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Spectroscopic insights into axial ligation and active-site H-bonding in substrate-bound human heme oxygenase-2

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Abstract

Heme oxygenases (HOs) are monooxygenases that catalyze the first step in heme degradation, converting heme to biliverdin with concomitant release of Fe(II) and CO from the porphyrin macrocycle. Two heme oxygenase isoforms, HO-1 and HO-2, exist that differ in several ways, including a complete lack of Cys residues in HO-1 and the presence of three Cys residues as part of heme-regulatory motifs (HRMs) in HO-2. HRMs in other heme proteins are thought to directly bind heme, or to otherwise regulate protein stability or activity; however, it is not currently known how the HRMs exert these effects on HO-2 function. To better understand the properties of this vital enzyme and to elucidate possible roles of its HRMs, various forms of HO-2 possessing distinct alterations to the HRMs were prepared. In this study, variants with Cys265 in a thiol form are compared with those with this residue in an oxidized (part of a disulfide bond or existing as a sulfonate moiety) form. Absorption and magnetic circular dichroism spectroscopic data of these HO-2 variants clearly demonstrate that a new low-spin Fe(III) heme species characteristic of thiolate ligation is formed when Cys265 is reduced. Additionally, absorption, magnetic circular dichroism, and resonance Raman data collected at different temperatures reveal an intriguing temperature dependence of the iron spin state in the heme–HO-2 complex. These findings are consistent with the presence of a hydrogen-bonding network at the heme's distal side within the active site of HO-2 with potentially significant differences from that observed in HO-1.

Keywords

Heme oxygenase; Heme; Magnetic circular dichroism; Resonance Raman

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Introduction

Heme is an iron-containing cofactor necessary for a variety of biological processes [1]. It is an integral component of the O₂ transport and storage proteins hemoglobin and myoglobin [2]. Heme is also found in the cytochromes involved in the electron transport chain used for ATP synthesis. Additionally, heme serves as a cofactor in many enzymes, such as cytochromes P450, which are monooxygenases that insert oxygen into a variety of biological substrates, and peroxidases, which catalyze oxidation–reduction reactions involving peroxides [3]. Heme also plays a regulatory role as a small molecule sensor in proteins such as FixL, in which O₂ binding to heme ultimately regulates gene expression, and soluble guanylate cyclase, which is activated by binding of NO to heme [4–6]. In spite of its importance, heme is toxic when it is free in the body. Free heme is a lipophilic molecule that can intercalate in, and thus destabilize, lipid bilayers of cell and organelle membranes [7,8]. It also generates reactive oxygen species that can damage lipids, proteins, and DNA [8,9]. A heme degradation pathway exists to prevent such damage. Heme is first oxidized by heme oxygenase (HO) to produce biliverdin, along with CO and iron [10,11]. The biliverdin is subsequently reduced by biliverdin reductase to bilirubin, which is less lipophilic and thus more readily excreted.

By catalyzing the first step in heme degradation, HO plays a vital role in maintaining proper heme homeostasis. Crystal structures of human forms of the enzymes have revealed that heme binds to HO in a highly conserved pocket of hydrophobic amino acid residues [12–14]. Several residues contribute to hydrogen-bonding interactions with peripheral substituents of the heme macrocycle to correctly orient the substrate within the active site. Additionally, the heme is ligated axially by a His residue from the protein and a solvent molecule. The reaction catalyzed by HO is intriguing in that heme serves as both a cofactor and a substrate, essentially catalyzing its own degradation [15]. Catalytic mechanisms have been proposed wherein O₂ coordinates axially to Fe, replacing solvent in the distal position, and subsequently attacks the α -methine carbon of the heme macrocycle [11].

Two HO isoforms, HO-1 and HO-2, exist that catalyze the same reaction but differ in several notable ways [16]. HO-1 exhibits inducible expression, whereas HO-2 is expressed constitutively. Also, whereas HO-1 is localized predominantly in the liver and spleen, where it serves to break down excess heme, HO-2 is present at high levels in the brain, where it is thought to contribute to cell signaling by formation of CO [16–18]. Structurally, the two isoforms have very similar, though not entirely identical, heme-binding regions but otherwise exhibit a number of significant differences, including a complete lack of Cys residues in HO-1 and the presence of three Cys residues as part of heme-regulatory motifs (HRMs) in HO-2 [19]. HRMs are also found in a few other heme proteins and are thought to bind heme or to otherwise regulate protein stability and/or activity [20–27]. For instance, the yeast gene transcriptional activator HAP1 is activated by heme binding to the Cys residue in its HRM, which blocks repressor binding [20]. Also, binding of heme to an HRM in δ -aminolevulinic acid synthase, the first enzyme in heme biosynthesis, inhibits transport of this enzyme into the mitochondria [21].

