Hmu O, a heme degradation enzyme in Corynebacterium diphtheriae, forms a stoichiometric complex with iron protoporphyrin IX and catalyzes the oxygen-dependent conversion of hemin to biliverdin, carbon monoxide, and free iron. Using a multitude of spectroscopic techniques, we have determined the axial ligand coordination of the heme-Hmu O complex. The ferric complex shows a pH-dependent reversible transition between a water-bound hexacoordinate high spin neutral pH form and an alkaline form, having high spin and low spin states, with a pK_a of 9. 1H NMR, EPR, and resonance Raman of the heme-Hmu O complex establish that a neutral imidazole of a histidine residue is the proximal ligand of the complex, similar to mammalian heme oxygenase. EPR of the deoxy cobalt porphyrin IX-Hmu O complex confirms this proximal histidine coordination. Oxy cobalt-Hmu O EPR reveals a hydrogen-bonding interaction between the O_2 and an exchangeable proton in the Hmu O distal pocket and two distinct orientations for the bound O_2. Mammalian heme oxygenase has only one O_2 orientation. This difference and the mixed spin states at alkaline pH indicate structural differences in the distal environment between Hmu O and its mammalian counterpart.

Biological heme degradation reactions in mammalian systems are catalyzed by a class of enzymes termed “heme oxygenase” (HO)(1–2). Mammalian amphipathic microsomal heme oxygenase isoforms, HO-1 and HO-2, catalyze the regiospecific oxidative degradation of iron protoporphyrin IX (heme hereafter) to biliverdin IXα, iron, and CO in the presence of NADPH-cytochrome P450 reductase, which serves as an electron donor (1–4). HO is not a hemeprotein by itself but utilizes heme as both a prosthetic group and a substrate, a property unique to this enzyme. In the HO catalytic cycle, HO first binds 1 equivalent of heme to form a hemin-HO complex. The first electron donated from the reductase reduces the hemin iron to the ferrous state. Then O_2 binds to form a metastable oxy complex. Further electron donation to the oxy complex initiates the three-step conversion of oxoheme to the ferric iron-biliverdin complex through the intermediates a-hydroxyxem and verdoxheme (2, 3, 5). In mammalian systems, HO is the enzyme responsible for excess hemin excretion and iron recycling (6). The product CO has been reported as a messenger molecule in vasodilation and neuronal transmission by activating soluble guanylyl cyclase under pathophysiological conditions (7–9).

The presence of heme degradation enzymes in pathogenic bacteria has been reported through genetic studies (10–12). Iron is essential for the survival of most bacteria. To circumvent the low concentration of free extracellular iron, bacteria have developed mechanisms by which they acquire iron from iron-containing proteins found in their hosts (10–12). In the pathogen Corynebacterium diphtheriae, the causative agent of diphtheria, a gene, hmu O, which encodes a 24-kDa protein responsible for heme utilization, has been discovered (12).

Hmu O, the hmu O-coded protein, is the first bacterial heme degradation enzyme isolated to date. Wilks and Schmitt (13) have constructed an Escherichia coli expression system of Hmu O and purified a 24-kDa protein that stoichiometrically binds hemin and converts it to biliverdin IXα and CO upon the addition of electron donors. Recently, we have demonstrated that a-meso-hydroxyheme and verdoxheme are intermediates of Hmu O catalysis (14). Although crystallization and preliminary x-ray diffraction analysis of the heme-Hmu O complex have been done to date (15), little is known about the Hmu O active-site structure. The electronic state and the coordination structure of the heme group of the complex have not been established. Elucidation of this is essential to understanding Hmu O catalytic mechanisms.

This paper reports the first axial coordination structure of the heme-Hmu O complex. By optical absorption, resonance Raman, EPR and NMR spectroscopy, we have examined the heme and cobalt porphyrin complexes of a purified, recombinant Hmu O expressed from a synthetic gene in E. coli (14). We have established that the proximal ligand of the heme-Hmu O complex is a neutral imidazole group of a histidine residue. EPR studies of the cobalt porphyrin complex show a hydrogen-bond formation between the bound O_2 and an exchangeable proton in the distal pocket of Hmu O. Although there exist some similarities between Hmu O and mammalian HO, we have found that the structure of the Hmu O distal pocket is different from that of mammalian HO.

