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Crystallization and preliminary X-ray analysis of the small component of 4-hydroxyphenylacetate 3-monooxygenase (HpaC) and its cofactor complex from *Thermus thermophilus* HB8

Seong-Hoon Kim,[a] Hideyuki Miyatake,[a] Tamao Hisano,[a] Naoto Ohtani[a] and Kunio Miki[a,b]*

[a]RIKEN Harima Institute/SPring-8, Koto 1-1-1, Mikazuki-cho, Sayo-gun, Hyogo 679-5148, Japan, and [b]Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Correspondence e-mail: miki@kuchem.kyoto-u.ac.jp

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The small component of 4-hydroxyphenylacetate 3-monooxygenase (HpaC) is an NADH oxidoreductase containing a flavin molecule as a cofactor. HpaC reduces a flavin molecule and reduced flavin is subsequently supplied to the large component of 4-hydroxyphenylacetate 3-monooxygenase (HpaB). The HpaC protein from *Thermus thermophilus* HB8 has been overexpressed in *Escherichia coli* and crystallized. During purification, the eluted HpaC protein solutions were separated into colourless and yellow-coloured fractions (*i.e.* apo-HpaC and HpaC–flavin complex, respectively). Crystals of apo-HpaC grown in 5%(*v*/*v*) isopropyl alcohol, 0.1 *M* HEPES–NaOH pH 7.0, 40%(*w*/*v*) polyethylene glycol (PEG) 4000 and 10%(*v*/*v*) glycerol diffracted X-rays to a resolution of 1.85 Å, whereas crystals of the HpaC–flavin complex grown in 20%(*w*/*v*) PEG 1000, 10%(*w*/*v*) PEG 8000 and 10%(*v*/*v*) glycerol diffracted X-rays to a resolution of 1.3 Å. Both crystals belong to the monoclinic system, space group $P2_1$, with similar unit-cell parameters. Selenomethionyl protein crystals of the HpaC–flavin complex grown under similar conditions to the native crystals diffracted X-rays to a resolution of 1.8 Å. They also belong to the monoclinic space group $P2_1$, but are not isomorphous to crystals of the HpaC–flavin complex of the native protein. MAD data for structure determination were successfully collected using these crystals.

1. Introduction

The catabolic pathway of aromatic compounds is one of the most important processes providing sources of carbon and energy in bacteria (Schreuder *et al.*, 1988; Arunachalam & Massey, 1994; Gibello *et al.*, 1997; Noh *et al.*, 2002). In the case of *Escherichia coli* W, 4-hydroxyphenylacetate (4-HPA) 3-monooxygenase converts 4-HPA to 3,4-dihydroxyphenylacetate (3,4-DHPA), the first intermediate of the catabolic pathway. Two components of 4-HPA 3-monooxygenase, the large and small components (HpaB, 58.7 kDa and HpaC, 18.6 kDa, respectively) are encoded by *hpaB* and *hpaC*, respectively (Cooper & Skinner, 1980; Prieto & Garcia, 1994; Prieto *et al.*, 1996; Galan *et al.*, 2001). The HpaB and HpaC proteins are classified into the two-component flavin-diffusible monooxygenase (TC-FDM) family (Galan *et al.*, 2000). HpaB introduces a hydroxyl group into the *ortho* position of the benzene ring of 4-HPA and HpaC is a flavin-containing NADH oxidoreductase. HpaC reduces flavin and reduced flavin is subsequently supplied to HpaB. The reaction scheme catalyzed by these two enzymes is shown in Fig 1. The flavin molecule has not been completely defined experimentally as a cofactor, but it has been suggested that HpaC may possess either FAD or FMN (Raju *et al.*, 1988; Prieto & Garcia, 1994; Gibello *et al.*, 1997; Galan *et al.*, 2000; Fontecave *et al.*, 1987). Genomic analysis revealed that *Thermus thermophilus* HB8 contains the open reading frame (ORF) of the large and small components (*hpaB* and *hpaC*, respectively) of 4-HPA 3-monooxygenase. The deduced amino-acid sequences of the HpaB (65.7 kDa) and HpaC (16.1 kDa) proteins have identities of 30 and 29%, respectively, to the corresponding proteins of the TC-FDM family.

