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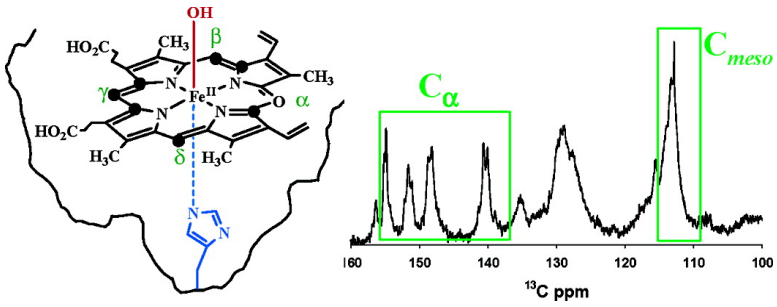

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The Ferrous Verdoheme−Heme Oxygenase Complex is Six-Coordinate and Low-Spin

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The degradation of heme in mammalian cells is catalyzed by the enzyme heme oxygenase (HO).[1] In the catalytic cycle of HO, the addition of one electron to the oxyferrous complex transforms the latter into an activated hydroperoxide (Fe^{III}−OOH) oxidizing species, which hydroxylates the α-meso carbon to produce α-meso hydroxyheme.[2,3] This species undergoes a subsequent O_2-dependent elimination of the hydroxylated α-meso carbon as CO, with the concomitant formation of verdoheme. Fe^{II}−verdoheme is then oxidized to Fe^{III}−biliverdin in a reaction that requires both O_2 and reducing agent.[2,4] To study the mechanism for the conversion of verdoheme to biliverdin, it is important to unambiguously establish the coordination state and electronic configuration of the verdoheme−HO complex. At present, there are two conflicting views regarding the coordination state of Fe^{II}−verdoheme: resonance Raman spectroscopic studies suggest that Fe^{II}−verdoheme is coordinated by a proximal histidine and a distal H_2O or OH^- ligand.[5] In contrast, an X-ray crystal structure suggests that the Fe^{II}−verdoheme complex is five-coordinate, with a proximal His as the sole axial ligand.[6]

Herein we report a novel biosynthetic and enzymatic approach for the efficient preparation of ^{13}C-labeled α-verdoheme, which facilitates the ^{13}C NMR spectroscopic characterization of the elusive verdoheme−HO complex. The ^{13}C NMR evidence indicates that Fe^{II}−verdoheme in HO is six-coordinate and diamagnetic ($S = 0$), a finding that underscores the chemical differences between ferrous heme and ferrous verdoheme and argues for the coordination of OH^- in the distal site.

The preparation of ^{13}C-labeled verdoheme starts with the biosynthesis of ^{13}C-labeled heme using methodology developed previously.[7,8] For the purposes of this study, we labeled heme at the meso (C_m) and pyrrole-α (C_α) carbons (Figure 1) to take advantage of the relatively straightforward correlations between core porphyrin carbon chemical shift and the coordination state and electronic configuration of metalloporphyrins.[8−11] Hence, addition of 5-^{13}C-aminolevulinic acid to a growing culture of *E. coli* expressing a gene coding for outer mitochondrial membrane cytochrome b_5 (OM b_5) results in the accumulation of OM b_5 harboring ^{13}C-labeled heme.[7] ^{13}C-Labeled heme is then extracted[12] from OM b_5 and reconstituted into HO from *Neisseria meningitides* (*nm*-HO). The ^{13}C-labeled heme *nm*-HO complex (3.5 mL, 0.5 mM, in deuterated phosphate buffer $\mu = 0.1$, pH 7.6) was quantitatively oxidized to Fe^{III}−α-verdoheme−HO by the addition of 8 equiv of H_2O_2. The Fe^{III}−α-verdoheme−HO complex, which exhibits a band at 666 nm (Figure S1), is stable for a few hours in the presence of air, if unreacted H_2O_2 is eliminated by the addition of catalase. However, the intensity of the 666 nm band decreases significantly if the complex is left overnight, whether O_2 is present or not. The product of this slow decomposition has not been characterized, but it is not biliverdin. To avoid decomposition, it is important to reduce this complex to Fe^{II}−α-verdoheme−HO, which was accomplished by the addition of 3 equiv of sodium ascorbate under strict anaerobic conditions. Formation of the Fe^{II}−α-verdoheme−HO complex is accompanied by a red shift of the visible band to 690 nm (Figure S1). The Fe^{II}−α-verdoheme−HO complex (verdo-HO hereafter), which is stable in the absence of O_2, was concentrated to a final volume of 0.5 mL by continuous purging with argon, transferred to an NMR tube under anaerobic conditions, and then sealed. If argon is replaced by CO, the corresponding Fe^{II}−CO complex is formed (verdo-HO−CO), which exhibits a UV−vis spectrum with a peak at 637 nm (Figure S1).

