Histidine 20, the Crucial Proximal Axial Heme Ligand of Bacterial Heme Oxygenase Hmu O from Corynebacterium diphtheriae*§

The hemin complex of Hmu O, a 24-kDa soluble heme degradation enzyme in Corynebacterium diphtheriae, is coordinated axially to a neutral imidazole of a proximal histidine residue in Hmu O. To identify which of the eight histidines in Hmu O is the proximal heme ligand, we have constructed and expressed the plasmids for eight His → Ala Hmu O mutants. Reconstituted with hemin, the active site structures and enzymatic activity of these mutants have been examined by EPR, resonance Raman, and optical absorption spectroscopy. EPR of the NO-bound ferrous heme-Hmu O mutant complexes reveals His$^{20}$ as the proximal heme ligand in Hmu O, and this is confirmed by resonance Raman results from the ligand-free ferrous heme-H20A. All eight His → Ala mutants bind hemo stoichiometrically, proving that none of the histidines is essential for heme-Hmu O formation. However, His$^{20}$ is crucial to Hmu O catalysis. Its absence by point mutation has inhibited the conversion of hemin to biliverdin. The ferric heme-H20A complex is pentacoordinate. Resonance Raman of the CO-bound ferrous heme-H20A corroborates this and reveals an Fe-C-O bending mode, δ(Fe-C-O)$^\dagger$, the first reported for a pentacoordinate CO-bound hemeprotein. The appearance of δ(Fe-C-O)$^\dagger$ in C. diphtheriae Hmu O H20A but not mammalian HO-1 mutant H25A indicates that the heme environment between the two heme oxygenases is different.

Pathogenic bacteria require free iron from their hosts to survive and multiply, and the degree of their virulence is dependent on free iron availability (1–4). Unfortunately for the invading pathogens, the majority of the host iron is intracellular, and free soluble iron is not readily available under physiological conditions (5). Various strategies have been developed by the pathogens to acquire iron, among which is the production of low molecular weight chelators called siderophores that bind soluble ferric iron with high affinity (6). Other iron uptake mechanisms utilize host iron compounds such as hemoglobin, hemopexin, and heme itself (3, 6). In these systems, the free heme or hemoeproteins are recognized by bacterial cell surface receptors, which transport heme groups through the cell outer membrane into the periplasmic space (3, 7–9). From there, periplasmic receptors bind to the heme and shuttle it to heme-specific permeases through which heme is transported into the cytoplasm. Once engulfed into the bacterial cell interior, iron may be liberated from the hemoeprotein by a heme degrading enzyme (3, 10, 11). In the pathogen Corynebacterium diphtheriae, the causative agent of diphtheria, Hmu O, a prokaryotic heme oxygenase expressed from the hmu O gene, has been identified to be responsible for heme degradation (11).

The 24-kDa soluble Hmu O catalyzes the oxygen-dependent conversion of hemin (iron protoporphyrin IX$a$) to biliverdin IX$a$, CO, and free iron in the presence of an electron donor (12, 13). This conversion occurs in three consecutive mono-oxygenation cycles with α-meso-hydroxyheme and verdoxheme as intermediates (13). The Hmu O-catalyzed heme degradation pathway is analogous to that of mammalian heme oxygenase (HO)$^3$ isozymes 1 and 2 (14–18). Although crystallization and preliminary x-ray diffraction analysis of the heme-Hmu O complex have been reported (19), information about the structure of Hmu O and its heme derivatives remains scarce. Via a multitude of spectroscopic techniques, the proximal ligand of the heme-Hmu O complex has been established as a neutral imidazole of a histidine residue (20), but the identity and the role of this histidine residue remain to be determined.

