Acta Cryst. (1998). D**54**, 1017–1019

Crystallization and preliminary X-ray diffraction studies on the water soluble form of rat heme oxygenase-1 in complex with heme

YOSHIAKI OMATA,[a] SHINYA ASADA,[b] HIROSHI SAKAMOTO,[a] KEIICHI FUKUYAMA[b] AND MASATO NOGUCHI[a]* *at [a]Department of Medical Biochemistry, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan, and [b]Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan. E-mail: mnoguchi@med.kurume-u.ac.jp*

(*Received 1 November 1997; accepted 26 February 1998*)

Abstract

The water-soluble portion of rat heme oxygenase-1 which lacks 22 hydrophobic amino-acid residues at the C-terminus was expressed in *E. coli* and crystallized in the form of a complex with heme by the vapor-diffusion method using polyethylene glycol 4000 as the precipitant. The crystals belong to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell dimensions of $a = b = 56.7$, $c = 186.7$ Å. The crystal contains one heme–heme oxygenase-1 complex in an asymmetric unit and diffracts X-rays beyond 3.0 Å resolution with a conventional X-ray source.

1. Introduction

Heme oxygenase (HO), a microsomal enzyme which oxidizes protoheme to biliverdin IXα, plays a key role in physiological heme metabolism. The first step of heme degradation catalyzed by HO is the oxidation of heme to α-hydroxyheme, requiring O_2 and reducing equivalents from NADPH-cytochrome P450 reductase (fp_2) (Yoshida, *et al.*, 1981; Yoshinaga *et al.*, 1990). The second step is the formation of verdoheme with concomitant release of hydroxylated α-*meso*-carbon as CO; this step is also O_2 dependent, but the opposed results have been reported concerning the necessity of reducing equivalents from fp_2 (Liu *et al.*, 1997; Matera *et al.*, 1996). In the third step, biliverdin is formed from verdoheme in a reaction which requires O_2 and reducing equivalents from fp_2 (Yoshida & Noguchi, 1984). Two isoforms of HO, HO-1 and HO-2, are known. HO-1 is highly expressed in spleen and liver and is in charge of degradation of heme derived mainly from senescent erythrocytes. HO-1 is inducible by heme itself and various xenobiotics. In contrast, HO-2 is fully refractory to the inducers for HO-1 and is constitutively expressed in brain and testis (for review, see Abraham *et al.*, 1996). In the past decade cDNAs for both forms were isolated from several sources and sequenced. Rat HO-1 (32 kDa) consists of 289 amino acids possessing a hydrophobic membrane-binding domain at the C-terminus. The proximal ligand to heme bound to rat HO-1 was established to be His25 (Ito-Maki *et al.*, 1995), but the distal ligand has not yet been identified. To investigate further the heme-degradation chemistry that is still controversial and also to learn about the oxygen-activation mechanism of HO, information on the three-dimensional structure of HO is indispensable. A water-soluble 30 kDa fragment that was obtained by proteolytic digestion of the full-length HO-1 has been proved to retain the ability to catalyze biliverdin formation (Ishikawa *et al.*, 1991). Hence, we constructed an expression vector carrying a truncated HO-1 gene (Met1–Pro267), which was completely identical to the corresponding sequence of the rat HO-1, and expressed a large amount of the water-soluble form of HO-1, which was fully active as the membrane-bound forms. Further we obtained crystals of the HO-1 enzyme in complex with heme (heme–HO-1 complex) suitable for high-resolution X-ray analysis. Here we describe the crystallization and preliminary X-ray diffraction analysis of the heme–HO-1 complex.

2. Experimental and results

2.1. *Expression and purification of HO-1*

An expression vector carrying the *HO*-1 gene was constructed according to the method of Wilks & Ortiz de Montellano (1993) with slight modifications. The gene encoding the water-soluble portion of HO-1 which lacks 22 amino acids at the C-terminus was amplified by the polymerase chain reaction from rat spleen cDNA library (Clontech) as a template. The sense primer (25 b) including an *Nde*I restriction site at the initiation codon, and the antisense primer (25 b) including a termination codon at Leu268 and a consecutive *Sal*I restriction site were utilized to obtain a truncated HO-1 (Met1–Pro267). The DNA obtained was inserted into pUC18 and screened in *E. coli* JM109. The cloned vector which had an identical sequence with the rat *HO*-1 gene (Shibahara *et al.*, 1985) was digested with *Nde*I and *Sal*I. The expression vector was obtained by ligating the digested fragment to pBAce (Craig *et al.*, 1991) which had *pho*A promoter, followed by transfection into *E. coli* JM109.