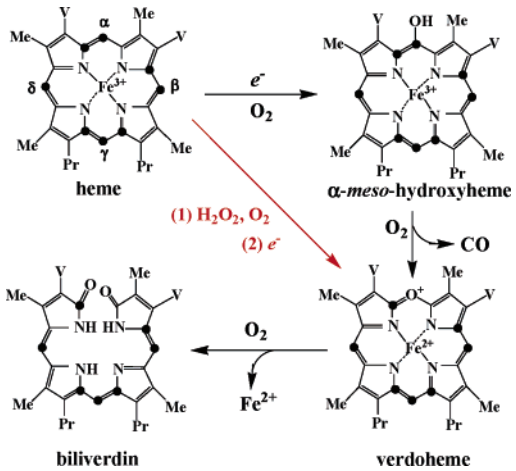

Figure 1. Oxidation of heme to biliverdin by HO. H_2O_2 oxidizes heme-HO to hydroxyheme-HO, which is rapidly converted to FE^{III}−verdoheme−HO by O_2. Ascorbate was used to reduce Fe^{III}−verdoheme to Fe^{II}−verdoheme. The C_m and C_α carbons (black dots) were labeled with ^{13}C.

The ^{13}C NMR spectrum of verdo-HO−CO (Figure 2A), a low-spin diamagnetic ($S = 0$) complex, shows a set of four peaks between 138 and 155 ppm. These resonances originate from each of the four ^{13}C-labeled C_α carbons shown in Figure 1. Three of the labeled C_α positions are next to a ^{13}C-labeled C_m, which gives rise to the three doublets between 138 and 145 ppm, whereas the C_α next to the oxygen atom is a singlet at 154 ppm. The C_m resonances, which are less resolved from one another than the C_α resonances, are located at ca. 112 ppm. The verdoheme C_α and C_m resonances are absent in the control ^{13}C NMR spectrum of verdo-HO−CO harboring nonlabeled verdoheme (Figure 2C). As expected for a diamagnetic complex, the C_α and C_m chemical shifts from verdo-HO−CO are similar to those obtained from Zn−protoporphyrin.[13] The UV−vis spectrum of the sample contained in the NMR tube (shown next to the NMR spectrum) was obtained by coating the wall of the NMR tube with the solution; this spectrum corroborates that the ^{13}C NMR spectrum originates from verdo-HO−CO.

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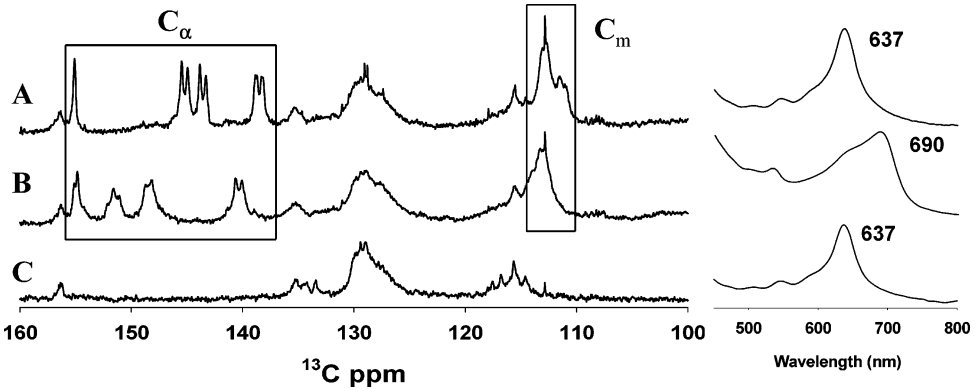
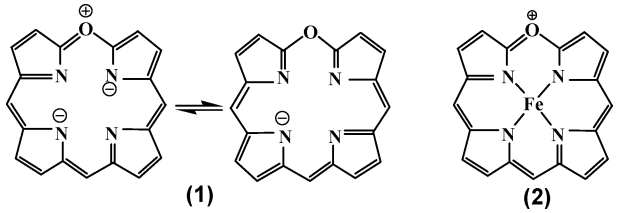


Figure 2. ^{13}C NMR spectra (13 °C, pH 7.6) of (A) ^{13}C-labeled verdo-HO−CO, (B) ^{13}C-labeled verdo-HO, and (C) verdo-HO−CO. The UV−vis spectra were obtained by coating the wall of the NMR tube with the corresponding solutions.

