Heme Oxygenase His25Ala Mutant: Replacement of the Proximal Histidine Iron Ligand by Exogenous Bases Restores Catalytic Activity

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Heme oxygenase catalyzes the NADPH- and cytochrome P450 reductase-dependent conversion of heme to biliverdin (Figure 1).[1] Heme oxygenase is a membrane-bound protein, but water soluble, catalytically active versions of the rat[2] and human[3] (hHO-1) enzymes without the membrane-binding domain have been expressed in *Escherichia coli*. Histidine-25 has been identified as the fifth iron ligand in the hHO-1:heme complex by resonance Raman (RR) and site specific mutagenesis.[4-6] Replacement of His-25 by an alanine yields a catalytically inactive protein that binds heme without providing a strong axial iron ligand.[6] This suggests that the H25A hHO-1 mutant has a cavity below the heme iron atom due to replacement of the histidine by an alanine. We report here that exogenous imidazoles bind in this cavity, coordinate to the iron, and restore catalytic activity. The ability to replace the proximal iron ligand by groups other than those in the conventional library of amino acids makes possible a general examination of the role of the proximal iron ligand in heme oxygenase catalysis and, more generally, in hemoprotein function.[7-10]

The H25A hHO-1:heme complex (λ_{max} = 398 nm)[11] is reduced by dithionite under an atmosphere of CO to the ferrous–CO complex with a blue-shifted (relative to wild type) Soret maximum at 411 nm and α/β bands at 566 and 533 nm.[6] Aerobic passage through Sephadex G25 regenerates the ferric state rather than, as with the native enzyme, resulting in displacement of CO by O_2 to give the ferrous dioxygen complex. The formation of a highly autooxidizable ferrous dioxygen complex is implied. Addition of NADPH (50 μM) to a solution of the hH25A HO-1:heme complex (19 μM) and cytochrome P450 reductase (5 μM) under a partial atmosphere of CO fails

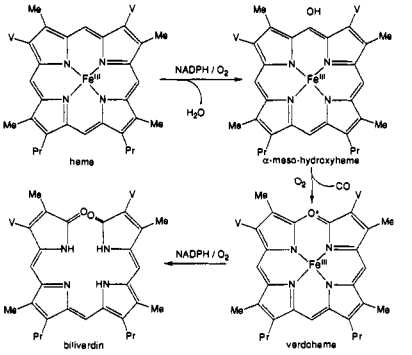

Figure 1. Transformations catalyzed by heme oxygenase.

to produce the shift and decrease in intensity of the Soret band or the increase in the 640–680 nm absorbance due to formation of the verdoheme–CO complex characteristic of normal turnover (Figure 2, upper panel).[2a] The heme is slowly destroyed, presumably by H_2O_2 generated by the reductase, but no more than a trace of biliverdin is detected by HPLC analysis.[12]

When 20 equiv of imidazole (Im) is added to H25A hHO-1, the Soret band shifts from 398 to 406 nm (versus wild type at 404 nm).[13] Furthermore, reduction by NADPH–cytochrome P450 reductase under an atmosphere of CO gives a Soret band at 419 nm and α/β bands at 566 and 533 nm identical to those of the wild-type ferrous hHO-1 CO complex. As the CO is displaced by O_2, the spectrum of the H25A hHO-1 dioxygen complex (λ_{max} = 412 nm) is observed and then is seen to decrease concomitantly with the appearance of absorption at 680 nm due to biliverdin formation (Figure 2, lower panel). HPLC analysis[12] shows that a single product is formed that coelutes with authentic α-biliverdin and has an absorption spectrum identical to that of α-biliverdin. The same results are obtained with *N*-methyl- or 4-methylimidazole, but addition of the sterically hindered 2-methylimidazole does not alter the visible spectrum and does not promote catalytic turnover of the protein. The ability of *N*-methylimidazole to support catalytic activity establishes, as proposed earlier, that deprotonation of the histidine iron ligand is not important for catalysis.[4]