The primary sequence of Hmu O, only approximately 30% identical to the first 221 amino acids of the 33-kDa human HO-1, contains eight histidines (11), one of which is located within an 18-residue stretch that is highly similar to the region between Leu$^{129}$ and Val$^{146}$ of eukaryotic HO-1 upon the alignment of Hmu O His$^{128}$ to HO-1 His$^{132}$ (11). Because His$^{132}$ of HO-1 has been determined not to be the axial ligand of the heme iron (21), by analogy, it is unlikely that His$^{128}$ of Hmu O is the proximal axial ligand either. Of the remaining seven histidines in Hmu O, His$^{20}$ is most likely the proximal ligand because of its aligned position to His$^{25}$ of HO-1, which is the proximal axial ligand of mammalian HO-1 (22, 23).

To identify which histidine is the proximal ligand of heme-Hmu O, we have made eight Hmu O mutants, each having one...

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1 The abbreviations used are: HO, heme oxygenase; Im, imidazole.
of the eight histidine residues replaced by Ala. By EPR, resonance Raman, and optical absorption spectroscopy, we have examined each of their active site structure and enzymatic activity. Here we report that His20 is the proximal iron ligand of heme-Hmu O. Although none of the eight histidines is essential to the formation of the heme-Hmu O complex, His20 is important to Hmu O catalysis and heme catabolism. We also find the heme complex of the H20A mutant (heme-H20A) to be pentacoordinate, the ferrous CO form of which gives indications of structural differences in the distal heme pocket as compared with its mammalian counterpart.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids of His → Ala Mutants of Hmu O**—Construction of pMWHmu O, the synthetic expression plasmid for Hmu O wild type, is described elsewhere (13). The full-length synthetic hmu O gene was designed with unique restriction sites convenient for cassette mutagenesis. To synthesize the expression plasmid of mutant H20A, pMWHmu O H20A, the wild type pMWHmu O was excised with StuI and Apal, and the resulting fragment of the digest was gel purified. A BsiHKAI/Apal (75/566) fragment of pMWHmu O was prepared by digesting pMWHmu O with BsiHKAI and Apal and followed by gel purification. A synthetic 54-residue nucleotide, 5'-CTCTGCGTCGCGC/CTCTGAGTCGCGCAGGCAGAGCATGAAACGAGCA/CAAAG-3', containing the codon for Ala20, as indicated by the underlined bases, was phosphorylated with T4 polynucleotide kinase. Then it was annealed with its phosphorylated complementary nucleotide to make a double-stranded DNA. The 5' and 3' ends of this double-stranded DNA were ligated to the StuI site of the larger StuI/Apal fragment and the BsiHKAI site of the BsiHKAI/Apal fragment, respectively. Finally, this overall DNA was ligated to the Apal site of the StuI/Apal fragment to form pMWHmu O H20A.

Seven other oligonucleotides, 17–48 nucleotides in length, each with the codon for one of the seven His changed to that for Ala (e.g., H25A, H128A, H192A, H192A, and H205A) were synthesized. Procedures similar to those described above for the construction of pMWHmu O H20A, but at different restriction sites and with their corresponding endonucleases, were applied to produce pMWHmu O H25A, pMWHmu O H128A, pMWHmu O H129A, pMWHmu O H150A, pMWHmu O H162A, pMWHmu O H192A, and pMWHmu O H205A. All final coding sequences of these mutant plasmids were checked with an Applied Biosystems 310 DNA sequencer.

**Protein Expression and Purification**—Expression and purification of the Hmu O mutants, except H128A, are described elsewhere (13). All of the mutants were cultured in Escherichia coli in the same manner, but H128A was expressed in inclusion bodies, differing from the other mutants that were expressed in the soluble form. The extraction of H128A was similar to that of rat HO-1 mutant H133A (21). All protein expression and purity were analyzed by SDS-polyacrylamide gel electrophoresis on 10–20% gradient gels. The purified proteins were stored at 77 K until use.

**Preparation of the NO and CO Forms of Ferrous Heme-Hmu O Mutant Complexes**—Hemin reconstitution of each purified Hmu O His → Ala mutant was performed as described previously (13). The hemin complex of each mutant was reduced with sodium dithionite and ligated to the codon for one of the seven His changed to that for Ala (e.g., H128A, H192A, and H205A). All these final coding sequences of these mutant plasmids were checked with an Applied Biosystems 310 DNA sequencer.

