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Covalent heme attachment to the protein in human heme oxygenase-1 with selenocysteine replacing the His25 proximal iron ligand

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Abstract

To characterize heme oxygenase with a selenocysteine (SeCys) as the proximal iron ligand, we have expressed truncated human heme oxygenase-1 (hHO-1) His25Cys, in which Cys-25 is the only cysteine, in the *Escherichia coli* cysteine auxotroph strain BL21(DE3)*cys*. Selenocysteine incorporation into the protein was demonstrated by both intact protein mass measurement and mass spectrometric identification of the selenocysteine-containing tryptic peptide. One selenocysteine was incorporated into approximately 95% of the expressed protein. Formation of an adduct with Ellman's reagent (DTNB) indicated that the selenocysteine in the expressed protein was in the reduced state. The heme-His25SeCys hHO-1 complex could be prepared by either (a) supplementing the overexpression medium with heme, or (b) reconstituting the purified apoprotein with heme. Under reducing conditions in the presence of imidazole, a covalent bond is formed by addition of the selenocysteine residue to one of the heme vinyl groups. No covalent bond is formed when the heme is replaced by mesoheme, in which the vinyls are replaced by ethyl groups. These results, together with our earlier demonstration that external selenolate ligands can transfer an electron to the iron (Jiang, Y., Ortiz de Montellano, P.R., *Inorg. Chem.*, 47, 3480-3482 (2008)), indicate that a selenyl radical is formed in the hHO1 His25SeCys mutant that adds to a heme vinyl group.

Keywords

heme oxygenase; selenocysteine; heme covalent binding; selenium-iron ligation; electronic spectra; mass spectrometry

1. Introduction

Human heme oxygenase-1 (hHO-1) catalyzes the NADPH- and cytochrome P450 reductase (CPR)-dependent conversion of heme to biliverdin, a multistep process in which the enzyme first oxidizes heme to α -meso-hydroxyheme, then via loss of CO to verdoheme, and finally to iron biliverdin (1,2). Reduction of the iron biliverdin to the ferrous state by electron transfer from CPR results in product dissociation from the enzyme (3). The three products of the reaction are consequently CO, ferrous iron, and biliverdin. Furthermore, this multistep transformation is unusual in that the enzyme utilizes heme as both a prosthetic group and a substrate. The first of the three oxidative steps, the conversion of heme to α -meso-hydroxyheme, superficially resembles a cytochrome P450-catalyzed monooxygenation, but

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the hydroxylation is mediated by a different mechanism. The ferric hydroperoxide [Fe(III)-OOH] intermediate, which in a cytochrome P450 (P450) reaction is a precursor of the ferryl [formally Fe(V)=O] hydroxylating species (4), is actually responsible for the porphyrin hydroxylation in heme oxygenase (5,6). Hydrogen bonding networks and the nature of the proximal ligand to the heme iron atom appear to be important factors in channeling the ferric hydroperoxide intermediate into the appropriate reaction pathway (2,7,8).

A cysteine thiolate is invariably the proximal iron ligand in all P450 enzymes, whereas the proximal ligand in all the heme oxygenases is a histidine. In hHO-1, His-25 has been identified as the proximal iron ligand by site-directed mutagenesis (9), resonance Raman spectroscopy (9), and X-ray crystallography (10). In an earlier effort to evaluate the role of the proximal ligand on heme oxygenase catalysis, we used site specific mutagenesis to replace His-25 in hHO-1 by a cysteine (11). Coordination of the thiolate to the iron in the ferric state of the resulting H25C mutant was confirmed by resonance Raman spectroscopy combined with labeling of the iron with ^{54}Fe , but reduction of the iron resulted in protonation, dissociation, or replacement of the thiolate ligand, as indicated by the observation of a ferrous-CO absorption maximum at 412 nm rather than at the 450 nm expected for thiolate ligation. This hHO-1 H25C mutant was devoid of heme oxygenase activity. Although it still accepts electrons from CPR, its normal redox partner, it uses them in an uncoupled manner to reduce O_2 to H_2O_2 .

The atomic radius of the selenocysteine selenium atom (1.17 Å) is only slightly larger than that of the sulfur in cysteine (1.04 Å), but the selenocysteine pKa is ~5.2 whereas that of cysteine is ~8.3 (12,13). Thus, at pH 7, selenocysteine is ~98% ionized whereas less than 5% of cysteine is ionized. Replacement of the cysteine by a selenocysteine in the hHO-1 H25C mutant might therefore favor coordination of the fully ionized selenocysteine ligand to the iron, whereas coordination of the thiolate rather than protonated thiol to the iron depends on whether the protein environment can lower its pKa value. Coordination of either a thiolate or selenolate to the ferrous heme iron should give the spectroscopic signature of a cytochrome P450 enzyme – i.e., a Soret maximum for the ferrous-CO complex at ~450 nm. In view of the fact that hHO-1 normally accepts electrons from CPR and binds and activates oxygen (1,2), the protein might preferentially produce a ferryl species and thus acquire a P450-like monooxygenase activity.

Insertion of selenocysteine into a protein sequence through genetic code manipulations is a complex cotranslational process not easily adapted to recombinant protein expression. Selenocysteine, the 21st amino acid in the genetic code, is encoded by a predefined UGA codon that normally functions as a stop codon (13,14). Decoding of UGA as selenocysteine requires the assistance of a species-specific selenocysteine insertion sequence (SECIS) element in the mRNA and the involvement of several proteins dedicated to selenocysteine incorporation (15). Both the species specificity of the SECIS element and the complexity of the cotranslational process greatly constrain recombinant selenoprotein synthesis. An alternative approach is to use a cysteine auxotrophic strain of *Escherichia coli* that makes no cysteine, enabling the incorporation of exogenously provided selenocysteine into the protein (16). This approach, which exploits the fact that selenocysteine competes with cysteine in the aminoacylation reaction of cysteinyl-tRNA ligase, has been successfully utilized in the expression of a number of selenoproteins in which selenocysteine incorporation ranged from 70% to over 91% (16-19). In contrast to the insertion of selenocysteine through genetic code manipulations, the cysteine auxotroph approach lends itself to recombinant protein expression. However, seleno-hemoprotein expression in the cysteine auxotroph is subject to the following limitations: (1) the availability of appropriate cell strains; (2) inhibition of cell basal expression under the selenocysteine incorporation protocol; and (3) non-specific replacement of all cysteine residues in the protein. The approach is therefore most appropriate for well-expressed proteins with one or two cysteine residues. The hHO-1 H25C mutant satisfies these conditions, as Cys-25 is the only cysteine residue in a well-expressed protein.

Here we report expression of the selenocysteine-substituted hHO-1 H25C mutant in an *E. coli* cysteine auxotroph and its characterization. The selenocysteine in this protein was found under reducing conditions to form a covalent bond with a vinyl group of the heme, presumably *via* a process that involves generation of the selenyl radical by electron transfer to the heme iron atom and addition of the resulting selenyl radical to a heme vinyl group.

