Cloning and Expression of cDNA for Soluble Form of Rat Heme Oxygenase-1

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Summary: Heme oxygenase catalyzes the oxidation of heme to biliverdin and carbon monoxide. The gene encoding the truncated soluble rat heme oxygenase-1 (Met1-Pro267) was cloned. The enzyme protein was expressed in E. coli JM109 and purified to homogeneity. The molecular weight of the recombinant enzyme was 30 kDa as assessed by SDS-polyacrylamide gel electrophoresis. From a 3-L culture, about 90 mg of the purified enzyme was routinely obtained. The dependency of the heme oxygenase reaction catalyzed by the soluble enzyme on the NADPH-cytochrome P-450 reductase concentrations and the effect of catalase on the reaction were examined to compare with the purified membrane-bound form of heme oxygenase-1 (Yoshida and Kikuchi, 1978b). The activity of the soluble enzyme was inhibited at high concentrations of NADPH-cytochrome P-450 reductase and the inhibition was not alleviated by addition of catalase unlike the membrane-bound form. The ferric iron of the heme-heme oxygenase complex was in a typical high spin state at acidic to neutral pH (pH 6.5-7.0), but conversion to low spin state was observed at basic pH (pH 9-10). The heme bound to heme oxygenase was converted to biliverdin at a stoichiometric ratio of unity in the presence of NADPH-cytochrome P-450 reductase system. During the heme degradation of the heme-heme oxygenase complex under atmospheric oxygen, several intermediates, that is, oxygenated heme and verdoheme, were spectrally discriminated.

Key words: heme oxygenase-1, heme degradation, biliverdin, bilirubin, hydroxyheme, verdoheme, electron spin resonance

Introduction

Protopheme is catabolized to biliverdin IXα by the microsomal heme oxygenase system consisting of two proteins, heme oxygenase (HO) and NADPH-cytochrome P-450 reductase at the expense of NADPH and dioxygen (Tenhunen et al. 1969). HO is not a heme protein by nature, but it easily binds with heme to form an

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1 Abbreviations used are: HO, heme oxygenase; PCR, polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; ESR, electron spin resonance.
enzyme-substrate complex (heme-HO complex). Studies with heme-HO complex revealed that the heme degradation reaction proceeds in the following way (for review, see Kikuchi and Yoshida, 1980). First, ferric heme binds to the HO protein to form a ferric heme-HO complex and the ferric heme is then reduced to the ferrous state by the NADPH-cytochrome P-450 reductase, followed by binding with dioxygen to form an oxygenated heme-HO complex. The dioxygen bound to heme iron appears to be activated by another electron from the NADPH-cytochrome P-450 reductase, and the dioxygen attacks the $\alpha$-methene carbon to yield $\alpha$-hydroxyheme. Next the $\alpha$-hydroxyheme is converted to a verdoheme-like intermediate which was originally designated as 688-nm substance because of its characteristic absorption at 688 nm in the ferrous state (Yoshida et al. 1980a; Noguchi et al. 1981; Yoshida and Noguchi, 1984). Another dioxygen and probably another electron are required for the formation of this verdoheme-like intermediate and at this step the $\alpha$-methene carbon is liberated as carbon monoxide (Yoshida et al. 1982). The intermediate is then further oxidized to a biliverdin-iron complex with consumption of another dioxygen. Finally, release of iron from the biliverdin-iron complex yields biliverdin.

The possible chemical sequence of heme degradation by the HO system is

Scheme 1. The possible intermediates during the degradation of heme into biliverdin by the HO system.
depicted in Scheme 1.

The HO enzyme has been purified from several sources, including pig spleen (Yoshida and Kikuchi, 1978a), bovine spleen (Yoshinaga et al. 1982) and chicken liver (Bonkovsky et al. 1990). The cDNAs for the rat (Shibahara et al. 1985), human (Yoshida et al. 1988), and chicken (Evans et al. 1991) enzymes have been isolated and sequenced. Two forms of the enzyme, known as HO-1 and HO-2, have so far been identified; the HO-1 enzyme is induced by a number of metals such as cobalt, cadmium and tin (Maines and Kappas, 1977) as well as by a host of unrelated chemicals including hemin (Tenhunen et al. 1968), phenylhydrazine (Maines and Veltman, 1984) and bromobenzene (Guzelian and Elshourbagy, 1979). The HO-1 enzyme is highly expressed in spleen and liver and is believed to be in charge of degradation of the hemoglobin heme derived from senescent erythrocytes. In contrast, the HO-2 enzyme is fully refractory to the inducers for HO-1 and is constitutively expressed in many tissues (Braggins et al. 1986; Maines et al. 1986; Trakshel et al. 1986).

