Heme Oxygenase Active-Site Residues Identified by Heme−Protein Cross-Linking during Reduction of $CBrCl_3$[†]

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ABSTRACT: The reduction of $CBrCl_3$ by the heme−heme oxygenase complex forms dissociable and covalently bound heme products. No such products are formed with mesoheme in which the heme vinyl substituents are replaced by ethyl groups. The dissociable heme products are chromatographically similar but not identical to those obtained in the analogous reaction with myoglobin. Tryptic digestion of the heme−protein adduct and Edman sequencing and mass spectrometric analysis of the heme-linked peptide identify His-25, the proximal iron ligand, as the alkylated residue. Reaction of $CBrCl_3$ with the heme complexes of the T135V mutant and a Δ^{221} C-terminal truncated protein yields heme-linked peptides in addition to that from the wild-type reaction. The sequence of the principal labeled peptide from the T135V reaction, ^{205}TAFLLNIQLFEELQELLTHDTK^{226}, and the lability of the adduct suggest the heme is attached to one of the carboxylic acid residues. A carboxylic acid residue is probably also labeled in the modified peptide ^{49}LVMASLYHIYVALEEEIER^{67} from the Δ^{221} truncated protein. Thus, addition of the reductively generated trichloromethyl radical to a heme vinyl group produces a species that alkylates active-site residues. The changes in the alkylated residue caused by the Thr-135 mutation or truncation of the protein places residues in the sequences 49−67 and 205−226 within the active site. Furthermore, this is the first demonstration that heme oxygenase, like cytochrome P450, may catalyze the reductive metabolism of halocarbons and thus contribute to the toxicity of these agents.

Heme oxygenase catalyzes the NADPH- and cytochrome P450 reductase-dependent conversion of heme to biliverdin and carbon monoxide (*1, 2*). Two isoforms of heme oxygenase are known, an inducible form (HO-1) found in highest concentrations in the liver and spleen (*3, 4*), and a constitutive form (HO-2) found in highest concentrations in the brain and testes (*5, 6*). Heme oxygenase has recently come under intense scrutiny due to its role in heme homeostasis and the putative role of carbon monoxide as an endogenous neuromodulator and/or vasodilator (*7*). The recent expression in *Escherichia coli* of truncated forms of rat and human HO-1 has provided fully active, soluble forms of these proteins for detailed structural and mechanistic studies (*8*−*10*). Expression of soluble, readily purified human HO-1 was achieved by deletion of the 23 carboxy-terminal amino acids that serve as the membrane anchor for the native protein (*8, 9*). Spectroscopic and site-specific mutagenesis studies of the resulting 265 amino acid protein, which is identified here as hHO-1,[1] have established that the ligands to the heme in the protein are His-25 and a water molecule (*11*−*13*). Similar studies suggest that the water molecule coordinated to the iron in hHO-1 may be stabilized by His-132, a highly conserved residue, although the evidence on this point is contradictory (*14, 15*). No other active-site residues have been identified.

The reactions of $CBrCl_3$, CCl_4, and similar halocarbons with heme proteins under reducing conditions produce haloalkyl radicals (*16*−*20*). This radical production is of toxicological interest because it is the activating step in the hepatotoxicity of such agents (*21, 22*). Cytochrome P450 enzymes, in particular, actively catalyze the formation of carbon radicals from halocarbon substrates and are concomitantly inactivated (*17, 22*). Work with myoglobin and hemoglobin has established that, under anaerobic conditions, these proteins also catalyze the reduction of agents such as $CBrCl_3$ (*23*−*25*). The reaction of $CBrCl_3$ with sperm whale myoglobin has been well-characterized and shown to yield both dissociable heme products and heme−protein adducts (*23, 24*). The dissociable heme products were characterized by NMR and mass spectrometric techniques and were shown in all cases to result from addition of a halocarbon radical to one of the heme vinyl groups (*26*). Enzymatic hydrolysis and sequencing of the heme−protein adduct identified the

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of iron oxidation or ligation states; hHO-1, human heme oxygenase isoform 1 truncated by 23 amino acids, leaving a protein of 265 amino acids; wild-type hHO-1 refers to the Δ^{265}-truncated protein); Δ^{233}-hHO-1, human heme oxygenase isoform 1 truncated to a protein of 233 amino acids; Δ^{226}-hHO-1, human heme oxygenase isoform 1 truncated to a protein of 226 amino acids; Δ^{221}-hHO-1, hHO-1 truncated to a protein of 221 amino acids; Δ^{215}-hHO-1, human heme oxygenase isoform 1 truncated to a protein of 215 amino acids; HPLC, high-pressure liquid chromatography.

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proximal iron ligand (His-93) as the residue alkylated by the heme after its activation by addition of a trichloromethyl radical to one of its vinyl groups (*24*). The finding that the alkylation reaction was limited to an active-site residue suggests that the reaction with $CBrCl_3$ may be used to identify active-site residues in hemoproteins of unknown structure.

In the studies reported here we have truncated human HO-1 to determine the minimum length of the carboxyl terminus compatible with the retention of catalytic activity. We have simultaneously investigated the reaction of HO-1 with $CBrCl_3$ under anaerobic conditions and have successfully used it to identify active-site peptides and residues. The results advance our understanding of the active site of the enzyme and demonstrate for the first time that heme oxygenase may function as a catalyst in the activation of halocarbons to toxic radical species.

