Role of Cysteine Residues in Heme Binding to Human Heme Oxygenase-2 Elucidated by Two-dimensional NMR Spectroscopy*

Received for publication, May 5, 2012, and in revised form, August 16, 2012. Published, JBC Papers in Press, August 24, 2012, DOI 10.1074/jbc.M112.378042

Fatbardha Varfaj, Jed N. Lampe, and Paul R. Ortiz de Montellano

From the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94158-2517

Human heme oxygensases 1 and 2 (HO-1 and HO-2) degrade heme in the presence of oxygen and NADPH-cytochrome P450 reductase, producing ferrous iron, CO, and biliverdin. HO-1 is an inducible enzyme, but HO-2 is constitutively expressed in selected tissues and is involved in signaling and regulatory processes. HO-2 has three cysteine residues that have been proposed to modulate the affinity for heme, whereas HO-1 has none. Here we use site-specific mutagenesis and two-dimensional NMR of L-[13C]cysteine-labeled proteins to determine the redox state of the individual cysteines in HO-2 and assess their roles in binding of heme. The results indicate that in the apoprotein, Cys282 and Cys265 are in the oxidized state, probably in an intramolecular disulfide bond. The addition of a reducing agent converts them to the reduced, free thiol state. Two-dimensional NMR of site-specific mutants reveals that the redox state of Cys265 and Cys282 varies with the presence or absence of other Cys residues, indicating that the microenvironments of the Cys residues are mutually interdependent. Cys265 appears to be in a relatively hydrophilic, oxidizable environment compared with Cys282 and Cys282. Chemical shift data indicate that none of the cysteines stably coordinates to the heme iron atom. In the oxidized state of the apoprotein, heme is bound 2.5-fold more tightly than in the reduced state. This small difference in heme affinity between the oxidized and reduced states of the protein is much less than previously reported, suggesting that it is not a significant factor in the physiological regulation of cellular heme levels.

Mammalian heme oxygenases are enzymes that, in the presence of O2 and NADPH-cytochrome P450 reductase, degrade Fe(III) protoporphyrin IX (heme) to biliverdin IXα, Fe2+ and carbon monoxide (CO). The heme oxygenases are the only enzymes in mammals that physiologically degrade heme and are therefore essential for the maintenance of heme and iron homeostasis. In addition, the three products generated from heme oxygenase (HO)-2 catalysis have important biological functions. The iron is recycled because only ~3% of the amount of iron required daily is obtained from the diet. CO serves as a signaling molecule exhibiting anti-apoptotic, anti-inflammatory, and anti-proliferative properties (1–3). Finally, in mammals, biliverdin is reduced by biliverdin reductase to the potent antioxidant bilirubin, which is subsequently conjugated with glucuronic acid and excreted.

There are two functional human HO isoforms, heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2). HO-1 is an inducible form expressed at high levels in the liver and spleen, whereas HO-2 is a constitutive form expressed at high levels in the brain and testis (4). The primary physiological functions proposed for HO-2 are O2 sensing through regulation of the activity of the BK channel, a Ca2+-activated potassium channel (5), and the generation of CO that serves as a neurotransmitter (6).

Mammalian HO-1 and HO-2 share ~45% sequence identity and have very similar core structures (7, 8). Both HO isoforms are bound to the membrane via their C-terminal region. The major difference between these isoforms is the presence of heme regulatory motifs (HRMs) in HO-2 but not in HO-1. There are three HRM motifs in HO-2: HRM1, consisting of Lys264-Cys-Pro-Phe-Tyr-Ala269; HRM2, consisting of Ser281-Cys-Pro-Phe-Arg-Thr286; and HRM3, consisting of Gln126-Cys-Pro-Lys-Ala-Ala311. The HRM motifs have been found in a few other proteins whose functions are heme-dependent, such as heme-regulated initiation factor 2α kinase (9, 10), yeast transcription factor HAP1, which regulates the transcription of many genes in response to oxygen availability (11), cytochrome c heme lyase of Saccharomyces cerevisiae (12, 13) and Neurospora crassa (14), human erythroid δ-aminolevulinate synthase, which is the rate-limiting enzyme in heme biosynthesis (15), and Escherichia coli catalase (16). The HRM motifs in HO-2 have been proposed to control the binding affinity of the

* This work was supported, in whole or in part, by National Institutes of Health Grant DK30297.

1 To whom correspondence should be addressed: Dept. of Pharmaceutical Chemistry, University of California, 600 16th St., San Francisco, CA 94158-2517. Tel.: 415-476-2903; Fax: 415-502-4728; E-mail: ortiz@cgl.ucsf.edu.

2 The abbreviations used are: HO, heme oxygenase; HRM, heme regulatory motif; TCEP, tris(2-carboxyethyl) phosphine solution, neutral pH; NBD-Cl, 4-chloro-7-nitro-2,1,3-benzoxadiazole; HSQC, heteronuclear single quantum coherence.
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protein for heme and the spin state of the heme iron (17). This affinity was reported to be regulated by the presence or absence of a disulfide bond between Cys265 and Cys282 located in HRM1 and HRM2, respectively (17). Reduction of this disulfide bond reportedly resulted in an ~10-fold decrease in the HO-2 affinity for heme, suggesting a possible role for the HRMs in regulating the cellular levels of free heme, CO, iron, and biliverdin. There is no structural information on the region comprising Cys265 and Cys282 because it is missing in the HO-2 crystal structure (8). Furthermore, no NMR structural data have been reported to date. However, NMR spectroscopy has been successfully employed in monitoring the redox state of Cys residues and protein conformational changes upon ligand binding or binding of other proteins. The NMR chemical shift of cysteine is sensitive to the redox state of the thiol and can be diagnostic of disulfide bond formation (18). In this study, we utilized two-dimensional (1H,13C) NMR spectroscopy to shed light on the involvement of the cysteine residues of HO-2 in heme binding and catalysis. The HO-2 protein was labeled with l-[3-13C]cysteine, and two-dimensional heteronuclear single quantum coherence (HSQC) NMR spectra were recorded in the presence and absence of heme. Our results suggest that the Cys residues are not directly involved in heme binding, and the presence or absence of the intramolecular disulfide bond results in only a 2.5-fold, rather than 10-fold, difference in the binding affinity of HO-2 for heme.