**EXPERIMENTAL PROCEDURES**

Expression and purification of the recombinant Hmu O and its reconstitution with hemin are described elsewhere (14). The ferrous NO complexes were obtained as described previously (16). The cobalt porphyrin-Hmu O (cobalt-Hmu O) complex was prepared as described.
have confirmed the pH-dependent spin state changes deduced from the aforementioned optical absorption measurements. In the region of the Raman spectrum containing the porphyrin skeletal modes are lines whose frequencies characterize the spin and coordination states of the hemin iron (18). Specifically, the lines assigned as $v_2$ and $v_3$ are located in the regions of 1580–1590 and 1500–1510 cm$^{-1}$ for hexacoordinate low spin iron, 1560–1565 and 1475–1485 cm$^{-1}$ for hexacoordinate high spin iron, and 1570–1575 and 1490–1500 cm$^{-1}$ for pentacoordinate high spin iron. As shown in spectrum A of Fig. 2, at pH 7, the $v_2$ and $v_3$ for hemin-Hmu O are 1563 and 1483 cm$^{-1}$, respectively, indicating a hexacoordinate high spin iron. At pH 9 (spectrum B, Fig. 2), these high spin Raman lines become weaker as the lines characteristic of a hexacoordinate low spin iron become apparent, as indicated by the $v_2$ and $v_3$ lines at 1579 and 1504 cm$^{-1}$, respectively. The presence of both high spin and low spin Raman lines in the pH 9 spectrum is consistent with the results of the optical absorption spectra described above.

EPR spectra of the ferric heme-Hmu O complex are shown in Fig. 3. The complex at pH 7 (spectrum A) exhibits an EPR spectrum of an axial symmetry typical of ferric high spin hemeproteins ($g = 6$ and 2). The $g = 2$ region spectrum recorded in buffered H$_2$O (45% enrichment) is compared with that in H$_2^{18}$O in the inset of Fig. 3. Substitution of H$_2^{18}$O with the partially enriched H$_2^{17}$O induces a broadening of the g = 2 signal. This broadening is due to the hyperfine interaction caused by the nuclear spin 5/2 of the iron-bound$^{17}$O. A similar phenomenon has been observed in metmyoglobin (metMb), in which H$_2$O is coordinated to the hemin iron as the sixth ligand (19). However, such a broadening has not been observed in pentacoordinate horseradish peroxidase because of the absence of a coordinated H$_2$O (19, 20). Thus, our EPR results show that the sixth ligand of the heme-Hmu O complex is H$_2$O.

The EPR spectrum of hemin-Hmu O at pH 9 (spectrum B, Fig. 3) demonstrates both high spin and low spin species. Consistent with the aforementioned optical absorption and resonance Raman results, the high spin species is the same as that at pH 7. However, two types of low spin forms are detected. The major form is characterized by g values of 2.67, 2.21, and 1.80, whereas the minor form has g values of 2.72, 2.16, and 1.76. The former set of g values is close to that of the OH-bound low spin species reported for the HO-1 complex (14), the ferric iron of the hemin-Hmu O complex has been postulated to be hexacoordinate high spin. At alkaline pH (spectrum B, Fig. 1), the intensity of the Soret and visible peaks decreases, and new peaks at 540 and 575 nm emerge. The latter peaks are similar to those of the low spin hydroxide form of the mammalian HO-hemin complex (16). The pH-dependent spectral change observed is reversible between pH 6 and 10 and characterized by a $pK_a$ of 9.0, as shown in the inset of Fig. 1. In contrast to mammalian HO, which is predominantly in the hydroxide-bound (OH-bound) low spin form at alkaline pH, the hemin-Hmu O complex still retains a partially high spin form.