EXPERIMENTAL PROCEDURES

General Methods. Plasmid purification, sequencing, subcloning, and bacterial transformations were carried out by standard procedures (*27*). A polyclonal rabbit anti-rat HO-1 antibody was raised against the truncated, soluble rat HO-1 protein as reported elsewhere (*28*). Deionized, doubly distilled water was used for all experiments. Oligonucleotide synthesis was carried out at the Biomolecular Resource Center of the University of California at San Francisco on an Applied Biosystems 380B DNA synthesizer. HPLC was done on a Hewlett-Packard Series II 1090 liquid chromatograph. *Escherichia coli* strain DH5α [F′ *ara* Δ*(lac*−*proAB) rpsL* ϕ80d *lacZ*ΔM15 *hsdR17*] was used for the expression of the hHO-1 constructs.

Construction of the hHO-1 T135V Mutant and hHO-1 Truncated Proteins. The hHO-1 T135V mutant was generated using the Altered Sites II mutagenesis kit (Promega, Madison, WI). Antibiotic selection (ampicillin) was used to obtain a high frequency of mutants. Transformants were screened by restriction digestion and confirmed by sequence analysis. The truncated hHO-1 constructs were generated by PCR with the following forward primer hHO-1N encoding the start codon and *Nde*I site and the corresponding reverse primers (the reverse primer nomenclature corresponds to the amino acid residue after which the stop codon TTA is inserted): hHO-1N (5′-GGAGAAAATCAT*ATG*GAGCGTCCGCAACCCGAC-3′), hHO-233 (5′-GCCCGCTGGCGAAGCTCGAG*TTA*CCGTGAGGGGCTCTG-3′), hHO-226 (5′-TGGTGCCCGTGAGGGGTCGAC*TTA*CTTGGTGTCATGGGT-3′), hHO-221 (5′-CTGGTCCTTGGTGTCGAC*TTA*CAGCTCCTG-3′), and hHO-215 (5′-ATGGGTCAGCAGCTCGTCGAC*TTA*CTCAAAGAGCTG-3′).

The reactions contained pBHO-1 (50 ng), the forward primer hHO-1N (150 pmol) and the respective reverse primer (150 pmol), 3 μM dNTPs and 1 μL (10 Units) Vent DNA polymerase in a final volume of 50 μL of 20 mM Tris-HCl (pH 8.88), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, and 0.1% Triton X-100. The annealing and extension cycles were as follows: 94 °C for 10 min (1 cycle), 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min (30 cycles), and 72 °C for 10 min (1 cycle). Following gel purification, the amplified product was digested with *Nde*I and *Sal*I and cloned into pBAce for sequence analysis and expression.

Expression, Purification, and Characterization of Heme Oxygenase Mutants and Truncated Proteins. The hHO-1, hHO-1 T135V, and Δ^{233}-, Δ^{226}-, and Δ^{221}-hHO-1 proteins were expressed and purified as previously described for the wild-type protein (*8, 9*). The hHO-1, hHO-1 T135V, and Δ^{233}-, Δ^{226}-, and Δ^{221}-hHO-1−heme complexes were also purified as previously described for the wild-type protein (*8, 9*). The hHO-1−mesoheme complex was prepared by the same method using ferric mesoheme. The spectra of the complexes in 100 mM potassium phosphate buffer (pH 7.4) were recorded on a Hewlett-Packard 8450A spectrophotometer.

Spectroscopic Assay of Heme Oxygenase Activity. Heme oxygenase activity was assayed as previously described (*8, 9*). The assays contained the appropriate heme oxygenase (3 μg, 0.1 nmol), 15 μM hemin, 1 μM bovine serum albumin, an excess of partially purified biliverdin reductase (rat 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). 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}.

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 $CHCl_3$. The organic layer was washed twice with water and dried over anhydrous Na_2SO_4. The $CHCl_3$ was then removed under argon and the residue was resuspended in the HPLC solvent. HPLC was carried out on a Partisil ODS 3−5 μM reverse-phase column (4.6 × 250 mm) from which the biliverdin isomers were eluted in 50:50 (v/v) acetone/20 mM formic acid (J. C. Lagarias, private communication). The products of the hHO-1 truncated protein reactions were run against known standards.

Reaction of the Heme Complexes with hHO-1, T135V hHO-1, and Δ^{221}-hHO-1 with $CBrCl_3$. The heme complexes of hHO-1, T135V hHO-1, and Δ^{221}-hHO-1 (0.3 μmol in 5 mL) were made anaerobic by purging with argon. The ferrous−deoxy complex was formed by adding sodium dithionite (1 mM) dropwise through a gastight syringe. Formation of the ferrous−deoxy complex was judged to be complete when the solution changed from brown to red. The reaction was started by adding $CBrCl_3$ (3.4 μmol). Following 12 h with stirring at room temperature, the reaction was quenched by the addition of $K_3Fe(CN)_6$ to a final concentration of 110 μM to oxidize any ferrous heme remaining in solution. The reaction mixture was acidified to pH 2.2 with HCl and extracted with an equal volume of ice-cold 2-butanone. The resulting aqueous phase contained a mixture of apoprotein and heme−protein adducts. Exactly the same procedure was used to cross-link the heme to the protein in myoglobin.

Trypsin Digestion of the HO-1 Heme Adducts. Following extraction of the dissociable heme products, the aqueous phase was lyophilized and resuspended in 50 mM Tris (pH 8.0) to a final concentration of 5 mg/mL protein. Trypsin (10 mg/mL) in 0.1 M HCl was added to a final concentration of 1% (w/w) and the protein was digested at 37 °C for 4 h.