MATERIALS AND METHODS

Chemicals—Hemin chloride, NADPH, BSA, EDTA, ampicillin, lysozyme, isopropyl β-D-thiogalactoside, amino acids, nucleosides, PMSF, potassium phosphate monobasic and dibasic, and diamide all were purchased from Sigma-Aldrich. The disulfide bond-reducing tris(2-carboxyethyl) phosphine solution, neutral pH (TCEP) was purchased from Thermo Scientific. EDTA- and Ca2+-free protease inhibitor mixture was purchased from Roche Applied Science. Luria-Bertani (LB) medium was purchased from Fisher, 4-chloro-7-nitro-2,1,3-benozoxadiazole (NBD-Cl) from Molecular Probes, and l-[3-13C]cysteine from Cambridge Isotope Laboratories.

Enzymes—Truncated human HO-2 lacking the last 23 C-terminal residues was expressed and purified according to the procedure described below. Rat biliverdin reductase (19) and human NADPH-cytochrome P450 reductase (20) were expressed in E. coli and purified as previously described. Lysozyme was purchased from Sigma-Aldrich.

DNA Construct of Human HO-2—The human HO-2 cDNA utilized in this study codes for residues 1–293 and thus lacks the 23 amino acids of the C terminus membrane binding region. In order to facilitate HO-2 protein purification, six histidine residues were inserted at the C terminus. The HO-2 mutants C127A, C265A, C282A, C127A/C265A, C127A/C282A, C265A/C282A, and C127A/C265A/C282A were prepared by introducing Cys to Ala mutations using a QuikChange™ site-directed mutagenesis kit (Agilent Genomics, La Jolla, CA). In addition, Met28 and Met30 were mutated to alanine in concert with Cys to Ala mutations to eliminate the presence of the alternative truncated HO-2 species consisting of either residues 28–293 or 30–293. Translation of these truncated species most likely results from the Shine-Delgarno sequence present at residues 24 and 25. Introduction of the Met to Ala double mutation did not affect HO-2 binding or activity. Mutations were introduced into the HO-2 cDNA in the pBAce cloning vector by PCR using forward and reverse primers (Invitrogen). Finally, the HO-2 gene was transferred into a pET21a expression vector (Novagen).

Expression and Purification of [13C]Cys-labeled HO-2 Mutant Proteins—The HO-2 expression construct was transformed into the cysteine auxotroph E. coli strain BL21(DE3)cysE51 and plated on M9 minimal medium plates supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 50 μg/ml cysteine. A single colony was inoculated in 50 ml of LB medium supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 50 μg/ml cysteine. The overnight culture was centrifuged at 4,000 × g for 15 min at 4 °C, and the cell pellet was resuspended in M9 minimal medium supplemented with 19 amino acids and l-[3-13C]cysteine in place of unlabeled Cys, nucleosides, MgSO4, CaCl2, biotin, nicotinamide, thiamine, a trace element solution containing H3BO3, CoCl2, CuCl2, MnCl2, ZnCl2, FeCl3, and ampicillin and kanamycin, as described previously (21).

Cells were grown at 37 °C until an A600 of 0.6–0.8 was reached. Protein expression was induced with isopropyl β-D-thiogalactoside (final concentration of 0.5 mM) for an additional 16 h at 30 °C. Cells were harvested by centrifugation at 4,000 × g for 20 min, and the cell pellet was resuspended in 100 mM potassium P, pH 7.4, buffer containing 2 mM EDTA, 2 mM PMSF, and a protease inhibitor mixture tablet. Cell lysis was performed by adding lysozyme (15 mg/liter) and stirring the solution for 30 min at 4 °C, followed by sonication using a Bronson sonicator (seven cycles, 1 min on, 1 min off at 50% power). The cell debris was separated from the soluble protein by ultracentrifugation at 150,000 × g for 1 h at 4 °C. The supernatant was filtered (0.2 μm) and loaded onto a 5-ml HisTrap column (GE Healthcare), which was equilibrated with 50 mM potassium P, 1 mM EDTA, and 30 mM imidazole buffer, pH 7.4. The column was washed with five column volumes of 50 mM potassium P, 1 mM EDTA, and 40 mM imidazole, pH 7.4, buffer, and the protein was eluted with 50 mM potassium P, pH 7.4, 1 mM EDTA, using a 0–500 mM imidazole gradient. After dialysis, the protein was consecutively loaded onto SP-Sepharose and Q-Sepharose columns (Amersham Biosciences), both pre-equilibrated with 50 mM potassium P, pH 7.4, 2 mM EDTA, and 1 mM NaCl buffer. The eluted protein was further dialyzed against 50 mM potassium P, pH 7.4, buffer containing 1 mM EDTA. Protein was eluted from the Q-Sepharose column with a 300-ml linear gradient of 50–400 mM potassium P, pH 7.4, 1 mM EDTA, 400 mM NaCl buffer. The eluted HO-2 protein was subject to electrophoresis on a 4–12% SDS-polyacrylamide gel, which was visualized by SimplyBlue gel staining (Invitrogen) and was determined to be greater than 90% pure. Fractions containing the protein were combined and concentrated using 10,000 MWCO concentrators (Millipore). The protein was further dialyzed against 50 mM potassium P, pH 7.4, buffer. Protein concentration was determined using the Bradford assay.

Expression and Purification of Unlabeled HO-2 Mutant Proteins—The HO-2 expression vector was transformed into BL21(DE3) E. coli cells and plated on LB plates supplemented with 100 μg/ml ampicillin. A single colony was grown in 5 ml
and later in 3 liters of LB medium supplemented with 100 μg/ml ampicillin at 37 °C. The protein expression was induced with isopropl β-d-thiogalactoside (final concentration of 0.5 mM) when A490 reached 0.6–0.8. Protein expression was carried out for 16 h at 30 °C. Cells were harvested by centrifugation at 4,000 × g for 15 min at 4 °C using a Beckman Coulter centrifuge, and the protein was purified using the same protocol as the 13C-labeled proteins described above.

