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Crystallization and preliminary X-ray diffraction analysis of fatty-acid hydroxylase cytochrome P450BSβ from *Bacillus subtilis*

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Cytochrome P450 isolated from *Bacillus subtilis* (P450BSβ; MW 48 kDa) catalyzes the hydroxylation of long-chain fatty acids at the α and β positions using H_2O_2 as an oxidant. Crystals of the substrate-free form of P450BSβ belonging to the trigonal space group $P3_221$ or $P3_121$ were obtained by the sitting-drop vapour-diffusion method using a precipitate solution consisting of 10%(*w*/*v*) PEG 4000 and 50 m*M* MES pH 6.8. Another crystal form, belonging to the rhombohedral space group *R*3 or *R*32, was obtained from precipitate solution consisting of 10% PEG 4000, 0.15 m*M* magnesium acetate and 50 m*M* MES pH 6.5 in the presence of 2 m*M* myristic acid (substrate). Using synchrotron radiation, both P450BSβ crystals diffracted to 2.5 Å resolution. Bijvoet and dispersive anomalous difference Patterson maps show a clear peak corresponding to the haem iron.

1. Introduction

Cytochrome P450BSβ was first identified as the *ybd*T gene product from *B. subtilis* and is a 48 kDa protein of unknown function (Kunst *et al.*, 1997). A database homology search indicated that the amino-acid sequence of this gene product exhibits 44% identity to that of a cytochrome P450 isolated from *Sphingomonas pautimobilis* (P450SPα; Matsunaga *et al.*, 1999). Now, both P450SPα and P450BSβ belong to the P450 superfamily and are given the systematic names CYP152B1 and CYP152A1, respectively (Matsunaga *et al.*, 2000). P450SPα catalyzes the hydroxylation of the α-carbon position of long-chain fatty acids, for example myristic acid, using hydrogen peroxide (H_2O_2) instead of using O_2, two electrons and two protons (Imai *et al.*, 2000). As expected from the high homology of its primary sequence to that of P450SPα, P450BSβ is also involved in the hydroxylation reaction of fatty acids by H_2O_2, in which both the α and β positions are hydroxylated but the amount of the β-hydroxyl product is slightly higher than that of the α-hydroxyl product (Matsunaga *et al.*, 1999). It is suggested from this site-specificity of hydroxylation that the α and β positions of fatty acids are located very close to the active site (the haem iron) of the P450 enzyme.

The substrate–enzyme interaction is unique to P450SPα and P450BSβ, as other P450s catalyze hydroxylation at the CH_3-terminus (ω position) or neighbouring positions of the alkyl chain of the fatty acid. In the crystal structure of the substrate-bound form of P450 from *B. megaterium* (P450BM3), it was found that the substrate (palmitoleic acid) binds to the enzyme through electrostatic interaction of the carboxylate with the ε-amino group of Lys244 located at the protein surface and through the hydrophobic interaction of the long alkyl chain with the hydrophobic protein interior (Li & Poulos, 1997). Therefore, the CH_3-terminus (ω position) of the alkyl chain of the fatty acid is located close to the haem iron, consistent with the site-specificity of the reaction. In order to examine the interaction of substrates with P450BSβ, we have attempted to produce crystals of the enzyme in the presence or absence of fatty acids for crystallographic analyses. This paper describes the crystallization and preliminary X-ray data of P450BSβ.

2. Materials and methods

Recombinant P450BSβ expressed in the *Escherichia coli* BL21 expression system we constructed was purified according to the method reported previously (Matsunaga *et al.*, 2001). The purified protein was homogeneous as judged by SDS–PAGE and isoelectric focusing gel electrophoresis. For crystallization, the enzyme solution in 50 m*M* MES buffer at pH 6.7 was concentrated to 20 mg ml^{-1} ($\sim$0.42 m*M*).