HPLC Analysis of the Heme Adducts. Prior to tryptic digestion, the aqueous phase containing the hHO-1 apoprotein and heme-bound protein was subjected to HPLC analysis on a C_4 column (4.6 × 250 mm from Vydac) using a 25

min linear gradient from 20% to 50% solvent B (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile) at a flow rate of 1.0 mL/min. After tryptic digestion, the peptides were separated using the same column and solvents described above with the following gradient protocol: 0−60% solvent B over 60 min, 60−100% solvent B from 60 to 65 min, and 100% solvent B from 65 to 70 min. The eluent was monitored at 400, 280, and 215 nm.

Edman Sequencing of Heme−hHO-1 Peptides. The amino acid sequences of the peptides were obtained on an Applied Biosystems model 470A protein sequencer by the Biomolecular Resource Center, University of California, San Francisco.

Mass Spectrometric Characterization of Heme−hHO-1 Peptides. The MALDI experiments were carried out on a Voyager Elite MALDI-TOF (time-of-flight) mass spectrometer (PerSeptive Biosystems, Framingham, MA), in reflectron mode, with delayed extraction, using the following parameters: accelerating voltage, 20 kV; grid voltage, 70%; guide wire voltage, 0.05%; delay time, 185 ns; and with 4-hydroxy-α-cyanocinnamic acid (Hewlett-Packard, Palo Alto, CA) as the matrix. In linear mode the accelerating voltage was 20 kV, the grid voltage was 94%, the guide wire voltage was 0.05%, and the delay time was 125 ns.

The electrospray experiments were carried out on a Sciex API 300 triple-quadrupole mass spectrometer. The samples were injected in methanol:water (1:1) acidified with 1% acetic acid. For the CID experiment air was used as the collision gas.

RESULTS

Expression and Purification of the Truncated hHO-1 Constructs. Earlier studies demonstrated that the human heme oxygenase without the membrane binding domain is expressed in high yield in *E. coli* as a soluble, catalytically active protein (*8, 9*). This solubilization was achieved by deleting 23 amino acids from the carboxy terminus to give hHO-1, formally a Δ^{265}-truncated protein. To determine to what extent the carboxy terminus of the protein can be further trimmed back without compromising catalytic activity, we have prepared cDNAs coding for the Δ^{233}-, Δ^{226}-, Δ^{221}-, and Δ^{215}-hHO-1 truncated proteins. The purpose of this truncation exercise was to help define residues involved in the catalytic mechanism or in interactions with cytochrome P450 reductase, as well as to obtain proteins with better crystallization properties. The Δ^{233}-, Δ^{226}-, and Δ^{221}-hHO-1 proteins are expressed in *E. coli* at levels comparable to that of the wild type, whereas the Δ^{215}-hHO-1 construct gives no detectable protein as judged by western blot analysis. The Δ^{233}-, Δ^{226}-, and Δ^{221}-hHO-1 proteins, purified by the protocol reported previously for hHO-1 (*8, 9*), were obtained in highly purified form in yields of 20−30 mg/L of cells.

The spectra of the heme complexes of the three truncated proteins were identical to those of the parent heme−hHO-1 complex. The Soret maximum of all these ferric hemeproteins is at 404 nm, and the Soret maxima of the ferrous−dioxygen and ferrous−carbon monoxide complexes are at 410 and 419 nm, with α,*β*-bands at 570 and 540 nm and at 568 and 578 nm, respectively. The specific activities of the three new mutants are approximately 50% that of the original hHO-1 construct, which, in turn, has an activity similar to that of the native enzyme (Table 1). Spectroscopic measurements of the binding of heme to the Δ^{226} protein carried out as previously described yield a K_d value for heme binding of ∼1 μM, a value essentially identical to that for the wild-type protein (*29*). HPLC analysis of the product formed by the three proteins shows that it is exclusively α-biliverdin (not shown). Thus, the truncations do not alter the spectroscopic properties of the heme ligand or the regiospecificity of the heme cleavage reaction but modestly decrease the rate of the reaction.

Table 1: Catalytic Activities and Regiospecificities of the Truncated HHO-1 Proteins

protein	rate (nmol min^{-1} mg^{-1})	activity (%)	biliverdin regioisomer
hHO-1	49	100	α
Δ^{233}	27	56	α
Δ^{226}	23	46	α
Δ^{221}	26	54	α
Δ^{215}			

Formation of the Heme−hHO-1 Protein Adduct. Osawa and co-workers have shown that reductive reaction of $CBrCl_3$ with myoglobin results in addition of a trichloromethyl radical to the heme group and, to some extent, in subsequent covalent binding of the heme to the protein (*23−26*). In principle, reductive heme−protein cross-linking can be used to identify active-site residues in a protein of unknown structure, although the reaction has not been used for this purpose. We report here an investigation of the anaerobic reaction of heme oxygenase with $CBrCl_3$.

The HPLC profiles obtained when the heme−hHO-1 complex is analyzed before and after anaerobic reaction with $CBrCl_3$ show that the heme group is readily modified by the halocarbon. Acidification and chromatography of the unreacted heme−hHO-1 complex shows that the heme (monitored at 400 nm) readily dissociates as a single peak from the protein (monitored at 215 nm) (not shown). The HPLC profile obtained after reaction with $CBrCl_3$ is considerably more complex than the single-peak profile obtained with the unreacted protein. A number of new dissociable heme peaks, labeled 1−4 in Figure 1 (middle panel), and a new protein peak that retains heme absorbance at 400 nm are observed. The dissociable heme products and the residual heme can be extracted from the aqueous phase with 2-butanone but the remaining heme absorbance is irreversibly bound to the protein and cannot be extracted. If the approximation is made that the molar absorbance is not altered by covalent attachment to the protein, approximately 85% of the heme in the original complex is covalently bound to the protein.

