

# Spectral Analysis of Lactoperoxidase

## EVIDENCE FOR A COMMON HEME IN MAMMALIAN PEROXIDASES\*

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**The identity of the non-extractable heme of mammalian lactoperoxidase (LPO) has remained unsolved for over 40 years. Accepted possibilities include a constrained heme b or an 8-thiomethylene-modified heme b. Recent studies of myeloperoxidase (MPO) (Fenna, R., Zeng, J., and Davey, C. (1995) *Arch. Biochem. Biophys.* 316, 653–656; Taylor, K. L., Strobel, F., Yue, K. T., Ram, P., Pohl, J., Woods, A. S., and Kinkade, J. M., Jr. (1995) *Arch. Biochem. Biophys.* 316, 635–642) suggest possible prosthetic group similarities between MPO and LPO. To address heme identity for LPO, we used comparative magnetic circular dichroism (MCD) spectroscopy of LPO versus myoglobin (Mb), horseradish peroxidase (HRP), and MPO. MCD spectra of native  $\text{Fe}^{3+}$ -LPO and  $\text{Fe}^{3+}$ -CN<sup>−</sup>-LPO are ~10 nm red shifted from analogous forms of Mb and HRP, including the formate-Mb adduct. MCD spectra of native LPO and MPO are opposite in sign, and MCD spectra of their cyanoadducts also differ. These data indicate the LPO heme is distinct from heme b of Mb and HRP as well as from “heme m” of MPO. From this work and literature analysis, we suggest that the non-extractable “heme l” of LPO has the two vinyl groups of heme b but lacks the 2-sulfonium-vinyl linkage of heme m. The observed red shifts in LPO spectra may derive from ester linkages to protein as for MPO. Strong spectral analogies between LPO and mammalian peroxidases (e.g. from saliva, eosinophils, thyroid, intestine) indicate similar prosthetic heme moieties.**

Lactoperoxidase (LPO,<sup>1</sup> EC 1.11.1.7) is a mammalian peroxidase that is the product of exocrine gland secretion, e.g. into milk, saliva, and tears (1–3). Its normal physiological role appears to be as a bactericide, converting thiocyanate to hypothiocyanate in an  $\text{H}_2\text{O}_2$ -dependent reaction. The bactericide function of LPO is also of primary importance for the closely related salivary peroxidase (SPO) (4). LPO has recently been found in human colostrum and may be a useful criterion for distinguishing between hormone-dependent and hormone-in-

dependent types of mammary cancer (5). Yet another functional role for LPO may be in the degradation of catecholamines such as norepinephrine (6). Finally, soluble LPO is both a functional and a spectral model for membrane-bound thyroid peroxidase (TPO) (3, 7).

The series of mammalian peroxidases, including LPO, SPO, TPO, eosinophil peroxidase, intestinal peroxidase, and myeloperoxidase (MPO), have related protein primary structures (Refs. 8 and 9, and references therein) and prosthetic heme moieties that are not readily extracted by conventional approaches (1–3). The proteins also display spectral signatures that are distinct from those of known heme groups (Table I) (1–3). As a consequence, the structural identity of the heme moieties of mammalian peroxidases has been a subject of considerable controversy.

Proposed structures for the prosthetic group of LPO are shown in Fig. 1. Heme b (Fig. 1A) is found in many common heme proteins. These include myoglobin (Mb) and hemoglobin, the P450 enzymes, catalases, and peroxidases from yeast, plant, and fungal sources (cytochrome *c* peroxidase, horseradish peroxidase (HRP), lignin peroxidase, and chloroperoxidase). Evidence for heme b (Structure A) as the heme of LPO came from Pronase digestion of the enzyme (17). Sievers (17) proposed that the anomalous electronic absorption spectrum of LPO and its pyridine hemochrome derivative, relative to those of known heme b-containing proteins (see Table I), arose from an unusually constrained active-site pocket surrounding the heme. The 8-thiomethylene-substituted derivative of iron PPIX (Fig. 1B) was proposed as the LPO heme by Clezy and colleagues (18) following treatment of LPO with 2-mercaptoethanol and urea. This hypothesis would explain the lack of ready extractability for the LPO heme moiety but does not fully address observed spectral variations from heme b.

