Axial Ligation States of Five-Coordinate Heme Oxygenase Proximal Histidine Mutants, as Revealed by EPR and Resonance Raman Spectroscopy

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The substitution of the proximal iron ligand in heme proteins with a noncoordinating amino acid (e.g. Ala or Gly) to create a heme pocket capable of binding exogenous ligands has been applied effectively to study the stereochemical influences of the proximal ligand on heme cavity and reactivity.[1] For heme proteins whose proximal His ligand has been mutated, typically exogenous imidazole (Im) is added to assess the success of restoring properties of the wild-type (WT) protein.[1] However, studies on the ligation states of mutant heme proteins devoid of innate proximal His and of exogenous ligands (e.g. Im) have been scarce, limited to only myoglobin (Mb)[2a] and cytochrome C peroxidase (CCP)[2b] mutants.

In the present study, we have examined the ligation states of a different class of heme complexes known as "heme oxygenase" (HO). Found in eukaryotes[3] and prokaryotes,[4] HO is not a heme protein per se, but binds heme (1:1) and uses the heme as its catalytic site for heme catabolism.[3,5] Similar to Mb and CCP, the proximal iron ligand in both mammalian[6] and bacterial[7] HO is a His residue. In both HO systems, the proximal His is crucial for the conversion of heme to biliverdin and extrication of the heme iron.[6,8] In HmuO, the first bacterial HO isolated,[4] His20 is the proximal iron ligand.[8] Its replacement by an Ala yields a protein, the HmuO mutant H20A, that fails to catalyze heme degradation to biliverdin.[8] Here we provide the first definitive report of the ligation states of the five-coordinate (5c) ferric (Fe^{3+}) heme-H20A complex[8] in both neutral and alkaline environments and devoid of exogenous Im. Moreover, we resolve the ambiguities regarding the axial ligand of the Fe^{3+} heme complex of mammalian HO-1 proximal His mutant H25A.[6a,9]

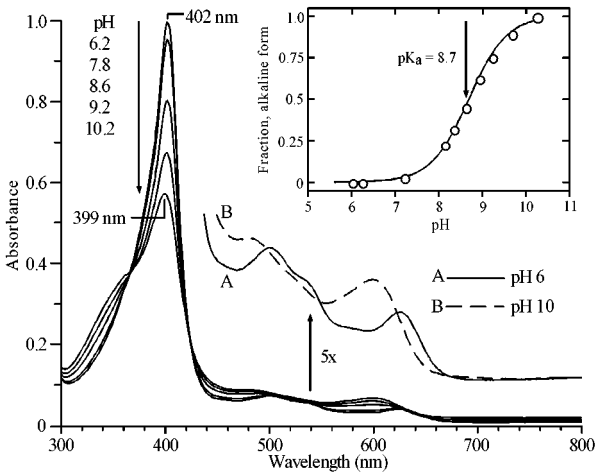

Figure 1. UV−vis spectra of Fe^{3+} heme-H20A between pH 6 (spectrum A) and 10 (spectrum B). The fraction in the alkaline form (inset) is calculated from pH-dependent absorbance changes at 402 nm (○) and fitted to the $n = 1$ Hendersen−Hasselbalch equation.

The UV−vis spectrum of Fe^{3+} heme-H20A at pH 6 (spectrum A, Figure 1) exhibits bands at 402, 500, and 622 nm that are indicative of a high-spin (HS) Fe^{3+} heme. At pH 10 (spectrum B, Figure 1), a new species occurs with bands at 399, 480, and 600 nm, replacing those observed at pH 6. The absence of low-spin (LS) bands, typically found near 575 and 540 nm, indicates that the heme iron remains HS. This contrasts the Fe^{3+} heme-HmuO WT complex, which is both HS and LS at pH 10.[7] The pH-dependent spectral change observed for Fe^{3+} heme-H20A is reversible between pH 6 and 10 and characterized by a pK_a of 8.7 (inset, Figure 1), 0.3 unit lower than that for the six-coordinate (6c) Fe^{3+} heme-HmuO WT.[7] The conversion of Fe^{3+} heme-H20A from an acidic to an alkaline form offers evidence of water/hydroxide transition in the H20A distal heme pocket. In HO-1 WT, deprotonation of a H_2O molecule bound to the heme at the 6th coordinate position to an OH^- is proposed to occur via ionization by a long-range hydrogen-bond (H-bond) network located in the distal heme pocket.[10] The closeness of the pK_a values between Fe^{3+} heme-H20A and heme-HmuO WT suggests that the group responsible for deprotonation in H20A is similar to that in WT and that H_2O is bound to the distal side of the heme iron of the 5c Fe^{3+} heme-H20A at neutral pH.[11]

