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

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Background: Heme oxygenase-2 (HO-2) containing three cysteines is involved in signaling pathways.

Results: The cysteines in HO-2 interact with each other, but their redox state only modestly alters heme affinity.

Conclusion: Changes in the redox state of the cysteines do not significantly control heme oxidation rates.

Significance: The HO-2 cysteine residues play roles in interactions related to the role of HO-2 in regulatory processes.

Human heme oxygenases 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-[3-¹³C]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 Cys¹²⁷ 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 IX α , 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-Ala¹³¹. The HRM motifs have been found in a few other proteins whose functions are heme-dependent, such as heme-regulated initiation factor 2 α 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 -aminolevulinic 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

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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.

Cysteine Residues and Heme Binding in Heme Oxygenase-2

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-benzoxadiazole (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 QuikChangeTM 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^β]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 A_{600 nm} 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_i, 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_i, 1 mM EDTA, and 30 mM imidazole buffer, pH 7.4. The column was washed with five column volumes of 50 mM potassium P_i, 1 mM EDTA, and 40 mM imidazole, pH 7.4, buffer, and the protein was eluted with 50 mM potassium P_i, 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_i, 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_i, 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_i, 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 $\mu\text{g}/\text{ml}$ ampicillin at 37 °C. The protein expression was induced with isopropyl β -D-thiogalactoside (final concentration of 0.5 mM) when A_{600} reached 0.6–0.8. Protein expression was carried out for 16 h at 30 °C. Cells were harvested by centrifugation at $4,000 \times g$ for 15 min at 4 °C using a Beckman Coulter centrifuge, and the protein was purified using the same protocol as the ^{13}C -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, 15 μM hemin, 0.1 M potassium P_i , pH 7.4, buffer supplemented with 10 mg/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 $\text{mm}^{-1} \text{cm}^{-1}$. The specific activity of the WT and mutant HO-2 proteins is expressed in nmol/min/mg.

Mass Spectrometric Analysis of [$^{13}\text{C}^\beta$]Cys-labeled HO-2 Protein—Incorporation of the [$^{13}\text{C}^\beta$]Cys-labeled amino acid into the HO-2 protein was specifically verified for the A127/ $^{13}\text{C}265/^{13}\text{C}282$ 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 $\mu\text{m} \times 150\text{-mm}$ 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 $\mu\text{l}/\text{min}$. 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 of 400–1800 for 1 s. The most abundant, multiply charged resonance in the survey scan was selected for collision-induced dissociation. MS/MS spectra were acquired for 3 s over an m/z range from 59 to 1800. Spectra were analyzed using Data Explorer software (Applied Biosystems, Foster City, CA).

$^1\text{H},^{13}\text{C}$ HSQC NMR Measurements—Two-dimensional $^1\text{H},^{13}\text{C}$ HSQC spectra were acquired on a Bruker 800-MHz spectrometer (Bruker Avance, Bremen, Germany) equipped with a $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ triple resonance Coldprobe™ 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 (^{13}C), 128 complex points in 1, and a 1-s recycle time. The ^{13}C chemical shifts were calibrated to the reference (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) indi-

rectly. Each NMR sample consisted of $\sim 300 \mu\text{l}$ of a 130–300 μM concentrated solution of [^{13}C]Cys-labeled HO-2 protein in a 50 mM potassium P_i , pH 7.4, buffer with 10% D_2O 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,

$$IF = IF_0 + \Delta IF \frac{(K_D + P_t + L_t) - \sqrt{(K_D + P_t + L_t)^2 - 4L_tP_t}}{2P_t} \quad (\text{Eq. 1})$$

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, IF_0 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 N NaOH and 0.1 M potassium P_i , 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 $\text{mm}^{-1} \text{cm}^{-1}$. 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

Cysteine Residues and Heme Binding in Heme Oxygenase-2

TABLE 1

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

NMR chemical shifts (^{13}C , ^1H) for the oxidized and reduced [^{13}C]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 ^{13}C for labeled cysteine. ND, not determined; ppm, chemical shift in parts per million of the spectrometer frequency.

HO-2 protein	Without TCEP			With TCEP			Specific activity <i>nmol/min/mg</i>
	Reduced [^{13}C]Cys <i>ppm</i>	Oxidized [^{13}C]Cys <i>ppm</i>	K_d for heme <i>nM</i>	Reduced [^{13}C]Cys <i>ppm</i>	Oxidized [^{13}C]Cys <i>ppm</i>	K_d for heme <i>nM</i>	
$^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$		40.5:3.11 40.5:2.92	57	28.0:2.82		146	28
A/ $^{13}\text{C}/^{13}\text{C}$		40.5:3.11 40.5:2.92	90	28.0:2.82		111	34
$^{13}\text{C}/\text{A}/\text{A}$			105			ND	29
$^{13}\text{C}/\text{A}/^{13}\text{C}$		38.3:3.05 38.3:2.76	102	28.0:2.84 28.0:2.80 (doublet)	38.3:3.05 38.3:2.76	316	26
$^{13}\text{C}/^{13}\text{C}/\text{A}$	28.0:2.82	38.7:3.03 38.7:2.68	45	28.0:2.82	38.7:3.03 38.7:2.68	373	39
A/A/ ^{13}C	28.0:2.84 28.0:2.80 (doublet)		31	28.0:2.84 28.0:2.80 (doublet)		26	21
A/ $^{13}\text{C}/\text{A}$		38.7:3.04 38.7:2.68	103	28.0:2.82		77	29
A/A/A			54			47	36

protein solution using a gas-tight syringe (Hamilton, Reno, NV). The K_D values of the reduced HO-2 for heme were calculated using the same method as that for the HO-2 titration done in the presence of air as described above.

