Expression and Characterization of Truncated Human Heme Oxygenase (hHO-1) and a Fusion Protein of hHO-1 with Human Cytochrome P450 Reductase†

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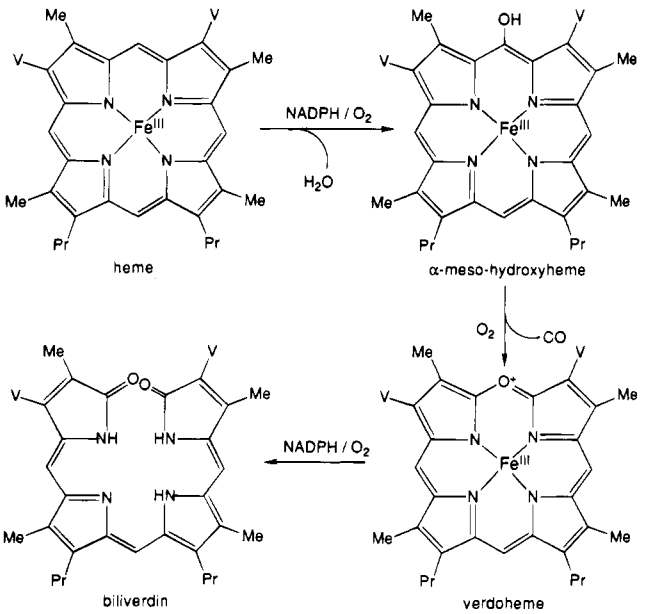
ABSTRACT: A human heme oxygenase (hHO-1) gene without the sequence coding for the last 23 amino acids has been expressed in *Escherichia coli* behind the *pho A* promoter. The truncated enzyme is obtained in high yields as a soluble, catalytically-active protein, making it available for the first time for detailed mechanistic studies. The purified, truncated hHO-1/heme complex is spectroscopically indistinguishable from that of the rat enzyme and converts heme to biliverdin when reconstituted with rat liver cytochrome P450 reductase. A self-sufficient heme oxygenase system has been obtained by fusing the truncated hHO-1 gene to the gene for human cytochrome P450 reductase without the sequence coding for the 20 amino acid membrane binding domain. Expression of the fusion protein in pCWori$^+$ yields a protein that only requires NADPH for catalytic turnover. The failure of exogenous cytochrome P450 reductase to stimulate turnover and the insensitivity of the catalytic rate toward changes in ionic strength establish that electrons are transferred intramolecularly between the reductase and heme oxygenase domains of the fusion protein. The V_{max} for the fusion protein is 2.5 times higher than that for the reconstituted system. Therefore, either the covalent tether does not interfere with normal docking and electron transfer between the flavin and heme domains or alternative but equally efficient electron transfer pathways are available that do not require specific docking.

Heme oxygenase catalyzes the NADPH- and cytochrome P450 reductase-dependent oxidation of heme[1] to biliverdin via a reaction sequence that involves α-*meso*-hydroxylation, extrusion of the α-*meso*-carbon as carbon monoxide with concomitant formation of verdoheme, and oxidative conversion of verdoheme to biliverdin (Scheme 1) (Tenhunen et al., 1969; Maines, 1992; Schmid & McDonagh, 1979). Biliverdin, the end product of heme catabolism, is reduced by biliverdin reductase to bilirubin, which is then conjugated with glucuronic acid and excreted (Maines, 1992; Schmid & McDonagh, 1979). Expression of the glucuronyl transferase is often deficient at birth, giving rise to neonatal jaundice and the potential for neurological damage (Hyman et al., 1969). Inhibition of heme oxygenase is therefore one avenue that has been pursued in the treatment of neonatal jaundice (Maines & Trakshel, 1992; Kappas et al., 1988; Drummond & Kappas, 1981). Recent reports suggest, furthermore, that the carbon monoxide produced by heme oxygenase in the brain may play a signaling role analogous to that of nitric oxide (Verma et al., 1993; Zhuo et al., 1993; Steven & Wang, 1993).

Scheme 1: Heme Oxygenase-Catalyzed Conversion of Heme to Biliverdin



Two forms of heme oxygenase are known. The enzyme responsible for catabolism of most of the body burdern of heme, known as HO-1, predominates in the liver, spleen, and most other tissues, and is induced by a variety of exogenous stimuli (Maines, 1992). The second form of the enzyme, known as HO-2, is not inducible, and is found in particularly high concentrations in brain and testes (Maines et al., 1986; Maines, 1988). The human HO-1 (hHO-1) and

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of the oxidation and ligation state of the iron; hHO-1, human HO-1 heme oxygenase; HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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HO-2 genes share approximately 40% sequence identity, whereas the rat and human HO-1 genes are approximately 80% identical (McCoubrey et al., 1992). Most of what is known about the structure and mechanism of heme oxygenases is based on model studies or studies of enzymes other than that from humans. hHO-1 is a 32 kDa protein that, like other HO-1 enzymes, is anchored to the membrane by a C-terminal lipophilic peptide of approximately 23 amino acids (Yoshida et al., 1988a,b). Catalytic turnover of the enzyme requires reducing equivalents from NADPH−cytochrome P450 reductase, a flavoprotein that is also anchored to the membrane by a terminal lipophilic domain. Absorption, resonance Raman, NMR, and site-specific mutagenesis studies of the rat enzyme indicate that heme is coordinated in that enzyme to His-25 (Wilks & Ortiz de Montellano, 1993; Sun et al., 1993, 1994; Hernández et al., 1994; Ishikawa et al., 1992).