**RESULTS**

**EPR Forms of the Heme-Hmu O Complex**—Fig. 1 shows the pH-dependent changes in the optical absorption spectrum of the hemin-Hmu O complex between pH 6 and 10. At pH 6 (spectrum A, Fig. 1), the complex has a Soret peak at 404 nm and bands at 500 and 630 nm. The spectrum is similar to that of the hemin complex of mammalian HO (16). Based on the position (404 nm) and the extinction coefficient ($150 \text{mM}^{-1}\text{cm}^{-1}$) of the Soret peak (14), the ferric iron of the hemin-Hmu O complex has been postulated to be hexacoordinate high spin. At alkaline pH (spectrum B, Fig. 1), the intensity of the Soret and visible peaks decrease, and new peaks at 540 and 575 nm emerge. The latter peaks are similar to those of the low spin hydroxide form of the mammalian HO-hemin complex (16). The pH-dependent spectral change observed is reversible between pH 6 and 10 and characterized by a $pK_a$ of 9.0, as shown in the inset of Fig. 1. In contrast to mammalian HO, which is predominantly in the hydroxide-bound (OH-bound) low spin form at alkaline pH, the hemin-Hmu O complex still retains a partially high spin form.

**Ferric Forms of the Heme-Hmu O Complex**—Fig. 1 shows the pH-dependent changes in the optical absorption spectrum of the hemin-Hmu O complex between pH 6 and 10. At pH 6 (spectrum A, Fig. 1), the complex has a Soret peak at 404 nm and bands at 500 and 630 nm. The spectrum is similar to that of the hemin complex of mammalian HO (16). Based on the position (404 nm) and the extinction coefficient ($150 \text{mM}^{-1}\text{cm}^{-1}$) of the Soret peak (14), the ferric iron of the hemin-Hmu O complex has been postulated to be hexacoordinate high spin. At alkaline pH (spectrum B, Fig. 1), the intensity of the Soret and visible peaks decrease, and new peaks at 540 and 575 nm emerge. The latter peaks are similar to those of the low spin hydroxide form of the mammalian HO-hemin complex (16). The pH-dependent spectral change observed is reversible between pH 6 and 10 and characterized by a $pK_a$ of 9.0, as shown in the inset of Fig. 1. In contrast to mammalian HO, which is predominantly in the hydroxide-bound (OH-bound) low spin form at alkaline pH, the hemin-Hmu O complex still retains a partially high spin form.

**FIG. 2.** Resonance Raman spectra of the hemin-Hmu O complex in 50 mM phosphate buffer, pH 7 (spectrum A), and 50 mM borate buffer, pH 9 (spectrum B), in the 1300–1750 cm$^{-1}$ region.

**FIG. 1.** Optical absorption spectra of the hemin-Hmu O complex between pH 6 (spectrum A) and 10 (spectrum B) at 20 °C. Inset, fraction of the alkaline form calculated from pH-dependent changes in absorbance at 404 nm. The symbols (C) are experimental values, and the curve is drawn by a least-square fitting to the $n = 1$ Henderson-Hasselbalch equation.
amplitude of the high spin species is reduced, whereas that of
the hydroxide low spin species is increased (data not shown).

In Fig. 4, the resolved portion of the $^1$H NMR spectrum of the
hemin-Hmu O complex in 90% H$_2$O and 10% D$_2$O is compared
with that of metMb. The NMR spectrum of the Hmu O complex
has resolved signals at 75, 74, 67, and 62 ppm with areas of
three protons. These are manifestations of the heme methyl
groups. The chemical shift pattern of these methyl peaks is
different from that of metMb, which exhibits heme methyl
resonances from 52 to 90 ppm (89.8, 83.3, 72.0, and 52.4 ppm).

Different from Hmu O, the rat HO-1 hemin complex reported
by La Mar and co-workers (21) has a complicated hyperfine-
shift pattern in the 60–90-ppm region because of the hemin-
orientational disorder about the $\alpha, \gamma$-meso axis.

