Replacement of the Distal Glycine 139 Transforms Human Heme Oxygenase-1 into a Peroxidase*

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The human heme oxygenase-1 crystal structure suggests that Gly-139 and Gly-143 interact directly with iron-bound ligands. We have mutated Gly-139 to an alanine, leucine, phenylalanine, tryptophan, histidine, or aspartate, and Gly-143 to a leucine, lysine, histidine, or aspartate. All of these mutants bind heme, but absorption and resonance Raman spectroscopy indicate that the water coordinated to the iron atom is lost in several of the Gly-139 mutants, giving rise to mixtures of hexacoordinate and pentacoordinate ligation states. The active site perturbation is greatest when large amino acid side chains are introduced. Of the Gly-139 mutants investigated, only G139A catalyzes the NADPH-cytochrome P450 reductase-dependent oxidation of heme to biliverdin, but most of them exhibit a new H2O2-dependent guaiacol peroxidation activity. The Gly-143 mutants, all of which have lost the water ligand, have no heme oxygenase or peroxidase activity. The results establish the importance of Gly-139 and Gly-143 in maintaining the appropriate environment for the heme oxygenase reaction and show that Gly-139 mutations disrupt this environment, probably by displacing the distal helix, converting heme oxygenase into a peroxidase. The principal role of the heme oxygenase active site may be to suppress the ferryl species formation responsible for peroxidase activity.

Heme oxygenase (HO), also known as hsp32, catalyzes the NADPH- and cytochrome P450 reductase-dependent oxidation of heme to biliverdin IXα, iron, and CO, each of which has important biological properties (1). Biliverdin is reduced by biliverdin reductase to bilirubin, which is then excreted as the glucuronic acid conjugate (2). The excretion of bilirubin is frequently impaired in newborn children due to a lag in the expression of the genetic glucuronyl transferase deficiency (3). High concentrations of unconjugated bilirubin are neurotoxic, and the prevention of its accumulation through phototherapy or the inhibition of HO is of critical importance (4–6). However, low concentrations of biliverdin and bilirubin appear to provide important antioxidant protection (7, 8). The iron released by HO is normally recycled and represents the major source of this metal in iron homeostasis, but increased iron release due to elevated heme oxygenase activity can trigger enhanced lipid and protein peroxidation (8, 9). Finally, CO appears to play a role as a signaling molecule akin to that of nitric oxide (10–12). A role for CO in signaling pathways has received strong support from studies of HO knockout mice (13, 14).

The existence of two HO isoforms, HO-1 and HO-2, is well established (15–17), and a third isoform whose significance is unclear has been reported (18). HO-1 is induced by chemical agents and a variety of stress conditions, and is found in highest concentration in the spleen and liver. In contrast, HO-2 is not induced by exogenous stimuli and is found in highest concentrations in the brain and testes. The HO enzymes are membrane-bound proteins (19, 20), but water-soluble, catalytically active versions of rat and human HO-1 without the C-terminal 23-amino acid membrane anchor have been expressed in Escherichia coli (21–23). Truncated forms of HO-1 have been used to obtain crystal structures of both the human and rat enzymes (24, 25).

The truncated human hHO-1 crystal structure shows that the heme pocket is primarily formed by two helices, one on the proximal and the other on the distal side of the heme (24). The proximal helix includes His-25, the residue shown to be the proximal iron ligand by site-directed mutagenesis and resonance Raman (RR) studies (26–30). These spectroscopic studies indicate that His-25 is coordinated to the iron as a neutral rather than strongly hydrogen-bonded or deprotonated imidazole (26, 30). Replacement of His-25 by an alanine produces a catalytically inactive protein to which heme binds without the detectable coordination of a strong axial iron ligand (27). However, the proximal iron coordination and the catalytic activity can be restored in the H25A mutant by the addition of exogenous imidazole (28). The proximal histidine ligand is therefore catalytically important, but its precise orientation relative to the heme and its low imidazole character do not appear to critically influence catalysis.