3. Results and discussion

3.1. Crystallization of P450BSβ in the absence of substrate

A single crystal of a substrate-free form of P450BSβ in the ferric resting state (crystal 1)

was obtained by the vapour-diffusion method using the sitting-drop technique. Crystals were grown at 293 K in 50 m*M* MES solution pH 6.8 using 10%(*w*/*v*) PEG 4000 as a precipitant. The initial droplets contained 1.0 µl protein solution (20 mg ml^{-1}) and 1.0 µl precipitant solution and were equilibrated against a reservoir containing 1 ml the precipitant solution. Crystals were obtained after one week. Crystals grown under these conditions reached their maximum size within 7 d; their typical dimensions are approximately 0.15 × 0.15 × 0.1 mm (Fig. 1*a*).

3.2. Diffraction data collection and molecular-replacement (MR) analysis

Diffraction data were collected from the crystals of P450BSβ using the synchrotron-radiation source at the RIKEN beamline (BL44B2) station, SPring-8, Harima, Japan. Intensity data were measured with a MAR CCD detector, which was mounted on a Huber alignment table. The cryogenic head of the Rigaku Cryosystems cryostream was mounted close to the goniometer head. The crystals were mounted with the *c* axis as the axis of rotation; its distance from the CCD detector was 230 mm. Measurements were performed at 90 K; the wavelength of the incident X-ray was 0.7 Å. Diffraction data were integrated and scaled with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

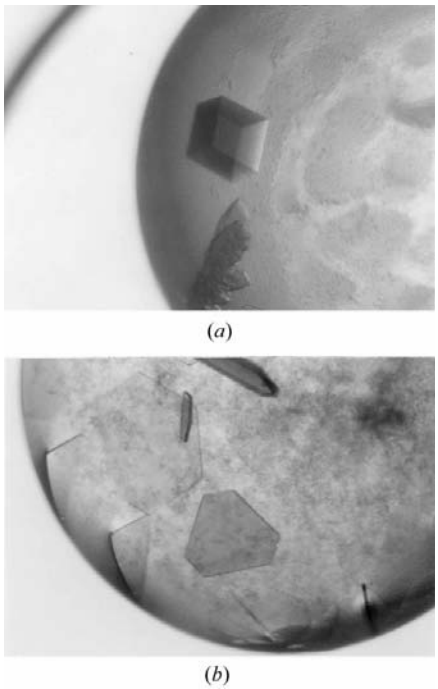

Figure 1
A single crystal of cytochrome P450BSβ in (*a*) substrate-free and (*b*) myristate-bound form.

The crystal of the substrate-free P450BSβ diffracted to a resolution of 2.5 Å and was found to belong to the trigonal space group $P3_221$ or $P3_121$, with unit-cell parameters $a = b = 156.6$ (2), $c = 112.9$ (1) Å. Assuming three molecules of the enzyme per asymmetric unit, the crystal volume per protein mass (V_M) is calculated to be 2.77 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 55%. Data-collection statistics are summarized in Table 1.

Until now, nine crystal structures of cytochrome P450 enzymes have been reported. The overall fold of each enzyme is basically similar. Therefore, we tried to analyze the structure of P450BSβ with the molecular-replacement (MR) method using the coordinates of P450s available thus far, but have not succeeded.

3.3. Multiwavelength anomalous dispersion (MAD) analysis and position of the haem Fe atom from anomalous dispersion

As P450BSβ contains one haem iron per protein molecule, we collected additional diffraction data using the multiwavelength anomalous dispersion (MAD) method in order to obtain initial phase information for structural determination. Prior to collection of the diffraction data, the X-ray fluorescence spectrum of the P450BSβ crystal was measured in order to determine the absorption edge of the haem iron using an Si-Pin photodiode X-ray detector (Amptec Inc. XR-00CR). Three data sets for MAD calculation were collected with wavelengths of 1.738 (peak), 1.742 (edge) and 1.754 Å (remote) using a single crystal. No serious radiation damage to the crystal was detected during the data collection at the four different wavelengths. The distance between

Table 1
Crystal parameters and data reduction of P450BSβ.

Values in parentheses refer to the highest resolution shell.

