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Crystallization and preliminary X-ray diffraction analysis of a rat biliverdin reductase

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Biliverdin reductase (BVR) catalyzes the final step of haem degradation and converts biliverdin to bilirubin using NAD(P)H as an electron donor. This paper deals with the first crystallization and preliminary crystallographic study of recombinant rat BVR expressed in *Escherichia coli*. Crystals of BVR were obtained by the sitting-drop vapour-diffusion method. Using synchrotron radiation at station BL44B2 of SPring-8, Japan, BVR diffraction data were collected to 1.6 Å resolution. Crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 58.89, b = 70.41, c = 87.76 Å. The complete determination of the crystallographic structure is currently in progress using MAD (multiwavelength anomalous diffraction) data from an Ir-derivative crystal.

1. Introduction

The haem (Fe-protoporphyrin IX) degradation is catalyzed by haem oxygenase to give biliverdin, the open tetrapyrrole, as a product (Tenhunen *et al.*, 1969; Kikuchi & Yoshida, 1980; Maines, 1988). Subsequently, the biliverdin is reduced to bilirubin by biliverdin reductase (BVR; Singleton & Laster, 1965; Tenhunen *et al.*, 1970; Noguchi *et al.*, 1979; Kutty & Maines, 1981; Fang & Lai, 1987); it is an end product of the haem degradation in mammals. BVR was first purified from rat liver and appears as a homogeneous protein with an estimated molecular weight of 34 kDa as estimated by SDS–PAGE. The enzyme displays a high degree of specificity for reduction, utilizing one proton and two electrons, of the γ-meso bridge of biliverdin, but exhibits no other oxidoreductase activities. To elucidate the molecular and catalytic properties, detailed biochemical studies have been carried out.

BVR uniquely has two distinct ranges of pH optima for its reaction, using a different cofactor as an electron donor in each pH range; NADH at pH lower than 6.75, but NADPH at pH 8.7 (Kutty & Maines, 1981). Little is known about the electron transfer from these cofactors to the substrate. Since BVR does not require any cofactors other than NAD(P)H in the enzymatic reduction to bilirubin, the enzyme should receive electrons directly from NAD(P)H. Therefore, a ternary complex comprised of BVR, biliverdin and NAD(P)H should be formed during the enzymatic reaction (Bell & Maines, 1988; Frydman *et al.*, 1990). In addition, it has also been suggested that the three cysteine residues (Cys73, Cys280 and Cys291) of rat BVR are involved in enzyme activity and in binding of substrate and cofactor, as evidenced by chemical modification of these three residues (McCoubrey & Maines, 1994).

However, the nature of the interactions of substrates and cofactors with BVR, including the mechanism of the electron transfer, are not yet understood. An X-ray crystallographic study to elucidate the three-dimensional structure is required in order to discuss the binding motif of the ternary complex and the mechanism of electron transfer in more detail. However, crystallographic data from BVR are not yet available. In this paper, we report the first crystallization and preliminary crystallographic study of rat BVR expressed in *E. coli*.

2. Material and methods

E. coli BL21 cells transformed with the T7 promoter-based expression vector, which contained the rat BVR gene, were grown in LB-Amp media. After approximately 16 h of cultivation at 310 K, the cells were harvested and resuspended in 50 m*M* Tris buffer pH 7.2 containing 2 m*M* EDTA. After the addition of 2 mg of lysozyme per 1 g wet weight of *E. coli*, the mixture was stirred for 30 min followed by sonication. The lysate was centrifuged and the resulting supernatant was recovered for further purification. Solid ammonium sulfate was added to the supernatant. The precipitate obtained at a 45–65% saturation of ammonium sulfate was collected by centrifugation and dissolved in 20 m*M* phosphate buffer pH 7.4. Subsequent purification of the dissolved protein included gel filtration, DEAE-cellulose

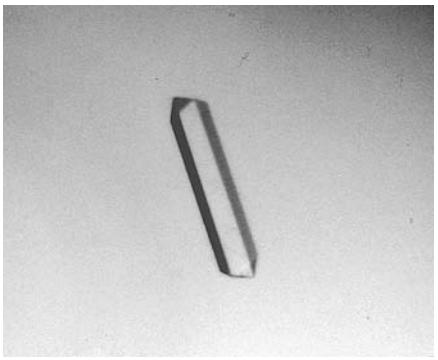

Figure 1
A single crystal of rat biliverdin reductase (BVR) grown from 2.0 *M* ammonium sulfate, 0.1 *M* sodium/potassium tartrate in 0.1 *M* sodium citrate buffer at pH 6.5. Approximate dimensions of the crystals are 0.6 × 0.2 × 0.2 mm.

