Disruption of an Active Site Hydrogen Bond Converts Human Heme Oxygenase-1 into a Peroxidase*

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The crystal structure of heme oxygenase-1 suggests that Asp-140 may participate in a hydrogen bonding network involving ligands coordinated to the heme iron atom. To examine this possibility, Asp-140 was mutated to an alanine, phenylalanine, histidine, leucine, or asparagine, and the properties of the purified proteins were investigated. UV-visible and resonance Raman spectroscopy indicate that the distal water ligand is lost from the iron in all the mutants except, to some extent, the D140N mutant. In the D140H mutant, the distal water ligand is replaced by the new His-140 as the sixth ligand, giving a bis-histidine complex. The D140A, D140H, and D140N mutants retain a trace (<3%) of biliverdin forming activity, but the D140F and D140L mutants are inactive in this respect. However, the two latter mutants retain a low ability to form verdoheme, an intermediate in the reaction sequence. All the Asp-140 mutants exhibit a new peroxidase activity. The results indicate that disruption of the distal hydrogen bonding environment by mutation of Asp-140 destabilizes the ferrous dioxygen complex and promotes conversion of the ferrous hydroperoxo intermediate obtained by reduction of the ferrous dioxygen complex to a ferryl species at the expense of its normal reaction with the porphyrin ring.

Heme oxygenase (HO) catalyzes the regiospecific oxidation of heme to α-biliverdin, CO, and free iron (1). All of the oxidative steps in the heme catabolic pathway have been extensively studied over the past 30 years, but significant gaps still exist in our understanding of this enzyme system. During its catalytic cycle, HO consumes 3 eq of O₂ and 7 reducing eq supplied by NADPH-cytochrome P450 reductase (P450 reductase) (2). The enzyme catalyzes a sequence of reactions that includes the conversion of heme to verdoheme, and verdoheme to α-biliverdin (Fig. 1). The intermediates remain bound to the enzyme throughout the catalytic cycle until α-biliverdin is produced and released. It is remarkable that HO can catalyze such a diverse set of reactions, because they involve the oxidation of compounds that possess different electronic and coordination properties and that have different reactivities with O₂. This enzyme is also distinguished from all other hemoproteins in that the heme serves as the prosthetic group and substrate, and the first oxidizing species appears to be a ferric hydroperoxide (Fe(III)-OOH) rather than ferryl oxene (Fe(V)=O) intermediate (3). These characteristics suggest that unique interactions exist between the heme, the iron-bound O₂, and the amino acid residues within the active site of the enzyme.

In humans, HO exists in two well established forms, HO-1 and HO-2, that share moderate (~45%) amino acid sequence identity but vary in their inducibility and localization (4). HO-1, also known as heat shock protein 32, is highly inducible and is the major form present in the spleen, whereas HO-2 is a constitutive enzyme that is found in highest concentration in the brain and testes (5). Under normal conditions, HO-2 predominates by ~2-fold over HO-1 in the liver (4). However, HO-1 can be up-regulated about 100-fold in response to various oxidative stress agents and thus can be the dominant form in that tissue. Due to their respective physiological locations, HO-1 is considered to be important for heme homeostasis, and HO-2 is thought to be primarily responsible for the effects of CO as a neurotransmitter (4). Despite these differences, HO-1 and HO-2 exhibit similar heme coordination and electronic properties and catalyze the same set of reactions. By virtue of their ability to catabolize the oxidant heme and to produce the antioxidant biliverdin, both enzymes are intricately involved in providing protection against the formation of reactive oxygen species and heme-mediated lipid peroxidation.

There is considerable interest in elucidating those factors that control the reactivity of HO, because of its unique and intrinsically interesting catalytic mechanism and its role in several important physiological processes. HO is a membrane-bound protein, but truncated, water-soluble, fully active forms of human (hHO-1) and rat HO-1 have been expressed in Escherichia coli (3, 7, 8). Furthermore, the x-ray crystal structures of truncated versions of human and rat HO-1 have recently been determined (9, 10). The rat and human HO-1 isozymes share a high degree (~80%) of sequence identity (4). Analysis of the hHO-1 crystal structure indicates that Asp-140, a residue within the distal heme pocket, could facilitate catalytic activity by hydrogen bonding to a water molecule that could, in turn, hydrogen bond with the putative Fe(III)-OOH species (Fig. 2). In the present study, Asp-140 was replaced by amino acids that...
vary in size, charge, and hydrophobicity, and the resulting mutant proteins were characterized with regard to their HO and peroxidase activities. The results clearly establish that Asp-140 is of critical importance to the outcome of the catalytic cycle.