The earlier work on the reductive reactions of myoglobin and hemoglobin with $CBrCl_3$ identified a number of heme products, all of which appear to arise from addition of a trichloromethyl radical to a heme vinyl group. The vinyl groups are similarly critical for the reaction of the halocarbon with heme oxygenase. If mesoheme, in which the two protoporphyrin IX vinyl groups are replaced by ethyl groups, is substituted for heme in the reaction, no modified hemes or heme−protein adducts are obtained (Figure 1, bottom chromatogram). The mesoheme−hHO-1 complex reacts with the $CBrCl_3$ but no protein adducts are formed because the vinyl groups are required for reaction with the $CCl_3^{\bullet}$

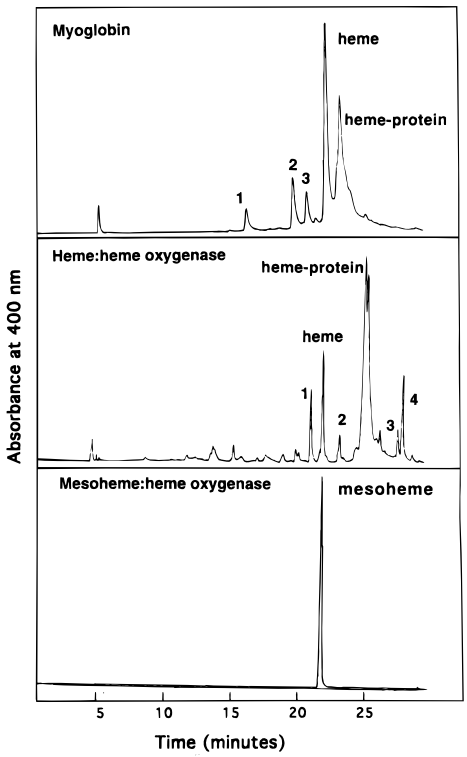

FIGURE 1: Comparison of the HPLC retention times of the heme-derived products formed in the reactions of sperm whale myoglobin (top), heme−hHO-1 (middle), and mesoheme−hHO-1 (bottom) with $CBrCl_3$ under anaerobic, reductive conditions. The HPLC traces were obtained prior to extraction of the samples with butanone. The principal diffusible heme-derived products are numbered and the residual heme and protein-bound heme are labeled.

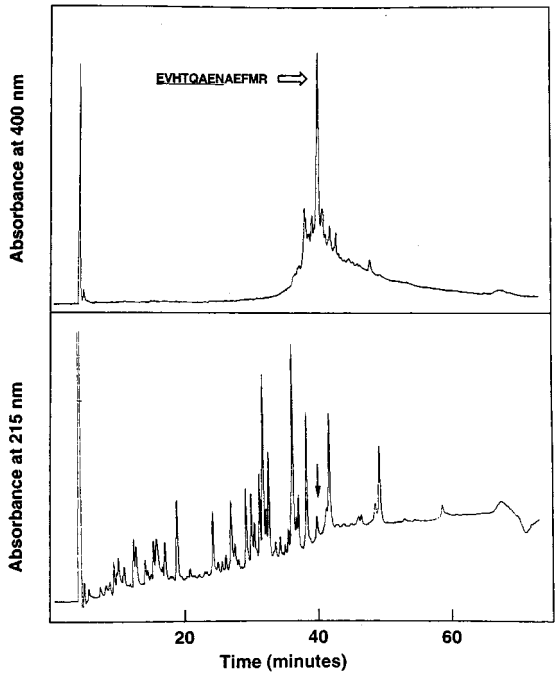

FIGURE 2: Tryptic digest of the protein after reductive reaction of the heme−hHO-1 complex with $CBrCl_3$. Upper panel: Chromatogram monitored at 400 nm. The arrow indicates the heme-labeled peptide and the sequence determined for it is indicated. Lower panel: Chromatogram monitored at 215 nm. The peak corresponding to the heme−peptide is indicated by the arrow.

radical. Despite this similarity, the modified hemes generated in the reaction of the heme−hHO-1 complex with $CBrCl_3$ differ from those obtained in the analogous reaction with myoglobin. HPLC comparison of the products obtained with the two proteins shows that the heme oxygenase reaction gives a larger number of products and that most of the heme oxygenase products have retention times similar, but not identical, to those of the products from the myoglobin reaction (Figure 1). The exact structures of the dissociable heme products have not been pursued because they would provide little information on the heme oxygenase active site, but the failure of the ethyl-substituted heme to give such products shows that they are closely related to the adducts obtained in the myoglobin reaction.

Characterization of the Heme−hHO-1 Adduct. The aqueous fraction that remained after extraction of the dissociable heme products with 2-butanone was subjected to tryptic digestion. HPLC analysis of aliquots taken at various time points showed that after 4 h a principal peptide with absorbance in the 400 nm region and a retention time of 39 min is obtained (Figure 2). Analysis of the peptide by automated Edman degradation provided the sequence ^{23}-EVHTQAEN^{30}, which unambiguously identifies the tryptic peptide as EVHTQAENAEFMR. The yield for each amino acid in the sequencing run was as follows (in picomoles): E (26), V (10), H (2), T (8), Q (8), A (8), E (5), and N (2). In some sequencing runs, the two preceding residues, ^{21}TL^{22}, also appeared to be present in the peptide but these may have been due to a contaminating peptide. A low value for the histidine residue in the peptide identifies it as the site to which the heme is covalently bound. The histidine in question, His-25, has been identified by other methods as the proximal ligand to the heme iron atom (*11*−*13*). The reaction of $CBrCl_3$ with wild-type hHO-1 thus results, like the myoglobin and hemoglobin reactions, in covalent heme attachment to the proximal histidine ligand.

