is catalyzed by an NAD(P)-dependent β-keto acyl carrier protein reductase (BKR), which reduces the keto group of a β-keto acyl ACP substrate to its hydroxy product (Sheldon *et al.*, 1992).

The *E. coli* BKR has been isolated and has been shown to be NADP-dependent (Alberts *et al.*, 1964). It has been identified as the product of the *fabG* gene in *E. coli* (Rawlings & Cronan, 1992) and is composed of 244 amino acids and has a monomer molecular weight of ~25.5 kDa. Gel-filtration studies suggest that it is a tetramer in solution (N. C. Thomas, unpublished work). It can catalyze the conversion of ACP-, CoA- and pantetheine-bound substrates (Alberts *et al.*, 1964) up to a chain length of 16 carbon units in a reaction which takes the form,

$$R - CO - CH_2 - CO - S - ACP + NADPH \rightleftharpoons R - CH(OH) - CH_2 - CO - S - ACP + NADP^+.$$

The product of the reduction is the D-(−)-β-hydroxyacyl isomer (Alberts *et al.*, 1964). BKR can also catalyze the reduction of the β-keto group of unsaturated acyl chains during the biosynthesis of unsaturated fatty acids in *E. coli* (Birge & Vagelos, 1972).

Sequence-comparison studies have shown the BKR enzyme is very similar to another component enzyme of the same lipid biosynthetic pathway, enoyl acyl carrier protein reductase (ENR) (Rafferty *et al.*, 1995), which catalyzes the reduction of an enoyl substrate at a later stage in the fatty-acid-chain elongation cycle. The two enzymes thus appear to have evolved from a common ancestral lipid oxidoreductase enzyme despite the fact that BKR is responsible for catalyzing the reduction of a C=O double bond whereas ENR catalyzes a C=C double bond reduction.

Further sequence comparison studies, coupled with the structure determination of ENR from *Brassica napus* (Rafferty *et al.*, 1995), have led to the discovery that BKR belongs to a family of enzymes, including 3α,20β-hydroxysteroid dehydrogenase (HSD) (Ghosh *et al.*, 1994), dihydropteridine reductase (Varughese *et al.*, 1992) and UDP-galactose-4-epimerase (Bauer *et al.*, 1992), with quite diverse function but which utilize conserved lysine and tyrosine residues as part of their catalytic machinery (Persson *et al.*, 1991; Labesse *et al.*, 1994). The enzymes of the superfamily are proposed to have a similar underlying protein fold that is modified in accordance with the nature of their substrates. The exact disposition of the putative catalytic lysine and tyrosine residues relative to the substrates and cofactors is believed to vary subtly depending upon the precise details of the reaction being catalyzed. The structure determination of BKR will give a greater understanding of the catalytic mechanism involved in the reduction of the β-keto acyl substrates and the requirements for the alignment of the catalytic residues. It will also permit a unique insight into the evolution of enzymes of a biosynthetic pathway and provide valuable information into lipid-substrate specificity.

2. Materials and methods

β-Keto reductase was purified from an overexpressing *E. coli* strain BL21(DE3) which had been transformed with the pETNEβ1 plasmid encoding the gene for *E. coli* β-keto reductase. The pETNEβ1 plasmid had been constructed from the overexpression plasmid pET-11a (Novagen) using PCR

primers designed to amplify and enable ready insertion and ligation of the coding sequence for the putative *E. coli* BKR gene, *fabG* (Rawlings & Cronan, 1992). Cells were grown at 310 K under ampicillin selection until an OD_{600} of 0.8 a.u. was achieved and then BKR overexpression was induced with 100 μM IPTG for a further 3 h before the cells were harvested by centrifugation and frozen. Lysis was achieved by a repeated freeze-thawing method after resuspension of the cells in 25 mM Na_2PO_4 buffer, pH 8.0, 1 mM DTT (buffer A) and debris removed by centrifugation at 30 000g for 15 min. The resulting crude cell extract showed a high level of BKR activity in an optical assay employing the substrate analogue acetoacetyl-CoA (Sheldon *et al.*, 1990). It was then loaded onto a 50 ml HiLoad Q column (Pharmacia) equilibrated in buffer A, washed with buffer A and then eluted from the column using a 60 ml linear gradient over the concentration range 0–1.0 M NaCl in buffer A at 5 ml min^{-1}. The BKR enzyme was found in fractions with a NaCl concentration of ~0.25 M. These fractions were dialyzed against 25 mM Na_2PO_4 buffer, pH 7.0, 1 mM DTT and then re-applied to the HiLoad Q column equilibrated with the same concentration of buffer but at pH 7.2. The BKR activity bound to the column and could be subsequently eluted with buffer at pH 7.0. The final BKR enzyme preparation was >99% pure as estimated by SDS–PAGE. N-terminal sequencing of the pure protein confirmed the first six residues corresponded to those deduced from the *fabG* gene sequence.