**EXPERIMENTAL PROCEDURES**

**Materials**—H$_2$O$_2$ (30%), NADPH, ampicillin, isopropyl-β-D-thiogalactopyranoside, hemin, bovine serum albumin, sodium dithionite, and guaiacol were obtained from Sigma. High purity argon (99.998%), CO (99.95), and O$_2$ (99.9%) were obtained from Matheson (Newark, CA) and Aldrich.

**Enzymes**—Catalase was from Sigma. Rat P450 reductase was expressed and purified from bacterial cultures according to published procedures (11). Biliverdin reductase was also expressed and purified according to a published procedure (12) with the following modifications: (i) BL-21 cells were used for expression in LB-Amp media; (ii) cells were grown at 30 °C for 8 h after addition of isopropyl-β-D-thiogalactopyranoside; (iii) the Ni(II) column was washed with 20 mM Tris-HCl (pH 8.0) containing 5 mM imidazole; and (iv) biliverdin reductase was eluted from the Ni(II) column with 20 mM Tris-HCl (pH 8.0) containing 100 mM imidazole.

**Expression and Purification of hHO-1 and Asp-140 Mutants**—Wild-type hHO-1 and its Asp-140 mutants were expressed using *E. coli* strain DH5α (F′araDlac-proAB) and the hHO-1 construct encoding the human liver protein lacking the 23 C-terminal amino acids (7). The general sequence for the mutant primers that were employed was 5′-ACC CGC TAC CTG GGG XXX 3′ (Life Technologies, Inc., Cell Culture Facility, University of California, San Francisco), where XXX denotes the nucleotides encoding the desired mutation. The hHO-1 Asp-140 mutants were generated using the polymerase chain reaction and QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Plasmid purification, subcloning, and bacterial transformations were carried out by standard procedures (13). Antibiotic selection using ampicillin afforded a high frequency of mutants. Transformants were confirmed by sequence analysis (Biomolecular Resource Center, University of California, San Francisco). The hHO-1 proteins were expressed, purified, and reconstituted with hemin according to published procedures (3, 7) except that, after reconstitution, a hydroxyapatite chromatographic step using 75 mM potassium phosphate (pH 7.4) as the elution buffer was found to be optimal. The proteins were obtained with yields ranging from 0.5 to 5 mg of purified protein per liter. All experiments using the purified proteins were performed in triplicate in 100 mM potassium phosphate buffer (pH 7.4) (standard buffer) unless otherwise stated.

**Spectral Characterization**—The UV-visible spectra of the hHO-1 proteins were recorded in standard buffer on a Hewlett-Packard 8452A diode array spectrophotometer. The ferrous carbon monoxide (Fe(II)-CO) complexes were formed by saturating the solutions with CO by bubbling with the gas for 1 min followed by reduction of the Fe(III) complexes with a few grains of sodium dithionite. The ferrous dioxygen (Fe(II)-O$_2$) complexes were formed by bubbling the samples containing the Fe(II)-CO complexes with O$_2$ for up to 30 s. The pK$_a$ value of the water ligand coordinated to the iron (if present) of hHO-1 and its Asp-140 mutants was determined by recording the UV-visible spectrum over the pH range 6–11.