The HPLC-purified labeled tryptic peptide was further analyzed by matrix-assisted laser desorption ionization (MALDI) and electrospray mass spectrometry. The MALDI spectrum in both linear and reflectron modes showed ions at *m*/*z* 616.2, 732.1, 1561.68, and 2292.63 (ions listed as monoisotopic masses) (Figure 3). The electrospray analysis yielded ions at *m*/*z* 765.9 (3+) and 1147.9 (2+), and thus, an average molecular weight of 2294.3 Da (data not shown). On the basis of these results we conclude that the lower mass ions in the MALDI spectra represent prompt (in-source) fragments. The ion at *m*/*z* 616 may correspond to an Fe(II)−heme complex (minus CCl_3), while the ion cluster starting at *m*/*z* 732, based on the isotope distribution, must be the modified (3 Cl-containing) heme−Fe(III) and heme−Fe(II) complex mixture (data not shown). The ion at *m*/*z* 1561.68 is the MH^+ for the nonmodified peptide (MH^+_{calc} = 1561.8, for Glu^{23}-Arg^{35}). The isotope cluster of the labeled peptide shows the presence of the modified heme and Fe(III) in a single charged species (Figure 4). In the electrospray spectrum the cleavage products were not observed. To determine the site of modification the triply charged ion of the labeled peptide was subjected to low-energy collision-induced dissociation (CID) analysis. The CID spectrum features an abundant ion at *m*/*z* 734 (modified heme−Fe complex) and an almost complete series of C-terminal sequence ions (up to Y_{10}) confirming the identity of the

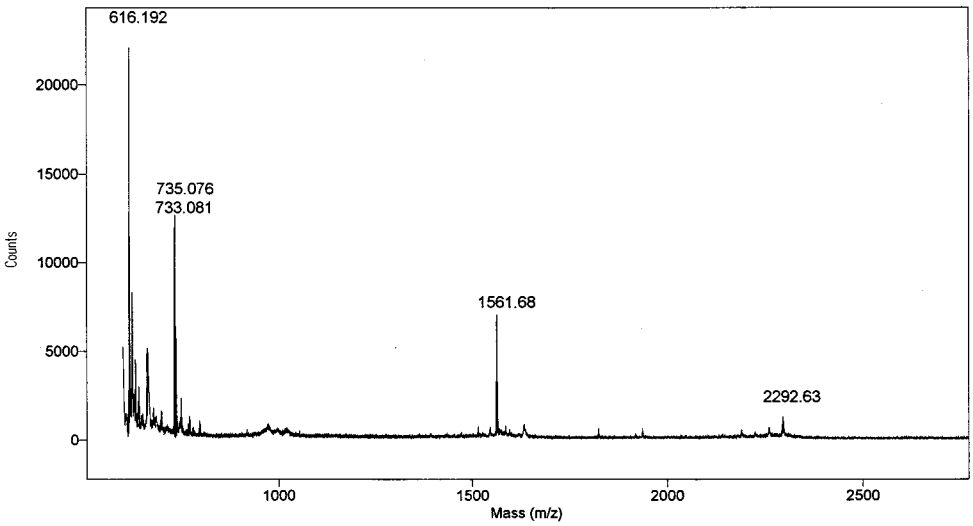

FIGURE 3: MALDI mass spectrum of the HPLC-purified, labeled hHO-1 tryptic peptide. This spectrum was acquired in reflectron mode, with delayed extraction, and 4-hydroxy-α-cyanocinnamic acid as the matrix. Masses indicate the most abundant peak(s) in the isotope clusters, except the modified peptide, for which the monoisotopic mass is displayed.

peptide, but no peptide fragment was detected bearing the label (data not shown).

Characterization of the Adduct with the T135V Mutant. The reductive labeling of heme oxygenase with $CBrCl_3$ has also been examined after mutation of Thr-135 to a valine. Thr-135 was selected for mutagenesis because it is polar and close to His-132, a highly conserved residue that may be located close to the iron atom on the distal side of the heme (*14, 15*). Reductive reaction of the heme−T135V hHO-1 complex with $CBrCl_3$ followed by HPLC indicates that approximately 75% of the heme is covalently bound if it is assumed that the molar extinction coefficient for the heme is not altered by covalent binding (not shown). Extraction with butanone to remove dissociable heme products followed by tryptic digestion provided not only the heme-labeled peak at ∼39 min obtained with the wild-type protein but also a prominent heme-labeled peak with a retention time of 46 min (Figure 5). Analysis of the new peptide by Edman degradation provided the sequence TAFLLNIQLFEELQEL, which corresponds to the tryptic peptide ^{205}TAFLLNIQL-FEELQELLTHDTK^{226} (Figure 5). No loss of an amino acid was observed in the sequenced section of the peptide, which suggests that the alkylation site lies between residues 220 and 226. However, the heme−peptide link is labile under the acidic HPLC and amino acid sequencing conditions. This lability, which is not observed with the histidine-labeled peptide, suggests that the heme may be linked to the protein via an ester linkage. Thus, although the normal levels of the residues Glu-215, -216, and -219 obtained in the sequencing experiments would seem to eliminate these residues as the site of the alkylation, this conclusion may not be valid because the heme label may be lost due to the acid sensitivity of the heme-peptide link.