For metMb, the single exchangeable proton resonance at 102
ppm is assigned to a proximal histidine (His) imidazole N$_1$H
(22). In the hemin-Hum O spectrum, a broad single proton
resonance is discerned at 99 ppm. This signal is absent when the
measurement is conducted in buffered D$_2$O (data not shown). The appearance of the single proton peak in H$_2$O but
not in D$_2$O solution attests that it is an exchangeable proton
strongly coupled to the ferric iron but not associated with the
porphyrin itself. Because the chemical shift is similar to that
for the axial His imidazole of metMb, this resonance at 99 ppm
for the hemin-Hmu O complex is deduced to be from the prox-
imal imidazole N$_1$H proton. The implication of this deduction is
that the proximal ligand of the heme iron in Hmu O is an

**Fig. 3.** EPR spectra of the hemin-Hmu O complex at pH 7
(spectrum A) and 9 (spectrum B). The inset of the pH 7 spectrum (A)
shows EPR spectra of the $g = 2$ signal of hemin-Hmu O in H$_2$O$_{16}$O (solid
line) and H$_2$O$_{17}$O (broken line). For the pH 9 spectrum (B), the low spin
region is expanded 4-fold. All pH 7 spectra were recorded at 5 K, 1 mW
microwave power, and 0.1 mT field modulation at 100 kHz, whereas the pH
9 spectrum was at 8 K and 0.2 mW.

**Fig. 4.** 600-MHz $^1$H NMR spectra of metMb and hemin-Hmu O
in 90% H$_2$O, 10% D$_2$O of 0.1 M phosphate buffer, pH 7, at 25 °C.
Peaks indicative of heme methyl groups are labeled a.

**Fig. 5.** EPR spectra of the $^{14}$NO (spectrum A) and $^{15}$NO (spec-
trum B) forms of the ferrous heme-Hmu O complex and of the
$^{15}$NO form of the ferrous heme-HO complex (spectrum C). Measure-
ments were done at 25 K, 0.2 mW microwave power, and 0.05 mT
field modulation at 100 kHz.

imidazole of a His residue.

**Ferrous Forms of the Heme-Hmu O Complex**—EPR spectra of the
nitric oxide ($^{14}$NO and $^{15}$NO)-bound ferrous heme-Hmu O
complexes are shown in spectra A and B of Fig. 5, respectively,
along with the spectrum of the $^{15}$NO-bound rat HO-1 complex
(spectrum C, Fig. 5). The Hmu O complexes have spectra typi-
cal of NO hemeproteins with rhombic symmetry ($g_1 = 2.082, g_2$
A comparison of the $^{14}$NO and $^{15}$NO spectra demonstrates that the doublet splitting associated with a coupling constant of 3.0 mT shown in the $^{15}$NO spectrum is due to the nuclear spin 1/2 of the $^{15}$N of the bound $^{15}$NO. Thus, the triplet hyperfine splitting ($A = 0.68$ mT) associated with the $g = 2.004$ component arises from the nuclear spin 1 of the $^{14}$N of the axial ligand trans to the bound NO (23). This firmly establishes that the proximal ligand of the heme-Hmu O complex is a nitrogenous base, most likely an imidazole group of His. Further support of this is given by the resonance Raman spectrum of the ligand-free ferrous heme-Hmu O (Fig. 6), which shows a line at 221 cm$^{-1}$, the characteristic frequency of an iron-histidine stretching mode in pentacoordinate ferrous hemeproteins (24). For the rat HO-1 complex, the iron-histidine stretching mode is observed at 218 cm$^{-1}$ (25). These results on the ferrous forms of the enzyme are consistent with the imidazole ligation deduced from NMR of the ferric form described above.

The CO-bound form of the ferrous heme-Hmu O complex exhibits a resonance Raman spectrum (Fig. 7) with $v_2$ and $v_3$ lines at 1583 and 1498 cm$^{-1}$, respectively, indicating a CO-bound low spin heme. Three carbon isotope-sensitive bands are found for the CO-bound Hmu O complex when the $^{12}$CO and $^{13}$CO (spectra A and B, Fig. 7, respectively) derivatives are compared. Following the previous mode assignment done on the CO-bound rat HO-1 complex (25), the line at 508 cm$^{-1}$ can be assigned to the Fe-CO stretching mode, 572 cm$^{-1}$ to the Fe-C-O bending mode, and 1962 cm$^{-1}$ to the C-O stretching mode (spectrum C, Fig. 7).