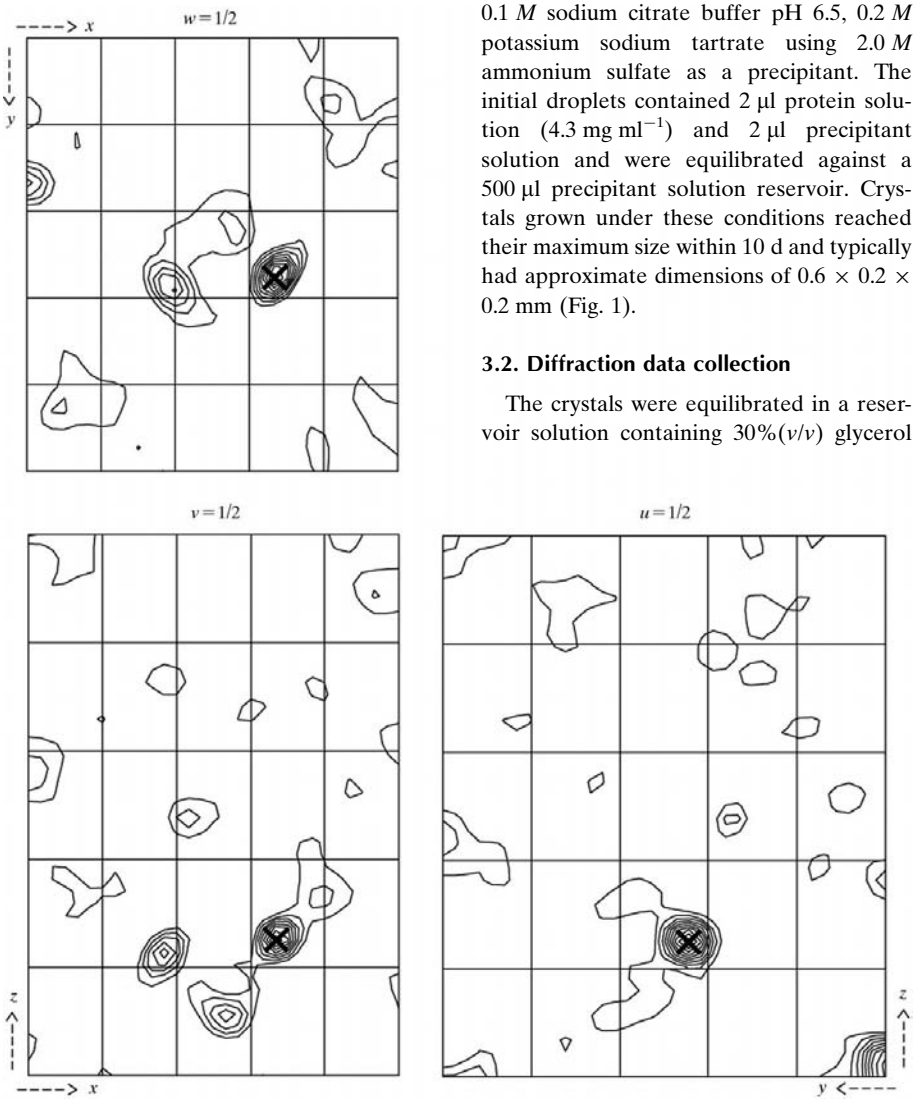

Figure 2
Bijvoet difference Patterson maps using the data collected at 1.1053 Å. Diffraction data in the resolution range 30–2 Å were used for calculation. Cross symbols correspond to Ir–Ir self-vectors. The position of the Ir atom is refined as (0.081, 0.139, 0.062) by vector-space refinement.

chromatography and hydroxyapatite column chromatography. Fractions containing the BVR protein were pooled. Protein homogeneity was checked with SDS–PAGE and isoelectric focusing electrophoresis. The specific activity was comparable to rat BVR purified from rat liver (Noguchi *et al.*, 1979). For crystallization, the phosphate buffer of the purified enzyme was changed to 50 m*M* Tris–HCl buffer pH 7 with 0.2 *M* $(NH_4)_2SO_4$. The enzyme solution was concentrated to ∼4 mg ml^{-1}.

3. Results and discussion

3.1. Crystallization of rat BVR

Rat BVR was crystallized by the vapour-diffusion method using the sitting-drop technique. Crystals were grown at 283 K in 0.1 *M* sodium citrate buffer pH 6.5, 0.2 *M* potassium sodium tartrate using 2.0 *M* ammonium sulfate as a precipitant. The initial droplets contained 2 µl protein solution (4.3 mg ml^{-1}) and 2 µl precipitant solution and were equilibrated against a 500 µl precipitant solution reservoir. Crystals grown under these conditions reached their maximum size within 10 d and typically had approximate dimensions of 0.6 × 0.2 × 0.2 mm (Fig. 1).

3.2. Diffraction data collection

The crystals were equilibrated in a reservoir solution containing 30%(*v*/*v*) glycerol before flash-freezing in a gaseous N_2 stream at 100 K. Diffraction data were obtained from the BVR crystal using synchrotron radiation of wavelength 0.6 Å at the BL44B2 station, SPring-8, Japan (Adachi *et al.*, 1996). Intensity data were collected on a MAR CCD165 detector system. 180° of data were collected with an oscillation angle of 1°. The crystal-to-detector distance was set to 150 mm. Diffraction data were indexed and integrated with *MOSFLM* (Leslie, 1994) and were scaled with *SCALA* (Collaborative Computational Project, Number 4, 1994). The resolution limit of the native data set was better than 1.6 Å.