The magnetic properties of the intermediates appearing during the heme degradation reaction are still poorly understood and the structural studies of the enzyme have never been performed. A relatively large amount of HO protein, however, is needed for such studies on the heme-HO complex. An early attempt (Ishikawa et al. 1991) to obtain the entire HO protein including the C-terminal membrane-binding region from E. coli strains transfected with expression vectors carrying the full length HO gene was hampered by insertion of the expressed enzyme into bacterial membranes and also by partial proteolytic digestion leading to production of a variety of fragments inside the bacterial cells. Recently Wilks and Ortiz de Montellano (1993) have constructed an expression vector carrying a truncated HO-1 gene (Met1-Thr266) without the sequence coding for the last 23 amino acids and expressed a soluble and catalytically active HO-1. But the protein had two amino acid mutations of Ser262Arg and Ser263Leu. In the present study we report the construction of an expression vector carrying a truncated HO-1 gene (Met1-Pro267) which is completely identical to the corresponding sequence of the rat HO-1 protein, and the characterization of the expressed protein.

Materials and Methods

Cloning of cDNA of rat HO-1

The strategy for cloning of the HO-1 cDNA is outlined in Scheme 2. A λgt10 rat spleen cDNA library (Clontech) was screened by PCR to obtain a cDNA fragment encoding the soluble region of the enzyme. The PCR amplification was carried out with a sense primer of 5'C AGT CCC CAT ATG GAG CGC CCA CAG3' and an antisense primer of 5'GAC CCA GTC GAC GTA CCG TGT CTG G3'. The primer pair was designed as described previously (Wilks and Ortiz de Montellano, 1993) with slight modifications. In the sense primer a Nde I site (underlined) is inserted just prior to the initiation codon 5'ATG3', while in the antisense primer a termination codon 5'TTA3' and a Sal I site (underlined) are inserted in tandem.
just after the codon 5’CGG’ (Pro267). Thus a DNA fragment of 826bp, which lacked the hydrophobic membrane-binding region consisting of 22 amino acids at the C-terminus, was obtained. The DNA fragment was trimmed by the Klenow fragment of T4 DNA polymerase to create blunt ends and was inserted into the Sma I site in the multiple cloning site of pUC18 vector. The recombinant pUC18 was transfected into E. coli JM109. The transformed E. coli colonies were selected by X-gal blue-white screening on LB-broth agarose plates.

The plasmid DNA carrying the HO gene in a proper direction was selected by restriction digestion with Nde I, Sal I and EcoR I, and sequenced by the dideoxy chain termination method (Sanger et al. 1977) using a DNA sequencer (ALF™ II DNA sequencer, Pharmacia Biotech). The nucleotide sequence was proved to be completely identical with the sequence of the soluble region of rat HO-1 as previously reported (Shibahara et al. 1985). The open reading frame fragment encoding Met1-Pro267 was excised by Nde I and Sal I and ligated to the Nde I and Sal I sites of an expression vector pBAce. The pBAce vector has a pho A promoter just upstream to the cloning site, and the activity of this promoter is greatly enhanced under a low phosphate condition (Craig et al. 1991). After transfection into E. coli JM 109, the pBAce-derived vector carrying the truncated HO-1 gene was identified by digestion with restriction enzymes and sequencing. The expression vector thus obtained was named pBA-THO1rs. The transformed E. coli was suspended in LB broth containing 25% glycerol, divided into 0.5-ml portions in 1.5-ml Eppendorf tubes and stored at −80°C until use.