The distal active site helix in hHO-1 is kinked by about 50° directly over the heme (Fig. 1) (24). Unlike the myoglobins and peroxidases, the distal HO-1 active site does not have a histidine or other obvious polar residue positioned to stabilize iron-bound ligands. The only polar moieties that appear to be sufficiently close to directly hydrogen bond to the distal water
Heme Oxygenase Gly-139 and Gly-143 Mutants

lign are the carbonyl oxygen and amide nitrogen of Gly-139 and Gly-143, respectively (24). If the α-helical hydrogen bond involving these residues does not remain intact, Gly-139 and Gly-143 may be able to form polar contacts with the iron-ligand ligands. In addition, these conserved glycine residues directly contact the heme and may provide the flexibility required to allow the distal helix to approach closer to the heme than it does in the myoglobin and peroxidases (24). The flexibility of the distal helix is thought to enable the opening and closing of the active site required to bind heme and release biliverdin.

To determine the importance of the two conserved glycine residues in hHO-1-catalyzed heme oxidation, we have constructed, expressed, and characterized a series of mutants in which the glycines have been replaced by amino acids with larger aliphatic, aromatic, or charged side chains. The structural, and catalytic properties of these hHO-1 mutants establish that Gly-139 and Gly-143 are critical determinants of the catalytic outcome.

EXPERIMENTAL PROCEDURES

Materials—H2O2 (30%), NADPH, heme, bovine serum albumin, sodium dithionite, sodium ascorbate, guaiacol, and phenol were obtained from Aldrich or Sigma. High purity argon (99.999%), CO (99.95%), and O2 (99.9%) were obtained from Matheson and Aldrich. All chemicals were used without further purification.

Enzymes—Human NADPH-cytochrome P450 reductase and rat biliverdin reductase were expressed and purified according to previously described protocols (32, 33).

Expression and Purification of hHO-1 and Its Mutants—The HO-1 construct that was used encoded the human liver protein without the C-terminal amino acids (22). Oligonucleotide synthesis was carried out by the Biomolecular Resource Center of the University of California at San Francisco on an Applied Biosystems 380B DNA synthesizer. The construct that was used encoded the human liver protein without the 23 C-terminal amino acids (22). Oligonucleotide synthesis was carried out by the Biomolecular Resource Center of the University of California at San Francisco on an Applied Biosystems 380B DNA synthesizer. The hHO-1 Gly-139 and Gly-143 mutant constructs were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Antibiotic selection (ampicillin) was used to obtain a high frequency of mutants. Transformants were screened by restriction digestion and confirmed by sequence analysis. Plasmid purification, sequencing, subcloning, and bacterial transformations were carried out by standard procedures (34). E. coli strain DH5α (F ara proAB lacIq pep4 dcm L-lys), B800 lacZaD M15 hisd R7) was used for expression of the hHO-1 constructs. The hHO-1 proteins were purified and reconstituted with heme using previously reported methods with a yield of purified protein of 3–4 mg ml−1 (21). All experiments using the purified proteins were performed in 100 mM potassium phosphate, pH 7.4 (standard buffer), unless otherwise stated.

Spectral Characterization of hHO-1 and Its Mutants—The UV-visible spectra of hHO-1 and the hHO-1 mutants were recorded in the standard buffer on a Cary Varian Model 1E spectrophotometer. The FeIII and FeIII-CO complexes were formed by reduction of the FeIII complex with sodium dithionite or anaerobically with NADPH and cytochrome P450 reductase, in the absence or presence, respectively, of bubbling in the solution.

RR Spectroscopy—Typical enzyme concentrations for RR experiments were 150–250 μM in standard buffer solution. Microcon 10 ultrafiltration devices (Amicon) were used for buffer and water exchange. A final enrichment of 80% 18O or 2H-labeled water (95% 18O, Cambridge Isotope Laboratory; 99.9% 2H, Aldrich) was achieved in the experiments using labeled water. Reduction to the FeII state was achieved by adding microliter aliquots of a 10 mM sodium dithionite solution to an argon-purged sample in the Raman capillary cell and was monitored by UV-visible spectroscopy in the same cell (35). 13CO (CP grade, Air Products) and 13CO (99% 13C, 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 spectrograph (set at 0.67 m with variable gratings) equipped with a Princeton Instruments liquid-N2-cooled charge-coupled device detector (LN1100PB). Kaiser Optical superetch filters were used to attenuate Rayleigh scattering. Excitation sources consisted of an Innova 302 krypton laser (413 nm) and a Liconix 4240NB He/Cd laser (442 nm). Spectra were collected in a 90°-scattering geometry on samples at room temperature with a collection time of a few minutes, whereas longer times and a back-scattering geometry were used for low-temperature experiments. Frequencies were calibrated relative to indene and CCl4 standards and are accurate to ± 1 cm−1. CCl4 was also used to check the polarization conditions. Optical absorption spectra of the Raman samples were obtained on a PerkinElmer Lambda 9 spectrophotometer to monitor the samples (fully oxidized, fully reduced, and reduced CO complex) both before and after laser illumination.