2. Materials and methods

2.1. Chemicals

Amino acids and *L*-selenocystine were purchased from Sigma-Aldrich. Tri(2-carboxyethyl) phosphine (TCEP) was purchased from Molecular Probes (Eugene, OR). Mesoheme was from Frontier Scientific, Inc. (Logan, Utah). Unless specified, all other reagents and biochemicals were purchased from Fisher Scientific. Water was purified with a Milli-Q purification system (Millipore).

2.2. Gene construction

The gene sequence coding for a 6×His-tag was attached to the *hHO-1* H25C gene C-terminus by PCR. The PCR was carried out using a pBac/*hHO-1*/H25C plasmid as a template and the primers 5'-

CAGGAAACAGGATCCATCGATGCTTAGGAGGTCATATGGAGCGTCCGCAA
CCCGAC-3' and 5'-

CTGCAGGTCGACAAGCTTTTAATGGTGATGGTGATGGTGAGCCT

GGGAGCGGGTGTG-3'. The underlined letters in the downstream primer encode a C-terminal 6×His tag. The PCR product was purified by 1% (w/v) agarose gel electrophoresis, digested directly using *Nde* I and *Hind* III (New England Biolabs), and ligated into a similarly cut pET21a(+) vector (Invitrogen) to give a new plasmid. The resulting product was transformed into *Escherichia coli* DH5α cells for ampicillin screening and the resulting construct was sequenced.

2.3. Cell strain and media

The cysteine auxotrophic *E. coli* host cell BL21(DE3) *selB::kan cys51E* (referred to as BL21 (DE3)*cys*) was kindly provided by Dr. Marie-Paule Strub (National Institute of Health, Bethesda, MD) (16,18). The cysteine biosynthetic pathway in this cell type is blocked by a mutation in the *cysE* gene encoding for a serine O-acetyltransferase (16). Competent cells were prepared by a modified RbCl₂ method. The cell growth medium (1 L) was prepared according to the literature with slight modifications (18). A solution containing NaOAc (1 g), NH₄Cl (2 g), K₂HPO₄ (10 g), sodium succinate (2.7 g), glycerol (8 mL), and Milli-Q water (800 mL) was autoclaved and cooled to room temperature. The amino acid (–Cys) solution (200 mL) contained Ala, Arg, Gln, Glu, Gly, Ser (400 mg ea.), Asp, Met (250 mg ea.), Asn, His, Ile, Leu, Lys, Pro, Thr, Tyr (100 mg, ea.), and Phe, Trp, Val (50 mg ea.). The amino acid solution was autoclaved separately and then added to the buffer solution. The medium was then supplemented with the following filter sterilized solutions: Cys (50 mg), MgSO₄ (2 mM), CaCl₂ (0.1 mM), biotin (0.5 mg), nicotinamide (100 mg), thiamine (50 mg), and a trace element solution (1 mL) containing H₃BO₃ (40 μM), CoCl₂ (3 μM), CuCl₂ (0.1 μM), MnCl₂ (8 μM), ZnCl₂ (1 μM), and FeCl₃ (10 μM). The cell expression medium (1 L) was prepared in the same manner except that cysteine was not added and MgSO₄ was replaced by Mg(OAc)₂ (3 mM). The cell wash buffer (1 L) contained NaOAc (1 g), K₂HPO₄ (10 g), and Milli-Q water (1 L). The solution was autoclaved and pre-chilled to 4 °C before use.

2.4. Expression of the heme-bound hHO-1 H25C and H25SeCys proteins

Cell expression was carried out on a 1 L scale in a 2.8 L-Fernbach flask. The BL21(DE3)cys competent cells were transformed with the pET21a/hHO-1/H25C gene and a single colony was selected for overnight growth in LB medium (50 mL) containing ampicillin (100 mg/L), kanamycin (50 mg/L), and Cys (50 mg/L) at 37 °C with shaking at 220 rpm. The overnight culture was centrifuged at $5,000 \times g$ for 5 min at 4 °C and the pellet was resuspended in the cell growth media (1 L) supplemented with Cys (50 mg/L). The culture was grown at 37 °C with shaking at 220 rpm. At OD_{600 nm} of ~1.5 (after about 5 h), isopropyl β -D-1-thiogalactopyranoside (IPTG) (1 mM) was added, followed by chloramphenicol (10 μ g/mL) 10 min later. After 5 min, the cells were cooled on ice and sedimented in sterilized 1 L-bottles by centrifugation at $5,000 \times g$ for 15 min at 4 °C. The cell pellet was washed twice with pre-chilled cell wash buffer (1 L) and resuspended in the expression medium (1 L) supplemented with L-selenocystine (100 mg) for the hHO-1 H25SeCys mutant or Cys (50 mg) for the H25C mutant, rifampicin (400 mg), and hemin (15 mg). The expression was induced by adding IPTG (0.5 mM) and continued at 37 °C with shaking at 220 rpm for 12-14 h. The cells were then harvested, washed twice with ice-cooled phosphate buffer (20 mM, pH 7.5, 1 mM EDTA), and stored at -80 °C. The wet cell pellet weighed 4-5 g.

2.5. Purification of the heme-bound hHO-1 H25C and H25SeCys

The frozen cell pellet was thawed on ice and resuspended in 10 mL of buffer A (50 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl, 10% (v/v) glycerol, 20 mM imidazole, and 2 mM phenylmethylsulphonyl fluoride (PMSF) containing 5 mM β -mercaptoethanol. After addition of lysozyme (1 mg/mL), the cell suspension was stirred for 30 min at 4 °C. The cells were disrupted by sonication using a Branson sonicator (3 $\times$ 2-min bursts at 50% power, with 2 min cooling on ice between each burst). Cell debris was removed by centrifugation at $100,000 \times g$ for 1 h at 4 °C. The supernatant solution was loaded onto a 5-mL HisTrap HP column (GE) equilibrated with buffer A. The column was washed with buffer A and the protein was eluted with buffer B (same as buffer A except 200 mM imidazole). Fractions of deep brownish color were collected and dialyzed at 4 °C against buffer C (50 mM sodium phosphate buffer, pH 7.4, 10% (v/v) glycerol, and 0.1 mM EDTA). The protein solution was concentrated to >200 μ M using an Amicon Ultra 10,000 Da centrifugal filter (Millipore). A fraction of the protein was further purified by fast protein liquid chromatography (FPLC) (Amersham) using a Superdex HR75 size-exclusion column (isocratic gradient of 50 mM phosphate buffer, pH 7.5, 150 mM NaCl, and 0.1 mM EDTA). The fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and those showing hHO-1 H25SeCys (or H25C) band were pooled and concentrated as described above.

For purification under anaerobic conditions, buffers A, B, and C were prepared in the glovebox according to the description in section 2.10. After cell disruption and ultracentrifugation, the protein solution was transferred into the glovebox and the purification proceeded as described above except that the FPLC purification was not done.

2.6. Expression and purification of the hHO-1 H25SeCys apoprotein

The hHO-1 H25SeCys apoprotein was expressed in the same manner as described above for the heme-bound hHO-1 H25SeCys with the following modifications: (a) Both the cell growth and expression media were stirred with Chelex-100 resin (10 g/L) for 2 h to remove the traces of iron in the media (20) and the resin was removed by passage through a 0.2 μ m membrane (Millipore) prior to autoclaving; (b) FeCl₃ was not added to the media; and (c) hemin was not added in the expression medium.