Determining the Redox State of A/ $^{13}\text{C}/\text{A}$ and A/A/ ^{13}C HO-2 Proteins by NBD-Cl—The redox state of Cys residues for the oxidized and reduced HO-2 was determined using the NBD-Cl fluorescent probe. The latter reacts with the protein Cys-SH group forming Cys-S-NBD and Cys-SOH forming the Cys-S(O)-NBD product, both of which have a characteristic absorbance at 420 and 347 nm, respectively. The reduced HO-2 protein was obtained by incubating it with a 10-fold excess of TCEP for 30 min in the glove box. TCEP was later removed using 0.5-ml 10,000 MWCO centrifugal filter units. Both the oxidized and reduced HO-2 proteins (20 μM) were then reacted with 500 μM NBD-Cl (25-fold excess) for 45 min at 22 $^\circ\text{C}$. The unbound NBD-Cl was removed by buffer exchanging three times using 0.5-ml 10,000 MWCO centrifugal filter units. The absorption spectra of the oxidized and reduced HO-2 were then recorded under anaerobic conditions on a Cary 50 spectrophotometer (Varian).

RESULTS

Characterization of [^{13}C]Cys-labeled HO-2 Mutant Proteins— [^{13}C]Cys-labeled HO-2 mutant proteins were expressed in M9 minimal medium using BL21(DE3)cysE51 auxotrophic cells as described under "Materials and Methods." The proteins expressed were $^{13}\text{C}127/\text{A}265/\text{A}282$ ($^{13}\text{C}/\text{A}/\text{A}$), A127/ $^{13}\text{C}265/\text{A}282$ (A/ $^{13}\text{C}/\text{A}$), A127/A265/ $^{13}\text{C}282$ (A/A/ ^{13}C), A127/ $^{13}\text{C}265/\text{A}282$ (A/ $^{13}\text{C}/^{13}\text{C}$), $^{13}\text{C}127/\text{A}265/\text{A}282$ ($^{13}\text{C}/\text{A}/\text{A}$), $^{13}\text{C}127/\text{A}265/\text{A}282$ ($^{13}\text{C}/\text{A}/^{13}\text{C}$), and $^{13}\text{C}127/\text{A}265/\text{A}282$ ($^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$), in which one, two, or three Cys residues were ^{13}C -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 ^{13}C 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 [^{13}C]Cys into HO-2 was specifically verified for the A/ $^{13}\text{C}/^{13}\text{C}$ protein by LC/MS/electrospray ionization mass spectrometric analysis. Although the difference of only 1 mass unit between ^{12}C and ^{13}C isotopes poses a challenge in accurately determining the efficiency of incorporation of the [^{13}C]Cys amino acid, the data clearly revealed the presence of both the [^{12}C]Cys and [^{13}C]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 , ^{13}C HSQC) spectroscopic analysis of the $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ 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 $^{13}\text{C} = 40.5$ ppm, $^1\text{H} = 3.11$ ppm and the second at $^{13}\text{C} = 40.5$ ppm, $^1\text{H} = 2.92$ ppm, partially overlapping a non-cysteine-related resonance at $^{13}\text{C} = 42$ ppm, $^1\text{H} = 2.93$ ppm. The non-cysteine-related resonance was observed in the spectra of all [^{13}C]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 $^{13}\text{C} = 42$ ppm, $^1\text{H} = 2.93$ ppm derives from the abun-

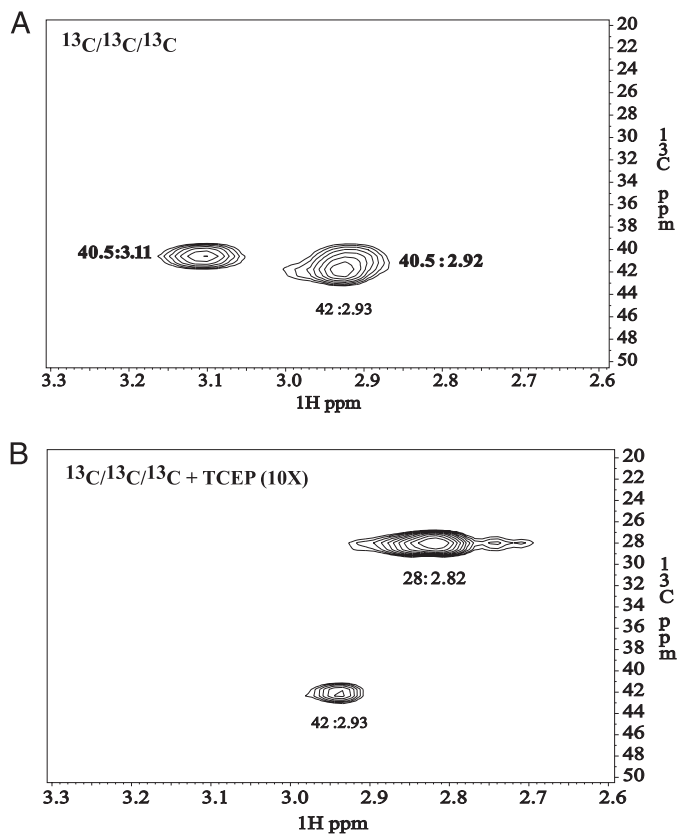


FIGURE 1. Two-dimensional NMR spectra of $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ HO-2 apoprotein. A, protein (250 μM) alone. B, protein in the presence of a 10-fold excess (2.5 mM) of TCEP. Spectra were recorded at 298 K in 50 mM potassium P_i , pH 7.4, 10% D_2O buffer. ppm, chemical shift in parts per million of the spectrometer frequency.