The HpaC protein from *T. thermophilus* HB8 is a single polypeptide chain consisting of 149 amino-acid residues. It has been suggested that HpaC contains two cofactor [*i.e.* flavin and NAD(P)H] binding sites (Fontecave *et al.*, 1987; Raju *et al.*, 1988), but very little is known about its reaction mechanism. In order to gain a better understanding of the structure–function relationship of the HpaC protein, we initiated structure determination by X-ray crystallography. Here, we describe the crystallization and preliminary X-ray diffraction analysis of the HpaC protein from *T. thermophilus* HB8.

2. Materials and methods

2.1. Purification of HpaC

The ORF of HpaC was amplified by PCR using *T. thermophilus* HB8 genomic DNA as a template and the two following oligonucleotide primers containing artificial *Nde*I and *Bgl*II sites (in bold): 5′-ATAT**CATATG**-AAAGAGGCCTTCAAGGAGGCCCTCG CCC-3′ and 5′-ATAT**AGATCT**TTATTAC-GATGGCCATACCAACCTCCTGTAG-3′. The PCR product was digested with *Nde*I and *Bam*HI and was ligated into the pET11a expression plasmid (Novagen), in which the *hpaC* gene is under control of the T7 promoter. Cells were grown at 310 K in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin. Protein expression was induced without IPTG in *Escherichia coli* BL21 (DE3) for 24 h. Cells were harvested by centrifugation at 2000*g* for 10 min at 277 K and the cell pellets were resuspended in lysis buffer (50 m*M* NaCl, 20 m*M* Tris–HCl pH 8.0, 5 m*M* 2-mercaptoethanol) and disrupted by sonication. The cell lysates were clarified by centrifugation at 200 000*g* for 60 min at 277 K. The supernatant was heated to 343 K for 10 min and centrifuged at 65 300*g* for 60 min. The supernatant was further purified by six column-chromatography steps, using Resource PHE, Resource Q, Hydroxyapatite CHT2, HiLoad 16/60 Superdex 75, Resource Q and HiPrep 26/10 Desalting columns (all columns were obtained from Pharmacia Biotech, except Hydroxyapatite CHT2, which was from Bio-Rad). The resulting protein was separated into two fractions in the second step of the column chromatography. One fraction was colourless and the other fraction was yellow. The yellow fraction showed the characteristic absorption spectrum unique to flavin molecules at 450 nm. The colourless and yellow fractions were assigned as containing the apo-HpaC protein and its flavin complex, respectively. In this purification protocol, 9.74 mg HpaC–flavin complex and 0.86 mg apo-HpaC protein were obtained from 23.2 g of wet cells. For crystallization, purified apo-HpaC and HpaC–flavin complex were concentrated to 12 and 25 mg ml^{-1} [containing 1 m*M* dithiothreitol (DTT) and 20 m*M* Tris–HCl pH 8.0] using an ultrafiltration membrane (Vivaspin 20, Vivascience), respectively.

In the case of selenomethionine-labelled HpaC, cells were grown at 310 K in LeMaster medium (LeMaster & Richards, 1985) containing 50 µg ml^{-1} selenomethionine, 1.0%(*w*/*v*) lactose, 50 µg ml^{-1} ampicillin and vitamin mixture (Kao and Michayluk Vitamin Solution, Sigma) in place of methionine and glucose. Protein expression was induced without IPTG in *E. coli* B834 (DE3) for 24 h. Cells were harvested by centrifugation at 2000*g* for 10 min at 277 K and the cell pellets were resuspended in lysis buffer (50 m*M* NaCl, 20 m*M* Tris–HCl pH 8.0, 5 m*M* 2-mercaptoethanol) and disrupted by sonication. The cell lysates were clarified by centrifugation at 200 000*g* for 60 min at 277 K. The supernatant was heated to 343 K for 10 min and centrifuged at 65 300*g* for 60 min. The supernatant was purified by five steps of column chromatography, using Resource PHE, Resource Q, Hydroxyapatite CHT2, HiLoad 16/60 Superdex 75 and HiPrep 26/10 desalting columns. In this case, the purified protein solution was yellowish. In this protocol, 1.5 mg of selenomethionine-labelled protein was obtained from 23.5 g of wet cells. The purified selenomethionine-labelled HpaC protein was concentrated to 7.1 mg ml^{-1} (containing 1 m*M* DTT and 20 m*M* Tris–HCl pH 8.0) using an ultrafiltration membrane (Vivaspin 20, Vivascience).