At present, the roles that HRMs play with respect to HO-2 function are incompletely understood; however, it has been shown through mutagenesis studies that the HRMs are not necessary for catalysis [19]. Recently, it was proposed that the HRMs act as a so-called redox switch to regulate HO-2 heme-binding affinity [28]. Studies of various forms of HO-2 possessing distinct alterations (truncations or Cys to Ala mutations) to the HRMs provided evidence that a disulfide bond exists between the Cys residues of the two C-terminal HRMs (Cys265 and Cys282) that can be reduced to provide a free thiol(ate) group (Cys265) capable of coordinating to the heme iron. The disulfide-reduced HO-2 species were shown to have significantly lower binding affinities for Fe(III) heme than their counterparts containing intact

disulfide bonds. Further characterization of these species by absorption, magnetic circular dichroism (MCD), and resonance Raman spectroscopies presented herein confirms the presence of a Cys-ligated heme species. Additionally, these spectroscopic studies reveal an intriguing temperature dependence of the spin state of the Fe(III) ion in the heme–HO-2 complex, consistent with the possibility that, as observed in HO-1, a hydrogen-bonding network exists at the heme's distal site in HO-2, which could play an important role in the catalytic mechanism employed by this enzyme. Moreover, because this temperature-dependent behavior has only been observed in HO-2, our data suggest that small, but potentially significant, differences exist between the active sites of these two HO isoforms.

Materials and methods

Sample preparation

Samples of three HO-2 variants were used for the experiments described herein. Each variant was truncated to remove the membrane-binding region (amino acid residues 289–316) because the full-length protein is less stable and cannot be prepared at sufficiently high concentrations for these spectroscopic experiments; this truncated protein is hereafter referred to as HO-2 Δ . The variants studied include HO-2 Δ , C127A/C282A HO-2 Δ , and C127A HO-2 Δ^* , where HO-2 Δ^* indicates the removal of amino acid residues 265–316, which contain the C-terminal HRMs. Each species was characterized in the as-isolated [i.e., containing Fe(III) heme and a disulfide bond between C265 and C282 in the case of HO-2 Δ] and dithiothreitol (DTT)-reduced [wherein Cys residues are reduced to the thiol form but the iron remains in the Fe(III) state] forms. The HO-2 Δ and C127A HO-2 Δ^* variants were also studied in the Fe(II) heme form. To prepare these samples, the protein was suspended in buffer containing 50 mM Tris (hydroxymethyl)aminomethane (Tris)–HCl and 50 mM KCl (pH 7.5). DTT-reduced protein was prepared by incubating 150 μ M as-isolated protein with 10 mM DTT on ice for 1 h then extensively dialyzing with buffer in an anaerobic chamber (Vacuum Atmospheres, Hawthorne, CA, USA). The number of free thiol groups in the DTT-reduced HO-2 Δ variants was determined using 5,5'-dithiobis(2-nitrobenzoate) as described previously [28]. The protein–heme complexes were prepared by incubating purified HO-2 Δ or its variants with a 1.5-fold molar excess of a freshly prepared Fe(III) heme stock solution containing 5–10% dimethyl sulfoxide and 100 mM NaOH to prevent heme aggregation. This stock solution was passed through a 0.22- μ m filter prior to addition to HO-2 to ensure that no heme aggregates were present. Excess heme was immediately removed from the reconstituted protein samples by two cycles of chromatography with a Bio-Spin6 column (Bio-Rad, Hercules, CA, USA). During a previous optimization of this method it was found that the final heme to protein ratio is approximately 1:1, as verified by the pyridine hemochrome assay [29]. All samples were examined spectroscopically after chromatography to verify the absence of unbound heme, the presence of which would give rise to a characteristic broad absorption band at 385 nm. Addition of Fe(III) heme was carried out in an anaerobic chamber in the case of the DTT-reduced HO-2 variants. Fe(II) heme was prepared by adding freshly prepared sodium dithionite (2 mM final concentration) to a stock solution of 200 μ M Fe(III) heme in 20% dimethyl sulfoxide in the anaerobic chamber. This was then added to protein anaerobically as described above. Protein concentrations were determined using the rose Bengal method [30]. Samples contained 15–20 μ M heme-bound protein, and all samples used for low-temperature absorption and MCD experiments also contained approximately 60% (v/v) glycerol. In preparation for spectroscopic experiments, the samples were thawed on ice, transferred into MCD sample cells or NMR tubes, and frozen in liquid N₂. All DTT-reduced samples and Fe(II)-heme-containing samples were handled under an atmosphere of N₂ (gas).

Spectroscopy

Room-temperature absorption spectra were obtained using a Varian Cary 5e spectrophotometer. The sample compartment was purged with N₂ (gas) when collecting data for the DTT-reduced and Fe(II)-heme-containing samples. Low-temperature (4.5 K) absorption and MCD spectra and 277 K MCD spectra were obtained using a JASCO J-715 spectropolarimeter in conjunction with an Oxford Instruments SM4000-8T magnetocryostat. The MCD spectra presented herein were obtained by subtracting the -7 T data from the +7 T data.