Detailed studies of the mechanism and function of heme oxygenase have been difficult to carry out due to the intractability of the membrane-bound protein and, in the case of the human enzyme, the unavailability of source material. We recently expressed rat HO-1 in *Escherichia coli* as a truncated protein without the 23 C-terminal amino acid membrane anchor and showed that the protein is soluble, easily purified, and catalytically-active (Wilks & Ortiz de Montellano, 1993). We report here expression and characterization of the corresponding truncated human protein. More importantly, we describe high-yield expression of a soluble, catalytically self-sufficient protein in which truncated hHO-1 has been fused to truncated cytochrome P450 reductase, and demonstrate that electrons are efficiently transferred intramolecularly within the fusion protein.

EXPERIMENTAL PROCEDURES

General Methods. Plasmid purification, subcloning, and bacterial transformations were carried out as previously described (Wilks & Ortiz de Montellano, 1993). Deionized, doubly distilled water was used for all experiments. Oligonucleotides were synthesized at the Biomolecular Resource Center of the University of California at San Francisco using an Applied Biosystems 380B DNA synthesizer. HPLC was done on a Hewlett Packard Series II 1090 liquid chromatograph.

Bacterial Strains. E. coli strain DH 5α [F′, *ara* Δ(*lac-proAB*) rpsL ø80d*lacZ*ΔM15 *hsd* R17] was used for the expression of heme oxygenase and *E. coli* strain CJ 236 [*dut*-1, *ung*-1, *thi*-1, *rel* A-1; pCJ105(Cm^r)] for the isolation of uracil-laden DNA for mutagenesis.

Construction of pBH01, pCHO-1, and Fusion Vectors. The expression vector for truncated hHO-1 was constructed in pBAce (Craig et al., 1991). The truncated gene cloned into pBAce was obtained by PCR from the full-length gene in an Okayama−Berg vector provided by Shibahara et al. (1985). The 5′-sense oligonucleotide primer (5′-CACCG-GCCATATGGAGCGTCCGCAA-3′) coded for an *Nde*I site at the N-terminus. The 3′-antisense oligonucleotide primer (5′-CATCGGTCGACTTAAGCCTGGGAG-3′) encoded the termination codon TAA at the proline 23 amino acids from the C-terminus immediately preceding a *Sal*I site. The reaction conditions were as described for the rat heme oxygenase (Wilks & Ortiz de Montellano, 1993). The target fragment was gel-purified and cloned into pBAce to give vector pBHO1 as previously described (Wilks & Ortiz de Montellano, 1993). Transformants were screened by restriction digestion and confirmed by sequencing.

For later experiments, the gene was subcloned (*Nde*I/*Xba*I) into the pCWori$^+$ vector for expression under control of the *lac* operon (Muchmore et al., 1989). The NADPH−cytochrome P450 reductase gene was obtained within the pECE expression vector as a fusion protein with the cytochrome $P450_{scc}$ gene cloned at the 5′-end of the fusion (Black et al., 1994). At the C-terminus of the $P450_{scc}$ gene is the linker sequence coding for Thr-Asp-Gly-Thr-Ser followed by the P450 reductase gene minus the sequence coding for the 20 N-terminal amino acids. The stop codon at the C-terminus of the hHO-1 gene was replaced by PCR with the Thr-Asp-Gly-Thr-Ser linker containing an *Spe*I site. The N-terminus of hHO-1 was PCR-amplified with a *Kpn*I site prior to the *Nde*I site at the initiation codon. The hHO-1 was then subcloned as a *Kpn*I/*Spe*I fragment into the pECE vector, replacing the $P450_{scc}$ cDNA. The full-length fusion (2.8 kb) was sequenced and subcloned into the *Nde*I/*Xba*I sites of pCWori$^+$ to give vector pCHO-1 that was used for expression in *E. coli*.