Cobalt Porphyrin-Hmu O Complex—EPR of the deoxy form of the cobalt porphyrin-Hmu O complex (cobalt-Hmu O) displays a spectrum of axial symmetry typical of a pentacoordinate cobalt(II) complex with a nitrogenous base axial ligand (spectrum not shown). The $g_{//}$ signal of deoxy-cobalt-Hmu O has an octuplet hyperfine structure due to the hyperfine interaction with the $^{59}$Co nucleus. Each of the hyperfine lines is split further into a triplet because of the hyperfine interaction with a nitrogen atom in the proximal axial ligand. The $g$ values and hyperfine coupling constants are estimated as $g_{//} = 2.33$, $g_{\perp} = 2.03$, $A_{//}(^{59}\text{Co}) = 7.8$ mT, and $a_{\perp}(^{14}\text{N}) = 1.8$ mT.

As shown in spectrum C of Fig. 8, the EPR spectrum of oxy cobalt-Hmu O, recorded at 25 K, exhibits free radical-type absorption around $g = 2$, similar to other oxygenated forms of cobalt (II) complexes including cobalt-Mb and cobalt-HO (spectra A and B, Fig. 8, respectively) (17, 26). Consistent with the oxy forms of cobalt-Mb and cobalt-HO, cobalt-Hmu O dissolved in buffered $\text{D}_2\text{O}$ (spectrum D, Fig. 8) exhibits a narrower hyperfine structure than in buffered $\text{H}_2\text{O}$ (spectrum C, Fig. 8). In comparison to HO, the spacing and the amplitude of the hyperfine structure associated with the $g_{//}$ signal ($g \sim 2.1$) are
irregular in the cobalt-Hmu O spectrum. Based on EPR analyses of oxy cobalt-Mb, which show two paramagnetic species because of the different orientations of the bound O₂ (27), we attribute the oxy cobalt-Hmu O spectral irregularity to the two distinct bound O₂ orientations present in oxy cobalt-Hmu O. This is different from oxy cobalt-HO, which has only one bound O₂ orientation (26). Assuming two paramagnetic species for oxy cobalt-Hmu O, the following EPR parameters have been calculated by spectral simulation. For species I, \( g_1 = 2.100, g_2 = 2.008, \) and \( g_3 = 1.999 \), \( A_1 = 1.72 \text{ mT}, A_2 = 0.92 \text{ mT}, \) and \( A_3 = 0.85 \text{ mT}. \) For species II, \( g_1 = 2.108, g_2 = 2.008, \) and \( g_3 = 1.991, A_1 = 1.72 \text{ mT}, A_2 = 0.92 \text{ mT}, \) and \( A_3 = 0.85 \text{ mT}. \) Giving equal contribution to each of the paramagnetic species, our simulated EPR spectrum (spectrum E, Fig. 8) matches the experimental spectrum (spectrum C, Fig. 8). The two different orientations of the bound O₂ are almost equally populated.

**DISCUSSION**

**Axial Ligands and Coordination States**—EPR spectra of the ferrous NO complex of heme-Hmu O (Fig. 5) and deoxy cobalt-Hmu O (Fig. 8) demonstrate a nitrogenous base as the proximal ligand of Hmu O. Moreover, the single exchangeable \(^1\text{H} NMR signal at \( 99 \text{ ppm} \) in the hemin-Hmu O complex (Fig. 4) is attributable to the proximal His imidazole \( N_1 \text{H} \) and provides evidence of an imidazole coordination. Resonance Raman scattering of the ligand-free ferrous heme-Hmu O yields a band at 221 cm\(^{-1} \) (Fig. 6), a frequency unique to the iron-histidine stretching mode of pentacoordinate ferrous hemeproteins. The observations from these three different techniques conclusively establish that the proximal axial ligand of the heme-Hmu O complex is an imidazole of a His residue.