dant methylene (CH_2) 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 ^{13}C dimension is >35 ppm and in the reduced state if it is <32 ppm. The two resonances observed at $^{13}\text{C} = 40.5$ ppm (Fig. 1A) suggest that two Cys residues in the $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ protein are in the oxidized state, probably in a disulfide bond. To confirm that these two resonances correspond to oxidized Cys residues, the $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ 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 $^{13}\text{C} = 40.5$ ppm disappear, and a large, line-broadened resonance appears at $^{13}\text{C} = 28$, $^1\text{H} = 2.82$ ppm (Fig. 1, A 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 $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ 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/ $^{13}\text{C}/^{13}\text{C}$ protein, in which Cys-127 is mutated to Ala, revealed the same resonances as the $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ protein (Table 1), indicating that the two resonances at $^{13}\text{C} = 40.5$ ppm correspond to Cys²⁶⁵ and Cys²⁸². The

same resonances were also observed for the reduced (by 10-fold excess TCEP) $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ and A/ $^{13}\text{C}/^{13}\text{C}$ proteins, as shown by the presence of a resonance at $^{13}\text{C} = 28$ 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 $^{13}\text{C} = 42$ ppm, as expected, was also observed with both of these mutants. In efforts to separate this resonance from the resonance at $^{13}\text{C} = 40.5$ ppm, $^1\text{H} = 2.92$ ppm deriving from the labeled Cys, the spectrum of the A/ $^{13}\text{C}/^{13}\text{C}$ 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 $^{13}\text{C} = 40.5$ ppm for both the $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ and A/ $^{13}\text{C}/^{13}\text{C}$ proteins to Cys²⁶⁵ and Cys²⁸² is confirmed by the NMR spectrum of the $^{13}\text{C}/\text{A}/\text{A}$ protein in the absence and presence of TCEP (Table 1). The spectrum of this protein only exhibited the spurious peak at $^{13}\text{C} = 42$ 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 $^{13}\text{C}/\text{A}/\text{A}$ and $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ proteins suggests that its molecular motion is highly constrained.

To specifically determine which of the two resonances at $^{13}\text{C} = 40.5$ ppm corresponds to Cys²⁸², the two-dimensional NMR spectrum of the A/A/ ^{13}C protein was obtained. The spectrum revealed the presence of a doublet at $^{13}\text{C} = 28$ 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/ ^{13}C 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 $^{13}\text{C}^\beta$ carbon. Interestingly, Cys²⁸² is in the oxidized state in the presence of a second Cys, either Cys¹²⁷ ($^{13}\text{C}/\text{A}/^{13}\text{C}$) or Cys²⁶⁵ (A/ $^{13}\text{C}/^{13}\text{C}$), or when both Cys¹²⁷ and Cys²⁶⁵ ($^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$) are present (Table 1). The spectrum for the $^{13}\text{C}/\text{A}/^{13}\text{C}$ protein revealed two resonances in the oxidized Cys region at $^{13}\text{C} = 38.3$ ppm (Fig. 3A and Table 1). These resonances are in the same region as the resonances observed for the A/ $^{13}\text{C}/\text{A}$ protein (Fig. 4A and Table 1). Upon the addition of TCEP to $^{13}\text{C}/\text{A}/^{13}\text{C}$ HO-2, the intensity of the two resonances decreases with concomitant appearance of a doublet resonance in the reduced Cys region at $^{13}\text{C} = 28$ ppm, which is in very close proximity to the one observed for the reduced A/ $^{13}\text{C}/^{13}\text{C}$ and $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ proteins. This indicates that Cys²⁸² is oxidized in $^{13}\text{C}/\text{A}/^{13}\text{C}$ HO-2, either forming an intramolecular disulfide bond with Cys¹²⁷ or, less likely, existing in the sulfenic acid form. Interestingly, the $^{13}\text{C}/\text{A}/^{13}\text{C}$ HO-2 protein is only partially reduced by