The apoprotein was purified in the same manner as described above for the heme-bound hHO-1 H25SeCys with the following modifications: (a) Ellman's reagent (10 mM) was added to the

cell lysis buffer; (b) The dialysis buffer was 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA); and (c) After dialysis, the protein solution was loaded onto a QA-52 column (Whatman) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA. The protein was eluted with a linear gradient of 0 - 100 mM KCl in the same buffer. The fractions were analyzed by SDS-PAGE and those showing the hHO-1 H25SeCys band were pooled and concentrated as described above. The purified protein was the hHO-1 H25SeCys-TNB adduct in which the selenocysteine was modified by addition of TNB (5-thio-2-nitrobenzoic acid).

The following experiments were conducted in the glovebox. Typically, a solution of the hHO-1 H25SeCys-TNB adduct (0.3 nmol) in 2.5 mL of 10 mM potassium phosphate buffer (pH 7.4) was treated with *tris*-(2-carboxyethyl)phosphine (TCEP, 10 mmol) at room temperature for 30 min. The solution was then loaded onto a PD-10 column equilibrated with 10 mM potassium phosphate buffer (pH 7.4). The protein was eluted with 3.5 mL of the same buffer and the yellow TNB fraction was eluted with another 5 mL of buffer. The amount of TNB was estimated by measuring the optical absorbance at 410 nm ($\epsilon_{410\text{ nm}} = 0.19\text{ mM}^{-1}\text{cm}^{-1}$).

2.7. Reconstitution of the hHO-1 H25SeCys apoprotein with heme or mesoheme

Reconstitution and purification of the hHO-1 H25SeCys heme complex followed previously described protocols (21,22). Briefly, the hHO-1 H25SeCys apoprotein was incubated with heme or mesoheme (1.5 equiv) in 10 mM phosphate buffer (pH 7.4) at room temperature for 3 h. The protein solution was then loaded onto a hydroxyapatite column (Biorad). After the column was washed with 10 mM phosphate buffer (pH 7.4), the protein was eluted with a linear gradient of 10 - 100 mM phosphate buffer (pH 7.4).

2.8. Expression, purification, and heme reconstitution of the hHO-1 H25C apoprotein

Expression and purification of the hHO-1 H25C apoprotein from *E. coli* DH5 α cells followed a published protocol except that the cell incubation temperature was changed from 30 °C to 37 °C (11,21). Expression of the hHO-1 H25C apoprotein from the BL21(DE3)cys cells was carried out in the cell growth medium (+Cys) as described in section 2.3 and cells were induced by adding IPTG (0.5 mM) at OD₆₀₀ of ~1.0, followed by expression at 37 °C for 12-14 h. The protein was purified according to the same procedure as described in section 2.6 except that Ellman's reagent was not added to the cell lysis buffer. Reconstitution of the hHO-1 H25C apoprotein with heme and the following purification of the complex were done by the same procedure as described in section 2.7.

2.9. Optical absorption spectroscopy

Optical spectra were recorded on a CARY UV-visible scanning spectrophotometer (Varian) in 100 mM potassium phosphate buffer (pH 7.4) at 23 °C. The hHO-1 H25C concentration was estimated by the Bradford method or from the extinction coefficient ($140\text{ mM}^{-1}\text{cm}^{-1}$) of the Soret absorption maximum of the wild-type heme-hHO-1 complex (21). Formation of the ferrous CO complex was achieved by bubbling CO gas (Airgas, CA) into the ferric enzyme solution for approximately 30 s through a septum-sealed cuvette prior to the injection of 1 mM sodium dithionite using a gas-tight syringe (Hamilton, Reno, NV). The reduced pyridine hemochromogen assay was carried out according to the method in the literature (23).

2.10. Anaerobic techniques

Anaerobic experiments were done in a glovebox (Unilab, Mbraun Inc., Stratham, NH). The buffer was boiled for 5 min, cooled to <50 °C while bubbling argon through the solution, and then further cooled to room temperature with stirring in the glovebox overnight before use. The oxygen level in the glovebox was monitored by an oxygen sensor and was less than 2 ppm.

UV-visible spectra were recorded on a UV-visible scanning spectrophotometer (Agilent 8453) in the glovebox.

2.11. Covalent heme binding in the hHO-1 H25SeCys heme complex

Covalent heme attachment was observed when the HisTrap column-purified heme-bound hHO-1 H25SeCys was incubated with β -mercaptoethanol (100 mM) under anaerobic conditions. The buffer was 50 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl, 10% (v/v) glycerol, 200 mM imidazole.

Formation of the covalent heme adduct of the reconstituted hHO-1 H25SeCys heme complex was examined in the glovebox. Equal samples of the hHO-1 H25SeCys heme (or mesoheme) complex ($\sim 10 \mu\text{M}$) in 100 mM phosphate buffer (pH 7.4) containing 5 mM DTT (dithiothreitol) were incubated in parallel with each of the following reagents: (a) water; (b) sodium dithionite (1 mM); (c) imidazole (100 mM); and (d) sodium dithionite (1 mM) and imidazole (100 mM). The parallel samples were incubated at room temperature for from 4-14 h and analyzed by HPLC at different time intervals to quantitate covalent heme adduct formation.

2.12. HPLC analysis of covalent heme

HPLC analysis was performed with an Applied Biosystems Poros R2 C₁₈ column (4.6 $\times$ 150 mm, 10 μm) fitted with a guard column. Solvent A was water containing 0.1% trifluoroacetic acid, and solvent B was acetonitrile containing 0.1% trifluoroacetic acid. The gradient program consisted of linear segments with 30% B (0-3 min), from 30% to 50% B (3-8 min), 50% B (8-9.5 min), from 50% to 95% B (9.5-13 min), 95% B (13-18 min), from 95% to 30% B (18-20 min), and 30% B (20-22 min) at a flow rate of 1.5 mL/min. The eluent was monitored at 280 and 400 nm.

2.13. Mass Spectrometry

For peptide analysis in solution digestion was carried out with trypsin at 37 °C for 4 h. Prior to the digestion the protein was denatured in 25% acetonitrile, at 56 °C. The peptides were fractionated on a reversed phase nanocolumn (C₁₂, 75 μm $\times$ 15 mm). The HPLC system was a Dionex Ultimate with Famos autosampler. The flow rate was $\sim 300 \text{ nL/min}$. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. The column was equilibrated with 5% solvent B. A linear gradient was then developed up to $\sim 35\%$ B in 30 min. The eluting peptides were analyzed by LC/MS/MS on a QqTOF mass spectrometer (QSTAR XL, Applied Biosystems) in a data-dependent fashion: 1 sec surveys were followed by 3 sec CID experiments on computer-selected multiply charged ions. ProteinProspector v4.25.2 was used for data analysis.