The amino acid sequence of Hmu O contains eight histidines (12), one of which is within an 18-residue stretch that is highly similar to the region between Leu-129 and Val-146 of eukaryotic HO-1 when His-126 of Hmu O is aligned to His-132 of HO-1 (12). Since His-132 of HO-1 has been determined not to be the axial ligand of the heme iron (28), it is unlikely that His-126 of Hmu O is the proximal axial ligand either. Of the remaining 7 histidines in Hmu O, His-20 is potentially the proximal ligand because of its aligned position to His-25 of HO-1, which is the proximal axial ligand of mammalian HO-1 (29, 30).

As described above, an imidazole group of a His is coordinated to the iron of the heme-Hmu O complex. Whether it is a neutral imidazole or an imidazolate anion can be assessed by the optical absorption and resonance Raman spectra described earlier, spectra that resemble those of other H₂O-bound hexacoordinate high spin hemeproteins. Based on the similarities of the low spin components distinguished in the optical absorption, resonance Raman, and EPR spectra of the hemin-Hmu O complex (Figs. 1–3, respectively) to those of rat HO-1 at alkaline pH, we infer that hydroxide is the axial ligand of the low spin Hmu O species at alkaline pH.

**Effect of pH on the Hemin-Hmu O Complex**—Acid-base transitions observed in ferric hemeproteins with a H₂O ligand are generally correlated to a change in the protonation status of an amino acid residue in the distal heme pocket. The distal pocket residue forms a hydrogen bond with the bound-H₂O ligand, and deprotonation of the distal residue, such as His in globins, causes ionization of the iron-bound H₂O. Consequently, this results in the hydroxide form (34). In mammalian HO-1, the group responsible for the ionization is not directly linked to the bound H₂O (25, 35) but is proposed to be by a long range hydrogen bond network via H₂O molecules. Our results on hemin-Hmu O show the presence of an ionizable group that is responsible for the acid-base transition. Because the observed \( pK_a \) of 9.0 for the hemin-Hmu O complex is higher than the \( pK_a \) values of 7.6 and 8.5 for mammalian HO-1 and HO-2, respectively (16, 36), ionization of the bound H₂O is less favorable in Hmu O. The distal environment responsible for the acid-base transition in Hmu O appears to be different from that in mammalian HO.

The pH titration plot of hemo-Hmu O (inset of Fig. 3) indicates that the transition to the alkaline form is nearly complete at pH 10 (theoretically >90% complete). However, the optical absorption spectrum taken at pH 10 has a considerable amount of the high spin species in addition to the low spin. The alkaline form of the Hmu O complex appears to be a statistical mixture of the low spin OH- and high spin H₂O-bound forms. This is different from mammalian HO isoforms, the alkaline forms of which are predominantly in the low spin state (16, 36). One possible reason for this mixed spin state in Hmu O is that the hydrogen bonding between the distal ionizable group and the bound H₂O is weaker than that in mammalian HO. Consequently, the deprotonation efficiency of the distal residue is reduced. The distal pocket structure of Hmu O, as mentioned above, is most likely different from that of eukaryotic HO isoforms.

**Interactions between Heme and Hmu O as Evaluated by \(^1\text{H} NMR**—The hemin-Hmu O complex exhibits four resolved heme methyl peaks in the low-field window, indicating a single dominant molecular species in solution. In contrast, the high spin NMR spectra of rat HO-1 and human HO-2 complexes show unresolved overlapping peaks between the 80–60 ppm region because of the heme orientational disorder about the \( \alpha, \gamma- \text{meso axis (21).} \) The initial assembly of rat HO-1 with heme yields an equal population of two species with different heme orientations about the \( \alpha, \gamma- \text{meso axis.} \) Even after equilibrium has been attained, the two isomers remain with the population ratio of 2:3 (21). For Hmu O, the presence of one dominant species suggests that the protein favors one particular orientation in its incorporation of heme. The mechanism by which the Hmu O apoprotein recognizes the most favorable heme orientation to yield a stable enzyme-hemin complex but a reactive species upon the oxidative addition of electrons remains to be elucidated.