The wild-type reduced heme:hHO-1 complex has been identified by RR as having a five-coordinate, high-spin (5cHS) heme in which the iron is coordinated to the imidazolyl side chain of His 25.[4-6] The RR spectrum of ferrous H25A hHO-1 without added imidazole is also indicative of a predominantly 5cHS heme.[6] The exact identity of the fifth ligand in the mutant is not known, however, but is a weak ligand such as a water molecule. In fact, a small amount of four-coordinate, intermediate-spin species was also detected.[6]

Upon anaerobic addition of imidazole to ferrous H25A hHO-1, a new 5cHS species is formed (Im:enzyme ratios ≤50) as judged by RR.[14] With *N*-methylimidazole (*N*-MeIm), a 5cHS

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(1) Recent reviews: (a) Maines, M. D. In *Heme Oxygenase–Clinical Applications and Functions*; CRC Press: Boca Raton, 1992. (b) Beale, S. I. *Chem. Rev.* **1993**, *93*, 785.

(2) (a) Wilks, A.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993**, *268*, 22357. (b) Ishikawa, K.; Sato, M.; Ito, M.; Yoshida, T. *Biochem. Biophys. Res. Commun.* **1992**, *182*, 981.

(3) Wilks, A.; Black, S.; Miller, W.; Ortiz de Montellano, P. R. *Biochemistry*, in press.

(4) Sun, J.; Wilks, A.; Ortiz de Montellano, P. R.; Loehr, T. M. *Biochemistry* **1993**, *32*, 14151.

(5) Takahashi, S.; Wang, J.; Rousseau, D. L.; Ishikawa, K.; Yoshida, T.; Takeuchi, N.; Ikeda-Saito, M. *Biochemistry* **1994**, *33*, 5531.

(6) Sun, J.; Wilks, A.; Ortiz de Montellano, P. R.; Loehr, T. M. *Biochemistry* **1994**, *33*, 13734–13740.

(7) Pioneering work on the restoration of catalytic activity by exogenous amines was reported for an aspartyl aminotransferase Lys258Ala mutant.[8] The replacement of a histidine metal ligand by exogenous imidazoles was first performed with the His117Gly mutant of *Ps. aeruginosa* azurin[9] and, more recently, the His93Gly mutant of myoglobin.[10]

(8) Toney, M. D.; Kirsch, J. F. *Science* **1989**, *243*, 1485–1488.

(9) den Blaauwen, T.; Canters, G. W. *J. Am. Chem. Soc.* **1993**, *115*, 1121.

(10) DePillis, G. D.; Decatur, S. M.; Barrick, D.; Boxer, S. G. *J. Am. Chem. Soc.* **1994**, *116*, 6981.

(11) The Soret band in the wild-type truncated hHO-1:ferric heme complex is at 404 nm.

(12) Enzyme reactions (1 mL), acidified with 200 μL of 5 N HCl and 400 μL of glacial acetic acid, were extracted with $CHCl_3$, and the organic phase was washed twice with water, dried over anhydrous Na_2SO_4, and taken to dryness under a stream of argon. The residue, taken up in 300 μL of HPLC solvent, was analyzed on a Partisil ODS 3–5 μm reverse phase column (4.6 × 250 mm) eluted with 45:55 (v/v) acetone–20 mM formic acid at a flow rate of 0.5 mL/min (detector at 380 nm). The biliverdin isomers eluted in the order α, δ, β, and γ with retention times of, respectively, 15, 16, 17, and 23 min. The HPLC system was developed by J. Clark Lagarias (private communication).

(13) Addition of a 50:1 ratio of Im to wild-type hHO-1 shifts the Soret band to 436 nm, presumably due to coordination of imidazole at the distal iron site. The same spectrum is obtained with the H25A mutant at a 100:1 rather than 20:1 ratio of Im to enzyme.