Cysteine Residues and Heme Binding in Heme Oxygenase-2

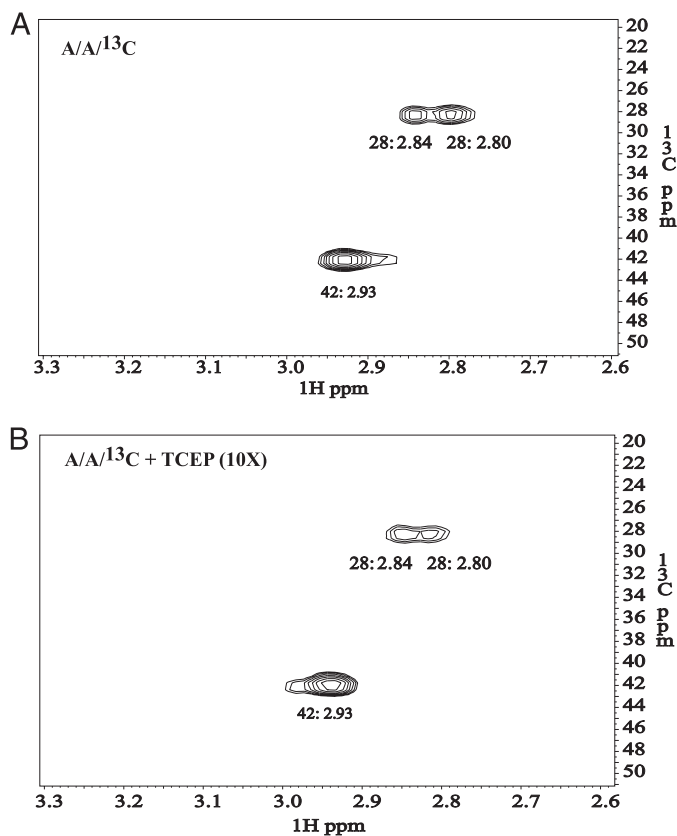


FIGURE 2. Two-dimensional NMR spectra of A/A/ ^{13}C HO-2 apoprotein. A, protein alone (150 μM). B, protein in the presence of 10-fold excess (1.5 mM) of TCEP. Spectra were recorded at 298 K in 50 mM potassium P_i , pH 7.4, 10% D_2O buffer.

TCEP, as indicated by the presence of two resonances at $^{13}\text{C} = 38.3$ ppm and a doublet resonance at $^{13}\text{C} = 28$ 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 $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$, A/ $^{13}\text{C}/^{13}\text{C}$, $^{13}\text{C}/^{13}\text{C}/\text{A}$, and A/ $^{13}\text{C}/\text{A}$, 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 $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ and A/ $^{13}\text{C}/^{13}\text{C}$ proteins revealed the presence of two resonances at $^{13}\text{C} = 40.5$ ppm that correspond to either Cys²⁶⁵ or Cys²⁸² (Table 1). These resonances are shifted

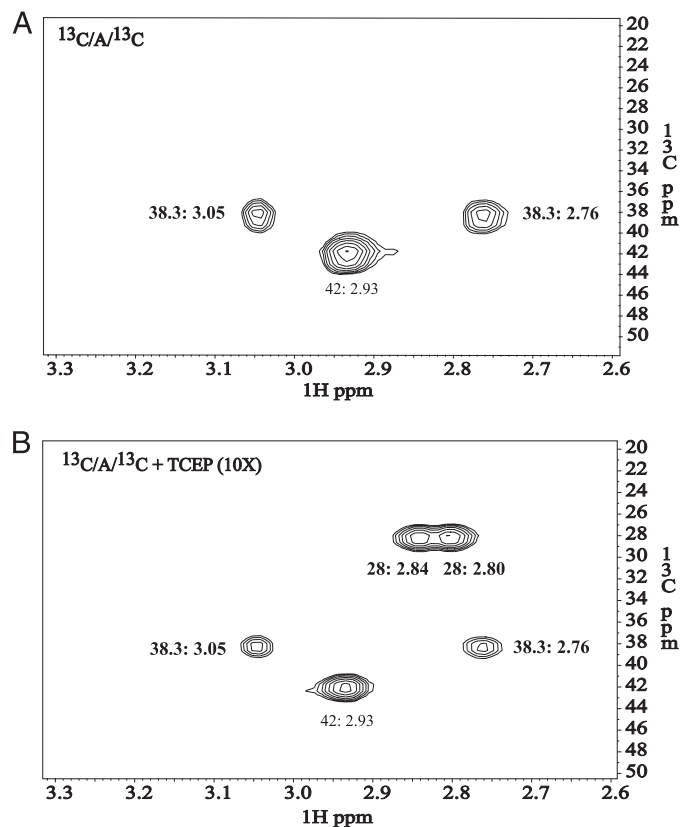


FIGURE 3. Two-dimensional NMR spectra of $^{13}\text{C}/\text{A}/^{13}\text{C}$ HO-2 apoprotein. A, protein alone (300 μM). B, protein in the presence of a 10-fold excess (3 mM) of TCEP. Spectra were recorded at 298 K in 50 mM potassium P_i , pH 7.4, 10% D_2O buffer. ppm, chemical shift in parts per million of the spectrometer frequency.

to $^{13}\text{C} = 38.7$ ppm in both A/ $^{13}\text{C}/\text{A}$ (Fig. 4) and $^{13}\text{C}/^{13}\text{C}/\text{A}$ (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/ $^{13}\text{C}/\text{A}$ protein suggest that Cys²⁶⁵ is either involved in an intermolecular disulfide bond or is present as a sulfenic acid. A control experiment with the [^{13}C]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 $^{13}\text{C} = 40.5$ ppm correspond to the two β protons bound to the ^{13}C 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/ $^{13}\text{C}/\text{A}$ protein, the latter was incubated with a 10-fold excess of TCEP for 30 min. The reduced protein exhibited a new resonance at $^{13}\text{C} = 28$ ppm with disappearance of the two resonances at $^{13}\text{C} = 38.7$ 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/ $^{13}\text{C}/\text{A}$ HO-2 mutant (17). The presence of only one resonance at $^{13}\text{C} = 28$ ppm for the reduced A/ $^{13}\text{C}/\text{A}$ protein suggests that the two protons bound to the $^{13}\text{C}^\beta$ carbon experience similar chemical environments.