Most recently, Fenna and colleagues (9) and Taylor *et al.* (19) have determined the structure of the “heme m” prosthetic group of native MPO (Fig. 1C). These studies clearly identified the prosthetic group as an iron porphyrin, resolving a long standing controversy where unusual spectral properties of MPO were suggested to derive from an (atypical) iron chlorin prosthetic moiety (for example, Ref. 13 and references therein). Fenna *et al.* (9) suggested that close similarities in the protein sequences of MPO and LPO made it possible that LPO could have heme-protein linkages and a heme structure related to that of MPO.

Each of the three proposals for the native heme moiety of LPO (Fig. 1) has had its supporters. Evidence for the identity of the LPO heme has come from a variety of different experimental approaches. These include resonance Raman (20, 21), NMR (22, 23), and EPR (24) spectroscopy, as well as other types of biochemical analyses (25, 26).

Two additional factors of importance with respect to the heme of native LPO are 1) the nature and identity of the 5th axial ligand to the heme moiety and 2) the presence or absence

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<sup>1</sup> The abbreviations used are: LPO, lactoperoxidase; SPO, salivary peroxidase; TPO, thyroid peroxidase; MPO, myeloperoxidase; heme, iron porphyrin; heme b, iron protoporphyrin IX (iron PPIX); Mb, myoglobin; HRP, horseradish peroxidase; CN<sup>−</sup>, cyanide; MCD, magnetic circular dichroism.

of a 6th ligand. The 5th ligand, donated by the protein, is known to have a considerable effect on observed spectral properties as well as on functional characteristics. The classical example is  $\text{Fe}^{2+}$ -CO-P450 (Soret band at  $\sim 450$  nm) in contrast with  $\text{Fe}^{2+}$ -CO-Mb (Soret at  $\sim 420$  nm), where the observed differences derive from an axial S-Cys<sup>-</sup> ligand in the case of P450 and an axial N-His ligand in the case of Mb (e.g. Ref. 27). The S-Cys<sup>-</sup> 5th ligand of P450s has an effect on all of the spectral properties of these enzymes relative to the analogous forms of other heme proteins (28–32). Yet other examples are the N-His<sup>-</sup> 5th ligand of HRP, cytochrome *c* peroxidase, and presumably other peroxidases (33) and the O-Tyr<sup>-</sup> 5th ligand of catalases (Ref. 34, and references therein). The 5th ligand to the heme of LPO is either a histidine (35) or the deprotonated N-His<sup>-</sup> common to many peroxidases (Refs. 22, 24, 36, and 37, and references therein). The 6th ligand of LPO has variously been reported to be an unusual formate (35) or  $\text{H}_2\text{O}$  (20, 21). Some assignment inconsistencies exist such as a reported 5-coordinate structure (37, 38), whereas the body of other evidence indicates that native LPO is 6-coordinate (20, 21, 39).

In this work, we have addressed the heme moiety of LPO with respect to the structural proposals in Fig. 1. Our approach is comparative spectral analysis of LPO and the analogous forms of: 1) Mb and HRP, both of which have heme b and, respectively, either N-His or N-His<sup>-</sup> 5th ligands; and 2) MPO, which has the novel heme m and N-His<sup>-</sup> ligation (9, 19). These proteins were examined in both their native and in the  $\text{Fe}^{3+}$ -CN<sup>-</sup> forms, using MCD and electronic absorption spectroscopy. MCD spectroscopy is able to distinguish easily between 5- and 6-coordinate ferric heme systems (16, 27, 34) and is sensitive to heme-bound water (16, 34). Thus we directly compare LPO properties with those of heme systems proposed to have identical prosthetic heme moieties, identical axial ligation, or identity in both heme group and axial ligands.