**Expression and Purification of the Truncated HO-1**

The low phosphate medium for induction of the bacterial pho A promoter consists of 50 mM NaCl, 40 mM 3-[N-morpholino]propane-sulfonic acid (MOPS), 4 mM tricine, 9.5 mM NH₄Cl, 0.52 mM MgCl₂, 0.28 mM K₂SO₄, 10 μM FeSO₄, 0.5 μM CaCl₂, 0.4 μM H₂BO₃, 80 mM MnCl₂, 30 mM CoCl₂, 10 mM CuSO₄, 10 mM ZnSO₄, 3 nM (NH₄)₆Mo₇O₂₄, 0.2% casamino acids, 75 mg/l ampicillin, 0.1 mM phosphate (an equimolar mixture
Fig. 1. SDS-PAGE analysis. Lane A, molecular weight markers; B, cell lysate of *E. coli* JM109 transfected with control pBAce vector; C, cell lysate of *E. coli* JM109 transfected with pBA-THO1rs; D, purified HO-1.

of Na₂HPO₄ and NaH₂PO₄), 1.5 μM thiamin and 20 mg/l adenine. A 20-ml inoculum in the low phosphate induction medium was prepared from the transformed *E. coli* JM109. At the mid-log phase, a 100-μl aliquot was inoculated into 330 ml of the same culture medium. The cells were grown at 30°C with a rotary shaker at 300 rpm for 18-24 hs and the media became green during the incubation period. A protein of molecular mass of about 30 kDa was expressed in large amounts in the cultures (Fig. 1C). Culturing for more than 24 hs resulted in partial proteolysis of the 30 kDa form to a 28 kDa protein. Three liters of the expression medium were combined and used for the following enzyme purification. The purification manipulations were carried out at 4°C. The harvested cells were sonicated in 50 mM Tris-HCl (pH 8.0) containing 6 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, and 1 mM phenylmethylsulfonylfluoride, and centrifuged at 15,000 × g for 60 min. Ammonium sulfate was added to the supernatant to a final concentration of 20% saturation and the solution was gently mixed for 60 min with a seesaw mixer. Following centrifugation (15,000 × g for 60 min), the ammonium sulfate concentration was raised to 50% saturation. The 20-50% precipitates were collected by centrifugation and resuspended in 10 mM potassium phosphate (pH 7.4). The sample was divided into two parts, separately applied on two hydroxylapatite columns (2.5×10 cm) and eluted by a 400-ml linear gradient from 10 mM to 0.2 M potassium phosphate (pH 7.4). The HO-active fractions were combined and dialyzed against 10 mM potassium phosphate (pH 7.4). The dialyzed sample was diluted with the same volume of 40 mM Tris-HCl buffer (pH 8.0) and applied onto a Poros HQ 10 column (10-ml bed volume) connected to a ConSep LC-100 system (PerSeptive Biosystems) equilibrated with 20 mM Tris-HCl (pH 8.0). The column was washed with 50 ml each of the same buffer and 20 mM Tris-HCl (pH 7.5) successively. The protein was eluted at a flow rate of 5 ml/min with a linear gradient of KCl (0-62.5 mM) in 20 mM
Tris-HCl (pH 7.5) over 20 min and with 62.5 mM KCl in the same buffer for 20 min. Then the KCl concentration was raised linearly up to 250 mM over 20 min. The HO activities emerged from the column around the end of the first gradient.

Assay procedures

The HO activity was assayed photometrically as previously described (Yoshida and Kikuchi, 1978a). Briefly, the standard reaction mixture, if not otherwise specified, contained an appropriate amount of HO preparation, 15 μM hemin, 1 μM bovine serum albumin, an excess amount of partially purified biliverdin reductase (Noguchi et al. 1979), 0.5 unit of purified NADPH-cytochrome P-450 reductase (Yasukochi and Masters, 1976), and 100 mM Hepes-KOH (pH 7.5) in a final volume of 0.2 ml. The measurements were carried out at 37°C using a JASCO V-560 UV/VIS spectrophotometer. The reaction was initiated by addition of NADPH at a final concentration of 150 μM. NADPH was omitted from the blank cuvette. The amount of bilirubin formed was calculated using a difference extinction coefficient of 43.5 mM⁻¹cm⁻¹ at 468 nm. A unit of heme oxygenase activity is defined as the amount of enzyme catalyzing the formation of 1 nmol bilirubin per h. A unit of NADPH-cytochrome P-450 reductase is defined as the amount of enzyme that catalyzes the reduction of 1 μmol cytochrome c in 1 min. Protein concentration was measured by the method of Bradford using the dye reagent protein assay kit (Bio-Rad) and bovine serum albumin as standard (Bradford, 1976). Hemin concentration was determined by pyridine hemochrome method (Furhop and Smith, 1975). The X-band ESR spectroscopy was performed using a JEOL-FE3X spectrometer equipped with a JEOL liquid helium cryostat (ESLTR3X).