Expression and Purification of the Truncated Heme Oxygenase. Expression and purification of the truncated heme oxygenase were carried out as previously described for the rat heme oxygenase (Wilks & Ortiz de Montellano, 1993). A 3 mL inoculum in LB-amp induction media was prepared from plates with fresh colonies of transformed *E. coli* DH5αF′ cells. Two-liter cultures were inoculated (2 mL) from the overnight cultures, grown at 37 °C to an OD_{600} of 0.3−0.5, and induced with a final 1 mM concentration of IPTG. The cells were grown at 30 °C for 18 h or until the media became green. Expression of the protein for periods over 24 h results in partial proteolysis of the 30 kDa form to a 28 kDa protein. The harvested cells were lysed in 50 mM Tris buffer (pH 8.0) containing 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), antipain (2 μg/mL), and leupeptin (1 μg/mL). The cells were then spun at 27000*g* for 60 min. Ammonium sulfate was added to the resulting supernatant to a final concentration of 30% of saturation, and the solution was stirred for 60 min. Following centrifugation (27000*g* for 20 min), the ammonium sulfate concentration was raised to 60% of saturation. The 30−60% pellets were collected and resuspended in 10 mM potassium phosphate buffer (pH 7.7) (buffer A). The protein was applied to a Mono Q (HR 10/10) column, and the protein was eluted with a step gradient of 10 mM potassium phosphate (pH 7.7) containing 250 mM KCl (buffer B). The gradient was increased linearly from 0 to 25% buffer B, and then increased linearly to 100% buffer B. The fractions in the first half of the gradient containing heme oxygenase activity were pooled and dialyzed against buffer A.

Expression and Purification of the Recombinant Heme Oxygenase/Cytochrome P450 Reductase Fusion Protein. The cells were expressed, grown, and lysed, and the lysate was fractionated by ammonium sulfate, as described above for truncated hHO-1. The precipitate was resuspended in 10 mM potassium phosphate (pH 7.4) and dialyzed against the same buffer. Following dialysis, the protein was loaded onto a 2′,5′-ADP−Sepharose column (10 × 50 mm) equilibrated with 10 mM potassium phosphate (pH 7.4). The column was washed sequentially with 5 volumes of the same buffer,

10 volumes of 100 mM potassium phosphate buffer (pH 7.4), and 5 volumes of 10 mM potassium phosphate buffer (pH 7.4). The protein was eluted with 10 mM potassium phosphate buffer (pH 7.4) containing 2.5 mM 2′-AMP. The fractions containing the activity were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 7.4).

Spectroscopic Assay of Heme Oxygenase Activity. Heme oxygenase activity was assayed as previously described (Wilks & Ortiz de Montellano, 1993). The assays contained heme oxygenase (3 μg, 0.1 nmol), 15 μM hemin, 1 μM bovine serum albumin, an excess of partially purified biliverdin reductase (rat liver cytosol), and purified rat liver cytochrome P450 reductase (0.3 nmol) in a final volume of 1 mL of 100 mM potassium phosphate buffer (pH 7.4). In the case of the fusion protein, the reductase was excluded from the reaction unless specifically stated. Assays to determine kinetic constants were carried out under the same conditions as above but contained between 0.5 and 100 μM heme. Rat liver cytochrome P450 reductase was purified as reported by Yasukochi and Masters (1976). The reaction was initiated by adding NADPH to a final concentration of 100 μM. The rate of bilirubin formation at 37 °C was monitored at 468 nm and calculated using an extinction coefficient of 43.5 mM^{-1} cm^{-1}.

Purification of the Heme/Heme Oxygenase Complex. The truncated hHO-1/heme complex was prepared essentially as described previously (Wilks & Ortiz de Montellano, 1993). Hemin was added to FPLC-purified hHO-1 to give a final 2:1 heme:protein ratio. The sample was applied to a Bio-Gel HTP column (15 × 60 mm) pre-equilibrated with buffer A. The column was then washed in the same buffer until no heme was detected in the eluent by a UV monitor set at 402 nm. The truncated hHO-1/heme complex was finally eluted with 110 mM potassium phosphate buffer (pH 7.4). Protein purity was checked by SDS−PAGE on 12.5% polyacrylamide gels (Laemmli, 1970). The fusion protein was reconstituted with heme in an identical manner.

Absorption Spectroscopy. The spectra of the heme/heme oxygenase complex were recorded on a Hewlett-Packard 8450 A spectrophotometer. The reduced ferrous CO complex was formed by addition of dithionite to a carbon monoxide-saturated solution of the ferric complex. The ferrous O_2 complex was obtained, as previously described (Yoshida & Kikuchi, 1979), by passing the carbon monoxide complex down a Sephadex G25 column preequilibrated with 10 mM potassium phosphate buffer (pH 7.4).

Reaction of hHO-1 and the Fusion Protein with NADPH. Cytochrome P450 reductase (42 μM, 3:1 reductase:hHO-1 ratio) and NADPH (14 μM) were added to a cuvette containing a solution of the hHO-1/heme complex (14 μM) in 0.1 M potassium phosphate buffer (pH 7.4) presaturated with carbon monoxide by bubbling with the gas. The progress of the reaction was monitored by UV spectroscopy. After approximately 10 min, at which point no further change occurred in the spectrum, pyridine (20% final concentration) was added. The mixture was extracted with chloroform and the product isolated by removing the organic solvent under a stream of nitrogen. Under these conditions, the reaction is arrested at the verdoheme stage. In some experiments, the verdoheme intermediate accumulated in the presence of carbon monoxide was not extracted but was allowed to continue to the fully oxidized product by displacing the carbon monoxide with oxygen in the presence of NADPH before isolating the product for analysis. The product of the fusion protein reaction was monitored in an identical fashion minus the addition of exogenous cytochrome P450 reductase.