The protein concentration was determined using the absorption measurement with a calculated molar absorption coefficient (ε_{280} = 10 185; obtained from the sum of the ε_{280} values of the one tryptophan and three tyrosine residues of HpaC). The purity of the resulting sample was confirmed by SDS–PAGE and the gels were stained with Coomassie Brilliant Blue (CBB). SDS–PAGE was carried out under reducing conditions using a 12.5% separation gel, as described previously (Laemmli, 1970).

2.2. Crystallization

The crystallization was performed by the hanging-drop vapour-diffusion method. The protein solution was allowed to equilibrate against 300 µl of reservoir solution at 293 K. Each droplet was formed by mixing equal volumes (2–5 µl) of protein solution and reservoir solution. The crystallization was initially carried out using Crystal Screens I and II (Hampton Research) and JBScreen (Jena BioScience). After the initial crystallization trials using these screening kits, the chosen crystallization conditions were optimized by employing finer intervals of pH and precipitant concentration.

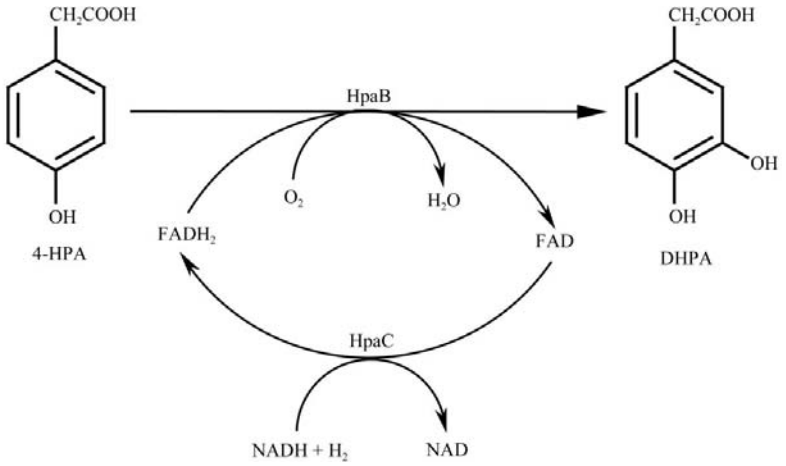

Figure 1
Reaction catalyzed by HpaB and HpaC.

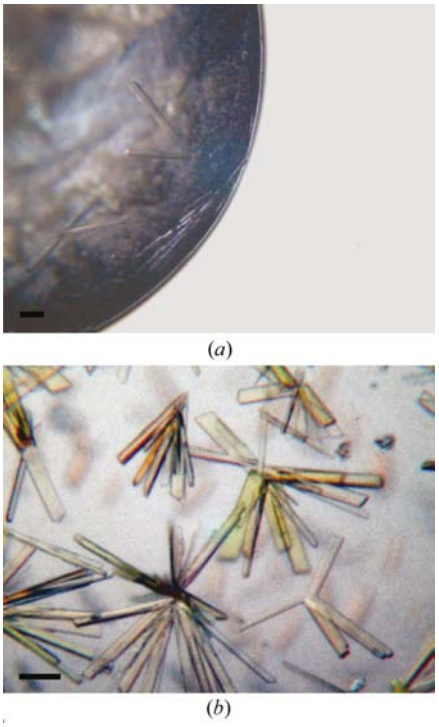

Figure 2
Crystals of HpaC. The bar corresponds to 0.1 mm. (*a*) Apo-HpaC, (*b*) HpaC–flavin complex.

