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Crystallization and preliminary X-ray analysis of native and selenomethionine 2-hydroxybiphenyl 3-monooxygenase

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2-Hydroxybiphenyl 3-monooxygenase (HbpA; EC 1.14.13.44) from *Pseudomonas azelaica* HBP1 was produced in *Escherichia coli* both as native and SeMet-labelled protein. The two enzymes were purified to homogeneity and crystallized by the hanging-drop vapour-diffusion method. The crystals belong to the monoclinic space group $C2$, with unit-cell parameters $a = 108.6$, $b = 196.8$, $c = 79.3$ Å, $\beta = 97.7^\circ$ for the native protein and $a = 108.3$, $b = 196.8$, $c = 79.0$ Å, $\beta = 97.8^\circ$ for SeMet HbpA. Crystal-packing considerations led to the assumption of two HbpA subunits per asymmetric unit, which corresponds to a V_M value of 3.3 Å^3 Da^{-1} and a solvent content of 62%. The crystals were radiation-sensitive and only had a lifespan of about 120 s when exposed to synchrotron radiation on an undulator beamline. To obtain complete data sets, data were collected from 23 native and 26 derivative crystals. The high-resolution limit was 2.0 Å for native and 2.25 Å for SeMet HbpA.

1. Introduction

Hydroxylation reactions are among the most widespread enzymatic activities, occurring in all life forms from bacteria to humans. The elucidation of how enzymes that catalyze such reactions handle the three required substrates (molecular oxygen, the electron donor and the substrate to be hydroxylated) remains a challenge (Holland & Weber, 2000). Despite the limited information base, hydroxylases have an enormous biosynthetic value owing to their ability to carry out unique oxidations, usually with high regioselectivity and enantioselectivity, that extend well beyond the toolkits of classical organic synthesis (Duetz *et al.*, 2001).

2-Hydroxybiphenyl 3-monooxygenase (EC 1.14.13.44; HbpA) is the first enzyme of the 2-hydroxybiphenyl degradation pathway of *Pseudomonas azelaica* HBP1 (Kohler *et al.*, 1988). HbpA is able to catalyse the *ortho*-hydroxylation of different 2-substituted phenols to the corresponding catechols using NADH as electron donor (Fig. 1) (Kohler *et al.*, 1988; Suske *et al.*, 1997). HbpA has a homotetrameric structure with a total mass of 256 kDa and each subunit contains a non-covalently but tightly bound FAD (Suske *et al.*, 1997). The catalytic mechanism has been investigated by spectroscopic techniques and the enzyme kinetics have been reported in detail (Suske *et al.*, 1999). Owing to its conserved sequence motifs for FAD/NADH binding, HbpA was classified as a flavoprotein aromatic hydroxylase (Eppink *et al.*, 1997). Of this large family, only two three-dimensional structures have thus far been solved: those of phenol 2-monooxygenase and *p*-hydroxybenzoate hydroxylase (Enroth *et al.*, 1998; Schreuder *et al.*, 1989; Wierenga *et al.*, 1979). However, both of these enzymes are homodimers and show only small overall homology with HbpA.

2-Hydroxybiphenyl 3-monooxygenase has proven its potency as an efficient biocatalyst in biotechnological organic synthesis. Using this enzyme, different 3-substituted catechols were produced *in vivo* and *in vitro* (Held *et al.*, 1998, 1999; Schmid *et al.*, 2001). In addition, directed enzyme evolution was successfully used to change the substrate specificity of HbpA and to enable the synthesis of compounds that could not be produced using the wild-type enzyme (Meyer *et al.*, 2002, 2003).

We have initiated structural studies for two main reasons: (i) to further investigate the catalytic mechanism of HbpA with special reference to the variants obtained by directed evolution and (ii) to rationally design novel biocatalysts for bioorganic synthesis. In this study, we report the produc-

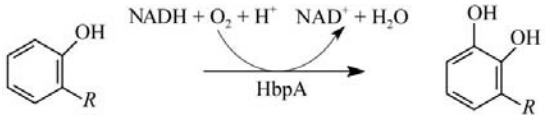

Figure 1
Reaction catalysed by 2-hydroxybiphenyl 3-monooxygenase. *R* = phenyl, 2-hydroxyphenyl, methyl, ethyl, propyl, isopropyl, butyl, *sec*-butyl, fluoro, chloro, bromo or iodo.

tion of native and selenomethionine HbpA crystals, the initial step towards reaching these goals.