**HO-2 Protein Activity Assay**—The activity of the HO-2 mutant proteins was determined by monitoring the rate of bilirubin formation at 468 nm using a Spectramax® UV-visible plate reader (Molecular Devices, Sunnyvale, CA) as described previously (22, 23). Reaction mixtures (200 μl) consisted of 0.4 μM HO-2, 1 μM NADPH-cytochrome P450 reductase, 4 μM biliverdin reductase, 0.5 μM heme, 0.1 mM potassium Pi, pH 7.4, buffer supplemented with 10 μg/ml BSA. The reactions were incubated at 22 °C for 2 min prior to the addition of 400 μM NADPH to initiate the reaction. The initial rate of bilirubin formation was calculated using an extinction coefficient of 43.5 mM cm−1. The specific activity of the WT and mutant HO-2 proteins is expressed in nmol/min/mg.

**Mass Spectrometric Analysis of [13C]Cys-labeled HO-2 Protein**—Incorporation of the [13C]Cys-labeled amino acid into the HO-2 protein was specifically verified for the A127/13C265/13C282 mutant by LC/MS/electrospray ionization mass spectrometric analysis. The protein was loaded on an SDS-polyacrylamide gel (4–12% gradient) and visualized by gel staining with SimplyBlue Safe Protein Stain. The HO-2 protein band was excised and subjected to in-gel digestion according to a published protocol (24, 25). The extracted tryptic peptide mixture was separated by nano-flow liquid chromatography. The HPLC system employed was an Eldex MicroPro pumping system equipped with a 100 μM × 150-mm monolithic C18 column (Phenomenex Onyx). The mobile phase A consisted of 0.1% formic acid in water, whereas mobile phase B consisted of 0.1% formic acid in acetonitrile. After equilibration of the column at 5% B for 20 min, 1 μl of the extracted peptides was injected at a flow rate of 0.5 μl/min. After loading, the peptides were eluted by increasing the organic content linearly from 5 to 60% B over 35 min and then held at 35% B for 5 min. The eluent from the reverse phase column was coupled on-line to a microspray ion source attached to a QSTAR-XL hybrid QqTOF mass spectrometer (Applied Biosystems) operated in positive ion mode. MS Survey scans were acquired over an m/z range from 59 to 1800. Spectra were analyzed using Data Explorer software (Applied Biosystems, Foster City, CA).

**1H,13C HSQC NMR Measurements**—Two-dimensional 1H,13C HSQC spectra were acquired on a Bruker 800-MHz spectrometer (Bruker Avance, Bremen, Germany) equipped with a 1H/13C/15N triple resonance ColdprobeTM with actively shielded pulse field gradients at 25 °C unless otherwise stated. Spectra were acquired with 60 scans using spectral widths of 8385 Hz (1H) and 1600 Hz (13C), 128 complex points in 1, and a 1-s recycle time. The 13C chemical shifts were calibrated to the reference (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) directly. Each NMR sample consisted of ~300 μl of a 130–300 μM concentrated solution of [13C]Cys-labeled HO-2 protein in a 50 mM potassium Pi, pH 7.4, buffer with 10% D2O added as a lock solvent placed in a Shigemi tube. In the experiments with the reduced HO-2 protein, TCEP was added to a final concentration of 10-fold relative to the HO-2 protein. For the heme titration experiments, aliquots of a heme stock solution were added sequentially to reach the desired final concentrations for each experiment and incubated for 30 min, and then the spectrum was recorded. Data processing and analysis were performed using NMRPipe software (26).

**Tryptophan Fluorescence Quenching**—The fluorescence spectra were recorded on a Fluorolog 3 spectrofluorimeter (Horiba Jobin Yvon) at 22 °C. The HO-2 protein has one tryptophan residue, Trp121. The tryptophan was excited at 290 nm, and the emission spectrum was recorded at 310–500 nm with a maximum emission value at 338 nm. The excitation and emission slits were set at 8 nm. The emission spectrum of tryptophan fluorescence was measured at increasing concentrations of heme ligand. The fluorescence intensity of the 338 nm emission was plotted against the concentration of heme, and the data obtained were fitted to the quadratic equation,

\[
IF = IF_0 + \Delta IF \left( \frac{(K_D + P_t + L_t) - \sqrt{(K_D + P_t + L_t)^2 - 4L_tP_t}}{2P_t} \right)
\]

(Eq. 1)

using non-linear least square regression analysis to calculate the dissociation constant of HO-2 for heme, where IF is the fluorescence intensity at a given heme concentration, IF₀ is the fluorescence intensity in the absence of ligand, ΔIF is the change in fluorescence intensity on ligand binding, K_D is the dissociation constant, and P_t and L_t are the total protein and ligand concentrations, respectively. The K_D values represent the averages of two experiments conducted with separate samples. Best fit curves were generated with GraphPad Prism (GraphPad Software, San Diego, CA).

**Anaerobic Measurements**—The binding affinity of the reduced HO-2 for heme was determined by tryptophan fluorescence quenching after reduction of the Cys residues. The experiments were done under anaerobic conditions using a cuvette sealed with a septum (Hellma). The HO-2 protein solution and buffers were bubbled with argon to remove the oxygen. Furthermore, buffers were kept stirring in a glove box (Unilab) overnight prior to use. The oxygen level in the glove box was monitored by an oxygen sensor and kept at <2 ppm. The heme stock was prepared in the glove box using 0.1 M NaOH and 0.1 M potassium Pi, pH 7.4, solutions that were degassed prior to use. The heme solution was filtered using a 0.2-μm filter (Millipore), and its concentration was determined by the absorbance value at 385 nm using an extinction coefficient of 6.22 mm⁻¹ cm⁻¹. The reduced HO-2 protein was obtained by incubating it with 10-fold TCEP for 30 min. TCEP was later removed using 500-μl centrifugal filter units of 10,000 MWCO (Millipore). The reduced HO-2 protein was placed in a 1-ml anaerobic cuvette sealed with a septum in the glove box. A second sealed cuvette containing buffer was used as a blank. The heme solution was also kept in an air-tight bottle and added to the HO-2
proteins were measured to ensure that their structures and catalytic properties were similar to those of the corresponding unlabeled proteins. The specific activity was measured using a coupled enzymatic assay in which the formation of bilirubin is quantified, and the catalytic activities of the HO-2 variants were all obtained as described under “Materials and Methods.” The abbreviated nomenclature for the HO-2 proteins indicates the residue at positions 127, 265, and 282, in that order, with an A for alanine and 13C for labeled cysteine. ND, not determined; ppm, chemical shift in parts per million of the spectrometer frequency.