Determination of hHO-1 Activity by Measuring Bilirubin Formation—A solution of hHO-1 or one of its mutants (1 μM) and NADPH (500 μM) reductase (1 μM), biliverdin reductase (4 μM), heme (30 μM), and bovine serum albumin (1 μM) in standard buffer was preincubated for 3 min at 37 °C. The reaction was initiated by the addition of NADPH (500 μM) and the formation of bilirubin was monitored at 468 nm.

Reaction of the hHO-1 Mutants with NADPH-Cytochrome P450 Reductase—Cytochrome P450 reductase (2 μM) and the hHO-1 mutant (5 μM) in standard buffer (final incubation volume, 500 μl) were preincubated at 23 °C in a cuvette. The reaction was initiated by addition of NADPH (1 mM), and the progress of the reaction was monitored with UV-visible spectroscopy. When no further changes were observed (~20 min), the incubation was extracted and the biliverdin region formed was determined by HPLC as described previously (21).

RESULTS

Expression, Purification, and Characterization of the hHO-1 Mutants—The G139A, G139L, G139F, G139W, G139H, G143A, G143L, G143F, G143W, and G143H hHO-1 mutants were expressed and purified as described previously for wild-type hHO-1 (21). Each mutant was obtained in a yield of approximately 3–4 mg of purified protein per liter and was judged to be >95% pure by SDS-polyacrylamide gel electrophoresis. The FeIII heme complexes of the mutants exhibited slightly different Soret absorption maxima, with a low value of 384 nm for the G143H mutant (Table I) and a high value of 411 nm for the G139F mutant (Fig. 2). The absorption spectrum of the Gly-139 (Figs. 2 and 3) and Gly-143 mutants (not shown) had FeIII, FeIII-O2, and FeIII-CO Soret maxima at 430, 410, and 420 nm, respectively. These values are similar to those for wild-type hHO-1 (21, 26). These spectroscopic results suggest that the heme coordination and ligation states for the wild-type and mutant hHO-1 enzymes are the same in the FeIII, FeIII-O2, and FeIII-CO heme complexes.
Fe$^{3+}$-CO states but differ in the resting Fe$^{3+}$ state.

As previously reported, the Soret maximum for wild-type hHO-1 undergoes a red shift with a $pK_a$ value of $\sim 8$ (Table I (26)). In contrast, the Soret maxima of the Gly-139 mutants did not shift at pH values up to 11 and those of the Gly-139 mutants exhibited varying responses as the pH was increased. The Soret band of the G139A mutant underwent a red shift with a $pK_a$ of $\sim 7$, whereas those of the G139H and G139F mutants underwent a red shift with $pK_a$ values between 6 and 7. The G139D and G139W mutants did not exhibit a pH-dependent Soret shift, and the G139L mutant exhibited anomalous behavior in that it shifted to the red with a $pK_a$ of 7–8 but then appeared to shift back above pH 9. These results suggest that the G139A, G139H, and G139L mutants retained the distal water ligand characteristic of wild-type hHO-1, but its properties were modified by the alterations in its environment caused by the mutations. In contrast, the G139D, G139W, and Gly-143 mutants did not appear to retain a distal water ligand.

**RR of the Gly-139 hHO-1 Mutants**—RR spectra of the Fe$^{3+}$ wild-type, G139A, and G139L heme complexes were obtained with Soret excitation (Fig. 4). The $v_2$ modes at 1483 and 1503, and $v_3$ modes at 1565 and 1582 cm$^{-1}$ observed in the wild-type enzyme are characteristic of a six-coordinate high spin/low spin mixture (6cHS/6cLS) with a predominance of the HS state (26, 29). In the mutant proteins, the 6cHS/6cLS equilibrium was perturbed and a pentacoordinate high spin (5cHS) species emerged with $v_3$ at 1492 and $v_2$ at 1572 cm$^{-1}$ (Fig. 4). In fact, this 5cHS species dominated the G139L RR spectrum (Fig. 4). The RR data indicate that as bulkier side chains were introduced into the heme distal pocket, the distal water ligand bound to the heme iron in the wild-type enzyme (26, 29) was destabilized.