Resonance Raman data were obtained by exciting the samples with 406.7- or 413.1-nm light from a Kr⁺ ion laser (Coherent I-302C). The power of the incident light was approximately 25 and 15 mW for low-temperature (77 K) and 277 K experiments, respectively. Light scattered at approximately 135° from the sample was dispersed by a triple monochromator (Acton Research SpectraPro) with a 2,400 grooves per millimeter grating and detected with a liquid-N₂-cooled back-illuminated CCD camera (Princeton Instruments Spec-10 100BR, 1,340 × 100 pixels). The NMR tubes containing the samples were immersed in liquid N₂ or an ice/H₂O bath during data collection.

Results

Fe(III)-heme-bound HO-2 samples

Absorption and MCD data—Room-temperature absorption spectra were obtained for the as-isolated [i.e., Fe(III)-heme-bound] truncated form of HO-2 (HO-2 Δ) and two variants (C127A/C282A HO-2 Δ , containing only Cys265 that is at least partially oxidized to its sulfenate form, and C127A HO-2 Δ^* , lacking all three Cys residues) that possess changes to the C-terminal HRMs [28]. The absorption spectrum of each of these species exhibits the Soret feature at approximately 403 nm and distinct charge transfer bands at approximately 500 and 631 nm (Fig. 1, broken lines), typical of high-spin Fe(III) heme complexes [31]. These data are consistent with the results obtained in previous studies of HO-2 Δ at neutral pH [28,32,33]. On the basis of the close resemblance of their absorption spectra, it can be concluded that at room temperature all three variant species investigated feature similar heme environments. Interestingly, however, significant spectral changes were observed for all three samples upon cooling to low temperature. In each case the absorption spectrum at 4.5 K (Fig. 1, solid lines) consists of a Soret band redshifted to 411 nm and α and β bands at approximately 560 and 533 nm, respectively, indicative of a six-coordinate low-spin Fe(III) heme complex [31].

A curious difference between our absorption spectra and those published previously for a low-spin form of Fe(III)-HO-2 obtained at pH 9.5 [32] is the position of the α band, which is blueshifted by about 15 nm in our spectra. This upshift of the α band is consistent with a neutral nitrogen-donor molecule (such as a His residue) serving as a second axial ligand to the Fe(III) heme. We do not yet have definitive evidence for the identity of the sixth ligand in our HO-2 species, so it is possible that OH⁻ is in fact still bound and the change in the absorption spectrum bears no real significance. However, it seems likely that our data reflect the presence of a neutral (perhaps nitrogen-donor) ligand instead of the hydroxide ion observed for the HO-2-bound low-spin Fe(III) heme formed at high pH. This latter possibility is also supported by EPR data obtained for this species (Fig. S1), which clearly reveal the presence of a low-spin Fe(III) heme species that exhibits *g* values characteristic of a His/nitrogen-donor axial ligand set (*g* = 2.87, 2.25, 1.64).

To explore the effects of reducing the disulfide bond between the Cys residues of the C-terminal HRMs (C265 and C282), absorption spectra were also collected for the DTT-reduced counterparts of the samples described above (Fig. 2). All three species still contain Fe(III)

heme, but the disulfide bond and sulfenate moiety have been reduced to thiol groups in HO-2 Δ and C127A/C282A HO-2 Δ , respectively. After reduction, these samples contained approximately 3.00 and 0.82 thiols per protein, respectively, as determined by the 5,5'-dithiobis (2-nitrobenzoate) assay (compared with 0.09 thiols per protein for as-isolated C127A/C282A HO-2 Δ , which contains a sulfenate at Cys265 that is reduced to a thiol in the reduced protein). Like the data for the as-isolated samples, the absorption spectra of the DTT-reduced samples are characteristic of high-spin Fe(III) heme species at room temperature and low-spin Fe(III) heme species at low temperature. However, whereas the absorption spectra for the as-isolated samples are virtually identical to one another, some small differences are observed in the spectra of these DTT-reduced samples. Specifically, a shoulder in the Soret region (approximately 428 nm) is observed in the spectra of the DTT-reduced HO-2 Δ and C127A/C282A HO-2 Δ samples. The position of this new feature does not change as a function of temperature. These results suggest the presence of a second heme species in the DTT-reduced samples that remains low spin at all temperatures (see Fig. 5, top).