Analysis of the selenocysteine-containing peptide-heme adducts by MALDI-TOF-TOF MS (matrix-assisted laser desorption ionization-time-of-flight tandem mass spectrometry) was carried out as follows. A 1 μL aliquot of the peptide/matrix mixture was spotted onto a MALDI target plate using the drying droplet method. The 4700 mass analyzer (Applied Biosystems) was calibrated in the range of 500-4500 Da with a peptide mass calibration kit (Sigma), used according to the manufacturer's instructions. Data were collected in the same mass range using an average of at least 100 laser shots. Spectra were analyzed using Data Explorer software (Applied Biosystems).

For intact protein mass measurements, the samples were diluted from the nominal concentration to $\sim 5 \mu\text{M}$ by addition of 0.1% formic acid in water. A 5 pmol aliquot of the protein was loaded onto a Phenomenex Jupiter C4 column for 10 min at a flow rate of approximately 0.5 $\mu\text{L/min}$ at a mobile phase concentration of 5% B (mobile phase A = 0.1% formic acid in H₂O, mobile phase B = 0.1% formic acid in acetonitrile). The organic content

was then raised to 65% B over 3 min and held at 65% B for an additional 12 min. The eluent from the column was directed into a QqTOF (quadrupole-orthogonal acceleration-time-of-flight) hybrid tandem mass spectrometer (QSTAR-XL, Applied Biosystems/MDS Sciex). MS spectra were acquired from m/z 500-1700. Charge state envelopes were deconvoluted using the Bayesian Protein Reconstruct algorithm within the BioAnalyst 2.0 suite of programs (Applied Biosystems/MDS Sciex).

3. Results

3.1. Expression and purification of the hHO-1 H25SeCys mutant

The hHO-1 H25SeCys mutant was expressed using the pET21a/hHO-1 H25C gene transformed BL21(DE3)*cys* cysteine auxotrophic strain of *E. coli*. The expression process included cell growth in a cysteine-containing medium, cysteine wash-out, and subsequent overexpression of the desired protein in cells suspended in a selenocysteine-containing medium (16-19). The selenocysteine added to the cell expression medium was in the oxidized (CysSe-SeCys) state, as the reduced form is toxic to the cell and is not stable in air. The ability of the cell redox system to reduce the diselenide bond was recently confirmed (24). The hHO-1 H25SeCys mutant was expressed either as a heme complex or as the apoprotein by adding or removing hemin and the iron source in the culture media, respectively.

Heme-bound hHO-1 H25SeCys and the corresponding apoprotein were purified by two different protocols. The heme-bound H25SeCys was purified by nickel column affinity chromatography followed by FPLC on a size-exclusion column. The complex was found to be sensitive to air during the purification process. When the purification was conducted in the absence of reducing agents, the optical purity ratio (Reinheitzahl or R_z , defined as $A_{\text{Soret}}/A_{280\text{ nm}}$) for the complex was found to be 0.5, nearly 4-fold lower than that of the heme-bound hHO-1 H25C mutant ($R_z = \sim 1.8$). The low R_z value indicated that most of the protein did not contain heme. When the purification was conducted in the presence of β -mercaptoethanol, the R_z for the protein was initially ~ 1.3 , but this value gradually decreased under aerobic conditions. The low or decreasing R_z value for the protein presumably reflects degradation of the heme, presumably by hydrogen peroxide that is generated by oxidation of reducing agents in the buffer. These results indicate that the heme-bound hHO-1 H25SeCys is sensitive to oxidative conditions. This is supported by the observation that the R_z of the protein was stable with time when the purification was conducted in the glovebox under anaerobic conditions (Fig. 1). The protein yield, ~ 20 mg per liter of culture, was comparable to that of the wild-type hHO-1 (~ 35 mg/L) and significantly higher than that of the H25C mutant (2-3 mg/L) when expressed in DH5 α cells using a pBAce vector (11,21).

The hHO-1 H25SeCys apoprotein was purified with Ellman's reagent (5,5'-dithiobis(2-nitrobenzoate, DTNB) in the cell lysis buffer. Ellman's reagent was added to protect the selenocysteine residue in the protein from being oxidized by converting it to a selenenyl sulfide that could be cleaved subsequently by reduction. The same strategy was used previously in the purification of selenoGADPH (17). The hHO-1 H25SeCys-TNB adduct was purified by nickel column affinity chromatography followed by passage through a QA-52 anion exchange column. The purified protein had an absorption maximum at 340 nm attributable to the selenocysteine-TNB adduct (Fig. 2). Formation of the hHO-1 H25SeCys-TNB adduct, confirmed by mass spectrometry (see below), indicated that the selenocysteine residue in the expressed protein was in the reduced state. The purified apoprotein yield (~ 12 mg per liter of culture) was lower than that of the heme-bound hHO-1 H25SeCys, possibly as a result of the inclusion of an additional purification step and/or the effect of iron-depletion in the media.

3.2. Reconstituted hHO-1 H25SeCys heme complex

The reconstituted hHO-1 H25SeCys heme complex was obtained by reducing the hHO-1 H25SeCys-TNB adduct followed by reconstitution of the protein with heme. Reductive cleavage of the TNB adduct was achieved by anaerobically incubating the hHO-1 H25SeCys-TNB protein with TCEP in the glovebox. The completeness of the reaction was indicated by a shift of the absorption maximum from 340 nm to 325 nm (Fig. 2). The reaction was complete within minutes. The apoprotein and TNB in the buffer were then separated on a size-exclusion Pd-10 column, providing a protein fraction with an absorption maximum at 280 nm and a fraction of yellowish TNB with an absorption maximum at 410 nm. The identity of the eluted TNB was confirmed by comparing its UV-visible spectrum and HPLC retention time with those of an authentic standard. The molar ratio between the protein and TNB was ~1:1, suggesting that the selenocysteine residue in the hHO-1 H25SeCys was quantitatively modified by TNB. Reconstitution of the hHO-1 H25SeCys apoprotein with heme followed a previous protocol except that the reconstitution was conducted anaerobically in the glovebox (22,24). The reconstituted hHO-1 H25SeCys heme complex after hydroxyapatite column purification had an R_z of 1.8, the same as that of the reconstituted hHO-1 H25C heme complex.

3.3. Purity of the hHO-1 H25SeCys protein by SDS-PAGE

The purity of the hHO-1 H25SeCys protein was examined by SDS-PAGE. Interestingly, in contrast to the hHO-1 H25C mutant, the SDS-PAGE gel of the hHO-1 H25SeCys protein showed not only a monomer band of ~31 KDa, but also a dimer band of ~62 KDa despite the fact that the samples loaded onto the gel had been boiled with the reducing agent DTT (Fig. 3). The monomer and dimer bands were of roughly the same intensity. This result was observed for both the heme-bound H25SeCys protein and the H25SeCys apoprotein. Under the same conditions, the hHO-1 H25C mutant exhibited only a monomer band. Replacing the DTT with TCEP in the sample loading buffer gave the same result. Trypsin digestion and LC/MS analysis of both the hHO-1 H25SeCys monomer and dimer bands revealed that both bands contained a selenocysteine-containing peptide (see below). However, the monomer band contained both the corresponding cysteine and selenocysteine-peptides, whereas the dimer band only contained the selenocysteine-peptide. FPLC of the hHO-1 H25SeCys protein on a Superdex-75 size-exclusion column gave a single protein peak with the same retention time as the H25C protein, indicating that the H25SeCys protein was entirely monomeric (data not shown). These results indicate that the H25SeCys dimer observed by SDS-PAGE was generated as the protein was denatured and was due to oxidative formation of the difficultly reducible Se-Se bond between the selenocysteine residues in two denatured protein molecules.