Characterization of the Heme−Δ^{221}-hHO-1 Adduct. If the glutamic acids at positions 215, 216, and 219 are excluded, a strong candidate for the acid-labile alkylation site is Asp-224. The sequencing of the heme-linked peptide from the T135V reaction could not be extended as far as that residue and therefore provides no information concerning the possible role of Asp-224 as the alkylation site. To examine the possible involvement of Asp-224, the $CBrCl_3$ reaction was carried out with the Δ^{221}-hHO-1 truncated protein, in which Asp-224 is no longer present. Similar studies could not be carried out with the Δ^{215}-HO-1 protein, which would lack all the acidic residues, because the protein is not expressed in detectable amounts. The peptide profile for the Δ^{221}-truncated protein, obtained as described for the other proteins, exhibits as a minor component the 39 min peak (found in Figure 6 at 41 min) also observed in the wild-type reaction, and as the major component a 44 min peak distinct from the 46 min peak observed with the T135V mutant (Figure 6). Amino acid sequence analysis of the isolated peak identified the sequence LVMAS, which corresponds to the tryptic peptide ^{49}LVMASLYHIYVALEEEIER^{67}. This peak, as found for the T135V mutant peptide, is acid-labile and, like it, has a number of glutamic acid residues that are possible sites for the alkylation reaction.

DISCUSSION

Heme oxygenase is a unique hemoprotein in that the heme group serves as both the prosthetic group and the substrate for the enzyme. It is also unique in that recent studies, using ethylhydroperoxide as the oxidizing agent or *meso*-methyl- and *meso*-formyl-substituted hemes as the substrate, indicate that heme oxygenase operates by a singular mechanism involving electrophilic addition of an activated oxygen to the heme porphyrin ring (*30*−*32*). The unusual properties of the heme oxygenase reaction make characterization of its active site and identification of the residues involved in heme binding and oxidation of high interest. Earlier mutagenesis and spectroscopic studies have identified His-25 as the proximal iron ligand (*11*−*13*), a water molecule as the distal iron ligand (*11*−*13*), and His-132 as a possible distal active-site residue (*14, 15*).

The truncation and heme alkylation studies reported here confirm the identity of His-25 as the proximal iron ligand, indicate that Thr-135 is within or proximal to the active site, and place residues of two additional defined peptides within the active site of the enzyme. Mutation of Thr-135 to a valine changes the regiochemistry of the alkylation reaction observed during the enzyme-catalyzed reduction of $CBrCl_3$. Whereas His-25, the proximal iron ligand, is the only residue