In agreement with the absorption data described above, the low-temperature MCD spectra indicate the presence of predominantly low-spin Fe(III) heme at cryogenic temperatures in all of the as-isolated and DTT-reduced Fe(III)-heme-bound samples studied (see Figs. 3, 4, respectively). This is particularly evident from the peak position of the intense derivative-shaped Soret feature centered at approximately 411 nm and the presence of several weaker features in the spectral region between 430 and 600 nm [34]. Whereas the MCD spectra of all of the as-isolated and DTT-reduced C127A HO-2 Δ^* samples are identical, spectral differences are observed for the DTT-reduced HO-2 Δ and C127A/C282A HO-2 Δ samples. Although these latter spectra are quite similar to those of the analogous as-isolated samples, an additional shoulder is present at approximately 428 nm, corresponding to the shoulder seen in the absorption spectra, and subtle changes are also apparent in the α/β region. These data suggest that although the DTT-reduced samples still primarily contain the same Fe(III) heme species observed in the as-isolated samples, an additional species is present that exhibits distinct spectroscopic signatures (see Fig. 5, bottom). Note that on the basis of the close resemblance of the absorption and MCD data obtained for the DTT-reduced C127A/C282A HO-2 Δ and C127A HO-2 Δ^* samples (Figs. 2,4) it may appear that, as proposed previously [28], the redox state of the HRMs has no effect on the optical spectra of these species. However, a more careful inspection of these data clearly reveals that the C127A/C282A HO-2 Δ spectra differ from those of the C127A HO-2 Δ^* variant by the presence of an additional Soret feature at approximately 428 nm, which is particularly obvious in the low-temperature MCD spectrum (Fig. 4). Consequently, the former sample is spectroscopically more similar to the HO-2 Δ sample, each containing two distinct heme species (see Fig. S2). However, the relative concentration of the second, unique heme species is considerably lower in the C127A/C282A HO-2 Δ sample than in the HO-2 Δ sample. This difference can be seen quite clearly by comparing the absorption and MCD data of these and additional, independently prepared samples (Fig. S2). Thus, our room-temperature absorption spectra are, in fact, qualitatively similar to those reported previously [28], with small quantitative differences arising from a currently unknown variation in sample preparations. However, our current data clearly demonstrate that the redox state of the HRMs affects the HO-2 species present and that this change can be observed easily via absorption and MCD spectroscopies.

For the as-isolated C127A HO-2 Δ^* and DTT-reduced HO-2 Δ samples, MCD spectra were also collected at 277 K (Figs. 3, 4, broken lines). On the basis of the marked decrease in intensity of the negatively signed Soret feature and several spectral changes in the α/β region, it is reasonable to conclude that at 277 K the major heme species in as-isolated C127A HO-2 Δ^* contains a high-spin Fe(III) ion [34]. Alternatively, the 277 K MCD spectrum of DTT-reduced HO-2 Δ exhibits two Soret features instead of just one, suggesting that two distinct heme species are present at this temperature. One species exhibits spectral features similar to those of as-

isolated C127A HO-2 Δ^* , signifying the presence of high-spin Fe(III) heme. The spectral features due to the second species are nearly identical to those displayed by the second heme species observed at low temperature for this sample and C127A/C282A HO-2 Δ . Thus, these results confirm the conclusions drawn from the absorption data; namely, (1) the major heme species in all samples undergoes a spin-state conversion from high spin at room temperature to low spin at low temperature and (2) a second heme species is present in the DTT-reduced HO-2 Δ and C127A/C282A HO-2 Δ samples that exhibits redshifted Soret features and remains low spin at all temperatures.

Resonance Raman data—The resonance Raman spectra of the as-isolated and DTT-reduced HO-2 variants are presented in Figs. 6 and S3, respectively. The spectra of all of these samples are virtually identical. The oxidation-state ($\nu_4 = 1,371 \text{ cm}^{-1}$) and spin-state ($\nu_2 = 1,580 \text{ cm}^{-1}$, $\nu_3 = 1,504 \text{ cm}^{-1}$, $\nu_{10} = 1,637 \text{ cm}^{-1}$) marker bands observed for these species at low temperature are characteristic of six-coordinate low-spin Fe(III) heme [35–37]. At 273 K these bands are observed at lower frequencies ($\nu_4 = 1,368 \text{ cm}^{-1}$, $\nu_2 = 1,560 \text{ cm}^{-1}$, $\nu_3 = 1,480 \text{ cm}^{-1}$, $\nu_{10} = 1,617 \text{ cm}^{-1}$) that are typical of six-coordinate high-spin Fe(III) heme complexes [35–37]. These results are consistent with the absorption and MCD data presented already and provide further evidence that the spin state of the HO-2-bound Fe(III) heme is temperature-dependent. Additional resonance Raman data were collected for otherwise identical samples in the presence and absence of 60% (v/v) glycerol to determine if the temperature dependence of the Fe(III) heme spin state is due to differential freezing in the absence and presence of the glassing agent necessary for low-temperature absorption and MCD experiments. It is evident from the similarity between these spectra (Fig. S4) that this is not the case.

Fe(II)-heme-bound HO-2 samples

Absorption and MCD data—Because Fe(II) heme species are also involved in the reaction catalyzed by HOs, it is equally important to explore how the HRMs affect the Fe(II)-heme-bound form of HO-2. In previous studies of the HO-2 variants investigated here, only absorption and EPR spectroscopies were employed; thus, the Fe(II)-heme-bound forms of these species remained poorly characterized [28]. To address this issue, we also performed absorption and MCD spectroscopic studies of the Fe(II)-heme-bound HO-2 species. The absorption spectra of the Fe(II)-heme-bound HO-2 Δ and C127A HO-2 Δ^* samples are presented in Fig. 7. At room temperature the two species give rise to nearly identical spectra, each containing a broad Soret feature at approximately 430 nm consisting of two distinct components, as well as noticeable but broad and indistinct α and β bands. The presence of two bands in the Soret region suggests that these samples contain contributions from both high-spin and low-spin Fe(II) heme species [31]. At 4.5 K, the two Soret bands are still present, but instead of their having nearly equal intensities, the shorter-wavelength feature is now more dominant. Also, the α and β bands are more distinct at low temperature. These spectral changes are consistent with a higher proportion of low-spin Fe(II) heme at cryogenic temperature [31]. The fact that both HO-2 Δ , which contains three reduced Cys residues, and C127A HO-2 Δ^* , which lacks all of these Cys residues, give rise to virtually identical absorption spectra both at low temperature and at room temperature suggests that no significant structural differences, such as Cys ligation to the Fe(II) heme in the former sample, exist between these samples.