**RESULTS AND DISCUSSION**

**Identification of the Proximal Iron Histidine Ligand by EPR and Resonance Raman Spectroscopy**—To identify which of the eight histidines in Hmu O was the proximal axial ligand of the heme-Hmu O complex, each Hmu O His → Ala mutant was first reconstituted with heme. All eight mutants bound hemin stoichiometrically, the implication of which will be addressed later in this paper. Reduced to the ferrous state, each of the heme-Hmu O mutant complexes was coordinated with 15NO, forming a NO-bound ferrous heme complex. NO is a powerful EPR probe of the electronic structure of heme and its surrounding environment because it has an unpaired electron and an intrinsic nuclear spin. The hyperfine interactions between the bound NO and the neighboring heme environment are detectable by EPR.

Of all the hem-Hmu O mutant complexes, only heme-H20A has a 15NO-bound ferrous form different from that of the wild type complex. The EPR spectrum of the 15NO-bound heme-H20A (Fig. 1, spectrum b) is characteristic of a pentacoordinate 15NO heme species (26), whereas that of Hmu O (Fig. 1, spectrum a) is of a hexacoordinate NO-bound heme species with an imidazole of a heme residue as an axial ligand (20, 24). As previously demonstrated (20), the triplet hyperfine splitting (A = 0.68 mT) associated with the g = −2.004 component in the 15NO spectrum of the heme-Hmu O wild type complex arises from the nuclear spin 1 of the 14N of the axial ligand trans to the bound NO. The noticeable absence of the triplet splitting in the 15NO heme-H20A spectrum indicates that one of the coordination ligands of the heme iron is vacant, producing a pentacoordinate species. Thus, one consequence of the point mutation of His20 to Ala is the loss of a nitrogenous base as an axial heme ligand. From these observations, we conclude that His20 is the proximal axial ligand of the heme-Hmu O complex.

Resonance Raman scattering of the ligand-free ferrous heme-Hmu O wild type complex (20) yields a band at 221 cm−1, a frequency unique to the Fe-N(His) stretching mode of pentacoordinate ferrous hemeproteins. A comparison of the reso-
nance Raman measurements of the ligand-free ferrous heme-H20A and heme-Hmu O complexes (Fig. 2, spectra b and a, respectively) shows no band at 221 cm$^{-1}$ for the ligand-free ferrous heme-H20A. The absence of this band is due to the mutation of His20 to Ala and the consequential loss of a proximal histidyl iron ligand. Thus, the resonance Raman results described here further substantiate that His20 is the proximal heme iron ligand for Hmu O.

Spectroscopic Characterization of Ferric Heme-H20A—The superposition of the optical absorption spectra for the ferric heme complexes of mutant H20A (Fig. 3, solid line) and Hmu O wild type (Fig. 3, broken line) indicates a blue shift in the spectrum of hemin-H20A. The latter has a Soret maxima at 402 nm and absorption bands in the visible region at 500 and 622 nm. These spectral positions show that the ferric iron of hemin-H20A is high spin, similar to the hemin complex of mammalian HO-1 mutant H25A (22, 23). The Soret band of hemin-H20A is broader and more reduced in intensity than that of hemin-Hmu O, illustrating a change in the heme environment of hemin-H20A, a change induced by the single mutation. Yet this difference between the Soret region of Hmu O and H20A is considerably less drastic than that between the heme complexes of mammalian HO-1 and HO-1 mutant H25A (22, 23). The harvested E. coli cells transformed with the H20A and H128A plasmids are pale brown, whereas those containing the other six mutants are green like the cultured cells containing the expressed Hmu O wild type (13).

Role of Proximal Iron Ligand His$^{20}$ on Hmu O Activity—As mentioned earlier, all eight Hmu O His$^{3}$Ala mutants bind hemin stoichiometrically to form hemin-enzyme complexes and have optical absorption spectra similar to that of hemin-H20A-hemin complex, whose heme iron is predominantly pentacoordinate high spin at neutral pH also (22).

