Lactoperoxidase Heme Structure Characterized by Paramagnetic Proton NMR Spectroscopy

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All peroxidase enzymes isolated to date from plant and fungal sources have been found to contain an iron protoporphyrin IX (heme) prosthetic group.[1] In contrast, peroxidase enzymes from mammalian sources are known to utilize a structurally unique heme group that allows for a presumed covalent linkage to the protein matrix.[2−6] Covalent attachment renders the heme inaccessible to simple extraction methods, and structural characterization has thus posed a long-term challenge. The antimicrobial enzyme lactoperoxidase (LPO) has been the subject of numerous investigations focused on elucidation of the unique heme structure since the initial isolation of the protein in 1943.[1] Extraction attempts following cleavage of the heme−protein linkage by relatively harsh chemical means have resulted in either low yields or probable heme modification during the extraction process. These attempts have led to a variety of structural proposals including ester- and amide-linked hemes,[2,5] as well as the most recently proposed disulfide-bound heme.[6] Arguments have even been made for an unusually encapsulated unmodified iron protoporphyrin IX group in LPO.[7]

In order to circumvent the question of heme alteration during the extraction process, an approach was undertaken to obtain a heme group from LPO in its more "native" state with covalent linkage to the protein intact. Through the use of proteolytic enzymes and reverse phase HPLC separation, the bulk of the protein is removed, and heme peptide fractions have been isolated in quantity and purity suitable for characterization by paramagnetic proton NMR spectroscopy.

Preparation of LPO for optimal proteolytic hydrolysis without the use of thiol disulfide reducing reagents was accomplished by heat-denaturation of a deoxygenated solution of the enzyme. The 170 mg of denatured protein in 75 mL of 2 M urea solution at pH 8.0 with 100 mM phosphate buffer was subjected to proteolysis with 6.0 mg of trypsin for 20 h, followed by 6.0 mg of chymotrypsin for another 20 h. After precipitation of the heme-bound protein with ammonium sulfate, the peptide mixture was separated on a Sephadex G-25 column into a larger molecular weight red-colored heme fraction and a lower molecular weight green-colored heme fraction. HPLC of the green component with H_2O/CH_3CN/0.1% trifluoroacetic acid gradient elution on a Vydac 218TP reverse phase C_{18} column yielded several well-resolved heme−peptide fractions. Redigestion of the red-colored fraction gave an additional quantity of the green fraction with a similar chromatographic profile.

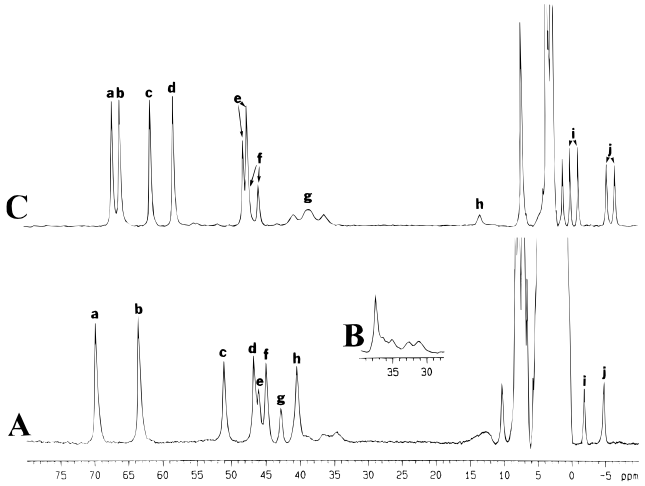

Figure 1. ^{1}H NMR spectra (600 MHz) of high-spin ferric bis(DMSO)−heme complexes in d_6-DMSO at 25 °C. (A) LPO heme−peptide (approximately 30 μM, 500 000 scans at 30 s^{-1}): (a) 69.6 ppm, CH_3; (b) 63.3 ppm, CH_3; (c) 51.0 ppm, α-propyl CH_2; (d) 46.6 ppm, hydroxy-CH_2; (e) 46.0 ppm, α-vinyl CH; (f) 44.8 ppm, α-propyl CH_2; (g) 42.7 ppm, α-vinyl CH; (h) 40.4 ppm, hydroxy-CH_2; (i) −1.9 ppm, trans β-vinyl CH; (j) −4.8 ppm, trans β-vinyl CH. (B) LPO heme peptide at 50 °C showing improved dispersion for meso CH proton signals. (C) Fe-protoporphyrin IX:[17] (a) 8-CH_3; (b) 5-CH_3; (c) 3-CH_3; (d) 1-CH_3; (e) 6,7-α-propyl CH_2; (f) 2,4-α-vinyl CH; (g) meso CH's; (h) COOH; (i) 2,4-β-vinyl cis CH; (j) 2,4-β-vinyl trans CH.