(14) RR spectra of ferrous H25A hHO-1 were obtained as a function of added imidazole or *N*-MeIm using the 441.6 nm line of a He/Cd laser for excitation. The samples were prepared from oxidized H25A hHO-1 by addition of the appropriate amount of imidazole or *N*-MeIm, followed by deoxygenation via argon purging and anaerobic reduction with dithionite in a septum-sealed glass capillary.

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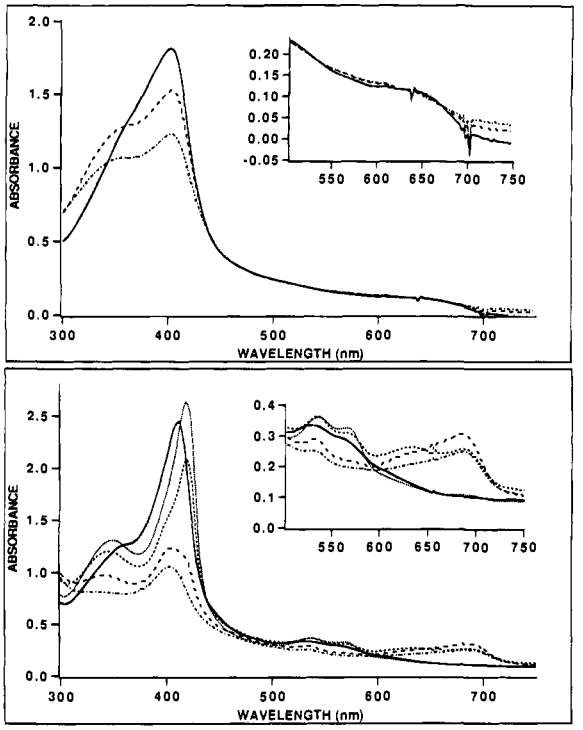

Figure 2. Reaction of the heme:H25A (hHO-1) complex with cytochrome P450 reductase and NADPH. The reactions were started under an initial atmosphere of CO, but the CO was allowed to dissipate with time. Upper panel: Spectra of the ferric H25A mutant (19 μM) (—); immediately following addition of cytochrome P450 reductase (5 μM) and NADPH (50 μM) (- - -); 5 min after addition of NADPH (— • —). Lower panel: Spectra of the ferric H25A mutant (19 μM) in the presence of 20:1 excess imidazole (—); following addition of cytochrome P450 reductase (5 μM) and NADPH (50 μM) (• • •); 3 min after addition of NADPH (- - -); 5 min after addition of NADPH (— — —); 10 min after the addition of NADPH (- • -). The heme loss observed in the upper panel occurs by a nonspecific process and does not give biliverdin.

species also dominates for ratios $\leq$100. Above these values, six-coordinate, low-spin (6cLS) products begin to appear, as indicated by changes in both absorption and RR spectra (not shown). The key to the identification of heme ligands comes from the low-frequency region of RR spectra where Fe—L_{axial} vibrations can be observed. Five-coordinate, high-spin ferrous hemes with imidazole ligands exhibit Fe—N_{Im} vibrational bands between 200 and 250 cm^{-1} that are maximally enhanced with ~440-nm excitation.[15,16] Thus, histidine ligation in the wild-type HO-1 complex is evident from the intense ν(Fe—N_{His}) vibration at ~216 cm^{-1} (Figure 3A).[4-6] This band is absent in the H25A mutant (Figure 3D). With the addition of a 50-fold molar excess of Im to the H25A protein, a new spectral feature emerges at ~228 cm^{-1}, with no significant changes in other RR bands (Figure 3C). We assign this band to ν(Fe—N_{Im}) of the 5cHS imidazole complex.