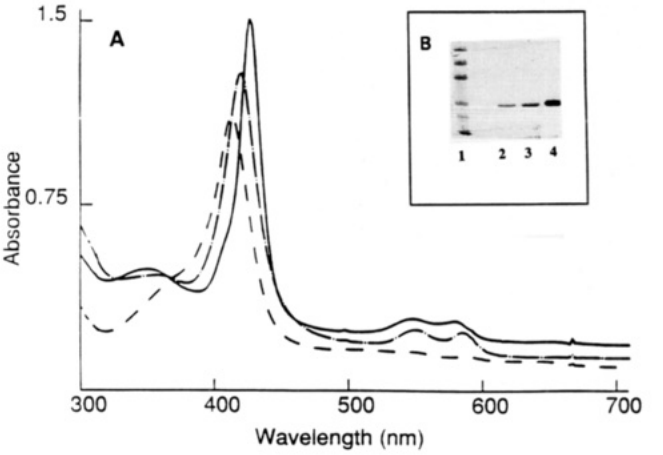

FIGURE 1: SDS−PAGE of purified truncated hHO-1 and absorption spectra of the hHO-1/heme complex. (A) Spectra are of (a) the ferric complex (- - -), (b) the ferrous dioxyheme complex (− • −), and (c) the ferrous carbon monoxide complex (—). (B) SDS−PAGE of purified truncated hHO-1: lane 1, molecular mass markers (top to bottom, 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa); lanes 2−4, purified truncated hHO-1 (2, 3, and 5 μg, respectively).

HPLC of the Products of the Heme Oxygenase Reaction. The reaction products were acidified by the addition of 200 μL of 5 N HCl and 400 μL of glacial acetic acid and extracted into chloroform. The chloroform layer was washed twice with water and dried over anhydrous sodium sulfate. The chloroform was removed under argon and the residue resuspended in HPLC solvent. HPLC was carried out on a Partisil ODS 3−5 μm reverse-phase column (4.6 × 250 mm). The biliverdin isomers were eluted in 45:55 (v/v) acetone/20 mM formic acid (J. Clark Lagarias, private communication). The products of the reactions of hHO-1 and the fusion protein were run against known standards.

RESULTS

Expression and Purification of Truncated hHO-1 and the hHO-1/Cytochrome P450 Reductase Fusion Protein. Expression of truncated hHO-1 in *E. coli* turned the media green due to an accumulation of biliverdin. The bacterial cells therefore have a reductase activity capable of supporting the catalytic turnover of hHO-1. This is consistent with recent reports that *E. coli* flavodoxin and flavodoxin reductase can substitute for cytochrome P450 reductase in supporting the catalytic turnover of cytochrome P450 (Jenkins & Waterman, 1994). Purification of the truncated hHO-1/heme complex yielded a protein that gave a single 30 kDa band on SDS−PAGE (Figure 1). The yield of the purified protein, based on an extinction coefficient at 405 nm of 140 $mM^{-1}cm^{-1}$, was 50 mg/L of cells. The purification stages and specific activities are shown in Table 1. The specific activity is somewhat lower than that reported for the truncated rat HO-1 enzyme (Wilks & Ortiz de Montellano, 1993), but this may be due to the use of rat rather than human cytochrome P450 reductase.

Purification of the hHO-1/cytochrome P450 reductase fusion protein gave an enzyme that migrated on SDS−PAGE as a 106 kDa protein (Figure 2). A minor impurity is present in the preparation that is not a proteolytic digestion fragment

Table 1: Purification of Recombinant, Truncated hHO-1[a]

fraction	protein (mg)	sp act. (nmol h^{-1} mg^{-1})	purification (*x*-fold)	activity yield (%)
cell supernatant	2280	87	1	100
40−60% $(NH_4)_2SO_4$	151	395	5	30
Mono-Q chromatography	105	1420	16	75

[a] The purification is reported for a starting volume of ~3 L of medium. Heme oxygenase activity was assayed as bilirubin formation in the coupled assay with biliverdin reductase (see Experimental Procedures). Ammonium sulfate precipitation causes a decrease in activity that is relieved by Mono-Q chromatography.

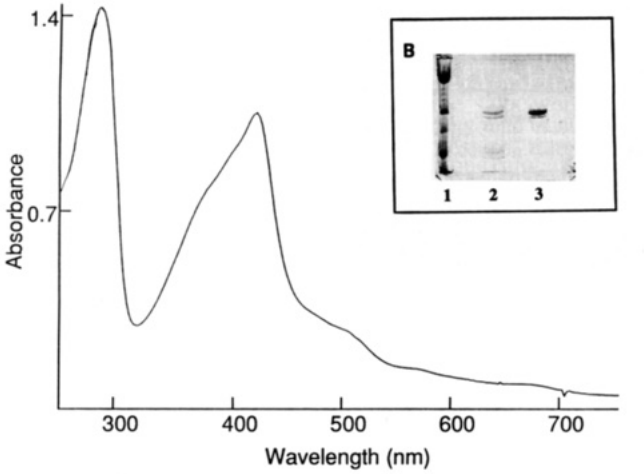

FIGURE 2: Absorption spectrum of the complex of heme with the hHO-1 fusion protein after passage through a hydroxylapatite column to remove excess heme. Inset: SDS−PAGE of the purified protein. Lane 1, molecular mass markers (from top to bottom, 205, 116.5, 80, and 49.5 kDa); lane 2, cell supernatant; lane 3, purified hHO-1 fusion protein.