The HPLC fraction with the largest 400 nm absorbance (approximately 40% of the total heme applied to the column) was collected and combined after repeated preparative separations and used for the spectroscopic characterization discussed herein.

Electronic spectra confirm the removal of most of the protein from the heme group, as the initial native enzyme Rz (A_{412}/A_{280}) = 0.85 was increased to an Rz (A_{400}/A_{280}) of 7.9. A hypsochromic shift of the Soret band from 412 nm in native LPO to 400 nm in the isolated heme−peptide is consistent with the displacement of the proximal histidine with a solvent ligand. As noted in earlier attempts to define the LPO heme structure,[6,7] the pyridine hemochrome spectrum of the heme−peptide is very similar to that of Fe-protoporphyrin IX.[8,9]

Although the optical absorbance spectra show little difference in the electronic properties of the LPO heme and Fe-protoporphyrin IX, the ^{1}H NMR spectra reveal distinct structural differences. The high-spin ferric form of the LPO heme in d_6-DMSO gave the NMR spectra in Figure 1A,B. Only two paramagnetically shifted methyl signals are apparent in the downfield region where they reside for Fe-protoporphyrin IX, and two extra methylene signals are found upfield from the methyl signals. Other resonances resolved in the high-spin LPO heme spectrum parallel those for Fe-protoporphyrin IX (Figure 1C) and can be assigned on that basis. These include the four meso protons resolved in a spectrum obtained at a temperature of 50 °C (Figure 1B).

NMR spectra of the low-spin ferric bis(cyano) LPO heme complex in 0.1% TFA/D_2O more clearly indicate the type of modification on this heme. Initially, a mixture of heme complexes was seen upon the addition of an excess of potassium cyanide. A period of several hours was necessary to convert

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(8) Pyridine hemochrome complexes for both Fe-protoporphyrin IX and the LPO heme were prepared by standard methods.[9] Values for band positions (λ_{max}) and relative intensites in parentheses (given as absorbance of band divided by absorbance of Soret band) are as follows: LPO heme, Soret = 422 nm (1.00), α = 560 nm (0.16), β = 526 nm (0.11); Fe-protoporphyrin IX, Soret = 420 nm (1.00), α = 556 nm (0.22), β = 524 nm (0.13).

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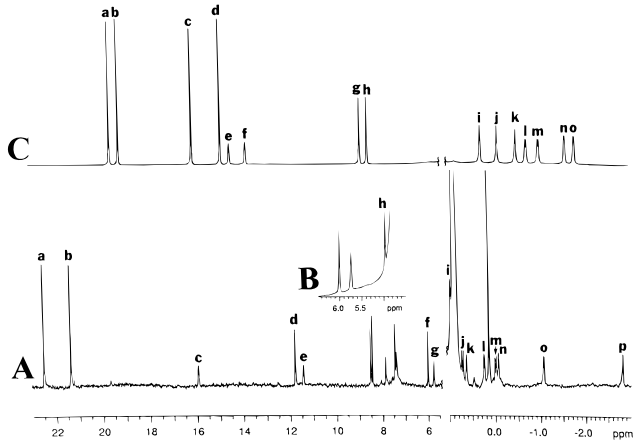

Figure 2. ^{1}H NMR spectra (600 MHz) of low-spin ferric bis(cyano)−heme complexes in D_2O with 0.1% d_1-TFA at 25 °C. (A) Peptide-free LPO heme (approximately 30 μM, 40 000 scans at 1 s^{-1}) with solvent presaturation: (a) 22.48 ppm, 8-CH_3; (b) 21.34 ppm, 3-CH_3; (c) 15.92 ppm, 4-α-vinyl CH; (d) 11.76 ppm, 7-α-propyl CH_2; (e) 11.42 ppm, 2-α-vinyl CH; (f) 6.00 ppm, 5-hydroxy-CH_2; (g) 5.74 ppm, 6-α-propyl CH_2; (i) 0.96 ppm, 6-β-propyl CH_2; (j) 0.71 ppm, 2-β-vinyl trans CH; (k) 0.62 ppm, meso CH; (l) 0.24 ppm, 2-β-vinyl cis CH; (m) −0.01 ppm, 4-β-vinyl trans CH; (n) −0.07 ppm, meso CH; (o) −1.06 ppm, 4-β-vinyl cis CH; (p) −2.97 ppm, α-meso CH. (B) LPO heme hydroxymethylene region from an experiment without solvent presaturation: (h) 4.97 ppm, 1-hydroxy-CH_2. (C) Fe-protoporphyrin IX:[18] (a) 8-CH_3; (b) 5-CH_3; (c) 3-CH_3; (d) 1-CH_3; (e) 2-α-vinyl CH; (f) 4-α-vinyl CH; (g) 6-α-propyl CH_2; (h) 7-α-propyl CH_2; (i) β-meso CH; (j) δ-meso CH; (k) α-meso CH; (l) 4-β-vinyl trans CH; (m) 2-β-vinyl trans CH; (n) 4-β-vinyl cis CH; (o) 2-β-vinyl cis CH.