The low-temperature MCD data obtained for these two samples (Fig. 8) allow for a more conclusive assignment of the Fe(II) heme species present. In agreement with the absorption data, the MCD spectra of the two samples are quite similar and exhibit characteristic features of both high-spin and low-spin Fe(II) heme species. The temperature-dependent features in the Soret region are due to the high-spin component; alternatively, the sharp derivative-shaped feature at 552 nm is characteristic of low-spin Fe(II) heme. More specifically, the position of

this latter feature is indicative of a low-spin Fe(II) heme species with His, rather than Cys, axial ligation [38–40]. This result provides further evidence against Cys ligation in the Fe(II)-heme-bound HO-2 species. Thus, the HRMs do not appear to have the same influence on the Fe(II)-heme-bound and Fe(III)-heme-bound HO-2 species.

Because five-coordinate Fe(II) heme proteins tend to be high spin even at low temperature, it follows that a sixth ligand must be present in the low-spin Fe(II) species we observe. Thus, it appears that our samples contain a mixture of five- and six-coordinate Fe(II) heme, with the relative amount of six-coordinate species increasing with decreasing temperature. Although it is clear from our data that one axial ligand to the Fe(II) heme is His rather than Cys, the identity of the sixth ligand is not yet established. There does not appear to be a good candidate for this ligand among the amino acid residues within the enzyme's active site, so the most likely ligand would be solvent ($\text{H}_2\text{O}/\text{OH}^-$) or a buffer molecule.

Discussion

Temperature dependence of the iron spin state

A particularly intriguing aspect of the spectroscopic data obtained in this study is that the Fe(III) spin state of the HO-2 species investigated changes as a function of temperature. This spin-state change is evidenced by the blue-shift of the Soret feature as well as a loss of discernible α/β features and the appearance of distinct charge transfer bands in the absorption spectra (Figs. 1, 2) when the temperature is increased. These absorption spectrum changes are accompanied by a loss of negative intensity in the Soret region and several changes in the 450–700-nm region in the corresponding MCD spectra (Figs. 3, 4) and marked shifts of the spin-state marker bands in the resonance Raman spectra (Figs. 6, S3). Collectively, the data obtained using these three complementary spectroscopic techniques provide compelling evidence that the ferric ion in the Fe(III) heme–HO-2 complex adopts a high-spin configuration at room temperature and a low-spin configuration at low temperature [31,34–37]. Although glycerol could, in principle, affect the protein structure in the frozen state, its presence in these HO-2 samples is not the cause of the observed spin-state change since the resonance Raman spectra obtained in the presence and absence of glycerol are virtually identical (Fig. S4). Alternatively, a temperature-dependant pK_a change or some other buffer effect could be responsible for the spin-state change upon freezing. However, the EPR data collected for samples prepared in different buffers appear virtually identical (not shown), strongly suggesting that the temperature dependence of the spin state of the Fe(III) heme–HO-2 complex is in fact intrinsic to this species.

Likewise, a comparison of room-temperature absorption with low-temperature absorption and MCD data for the Fe(II)-heme-containing HO-2 samples leads to the conclusion that the spin state of the ferrous ion exhibits a similar temperature dependence. As was seen with the Fe(III)-heme-bound HO-2 species, a higher proportion of low-spin Fe(II) heme is present at low temperature, whereas high-spin Fe(II) heme predominates at room temperature (Figs. 7, 8).

Clues as to what may cause this spin-state change come from a comparison with other heme proteins with active-site environments similar to that of HO-2. For instance, a similar change in spin state is observed in both HO isoforms and other heme proteins such as metmyoglobin and methemoglobin as a function of pH [2,32,41]. This change is thought to be the result of a change in the protonation state of the axial solvent ligand, from H_2O at low pH to OH^- at high pH. The acidity of the solvent ligand appears to be modulated by a hydrogen-bonding interaction with a distal amino acid residue, often a His like seen in met-myoglobin and methemoglobin. However, HOs lack a distal His residue, and instead possess a hydrogen-bonding network to a nearby Asp residue (see below). Additionally, some heme proteins, including the O_2 -sensing heme kinase FixL and the ring-cleaving enzyme indoleamine 2,3-

dioxygenase, exhibit a temperature-dependent spin state that is thought to be the result of a thermal equilibrium between high-spin and low-spin Fe(II) heme or Fe(III) heme, with the high-spin state being more favored at higher temperatures [4,42–44]. This phenomenon has been attributed to hydrogen-bonding interactions involving the axially bound solvent molecule (OH^-) of the heme moiety and nearby amino acid residues [2]. It has been suggested that a stronger hydrogen-bond donation to the axial OH^- ligand at high temperature makes this ligand more H_2O -like, resulting in a weaker ligand field experienced by the iron ion and, thus, favoring a high-spin configuration.