The transformed cells were cultured at 310 K in Luria–Bertani broth with rotation at 250 rev min^{-1}, and 1 ml of the overnight culture was inoculated into 10 l of the low-phosphate induction media (Craig *et al.*, 1991) maintained at 303 K. The cells were grown for 24 h with continuous aeration by bubbling, and harvested. The lysate was obtained from the cells with lysozyme (Sigma) and sonication in 50 m*M* Tris–HCl buffer (pH 8.0) containing 2 m*M* EDTA and 23 µg ml^{-1} phenylmethylsulfonyl fluoride, followed by centrifugation. Solid ammonium sulfate was added to the lysate, and 30–60% saturated ammonium sulfate fraction was dissolved with 20 m*M* potassium phosphate buffer (pH 7.4) and then dialyzed against the same buffer. Insoluble materials were removed by centrifugation, and the greenish dialyzed solution was applied to a hydroxylapatite (Bio-Rad) column (5 × 5 cm) equilibrated with 20 m*M* potassium phosphate buffer (pH 7.4). The column was washed with the same buffer until the green materials were removed, then eluted with a 20–150 m*M* linear gradient consisting of 500 ml each of potassium phosphate buffer (pH 7.4). The fractions containing expressed *HO*-1 as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis were combined and dialyzed against 10 m*M* Tris–HCl buffer (pH 7.4). The protein was applied to a POROS HQ column

Table 1. *Purification of the expressed HO-1 from 10 l culture media*

	Total protein (mg)	Total activity (unit†)	Specific activity (unit mg^{-1})	Yield (%)	Purification (fold)
Cell lysate	1620	2040000	1260	100	1
Ammonium sulfate	1080	2000000	1850	98	1.5
Hydroxylapatite	275	1360000	4930	67	3.9
POROS HQ	133	1100000	8300	54	6.6

† The activity was assayed according to the published procedure, and a unit of activity is defined as the amount of enzyme which produces 1 nmol of bilirubin h^{-1} (Yoshida & Kikuchi, 1978).

(10 ml) attached to ConSep LC100 (PerSeptive Biosystems) equilibrated with 10 m*M* Tris–HCl buffer (pH 7.4) at a flow rate of 5 ml min^{-1}. After washing with 50 ml of the same buffer, the protein was eluted with a linear gradient of 0–62.5 m*M* KCl in the same buffer. The fractions containing HO-1 were condensed by ultrafilteration and stored at 193 K until use.

A typical preparation of HO-1 is summarized in Table 1. A 10 l culture provides 100–130 mg of electrophoretically homogenous enzyme with 50% yield. The enzyme activity of expressed HO-1 was equal to or greater than the activity reported for the native enzyme which had been purified from pig spleen (Yoshida & Kikuchi, 1978) or rat liver (Yoshida & Kikuchi, 1979). The heme–HO-1 complex was prepared essentially as described previously (Yoshida & Kikuchi, 1979).

2.2. *Crystallization and X-ray diffraction studies*

Initial crystallization experiments of the heme–HO-1 complex were performed with the hanging-drop vapor-diffusion method at 293 K using Crystal Screen I (Hampton Research). The protein concentration was 25 mg ml^{-1}. In a typical experiment, 5 µl of the protein in 50 m*M* potassium phosphate buffer pH 7.0 was mixed with the same volume of the reservoir solution and equilibrated against 1 ml of the reservoir solution. Crystals were grown from one of the conditions (No. 22). Exploration of similar conditions established that the best crystals could be grown when 0.1 *M* Tris buffer (pH 8.5) containing 30% PEG 4000 and 0.2 *M* sodium acetate was used as the reservoir solution. Needle-shaped crystals appeared after a few days and reached a maximum size (*ca* 0.1 × 0.1 × 1.0 mm) in one week (Fig. 1).