3.4. Mass spectrometric analysis of the hHO-1 H25SeCys protein

Selenocysteine incorporation into the hHO-1 H25SeCys protein was confirmed by mass spectrometric analysis of both the intact protein and its peptide digests. When hHO-1 H25SeCys in buffer was digested with trypsin in the absence of a reducing agent, the selenocysteine-containing peptide (EVSeCysTQAENAEFMR) was found as a dimer with the methionine also oxidized to a sulfoxide. As shown in Fig. 4, the mass spectrum of the identified peptide matches perfectly both the calculated quadruple-charged mass, m/z 796.05 (the most abundant isotopic ion, not the monoisotopic ion) and the theoretical distribution based on natural isotopic abundance of chemical elements. The identity of this peptide was also verified by CID analysis (data not shown). The corresponding cysteine-containing peptide was not found in the digest mixture. Observation of the selenocysteine-peptide as a dimer is consistent with the observation of a dimer band on SDS-PAGE (Fig. 3). Both the hHO-1 H25SeCys monomer and dimer bands on the SDS-PAGE gel were cut from another gel, reduced, and alkylated with iodoacetamide, and were digested for LC/MS analysis. Other than the expected alkylated selenocysteine-peptide, the same peptide was also found with the selenocysteine

residue converted into a dehydroalanine. The formation of these peptides can be attributed to selenium elimination from the selenocysteine residue during the sample preparation protocol (25). While both cysteine and selenocysteine-containing peptides were found in the monomer band, only the selenocysteine-containing peptide was found in the dimer band. The ratio between the cysteine and selenocysteine-containing peptides in the monomer band could not be determined from the mass spectrometric data.

Selenocysteine incorporation into hHO-1 H25SeCys was confirmed and the incorporation yield was estimated by electrospray mass spectrometry (ESIMS) of the hHO-1 H25SeCys TNB adduct. The deconvoluted molecular mass of the hHO-1 H25SeCys-TNB was $31,336 \pm 3$ Da (Fig. 5), in complete agreement with that predicted for the adduct (31,336). The hHO-1 H25C-TNB adduct was not detected, as shown by the mass spectrometric raw data (Fig. 5 inert), which indicates that the SeCys containing mutant accounted for >95% of the total hHO-1 protein.

3.5. UV-visible spectroscopic analysis of the hHO-1 H25SeCys heme complex

The ferric heme-bound hHO-1 H25SeCys protein obtained from the cell expression system had a Soret maximum at 402 nm, a weak Q-band at ~532 nm, and a CT band at 620 nm (Fig. 1). Sodium dithionite reduction yielded the ferrous protein with a Soret maximum at 430 nm and Q-bands at 556 and 582 nm. The ferrous CO-bound form had a Soret maximum at 420 nm and Q-bands at 537 and 566 nm. Compared to the heme-bound H25SeCys protein obtained directly from the expression system, the reconstituted H25SeCys-heme complex had a broader UV-vis spectrum with a Soret maximum at 390 nm, blue-shifted by ~12 nm from that of the directly expressed heme complex (Fig. 1). However, the reconstituted H25SeCys heme complex had the same Soret maxima in the ferrous and ferrous-CO forms as the directly expressed heme-bound H25SeCys protein.

After heme reconstitution the hHO-1 H25C cysteine mutant expressed previously with a pBAce vector in DH5 α cells had a Soret maximum at 385 nm. The thiolate-iron ligation in the H25C heme complex was confirmed by resonance Raman spectroscopy and therefore the Soret band at 385 nm is a characteristic feature of the thiolate-ligated ferric complex (11). Compared to the H25C heme complex, the heme-bound H25SeCys protein exhibited a 17 nm red shift for the cell-expressed form and a 5 nm shift for the reconstituted form. Upon sodium dithionite reduction, both the H25C mutant and the two H25SeCys mutants had Soret maxima at 430 nm. The ferrous-CO forms of both the H25SeCys mutant proteins had Soret maxima at 420 nm, about 8 nm red-shifted from that of the H25C heme complex at 412 nm.

As the hHO-1 H25C mutant was obtained from a different expression system than the hHO-1 H25SeCys mutant, the Soret shift of the H25SeCys protein relative to H25C could stem from this difference in the expression systems. To examine this possibility, both the heme-bound hHO-1 H25C protein and the H25C apoprotein were expressed in BL21(DE3)*cys* under the same expression conditions as used for the heme-bound hHO-1 H25SeCys mutant and the H25SeCys apoprotein, respectively, except that the selenocysteine in the medium was replaced by cysteine. The UV-visible spectrum of the heme-bound hHO-1 H25C protein from the BL21(DE3)*cys* cells was found to be identical to that of the heme-bound H25SeCys mutant, both proteins having the same Soret maxima for the ferric, ferrous and ferrous-CO forms (data not shown). Interestingly, the Soret maxima of the hHO-1 H25C and H25SeCys heme complexes obtained from the BL21(DE3)*cys* expression system are similar to that of the proximal cavity mutant hHO-1 H25A, which has a Soret maximum at 400 nm (26). We therefore conclude that heme in these two complexes from BL21(DE3)*cys* cells was not ligated by the proximal Cys or SeCys ligand and the red shift of the Soret maximum of the hHO-1 H25SeCys heme complex from that of the hHO-1 H25C protein at 385 nm from DH5 α cells is due to a difference in the expression systems. However, the reconstituted hHO-1 H25C heme complex was found to

contain two fractions of proteins that were partially separated by chromatography on a hydroxyapatite column, one with a Soret maximum at 385 nm and the other at 410 nm (Data not shown). The presence of two fractions of proteins was also previously observed for other reconstituted hHO-1 His132 mutants (27). The former had the same Soret maxima as the reconstituted hHO-1 H25C heme complex from the DH5 α cells. Therefore, the ~5 nm red shift of the reconstituted hHO-1 H25SeCys heme complex from the H25C 385 nm species was not related to the expression system but could be a result of the replacement of Cys with SeCys at the protein active site. However, this small UV absorption change was found negligible when the UV-vis spectra of the two proteins were superimposed on each other, particularly because the absorption bands of both proteins around the Soret maxima were very broad.

3.6. Covalent binding of the heme in the hHO-1 H25SeCys heme complex

During purification of the heme-bound hHO-1 H25SeCys protein, we discovered that the heme was covalently bound to the protein after elution of the protein from the nickel column in the presence of β -mercaptoethanol. The UV-visible spectrum of the covalent heme hHO-1 H25SeCys complex had a narrower absorption peak with a Soret maximum at 402 nm, ~1 nm red-shifted from that of the non-covalently bound hHO-1 H25SeCys heme complex (Fig. 6). The Soret difference between the two complexes can be amplified by the binding of imidazole, which results in a red-shift of ~3 nm for the covalent heme complex relative to the non-covalently bound complex (Fig. 6 insert).