Although there is no obvious hydrogen-bonding amino acid residue adjacent to the distal solvent molecule in HOs, an examination of structural data for heme-bound human HO-1 reveals a short chain of H_2O molecules that link the iron ion to Asp and Arg residues [45]. Mutational studies of HO-1 provided evidence that this particular Asp residue (Asp140) and the precisely structured hydrogen-bonding network it supports are critical for proper HO function [45,46]. A similar hydrogen-bonding network has been identified in rat HO-1 and a HO from *Corynebacterium diphtheriae* (HmuO) [47–49]. Additionally, a recent X-ray crystallographic study has revealed that human HO-2 possesses an active site similar to that of HO-1 [12]. Although the crystal structure was obtained for a significantly truncated HO-2 variant, this form of the enzyme is catalytically active. Furthermore, most crystal structures available for HO-1 were obtained for proteins with similar truncations. Thus, it is reasonable to assume that the truncation leaves the active site intact, and that meaningful comparisons can be made with known structures of HO-1. To this end, it is intriguing to note that human HO-2 possesses an Asp residue whose position is similar to that of the Asp140 anchoring the active-site hydrogen-bonding network in HO-1 (see above).

Although the X-ray crystal structure of human HO-2 did not provide direct evidence for the existence of a hydrogen-bonding network between the Asp residue and a solvent molecule bound to the heme moiety, the many similarities between HO-1 and HO-2 suggest that this network likely exists. Our data are also consistent with this conclusion. Specifically, the observed temperature dependence of the iron spin state in HO-2^A can be explained in terms of small changes in hydrogen-bonding interactions, presumably involving a weakening of the hydrogen-bond donation to an axial ligand such as a solvent molecule or another hydrogen-bond-accepting ligand at low temperature so as to strengthen the Fe–ligand bond and stabilize low-spin iron. Although the low temperatures used in the experiments described above are not physiologically relevant and future studies of HO-2 species containing mutations of distal-site residues are needed to provide more conclusive evidence, this spin-state change clearly supports the possibility that a hydrogen-bonding network exists at the distal heme site, where O_2 is thought to bind during catalysis [11,28]. Presumably a hydrogen-bonding interaction similar to the one with the axial ligand in the resting enzyme will serve to stabilize and activate a heme-bound O_2 moiety in the early intermediates formed during the catalytic cycle of HO-2 in analogy to its proposed role in HO-1.

Despite these similarities between HO-1 and HO-2, however, the former does not exhibit a temperature-dependent spin-state change. Low-temperature EPR data of Fe(III) heme–HO-1 at neutral pH appear indicative of a high-spin species [41], and no evidence for a low-spin species at any temperature has been obtained under these conditions (note, however, that HO-1 does in fact also form a low-spin species, but its relative population is dependent only on pH and not on temperature). Because of the close similarity in structures of HO-1 and HO-2 and the presence of this hydrogen-bonding network in HO-1, the mere existence of such a network in HO-2 is not sufficient to explain the temperature-dependent behavior we observe for HO-2. However, HO-1 and HO-2 are not completely identical. They are reported to have different pK_a values for the conversion from high spin to low spin associated with the deprotonation of the axial solvent ligand (7.6 for HO-1, 8.5 for HO-2), and small structural differences are

apparent even within their active sites [12–14,32,41,45]. Instead of a mere deprotonation of, or an alteration of a hydrogen-bonding interaction with, an axial solvent ligand causing the observed spin-state change, our HO-2 species appear capable of undergoing a ligand exchange (perhaps involving a nitrogen donor) upon conversion to their low-spin Fe(III)-heme-bound form. The identity of this new ligand is not yet known, nor is its relevance, if any, to the catalytic mechanism of HO-2. However, this observation does bring to light new differences between the two HO isoforms. On the basis of these results, we conclude that small but important differences exist between the active sites of HO-1 and HO-2, which result in distinct temperature-dependent behaviors of the heme spin states.

Evidence for Cys ligation to Fe(III) heme

Although the necessity of HOs is undisputed, questions arise as to why two distinct forms of this enzyme are required. HO-1 appears well suited to fulfill its obvious role in heme degradation. It is localized primarily in liver and spleen tissue, where much of the heme catabolism occurs, and its transcription is regulated in part by cellular markers for free heme, such as the presence of a heme–hemopexin complex that serves to transport heme to hepatocytes, as well as oxidative stress, which can result from free heme [17]. In contrast, HO-2 is expressed constitutively and is found in high concentrations in other areas of the body, such as the brain [18]. It has been proposed that although HO-2 effectively degrades heme like HO-1 does, its primary role may be to generate CO, which can act as a cellular signaling molecule [17]. Understanding the differences between the two HO isoforms may help to more clearly elucidate the need for two forms of this enzyme and the distinct roles they may have.