**RR Spectroscopy**—Typical enzyme concentrations for RR experiments were 100–250 μM in standard buffer. Microcon 10 ultrafiltration devices (Amicon) were used for buffer and water exchange. A final enrichment of 80% $^{18}$O- or $^2$H-labeled water (95% $^{18}$O, Cambridge Isotope Laboratory, 99.9% $^2$H, Aldrich) was achieved in the experiments using labeled water. Reduction to the Fe(II) state was achieved by adding microliter aliquots of a sodium dithionite solution (10 mM) to an argon-purged sample in the Raman capillary cell and was monitored by RR spectroscopy in the same cell. $^{13}$CO (99% $^{13}$C, Cambridge Isotope Laboratory) and $^{12}$CO (99% $^{12}$C, Cambridge Isotope Laboratory) adducts were obtained by injecting CO through a septum into a closed capillary containing ∼20 μl of argon-purged, reduced enzyme.

RR spectra were obtained on a custom McPherson 2061/207 spectro-
allowed to proceed for 30 min. In other experiments, guaiacol (1 mM) was preincubated at 37 °C for 3 min. The reaction was initiated by the addition of NADPH (1 mM) (final incubation volume, 500 μM), catalase (200 units), hemin (30 μM), P450 reductase (1 μM), biliverdin reductase (4 μM) in standard buffer or in standard buffer that had been presaturated with CO to determine Fe(III)-verdoheme or Fe(II)-CO-verdoheme formation, respectively, for 0–100 s. Fe(II)-CO-verdoheme formation was monitored spectrophotometrically at 636–640 nm. The reaction was initiated by the addition of NADPH (1–2 eq) in standard buffer or in standard buffer that had been presaturated with CO. The initial rate of peroxidase activity was calculated using the value E₄₅₀ = 43.5 mM⁻¹ cm⁻¹ for the bilirubin product.

**HO-1 Biliverdin Activity Assay**—A solution of hHO-1 (1 μM) or an Asp-140 mutant (10 μM) and guaiacol (90 μM) in standard buffer was preincubated at 23 °C in a cuvette. The reaction was initiated by the addition of H₂O₂ (100 μM) to 100 mM). The regioisomer of biliverdin formed was determined by HPLC analysis. The values given are the average of three separate determinations (±S.D.). ND, none detected; WT, wild type.

| Enzyme | BR formation rate ± S.D. min⁻¹ (%) | Amount BR formed (%) | Biliverdin isomer formed |
|--------|----------------------------------|----------------------|-------------------------|
| WT     | 3.87 ± 0.150 (100)               | 100                  | α                       |
| D140A  | 0.07 ± 0.007 (1.8)               | 1.4                  | α                       |
| D140F  | ND (0)                           | 0                    | ND                      |
| D140H  | 0.01 ± 0.002 (0.2)               | 0.003                | ND                      |
| D140L  | ND (0)                           | 0                    | ND                      |
| D140N  | 0.11 ± 0.003 (2.8)               | 3.04                 | α                       |

**RESULTS**

**Expression, Purification, and Spectral Characterization of the Asp-140 Mutants**—The D140A, D140F, D140H, D140L, and D140N hHO-1 mutants were expressed and purified as described previously for wild-type hHO-1 (3, 7). Each protein was judged to be >95% pure by SDS-polyacrylamide gel electrophoresis. The Soret maxima of the Fe(III)-CO complex of the proteins ranged from a low of 402 nm for the D140A mutant to a high of 412 nm for the D140H mutant (Table I and Fig. 3). In contrast, the Asp-140 mutants exhibit the same Soret maxima at 410 nm.
tants is similar in the Fe(II)-O₂ and Fe(II)-CO states but differs in their Fe(III) resting states. The water coordinated to the iron as the sixth ligand in wild-type hHO-1 has a pKₐ of 8 as determined by the observation of a red shift in the Soret maximum and changes from a high spin (HS) to a low spin (LS) species in the RR spectra between pH 7 and 9 (15, 16). Similarly, the Soret maximum of the D140N mutant exhibited a small, but detectable, shift between pH 7 and 9. However, the Soret maximum of the other Asp-140 mutants did not shift at pH values up to 11 (Table I). These results indicate that the distal water ligand is at least partially retained in the D140N mutant but is completely absent from all the other Asp-140 mutants.