Covalent attachment of the heme to the protein in the hHO-1 H25SeCys protein was unambiguously confirmed by several methods previously used to characterize proteins with a covalently-bound heme group (28,29). First, acidified butanone did not extract the heme from the protein, in contrast to a control experiment which showed that the heme was completely removed under the same conditions from the H25C protein. Second, HPLC analysis showed coelution of the heme (monitored at 400 nm) with the protein (monitored at 280 nm) at 7.5 min for the H25SeCys protein, whereas the heme in the H25C protein was released from the protein and eluted earlier than the protein peak (Fig. 7). Coelution in this HPLC system has been used to demonstrate covalent heme attachment in several covalently linked heme proteins (30-32). Third, the reduced pyridine-hemochromogen assay of the protein showed a spectrum with maxima at 550 and 520 nm, ~5 nm blue-shifted relative to those of H25C (Fig. 8). These spectral changes are diagnostic of a reaction in which one of the heme vinyl groups is converted to a saturated alkyl substituent (28,33).

Mass spectrometry provided further evidence that the heme prosthetic group in hHO-1 H25SeCys was covalently linked to the protein. MALDI-TOF mass spectrometry of the tryptic digest of H25SeCys hHO-1 yielded a peptide with a mass of 2190.80 Da that matched both the calculated mass and the isotopic pattern for the EVSeCysTQAENAEFMR sequence with a covalently bound heme. The same peptide with an oxidized methionine residue was also detected (2206.80 Da) (Fig. 9). When this peptide was selected for Collision-Induced dissociation (CID) analysis in a MALDI-TOF-TOF mass spectrometer (4700 Mass Analyzer, Applied Biosystems), prominent ion-signals due to heme were observed, as well as a y-ion series (34) confirming the identity of the peptide (Fig. 9B). This confirms that the precursor peptide contains a heme adduct, which is not retained during CID conditions.

The conditions for formation of the covalent heme adduct in heme-bound hHO-1 H25SeCys were investigated with the reconstituted H25SeCys heme complex. Since formation of the covalent heme adduct was observed during nickel column purification of the H25SeCys heme complex, the components of the elution buffer were examined. Incubation of the heme complex under a series of conditions indicated that both a reducing agent (DTT or BME) and imidazole were required for covalent heme attachment. No covalent heme formation was observed for the H25C heme complex treated under the same conditions. This result indicates that the

selenocysteine is responsible for the formation of the covalent heme adduct in the heme-bound hHO-1 H25SeCys. Interestingly, no covalent heme attachment was observed when the protein was incubated with H₂O₂. This is consistent with previous observations that reducing conditions were required for covalent heme attachment in other proteins (28,35). To provide further information on the site of covalent attachment to the heme, the hHO-1 H25SeCys apoprotein was reconstituted with iron (III) mesoporphyrin (mesoheme) in which ethyl groups replace the 2- and 4-vinyl groups of normal heme. Based on the HPLC analysis and the pyridine hemochromogen assay (Data not shown), the mesoheme-bound H25SeCys did not form a covalent heme product under conditions that gave the normal covalent heme adduct. This result, together with the shift in the absorbance maximum of the pyridine hemochromogen spectrum, indicates that heme in the covalent heme hHO-1 H25SeCys complex is attached to the protein through one of its vinyl groups.

4. Discussion

We have investigated the incorporation of selenocysteine as the proximal ligand in the hHO-1 H25C mutant, a protein in which Cys-25 is the only cysteine residue, in order to (a) clarify the role of the proximal ligand in this enzyme system, and (b) characterize the interactions of a selenolate ligand with a heme iron atom. While selenoproteins are widely distributed in nature, a hemeprotein with a selenocysteine as the proximal ligand has not been reported in the refereed literature, although such a protein was cursorily reported in an abstract in 2006 (36). Selenocysteine is unique as a potential iron ligand because of (a) its lower pK_a, which means the thiolate form is the dominant one at physiological pH, (b) its greater electron donating properties, and (c) a size similar to that of cysteine itself.

To prepare the selenocysteine-ligated hHO-1 protein, we expressed the hHO-1 H25SeCys mutant in an *E. coli* cysteine auxotroph in the presence of selenocysteine. This cysteine auxotroph expression system was previously shown to allow the efficient replacement of a cysteine by a selenocysteine (16-19). Mass spectrometric analysis showed >95% incorporation of the selenocysteine into the protein, which was obtained in a yield comparable to that of the cysteine-containing protein. This extent of selenocysteine incorporation is on the high end relative to the previously reported 73% to over 91% incorporation into other proteins (16-19). A high level of selenocysteine incorporation was consistent with our identification of the selenocysteine-, but not cysteine-containing peptide in the mixture generated by trypsin digestion of the hHO-1 H25SeCys protein.

The observation that the hHO-1 H25SeCys protein migrated as both a monomer and a dimer on SDS-PAGE may stem from the presence of only one such residue in the protein, as this precludes the formation of internal diseleno or seleno-sulfide crosslinks. The dimer, however, is an artifact that results from denaturation of the protein when the sample is prepared for SDS-PAGE or mass spectrometric analysis, as FPLC indicates that the intact protein is entirely monomeric. The dimer is observed in the SDS-PAGE gel because the intermolecular Se-Se bond formed when the protein is denatured is not readily reduced by the β -mercaptoethanol in the electrophoresis mixture. LC/MS analysis of the tryptic peptides from the dimer band unambiguously confirms that the dimer derives entirely from the selenocysteine-containing protein.

Although the incorporation of selenocysteine in a properly folded protein is clearly established by our results, it is unclear whether the selenocysteine is stably coordinated to the heme iron in the hHO-1 H25SeCys mutant. The heme-bound hHO-1 H25SeCys and H25C mutants produced by the cysteine auxotroph system supplemented with selenocysteine or cysteine, respectively, have nearly identical UV-vis spectra in the ferric, ferrous, and ferrous-CO states. These spectra differ, however, from the spectra obtained earlier by expression of the hHO-1

H25C mutant using a pBAce vector in DH5 α cells and more closely resemble those of the proximal cavity hHO-1 H25A mutant (26). It is clear that the selenocysteine does not coordinate to the heme iron as a selenolate in the ferrous state, as a Soret maximum in the range of 450 nm would be expected from such coordination. The absence of this spectrum and its similarity to that of the H25A mutant, together with the fact that the ligand should be present as a selenolate due to its low pKa, clearly suggests that the selenocysteine is not coordinated to the iron in the ferrous state in the isolated protein. The coordination state in the ferric enzyme is more difficult to establish. Although the Soret maxima of the reconstituted hHO-1 H25SeCys and H25C heme complexes showed a small ~5 nm difference, the spectra of the two proteins were nearly identical when superimposed on each other, particularly because their absorption bands were very broad. Both mutants also have nearly identical UV-vis spectra in the ferrous and ferrous-CO states, indicating the absence of a selenolate ligand in the reconstituted hHO-1 H25SeCys heme complex in the ferrous state. In previous work we demonstrated that the cysteine sulfur did coordinate to the ferric heme iron in the H25C mutant, but dissociated in the ferrous state. This may also be true of the selenocysteine ligand, but the spectra of the ferric complexes are not sufficiently differentiated to unambiguously assert this.