2. Materials and methods

2.1. Expression and purification of HbpA and SeMet HbpA

Plasmid pAA1 (Meyer *et al.*, 2002), a pUC18 derivative harbouring the *hbpA* gene (Yanish-Perron *et al.*, 1985), was used for expression of HbpA and the selenomethionine-labelled protein (SeMet HbpA). For the synthesis of HbpA, the construct was transformed into *Escherichia coli* JM101 cells (Sambrook *et al.*, 1989), whereas SeMet HbpA was produced in *E. coli* LE392 (Sambrook *et al.*, 1989). The recombinant strains were grown in 3 l shaking flasks containing 1 l M9 mineral medium (Sambrook *et al.*, 1989) supplemented with 0.1%(*v*/*v*) MT trace-element solution (Lageveen *et al.*, 1988), 0.1 m*M* $CaCl_2$, 2 m*M* $MgSO_4$, 150 mg l^{-1} ampicillin and 0.5%(*w*/*v*) glucose. In addition, 0.001%(*w*/*v*) thiamine was added to *E. coli* JM101 cultures and 50 mg l^{-1} L-SeMet and 50 mg l^{-1} L-Trp were added to *E. coli* LE392 cultures. After incubation overnight at 303 K, 0.5%(*v*/*v*) glycerol was added to allow *hbpA* expression. To ensure plasmid maintenance, the addition of ampicillin was repeated and L-SeMet was again added to the *E. coli* LE392 culture. The *E. coli* JM101 cultures were harvested after 8 h, whereas the *E. coli* LE392 cultures were harvested after incubation overnight. Purification of HbpA and SeMet HbpA was performed as recently reported (Meyer *et al.*, 2002).

2.2. Screening for crystallization conditions

Initial screening of crystallization conditions was performed by the hanging-drop vapour-diffusion method using Hampton Research Crystal Screens I and II (Cudney *et al.*, 1994; Jancarik & Kim, 1991). The reservoir contained 1 ml precipitant solution and the drop consisted of 2 µl precipitant and 2 µl HbpA solution (10 mg ml^{-1}). Crystallization setups were allowed to equilibrate at 293 K for two weeks. Under these conditions, clusters of needle-shaped crystals formed using Crystal Screen II solutions No. 25 (1.8 *M* ammonium sulfate, 0.1 *M* MES pH 6.5, 0.01 *M* cobalt chloride) and No. 32 (1.6 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5, 0.1 *M* sodium chloride). Systematic optimization included investigation of ammonium sulfate concentrations in the range 0.4–2.1 *M*, testing of different organic buffers (MES, CHES, MOPS, TES, HEPES, PIPES) in the pH range 4.5–8 and application of several chloride salts (NaCl, $MgCl_2$, $MnCl_2$, KCl, LiCl, $CoCl_2$) at concentrations of 0.01–0.2 *M*. The optimized precipitant solution contained 1.6 *M* ammonium sulfate, 0.1 *M* sodium chloride and 0.1 *M* MES–NaOH pH 7.5 and was used for the crystallization of both native and SeMet-labelled HbpA.

Figure 2
Native HbpA crystal with dimensions of 0.1 × 0.2 × 0.4 mm.

Table 1
Data statistics for HbpA and SeMet HbpA crystals.

Values in parentheses refer to the outer resolution shell. The data set for HbpA consisted of 23 different crystals and that for SeMet HbpA of 26 crystals.