the heme complex to the one cyanide-bound species for which NMR spectra are shown in Figure 2A,B. It was subsequently found that the slow conversion is due to peptide removal from the heme upon addition of excess cyanide to the heme−peptide in water.[10] Hence, the resulting paramagnetic proton resonances in Figure 2A,B arise from a peptide-free heme group.

The presence of only two methyl groups on the porphyrin ring is confirmed in the low-spin ferric NMR spectra, as only two signals of appropriate intensity occur in the expected downfield region. The two unique methylene signals from this complex are identified just downfield from the residual water signal in an experiment without a solvent presaturation pulse (Figure 2B). Most of the paramagnetic proton resonances on the heme group were distinguished from signals arising from peptide protons by an inversion−recovery pulse sequence. All expected heme proton resonances are resolved except those from a meso-H and a β-propionate methylene, both of which would be expected near or beneath signals from the peptide portion in the diamagnetic envelope. The unresolved β-propionate methylene signal was identified at 1.96 ppm by a cross peak to the α-propionate methylene signal at 11.76 ppm in a COSY spectrum. Of particular note are the well-resolved signals from all three protons of both vinyl groups on the LPO heme, precluding the type of peptide linkage at these sites as discovered in myeloperoxidase[11,12] or cytochrome *c*.[13]

(10) An assumed peptide-free LPO heme group obtained by the acetic acid hydrolysis and proteinase K digestion procedure recently used to isolate a peptide-free heme group from myeloperoxidase[11] showed identical paramagnetic resonances for the low-spin ferric bis(cyano) LPO heme complex. However, the high-spin form of this peptide-free heme in d_6-DMSO gave a spectrum distinctly different from that displayed in Figure 1 for the heme−peptide species.

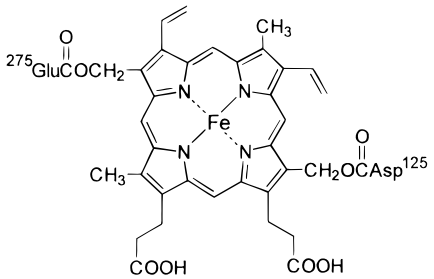

Figure 3. Proposed structure of the lactoperoxidase heme.

Aside from the absence of protein interaction with the heme vinyl groups, the resonances in these spectra argue for a structure otherwise much like the heme in myeloperoxidase (MPO). The two methylene peaks seen in both the high-spin and low-spin systems (clearly seen as singlets in the low-spin form) and the loss of two of the four expected heme methyl peaks found in Fe-protoporphyrin IX suggest substitutions at two heme methyl sites. This is consistent with the ester linkages at two hydroxylated methyl groups discovered in the recent X-ray analysis of human MPO.[12] One-dimensional steady-state NOE difference spectra collected for the peptide-free LPO heme in a viscous 4:1 d_6-DMSO:D_2O solvent mixture at low temperatures are consistent with only a 1- and 5-methyl-substituted derivative of Fe-protoporphyrin IX. NOE signals detected for pyrrole substituents upon irradiating each of the four meso-proton resonances, as well as those between two substituents on a given pyrrole, eliminate other possible structures.

A molecular ion of 648 mass (Fe-protoporphyrin IX + 2 O) was found in the FAB mass spectrum of the peptide-free LPO heme in both glycerol and 3-nitrobenzyl alcohol matrices. The LPO heme structure thus proposed on the basis of paramagnetic NMR and mass spectroscopy studies is illustrated in Figure 3. Given the amino acid sequence homology between human MPO and bovine LPO in the region of both the glutamate and aspartate responsible for these ester bonds,[14,15] as well as the similarity in the native enzyme paramagnetic proton NMR pattern,[16] it is likely that a similar linkage exists in LPO as shown in Figure 3.

A novel heme structure now tentatively identified for two mammalian peroxidases raises many questions related to biosynthesis, reactivity with thiourealene compounds, and modulation of redox activity by peripheral porphyrin substitutions.

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