In comparison to that of metMb, the heme methyl hyperfine shift pattern of hemo-Hmu O is less dispersed. Factors that affect the in-plane asymmetry in ferric high spin hemeproteins include zero-field splitting of the ferric iron and peripheral

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\(^{2}\) H. Fujii, and T. Yoshida, unpublished results.
heme-protein interactions (37, 38). Zero-field splitting is sensitive to the type of axial coordination (hexa or pentacoordinate) and varies little within a given coordination structure (38). Because both metMb and hemin-Hmu O have the same axial ligand structure, H₂O-Fe-neutral imidazole, the zero-field splitting of each is expected to be similar. Hence, zero-field splitting is not likely a major cause for the differences in the hyperfine shift pattern between Hmu O and metMb.

Heme-apoprotein interactions can affect the heme methyl shift by altering the electron-withdrawing ability of the 2-, 4-vinyl heme side chains. Resonance Raman measurements of heme-Hmu O show a band at 410 cm⁻¹ (Fig. 6). Because this is close to the mode at 411 cm⁻¹ for Mb (25), we interpret the heme-Hmu O band as the vinyl bending mode. Based on this, the conformation of the heme vinyl group in heme-Hmu O is expected to be similar to that in deoxy Mb. Thus, the electron-withdrawing ability of the heme vinyl group in heme-Hmu O is most likely the same as that in Mb.

Excluding zero-field splitting and peripheral heme-protein interactions, another possibility for the cause of the heme methyl hyperfine shift is the orientation of the proximal axial ligand. This is because the observed shifts of the β-pyrrole substituents are actually the balance of a large positive shift due to σ-delocalization and a smaller positive shift due to π-delocalization, since all five d orbitals are half-filled. Recently, Walker (39) suggested that the orientation of the axial imidazole strongly affects the methyl shift spread through electron donation from the filled dπ orbital of the high spin ferric iron. Consequently, this decreases the iron to pσ spin delocalization into the porphyrin ring. The smaller spin density distribution about the porphyrin in heme-Hmu O might result in a less dispersed methyl hyperfine shift. Thus, the orientation of the proximal imidazole ligand of heme-Hmu O might be different from that in Mb, thereby accounting for the less dispersed methyl hyperfine shift pattern in Hmu O. Because the heme methyls are not symmetrically placed, thereby interacting with the π orbitals of the heme to produce π spin densities that are dependent on the orientation of the proximal imidazole ligand (40), this orientation would affect the spread of the resonances.

Structure of the Oxy Form of Hmu O as Studied by Its Cobalt Derivative—When a paramagnetic ion is surrounded by other ions that have nuclear magnetic moments, such as protons, there is an appreciable magnetic dipole-dipole interaction. This interaction causes spectral line broadening. Deuteration can reduce the broadened line-width by a factor of 33% because of the conformation of the heme vinyl group in heme-Hmu O is expected to be similar to that in deoxy Mb.

In mammalian HO, the nature of the distal pocket plays a role in orienting the bound O₂ to a position that is favorable for the regiospecific hydroxylation of the α-meso carbon of the porphyrin ring and the subsequent formation of α-meso-hydroxyheme (26, 35, 44). Perhaps one of the two observed O₂ orientations for the oxy form of Hmu O is directed toward the α-meso position, whereas the other is away. The former would facilitate the transfer of the terminal oxygen atom to the α-meso carbon, hence its hydroxylation to form α-meso-hydroxyheme. In contrast, the latter orientation might perturb the hydrogen-bond interaction between the coordinated O₂ and a distal group in Hmu O so that the formation of the ferric hydroxy species is reduced or inhibited. This unfavorable O₂ orientation might explain the slower overall Hmu O catalytic rate in ascorbic acid-supported heme degradation and the low verdoheme recovery in the reaction of heme-Hmu O with H₂O₂ as compared with the corresponding reactions by mammalian HO (14).

One implication of the two O₂ orientations in the oxy form of Hmu O, in contrast to the single orientation in the oxy form of mammalian HO, is that there are structural differences between the distal pocket of Hmu O and that of its mammalian counterpart. The distal group(s) in Hmu O responsible for the orientation of the sixth ligand and the deprotonation of the peroxide in the H₂O₂ reactions described previously (14) and of the H₂O-bound hemin-Hmu O to the hydroxide form at alkaline pH could be different from that in mammalian HO.

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