2.3. X-ray data collection

Diffraction data were collected using a Rigaku R-AXIS V imaging-plate detector at the BL45XU beamline, SPring-8 (Harima, Japan). The crystals were flash-frozen in a nitrogen-gas stream at 90 K directly from a drop containing 10%(*w*/*v*) glycerol as a cryoprotectant and were maintained at 90 K during data collection. The oscillation angle used was 1.0° and the crystal-to-detector distance was set to 200 mm. A MAD data set was collected from one selenomethionine-labelled crystal. The wavelength was set to 1.0000 Å for the native apo-HpaC and HpaC–flavin complex crystals. Three wavelengths, corresponding to the maximum f'' (peak), the minimum f' (edge) and a reference wavelength (remote), were selected for the selenomethionine-labelled crystal, as based on the fluorescence spectrum of the Se atom in the crystals. The diffraction data were processed with the *HKL*2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Crystals of HpaC

Crystals of the apo-HpaC protein were obtained using condition No. C3 of the JBScreen 3 [20%(*w*/*v*) polyethylene glycol (PEG) 4000, 10%(*v*/*v*) isopropyl alcohol, 0.5 *M* HEPES–NaOH pH 7.5] and crystalline precipitants were also observed in solutions containing PEG 4000, HEPES–NaOH buffer and isopropyl alcohol. The optimization of these crystallization conditions led to well formed crystals which were grown from a solution made by mixing equal volumes of protein solution and reservoir solution containing 40%(*w*/*v*) PEG 4000, 5%(*v*/*v*) isopropyl alcohol, 0.1 *M* HEPES–NaOH pH 7.0 and 10%(*v*/*v*) glycerol as a cryoprotectant.

The crystals appeared within 2 d and reached final dimensions of 0.5 × 0.02 × 0.01 mm after 5–8 d (Fig. 2*a*). They diffracted X-rays to a resolution of 1.85 Å. The space group of apo-HpaC was determined to be $P2_1$, with unit-cell parameters a = 41.38, b = 43.93, c = 74.76 Å, β = 92.95°.

Crystals of the HpaC–flavin complex were initially grown at 277 and 293 K from condition No. 7 of Hampton Research Crystal Screen II [10%(*w*/*v*) PEG 1000, 10%(*w*/*v*) PEG 8000]. After numerous trials for optimizing the crystallization conditions, the best single crystals of the HpaC–flavin complex were obtained at 293 K from a solution made up of equal volumes of protein solution and reservoir solution consisting of 20%(*w*/*v*) PEG 1000, 10%(*w*/*v*) PEG 8000 and 10%(*v*/*v*) glycerol as a cryoprotectant. Crystals appeared within 2 d and reached final dimensions of 0.3 × 0.05 × 0.01 mm after one to two weeks (Fig. 2*b*). They diffracted X-rays to a resolution of 1.3 Å. The space group of the crystals was also determined to be $P2_1$, with unit-cell parameters a = 40.77, b = 42.84, c = 73.24 Å, β = 92.86°.

The crystals of the apo-HpaC protein and the HpaC–flavin complex are isomorphous to each other. The Matthews coefficient V_M value of each crystal was estimated to be 2.1 Å^3 Da^{-1}, assuming that two molecules of the HpaC protein are contained in an asymmetric unit (Matthews, 1968). The solvent content was calculated to be 41.1%, which is within the range of values typically observed for protein crystals. The crystallographic data and the diffraction data-collection statistics are summarized in Table 1.

Table 1
Data-collection statistics for HpaC crystals.

Values in parentheses are for the highest resolution shell.