Histidine 20, the Crucial Proximal Iron Ligand of Hmu O

FIG. 1. EPR spectra of the $^{15}$NO forms of the ferrous heme-Hmu O (spectrum a) and heme-H20A (spectrum b) complexes. Measurements were done at 25 K, 0.2 milliwatt microwave power, and 0.05 mT field modulation at 100 kHz.

FIG. 2. Low frequency region of the resonance Raman spectra of the ligand-free ferrous heme-Hmu O (spectrum a) and heme-H20A (spectrum b) complexes in 50 mM phosphate buffer, pH 7.

FIG. 3. Optical absorption spectra of the hemin-H20A (solid line) and hemin-Hmu O (broken line) complexes in 0.1 M phosphate buffer, pH 7, at 20 °C.

$^{15}$N(NO): A = 3.0 mT

$^{14}$N(His): A = 0.68 mT

$g_1 = 2.082$

$g_2 = 2.004$

$g_2 = 1.966$

$g_1 = 2.004$

$g_2 = 2.004$

$g_2 = 2.004$

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the consequence of which is the degradation of heme to biliverdin. Hence, the source of the green pigment in the cultured E. coli cells we have observed is biliverdin formed from the degradation of endogenous heme as catalyzed by the expressed Hmu O mutant protein and an E. coli reductase system such as flavodoxin-flavodoxin reductase. The absence of His(A), His(B), His(C), His(D), or His(E) by point mutation has not adversely affected the catalytic activity of the corresponding six mutants, thereby demonstrating that each of these particular six histidines by itself is not crucial to Hmu O catalytic activity. In contrast, the brown E. coli cells with the expressed H20A and H128A mutants would suggest that no biliverdin was formed, primarily because the expressed mutants are enzymatically inactive because of the single mutation of His to Ala.3

The catalytic efficacy of the mutants to bring about heme catabolism is evaluated further in terms of the rate of the formation of bilirubin, the decomposed product of biliverdin, and the single turnover of the bound hemin. Except H20A, the other seven Hmu O mutants yielded 700 nmol of bilirubin/mg of enzyme/h, comparable with Hmu O wild type. Only H20A generated no bilirubin. These results demonstrate that His20 is essential to bilirubin formation and, in general, to Hmu O catalysis. Previously (13) we have established that Hmu O, in the presence of oxygen and reducing equivalents from ascorbic acid, catalyzes the single turnover of heme-H20A with ascorbic acid, whereas that of heme-Hmu O is at 404 nm (B, spectrum a). After the addition of ascorbic acid, both Soret absorbances decrease with time. 2 h after the reaction started, the Soret of heme-Hmu O is replaced with a broad absorption band centered near 380 nm, and the absorbance at 680 nm increases concomitantly (B, spectrum b). In contrast, the Soret of heme-H20A remains and there is no distinct absorption band at 680 nm (A, spectrum b).

H20A mutant.

In both mammalian HO (18, 30) and prokaryotic Hmu O systems (13), H2O2 can substitute as both a source of reducing equivalents and dioxygen to support heme degradation partially and yield verdoheme as the final product. Fig. 6 presents a comparison between heme-H20A and heme-Hmu O when reacted with H2O2. In contrast to Hmu O (Fig. 6, A and B, respectively). In contrast to Hmu O (Fig. 6A, spectrum b), H20A reacts negligibly with H2O2, as indicated by the slight decrease of the Soret peak (Fig. 6A, spectrum b). No verdoheme is formed, as made evident by the lack of a verdoheme absorption peak at 680 nm (Fig. 6A, spectrum b).

The results described above demonstrate that the single mutation of His20 to Ala has altered the catalytic activity of Hmu O. This mutation might have caused protein unfolding, leading to activity dysfunction. To assess this possibility, we have measured the circular dichroism spectra of the heme complexes of Hmu O and mutant H20A and found them indistinguishable between 260 and 180 nm (data not shown). This result provides evidence that H20A has folded similarly to its wild type counterpart.