One noticeable difference between the two HO isoforms is the presence of three Cys residues as part of HRMs in HO-2 and the complete lack of Cys residues in HO-1 [19]. A few hemoproteins are known to contain HRMs that appear to play important roles in regulation or enzyme activity [20–27]. It is thought that these proteins, as well as HO-2, bind heme at the HRMs and that a Cys residue within the HRM serves as an axial ligand to the heme. For HO-2, it has been proposed that heme can bind to the HRMs as well as to His45 in the active site, resulting in a protein complex containing multiple heme molecules [19]. However, recent experiments have led to the conclusion that only one heme binds to each HO-2 protein, coordinating either to His45 in the active site or to Cys265 within one of the HRMs of DTT-reduced HO-2 species [28]. Interestingly, though, mutational studies of HO-2 have revealed that the HRMs are not necessary for enzyme activity or stability [19,28]. Nonetheless, recent studies have provided evidence that two of the three HRMs of HO-2 may constitute a redox switch that regulates the heme-binding affinity of HO-2 in response to the reducing nature of the protein environment [28].

The spectroscopic data obtained in our studies of various HO-2 species provide further support for the proposed Cys ligation to Fe(III) heme. The absorption and MCD spectra of as-isolated HO-2^Δ and C127A HO-2^{Δ*} are identical (Fig. 3); thus, the presence of Cys residues in the oxidized state of HO-2^Δ and C127A/C282A HO-2^Δ does not affect the binding of heme to the catalytic site (via His45). Interestingly, however, some spectral differences are observed among the DTT-reduced forms of the Fe(III)-heme-bound HO-2 species investigated, most notably the appearance of additional features for the samples that contain Cys265. Specifically, a low-energy shoulder at approximately 428 nm appears on the Soret feature in both the absorption and the MCD spectra (Figs. 2, 4) and small changes are discernible in the α/β region of the MCD spectra, signifying the presence of two distinct heme-bound protein species. The major species gives rise to spectra identical to those of the as-isolated HO-2 samples, indicating that it contains His-ligated Fe(III) heme. Alternatively, because the second (i.e., minor) species is observed exclusively in samples with free thiolate groups, which are known to coordinate to Fe(III) hemes [3], it likely possesses Cys-ligated Fe(III) heme.

Strong support for this assignment of the minor species in the DTT-reduced HO-2 samples investigated comes from a comparison with other spectroscopically characterized hemoproteins that are known to have Cys coordination [31,38,50–56]. As shown in Table 1, a characteristic feature defining Cys-bound Fe(III) heme species is a noticeably redshifted Soret band. Consistent with this trend, the Soret band of the minor HO-2 species (Fig. 5), which we propose to contain six-coordinate, Cys265-ligated low-spin Fe(III) heme, is redshifted by 17 nm relative to that of the major, His45-ligated Fe(III) heme species. From these data and previous EPR studies it seems clear that Cys serves as an axial ligand in this minor heme species and that this species contains six-coordinate, low-spin Fe(III) heme. The nature of the sixth ligand has not yet been definitively established, although a solvent molecule or the unknown nitrogen-donor ligand observed in the low-spin, His-ligated Fe(III) heme species discussed above are possible candidates. Interestingly, the appearance of the spectral features due to this minor Fe(III) heme species is concurrent with a marked decrease in the intensity of those associated with the His45-ligated form of HO-2. This observation suggests that the formation of the Cys-ligated Fe(III) heme species occurs at the expense of the His-bound form, corroborating a previous proposal that a single heme binds to each protein molecule [28].

In contrast, no differences are observed between the spectra of the various Fe(II)-heme-bound HO-2 species investigated, indicating that the presence of Cys residues does not affect the heme environment in the ferrous state. Consistent with this finding, the position of the MCD A-term feature associated with the low-spin Fe(II) heme fraction is characteristic of His rather than Cys ligation [39,51,52,57,58]. Thus, it appears that Cys265 competes with His45 as an axial ligand to ferric but not ferrous heme bound to HO-2.

It has recently been reported that the EPR spectra of the as-isolated HO-2 variants investigated here are all identical and that an additional species, with EPR signatures characteristic of thiolate-ligated low-spin Fe(III) heme, is present in the DTT-reduced HO-2^Δ and C127A/C282A HO-2^Δ species [28]. These findings are in excellent agreement with the conclusions drawn from the spectroscopic data obtained in the present study. One noticeable difference between the EPR spectra and our absorption, MCD, and resonance Raman data is that the former seem to suggest that the His-ligated heme bound to HO-2 contains predominantly high-spin Fe(III) at low temperature, whereas the latter data are consistent with mostly low-spin His-ligated Fe(III) heme. However, the EPR data clearly show that both high-spin and low-spin Fe(III) heme species are present in HO-2 at low temperature, though no quantitation of the relative amounts of each has been reported.