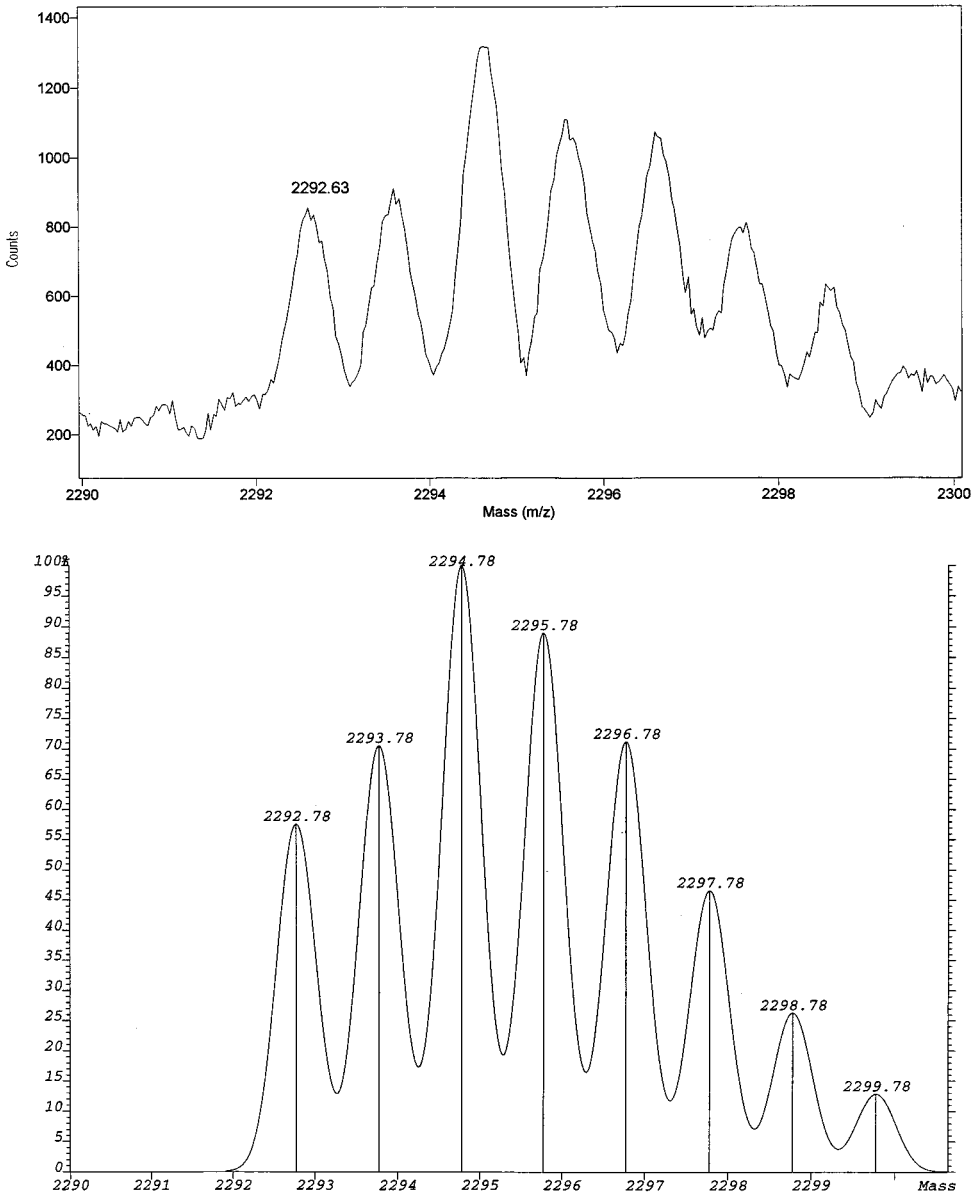

FIGURE 4: Upper panel: Molecular ion region of the labeled peptide from Figure 3. Lower panel: Calculated isotope distribution for the covalently labeled peptide−Fe(III)protoporphyrin IX complex with a single positive charge. The elemental composition of this compound is $C_{100}H_{131}SCl_3Fe$.

alkylated in wild-type hHO-1 (Figure 2), a second alkylation site dominates the reaction in the case of the T135V mutant (Figure 5). Alteration of the regiospecificity of heme alkylation by mutation of Thr-135 establishes that this residue is either in the active site or strongly influences the structure of the active site. The principal residue alkylated by the heme in the reductive reaction of Δ^{221}-hHO-1 with $CBrCl_3$ is different from that in the wild-type protein, indicating that truncation of the protein causes subtle active-site changes that expose a residue other than the proximal His-25 to alkylation. It is unclear whether the residues alkylated in the T135V and Δ^{221}-HO-1 proteins fulfill a catalytic role, but they are clearly part of the active-site structure and contribute to the physical environment that promotes heme oxidation rather than the formation of a peroxidase- or P450-like ferryl species.

In addition to identifying active-site peptides and residues, the results suggest that at least one glutamate or aspartate residue from each of the peptides ^{205}TAFLLNIQLFEELQELLTHDTK^{226} and ^{49}LVMASLYHIYVALEEEIER^{67} is located within the active site (Figure 6). The inference that a carboxylic acid residue in each of these peptides is probably alkylated is based on the observation that their adducts, unlike the His-25 adduct, are acid-labile. Edman sequencing accounted fully for the three glutamic acid residues in the peptide from the T135V mutant but did not extend as far as the Asp-224. None of these four residues can be ruled out as the alkylation site, however, because the intact amino acids may be regenerated by hydrolytic removal of the alkylating group during the purification and/or sequencing steps. Identification of carboxylic acid residues close to the heme is of particular interest because NMR evidence suggests that a carboxylate residue may help to direct the regiospecificity of heme hydroxylation (*33*). If Asp-224 is the alkylated residue, it does not play a significant role in the catalytic process because the Δ^{221}-hHO-1 truncated protein, which does not have this residue, not only retains almost complete catalytic activity (Table 1) but also still catalyzes regiospe-

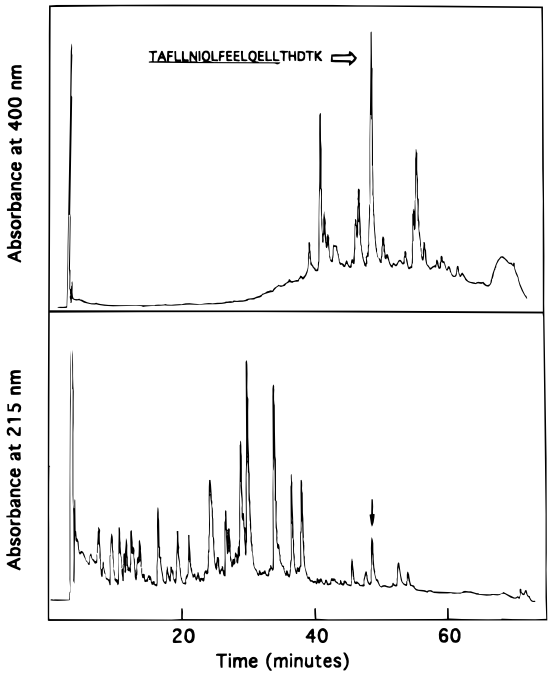

FIGURE 5: Tryptic digest of the protein after reaction of the heme−hHOT135V complex under reductive conditions with $CBrCl_3$. Upper panel: Chromatogram monitored at 400 nm. The heme-labeled peptide and the sequence that corresponds to it (the sequenced residues are underlined) are indicated. Lower panel: Chromatogram monitored at 215 nm. The peak corresponding to the heme-labeled peptide is indicated by the arrow.