For the crystallization trials, BKR protein samples stored as precipitate in 85% saturated ammonium sulfate, 25 mM sodium phopshate, pH 7.0, 1 mM DTT were dialysed against 10 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1 mM DTT. The concentration of the protein was adjusted to 10 mg ml^{-1} using an Amicon Centricon 10 microconcentrator by centrifugation at 3000g. Samples of the protein (10 µl) were mixed with an equal volume of a precipitant solution of poly(ethylene glycol) of average molecular weight 1450 in the range 15–25%(w/v) in 100 mM sodium citrate buffer, pH 4.5, and allowed to equilibrate by vapour diffusion with reservoirs of precipitant solution at 290 K. Crystals with a hexagonal-plate morphology and maximum dimensions 0.5 × 0.5 × 0.2 mm were obtained after approximately 1 week.

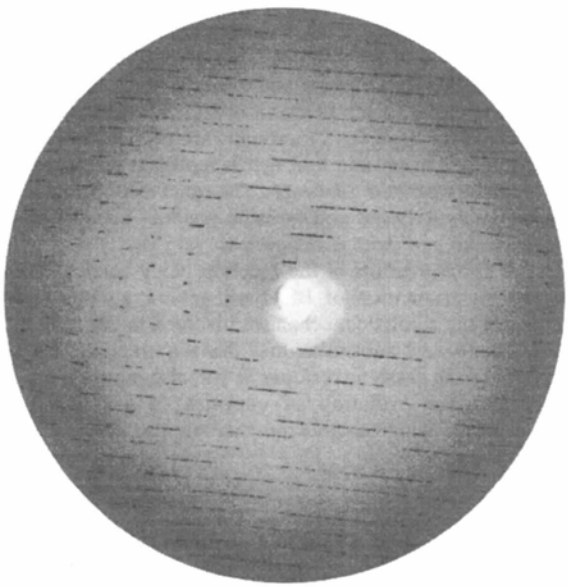

Fig. 1. An X-ray diffraction image taken at CCLRC Daresbury Laboratory on station 9.5 recorded on a large MAR image plate. The diffraction limit to the edge of the image is 3.5 Å and a rotation angle of 3° was used.

3. Results and discussion

Crystals were mounted in X-ray-transparent capillaries and precession photographs taken with home-laboratory sealed-tube X-ray sources. Although of poor quality, these preliminary photographs showed a clear sixfold pattern extending into the upper levels when the incident X-ray beam was normal to the hexagonal facet of the crystal and systematic

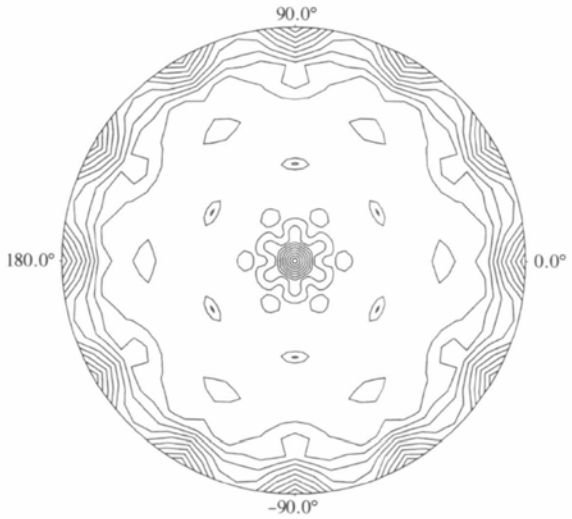

Fig. 2. A stereographic projection of the $\kappa = 180°$ section of the self-rotation function of BKR. The peaks corresponding to the crystallographic twofold axes can be seen at the perimeter of the plot ($\omega = 90°$) and occur at φ values of 0° and every 30° thereafter. Two further sets of six peaks occur at 60° intervals on φ related in pairs by 90° rotations on ω which correspond to the non-crystallographic dimer twofold axes.