Cys²⁶⁵ is also in the oxidized state in the $^{13}\text{C}/^{13}\text{C}/\text{A}$ protein (Fig. 5 and Table 1), which, as observed for A/ $^{13}\text{C}/\text{A}$ HO-2, has

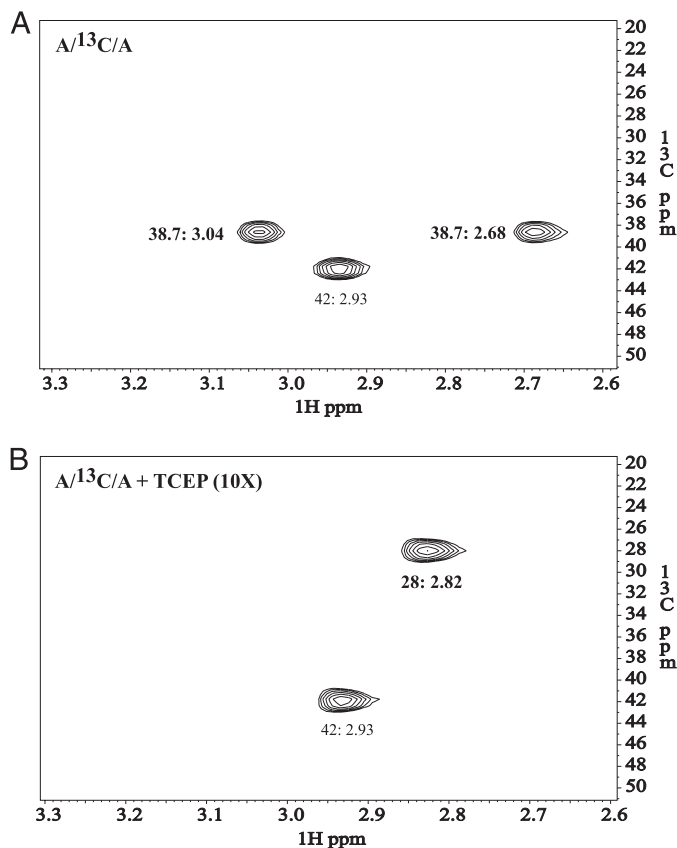


FIGURE 4. Two-dimensional NMR spectra of A/ ^{13}C /A HO-2 apoprotein. *A*, protein alone (130 μM). *B*, protein in the presence of a 10-fold excess (1.3 mM) of TCEP. Spectra were recorded at 298 K in 50 mM potassium P_i , pH 7.4, 10% D_2O buffer.

two resonances in the $^{13}\text{C} = 38.7$ ppm region. Interestingly, an additional strong resonance is observed at $^{13}\text{C} = 28$ 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 $^{13}\text{C} = 38.7$ ppm changes little, suggesting that either the Cys residue(s) is not fully accessible to TCEP, or its oxidation is irreversible. The resonance at $^{13}\text{C} = 28$ ppm is in the same location as the one observed for the A/ ^{13}C /A protein (Fig. 4*B* 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 $^{13}\text{C}/^{13}\text{C}$ /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.

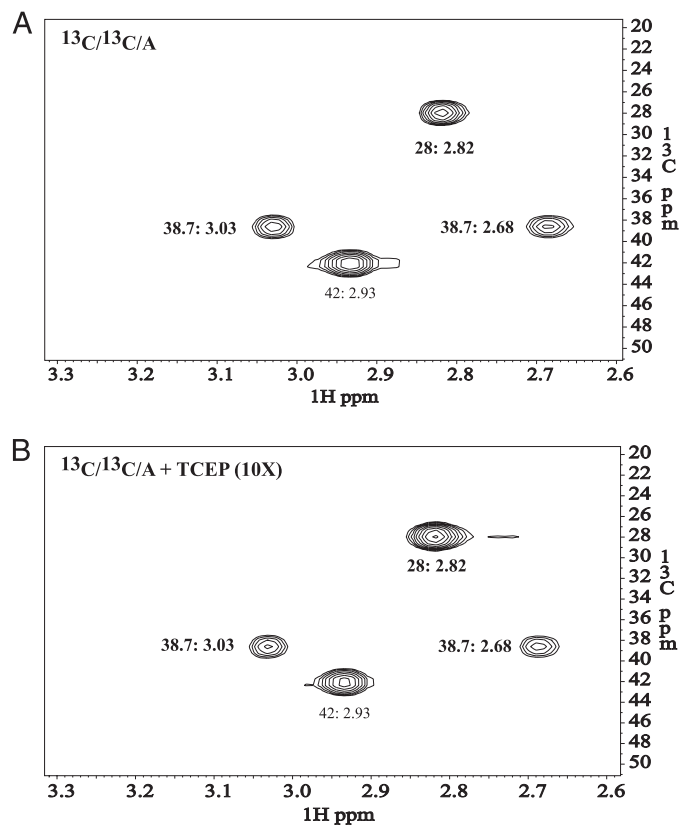


FIGURE 5. Two-dimensional NMR spectra of $^{13}\text{C}/^{13}\text{C}$ /A HO-2 apoprotein. *A*, protein alone (260 μM). *B*, protein in the presence of a 10-fold excess (2.6 mM) of TCEP. Spectra were recorded at 298 K in 50 mM potassium P_i , pH 7.4, 10% D_2O buffer.

TABLE 2

Summary of the two-dimensional NMR chemical shifts for the [$^{13}\text{C}^\beta$]Cys free amino acid in the absence and presence of diamide, NaOH, and heme

Shown are NMR chemical shifts ($^{13}\text{C}, ^1\text{H}$) of [$^{13}\text{C}^\beta$]Cys free amino acid (3 mg/ml) in the absence and presence of diamide (2.5 mM), NaOH (0.1 N, pH 12), or heme (300 $\mu\text{g}/\text{ml}$, pH 12). Spectra were recorded at 298 K in 50 mM potassium P_i , pH 7.4, 10% D_2O buffer. ppm, chemical shift in parts per million of the spectrometer frequency.