	Native HbpA	SeMet HbpA
Wavelength (Å)	0.979297	0.979297
Resolution range (Å)	20.0–2.0 (2.11–2.0)	20.0–2.25 (2.37–2.25)
Space group	*C*2	*C*2
Unit-cell parameters (Å, °)	a = 108.6, b = 196.8, c = 79.3, β = 97.7	a = 108.3, b = 196.8, c = 79.0, β = 97.8
Completeness (%)	92.2 (87.5)	99.7 (100)
Redundancy	4.9 (4.1)	6.1 (6.3)
$R_{\rm merge}$† (%)	9.4 (22.2)	11.0 (24.6)
$\langle I/\sigma(I)\rangle$ overall	4.8 (3.0)	4.4 (2.8)

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$, where I is the integrated intensity of a given reflection.

2.3. Diffraction experiments

X-ray diffraction experiments were performed at 100 K. To this end, the crystals were transferred directly into cryoprotectant solution consisting of 30%(*v*/*v*) glycerol, 1.6 *M* ammonium sulfate, 0.1 *M* NaCl, 0.1 *M* MES–NaOH pH 7.5. Both HbpA and SeMet HbpA data sets were measured at the protein crystallography beamline of the Swiss Light Source (SLS, PSI Villigen, Switzerland; http://sls.web.psi.ch). Data were recorded using a MAR CCD detector at 0.9793 Å, the peak wavelength of the SeMet HbpA derivative, for processing as SAD or SIRAS data. Integration was performed using *MOSFLM* (Leslie, 1992) and the *CCP*4 program suite was used for scaling (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

3.1. Expression and purification of SeMet HbpA

The catalytic activity of HbpA is completely inhibited by the presence of metals such as mercury, silver and platinum (Suske *et al.*, 1997). It is not known whether this inhibition is a consequence of impaired substrate binding or of structural changes in the substrate-binding pocket of the enzyme. Therefore, we decided to produce an SeMet HbpA heavy-atom derivative for phasing. HbpA contains 15 methionine residues per 64 kDa subunit, which results in an expected anomalous signal of approximately 9% of the total scattering of the crystal (Walsh *et al.*, 1999). We achieved synthesis of SeMet HbpA as 20% of the total protein in *E. coli* LE392. SeMet HbpA could be purified to homogeneity using the method developed for the native protein and showed 80% activity compared with that of HbpA. MALDI–TOF MS analysis of HbpA and SeMet HbpA indicated complete substitution of Met by SeMet. In addition, it revealed the post-translational excision of the N-terminal methionine, which is common in *E. coli* when, as in the case of HbpA, serine is the penultimate amino acid (Hirel *et al.*, 1989).

3.2. Crystallization

Crystals of native and SeMet HbpA were obtained within an equilibration time of 10 d (Fig. 2). They were coloured bright yellow, indicating the oxidized state of the flavin prosthetic group. Crystals grew to 0.8 mm in the longest dimension. However, it turned out that such large crystals were of inferior quality. The diffraction spots were often split and unshaped and accurate processing of the images was therefore not possible. In contrast, crystals with dimensions of about 0.1 × 0.2 × 0.4 mm showed clearer diffraction patterns, although to a lower resolution.

3.3. Diffraction experiments

Native crystals of HbpA diffracted to a resolution of 2.0 Å, whereas the resolution limit for SeMet HbpA crystals was 2.25 Å. Crystals from both protein types were isomorphous and belonged to the monoclinic space group *C*2. Based on two HbpA subunits per asymmetric unit, the crystal-

packing parameter V_M is 3.3 Å^3 Da^{-1}, which corresponds to a solvent content of 62% (Matthews, 1968).

Although the measurements were performed at 100 K, the HbpA and SeMet HbpA crystals were radiation-sensitive and showed structural damage after a total of 120 s exposure time, as indicated by degradation of the diffraction pattern. Therefore, data from 23 HbpA crystals were collected over 10° each with oscillations of 0.5°. 26 SeMet HbpA crystals, each collected over 12.5°, were used for the derivative data set. Detailed statistics of the merged data are listed in Table 1.

Localization of the selenium sites in large structures of SeMet-labelled proteins is still a challenge. However, computational advances facilitate this task and have allowed the successful elucidation of a number of large selenium substructures (Deacon & Ealick, 1999). We are confident that the recorded data will allow the positioning of the heavy atoms in SeMet HbpA.

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