RR of the Asp-140 Mutants—RR spectra of the Fe(III) state of wild-type hHO-1 and the Asp-140 mutant heme complexes were obtained with Soret excitation (Fig. 4). The ν₃ modes at 1483 and 1503 and ν₂ modes at 1565 and 1582 cm⁻¹ observed in wild-type hHO-1 are characteristic of a hexacoordinate high spin/low spin mixture (6cHS/6cLS) with a predominance of the HS state (15, 16). In D140N, the 6cHS species is still clearly observed, but a pentacoordinate high spin (5cHS) species emerges with ν₃ at 1491 cm⁻¹. This 5cHS configuration dominates the RR spectra of the Fe(III) state of the D140A, D140L, and D140F mutants (Fig. 4). When Asp-140 is replaced by a His, the Fe(III) state is mostly 6cLS as indicated by the intense and dominant ν₃ and ν₂ at 1503 and 1582 cm⁻¹, respectively, with only a minor 5cHS contribution (Fig. 4 and Table I). These results indicate that replacement of Asp-140 by other amino acids destabilizes the heme distal water ligand.

Of all the Asp-140 mutants examined by RR spectroscopy at alkaline rather than neutral pH, only the D140N mutant presents significant changes in the Fe(III) state (Fig. 5). A sub-population of the D140N mutant heme complex shows the pH-dependent 6cHS to 6cLS conversion observed for wild-type hHO-1 (Fig. 5). In the low frequency RR spectrum of the D140N mutant complex, a band at 549 cm⁻¹ downshifts by 32 cm⁻¹ in ¹⁸O-labeled water and is assigned to the ν(Fe-OH) of a 6cLS hydroxo adduct (Fig. 5). In wild-type hHO-1, the Fe(III)-OH vibration is observed at 546 cm⁻¹ (17). These results demonstrate that the D140N mutant partially retains the distal aqua/hydroxo ligand. Moreover, the similarity in the ν(Fe-OH) frequencies for both wild-type hHO-1 and the D140N mutant shows that the hydroxyl group is engaged in comparable H bond interactions in both distal pockets.

The Fe(III) state of the D140H mutant exhibits a red-shifted Soret maximum at 412 nm, and its RR spectrum confirms that

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**Table III**

Fe(III)-verdoheme and Fe(II)-CO-verdoheme formation by hHO-1 and its Asp-140 mutants in the presence of NADPH/P450 reductase

The initial rate of Fe(II)-CO-verdoheme and Fe(III)-verdoheme formation catalyzed by NADPH/P450 reductase under an atmosphere of CO or O₂, respectively. Fe(II)-CO-verdoheme and Fe(III)-verdoheme formation was monitored at 636–640 and 680–700 nm, respectively. The values given are the average of three separate determinations. ND, none detected. WT, wild type.

| Enzyme | Fe(III)-verdoheme | Fe(II)-CO-verdoheme |
|--------|------------------|--------------------|
|        | Initial rate    | Amount   | Initial rate | Amount |
| WT     | 100             | 100      | 100          | 100    |
| D140A  | 70.9            | 56.3     | ND           | ND     |
| D140F  | 0.8             | 2.5      | ND           | ND     |
| D140H  | 22.1            | 18.1     | ND           | ND     |
| D140L  | 35.2            | 35.4     | ND           | ND     |
| D140N  | 88.6            | 87.3     | ND           | ND     |
the heme adopts a 6cLS configuration (Fig. 4). Unlike wild-type hHO-1 and the D140N mutant, the D140H mutant shows no 18O-sensitive bands in the low frequency region (data not shown). After reduction with dithionite, the high frequency RR spectrum of the D140H mutant is indicative of an LS configuration, and its electronic absorption maxima at 425, 531, and 558 nm are reminiscent of the Fe(II) state of cytochrome \(b_558\) (18). These results support formation of a bis-histidine heme iron complex in the D140H mutant, although in the Fe(II) state the bis-histidine complex is labile, and the engineered histidyl ligand can be displaced by exogenous ligands such as CO and O\(_2\). The formation of a similar complex was recently reported in the high pH form of manganese peroxidase, where the distal histidine also acts as a labile iron ligand (19).