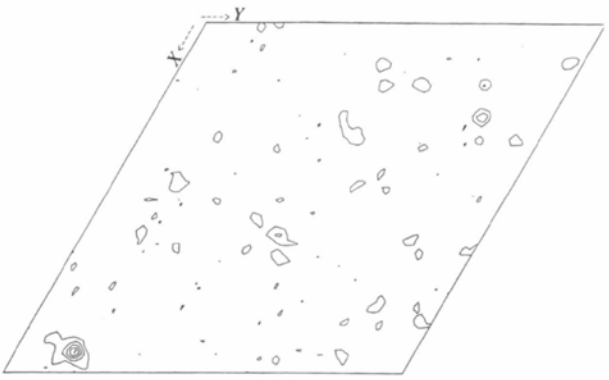

Fig. 3. A section from the translation function for BKR obtained using a dimer of HSD in space group $P6_122$. The section (at 0.34 of the cell on Z and showing the whole cell in X and Y) shows the major peak in the map which gave a clear solution consistent with packing considerations.

absences on other photographs where the crystal had been rotated by 90° were consistent with the presence of a sixfold screw axis. Measurements of the unit-cell parameters suggested dimensions of approximately $a = b = 70$ and $c \geq 350$ Å. Crystals were then mounted such that the crystallographic sixfold axis was along the capillary and a data set was collected by the rotation method with 3° rotations per frame at an X-ray wavelength of 1.2 Å on station 9.5 at the CCLRC Synchrotron Source, Daresbury Laboratory, using a large MAR image plate (Fig. 1). The crystals diffract to better than 3 Å resolution and accurate unit-cell dimensions were determined as $a = b = 67.9$, $c = 357.8$ Å, and thus the cell volume is 1.43×10^6 Å^3. Since BKR is believed to be a globular protein similar in overall fold to *B. napus* ENR a consideration of the packing of the ENR tetramer into the BKR unit cell is consistent with a sixfold screw axis along the *c* axis. Data were processed and analysed using the *MOSFLM* package (Leslie, 1992) and *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994) in space group $P6_1$ and gave a set 92% complete at 3.5 Å resolution with 47 688 measurements of 11 810 unique reflections with an overall multiplicity of 4.0 and a merging *R* factor of 3.8%. Calculation of a self-rotation function using the program *POLARRFN* (Kabsch, unpublished work; Collaborative Computational Project, Number 4, 1994) suggested the presence of additional twofold axes (Fig. 2) and indicated the space group to be one of the enantiomorphic pair $P6_122$ or $P6_522$ with a dimer in the asymmetric unit. This gives a value for V_m of 2.20 Å^3 Da^{-1} and a solvent content of 44%, which lies within the normal range for proteins (Matthews, 1977). The data were processed again in space group $P6_122$ and gave a set 92% complete at 3.5 Å resolution with 46 523 measurements of 6930 unique reflections with an overall multiplicity of 6.7 and a merging *R* factor of 4.0%.

An attempt is being made to determine phases for the BKR data by molecular replacement using the structure of HSD (preliminary results suggest this to be a slightly better model than the structure of ENR). A cross-rotation function has been calculated on data from 8 to 3.5 Å resolution with a 25 Å radius of integration using the program *POLARRFN* with a dimer model from the HSD structure (each of the three possible dimer combinations from the HSD tetramer were tested). A clear solution was obtained and a translation function was calculated using the program *TFFC* (Collaborative Computational Project, Number 4, 1994) with the dimer of HSD in both enantiomorphic space groups. A unique solution was obtained with a signal-to-noise ratio of 10.3σ for space group $P6_122$ (Fig. 3) and of 5.3σ for space group $P6_522$ which strongly indicates the correct space group to be $P6_122$. The packing was checked using the *FRODO* program (Jones, 1978). If necessary the phase information will be improved by the additional use of isomorphous replacement information arising from a heavy-atom derivative formed by the incorporation of selenomethionine into the native protein.

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