Cryogenic EPR[12] of Fe^{3+} heme-H20A at pH 7 (panel A, solid line, Figure 2) corroborates an Fe^{3+} HS species. However, unlike the 6c axially symmetric WT complex (panel A, broken line, Figure 2) which has g-values 6 and 2,[7] the 5c Fe^{3+} heme-H20A manifests a broadened $g \sim 6$ region, indicating not only axial ($g = 6$ and 2) but also rhombic ($g = 6.34$, 5.68, and ~2) Fe^{3+} HS species.[13] The substitution of $H_2{}^{16}O$ with partially enriched $H_2{}^{17}O$ (44% enrichment) induces a broadening in the $g = 2$ signal (inset, Figure 2.). This broadening is caused by the nuclear spin $^5/_2$ of the Fe-bound ^{17}O.[15] Our EPR experiments affirm that the oxygen atom of H_2O is the axial ligand of the acidic/neutral 5c Fe^{3+} heme-H20A.

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(11) RR spectra of heme-H20A at pH 7 showed a predominantly 5c HS species. However, a minor 6c HS form was detected, suggesting traces of a bis-H_2O complex.

(12) EPR spectra were obtained by a Bruker ESP-300 spectrometer operating at 9.45 GHz, 1 mW power, and 0.1 mT field modulation. An Oxford liquid He flow cryostat was used for measurements at 6 K.

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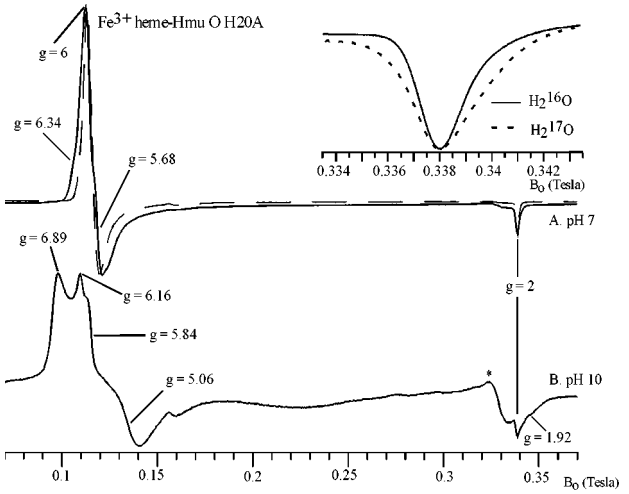

Figure 2. EPR spectra, taken at 6 K, of Fe^{3+} heme-H20A (panel A, solid line) and Fe^{3+} heme-HmuO WT (panel A, broken line) at pH 7, and of Fe^{3+} heme-H20A at pH 10 (panel B). The inset of panel A shows the EPR spectra of the $g = 2$ signal for Fe^{3+} heme-H20A in $H_2{}^{16}O$ (solid line) and $H_2{}^{17}O$ (dotted line) at pH 7. The asterisk denotes impurity signal from CuO in the cryostat.[14]

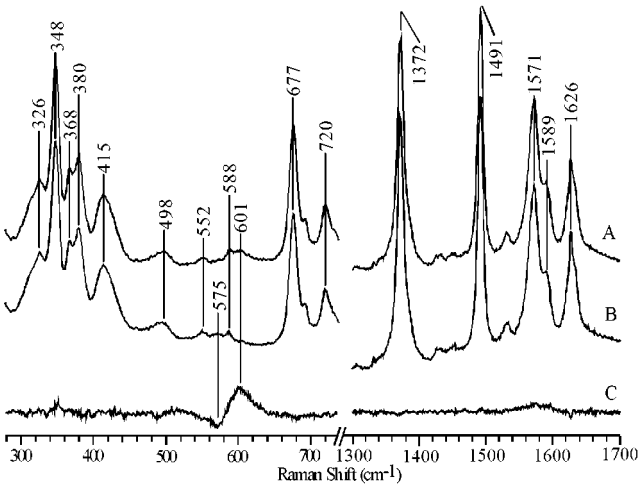

Figure 3. Resonance Raman spectra of Fe^{3+} heme-H20A in 50 mM CHES buffer, pH 10, prepared in $H_2{}^{16}O$ (spectrum A) and $H_2{}^{18}O$ (spectrum B). Spectrum C is the difference spectrum (A minus B).