Table 2: Purification of the Recombinant hHO-1/Cytochrome P450 Reductase Fusion Protein[a]

fraction	protein (mg)	sp act. (nmol h^{-1} mg^{-1})	purification (*x*-fold)	activity yield (%)
cell supernatant	2830	7	1	100
40−60% $(NH_4)_2SO_4$	630	13	2	40
2′,5′-ADP−Sepharose	27	606	83	79

[a] The purification is reported for a starting volume of ~1 L of medium. Ammonium sulfate fractionation caused a decrease in activity which was relieved on further purification. Heme oxygenase activity was assayed as bilirubin formation in the coupled assay with biliverdin reductase (see Experimental Procedures).

of the fusion protein because it does not cross-react with antibodies to either hHO-1 or human cytochrome P450 reductase. The yield was estimated to be 25 mg/L of cells using the same extinction coefficient as was used for truncated hHO-1. The purification stages and activities are shown in Table 2.

Properties of the Heme Complexes with Truncated hHO-1 and the hHO-1/Cytochrome P450 Reductase Fusion Protein. The Soret maximum of the ferric heme complex with truncated hHO-1, after removal of excess heme by passage through a hydroxylapatite column, is at 404 nm (Figure 1). Reduction of the ferric complex with dithionite under an atmosphere of carbon monoxide yields the ferrous carbon monoxide complex with a Soret band at 418 nm and well-defined α and β bands at 568 and 532 nm, respectively (Figure 1). Passage of the carbon monoxide complex through a Sephadex G25 column causes the Soret band to shift to 408 nm and the α and β bands to shift to 572 and 534 nm, respectively, as expected for conversion of the carbon monoxide complex to the ferrous dioxygen complex (Figure 1). These values are similar to those reported for the ferrous dioxygen complexes of native (Yoshida & Kikuchi, 1979) and recombinant (Wilks & Ortiz de Montellano, 1993) rat HO-1.

The complex of heme with the hHO-1/cytochrome P450 reductase fusion protein has a Soret maximum at 404 nm and a shoulder at 450−460 nm attributable to the flavin absorbance (Figure 2). The Soret band is broader than that for truncated hHO-1 (Figure 1). Addition of NADPH to the complex under an atmosphere of carbon monoxide generates a transient complex with a maximum at 408 nm that rapidly decays to a green species, presumably biliverdin (data not shown). We were unable under the conditions that yielded clear CO and O_2 complexes of the ferrous hHO-1/heme complex to obtain comparable spectra of the fusion protein/heme complex.

pH Dependence of the Spectra of the Ferric hHO-1/Heme and Heme/Fusion Protein Complexes. The absorption spectra of the complexes of heme with truncated hHO-1 and the hHO-1/cytochrome P450 reductase fusion protein at various pH values reveal a marked influence of pH on the heme axial coordination (Figure 3). The absorption spectra of the hHO-1 and fusion protein complexes are similar to that of metmyoglobin at pH 6.0, and similar pH-dependent changes are observed in all three proteins. As the pH is raised, the Soret band shifts to the red, and the absorption maximum at 632 nm indicative of a high-spin species decreases. These results suggest that the heme in the hHO-1 and fusion protein complexes, like that in metmyoglobin and rat HO-1 (Sun et al., 1993), is axially coordinated at pH 6.0 to an imidazole and a water molecule. Increasing the pH results in ionization of the water to a hydroxide, which is a stronger field ligand and causes a shift from high- to low-spin. Resonance Raman studies of this transition in rat HO-1 establish that the high- to low-spin shift occurs in that protein between pH 7.4 and 8.5 (Sun et al., 1993).

Catalytic Turnover of Truncated hHO-1 and the hHO-1/Cytochrome P450 Reductase Fusion Protein. The heme in the hHO-1/heme complex is quantitatively converted to biliverdin upon addition of NADPH and cytochrome P450 reductase (data not shown).[2] The turnover of the truncated hHO-1/heme complex in the presence of carbon monoxide was examined to determine if the reaction, as in the case of rat HO-1 (Wilks & Ortiz de Montellano, 1993; Yoshida et al., 1980), can be arrested at an intermediate stage by complexation with carbon monoxide. In effect, carbon monoxide causes accumulation of a biliverdin precursor (λ_{max} = 640 nm) spectroscopically identical to that obtained with the native enzyme (Figure 4A) (Wilks & Ortiz de Montellano, 1993; Yoshinaga et al., 1990). Furthermore, the spectrum obtained when the intermediate accumulated in the

[2] As found for the rat enzyme (Wilks & Ortiz de Montellano, 1993), reaction of truncated hHO-1 with H_2O_2 in the absence of NADPH and cytochrome P450 reductase results in the accumulation of verdoheme. H_2O_2 cannot be replaced in this reaction by *meta*-chloroperbenzoic acid, as the peracid oxidizes the enzyme to a ferryl species but does not detectably produce verdoheme or biliverdin.