Fig. 1. Crystals of heme–HO complex. The most developed direction of the crystal is the *c* axis.

Table 2. *Result of data collection of heme-HO-1 complex*

Resolution	$\langle F^2/\sigma(F^2)\rangle$	No. of unique reflections	Completeness (%)
15.0	21.0	77	87.5
10.0	19.7	154	96.9
7.5	18.4	272	96.5
5.0	11.8	1044	96.2
3.5	8.0	2604	93.4
3.0	2.5	1847	78.9
Total	7.9	5998	88.9

The crystal was sealed in a glass capillary tube with a small amount of mother liquor. Diffraction data were collected on the R-AXIS IV image-plate system (Rigaku) at room temperature using Ni-filtered Cu $K\alpha$ radiation. X-rays were focused with two perpendicular bent mirrors coated with nickel. Diffraction data recorded on the image plate were processed using the R-AXIS software (Higashi, 1990). The crystals belong to tetragonal system, with unit-cell dimensions $a = b = 56.7$, $c = 186.7$ Å. The Laue group was determined as $4/mmm$ by the analysis of equivalent reflections from the diffraction data. The observed systematic absences are compatible with space group $P4_12_12$ or $P4_32_12$. Assuming that one complex molecule is present in an asymmetric unit, the V_m value is 2.4 $Å^3$ Da^{-1} (Matthews, 1968). The result of a typical intensity measurement is shown in Table 2. The 24 773 observations merged to 5 998 unique reflections with an R_{merge} of 8.1%. Table 2 shows that the present crystals yield diffraction data to 3.0 Å resolution with conventional X-ray source and are suitable for high-resolution X-ray analysis. The search for heavy-atom derivatives and preparation of selenomethionyl HO-1 are under way.

We thank Dr T. Hidaka (Kurume University) for providing the expression vector. This work is supported by grant-in-aid for scientific research on the priority area (Molecular Biometallics) 08249104 (to MN) and 09235217 (to KF) from the Ministry of Education, Science, Sports, and Culture, Japan.

References

Abraham, N. G., Drummond, G. S., Lutton, J. D. & Kappas, A. (1996). *Cell. Physiol. Biochem.* **6**, 129–168.

Craig, S. P. III, Yuan, L., Kuntz, D. A., McKerrow, J. H. & Wang, C. C. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 2500–2504.

Higashi, T. (1990). *PROCESS: A Program for Indexing and Processing R-AXIS II Imaging Plate Data*, Rigaku Corporation, Tokyo, Japan.

Ishikawa, K., Sato, S. & Yoshida, T. (1991). *Eur. J. Biochem.* **200**, 161–165.

Ito-Maki, M., Ishikawa, K., Matera, K. M., Sato, M., Ikeda-Saito, M. & Yoshida, T. (1995). *Arch. Biochem. Biophys.* **317**, 253–258.

Liu, Y., Moënne-Loccoz, P., Loehr, T. M. & Ortiz de Montellano, P. R. (1997). *J. Biol. Chem.* **272**, 6909–6917.
Matera, K. M., Takahashi, S., Fujii, H., Zhou, H., Ishikawa, K., Yoshimura, T., Rousseau, D. L., Yoshida, T. & Ikeda-Saito, M. (1996). *J. Biol. Chem.* **271**, 6618–6624.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Shibahara, S., Müller, R., Taguchi, H. & Yoshida, T. (1985). *Proc. Natl Acad. Sci. USA*, **82**, 7865–7869.
Wilks, A. & Ortiz de Montellano, P. R. (1993). *J. Biol. Chem.* **268**, 22357–22362.
Yoshida, T. & Kikuchi, G. (1978). *J. Biol. Chem.* **253**, 4224–4229.
Yoshida, T. & Kikuchi, G. (1979). *J. Biol. Chem.* **254**, 4487–4491.
Yoshida, T. & Noguchi, M. (1984). *J. Biochem. (Tokyo)*, **96**, 563–570.
Yoshida, T., Noguchi, M., Kikuchi, G. & Sano, S. (1981). *J. Biochem. (Tokyo)*, **90**, 125–131.
Yoshinaga, T., Sudo, Y. & Sano, S. (1990). *Biochem. J.* **270**, 659–664.