Consistent with the aforementioned UV−vis spectra, the EPR spectrum of Fe^{3+} heme-H20A at pH 10 (panel B, Figure 2) shows no LS Fe^{3+} heme. Multiple HS species remain with two distinct sets of *g*-values at 6.89, 5.06, and 1.92 and 6.16, 5.84, and ∼2. High-frequency RR[16] of alkaline Fe^{3+} heme-H20A corroborates a predominantly 5c HS heme with intense porphyrin modes ν_2, ν_3, and ν_4 at 1571, 1491, and 1372 cm^{-1}, respectively (Figure 3). Low-frequency RR[16] reveals a Raman line at 601 cm^{-1} in $H_2{}^{16}O$ buffer (spectrum A, Figure 3) that is shifted to 575 cm^{-1} in $H_2{}^{18}O$ buffer (spectrum B, Figure 3). The difference spectrum (spectrum C, Figure 3) has a minimum and maximum at 575 and 601 cm^{-1} that we assign to the ν(Fe−^{16}OH) and ν(Fe−^{18}OH) stretching modes, respectively. This 26 cm^{-1} isotopic frequency shift is comparable to the theoretical value (24 cm^{-1}) expected for an isolated diatomic oscillator, with Fe and OH^- as the two oscillating units. The detection of a ν(Fe−OH) indicates that OH^- is the axial ligand of the alkaline 5c Fe^{3+} heme-H20A and is derived from the ionized H_2O ligand of the acidic/neutral Fe^{3+} heme-H20A. The absence of detectable 6c LS RR and EPR signals suggests that alkaline Fe^{3+} heme-H20A is monohydroxo, not bishydroxo.[13b]

The absence of a proximal ligand in 5c HS heme proteins tends to strengthen the Fe−OH bond, resulting in a ν(Fe−OH) typically between 534 and 578 cm^{-1},[2,13b,17] higher than that (450−560 cm^{-1}) for 6c LS and HS species.[18] Our ν(Fe−OH) of 601 cm^{-1} for the alkaline Fe^{3+} heme-H20A is the highest reported for 5c OH-bound heme complexes studied thus far. The OH^- axial ligand of complexes with ν(Fe−OH) between 575 and 578 cm^{-1} has been predicted to be in a hydrophobic environment and have negligible interactions with surrounding residues.[2a,17b] For alkaline Fe^{3+} heme-H20A, the upshift of ν(Fe−OH) and the absence of an observable D_2O shift in the RR measurements could indicate that OH^- is located in a polar environment and has strong H-bonding with its surrounding.[18] The possibility that the H-bond is between a dissociable group in the distal heme pocket and the oxygen atom of OH^- is excluded because this would weaken the Fe−OH bond and decrease ν(Fe−OH).[18,19] Instead, we propose that OH^- donates a H-bond to a nearby distal dissociable group and becomes oxo-like, leading to a higher ν(Fe−OH).[18] This oxo character could be partial at low temperatures because of a temperature-dependent shortening of the Fe−OH bond. This might be one reason for the multiple rhombic HS EPR signals observed earlier at 6 K.

We have also analyzed the mammalian HO-1 proximal His mutant H25A to clarify the identity of the ligand coordinated to its Fe^{3+} heme complex. Previous reports have proposed Glu as the axial ligand of Fe^{3+} heme-H25A at neutral pH.[6a,9] Our results show that its 5c HS acidic/neutral form converts to a different 5c HS alkaline form and this process is reversible (data not shown).[20] Cryogenic EPR measurements firmly establish H_2O as the axial ligand of Fe^{3+} heme-H25A at pH 7 (Supporting Information). OH^- is deduced to be the axial ligand of alkaline Fe^{3+} heme-H25A.

Overall, our study shows that, despite the absence of a proximal His/Im, heme cavities of the proximal His→Ala prokaryotic and eukaryotic HO mutants are still capable of ligand coordination. Having similar ligation states in neutral and alkaline environments, both 5c HO mutants have pH-dependent H_2O/OH^- transitions similar to their respective WT forms, indicating that the H_2O or OH^- is bound to the distal, not proximal, side of the Fe^{3+} heme. When bound with exogenous Im, both 5c HO mutants resume full catalytic activity.[1c,8] Hence, it is the absence of the proximal His ligand, not a collapse of the heme pocket structure, that is responsible for the inactivity of these HO proximal His mutants.

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Supporting Information Available: EPR spectra pf Fe^{3+} heme-H25A and Fe^{3+} heme-HO-1 WT (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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