[$^{13}\text{C}^\beta$]Cys free amino acid	Reduced [^{13}C]Cys		Oxidized [^{13}C]Cys	
	ppm		ppm	
Alone	27.8:3.03			
	27.8:2.98			
With diamide			40.5:3.34	
			40.5:3.13	
With NaOH	27.8:2.98			
With heme	27.8:3.02		40.0:3.30	
	27.8:2.93		40.0:3.07	

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 $^{3+}$ and His 45 -Fe $^{3+}$ 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

Cysteine Residues and Heme Binding in Heme Oxygenase-2

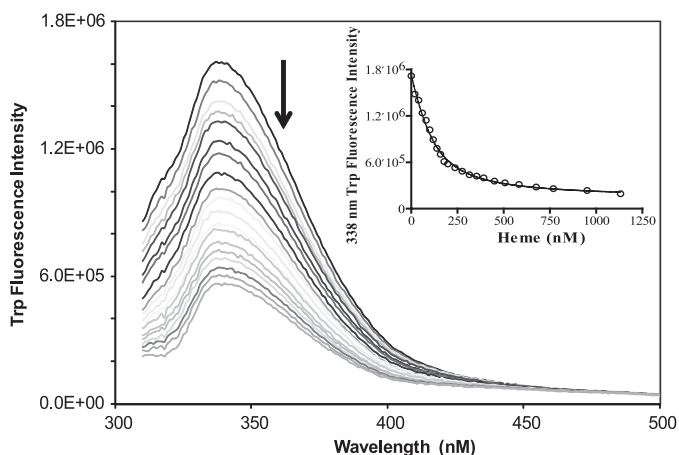


FIGURE 6. Heme titration of the HO-2 protein at increasing concentrations of heme. The tryptophan fluorescence spectra of the oxidized WT (100 nM) present in potassium P_i, pH 7.4, buffer upon the addition of 0–1130 nM heme is shown. The tryptophan residue was excited at the 290-nm wavelength, and fluorescence emission spectra were recorded at 22 °C. *Inset*, 338-nm tryptophan fluorescence intensity *versus* heme concentration data fitted to Equation 1, as described under “Materials and Methods.”

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 ¹³C = 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 ¹³C = 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 [¹³C]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_i, 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 [¹³C]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 ¹³C = 40 ppm (Table 2). Thus, heme causes oxidation of a Cys residue in the HO-2

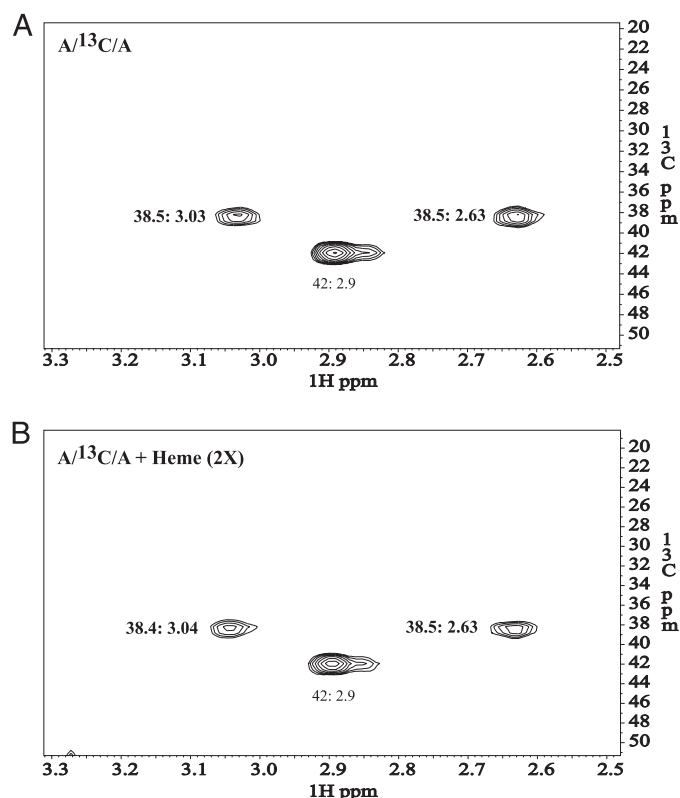


FIGURE 7. Heme titration of the oxidized A/¹³C/A HO-2 protein. A, oxidized protein (130 μM) alone. B, oxidized protein in the presence of a 2-fold excess (260 μM) of heme. The two-dimensional NMR spectra were recorded at 277 K.

protein. The pK_a 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 ¹³C/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 [¹³C]Cys resonances from the oxidized to the reduced Cys region. Only one resonance for the reduced protein is observed at ¹³C = 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-[3-¹³C]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 ¹H, ¹³C HSQC NMR spectroscopy in the presence and absence of the reducing agent TCEP. In addition, the NMR spectra for the oxidized and reduced proteins were recorded at increasing