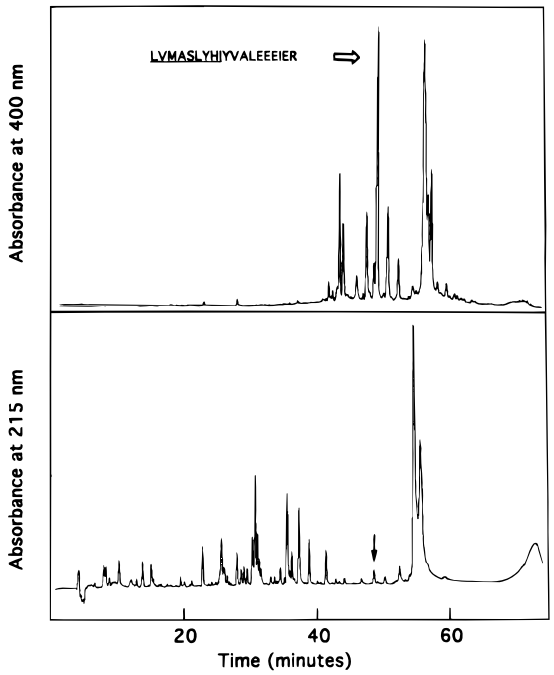

FIGURE 6: Tryptic digest of the protein after anaerobic, reductive reaction of the heme−Δ^{221}hHO-1 complex with $CBrCl_3$. Upper panel: Chromatogram monitored at 400 nm. The heme peptide and its corresponding sequence (the sequenced residues are underlined) are indicated. Lower panel: Chromatogram monitored at 215 nm. The peak corresponding to the heme−peptide is indicated by the arrow. The retention times vary slightly from one chromatogram to another. The peak at ∼41 min in this chromatogram corresponds to that at 39 min in Figure 2. The peaks at ∼55 min are due to incompletely digested protein and are not seen if highly active trypsin is used.

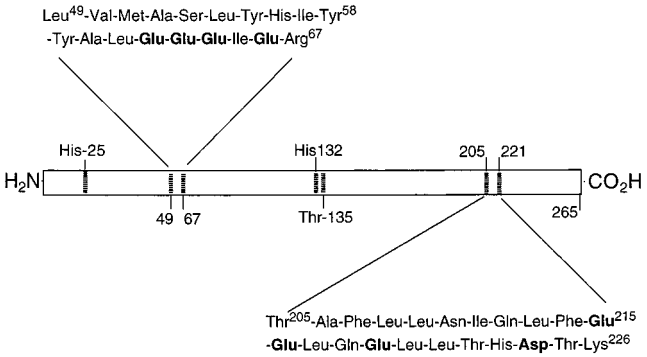

FIGURE 7: Schematic representation of the primary sequence of hHO-1, indicating the approximate location of the residues that are located within or close to the active site. The acidic residues in the two peptides are shown in boldface type. At least one of these residues in each peptide appears to be alkylated by the heme in one of the mutants and is therefore associated with the active site.

cific α-*meso*-oxidation of the heme. The three glutamic acid residues, however, remain as candidates for the group that may help to determine the reaction regiospecificity.

The finding that heme oxygenase can be truncated at the carboxyl terminus by up to 68 residues without altering the spectroscopic properties of the enzyme or decreasing the catalytic activity by more than 50% (Table 1) shows that the 68 residues play no key role in heme binding, interaction with cytochrome P450 reductase, or heme oxidation. The 23 terminal amino acids serve as a membrane anchor for the protein but no function is now apparent for the other 45 or so residues that link the membrane anchor to what is clearly the catalytic core of the protein. The active-site residues are located throughout the catalytic core (Figure 7), making it unlikely that other expendable domains are present in the protein. It is of interest in this context that the recently identified gene for a bacterial heme oxygenase codes for a protein of 215 amino acids comparable in length to the truncated mammalian proteins described here (*34*).

Although heme-protein cross-linking in the reductive reaction of $CBrCl_3$ with myoglobin and hemoglobin has been extensively studied (*23*−*26*), this is the first instance in which the reaction has been used to characterize the active site of a protein of unknown structure. As found with myoglobin (*24*), the alkylated residue in the wild-type hHO-1 reaction is His-25, the proximal iron ligand. In myoglobin, the histidine reacts with a heme vinyl substituent after addition of the trichloromethyl radical to the vinyl group (*23*−*26*). The structure of the heme moiety attached to the protein has not been explicitly determined in this study, but the fact that no adducts are formed when mesoheme is the substrate establishes that the alkylation also occurs at a heme vinyl group. Furthermore, the mass spectrum of the alkylated hHO-1 peptide indicates that it contains three chlorine atoms in addition to the heme, as expected from addition of a trichloromethyl radical. Alkylation of the proximal histidine by an activated heme vinyl group requires considerable displacement of the heme group relative to the imidazole side chain. A novel and helpful feature of the reaction as employed here is the use of mutations to shift the alkylation site to amino acid residues other than the proximal histidine. Mutation of Thr-135, a residue close in the primary sequence to the highly conserved His-132, and truncation of the protein to a length of 221 amino acids mediate such changes in the alkylated residue. The fact that the mutations result in

regioselective alkylation of one residue in addition to His-25 indicates that the alkylated residues are also within the active-site cavity. Mutagenesis in combination with heme labeling during the reductive metabolism of $CBrCl_3$ appears to be a useful method for the identification of hemoprotein active-site residues.

The reduction of $CBrCl_3$ is the first reductive reaction shown to be catalyzed by heme oxygenase. As found for cytochrome P450, the reductive metabolism of this halocarbon results in the production of reactive, presumably radical, species that inactivate the enzyme (*16*−*22*). The mass spectrum of the peptide obtained from the wild-type protein confirms the presence of three chlorine atoms in the heme−peptide adduct. The halocarbon radicals produced by cytochrome P450 are thought to play an important role in the toxicity of halocarbons such as $CBrCl_3$ and CCl_4. The present results suggest that heme oxygenase may contribute to this pathological process in tissues, such as the spleen, where the concentration of heme oxygenase is particularly elevated or in situations where the level of heme oxygenase is increased by inducing agents.

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