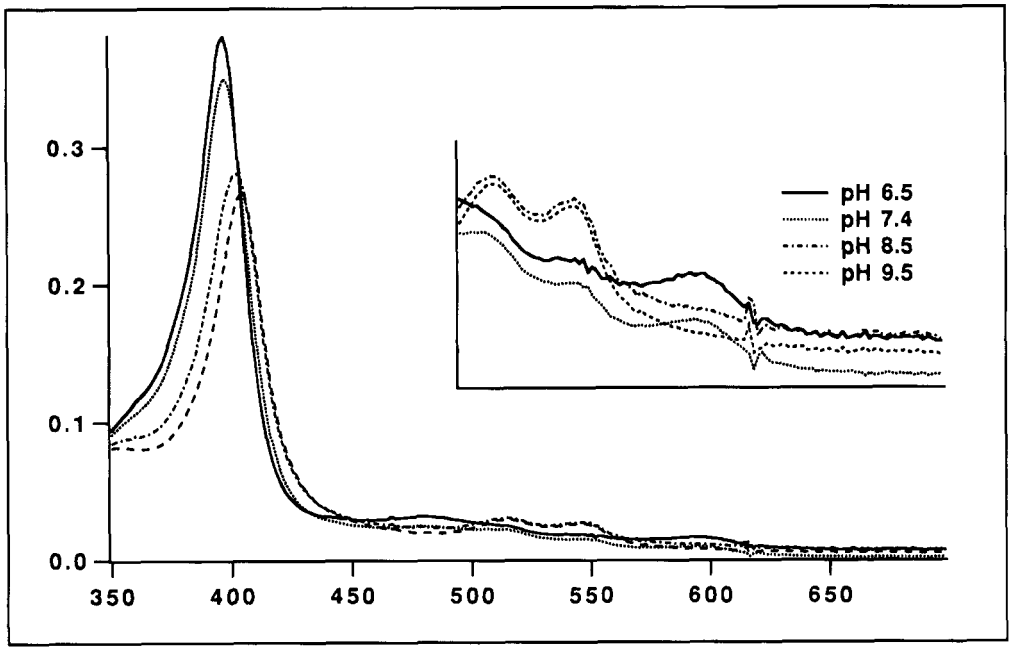

FIGURE 3: Absorption spectra of the ferric hHO-1/heme complex at the indicated pH values.

presence of carbon monoxide is treated with pyridine is characteristic of a verdoheme (Fe^{II})–pyridine complex (Figure 4B). Extraction of the product when the reaction is carried out under oxygen yields biliverdin (data not shown). The conversion of heme to biliverdin by the hHO-1/cytochrome P450 reductase fusion protein initiated by addition of NADPH is similarly arrested at the verdoheme state by carbon monoxide, as shown by the appearance of an absorption spectrum similar to that obtained with truncated hHO-1 (data not shown).

Kinetic Characterization of Truncated hHO-1 and the hHO-1/Cytochrome P450 Reductase Fusion Protein. The K_m value for truncated hHO-1 calculated from Hanes plots of enzyme activity ([heme]/v) versus heme concentration ([heme]) is 3.0 μM, in close agreement with those of the rat liver and bovine spleen enzymes (Yoshida & Kikuchi, 1980; Yoshinaga et al., 1982). The corresponding V_{max} value is 40 nmol h^{-1} (nmol of protein)$^{-1}$. The K_m value for the fusion protein determined by the same method is 6 μM, and the corresponding V_{max} value is 102 nmol h^{-1} (nmol of protein)$^{-1}$. The K_m and V_{max} values for the fusion protein are thus 2.0 and 2.5 times higher, respectively, than those for hHO-1.

To determine whether electron transfer in the fusion protein occurs via an intra- or intermolecular mechanism, the effect of exogenous cytochrome P450 reductase on the V_{max} was examined. The V_{max} was not increased by the addition of exogenous cytochrome P450 reductase (data not shown). Furthermore, increasing the ionic strength of the medium by raising the KCl concentration results in progressive loss of the catalytic activity of hHO-1 reconstituted with cytochrome P450 reductase (Figure 5). The catalytic activity of the fusion protein, however, exhibits a slight *increase* in activity as the ionic strength is raised (Figure 5).

DISCUSSION

An understanding of the structure, mechanism, and inhibition of human heme oxygenases is of importance in the context of human disease, but little direct information is available on these enzymes due to limited accessibility and the difficulties associated with their purification from microsomal membranes. We recently took a first step toward resolution of this problem by expressing rat heme HO-1 without the membrane binding domain in *E. coli* (Wilks & Ortiz de Montellano, 1993). The protein thus obtained is soluble, easily purified, and fully active. Encouraged by this result, we have constructed a gene coding for a truncated form of hHO-1 without the 23 amino acid membrane binding domain and have successfully expressed it in *E. coli*. As a consequence, we have been able, for the first time, to purify a human liver heme oxygenase to homogeneity (Figure 1, Table 1).