	Crystal 1				Crystal 2
Data set	Native	MAD data			Native
Wavelength (Å)	0.70	1.742	1.738	1.754	1.0
Resolution range (Å)	50–2.5	50–3.0	50–3.0	50–3.0	50–2.5
No. of reflections measured	469210	235326	236095	231599	360822
No. of unique reflections	48788	32196	32182	32024	54684
Completeness (%)	99.4 (96.2)	99.7 (97.6)	99.7 (97.4)	99.1 (91.5)	98.7 (88.0)
R_{merge}† (%)	4.7 (18.8)	6.2 (27.4)	5.4 (18.8)	9.2 (57.4)	5.1 (28.0)
Redundancy	9.6	7.3	7.3	7.2	6.6
Mean $\langle I/\sigma(I)\rangle$	14.1	10.7	14.7	6.4	18.9

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

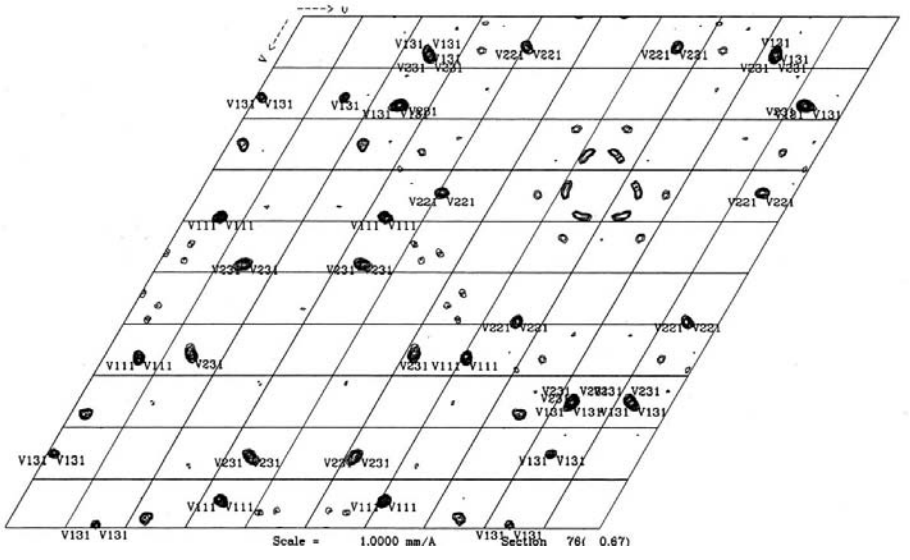

Figure 2
Bijvoet difference Patterson maps using the data collected at 1.738 Å. Diffraction data in the resolution range 10–3 Å were used for calculation. The labels correspond to haem iron–iron self vectors. The positions of the haem irons refined to (0.909, 0.485, 0.059), (0.508, 0.267, 0.058) and (0.904, 0.156, 0.053) by vector-space refinement.

the crystal and the detector was set to 200 mm. The data were processed as described above and the results are summarized in Table 1.

Fig. 2 shows the Harker section of the Bijvoet anomalous difference Patterson map using data collected at 1.738 Å. The maps show clear haem iron–iron self-vectors on the Harker section, with peak heights of more than 7σ. The Fe-atom positions were refined to (0.909, 0.485, 0.059), (0.508, 0.267, 0.058) and (0.904, 0.156, 0.053) by vector-space refinement (Collaborative Computational Project, Number 4, 1994).

3.4. Crystallization of P450BSβ in the presence of myristic acid

Another crystal form of P450BSβ in a ferric state (crystal 2) was obtained from a solution consisting of 10% PEG 4000, 0.15 m*M* magnesium acetate and 50 m*M* MES pH 6.5 in the presence of 2 m*M* myristic acid (Fig. 1*b*). In the absence of the fatty acid, no crystals were obtained from the same crystallization solution. The substrate-bound crystals have typical dimensions of approximately 0.25 × 0.25 × 0.07 mm. This crystal form diffracted with a resolution of 2.5 Å and was found to belong to the rhombohedral space group *R*3 or *R*32, with unit-cell parameters $a = b = 171.5$ (1), $c = 277.9$ (3) Å. The crystal volume per protein mass (V_M) was calculated to be 2.73 Å^3 Da^{-1} and the solvent content was approximately 55%, if six molecules are present in the asymmetric unit in the case of *R*3. We are now attempting to determine the structures of the two forms of P450BSβ using MAD data.

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