We recently reported formation of a complex of the proximal hHO-1 H25A cavity mutant with an external benzeneselenolate anion as the iron ligand (37). Interestingly, in this complex, the selenium transfers an electron to the heme iron atom, as demonstrated by partial formation of a ferrous-CO complex in the absence of other reducing agents. If the more electron donating benzylselenolate is introduced as the ligand, the conversion to the ferrous-CO complex is quantitative. In the presence of oxygen, the iron undergoes autoxidation, a reaction that oxidizes and consumes the external selenolate ligand, and the iron eventually reverts to the ferric state. Similar electron transfer to the iron does not occur when thiophenol is the externally provided ligand. Thus, the higher electron donating properties of the selenolate ligand result not only in coordination but also electron transfer from the selenium to the iron.

In the present situation, where the selenium ligand was from a selenocysteine residue in the protein sequence, electron transfer also apparently occurs (Scheme 1). However, in the H25SeCys protein, the selenyl radical that is formed adds to a vinyl group of the heme, resulting in covalent attachment of the heme to the protein. The addition clearly occurs at the vinyl group, as no covalent attachment is observed when the protein is reconstituted with mesoheme (ethyl instead of vinyl substituents) rather than heme. Based on the pyridine hemochromogen spectroscopic shift and the molecular mass of the tryptic peptide with the covalently bound heme, the vinyl group gives rise to a saturated bond linking the heme to the selenium, implying that a hydrogen atom is also incorporated into the product. Similar treatment of the normal H25C protein with a cysteine ligand does not result in covalent binding of the heme, demonstrating that this process is specifically a property of the selenocysteine ligand.

We have previously reported that reaction of ferrous hHO-1 with CBrCl₃ produces a CCl₃ radical that adds to one of the heme vinyl groups (30). The resulting radical adjacent to the porphyrin ring is oxidized by electron transfer to the ferric iron, giving a cation that is trapped by the proximal histidine ligand to give a His25-heme link. This finding shows that (a) the heme vinyl groups in hHO-1 can react with radical agents, and (b) the heme in the hHO-1 active site has a significant ability to move about, including to positions that place the vinyl group close to the proximal iron ligand. Covalent heme binding involving an adjacent Cys or Met mutation has been observed in other heme proteins (28,29,35). The ability of the selenolate moiety to transfer an electron to the heme iron, with consequent modification of the heme, may explain the failure to observe selenolate coordination to the heme iron in the ferrous state and therefore of a 450 nm-like absorption maximum for its CO complex.

The requirement for imidazole in formation of the heme-protein covalent bond may be due to a need for coordination of a ligand at the sixth iron site, possibly to prevent coordination of oxygen leading to autooxidation of the iron. However, we cannot exclude alternative roles, such as a donor of the hydrogen that quenches the radical expected from radical addition of the selenocysteine radical to the vinyl group.

In summary, it is clear from the present results that a hemoprotein selenocysteine iron ligand is unstable towards electron transfer to the heme iron atom, a process that can result in covalent heme binding of the heme to the protein via addition of a selenocysteine radical to a heme vinyl group. Interestingly, in work in progress we have substituted a selenocysteine for the normal cysteine in a P450 enzyme, have observed the expected Soret maximum in the 450 nm range, and have not observed the internal electron transfer seen with hHO-1 H25SeCys (unpublished results). Factors that control the redox potential balance in a P450 versus hHO-1 thus intervene in modulating the specific properties of the proximal ligand and its interaction with the iron atom.

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References

1. Ortiz de Montellano, PR.; Auclair, K. Porphyrin Handbook. Kadish, K.; Smith, K.; Guillard, R., editors. Vol. 12. Academic Press; New York: 2003. p. 183-210.
2. Unno M, Matsui T, Ikeda-Saito M. Nat Prod Rep 2007;24:553–570. [PubMed: 17534530]
3. Liu Y, Ortiz de Montellano PR. J Biol Chem 2000;275:5297–5307. [PubMed: 10681502]
4. Ortiz de Montellano, PR.; De Voss, JJ. Cytochrome P450: Structure, Mechanism, and Biochemistry. Vol. 3rd. Ortiz de Montellano, PR., editor. Kluwer Academic/Plenum Publishers; New York: 2005. p. 183-245.
5. Wilks A, Torpey J, Ortiz de Montellano PR. J Biol Chem 1994;269:29553–29556. [PubMed: 7961940]
6. Davydov RM, Yoshida T, Ikeda-Saito M, Hoffman BM, Amer J. Chem Soc 1999;121:10656–10657.
7. Li Y, Syvitiski R, Auclair K, Wilks A, Ortiz de Montellano PR, La Mar GN. J Biol Chem 2002;277:33018–33031. [PubMed: 12070167]
8. Syvitski RT, Li Y, Auclair K, Ortiz de Montellano PR, La Mar GN. J Am Chem Soc 2002;124:14296–14297. [PubMed: 12452690]
9. Sun J, Loehr TM, Wilks A, Ortiz de Montellano PR. Biochemistry 1994;33:13734–13740. [PubMed: 7947784]
10. Schuller DJ, Wilks A, Ortiz de Montellano PR, Poulos T. Nature Struct Biol 1999;6:860–867. [PubMed: 10467099]
11. Liu Y, Moënné-Loccoz P, Hildebrand D, Wilks A, Loehr TM, Mauk AG, Ortiz de Montellano PR. Biochemistry 1999;38:3733–3743. [PubMed: 10090762]
12. Jacob C, Giles GI, Giles NM, Sies H. Angew Chem Int Ed 2003;42:4742–4758.
13. Johansson L, Gafvelin G, Arnér ESJ. Biochim Biophys Acta 2005;1726:1–13. [PubMed: 15967579]
14. Atkins JF, Gesteland RF. Nature 2000;407:463–465. [PubMed: 11028985]
15. Jiang Z, Arner ES, Mu Y, Johansson L, Shi J, Zhao S, Liu S, Wang R, Zhang T, Yan G, Liu J, Shen J, Luo G. Biochem Biophys Res Commun 2004;321:94–101. [PubMed: 15358220]
16. Müller S, Senn H, Gsell B, Vetter W, Baron C, Böck A. Biochemistry 1994;33:3404–3412. [PubMed: 8136378]
17. Boschi-Muller S, Muller S, Van Dorsselaer A, Böck A, Branlant G. FEBS Lett 1998;20:241–245. [PubMed: 9845330]