Aside from the D140H mutant, the Fe(II) state of the Asp-140 mutants studied here presents high frequency RR spectra indicative of a 5cHS heme iron complex in the D140H mutant, although in the Fe(II) state the bis-histidine complex is labile, and the engineered histidyl ligand can be displaced by exogenous ligands such as CO and O\(_2\). The formation of a similar complex was recently reported in the high pH form of manganese peroxidase, where the distal histidine also acts as a labile iron ligand (19).

Catalytic Turnover of the Asp-140 Mutants—Under the standard conditions of our bilirubin activity assay (1 \(\mu\)M hHO-1 enzyme), all of the Asp-140 mutants studied here presents high frequency RR spectra indicative of a 5cHS heme configuration and display in the low frequency region \(\nu(Fe(II)-His)\) vibrations within 2 cm\(^{-1}\) of the 216-cm\(^{-1}\) value observed for wild-type hHO-1 (data not shown). Such Fe(II)-histidine stretching frequencies are consistent with a proximal histidine that retains the N-H proton and is only weakly or not hydrogen bonded (15, 16).

Catalytic Turnover of the Asp-140 Mutants—Under the standard conditions of our bilirubin activity assay (1 \(\mu\)M hHO-1 enzyme), all of the Asp-140 mutants were found to be catalytically inactive. However, by increasing the concentration of the Asp-140 mutants 10-fold, a small amount of activity was observed using the D140A, D140H, and D140N mutants. The initial rates and total amounts of bilirubin formation catalyzed by these three mutants were 1.8/1.4%, 0.2/0.003%, and 2.8/3.04%, respectively, of the wild-type hHO-1 activity (Table II). The spectroscopic changes observed using these mutants indicated that, similar to wild-type hHO-1, the Fe(III)-verdoheme (680–700 nm) and Fe(II)-verdoheme (636–640 nm) complexes were formed as intermediates prior to bilirubin formation (Fig.

**TABLE IV**

| Enzyme | \(K_m\) (mM) | \(V_{max}\) (\(\mu\)mol/min/mg) | Peroxidase activity (\% of WT) |
|--------|--------------|-------------------------------|-----------------------------|
| WT     | 0.8          | 0.3                           | 0.30 ± 0.07 (0.8)           |
| D140A  | 34.3         | 49.1                          | 35.60 ± 1.09 (100)          |
| D140F  | 20.3         | 6.8                           | 6.53 ± 1.22 (18.3)          |
| D140H  | 43.3         | 3.4                           | 2.44 ± 0.06 (6.9)           |
| D140L  | 25.9         | 9.4                           | 7.64 ± 0.52 (21.5)          |
| D140N  | 6.2          | 3.3                           | 3.09 ± 0.15 (8.7)           |

The values given are the average of three separate determinations (±S.D.). WT, wild type.
6). Even at the higher concentration, the D140F and D140L mutants were incapable of catalyzing bilirubin formation. However, the D140L mutant and, to a much lesser extent, the D140F mutant were able to form the verdoheme intermediate. HPLC analysis revealed that the α-isomer of biliverdin was formed from the D140A, D140H, and D140N mutants (Table II). This result established that the regiospecificity of the HO-1 catalytic mechanism was not altered by the Asp-140 amino acid substitutions.

The HO-1 reaction can be arrested at the Fe(II)-CO-verdoheme stage by pre-saturating the buffer with CO prior to the addition of NADPH. By using wild-type hHO-1, the sequence of events leading up to Fe(II)-CO-verdoheme formation includes generation of the heme-Fe(II)-CO and heme-Fe(II)-O₂ complexes. Under these conditions, the HO-1 reaction is dependent upon the affinity of the enzyme for H₂O₂ relative to the wild-type HO-1. Significant amounts (18–87% with respect to wild-type HO-1) of the D140A, D140H, D140L, and D140N mutants produced Fe(III)-verdoheme forming activity. The initial rate and amount of Fe(III)-verdoheme formation catalyzed by H₂O₂ (50 μM) was monitored at 680–700 nm and calculated relative to wild-type hHO-1 activity in the absence of guaiacol. The values given are the average of three separate determinations. Using 10 equivalents of H₂O₂ (100 μM) and 100 μM H₂O₂, only the D140F, D140H, and D140N mutants exhibited Fe(III)-verdoheme forming activity.