In accord with the above conclusion, addition of an aromatic side chain in the distal pocket in the G139F and G139W mutants strongly perturbed the heme configuration. In the Fe$^{3+}$ G139W heme complex, the major species was a 5cHS species, whereas in the Fe$^{3+}$ wild-type, G139H, and G139L complexes, the Fe$^{3+}$ HS configuration, but in the Fe$^{3+}$-CO complexes of the G139F and G139W mutants, the observation of multiple Fe–C–O vibrations indicates the existence of a heterogeneous active site environment (data not shown). Thus, it appears that the G139F and G139W mutations significantly perturbed the heme binding pocket and gave rise to several heme-protein conformers.

**TABLE I**

Heme oxygenase and peroxidase activities of the Gly-139 and Gly-143 mutants

| Enzyme   | Soret maximum | Soret band shift | Fe$^{3+}$ spin state, pH 7.0 | HO-1 activity | Peroxidase activity |
|----------|---------------|------------------|------------------------------|---------------|---------------------|
|          | nm            | $pK_a$           |                             | %             | s$^{-1}$             |
| WT       | 406           | $\sim$8         | 6cHS/6cLS                    | 100           | 0.0016              |
| G139A    | 409           | $\sim$7         | 6cHS/6cLS                    | 58            | 0.02                |
| G139L    | 403           | 7–8             | 5cHS                         | 3             | 0.05                |
| G139F    | 411           | 6–7             | 6cLS                         | 6cLS/6cHS     | 1                   | 0.05                |
| G139W    | 393           | None            | 5cHS                         | 1             | 0.02                |
| G139H    | 409           | 6–7             | 6cLS                         | 6cLS          | Not detected        | Not detected        |
| G139D    | 404           | None            | Not determined               | Not detected  | Not detected        | Not detected        |
| G143D    | 406           | None            | Not determined               | Not detected  | Not detected        | Not detected        |
| G143K    | 410           | None            | Not determined               | Not detected  | Not detected        | Not detected        |
| G143L    | 394           | None            | Not determined               | 2             | Not detected        | Not detected        |

* The dominant spin state is shown in bold letters.

**FIG. 2.** The UV-visible spectra of the mutant proteins in the Fe$^{3+}$, Fe$^{2+}$, and Fe$^{2+}$CO states. Solid lines, Fe$^{3+}$; dashed lines, Fe$^{2+}$; dashed and dotted lines, Fe$^{2+}$-CO.
the major species in this complex was 6cLS rather than the predominant HS state observed in the wild-type enzyme (Fig. 5). In the low frequency RR spectra of the G139H complex, a band at 508 cm$^{-1}$ downshifted by 4 cm$^{-1}$ in D$_2$O and 16 cm$^{-1}$ in 18O-labeled water and was assigned to the n(Fe$^\text{z}$OH) of a 6cLS hydroxo adduct (Fig. 5). The low frequency of the n(Fe$^\text{z}$OH) and small deuterium shift are indicative of hydrogen-bonding interactions between the hydroxo group and the distal pocket in the mutant. In wild-type HO-1, a higher pH is required to deprotonate the distal water ligand (Table I), and the Fe$^\text{z}$OH vibration is observed at 546 cm$^{-1}$ (29). The new imidazole side chain in the G139H mutant (Table I) is likely to hydrogen-bond the aqua/hydroxo distal ligand, lowering its $pK_a$ from ~8.0 in the wild-type (26, 29) to less than 7.0 in the G139H mutant.

The Fe$^{\text{III}}$ G139D heme complex was a 6cHS species (Fig. 5). However, raising the pH from 5.0 to 11.0 and incubating the enzyme in deuterated and 18O-labeled water had no effect on its RR spectrum at either room temperature or 90 K. Either the carboxylate side chain of Asp-139 in the G139D mutant replaced the distal water as the heme sixth ligand or the local pH in the distal pocket was so perturbed by the carboxylate group that deprotonation of a water ligand was prevented.