18. Strub MP, Hoh F, Sanchez JF, Strub JM, Böck A, Aumelas A, Dumas C. *Structure* 2003;11:1359–1367. [PubMed: 14604526]
19. Sanchez JF, Hoh F, Strub MP, Aumelas A, Dumas C. *Structure* 2002;10:1363–1370. [PubMed: 12377122]
20. Ghiladi RA, Knudsen GM, Medzihradsky KF, Ortiz de Montellano PR. *J Biol Chem* 2005;280:22651–22663. [PubMed: 15840564]
21. Wilks A, Ortiz de Montellano PR. *J Biol Chem* 1993;268:22357–22362. [PubMed: 8226746]
22. Yoshida T, Kikuchi G. *J Biol Chem* 1978;253:4224–4229. [PubMed: 96115]
23. Antonini, M.; Brunori, E. *Hemoglobin and Myoglobin and their Reactions with Ligands*. North Holland Publishers; Amsterdam: 1971.
24. Beld J, Woycechowsky KJ, Hilvert D. *Biochemistry* 2007;46:5382–5390. [PubMed: 17419591]
25. Ma S, Caprioli RM, Hill KE, Burk RF. *J Am Soc Mass Spectrom* 2003;14:593–600. [PubMed: 12781460]
26. Wilks A, Sun J, Loehr TM, Ortiz de Montellano PR. *J Am Chem Soc* 1995;117:2925–2926.
27. Matera KM, Zhou H, Migita CT, Hobert SE, Ishikawa K, Katakura K, Maeshima H, Yoshida T, Ikeda-Saito M. *Biochemistry* 1997;36:4909–4915. [PubMed: 9125512]
28. Metcalfe CL, Daltrop O, Ferguson SJ, Raven EL. *Biochem J* 2007;408:355–361. [PubMed: 17714075]
29. Metcalfe CL, Ott M, Patel N, Singh K, Mistry SC, Goff HM, Raven EL. *J Am Chem Soc* 2004;126:16242–16248. [PubMed: 15584761]
30. Wilks A, Medzihradsky KF, Ortiz de Montellano PR. *Biochemistry* 1998;37:2889–2896. [PubMed: 9485440]
31. Colas C, Ortiz de Montellano PR. *J Biol Chem* 2004;279:24131–24140. [PubMed: 15039425]
32. Hoch U, Ortiz de Montellano PR. *J Biol Chem* 2001;276:11339–11346. [PubMed: 11139583]
33. Daltrop O, Allen JWA, Willis AC, Ferguson SJ. *Proc Natl Acad Sci USA* 2002;99:7872–7876. [PubMed: 12060734]
34. Biemann K. *Methods in Enzymology* 1990;193:886–887. [PubMed: 2074849]
35. Barker PD, Ferrer JC, Mylrajan M, Loehr TM, Feng R, Konishi Y, Funk WD, MacGillivray RTA, Mauk AG. *Proc Natl Acad Sci USA* 1993;99:6542–6546. [PubMed: 8341666]
36. Gromov, I.; Garcia-Rubio, I.; Aldag, C.; Schweiger, A.; Hilvert, D. 6th European Federation of EPR Groups Meeting Abstract, OT3; 2006.
37. Jiang Y, Ortiz de Montellano PR. *Inorg Chem* 2008;47:3480–3482. [PubMed: 18376820]

5. Abbreviations

hHO-1	human heme oxygenase lacking the membrane binding sequence
P450	cytochrome P450
CPR	cytochrome P450 reductase
SeCys	selenocysteine
TCEP	tri(2-carboxyethyl)phosphine
IPTG	isopropyl β -D-1-thiogalactopyranoside
FPLC	

fast protein liquid chromatography

SDS-PAGE

sodium dodecyl sulfate polyacrylamide electrophoresis

TNB

5-thio-2-nitrobenzoate

Fig. 1.

UV-visible spectra of the hHO-1 H25SeCys heme complex from *E. coli* in the ferric, ferrous, and ferrous-CO states, and that of the reconstituted hHO-1 H25SeCys heme complex in the ferric state. The Soret maximum absorptions were 402 nm (ferric), 430 nm (ferrous), 420 nm (ferrous-CO) for the expressed H25SeCys heme complex and 390 nm (ferric) for the reconstituted H25SeCys heme complex. These spectra were recorded under anaerobic conditions (See 2.10).

Fig. 2.

UV-vis spectra of (a) the hHO-1 H25SeCys–TNB adduct, (b) the same protein solution after reduction by TCEP (*tris*-(2-carboxyethyl) phosphine), (c) the H25SeCys apoprotein, and (d) TNB (5-thio-2-nitrobenzoate) itself. The hHO-1 H25SeCys–TNB adduct in phosphate buffer (10 mM, pH 7.4) was treated with TCEP (10 mM) for 30 min and the protein and TNB were separated by a Pd-10 column using the same phosphate buffer. The absorption maxima were 280 and 340 nm for H25SeCys–TNB, 280 nm and 325 nm for H25SeCys–TNB+TCEP, 280 nm for H25SeCys, and 410 nm for TNB.

Fig. 3. SDS-PAGE of the heme-bound hHO-1 H25C and H25SeCys (MW ~31 kDa): Lanes (from left to right): 1, See Blue Plus 2 prestained protein standards; 2, H25C (~ 1 μ g); 3, H25SeCys (~ 1 μ g); 4, H25SeCys (~ 0.5 μ g); 5, and H25SeCys (~ 0.2 μ g). Samples were prepared by boiling a mixture of the protein (5 μ L), 4 $\times$ NuPage SDS loading buffer (5 μ L), 1 M DTT (5 μ L), and H₂O (5 μ L) for 5 min at 100 $^{\circ}$ C. The solution (8 μ L) was loaded onto a 4-12% BT Novex NuPage gel. The gel running buffer was NuPage MOPS SDS running buffer.

Fig. 4. Mass spectrum of the dimerized SeCys-containing peptide, (EVSeCysTQAENAEFM(O)R)₂, of hHO-1 H25SeCys obtained by trypsin digestion in solution (quadruple-charged, indicated as 4+). The calculated mass of this peptide dimer (4+) is 796.05 amu (the most abundant isotope). The identity of this peptide was confirmed by CID analysis (data not shown).

Fig. 5.

Deconvoluted mass spectrum of the hHO-1 H25SeCys-TNB protein adduct: with a measured MW of $31,336 \pm 3$ amu (Calculated MW: 31,336 amu). The insert shows the raw data.

Fig. 6.

UV-vis spectra of hHO-1 H₂₅SeCys covalent and noncovalent heme complexes in the absence and presence (insert) of imidazole. The Soret maximum was 401 nm for the noncovalent heme complex and 402 nm for the covalent heme complex in the absence of imidazole, and 410 nm and 414 nm in the presence of imidazole, respectively.

Fig. 7. HPLC traces of the hHO-1 H25SeCys covalent heme complex (A) and the H25C non-covalent heme complex (B). The protein component is monitored at 280 nm, and the heme group at 400 nm.

Fig. 8. Pyridine-hemochromogen spectra of the non-covalent hHO-1 H25C and covalent H25SeCys heme complexes. The absorption maxima were 555 and 525 nm for H25C and 550 and 520 nm for H25SeCys.

Fig. 9.
(A) MALDI-TOF mass spectrum of the heme-containing tryptic peptides EVSeCysTQAENAEFMR and EVSeCysTQAENAEFM(O)R of hHO-1 H25SeCys and **(B)** CID spectrum of the precursor at m/z 2190.80, indicating heme incorporation. The peptide sequence indicated the observed fragmentations.

Scheme 1.

Proposed mechanism for the covalent binding of the heme group in the hHO-1 H25SeCys heme complex.