Peroxidase Activity of Asp-140 Mutants—We reported previously that the Gly-139 hHO-1 mutants exhibit a peroxidase activity with H₂O₂ and guaiacol as substrates (20). After addition of H₂O₂ to the Asp-140 mutants, a significant amount of heme loss was observed with a corresponding increase in the formation of a species at 426 nm. Inclusion of guaiacol in the incubation prevented the heme loss and led to the formation of a species at 470 nm indicative of the guaiacol oligomerization. Furthermore, the 426 nm absorbing species represented a Compound II-like ferryl species. To ensure that the conditions used in the peroxidase assay were optimal, the Km and Vmax values for H₂O₂ for each Asp-140 mutant were determined (Table IV and Fig. 9). The Asp-140 substitutions led to a substantial decrease (8–119-fold) in the affinity of the enzyme for H₂O₂ relative to the wild-type enzyme. By using the optimized peroxidase assay, the Asp-140 mutants were found to possess peroxidase activities significantly greater (8–119-fold) than wild-type hHO-1 (Table IV), with the D140A mutant exhibiting the highest peroxidative activity. These results demonstrate that disruption of the putative Asp-140-dependent active site hydrogen bonding network converts hHO-1 into a peroxidase.

Reaction of the Asp-140 Mutants with H₂O₂—Under the standard conditions used for monitoring Fe(III)-verdoheme formation (10 μM enzyme and 100 μM H₂O₂), only the D140F, D140H, and D140N mutants were found to possess peroxidase activities significantly greater (8–119-fold) than wild-type hHO-1 (Table IV), with the D140A mutant exhibiting the highest peroxidative activity. These results demonstrate that disruption of the putative Asp-140-dependent active site hydrogen bonding network converts hHO-1 into a peroxidase.

FIG. 9. Eadie-Hofstee plot used for the determination of Kia and Vmax parameters of the D140A mutant and H₂O₂ in the peroxidase activity assay.

FIG. 10. Spectroscopic changes observed following the addition of H₂O₂ to initiate the Fe(III)-verdoheme formation using the D140A mutant in the absence (left) or presence (right) of guaiacol. Fe(III)-verdoheme formation is observed as an appearance of a band at 680–700 nm.

TABLE V

Fe(III)-verdoheme formation by hHO-1 and its Asp-140 mutants in the presence of H₂O₂

| Enzyme | Optimal [H₂O₂] | Fe(III)-verdoheme rate | Fe(III)-verdoheme amount |
|--------|----------------|------------------------|------------------------|
|        | μM             | (-)-Guaiacol           | (+)-Guaiacol           | (-)-Guaiacol | (+)-Guaiacol |
| WT     | 100            | 100                    | 105                    | 100          | 103          |
| D140A  | 500            | 0.6                    | 30.7                   | 1.2          | 30.5         |
| D140F  | 1000           | 3.3                    | 31.7                   | 6.8          | 25.0         |
| D140H  | 2500           | 17.7                   | 19.5                   | 17.9         | 19.2         |
| D140L  | 2500           | 4.8                    | 16.3                   | 8.1          | 33.0         |
| D140N  | 50             | 4.1                    | 30.3                   | 8.7          | 30.9         |

The initial rate and amount of Fe(III)-verdoheme formation catalyzed by H₂O₂ (50 μM to 2.5 mM) in the absence and presence of guaiacol (1 mM) was monitored at 680–700 nm and calculated relative to wild-type hHO-1 activity in the absence of guaiacol. The values given are the average of three separate determinations. Using 10 equivalents of H₂O₂ (100 μM), only wild type (WT) (100%) D140F (0.7%), D140H (1.7%), and D140L (0.4%) exhibited Fe(III)-verdoheme forming activity.
D140H, and D140L mutants were found to produce this intermediate, with initial rates of 0.7, 1.7, and 0.4%, respectively, of the wild-type hHO-1 rate. The D140A and D140N mutants did not catalyze Fe(III)-verdoheme formation under these conditions. It seemed likely that the relatively small amount of Fe(III)-verdoheme produced could be due to the decreased affinity of the Asp-140 mutants for H2O2. Therefore, a range of H2O2 concentrations was used in an attempt to optimize Fe(III)-verdoheme formation. The initial rates of Fe(III)-verdoheme formation from the D140F, D140H, and D140L mutants could be increased 5–10-fold at much higher (10–25-fold) H2O2 concentrations (Table V). Lower H2O2 concentrations were necessary to observe Fe(III)-verdoheme formation with the D140A and D140N mutants. The total amount of Fe(III)-verdoheme formed over the time course of the assay was found to correlate well with the increase in the initial rate of formation (Table V). Furthermore, the initial rate and total amount of Fe(III)-verdoheme formation increased significantly (3–51 and 4–30-fold, respectively) in the presence of guaiacol for a majority of the Asp-140 mutants (Table V and Fig. 10).