Catalytic Turnover of the hHO-1 Mutants—The G139A hHO-1 mutant retained 58% of the wild-type hHO-1 bilirubin forming activity, whereas all the Gly-139 and Gly-143 hHO-1 mutants exhibited little or no (~3%) activity (Table I). The G139A mutant formed biliverdin IX$\alpha$, the same biliverdin isomer formed by the wild-type protein (not shown). Addition of NADPH-cytochrome P450 reductase to the G139A hHO-1 mutant formed Fe$^{\text{III}}$-verdoheme-CO complexes. Addition of NADPH-cytochrome P450 reductase to the G139L, G139F, G139H, G143D, G143K, and G143L hHO-1 mutants also resulted in formation of the Fe$^{\text{III}}$-O$_2$ complex with a Soret
maximum at 410 nm and distinct α and β bands in the visible region (Fig. 3). However, with these proteins the verdoheme-CO complex characteristic of normal turnover was not observed, although gradual loss of the Soret bands due to the FeII-O2 complex and heme itself indicates that the heme was gradually destroyed. HPLC analysis revealed that no more than a trace of biliverdin IXα was produced by these mutants.

The G139W, G139D, or G139H hHO-1 mutants yielded FeII-CO but not detectable FeII-O2 complexes with NADPH-cytochrome P450 reductase (Figs. 2 and 3). These results show that the two roles of the heme group in heme oxygenase, as a prosthetic group and as a substrate, can be dissociated by mutations in the protein active site.

Reaction of the hHO-1 Mutants with H2O2—Addition of 1–2 eq of H2O2 to the G139A heme complex resulted, as with wild-type hHO-1, primarily in FeIII-verdoheme formation, as shown by the appearance of a broad absorption with a maximum at 680 nm (Fig. 6). In contrast, addition of 1–2 eq of H2O2 to the G139L and G139F heme complexes caused a red shift of the Soret band to 418 nm and the appearance of α and β bands at 544 and 580 nm, respectively, without an increase in absorption at 680 nm (Fig. 6). Under the same conditions, the absorption maximum of the G139D mutant underwent a similar but smaller red shift with the development of α- and β-bands. The FeIII enzyme was regenerated upon addition of guaiacol or ascorbic acid to these red-shifted species, as illustrated for the G139L mutant in Fig. 7. Furthermore, addition of guaiacol to the mutant hHO-1 enzyme prior to H2O2 addition prevented formation of the 418-nm species. The enzyme appeared to remain in the FeIII state, presumably because the rate of regeneration of the FeIII state was more rapid than the rate of formation of the 418-nm species. The enzyme appeared to remain in the FeIII state, presumably because the rate of regeneration of the FeIII state was more rapid than the rate of formation of the 418-nm species. However, when a 30-fold excess of H2O2 was added, a time-dependent increase in absorption at ~470 nm was observed due to guaiacol peroxidation products (Fig. 8). These results suggest that the 418-nm species is a Compound II-like ferryl species similar to that shown previously to be formed in the reaction of wild-type hHO-1 with acyl- and alkylhydroperoxides (21). Relative to the other Gly-139 hHO-1 mutants, a significantly smaller (or nonobservable) ferryl species was formed when 2 eq of H2O2 was added to the G139H or G139W hHO-1 mutants (Fig. 6). Addition of H2O2 to the Gly-143 mutants in the absence or presence of guaiacol resulted in bleaching of the heme without the observable formation of a ferryl species, guaiacol oligomers, or verdoheme.
the 470-nm absorbance was observed in these control experiments. The G139L, G139F, and G139D hHO-1 mutants had higher peroxidase activities than the G139A, G139W, or G139H mutants. Interestingly, the peroxidase activity was specific to the Gly-139 hHO-1 mutants, because the Gly-143 hHO-1 mutants did not catalyze the oxidation of guaiacol.

**DISCUSSION**

The x-ray crystal structures show that the distal portion of the hHO-1 active site, unlike that of the hemoglobin, myoglobin, and peroxidases, lacks a side chain capable of directly hydrogen bonding with ligands bound to the heme iron (24, 25). However, if the α-helical hydrogen bond between the amide nitrogen of Gly-139 and carbonyl oxygen of Gly-143 is weak, these backbone atoms may be able to hydrogen bond with bound ligands. In addition, these conformationally mobile glycine residues may provide the flexibility to the HO-1 active site required to allow heme to bind and biliverdin to dissociate (Fig. 1). By fulfilling these two functions, Gly-139 and/or Gly-143 could help to differentiate HO-1 from the myoglobins and peroxidases and confer upon the enzyme its efficient and specific ability to oxidize heme. To test these hypotheses, Gly-139 and Gly-143 hHO-1 mutants designed to evaluate the effects of side-chain steric bulk and polarity on hHO-1 activity were constructed, expressed, and characterized.