The substitution of an iron histidine or another metal ligand with exogenous Im has been carried out to restore catalytic activity to hemeproteins lacking a proximal ligand (31–33). In the case of heme-H20A, exogenous Im binds to the heme-H20A complex, and its coordination to the heme iron restores H20A

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3 In the case of H128A, however, the brown harvested cells may be because of its expression in inclusion bodies and consequent isolation from the bacterial reducing system.
Histidine 20, the Crucial Proximal Iron Ligand of Hmu O

activity. As illustrated in Fig. 7, the binding of exogenous Im to heme-H20A changes the initial absorption spectrum of the complex (Fig. 7, spectrum a) to one that resembles that of heme-Hmu O (Fig. 7, spectrum b). The Soret maxima, originally at 402 nm, has shifted to 404 nm. Reaction of the Im-bound heme-H20A with ascorbic acid under air results in heme degradation and yields an intermediate having an absorption spectrum with peaks at 400, 680, and 533 nm (Fig. 7, spectrum c). These peaks are characteristic of deoxy ferrous protoverdoheme IXa complexes (13, 34), and their appearances here suggest that a verdoheme complex has been formed from the Im-bound heme-H20A. As mentioned earlier, exogenous CO is capable of binding verdoheme to form a verdoheme-CO complex, the consequence of which is the arrest of the conversion of verdoheme to biliverdin (30, 35). When 100% CO is added to the reaction above, the resulting product renders an optical absorption spectrum (Fig. 7, spectrum d) with bands at 400, 533, and 635 nm, the latter of which is unique to verdoheme-CO (13, 30, 34, 35). Subsequently, when the CO is replaced with 100% O2, the spectrum of the reaction product, although still showing traces of residual verdoheme, loses the broad Soret band at 400 nm, and broad absorption bands centered near 380 and 680 nm appear (Fig. 7, spectrum e). The latter indicates that the initial heme has catabolized to biliverdin. These results altogether demonstrate that exogenous Im can restore the catalytic activity of H20A, specifically the conversion of heme to biliverdin with verdoheme as an intermediate. Moreover, they verify that the loss of enzymatic activity in H20A is not because of protein unfolding induced by a single mutation. If H20A had not folded properly, the heme degradation catalyzed by the Im-bound heme-H20A described above would not have taken place.

It must be noted that the amount of Im required to form an Im-bound heme-H20A complex that is capable of promoting catalytic turnover is significantly greater than that required for mammalian HO-1 mutant H25A. For the latter, only 50 equivalents of Im for 1 equivalent of heme-H25A is needed (33). In contrast, for 1 equivalent of heme-H20A, more than 3800 equivalents of Im are required. As stated earlier, the dissociation constant \( K_d \) for the binding reaction of heme-H20A to Im at pH 7 and 20 °C is \( \sim 27 \) mM. Thus, the binding affinity, as defined by \( K_d \), of Hmu O H20A for Im is significantly lower than that of mammalian HO-1 H25A. This finding provides further evidence that there is a structural difference between the heme pocket of Hmu O H20A and that of HO-1 H25A, an observation raised earlier in the comparison of the UV-VIS Soret regions of the heme complexes of both mutants with their respective wild type counterparts.

Overall, the results described above undeniably establish that His20 is essential to Hmu O catalysis. Previous studies on mammalian (18, 30) and prokaryotic heme oxygenases (13) have deduced the active intermediate in the first oxygenation step of HO catalysis, the conversion of heme to \( \alpha \)-hydroxyhe- min, to be a ferric hydroperoxide species. Because \( \alpha \)-hydroxyhemin is the immediate precursor to verdoheme, the inhibition of its formation consequentially prevents verdoheme formation also. The absence of verdoheme formation in the reaction of heme-H20A with reducing equivalents from ascorbic acid or \( \text{H}_2\text{O}_2 \) implies that His20 is specifically essential to the first oxygenation step of Hmu O catalysis.