The crystal was found to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 58.89, b = 70.41, c = 87.76 Å. Assuming one molecule of BVR per asymmetric unit (approximately 34 kDa per molecule), the crystal volume per protein mass (V_m) is calculated to be 2.7 Å^3 Da^{-1}, which is within the range of values observed in protein crystals (Matthews, 1968). This V_m value corresponds to a solvent content of approximately 51%. The reflection data have an $R_{\rm merge}$ value of 0.054 for 48 827 independent reflections derived from 360 587 total observations. The completeness of the data set is 99.9% at 38–1.60 Å. Data-collection statistics are summarized in Table 1.

It was reported that BVR purified from human liver contains Zn in an approximately 1:1 molar ratio (Maines *et al.*, 1996). Rat BVR might also be a zinc metalloprotein. We tried to collect additional diffraction data using the multiwavelength anomalous diffraction (MAD) method, in order to obtain initial phase information for structural determination. Prior to the diffraction data collection, the X-ray fluorescence spectrum of the rat BVR crystal was measured using the Si-PIN photodiode X-ray detector (AMPTEC INC. XR-100CR), but no Zn atom absorption edge was found around 9.6 keV in the spectrum. Zn was not found in the solution sample prepared for crystallization or that prepared from *E. coli* cultivated in the presence of $ZnCl_2$. Also, all efforts to introduce a Zn atom into the crystal by co-crystallization and soaking techniques failed. Unexpectedly our rat BVR does not contain a Zn atom; however, the activity assay of the sample showed its ability to convert biliverdin to bilirubin in the presence of both the cofactors NADH and NADPH. Thus, we concluded that the solution of the diffraction data can elucidate the three-dimensional structure of 'active' BVR.

Table 1
Crystal parameters and data reduction of rat BVR.

Values in parentheses are for the highest resolution shell (for final resolution 1.60 Å, this was 1.69–1.60 Å; for 2.0 Å final resolution it was 2.11–2.00 Å).

		MAD data		
	Native	λ1	λ2	λ3
Space group	$P2_12_12_1$	$P2_12_12_1$		
Unit-cell parameters (Å)	a = 58.89 (±0.04), b = 70.41 (±0.05), c = 87.76 (±0.02)	a = 58.93 (±0.02), b = 70.46 (±0.03), c = 88.04 (±0.05)		
Wavelength (Å)	0.6	1.1053	1.1058	1.1083
Resolution (Å)	38–1.6	30–2.0	30–2.0	30–2.0
Measured reflections	360587	179411	179866	179563
Unique reflections	48827	25416	25470	25444
Completeness (%)	99.9 (99.9)	99.9 (99.9)	99.9 (99.6)	99.9 (99.9)
R_{merge}† (%)	5.4 (29.3)	4.5 (13.4)	4.4 (14.1)	4.5 (12.7)
Multiplicity	7.4 (7.4)	7.1 (6.9)	7.1 (6.9)	7.1 (6.9)
Mean $\langle I/\sigma(I)\rangle$	10.1 (2.6)	8.8 (4.8)	9.0 (4.6)	8.9 (4.9)

† $R_{merge} = \sum\sum_i |I(h) - I(h)_i| / \sum\sum_i I(h)$, where $I(h)$ is the mean intensity after rejections.

3.3. Multiwavelength anomalous diffraction (MAD) analysis and determination of the heavy-atom position from anomalous dispersion measurement

A heavy-atom derivative for phase determination was obtained by soaking the crystals in 2.5 *M* Li_2SO_4 and 0.1 *M* sodium citrate buffer at pH 6.5 in the presence of 5 m*M* Na_3IrCl_6. We then attempted to solve the structure using the MAD method. MAD data were collected from a single Ir-derivative crystal at 100 K. Before mounting, the crystals were equilibrated in the Li_2SO_4 solution containing 30%(*v*/*v*) glycerol. The wavelengths of the infraction point (λ = 1.1058 Å) and the 'white line' (λ = 1.1053 Å) of the Ir L_{III} edge of the crystal were determined from the fluorescence spectrum. The remote wavelength was selected at λ = 1.1083 Å. The distance between the crystal and detector was set to be 120 mm. No serious radiation damage to the crystal was detected during the data collection at the three different wavelengths. The data were processed as described above and the results are summarized in Table 1.

Fig. 2 shows Harker sections of the Bijvoet anomalous difference Patterson map using data collected at 1.1053 Å. The maps show clear Ir–Ir self-vectors on the Harker sections with more than eight times the root-mean-square deviation of the maps. The Ir position was refined as (0.081, 0.139, 0.062) by vector-space refinement (Collaborative Computational Project, Number 4, 1994). The complete determination of the crystallographic structure is currently in progress using MAD data from an Ir-derivative crystal.

In this study, we have described the crystallization conditions and collected preliminary X-ray diffraction data for the rat BVR. Elucidation of the BVR structure will reveal the nature of the cofactor/substrate-binding site and provide structural information regarding the reduction mechanism of biliverdin to bilirubin in the haem degradation catalyzed by BVR.

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