Heme binds to truncated hHO-1 to give a complex whose spectroscopic properties in the ferric, ferrous carbon monoxy (Fe^{II}-CO), and ferrous dioxy (Fe^{II}-O_2) states (Figure 2) are identical to those of the native (Yoshida & Kikuchi, 1979) and recombinant rat liver enzymes (Wilks & Ortiz de Montellano, 1993). The spectra of truncated hHO-1 in all three states are similar to the corresponding three states of myoglobin, suggesting that the axial ligation of both proteins is similar. This is confirmed by spectroscopic observation of a high- to low-spin transition as the pH is increased very similar to that observed with metmyoglobin and rat HO-1 (Sun et al., 1993). In view of the electron absorption and resonance Raman evidence that the heme ligands in rat HO-1 are an imidazole and a water, it is highly likely that the same ligands are present in truncated hHO-1 (Sun et al., 1993). The spectroscopic studies indicate that catalytic turnover of hHO-1 under a partial atmosphere of carbon monoxide results in the accumulation of a verdoheme-like intermediate (Figure 3).[2] The active sites and catalytic sequences of rat HO-1 and hHO-1 thus appear to be very similar (Wilks & Ortiz de Montellano, 1993).

The spectroscopic and catalytic properties of truncated hHO-1 and the hHO-1/cytochrome P450 reductase fusion protein are similar with the exception that the ferrous dioxy and ferrous carbonmonoxy complexes of the fusion protein are less stable. Nevertheless, the catalytic process can be arrested at the verdoheme stage by carrying out the incuba-