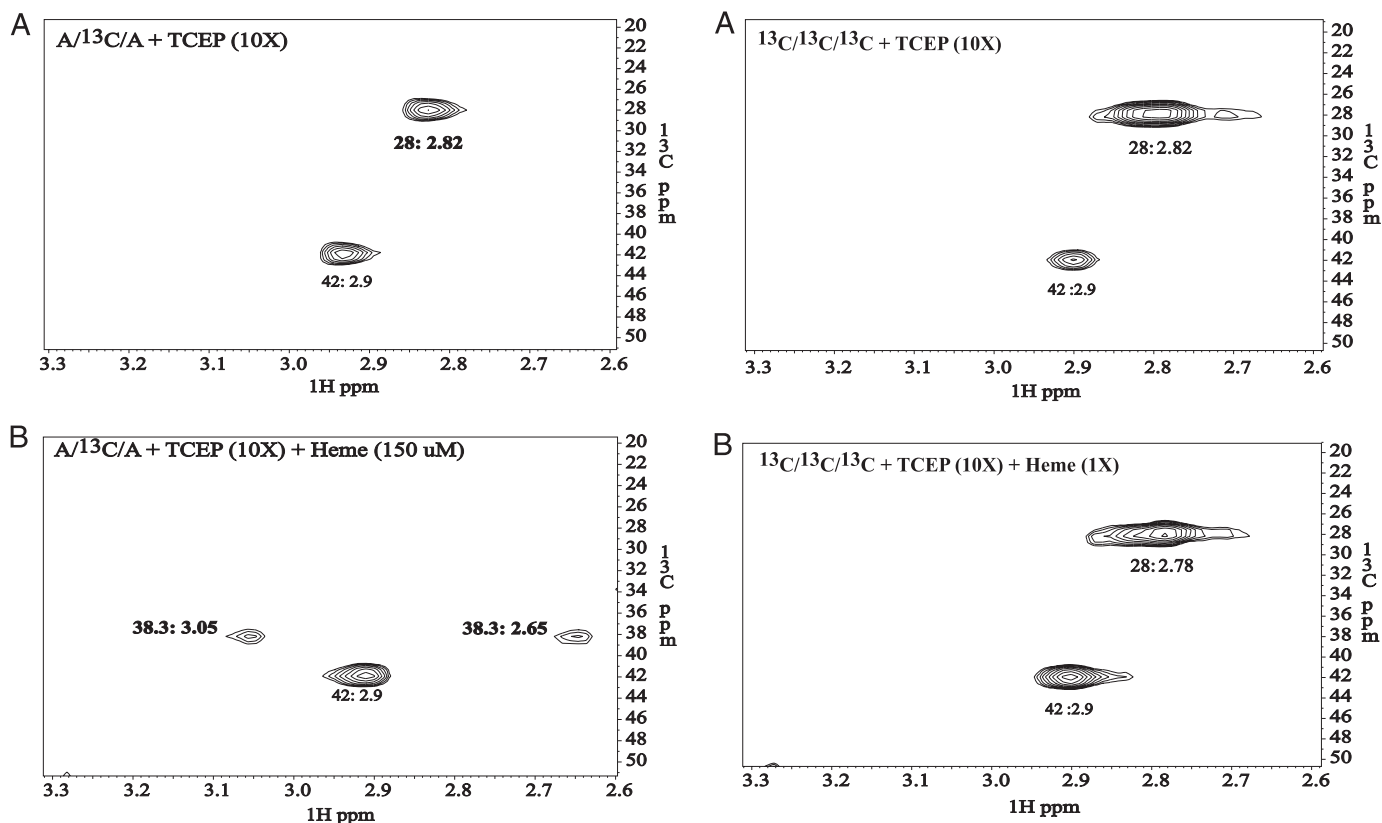


FIGURE 8. Heme titration of the reduced $A/^{13}\text{C}/A$ protein. *A*, reduced $A/^{13}\text{C}/A$ protein ($175\ \mu\text{M}$) alone. Reduced protein was obtained by incubating it with a 10-fold excess ($1.75\ \text{mM}$) of TCEP for 30 min. *B*, reduced $A/^{13}\text{C}/A$ protein in the presence of $150\ \mu\text{M}$ heme. The two-dimensional NMR spectra were recorded at 277 K.

FIGURE 9. Heme titration of the reduced $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ (WT) HO-2 protein. *A*, reduced $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ protein ($250\ \mu\text{M}$) alone. The reduced protein was obtained by incubating it with a 10-fold excess ($2.5\ \text{mM}$) of TCEP for 30 min. *B*, reduced $^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$ protein in the presence of a 1-fold excess ($250\ \mu\text{M}$) of heme. The NMR spectra were recorded at 277 K.

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.