Our data demonstrate obvious spectral differences between the hemes of LPO and Mb or HRP, and between the hemes of LPO and MPO, in both native and  $\text{Fe}^{3+}$ -CN<sup>-</sup> forms. Furthermore, the spectral differences do not derive from axial ligands, as shown by comparison of native LPO and Mb-formate. Thus, we suggest that the LPO heme (called here heme l) is distinct from either heme b or heme m, although it has similar peripheral substituents to each. Given the high degree of spectral and protein sequence agreement between mammalian peroxidases (8, 9, 19), we suggest that the heme l of LPO, or a very closely related heme, is likely to also be the heme of TPO, SPO, eosinophil peroxidase, and other mammalian peroxidases.

#### MATERIALS AND METHODS

Bovine LPO was a kind gift from Prof. Harold M. Goff, Department of Chemistry, University of Iowa. The enzyme was isolated and purified as described previously (36, 40–44). Enzyme concentration was determined using the published value:  $\epsilon$  (mM) at 412 nm = 114 (45, 46). Wild type, recombinant human Mb was prepared as described previously (16). HRP, Sigma type XII (highly purified Sigma type VI), was used as purchased.

All samples were examined in a 100 mM potassium phosphate buffer, pH 7.0. The cyanide adducts were prepared by addition of a 50–200-fold excess of a cyanide stock solution to a serum-stoppered cuvette ( $\sim 10$   $\mu\text{L}$  of a 0.5 M stock). The formate adduct of Mb was prepared by addition of a stock solution to a final concentration of 0.1 M (27).

The three protein samples and their various adducts were examined by electronic absorption spectroscopy in either 5-mm or 1-cm quartz Suprasil cuvettes using Varian-Cary 219, Hitachi U-2000, or Hitachi U-3200 spectrophotometers. MCD spectra were obtained on a Jasco J-720 spectropolarimeter fitted with a 1.5-tesla (15 kG) electromagnet in the sample compartment with the magnetic field direction parallel to the direction of light propagation. Following collection of the MCD/CD data, the samples were re-examined using electronic absorption spec-

troscopy.<sup>2</sup> In no case was an absorption spectral change greater than  $\sim 0.5\%$  observed.

The MCD/CD instrument was calibrated daily for intensity with ammonium *d*-10-camphor sulfonate (Jasco) and was calibrated bi-monthly with neodymium glass for wavelength. The MCD/CD data for each sample were obtained using the following experimental conditions: 1 nm bandwidth, 25 scan accumulation, 200 nm/min scan rate, 0.5 nm resolution, and 13.9 kG (1.39 tesla) magnetic field. Final MCD data were corrected for natural CD and for the buffer blanks using the Jasco software and were then corrected for concentration, light path, and magnetic field. The data presented in the figures have been smoothed using the Jasco software. Samples were maintained at  $\sim 14^\circ\text{C}$  during data collection by use of a custom-made flow-through cell holder and dedicated water bath (16, 32, 34).<sup>2</sup> Final MCD data are presented in units of  $\Delta\epsilon_M$  (moles/liter $\cdot\text{cm}\cdot\text{tesla}$ )<sup>-1</sup>, where  $\Theta_M$  (deg $\cdot\text{cm}^2\cdot\text{dmol}^{-1}\cdot\text{tesla}^{-1}$ ) =  $3300 \Delta\epsilon_M$ .

#### RESULTS AND DISCUSSION

The basic hypothesis on which this work rests is that structural identity results in spectral identity. Even relatively small structural changes can induce significant spectral effects. MCD spectroscopy is well known as a useful probe in the structural analysis of biological heme systems (e.g. Ref. 27 and references therein). One particular advantage is the low sample concentrations required relative to other biophysical methods. A second advantage lies in the accuracy of results, with general agreement between MCD and x-ray methods (e.g. Refs. 16 and 34). For example, MCD spectroscopy indicated heme-bound  $\text{H}_2\text{O}$  for ferric forms of His-64  $\rightarrow$  Gln and His-64  $\rightarrow$  Gly engineered recombinant mutant Mbs (pH 5.6); these data were shown to concur with results from x-ray analysis (16).<sup>2</sup> The sensitivity of MCD spectroscopy to heme-bound  $\text{H}_2\text{O}$  has also been useful in resolution of a controversy with regard to water occupancy in functional states of mammalian, fungal, and bacterial catalases (34).