**DISCUSSION**

The HO catalytic cycle that ultimately produces α-biliverdin proceeds via initial electrophilic addition of a reactive Fe(III)-OOH species to the α-meso-carbon of the heme group. This conclusion is based on the following: (i) ability of H2O2 to substitute for NADPH and P450 reductase in the production of verdoheme (3); (ii) formation of an α-ethoxyheme adduct upon exposure of hHO-1 to ethylhydroperoxide (21); (iii) electronic effects of meso-methyl and meso-formyl heme substituents on the reaction (22, 23); and (iv) direct spectroscopic observation of the Fe(III)-OOH complex and hydroxyheme intermediate following low temperature photoreduction (24). To define further the mechanism, it was of considerable interest to test the effect of point mutations introduced into the hHO-1 active site on the catalytic activity of the enzyme. We previously reported that the flexibility of the distal helix of hHO-1 is involved in controlling the reactivity of the enzyme (20). Two residues implicated by the crystal structure as critical for this flexibility were Gly-139 and Gly-143 (9). Therefore, several mutations were made at these residues in an effort to perturb the enzymatic reaction by altering the flexibility of the helix (20). Interestingly, the regiospecificity of the reaction was not perturbed, but the mutations did cause a substantial shift in the fate of the key Fe(III)-OOH species, favoring its conversion to a ferryl species at the expense of α-meso-hydroxylation of the heme. This change in the fate of the Fe(III)-OOH species caused a conversion of hHO-1 from an oxygenase to a peroxidase.

The hHO-1 x-ray crystal structure was critical for identifying other residues in the active site that might be essential for the highly regiospecific oxidation of the heme substrate. One such residue, Asp-140, appears to be well positioned to participate in a hydrogen bond network with a water molecule and the Fe(III)-OOH intermediate (Fig. 2). As shown here, wild-type hHO-1 has almost no peroxidase activity, but mutations at Asp-140 increase the peroxidative activity of the enzyme from 10 to more than 100-fold. In contrast, biliverdin production, as assayed by the subsequent formation of bilirubin, was greatly decreased with an almost complete (>97%) loss in the bilirubin formation activity relative to wild-type hHO-1. Despite this precipitous decrease in the ability to form biliverdin, the regiospecificity of the enzyme remained intact, and only the α-isomer of biliverdin was formed.

The individual steps leading up to bilirubin formation were examined to determine at which stage the normal catalytic cycle was disrupted. The relative amount of Fe(III)-verdoheme formed using the NADPH/P450 reductase system was significantly higher than that obtained using H2O2 even after optimization of the H2O2 concentration (Tables III and V). Addition of H2O2 to the Asp-140 mutants caused a gradual destruction of the heme that, compared with wild-type hHO-1, resulted in the formation of only a small amount of Fe(III)-verdoheme. This observation may reflect side reactions known to occur between H2O2 and heme that lead to fragmentation products other than biliverdin (25). In the present study, this abnormal heme destruction could be suppressed by the inclusion of guaiacol in the incubation, suggesting that the heme loss was catalyzed by a
ferryl rather than Fe(III)-OOH species. This conclusion derives from the fact that the production of Fe(III)-verdoheme is mediated by an Fe(III)-OOH intermediate (3), whereas the peroxidase activity is catalyzed by the ferryl species derived from this intermediate by cleavage of the O–O bond. Thus, it should be possible to suppress reactions that depend on the ferryl species by reducing it with guaiacol without altering reactions supported by the Fe(III)-OOH species, which is not expected to react with guaiacol. Indeed, when guaiacol is present, a significant increase in the amount of Fe(III)-verdoheme formed with H$_2$O$_2$ was observed (Table V).