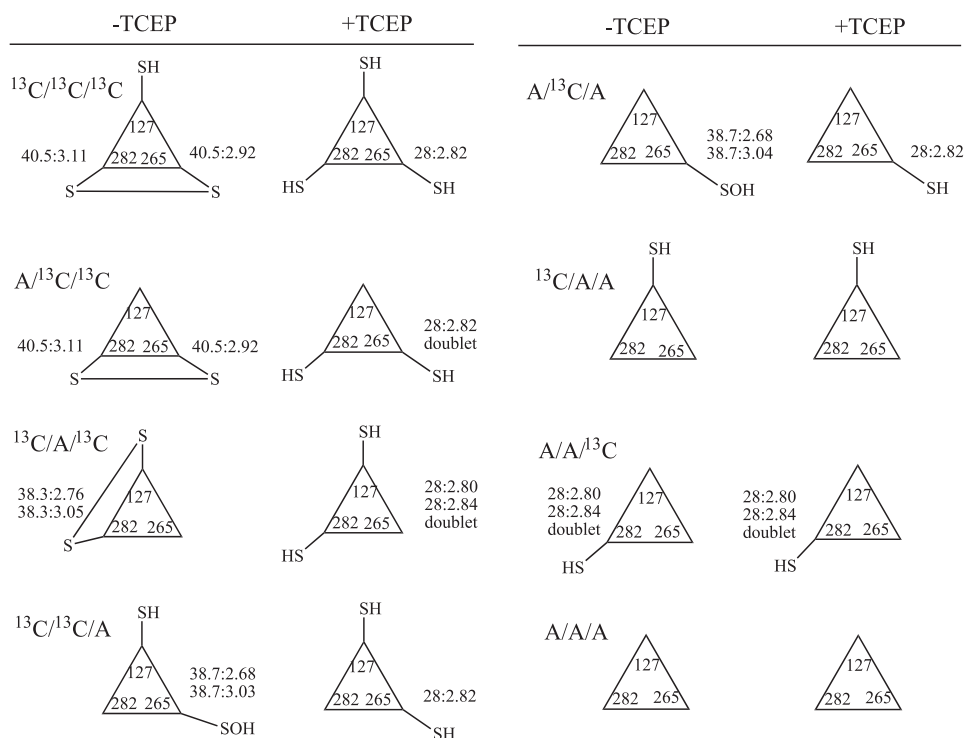
The NMR spectra of WT HO-2 ($^{13}\text{C}/^{13}\text{C}/^{13}\text{C}$) and the $A/^{13}\text{C}/^{13}\text{C}$ variant exhibited two resonances in the oxidized Cys region corresponding to Cys^{265} and Cys^{282} (Table 1). These resonances disappeared when TCEP was added, and a new resonance appeared in the reduced Cys region. These results indicate that in the WT protein, Cys^{265} and Cys^{282} reversibly form a disulfide bond, in accord with the results of the Ragsdale group (17).

The NMR spectra of the single and double [^{13}C]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, Cys^{282} exists in the reduced state when it is the only Cys present, as revealed by a doublet resonance at $^{13}\text{C} = 28\ \text{ppm}$ (Table 1). However, in the presence of Cys^{127} , it switches to the oxidized state, as illustrated by the appearance of two resonances in the oxidized Cys region for the $^{13}\text{C}/A/^{13}\text{C}$ protein (Table 1). This indicates that Cys^{127} can form a disulfide bond with Cys^{282} when Cys^{265} 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, Cys^{282} must be in close proximity to both Cys^{265} and Cys^{127} (Scheme 1).

Cys^{265} 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 Cys^{282} and Cys^{127} , but in the absence of these two Cys residues (*i.e.* $A/^{13}\text{C}/A$), Cys^{265} is still in an oxidized state. The nature of the oxidized sulfur differs, however, as shown by a shift of its two resonances from $^{13}\text{C} = 40.5\ \text{ppm}$ in the WT protein to $^{13}\text{C} = 38.7\ \text{ppm}$ in the $A/^{13}\text{C}/A$ 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 Cys^{265} 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). Cys^{265} therefore appears to be present in the $A/^{13}\text{C}/A$ variant as the sulfenic acid. The sulfenate form of a Cys residue is inherently unstable, which suggests that the local environment surrounding Cys^{265} protects it from either further oxidation to the sulfinic or sulfonic acid or attack by nucleophiles. This sequestration may explain the presence of two resonances for the $A/^{13}\text{C}/A$ protein, which presumably arise from the location of the two protons attached to the $^{13}\text{C}^{\beta}$ carbon in slightly different chemical environments. The microenvironment of Cys^{265} is modulated in the presence of Cys^{127} , as indicated by the presence of an

Cysteine Residues and Heme Binding in Heme Oxygenase-2



SCHEME 1. The redox states of Cys¹²⁷, Cys²⁶⁵, and Cys²⁸² in HO-2 WT (¹³C/¹³C/¹³C) and its Cys to Ala mutant apoproteins in the absence and presence of TCEP. S, thiolate; SH, thiol, SOH, sulfinic acid; S–S, disulfide bond. The corresponding [¹³C]Cys NMR signals are indicated.

additional strong resonance at ¹³C = 28 ppm for the ¹³C/¹³C/A versus A/¹³C/A proteins (Table 1).

The difference in the redox states of Cys²⁶⁵ and Cys²⁸² 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 p*K*_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.

Cys²⁶⁵ in the A/¹³C/A 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 Cys²⁶⁵ oxidation was observed during heme titration of the reduced A/¹³C/¹³C and ¹³C/¹³C/¹³C proteins, a finding that confirms that the local environment of Cys²⁶⁵ is altered by the absence of Cys²⁸². Cys oxidation is caused by heme and not by an increase in the pH of the solution, as demonstrated by NMR studies of free, ¹³C-labeled cysteine incubated aerobically with either NaOH (pH 12) or heme (pH 12). Interestingly, Cys²⁶⁵ was proposed to replace the His⁴⁵ 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 A/¹³C/A 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 Cys²⁶⁵ in the A/¹³C/A protein was first reduced by TCEP, titration with ferric heme caused the Cys²⁶⁵ signal in the reduced Cys 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 Cys²⁶⁵ thiol group.

Tryptophan fluorescence quenching measurements of the binding affinity of the WT and mutant HO-2 proteins for heme revealed an ~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 followed by six His *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

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 ($K_d = 57$ 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 Cys²⁶⁵ and Cys²⁸² in WT HO-2 and show that the K_d of HO-2 for heme is 0.057 μ M in the presence and 0.146 μ 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 μ 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 intraorganellar 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.

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