**TABLE 1**

Summary of the two-dimensional NMR chemical shifts for the oxidized and reduced [13C]Cys-labeled HO-2 apoproteins, their binding affinities for ferric heme, and their catalytic activities

NMR chemical shifts (13C, 1H) for the oxidized and reduced [13C]Cys-labeled HO-2 apoproteins, their binding affinities for ferric heme obtained by tryptophan fluorescence quenching, and the catalytic activities of the HO-2 variants were all obtained as described under “Materials and Methods.” The abbreviated nomenclature for the HO-2 proteins indicates the residue at positions 127, 265, and 282, in that order, with an A for alanine and 13C for labeled cysteine. ND, not determined; ppm, chemical shift in parts per million of the spectrometer frequency.

**RESULTS**

**Characterization of [13C]Cys-labeled HO-2 Mutant Proteins**—[13C]Cys-labeled HO-2 mutant proteins were expressed in M9 minimal medium using BL21(DE3)csy51 auxotrophic cells as described under “Materials and Methods.” The proteins expressed were 13C127/13C265/13C282 (A/13C/13C), A127/13C265/13C282 (A/13C/A), A127/13C265/13C282 (A/13C/A), A127/13C265/13C282 (13C/13C/13C), A127/13C265/13C282 (13C/A/13C), A127/13C265/13C282 (13C/A/13C), A127/13C265/13C282 (13C/A/13C), and A127/13C265/13C282 (13C/A/13C), in which one, two, or three Cys residues were 13C-isotopically labeled. The other Cys residue(s) normally present in HO-2 was mutated to an Ala. In the abbreviated nomenclature shown, the residues at positions 127, 265, and 282 are listed, in that order, with an A for alanine and C or 13C for unlabeled or labeled cysteine. The specific activities of these proteins were measured to ensure that their structures and catalytic properties were similar to those of the corresponding unlabeled proteins. The specific activity was measured using a coupled enzymatic assay in which the formation of bilirubin is monitored at 468 nm, as described under “Materials and Methods.” All of the isotopically labeled mutant HO-2 proteins had specific activities similar to those of both the WT (28 nmol/min/mg) and the corresponding unlabeled, mutant proteins, confirming that they are fully active (Table 1).

**Incorporation of [13C]Cys into HO-2** was specifically verified for the A/13C/13C, 13C/13C/13C, and A/A/A HO-2 proteins by LC/MS/electrospray ionization mass spectrometric analysis. Although the difference of only 1 mass unit between 12C and 13C isotopes poses a challenge in accurately determining the efficiency of incorporation of the [13C]Cys amino acid, the data clearly revealed the presence of both the [12C]Cys and [13C]Cys amino acids in the protein, with the latter constituting more than 50% of the mixture (data not shown).

**Redox State of the HO-2 Cys Residues by Two-dimensional NMR Spectroscopy**—To determine the redox state of the native HO-2 apoprotein, two-dimensional NMR (1H,13C HSQC) spectroscopic analysis of the 13C/13C/13C protein, in which all three Cys residues are isotopically labeled, was performed. The spectrum revealed the presence of two major resonances (Fig. 1A and Table 1): one at 13C = 40.5 ppm, 1H = 3.11 ppm and the second at 13C = 40.5 ppm, 1H = 2.92 ppm, partially overlapping a non-cysteine-related resonance at 13C = 42 ppm, 1H = 2.93 ppm. The non-cysteine-related resonance was observed in the spectra of all [13C]Cys-isotopically labeled (Figs. 1–5) and unlabeled HO-2 proteins, including that of the A/A/A HO-2 mutant lacking all Cys residues. Therefore, none of the Cys residues is responsible for this particular resonance. Additional control experiments ruled out the possibility that the resonance derived from contaminants left behind from the protein purification. The most likely explanation is that the spurious resonance at 13C = 42 ppm, 1H = 2.93 ppm derives from the abun-
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...dant methylene (CH₂) groups present in the protein (27). Based on the rules established by Sharma and Rajarathnam (18), the position of the Cys resonance in the spectrum is an indicator of its oxidation state, with the Cys in the oxidized state if the chemical shift in the ¹³C dimension is >35 ppm and in the reduced state if it is <32 ppm. The two resonances observed at ¹³C = 40.5 ppm (Fig. 1A) suggest that two Cys residues in the ¹³C/¹³C/¹³C protein are in the oxidized state, probably in a disulfide bond. To confirm that these two resonances correspond to oxidized Cys residues, the ¹³C/¹³C/¹³C protein was incubated with a 10-fold excess of TCEP reducing agent for 30 min prior to recording the two-dimensional NMR spectrum. The two resonances at ¹³C = 40.5 ppm disappear, and a large, line-broadened resonance appears at ¹³C = 28, ¹H = 2.82 ppm (Fig. 1A and B), confirming that two Cys residues in HO-2 are indeed in the oxidized state. The appearance of a single intense resonance in the reduced sample suggests that the two reduced Cys residues are in a similar chemical environment. Interestingly, a third distinct Cys resonance in the ¹³C/¹³C/¹³C protein spectrum is missing; thus, single and double HO-2 mutants were also examined as described below, and their NMR resonances are summarized in Table 1.