Resonance Raman Spectroscopy of the CO Forms of Ferrous Heme-H20A—Further characterization of the structure of heme-H20A was obtained by studying its CO derivatives via resonance Raman spectroscopy. Despite its lack of enzymatic

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4 The spectrum of the Im-bound heme-H20A has a band at 530 nm, which is attributed to a slight trace of a low spin bis-Im complex.

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**FIG. 6.** Reactions of the hemin-H20A (A) and hemin-Hmu O (B) complexes with \( \text{H}_2\text{O}_2 \). The spectra were recorded immediately before the addition of \( \text{H}_2\text{O}_2 \) (A and B, spectra a, respectively) and 1, 5, 10, 20, 30, and 76 min (A and B, spectra b, respectively) after the addition of 3 equivalents of \( \text{H}_2\text{O}_2 \).

**FIG. 7.** Reaction of ascorbic acid with a heme-H20A complex ligated with exogenous imidazole. Sufficient Im is added to the initial heme-H20A complex (spectrum a) to form an Im-bound heme-H20A complex (spectrum b). This coordination is indicated in the shift of the Soret peak from the initial 402 nm to 404 nm. Reaction of the Im-bound heme-H20A with ascorbic acid under air causes heme degradation, as is evident by the decrease of the Soret absorbance, and yields an intermediate with an absorption peak at 680 nm (spectrum c). The addition of 100% CO to this intermediate results in a reaction product with a distinct absorption peak at 635 nm (spectrum d). Replacement of the CO with 100% \( \text{O}_2 \) yields a final product with broad absorption bands centered near 380 and 680 nm (spectrum e). The shoulder near 635 nm in the final reaction product spectrum is because of residual verdoheme. The total reaction period is 2 h.
activity, the H20A mutant allows CO coordination to its heme complex. The CO-bound ferrous heme-H20A, similar to its wild type counterpart, exhibits a resonance Raman spectrum (Fig. 8) with $v_2$ and $v_3$ lines at 1582 and 1497 cm$^{-1}$, respectively, indicating a CO-bound low spin heme (25). Three carbon isotope-sensitive bands are apparent for the CO-bound heme-H20A when the $^{12}$CO and $^{13}$CO (Fig. 8, spectra a and b, respectively) derivatives are compared. Following the mode assignment given to the CO adducts of HO-1 H25A (22) and Hmu O (20), the line at 1962 cm$^{-1}$ can be assigned to the C-O stretching mode ($v$(CO)), 568 cm$^{-1}$ to the Fe-C-O bending mode ($v$(Fe-C-O)), and 535 cm$^{-1}$ to the Fe-CO stretching mode ($v$(Fe-CO)) (Fig. 8, spectrum c). The positions of the $v$(CO) and $v$(Fe-CO) stretching modes are similar to those reported for the CO-bound H1-O H25A-heme complex (22).

When $v$(Fe-CO) is plotted against $v$(CO) for CO-bound hemeproteins, a linear correlation with a negative slope is observed (36–38). The CO complexes of hemeproteins possessing the same proximal ligand share the same slope. For the CO-bound heme-H20A, the position of its $v$(Fe-CO)$v$(CO) deviates significantly from the line formed for hemeproteins with a proximal histidine ligand. Instead it falls onto the line specific for pentacoordinate CO adducts with no proximal ligand (38). This result not only validates the absence of a proximal iron histidine in heme-H20A but also provides support that the CO derivative of heme-H20A is pentacoordinate.

The $v$(CO) stretching mode of the CO derivatives of hemeproteins is sensitive to the environment of the bound CO (36–38). Studies on the CO complexes of myoglobin and its mutants having a neutral imidazole of histidine as a proximal ligand have shown that $v$(CO) can serve as a gauge of the electrostatic fields in the CO-binding site, which is the distal pocket in myoglobin (39). In the case of the CO-bound heme-Hmu O, where the CO ligand is in the distal heme pocket, its $v$(CO) is at 1962 cm$^{-1}$ (20). The $v$(CO) of the CO derivative of heme-H20A is nearly the same. This implies that the heme environment about the CO ligand is similar in both H20A and Hmu O. Hence, we deduce that CO is coordinated to heme-H20A in the distal pocket of H20A, as is the case in Hmu O.