All the mutants examined appeared to fold and bind heme normally, but the UV-visible (Fig. 2, Table I), pH titration (Table I), and RR (Fig. 4) data indicate that the distal water ligand coordinated to the FeIII heme iron in the wild-type enzyme is destabilized as bulky side chains are engineered into the heme distal pocket. The variations in the FeIII heme ligand and spin states of the mutants indicate that there are differences in their active site environments. A clear indicator of these differences is the absence of the characteristic shift of the Soret band as the pH is increased, which suggests that the distal water ligand is absent in all of the Gly-143 mutants. The changes in the iron coordination and spin state were more complex for the Gly-139 mutants (Table I). Three mutants (G139A, G139F, G139H) exhibited a normal red shift but with a lower pK_a value, one (G139L) an anomalous pH shift dependence, and finally two (G139W, G139D) no pH shift at all. As confirmed by the experiments described below, the water ligand is strongly perturbed in all but the G139A mutant.

RR spectroscopy provides additional information on the changes that occur at the heme iron atom in the Gly-139 mutants. Replacement of Gly-139 by a slightly larger alanine minimally perturbs the active site. Not only is the water ligand present, but it has a pK_a value similar to that of the wild-type and, as shown by RR spectroscopy, exists as a comparable mixture of 6cHS/6cLS states at neutral pH (Fig. 4). However, a small 5cHS component detectable in the G139A mutant is not present in the wild-type, so even the alanine substitution has some effect on the heme coordination and spin states. As Gly-139 is replaced by the larger leucine, phenylalanine, and histidine residues, the pK_a and other properties of the distal water ligand are more severely altered. The G139F mutant is a 6cLS/5cHS mixture, whereas the G139L mutant is primarily 5cHS and the G139H mutant is primarily 6cLS at neutral pH (Fig. 5). These alterations in the coordination and spin state of the iron may be due to steric interference with binding of the water ligand or perturbation of the hydrogen bonding and electrostatic interactions that stabilize the distal water ligand. In the case of the G139H mutant, the decreased pK_a of the distal water ligand and the observation of 1H- and 2H-isotope effects on a band assigned to the Fe-OH stretching mode (Fig. 5, inset) suggest that the histidine forms a hydrogen bond with the distal water ligand. In case of the G139D mutant, the observation of a 6cHS state in the RR spectrum (Fig. 5) implies that a weak ligand, at best, is coordinated to the 6th position on the iron. The absence of a water ligand is indicated by the fact that there is no pH-dependent shift in the Soret band of the FeIII protein (Table I) and by the absence of an isotope effect on the RR spectrum in 2H- or 18O-labeled water. These results are consistent with direct ligation of the Asp-139 carboxyl group to the iron. In the case of the G139W mutant, the observation of a 5cHS state by RR strongly suggests that the large side chain sterically prevents binding of a water molecule to the FeIII heme group.

All of the mutants, some of which are shown in Fig. 3, can be reduced by NADPH-cytochrome P450 reductase to give FeII and FeIII-CO complexes with UV-visible and RR spectra similar to those of the wild-type enzyme. However, the observation of multiple FeII-CO bands in the RR spectra of the mutants with the larger phenyl and tryptophan side chains indicates that they cause active site heterogeneity (spectra not shown). In contrast to formation of the FeII and FeIII-CO states, formation of the FeIII O2 complexes was not observed for all the mutants. The G139A, G139L (Fig. 3), G139F (Fig. 3), G139H (Fig. 3), G143D, G143K, and G143L hHO-1 mutants form detectable FeIII O2 complexes with NADPH-cytochrome P450 reductase, but the G139W (Fig. 3), G139D (Fig. 3), and G143H hHO-1 mutants do not. However, catalytic studies (vide infra) indicate that the failure to observe the FeIII O2 complex in some instances reflects its rapid decomposition rather than inability of the enzyme to form the complex. In all cases, the heme was completely destroyed upon exposure to NADPH-cytochrome P450 reductase but only the G139A mutant produced verdoheine and biliverdin. Clearly, of the mutants investigated here, only the G139A hHO-1 mutant is able to correctly reduce the FeIII O2 complex to the FeIII OOH species required for heme oxidation. In the case of the mutants that give a detectable FeIII O2 complex but did not form verdoheine or biliverdin, either the FeIII O2 complex autoxidizes too rapidly to allow formation of the FeIII OOH intermediate, or the FeIII OOH intermediate, once formed, is unable to react normally with the heme group. Failure of the FeIII OOH in the Gly-139 mutants to react normally with the heme reflects, as discussed below, its rapid cleavage to give a ferryl (FeIV =O) species. The abnormal heme degradation observed with these mutants presumably gives rise to propentdyopent and monopyrrole heme fragments by a general, but still not understood, mechanism that may be linked to the formation of a ferryl species (31).