The NMR spectrum of the A/¹³C/¹³C protein, in which Cys-127 is mutated to Ala, revealed the same resonances as the ¹³C/¹³C/¹³C protein (Table 1), indicating that the two resonances at ¹³C = 40.5 ppm correspond to Cys²⁶⁵ and Cys²⁸². The same resonances were also observed for the reduced (by 10-fold excess TCEP) ¹³C/¹³C/¹³C and A/¹³C/¹³C proteins, as shown by the presence of a resonance at ¹³C = 28 ppm (Table 1), supporting the suggestion that Cys²⁶⁵ and Cys²⁸² are in the oxidized form and are converted to the reduced state by TCEP. The non-Cys resonance at ¹³C = 42 ppm, as expected, was also observed with both of these mutants. In efforts to separate this resonance from the resonance at ¹³C = 40.5 ppm, ¹H = 2.92 ppm deriving from the labeled Cys, the spectrum of the A/¹³C/¹³C HO-2 protein was recorded following chemical denaturation with 7 M urea. A chemical shift of ~0.10 ppm up-field in the ¹H dimension was observed for all resonances, but separation of the second Cys from the spurious resonance was not achieved. In addition, post-processing of the NMR data using both Lorentzian and Gaussian functions did not result in their complete separation.

Attribution of the two resonances at ¹³C = 40.5 ppm for both the ¹³C/¹³C/¹³C and A/¹³C/¹³C proteins to Cys²⁶⁵ and Cys²⁸² is confirmed by the NMR spectrum of the ¹³C/A/A protein in the absence and presence of TCEP (Table 1). The spectrum of this protein only exhibited the spurious peak at ¹³C = 42 ppm, which indicates that the resonance corresponding to Cys¹²⁷ is present at an intensity below background level. Residue 127 is resolved in the HO-2 crystal structure, although the native Cys was substituted with an Ala. The side chain was partially surface-exposed, but this may not reflect the degree of exposure of the native Cys¹²⁷ residue. Lack of a detectable NMR signal for Cys¹²⁷ in the ¹³C/A/A and ¹³C/¹³C/¹³C proteins suggests that its molecular motion is highly constrained.

To specifically determine which of the two resonances at ¹³C = 40.5 ppm corresponds to Cys²⁸², the two-dimensional NMR spectrum of the A/A/¹³C protein was obtained. The spectrum revealed the presence of a doublet at ¹³C = 28 ppm (Fig. 2A and Table 1), suggesting that Cys²⁸² is in the reduced state when Cys¹²⁷ and Cys²⁶⁵ are not present. In accord with this, no change in the chemical shift was observed for the A/A/¹³C protein in the presence of TCEP (Fig. 2B and Table 1). Furthermore, the reduced state for this mutant was supported by absorbance measurements of the oxidized and reduced protein done in the presence and absence of NBD-Cl (data not shown). The doublet NMR signal is due to slightly different chemical environments for the two hydrogen atoms attached to the ¹³Cβ carbon. Interestingly, Cys²⁸² is in the oxidized state in the presence of a second Cys, either Cys¹²⁷ (¹³C/A/A/¹³C) or Cys²⁶⁵ (A/¹³C/A/¹³C), or when both Cys¹²⁷ and Cys²⁶⁵ (¹³C/A/A/¹³C) are present (Table 1). The spectrum for the ¹³C/A/A/¹³C protein revealed two resonances in the oxidized Cys region at ¹³C = 38.3 ppm (Fig. 3A and Table 1). These resonances are in the same region as the resonances observed for the A/¹³C/A/¹³C protein (Fig. 4A and Table 1). Upon the addition of TCEP to ¹³C/A/A/¹³C HO-2, the intensity of the two resonances decreases with concomitant appearance of a doublet resonance in the reduced Cys region at ¹³C = 28 ppm, which is in very close proximity to the one observed for the reduced A/¹³C/A/¹³C and A/¹³C/¹³C/¹³C proteins. This indicates that Cys²⁸² is oxidized in ¹³C/A/A/¹³C HO-2, either forming an intramolecular disulfide bond with Cys¹²⁷ or, less likely, existing in the sulfenic acid form. Interestingly, the ¹³C/A/A/¹³C HO-2 protein is only partially reduced by
TCEP, as indicated by the presence of two resonances at 13C/H11005 38.3 ppm and a doublet resonance at 13C/H11005 28 ppm (Table 1). This may reflect a limited access of TCEP to the Cys residue(s) or a partially irreversible oxidation of the Cys residue(s). Limited access by a reducing agent is supported by a previous study, in which the number of free thiols for this HO-2 mutant in the absence and presence of urea was 0.6 and 1.37 mol, respectively, and when the protein was initially treated with DTT, this number changed to 1.57 and 1.93 mol, respectively, in the absence and presence of urea (17). Furthermore, based on these findings, it was suggested that Cys282 has some ability to form a disulfide bond with Cys127 when Cys265 is absent (17). In order for Cys282 and Cys127 to form an intramolecular disulfide bond, they need to be within 2.05 Å of each other. Because the peptide region containing Cys265 and Cys282 is missing in the HO-2 crystal structure, the exact location of Cys282 relative to Cys127 cannot be determined, but their distance relationship can be defined.

To identify the resonance corresponding to Cys265, the spectra for all of the HO-2 proteins containing this residue, including 13C/13C/13C, A/13C/13C, 13C/13C/A, and A/13C/A, were compared (Table 1). Cys265 is in the oxidized state in all instances, whether it is the only cysteine in the protein or is in the presence of one or both of the other cysteines. The spectra for the 13C/13C/13C and A/13C/13C proteins revealed the presence of two resonances at 13C = 40.5 ppm that correspond to either Cys265 or Cys282 (Table 1). These resonances are shifted to 13C = 38.7 ppm in both A/13C/A (Fig. 4) and 13C/13C/A (Fig. 5) proteins, indicating that the chemical environment of Cys265 is altered in these single and double mutants compared with the WT HO-2 protein. The two resonances observed for the A/13C/A protein suggest that Cys265 is either involved in an intermolecular disulfide bond or is present as a sulfenic acid. A control experiment with the [13C]Cys-labeled free amino acid treated with an oxidizing agent (diamide) shows a shift of resonances from the reduced Cys to oxidized Cys region (Table 2), suggesting that the two resonances at 13C = 40.5 ppm correspond to the two β protons bound to the 13C carbon of the Cys involved in a disulfide bond with a second Cys residue or present in the sulfenic acid form. To determine the oxidation state of Cys265 in the A/13C/A protein, the latter was incubated with a 10-fold excess of TCEP for 30 min. The reduced protein exhibited a new resonance at 13C = 28 ppm with disappearance of the two resonances at 13C = 40.5 ppm (Fig. 4B), demonstrating that the oxidation of Cys265 is reversible, thus excluding the possibility of cysteine sulfinate or sulfonate forms. Furthermore, no intermolecular disulfide bond was detected in the SDS-PAGE and native gel analyses performed in the presence and absence of TCEP, in agreement with the published data for the A/C/A HO-2 mutant (17). The presence of only one resonance at 13C = 28 ppm for the reduced A/13C/A protein suggests that the two protons bound to the 13Cβ carbon experience similar chemical environments.