Preparation of heme-HO complex

Hemin chloride was mixed with the purified HO-1 at a molar ratio of 2:1 in 10 mM potassium phosphate (pH 7.4) and the mixture was applied on a hydroxylapatite column (2.5×3 cm) equilibrated with 10 mM potassium phosphate (pH 7.4). After washing with 20 times the column bed volume of the same buffer, heme-HO complex was eluted with 110 mM potassium phosphate (pH 7.4). The concentration of heme-HO complex was estimated at pH 7.4 with an extinction coefficient of 140 mM⁻¹cm⁻¹ at 405 nm (Yoshida and Kikuchi, 1978a).

Results and Discussion

Cloning, expression and purification of rat HO-1

A DNA fragment carrying the gene for the soluble region of rat HO-1 was amplified using PCR with the primer pair described under the Experimental Procedures section. The gene was expressed in E. coli JM109 with the aid of the expression vector pBAce, which has a highly active promoter, alkaline phosphatase promoter (pho A), just upstream of the cloning sites. HO-1 in the host strain JM109 was induced remarkably under a low phosphate condition (Fig. 1C). The induction of the HO-1 protein at high levels was also evident from the pale green color of the
culture medium and of the cell pellet due to biliverdin accumulated as reported previously (Ishikawa et al. 1991; Wilks and Ortiz de Montellano, 1993). The recombinant soluble HO-1 was purified from the cell lysate to homogeneity by a combination of ammonium sulfate fractionation and hydroxylapatite and ion exchange (Poros HQ10) column chromatographies. A typical purification result is summarized in Table 1. The final specific activity, 6500–7000 unit/mg protein in the standard reaction system, was very close to the activities of the microsomal HO-1 of rat spleen (Yoshida and Kikuchi, 1978a) and the soluble HO-1 (Wilks and Ortiz de Montellano, 1993). The purified HO-1 migrated as a single band and showed an apparent molecular mass of 30 kDa (Fig. 1D), which agreed with the molecular mass calculated from the nucleotide sequence. The HO-1 protein consists of 267 amino acid residues, Met1-Pro267, of the rat microsomal enzyme without any mutations. In the enzyme derived from the pBAHO30 vector prepared by Wilks and Ortiz de Montellano (1993), however, serine residues 262 and 263 are replaced by arginine and leucine, respectively. When the transformed JM109 cells were cultured for more than 24 hs at 37°C, a fragmented protein (28 kDa) appeared. It remains unclear whether or not the 28 kDa protein observed in this study is identical with the 28 kDa enzymes reported by other investigators (Ishikawa et al. 1991; Wilks and Ortiz de Montellano, 1993).

We subcloned the PCR product into pUC18 vectors in order to make use of the blue-white screening and of commercially available universal primers for sequencing. We were able to achieve the direct ligation of the fragment obtained by the digestion of the PCR product with Nde I and Sal I into the corresponding sites of pBAce.

### Characterization of the HO activity of the purified HO-1

Figure 2 shows the profile of pH dependence of the HO activity of the purified HO-1. The optimum pH was 7.5. Of the buffers examined, Hepes gave the highest activity. No HO activity was observed without NADPH. The maximum activity was obtained at 100 to 150 μM

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**TABLE 1.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein mg</th>
<th>Total activity unit</th>
<th>Specific activity unit/mg</th>
<th>Purification -fold</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated cell supernatant</td>
<td>1,169</td>
<td>755,000</td>
<td>646</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>20-50% (NH₄)₂SO₄</td>
<td>715</td>
<td>695,000</td>
<td>972</td>
<td>1.5</td>
<td>92</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>321</td>
<td>1,089,000</td>
<td>3,390</td>
<td>5.3</td>
<td>144</td>
</tr>
<tr>
<td>Poros HQ10</td>
<td>89</td>
<td>628,000</td>
<td>7,050</td>
<td>10.9</td>
<td>83</td>
</tr>
</tbody>
</table>
Fig. 2. The profile of pH dependence of HO activity of the purified HO-1. Assays were carried out with standard reaction mixtures containing 3.2 μg purified HO-1 except for the use of 100 mM Hepes-KOH (●), Tris-HCl (○) or potassium phosphate (△). The actual pH values, measured with the reaction mixtures at 37°C, were plotted.