This assignment is strengthened by the observation of a similar band at 216 cm^{-1} in the RR spectrum of the *N*-MeIm complex (Figure 3B). The 12 cm^{-1} downshift from ν(Fe—N_{Im}) to ν(Fe—$N_{N\text{-}MeIm}$) is attributable to a mass effect. Two different diatomic oscillator models have been described to account for ^{57}Fe and $^{15}N_{His}$ isotopic frequency shifts in deoxymyoglobin.[17] Applying these models to the present case, the observed ν(Fe—N_{Im}) at 228 cm^{-1} in the heme—imidazole complexes of H25A would be predicted to shift to 220 or 214 cm^{-1}, respectively, for the *N*-MeIm complex. The observed shift to 216 cm^{-1} for

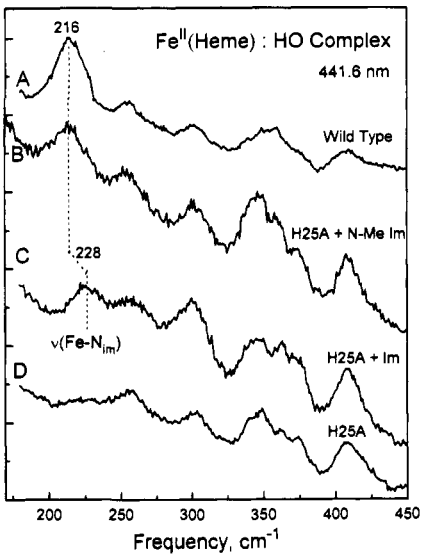

Figure 3. Low-frequency RR spectra with emphasis on the ν(Fe—N_{axial}) vibrations in ferrous forms of the heme:heme oxygenase complexes: (A) wild-type hHO-1; (B) H25A mutant protein plus 100 equiv of *N*-MeIm; (C) H25A mutant protein plus 50 equiv of Im; (D) H25A mutant protein with no exogenous ligand added.

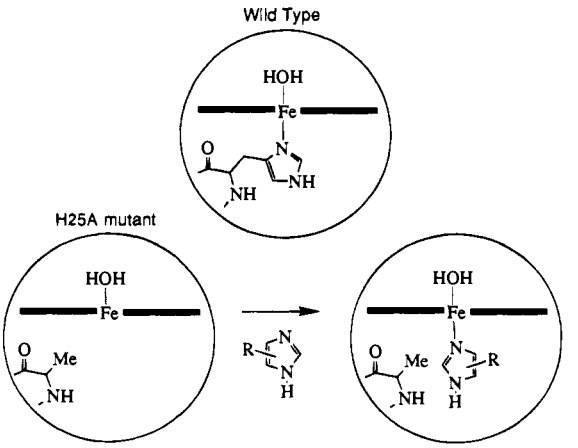

Figure 4. Schematic model of the binding of imidazole to the H25A mutant of hHO-1.

N-MeIm is within this range. Furthermore, the effective mass of *N*-MeIm more closely approximates that of histidine in the wild-type enzyme.

The appearance of ν(Fe—N) modes in the RR spectra of H25A hHO-1 containing exogenous imidazoles indicates that they coordinate to the heme iron. Restoration of heme axial ligation explains the restoration of heme oxygenase activity in the presence of imidazole and *N*-methylimidazole.

The results indicate that coordination of the heme to a strong proximal ligand is required for reduction of the heme iron by cytochrome P450 reductase and for the subsequent catalytic steps. This coordination can be provided in the H25A mutant by exogenous imidazole ligands, resulting in normal catalytic turnover (Figure 4). Four imidazoles have been examined as replacements for the normal histidine, but clearly a range of nonphysiological ligands can be examined to explore the role of axial ligation in hHO-1 function. The same approach can be used, in principle, to examine the catalytic roles of axial ligands in other hemoproteins.[7]

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(15) Yu, N.-T. *Methods Enzymol.* **1986**, *130*, 350.
(16) Smulevich, G.; Mauro, J. M.; Fishel, L. A.; English, A. M.; Kraut, J.; Spiro, T. G. *Biochemistry* **1988**, *27*, 5477.
(17) Wells, A. V.; Sage, J. T.; Morikis, D.; Champion, P. M.; Chiu, M. L., Sligar, S. G. *J. Am. Chem. Soc.* **1991**, *113*, 9655.