Cys265 is also in the oxidized state in the 13C/13C/A protein (Fig. 5 and Table 1), which, as observed for A/13C/A HO-2, has
two resonances in the $^{13}$C = 38.7 ppm region. Interestingly, an additional strong resonance is observed at $^{13}$C = 28 ppm that corresponds to the reduced state of either Cys$^{127}$ or Cys$^{265}$. In the presence of TCEP, the intensity of the reduced Cys resonance is increased, whereas that of the two resonances at $^{13}$C = 38.7 ppm changes little, suggesting that either the Cys residue(s) is not fully accessible to TCEP, or its oxidation is irreversible. The resonance at $^{13}$C = 28 ppm is in the same location as the one observed for the A/$^{13}$C/A protein (Fig. 4B and Table 1) in the presence of TCEP, consistent with the possibility that Cys$^{265}$ may be partially present in the reduced state. Comparison of the $^{13}$C/$^{13}$C/A and A/$^{13}$C/A proteins indicates that Cys$^{127}$ affects the local environment of Cys$^{265}$ and ultimately its redox state.

The redox state of Cys$^{265}$ was further examined by monitoring the absorbance spectrum of both the oxidized and reduced A/$^{13}$C/A proteins reacted with NBD-Cl under anaerobic conditions as described under “Materials and Methods.” The A/$^{13}$C/A protein revealed an absorbance peak at 347 nm, characteristic of Cys-S(O)-NBD, and at 420 nm when reduced with TCEP, characteristic of Cys-S-NBD product (data not shown). These results are similar to the NBD-Cl data previously reported for this mutant (17) and confirm that Cys$^{265}$ is oxidized and that its oxidation is reversible by TCEP, further corroborating the NMR data obtained in this study for the A/$^{13}$C/A protein.

Involvement of Cys$^{265}$ in Heme Binding—Reduction of the disulfide bond between Cys$^{265}$ and Cys$^{282}$ reportedly causes a 10-fold decrease in the binding affinity of HO-2 for heme (17). In our experience, the $K_d$ values determined using tryptophan fluorescence quenching for single, double, and triple Cys to Ala mutants differed by no more than 2.5-fold between the reduced and oxidized proteins (Fig. 6 and Table 1). Of particular interest, Cys$^{265}$ has been proposed to displace the His$^{45}$ axial ferric iron ligand at low temperatures when the Cys is in the reduced state (17). The assumption that equilibrium between Cys$^{265}$-Fe$^{3+}$/H$_2$O and His$^{45}$-Fe$^{3+}$/H$_2$O favored the former was supported by EPR data obtained at 10 K for the A/C/C and A/C/A mutants. To further test whether Cys$^{265}$ is involved in heme binding, we
monitored the chemical shifts of the oxidized and reduced A/13C/A mutant upon heme titration at 277 K, the lowest temperature accessible with the Bruker 800 spectrometer used in this study. The NMR spectrum for the A/13C/A apoprotein recorded at 277 K revealed the presence of two resonances at $^{13}$C/H11005 38.5 ppm (Fig. 7A) similar to those observed at 298 K (Fig. 4). Upon the addition of 30 and 260 μM (2-fold excess) heme, no significant changes were observed in the chemical shifts. The spectra of the reduced A/13C/A apoprotein in the presence of increasing concentrations of heme (0, 50, 150, and 350 μM (2-fold excess)) were also recorded. The protein was reduced with 10-fold TCEP for 30 min followed by heme titration. The reduced A/13C/A protein revealed a resonance at $^{13}$C/H11005 28 ppm that decreases in intensity with increasing concentrations of heme (Fig. 8). Furthermore, the two resonances in the oxidized Cys region start to reappear (Fig. 8B). This conversion of Cys265 from the reduced to the oxidized state upon heme addition could reflect interactions of the heme iron atom with Cys or an increase in the pH of the solution. The heme stock solution is alkaline, and its addition (1:7 (v/v) ratio of heme to protein solution) effectively increases the pH of the protein solution by 2 pH units. The effect of pH increase in the Cys oxidation was examined by monitoring the spectrum of the free $^{13}$C/Cys amino acid (3 mg/ml) upon the addition of 1:7 (v/v) 0.1 N NaOH (pH 12), where the final pH of the amino acid solution became ~9.5. The stock solution of 0.1 N NaOH used to dissolve heme is diluted 10-fold with potassium P, pH 7.4, buffer before being titrated into the protein solution. The NMR spectrum showed that the typical two resonances located in the reduced region coalesced into one (Table 2), suggesting that the increase in pH of the solution does not result in Cys oxidation. We also examined the effect of heme on Cys oxidation by monitoring the spectrum of the free $^{13}$C/Cys amino acid (3 mg/ml) upon the addition of one-seventh volume of heme stock solution (0.3 mg/ml final concentration). The spectrum revealed two new resonances in the Cys oxidized region at $^{13}$C = 40 ppm (Table 2). Thus, heme causes oxidation of a Cys residue in the HO-2 protein. The pKa of Cys265 in A/13C/A HO-2 is less than that of a regular Cys because it is already oxidized at pH 7.4 and, when reduced by TCEP, is quickly reoxidized even in the presence of 50 μM heme. This suggests that Cys265 is easily accessible in the A/13C/A protein and can interact with heme.