	Apo-HpaC	HpaC–FAD	MAD selenomethionine		
Wavelength (Å)	1.0000	1.0000	0.9790 (peak)	0.9795 (edge)	1.0000 (remote)
Space group	$P2_1$	$P2_1$	$P2_1$		
Unit-cell parameters (Å, °)	a = 41.38, b = 43.93, c = 74.76, β = 92.95	a = 40.77, b = 42.84, c = 73.24, β = 92.86	a = 47.00, b = 58.14, c = 53.39, β = 108.31		
Resolution (Å)	50–1.85 (1.92–1.85)	50–1.30 (1.35–1.30)	50–1.80 (1.86–1.80)		
No. of measurements	79298	202161	75415	69567	72261
No. unique reflections	23013	61498	49135	45864	45863
Completeness (%)	99.3 (99.4)	99.1 (98.9)	92.9 (95.7)	92.6 (94..2)	92.5 (95.1)
R_{merge}	0.061 (0.146)	0.068 (0.247)	0.060 (0.234)	0.061 (0.183)	0.058 (0.226)
Average $I/\sigma(I)$	26.7	17.3	21.6	23.4	24.6
Multiplicity	3.45	3.29	1.53	1.52	1.58

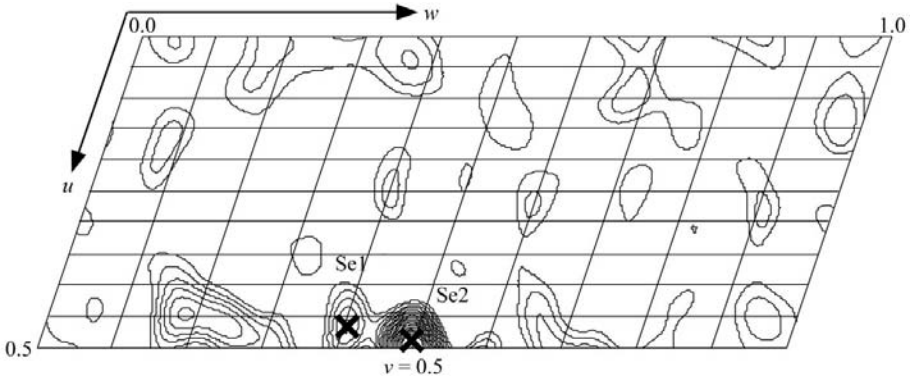

Figure 3
Harker section (v = 0.5) of the Bijvoet anomalous difference Patterson map calculated using diffraction data (>1σ; 40–2 Å resolution) collected at a wavelength of 0.9790 Å (peak data) for the selenomethionine-labelled crystal. The crosses correspond to Se-atom self-vectors.

3.2. MAD data collection of selenomethionine-labelled crystals

The crystallization of the selenomethionyl proteins was performed under the same conditions as used for the HpaC–flavin complex. The selenomethionine-labelled crystals diffracted X-rays to a resolution of 1.8 Å. They belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 47.00, b = 58.14, c = 53.39 Å, β = 108.31°; these crystals are not isomorphous to those of the HpaC–flavin complex. The Matthews coefficient V_M value calculated with two molecules per asymmetric unit is 2.1 Å^3 Da^{-1} (Matthews, 1968), which is similar to that calculated for the native complex. The X-ray absorption spectrum at the Se *K* edge was measured using one crystal for MAD data collection. Based on the fluorescence spectrum, two energy levels were chosen for the data collection; both energy levels were near the absorption edge of the Se atom: 12.1235 keV (λ = 0.9790 Å) and 12.0123 keV (λ = 0.9795 Å). The third energy level was set to 12.2345 keV (λ = 1.0000 Å) as a remote point. The crystallographic data and the MAD data-collection statistics for the selenomethionine-labelled crystals are also given in Table 1.

The Se-atom positions were obtained from a Patterson map calculated using the

observed anomalous differences as coefficients with the program *XTALVIEW* (McRee, 1999). A Harker section ($v = 0.5$) of the Bijvoet anomalous difference Patterson map of the peak data set showed two clear peaks corresponding to the two Se atoms out of the six expected in the asymmetric unit (Fig. 3). Atomic coordinates of the two clear Se sites [Se1 (0.2263, 0, 0.0877) and Se2 (0.2327, 0, 0.1979)] were refined by the maximum-likelihood phase refinement method. The initial electron-density map was calculated by *SOLVE* (Terwilliger & Berendzen, 1999) using these MAD data and model building is now under way.

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