It is noteworthy that the ^{13}C NMR spectrum of verdo-HO (Figure 2-B) is very similar to that of verdo-HO−CO, with the C_α resonances between 138 and 155 ppm and the C_m resonances at ca. 112 ppm. The nature of the sample giving rise to this NMR spectrum is corroborated in the UV−vis spectrum obtained by coating the wall of the NMR tube. The magnitudes of the ^{13}C chemical shifts unambiguously indicate that the Fe^{II} ion in verdo-HO is six-coordinate and diamagnetic. If the Fe^{II} ion in verdo-HO was five-coordinate, the complex should be high-spin and, therefore, exhibit C_α resonances near 900 ppm.[10,11]

It is also interesting to note that in contrast to the relatively sharp C_α resonances of verdo-HO−CO the corresponding resonances of verdo-HO are broader and appear to consist of more than a simple doublet caused by coupling among adjacent ^{13}C-labeled carbons. This is most clear in the 155 ppm resonance originating from the C_α adjacent to the oxo group. Although this resonance should be a singlet (as is the case in verdo-HO−CO), it clearly consists of at least two peaks. This multiplicity suggests that verdoheme in verdo-HO is in slow exchange between at least two (likely in-plane) conformations, a notion that is in agreement with observations made in the crystal structure of verdo-HO.[6]

The verdo-HO complex is diamagnetic and low spin in the experimentally accessible pH range (pH 7.1−10.1), as judged by ^{13}C NMR spectra identical to that shown in Figure 2B and invariant electronic absorption spectra in this pH range (Figure S2). Lowering the pH below 7.1 caused formation of precipitate, likely due to the release of verdoheme from HO, in a manner akin to the release of heme from HO at pH lower than ~6.7. The coordination number and spin state of verdo-HO are in stark contrast to the pentacoordinate and paramagnetic nature of the ferrous heme−HO complex and ferrous heme centers in general, which typically become hexacoordinate and diamagnetic only in the presence of O_2 or CO. Hence, the fact that ferrous verdo-HO is six coordinate and diamagnetic underscores the distinct coordination preferences observed with model complexes of verdoheme.[14] The ability of ferrous verdoheme to coordinate anionic ligands has been explained in the context that the oxoporphyrin ring of verdoheme is a monoanion (**1**) in its deprotonated form. In comparison, deprotonated porphyrins are dianionic. Hence, the overall positive charge of ferrous verdoheme (**2**) is neutralized by coordination of one anionic ligand, and the doubly positive charge of ferric verdoheme is neutralized by coordination of two anionic ligands.[14] Thus, the diamagnetic nature of the verdo-HO complex at pH 7.1 and above and the tendency of ferrous verdoheme model complexes to coordinate an anionic ligand argue in favor of coordination by a strong-field, anionic ligand (OH^-) in the distal site of verdo-HO. However, coordination of the weak-field H_2O ligand[15] cannot be completely ruled out at present.



We thus have demonstrated a strategy that permits selective and efficient ^{13}C labeling of reactive intermediates in the catalytic cycle of HO. The availability of these labeled intermediates, coupled to the wealth of information regarding coordination state and electronic structure afforded by core porphyrin carbon chemical shifts, will likely facilitate future spectroscopic characterization of other intermediates in heme metabolism, such as meso-hydroxyheme-HO and iron biliverdin−HO complexes. Herein, this strategy allowed us to demonstrate that the verdo-HO complex is hexacoordinate, thus settling contradictory observations made by resonance Raman and X-ray crystallography.

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Supporting Information Available: Electronic absorption spectra of the α-verdoheme−*nm*-HO complex in its ferric, ferrous, and ferrous-CO states. This material is available free of charge via the Internet at http://pubs.acs.org.

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