The distinct $\delta$(Fe-C) bending mode at 568 cm$^{-1}$ for mutant H20A is the first reported for a pentacoordinate CO-bound hemeprotein. Typically, it has not been observed in pentacoordinate heme-CO complexes because the Fe-C-O moiety assumes a linear conformation. The detection of an $\delta$(Fe-C-O) bending mode in H20A suggests that CO is coordinated to the heme iron in a nonlinear geometry. It is also possible that the environment about the bound CO has lost its 4-fold symmetry in terms of the electrostatic field, and under this circumstance, the $\delta$(Fe-C-O) bending mode can appear (38). Previous work by Loehr and co-workers (22) on mammalian HO-1 mutant H25A showed no $\delta$(Fe-C-O) bending mode, thus providing further evidence that its heme environment, in particular the distal pocket, is different from that of Hmu O H20A. Possibly in the case of H25A, the heme iron is protruded from the porphyrin plane toward the proximal pocket because the proximal axial His$^{25}$, which may be considered as an anchor that retains the heme iron within the porphyrin plane, is absent. Consequently, this causes the CO ligand to interact less with neighboring distal amino acids. In contrast, the single mutation of His$^{20}$ to Ala in Hmu O does not appear to have protruded the heme iron because the CO adduct of ferrous heme-Hmu O wild type has an $\delta$(Fe-C-O) bending mode (20) akin to that of mutant H20A. In both cases, the bound-CO conformation may be nonlinear and/or the distal environment about the CO is asymmetric and perturbed electrostatically. With neither effect present in HO-1 mutant H25A, as evident by the lack of an observable $\delta$(Fe-C-O) frequency, further investigation of the heme environments of Hmu O and mammalian HO is necessary to resolve the differences found in this study.