Fig. 3. The effects of catalase and NADPH-cytochrome P-450 reductase on the HO activity. Assays were carried out with the standard reaction mixtures containing 2.58 μg purified HO-1 in the presence (●) or absence (○) of 350 μg catalase. The amounts of NADPH-cytochrome P-450 reductase were varied as indicated.

NADPH. The HO activity increased with increasing concentrations of NADPH-cytochrome P-450 reductase, and reached the maximum around 0.5 unit/200 μl reaction mixture (~0.5 μM), and then decreased with further increases of the NADPH-cytochrome P-450 reductase concentration (Fig. 3). The concentration-dependent decrease of HO activity was not alleviated by the addition of catalase (Fig. 3) nor by increase of hemin and NADPH concentrations (data not shown). Previously we had shown that hydrogen peroxide, possibly produced by the NADPH-cytochrome P-450 reductase system during the heme degradation reaction, causes a considerable degree of nonspecific degradation of heme as well as inactivation of HO (Noguchi et al. 1983). Such hydrogen peroxide or the hydroxy radical derived from it, however, is probably not involved in the inhibition at high concentrations of NADPH-cytochrome P-450 reductase.

Heme-HO complex

The heme-HO complex was prepared as described in the Experimental Procedures section. As shown in Fig. 4, the spectrum of the complex displayed a Soret peak at 405 nm and bands at 500 and 630 nm at pH 6.5, indicating that the complex is in a ferric high spin state. Indeed, the ESR spectrum of the complex obtained at 7K (Fig. 5) showed typical high spin heme signals (g=6 and g=2). By raising the pH, conversion from the high to low spin state was observed as indicated by the absorption bands at 536 and 575 nm (Fig. 4). This spin con-
version was due to the replacement of the water ligand by hydroxide ion at the sixth coordination site (Takahashi et al. 1994).

As shown in Fig. 6, addition of a small amount of NADPH to the heme-HO complex in the presence of an excess amount of NADPH-cytochrome P-450 reductase resulted in a 5-nm red-shift of the Soret peak with a decrease of the absorption intensity, while the absorbances at 540, 575, 640 and 690 nm increased (spectrum 3), which indicated prompt formations of the oxygenated heme-HO complex and the verdoheme-like intermediate.

The absorption peaks at 410, 540 and 575 nm are characteristic for the ferrous dioxygen-bound heme of the heme-HO complex (Yoshida et al. 1980b) and the absorption bands at 690 and 640 nm are due to a ferrous verdoheme-like substance and its CO-bound form, respectively (Noguchi et al. 1981). The verdoheme-like compound had been discovered by us and designated 688-nm substance (Yoshida et al. 1980a; Noguchi et al. 1981; Yoshida and Noguchi, 1984). The chemical structure of this intermediate has not been unambiguously determined. Further addition of NADPH led
**Fig. 5.** ESR spectrum of the heme-HO complex. Measurement was carried out with a microwave power of 4 mW and 0.1 millitesla field modulation at 100 KHz at 7K. The heme-HO complex (0.1 mM) was dissolved in 0.1 M potassium phosphate at pH 6.5.

**Fig. 6.** Conversion of heme into biliverdin on the heme-HO complex.

The heme-HO complex (17 μM) in 0.2 ml of 110 mM potassium phosphate (pH 7.4) was placed in a cuvette and scanned (spectrum 1). An excess amount of NADPH-cytochrome P450 reductase (1.2 unit, 4 μl) was added (spectrum 2). NADPH was successively added in final concentrations of 9.7 μM (spectrum 3), 19.4 μM (spectrum 4), 37.7 μM (spectrum 5) and 58.0 μM (spectrum 6).
to decreases in absorption intensities of the four peaks (410, 540, 575 and 630 nm) and an accompanying transient increase of absorbance at 640 and 690 nm (spectrum 4). Finally the absorption spectrum reached the spectrum 6, a characteristic absorption spectrum of biliverdin IXα. The final product was converted to bilirubin IXα by addition of biliverdin reductase (data not shown). These results indicated that the recombinant enzyme obtained was a fully active HO enzyme able to catalyze the conversion of heme to biliverdin in the presence of the NADPH-cytochrome P-450 reductase system. With the recombinant HO-1 enzyme, detailed mechanistic studies of the HO reaction using ESR and stopped-flow spectroscopy are currently in progress.

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