All of the Asp-140 mutants could be reduced by NADPH and P450 reductase to form the characteristic Fe(II)-CO and Fe(II)-O$_2$ complexes (Fig. 11). In addition, although the amount was significantly less than that observed with wild-type hHO-1, the Asp-140 mutants were able to generate Fe(III)-verdoheme in the presence of P450 reductase and a few equivalents of NADPH. However, the Asp-140 mutants were unable to catalyze the formation of verdoheme in the presence of CO. In these experiments, the wild-type HO reaction is arrested at the Fe(II)-CO-verdoheme stage. Formation of this intermediate on the normal catalytic pathway is dependent on displacement of CO from the Fe(II)-CO-heme complex to form the Fe(II)-O$_2$-heme species. Clearly, the interactions favoring formation of the Fe(II)-O$_2$ intermediate from the Fe(II)-CO complex are disrupted by substitution of an Ala, Phe, His, Leu, or Asn for Asp-140. As a result, a much smaller proportion of the Fe(III)-OOH species is converted back to the Fe(II) state of the protein, and of H$_2$O$_2$ to the Fe(III) state of the enzyme, is mirrored in the Fe(III) state by loss of the water molecule that is normally coordinated as a sixth ligand to the iron atom. These findings support the inference from the x-ray crystal structure that Asp-140 participates in a critical hydrogen bonding network. Introduction of other amino acids at this position disrupts the favorable hydrogen bonding interactions, redirecting the HO catalytic cycle toward ferryl formation. As a consequence, the ferryl species promotes abnormal heme fragmentation or, in the presence of guaiacol, catalyzes the observed peroxidation reaction.

It is clear that the oxidation of heme to verdoheme and verdoheme to bilirubin is sensitive to the nature of the amino acid that replaces Asp-140 in the active site. For example, the oxidative steps leading from heme to verdoheme are significantly inhibited (65, 82, and 97%) in the D140F, D140H, and D140L mutants, respectively, and, as a result, no bilirubin formation is detected. On the contrary, mutation of Asp-140 to either Asn-140 or Ala-140 has less of an impact on the conversion of heme to verdoheme (13 and 44%, respectively), but produces a precipitous drop in the relative amounts of bilirubin formation (97 and 99%, respectively). It seems likely that the larger amino acids interfere with the binding and subsequent activation of O$_2$ in the hHO-1 active site, whereas the smaller amino acid substitutions simply reduce the efficiency of the reaction. This conclusion is most clearly illustrated by the RR data for the D140H mutant, which indicate that the distal histidine coordinates to the iron atom, giving a bis(histidine)-coordinated heme. As might be expected, his coordination significantly inhibits the binding of O$_2$ to the heme iron, thus accounting for the decrease in the amount of verdoheme formed. It has been reported that the redox potential of bis(histidine)-coordinated heme proteins is significantly lower than that of heme proteins in which only one of the iron ligands is a histidine (6, 26–28). This finding may explain the inability of the verdoheme that is formed from the D140H mutant to be reduced by P450 reductase and therefore to catalyze the oxidation of heme to biliverdin.

In summary, replacement of Asp-140 of wild-type hHO-1 by an Ala, Phe, His, Leu, or Asn significantly decreases bilirubin formation but increases peroxidative activity. These effects appear to be due largely to an alteration of the normal catalytic cycle that involved disruption of the O$_2$ binding facilitated by Asp-140. As a result, a much smaller proportion of the Fe(III)-OOH intermediate is directed toward electrophilic addition to the α-meso-carbon edge and, instead, is channeled by heterolytic O–O bond cleavage into the formation of a ferryl species. This drastic shift in the partitioning of the reactive intermediate formed by hHO-1 as a result of the mutations establishes the importance of Asp-140 as a catalytic residue and converts heme oxygenase into a peroxidase.

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