Supplementary Material

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Acknowledgments

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Fig. 1.

Room-temperature (*broken lines*) and 4.5 K (*solid lines*) electronic absorption spectra of as-isolated Fe(III)-heme-bound C127A heme oxygenase-2 (HO-2 Δ^*) (*top*), C127A/C282A HO-2 Δ (*middle*), and HO-2 Δ (*bottom*). Conditions: 15 μ M (HO-2 Δ , C127A HO-2 Δ^*) or 20 μ M (C127A/C282A HO-2 Δ) protein, 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 50 mM KCl (pH 7.5), 60% (v/v) glycerol

Fig. 2.

Room-temperature (*broken lines*) and 4.5 K (*solid lines*) absorption spectra of dithiothreitol (DTT)-reduced Fe(III)-heme-bound C127A HO-2^{Δ*} (*top*), C127A/C282A HO-2^Δ (*middle*), and HO-2^Δ (*bottom*). Conditions: 15 μM (HO-2^Δ, C127A HO-2^{Δ*}) or 20 μM (C127A/C282A HO-2^Δ) protein, 50 mM Tris-HCl, 50 mM KCl (pH 7.5), 60% (v/v) glycerol

Fig. 3.

The 277 K (broken line; $\times 30$ for comparison) and 8 K (solid lines) magnetic circular dichroism (MCD) spectra of as-isolated Fe(III)-heme-bound C127A HO-2 Δ^* (top), C127A/C282A HO-2 Δ (middle), and HO-2 Δ (bottom). Conditions: 15 μ M (HO-2 Δ , C127A HO-2 Δ^*) or 20 μ M (C127A/C282A HO-2 Δ) protein, 50 mM Tris-HCl, 50 mM KCl (pH 7.5), 60% (v/v) glycerol

Fig. 4.

The 277 K (*broken line*; $\times 30$ for comparison) and 4.5 K (*solid lines*) MCD spectra of DTT-reduced Fe(III)-heme-bound C127A HO-2 Δ^* (*top*), C127A/C282A HO-2 Δ (*middle*), and HO-2 Δ (*bottom*). Conditions: 15 μ M (HO-2 Δ , C127A HO-2 Δ^*) or 20 μ M (C127A/C282A HO-2 Δ) protein, 50 mM Tris-HCl, 50 mM KCl (pH 7.5), 60% (v/v) glycerol

Fig. 5. Absorption (*top*) and MCD (*bottom*) spectra representing the spectral contributions of the putative Cys265-ligated Fe(III)–HO-2 species. Spectra were obtained by subtracting data for the DTT-reduced Fe(III)-bound C127A HO-2 Δ^* species from those for the analogous HO-2 Δ species

Fig. 6.

The 273 K (*broken lines*) and 77 K (*solid lines*) resonance Raman spectra of as-isolated Fe (III)-heme-bound C127A HO-2^{Δ*} (*top*), C127A/C282A HO-2^Δ (*middle*), and HO-2^Δ (*bottom*), obtained with $\lambda_{\text{ex}} = 413.1$ nm. Conditions: 20 μM HO-2, 50 mM Tris-HCl, 50 mM KCl (pH 7.5)

Fig. 7. Room-temperature (*broken lines*) and 4.5 K (*solid lines*) absorption spectra of Fe(II)-heme-bound C127A HO-2 Δ^* (*top*) and HO-2 Δ (*bottom*). Conditions: 15 μ M HO-2, 50 mM Tris-HCl, 50 mM KCl (pH 7.5), 60% (v/v) glycerol

Fig. 8. The 277 K (*broken line*) and 4.5 K (*solid lines*) MCD spectra of Fe(II)-heme-bound C127A HO-2 Δ^* (*top*) and HO-2 Δ (*bottom*). Conditions: 15 μ M HO-2, 50 mM Tris-HCl, 50 mM KCl (pH 7.5), 60% (v/v) glycerol

Table 1

Position of the Soret band in the electronic absorption (*Abs*) and magnetic circular dichroism (*MCD*) spectra of various Fe(III)-heme-bound proteins with and without thiolate ligation

Protein	Soret band position (nm)		Axial ligands	References
	Abs	MCD		
Thiolate ligation (low spin)				
P450cam	417	418	Cys, H ₂ O	[50,51]
CooA	423	NA	Cys, Pro	[38]
Cystathionine β synthase	428	425	His, Cys	[52]
H93G myoglobin	425	425	Ethanethiol, imidazole	[31,53]
H66C cytochrome b_2	423	423	Cys, ?	[54]
HO-2 (minor)	428	425	Cys, ?	This work
Thiolate ligation (high spin)				
P450cam	391	397	Cys	[51]
No thiolate ligation				
C75S(A) CooA	415 (411)	NA	Pro, ?	[55,56]
Cytochrome b_2	413	413	His, His	[54]
HO-2 (major)	411	411	His, ?	This work

HO-2 heme oxygenase-2, *NA* not available

? Sixth ligand currently unknown