Heme titration of the reduced $^{13}$C/13C/13C (WT) protein was also performed. As mentioned previously, in the presence of TCEP, this protein shows a change in the chemical shift of the $^{13}$C/Cys resonances from the oxidized to the reduced Cys region. Only one resonance for the reduced protein is observed at $^{13}$C = 28.2 ppm (Fig. 9). Upon the addition of 50, 150, and 250 μM (2×) heme, no significant chemical shifts of the reduced resonance is observed and, furthermore, no conversion to the oxidized Cys region. Similar results were obtained for the reduced A/13C/13C protein upon the addition of 50, 150, 250, and 450 μM (2×) heme (data not shown), suggesting that Cys265 and Cys282 are in slightly different chemical environments when both residues are present in HO-2 versus the case when only one Cys is present.

**DISCUSSION**

In this study, we expressed all of the possible isotopically labeled HO-2 proteins in which one, two, or three Cys residues were labeled with L-[3-$^{13}$C]cysteine while the other Cys residues were replaced by Ala. The redox states of the Cys residues in these HO-2 variants were determined by two-dimensional $^1$H/$^{13}$C HSQC NMR spectroscopy in the presence of the reducing agent TCEP. In addition, the NMR spectra for the oxidized and reduced proteins were recorded at increasing...
concentrations of heme to examine the involvement of Cys residues in heme binding and catalysis. Finally, heme titration experiments of the oxidized and reduced HO-2 variants using tryptophan fluorescence quenching were performed to correlate the redox state of Cys residues with the affinity of each HO-2 variant for heme, with the ultimate goal of testing the putative involvement of an HO-2 thiol/disulfide redox switch in regulating the affinity of the enzyme for heme.

The NMR spectra of WT HO-2 (13C/13C/13C) and the A/13C/13C variant exhibited two resonances in the oxidized Cys region corresponding to Cys265 and Cys282 (Table 1). These resonances disappeared when TCEP was added, and a new resonance appeared in the reduced Cys region. These results indicate that in the WT protein, Cys265 and Cys282 reversibly form a disulfide bond, in accord with the results of the Ragsdale group (17).

The NMR spectra of WT HO-2 (13C/13C/13C) and the A/13C/13C variant exhibited two resonances in the oxidized Cys region corresponding to Cys265 and Cys282 (Table 1). These resonances disappeared when TCEP was added, and a new resonance appeared in the reduced Cys region. These results indicate that in the WT protein, Cys265 and Cys282 reversibly form a disulfide bond, in accord with the results of the Ragsdale group (17).

The NMR spectra of the single and double [13C]Cys-labeled HO-2 mutants demonstrate that the redox state of a given Cys residue is sensitive to the absence of one or both of the other Cys residues. For instance, Cys282 exists in the reduced state when it is the only Cys present, as revealed by a doublet resonance at 13C = 28 ppm (Table 1). However, in the presence of Cys127, it switches to the oxidized state, as illustrated by the appearance of two resonances in the oxidized Cys region for the A/13C/A variant (Table 1). This indicates that Cys127 can form a disulfide bond with Cys282 when Cys265 is absent. The formation of this disulfide bond is largely reversible because the addition of TCEP results in the appearance of NMR resonances in the reduced Cys region at the expense of the two resonances in the oxidized region. Thus, Cys282 must be in close proximity to both Cys265 and Cys127 (Scheme 1).

Cys265 in HO-2 is of particular interest because it exists in the oxidized state in all the HO-2 proteins containing this residue (Table 1). Not only is it involved in disulfide bonds with Cys282 and Cys127, but in the absence of these two Cys residues (i.e., A/13C/A), Cys265 is still in an oxidized state. The nature of the oxidized sulfur differs, however, as shown by a shift of its two resonances from 13C = 40.5 ppm in the WT protein to 13C = 38.7 ppm in the A/13C/A variant. Nevertheless, the oxidized thiol can be reduced, as shown by the changes in the NMR spectrum upon the addition of TCEP. The possibility that Cys265 forms an intermolecular disulfide bond in the absence of the other two Cys residues was ruled out by a native gel experiment, in agreement with previous results (17). Cys265 therefore appears to be present in the A/13C/A variant as the sulfenic acid. The sulfenate form of a Cys residue is inherently unstable, which suggests that the local environment surrounding Cys265 protects it from further oxidation to the sulfinic or sulfonic acid or attack by nucleophiles. This sequestration may explain the presence of two resonances for the A/13C/A protein, which presumably arise from the location of the two protons attached to the 13C6 carbon in slightly different chemical environments. The microenvironment of Cys265 is modulated in the presence of Cys127, as indicated by the presence of an
additional strong resonance at $^{13}\text{C} = 28$ ppm for the $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ versus $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ proteins (Table 1).

The difference in the redox states of Cys$^{265}$ and Cys$^{282}$ when one or the other is the only cysteine in the protein reflects differences in their microenvironments. This includes differences in the degree of burial of the thiol groups in a hydrophobic environment and differences in the electrostatic effect of neighboring charged groups. Charged residues can shift the $pK_a$ of SH groups (normally 8.5) up to 5 units, with positively charged residues decreasing it and negatively charged ones increasing it (28). In the absence of structural information on the regions of HO-2 that contain the cysteines, it is not possible to rationalize the differences in redox states of the two cysteines.

Cys$^{265}$ in the $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ variant is easily oxidized by small amounts of heme even in the presence of TCEP, as observed by NMR when the protein is titrated with heme. Oxidation of the thiol presumably involves electron transfer to the ferric heme, although ferrous heme was not detected in the system, presumably because it is aerobically reoxidized. A much slower rate of Cys$^{265}$ oxidation was observed during heme titration of the reduced $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ and $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ proteins, a finding that confirms that the local environment of Cys$^{265}$ is altered by the absence of Cys$^{282}$. Cys oxidation is caused by heme and not by an increase in the pH of the solution, as demonstrated by NMR studies of free, $^{13}\text{C}$-labeled cysteine incubated aerobically with either NaOH (pH 12) or heme (pH 12). Interestingly, Cys$^{265}$ was proposed to replace the His$^{45}$ axial ligand in the reduced HO-2 protein at very low temperatures (10 K) (17, 29). To determine if this ligand exchange occurs at something closer to physiological temperatures, we monitored the NMR spectrum of the oxidized $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ mutant at 277 K as heme was titrated into the solution. No changes were observed, indicating that the thiol does not form a stable complex with the heme iron atom. However, when Cys$^{265}$ in the $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ protein was first reduced by TCEP, titration with ferric heme caused the Cys$^{265}$ signal in the oxidized region to disappear as the two resonances in the oxidized region appeared, consistent with some form of transient interaction between the heme iron and the Cys$^{265}$ thiol group.