Although the G139A mutant retains approximately 60% of
the wild-type HO activity, it has also acquired a significant peroxidase activity (Table I). Several of the other Gly-139 mutants, all of which have lost their HO activity, also exhibit a new peroxidase activity. This activity is associated with the formation of a peroxidase-like Compound II species (Fig. 6). Catalytic turnover of G139A hHO-1 thus partitions between normal electrophilic attack of the Fe(III)-OOH intermediate on the porphyrin ring and cleavage of the O–O bond to give the ferryl species responsible for the peroxidase activity. The finding that the rate of verdoheme formation by the G139A mutant is the same whether guaiacol is present or absent is consistent with reduction by guaiacol of a ferryl species subsequent to divergence of the two catalytic pathways (data not shown). The HO and peroxidase reactions are thus parallel, independent processes that involve different fates for the Fe(III)-OOH intermediate. The finding that only the G139A with a small side chain retains HO activity indicates that the partition ratio between HO and peroxidase activities is sensitive to the size of the side chain at position 139. Side chains larger than an alanine suppress HO activity in favor of peroxidase activity, and very large side chains (e.g., G139W) suppress both activities (Table I).

The Gly-139 hHO-1 mutants possessing peroxidase activity react with H2O2 to form an Fe(III)OOH intermediate, which is converted to a Compound II-like ferryl species that oxidizes guaiacol. Because H2O2 is a two-electron oxidizing agent, it is likely that Compound II formation is accompanied by the formation of a protein radical. We have previously detected the formation of a protein radical in the reaction of wild-type HO-1 with alkyl and acylhydroperoxides (21). As already noted, the heme in the Gly-139 and Gly-143 mutants other than G139A hHO-1 is destroyed via an abnormal process on incubation with either H2O2 or NADPH-cytochrome P450 reductase. However, addition of guaiacol to the enzyme prior to incubation with H2O2 or NADPH-cytochrome P450 reductase protects the enzyme from abnormal heme destruction (Fig. 8). In view of the demonstration that guaiacol rapidly quenches the ferryl species, presumably quenches the protein radical, and also protects the heme from abnormal degradation, it appears that the abnormal degradation of the heme depends on the formation of a ferryl species.

In the peroxidases, a distal histidine acts as a general base to accelerate the binding of H2O2 to the iron atom by deprotonating it and as a general acid by protonating the terminal oxygen of the resulting Fe(III)OOH complex and thus facilitating helicryletic cleavage of the dioxygen bond to give the ferryl intermediate. However, the crystal structures show that in hHO-1 there is no distal histidine or other polar residue equivalent to that in the peroxidases (24, 25). The absence of a strong acid-base catalyst apparently suppresses oxygen-oxygen bond cleavage in favor of electrophilic reaction of the Fe(III)OOH with the porphyrin ring. The inverse correlation between the loss of HO activity and acquisition of peroxidase activity as a function of the size of the side chain suggests that steric effects play a role in the change in catalytic outcome for the Gly-139 mutants. The hHO-1 crystal structure indicates that, if the distal helix conformation is preserved, the side chains of the amino acids that replace Gly-139 will point directly toward the heme face (Fig. 9). Indeed, side chains larger than a methyl require either a conformational change in the distal helix or its vertical displacement to move it away from the heme face. Either of these structural adjustments would disrupt whatever hydrogen bonds exist between Gly-139 and the water, dioxy, or peroxo ligand bound to the heme iron atom. Displacement of the helix may also increase the water content and electrostatic environment of the active site.