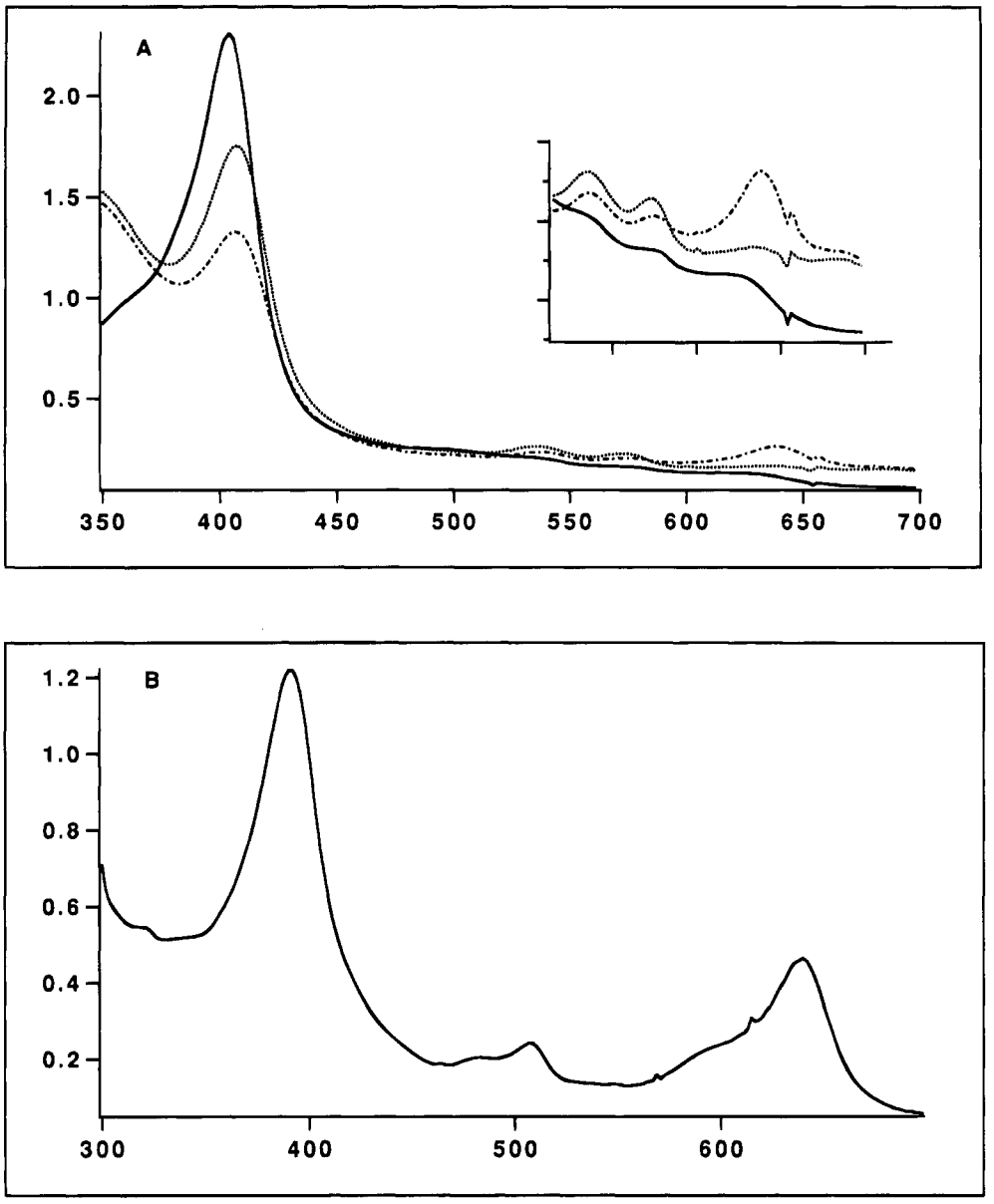

FIGURE 4: Conversion of heme to verdoheme by the truncated hHO-1 enzyme: (A) in the presence of cytochrome P450 reductase, NADPH, and carbon monoxide. Ferric enzyme prior to the addition of NADPH (—) and 5 min (•••) and 10 min (— • —) following the addition of NADPH. (B) Following the addition of pyridine 20:1.

tion under a partial atmosphere of carbon monoxide. The results indicate that deleting the membrane binding domains of hHO-1 and cytochrome P450 reductase and fusing the two truncated proteins via a five amino acid linker do not alter the nature or specificity of heme degradation.

The 2,5-fold higher V_{max} of the fusion protein over that of the reconstituted system indicates that the fusion protein is ultimately a more efficient catalyst. Although some of this difference in V_{max} could be due to the use of human cytochrome P450 reductase in the fusion protein and rat cytochrome P450 reductase in the reconstituted system, it is clear that electron transfer from the truncated reductase to the truncated heme oxygenase domains in the fusion protein is at least as efficient as electron transfer in an optimally reconstituted system. It is not possible to compare this efficiency with the efficiencies achieved in the fusion proteins of microsomal cytochrome P450 enzymes with cytochrome P450 reductase that have been reported because comparable V_{max} data have not been reported for those systems (Sakaki et al., 1994a,b; Fisher et al., 1992; Shet et al., 1993). It remains to be seen, therefore, whether the efficiency of electron transfer obtained with the cytochrome P450 fusion proteins is comparable to that obtained with heme oxygenase.

The protein−protein association required for electron transfer between two proteins can be controlled by ionic interactions between specific groups on the proteins, by interaction between complementary hydrophobic patches on the proteins, or by both electrostatic and hydrophobic forces. In general, ionic interactions between two proteins decrease and hydrophobic interactions increase as the ionic strength increases. Conflicting evidence has been presented in support of both types of mechanisms in the interactions of cytochrome P450 reductase with cytochrome P450 enzymes (Shen & Strobel, 1993; Voznesensky & Schenkman, 1994). The decrease in the catalytic activity of hHO-1 reconstituted with rat liver cytochrome P450 reductase suggests that the interaction between these two proteins includes a strong ionic component (Figure 5). On the other hand, the relative insensitivity of the catalytic rate of the fusion protein to changes in ionic strength clearly shows that ionic interactions

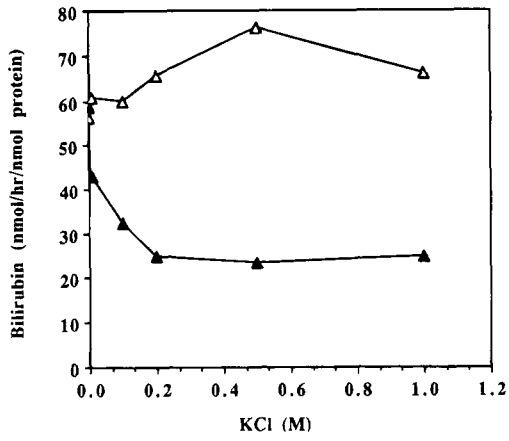

FIGURE 5: Salt effects on the catalytic turnover of truncated hHO-1 reconstituted with exogenous rat cytochrome P450 reductase (▲) and the fusion protein of hHO-1 with human cytochrome P450 reductase (△).

no longer play a dominant role in the intramolecular electron transfer process. The small initial increase in activity with increasing ionic strength suggests that hydrophobic interactions play some role in the process. The insensitivity to ionic strength clearly indicates that electron transfer does not involve intermolecular interaction of the cytochrome P450 domain of one fusion protein molecule with the reductase domain of another, since this interaction would be subject to the same ionic strength effects as the independent hemoprotein and flavoprotein interaction. The results also establish that the reductase is stable over the range of ionic strengths employed and therefore that the decrease in catalytic turnover of the reconstituted system is not due to denaturation of the reductase. The inference that electron transfer occurs via an intramolecular pathway in the fusion protein is supported by the finding that exogenous cytochrome P450 reductase does not increase the V_{max} for the fusion protein. The results indicate that either the critical regions of the heme binding and flavoprotein domains of the fusion protein retain the ability to interact normally or alternative routes exist in the fusion protein for electron transfer. Further comparative dissection of the catalytic turnover of truncated hHO-1 and the fusion protein should provide very useful information on electron transfer pathways in this and other hemoproteins.

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