Tryptophan fluorescence quenching measurements of the binding affinity of the WT and mutant HO-2 proteins for heme revealed an $\sim 2.5$-fold difference. This difference is minor compared with the 10-fold difference previously reported by the Ragsdale group (17) based on absorbance spectroscopy. There are two main differences between this study and that previously published. First, the length of HO-2 protein employed is different, residues 1–293 versus residues 1–288. The measurements obtained by the two groups of the oxidized WT HO-2 protein for heme reveal $K_d$ values of 57 and 33 nM, respectively. These results argue against a significant effect of the extra 5 residues (positions 289–293) of HO-2 in the binding affinity. In addition, our measurements of His-tagged versus non-tagged HO-2 proteins showed that His tag addition at the C terminus does not affect heme binding affinity or catalysis. The second difference is the technique used in the $K_d$ determination: tryptophan fluorescence quenching versus absorption spectroscopy. The main advantage of fluorescence quenching over absorption spectroscopy is the higher signal/noise ratio, which allows the measurements to be made with nanomolar rather than micromolar protein concentrations. Furthermore, accurate measurement of the binding affinity requires protein concentrations to be at levels lower than or near the $K_d$ value; otherwise, all of the added ligand will form a protein-ligand complex until saturation (30). The HO-2 protein concentration

**SCHEME 1.** The redox states of Cys$^{127}$, Cys$^{265}$, and Cys$^{282}$ in HO-2 WT ($^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$) and its Cys to Ala mutant apoproteins in the absence and presence of TCEP. S, thiolate; SH, thiol, SOH, sulfenic acid; S=S, disulfide bond. The corresponding $^{13}\text{C}$Cys NMR signals are indicated.
utilized in our fluorescence quenching measurements was 100 nM rather than the 8000 nM employed in the previous work, the former concentration being closer to the $K_d$ value of the WT ($K_d = 57 \text{ nM}$). In addition, our comparative measurements obtained with the two techniques showed that the affinity values determined by absorption spectroscopy were indeed severalfold higher than those obtained by fluorescence quenching.

In this study, we show that all three Cys residues in HO-2 are in close proximity and interact with each other. Most importantly, we establish the presence of an intramolecular disulfide bond between Cys$^{265}$ and Cys$^{282}$ in WT HO-2 and show that the $K_d$ of HO-2 for heme is 0.057 $\mu$M in the presence and 0.146 $\mu$M in the absence of this disulfide bond. The concentration of “free” heme in the cell is notoriously difficult to estimate because it is largely protein-bound, but it has been estimated to be in the range of 0.1 $\mu$M (31), a value consistent with the $K_d$ values reported here. The 2.5-fold difference in heme affinity for the proteins with and without the disulfide bond is small and would at most be responsible for a doubling of the rate of heme oxidation. This does not convincingly argue for a role of an HO-2 thiol/disulfide switch in regulating cellular heme and iron levels. In view of the location of the Cys thiol groups in HRM motifs that in other proteins are involved in regulation or intraorganelle transport, it is likely that the Cys residues in HO-2 are involved in some form of protein-protein interactions related to the signaling roles of the protein.

REFERENCES

1. Moore, R. A., Overhaus, M., Whitcomb, J., Iredi, E., Choi, A. M., Otterbein, L. E., and Bauer, A. J. (2005) Brief inhalation of low-dose carbon monoxide protects rodents and swine from postoperative ileus. *Crit. Care Med.* 33, 1317–1326
2. Otterbein, L. E., Soares, M. P., Yasamshita, K., and Bach, F. H. (2003) Heme oxygenase-1. Unleashing the protective properties of heme. *Trends Immunol.* 24, 449–455
3. Yachie, A., Niida, Y., Wada, T., Igarashi, N., Kaneda, H., Toma, T., Ohta, K., Kasahara, Y., and Koizumi, S. (1999) Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J. Clin. Invest.* 103, 129–135
4. Traksel, G. M., Kuty, R. K., and Maines, M. D. (1986) Purification and characterization of the major constitutive form of testicular heme oxygenase. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14848–14853
5. Williams, S. E., Woottton, P., Mason, H. S., Boul, J., Iles, D. E., Ricardi, D., Peers, C., and Kemp, P. J. (2004) Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science* 306, 2093–2097
6. Zakhary, R., Poss, K. D., Jaffrey, S. R., Ferris, C. D., Tonomura, S., and Snyder, S. H. (1997) Targeted gene deletion of heme oxygenase 2 reveals neural role for carbon monoxide. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14848–14853
7. Schuller, D. J., Wilks, A., Ortiz de Montellano, P. R., and Poulos, T. L. (1999) Crystal structure of human heme oxygenase-1. *Nat. Struct. Biol.* 6, 860–867
8. Bianchetti, C. M., Yi, L., Ragsdale, S. W., and Phillips, G. N., Jr. (2007) Comparison of apo- and heme-bound crystal structures of a truncated human heme oxygenase-2. *J. Biol. Chem.* 282, 37624–37631
9. Chen, J. J., Throop, M. S., Gehrke, L., Xue, C., Pal, J. K., Brodsky, M., and London, I. M. (1991) Cloning of the cDNA of the heme-regulated eukaryotic initiation factor 2a (eIF-2a) kinase of rabbit reticulocytes. Homology to yeast GCN2 protein kinase and human double-stranded RNA-dependent eIF-2a kinase. *Proc. Natl. Acad. Sci. U.S.A.* 88, 7729–7733
10. Ratei-Kolpin, M., Chefa, P. J., Hussain, Z., Hahn, J., Uma, S., Mats, R. L., and Chen, J. J. (2000) Two heme-binding domains of heme-regulated eu-