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REFERENCES

1. Woolridge, K. G., and Williams, P. H. (1993) *FEMS Microbiol. Rev. 12*, 325–348.
2. Tao, X., Schiering, N., Zeng, H., Ringe, D., and Murphy, J. R. (1994) *Mol. Microbiol. 14*, 191–197.
3. Lee, B. C. (1995) *Mol. Microbiol. 18*, 383–390.
4. Bullo, J. J., and Griffiths, E. (1999) *Iron and Infection* (Bullen, J. J., and Griffiths, E., eds) 2nd Ed., John Wiley & Sons, Ltd., Chichester, UK.
5. Otto, B. R., Verweij-van Vught, A. M. J. M., and MacLaren, D. M. (1992) *Crit. Rev. Microbiol. 18*, 217–231.
6. Griffiths, E., and Williams, P. (1999) in *Iron and Infection* (Bullen, J. J., and Griffiths, E., eds) 2nd Ed., pp. 87–212, John Wiley & Sons, Ltd., Chichester, UK.
7. Stojiljkovic, I., and Hancke, K. (1992) *EMBO J. 11*, 4359–4367.
8. Mills, M., and Payne, S. M. (1995) *J. Bacteriol. 177*, 3004–3009.
9. Braun, V. (1997) *Biol. Chem. 378*, 779–786.
10. Stojiljkovic, I., and Hancke, K. (1994) *Mol. Microbiol. 13*, 719–732.
11. Wilks, A., and Schmitt, M. P. (1997) *J. Biol. Chem. 272*, 835–841.
12. Chu, G. C., Katakurka, K., Zhang, X., Yoshida, T., and Ikeda-Saito, M. (1999) *J. Biol. Chem. 274*, 21319–21325.
13. Tenhunen, R., Harver, H. S., and Schmid, R. (1989) *J. Biol. Chem. 244*, 6388–6394.
14. Yoshida, T., and Kikuchi, G. (1978) *J. Biochem. 53*, 4224–4229.
15. Yoshida, T., and Kikuchi, G. (1979) *J. Biol. Chem. 254*, 4487–4491.
16. Maines, M. D. (1988) *FASEB J. 2*, 2557–2568.
17. Ishikawa, K., Takeuchi, N., Takahashi, S., Matera, K. M., Sato, M., Shibahara, S., Rousseau, D. L., Ikeda-Saito, M., and Yoshida, T. (1995) *J. Biol. Chem. 270*, 6345–6350.
18. Ishikawa, K., Park, S. Y., Shire, Y., Yoshida, T., and Ikeda-Saito, M. (1999) *J. Biol. Chem. 274*, 24490–24496.
19. Mansfield Matera, K., Zhou, H., Migita, C. T., Hubert, S. E., Ishikawa, K., Katakurka, K., Maeshima, H., Yoshida, T., and Ikeda-Saito, M. (1997) *Biochemistry 36*, 4909–4915.
20. Sun, J., Loehr, T. M., Wilks, A., and Ortiz de Montellano, P. R. (1994) *Biochemistry 33*, 13745–13746.
21. Ito-Maki, M., Ishikawa, K., Matera, K. M., Sato, M., Ikeda-Saito, M., and Yoshida, T. (1995) *Arch. Biochem. Biophys. 317*, 253–258.
22. Takahashi, S., Wang, J., Rousseau, D. L., Ishikawa, K., Yoshida, T., Host, J. R., and Ikeda-Saito, M. (1994) *J. Biol. Chem. 269*, 1010–1014.
23. Takahashi, S., Wang, J., Rousseau, D. L., Ishikawa, K., Yoshida, T., Takeuchi, N., and Ikeda-Saito, M. (1994) *Biochemistry 33*, 5531–5538.
24. Kon, H. (1976) *Biochim. Biophys. Acta 379*, 103–113.
25. Spiro, T. G., Stong, J. D., and Stein, P. (1979) *J. Am. Chem. Soc. 101*, 2648–2655.
26. Smulevich, G., Mauro, J. M., Fishel, L. A., English, A. M., Kraut, J., and Spiro,
Histidine 20, the Crucial Proximal Iron Ligand of Hmu O

T. G. (1988) *Biochemistry* **27**, 5477–5485
29. Jenkins, C. M., and Waterman, M. R. (1994) *J. Biol. Chem.* **269**, 27401–27408
30. Wilks, A., and Ortiz de Montellano, P. R. (1993) *J. Biol. Chem.* **268**, 22357–22363
31. den Blaauwen T., and Canters, G. W. (1993) *J. Am. Chem. Soc.* **115**, 1121–1129
32. De Pillis, G. D., Decatur, S. M., Barrick, D., and Boxer, S. G. (1994) *J. Am. Chem. Soc.* **116**, 6981–6982
33. Wilks, A., Sun, J., Loehr, T. M., and Ortiz de Montellano, P. R. (1995) *J. Am. Chem. Soc.* **117**, 2925–2926
34. Takahashi, S., Matera, K. M., Fujii, H., Zhou, H., Ishikawa, K., Yoshida, T., Ikeda-Saito, M., and Rousseau, D. L. (1997) *Biochemistry* **36**, 1402–1410
35. Yoshida, T., Noguchi, M., and Kikuchi, G. (1982) *J. Biol. Chem.* **257**, 9545–9548
36. Uno, T., Nishimura, Y., Tsuhsi, M., Makino, R., Izuka, T., and Ishimura, Y. (1987) *J. Biol. Chem.* **262**, 4549–4556
37. Yu, N.-T., and Kerr, E. A. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., ed) Vol. 3, pp. 39–95, John Wiley & Sons, Inc., New York
38. Ray, G. B., Li, X.-Y., Ibers, J. A., Sessler, J. L., and Spiro, T. G. (1994) *J. Am. Chem. Soc.* **116**, 162–176
39. Phillips, G. N., Teodora, M. L., Li, T., Smith, B., and Olson, J. S. (1999) *J. Phys. Chem. B* **103**, 8817–8829