In this work, we use direct spectral comparison for evaluation of heme b and heme m as potential prosthetic groups for LPO. If native LPO has a heme b prosthetic group, its spectra should be similar to those of either Mb or HRP, depending on the 5th (axial) ligand (N-His or N-His<sup>-</sup>, respectively) to the LPO heme group and its coordination state (6-coordinate, high spin or 5-coordinate, high spin, respectively). If ferric LPO has heme b with a carboxylate 6th ligand (35) causing the observed red shifting of the electronic absorption spectrum (Table I), then one would expect the formate adduct of Mb to display a close spectral similarity.

Yet another data set addressing the heme moiety of native LPO comes from preparation and analysis of the low spin, 6-coordinate  $\text{Fe}^{3+}$ -CN<sup>-</sup> adducts of LPO, Mb, HRP, and MPO. With all the proteins having CN<sup>-</sup> as 6th ligand and N-His or N-His<sup>-</sup> as 5th ligand, only differences in the respective heme moieties could explain any observed spectral variation. Therefore, we would expect  $\text{Fe}^{3+}$ -CN<sup>-</sup>-LPO to display close spectral similarity to  $\text{Fe}^{3+}$ -CN<sup>-</sup>-Mb or -HRP if the LPO moiety is heme b or to  $\text{Fe}^{3+}$ -CN<sup>-</sup>-MPO if the LPO heme moiety is heme m.

#### Electronic Absorption Spectra

Electronic absorption bands of LPO, Mb, and HRP in their native ferric, ferric-cyano, and ferric-fluoro forms are listed in Table II. As is clear from literature data in Table I and Table II, the electronic absorption spectrum of native LPO is distinct from that of known heme systems such as heme m of MPO (Fig. 1C) or heme b of Mb and HRP (Fig. 1A). As discussed above, LPO is commonly accepted to be high spin, 6-coordinate in the native state (20, 21, 39, 47).

In the electronic absorption spectra, the high spin marker

<sup>2</sup> L. A. Andersson, S. A. Bylka, Y. Dou, and M. Ikeda-Saito, manuscript in preparation.

TABLE I  
Absorption spectral features

Protein <sup>a</sup>	Absorption bands					Spin/coordinate state <sup>b</sup>	Heme <sup>c</sup>	Ref.
	Soret	Visible						
LPO	413	500	550	600	640	HS, 6	l	This work, 7
SPO	413	500	550	600	640	HS, 6	l	4
TPO	413	500	543	586	636	HS, 6	l	7
IPO	414	505	545	596	640	HS, 6	l	7, 10
MPO	428					HS, 6	m	11, 12
HRP	403	497			641.5	HS, 5	b	This work
CPO	403	515	542		650	HS, 5	b	13, 14
LIP	407	500			632	HS, 6	b	15
MetMb	409.5	505			635	HS, 6	b	This work, 16

<sup>a</sup> IPO, intestinal peroxidase; CPO, chloroperoxidase; LIP, lignin peroxidase.<sup>b</sup> HS, high spin.<sup>c</sup> Heme l, Fig. 5; heme m, Fig. 1C; heme b, Fig. 1A.TABLE II  
Electronic absorption spectral features for ferric LPO, Mb, HRP, and their cyano and fluoro adducts

	Native ferric, pH 7.0		Cyano adduct		Fluoro adduct	
LPO						
	412	1.0 <sup>a</sup>	345	0.32		
	497	0.097	431	1.0	411.5	1.0
	542	0.083	557	0.132		
	592	0.071	590	0.091	585	0.090
	626	0.061			612	0.048
Mb						
	409.5	1.0	365.5	0.317		
	503	0.067	423.5	1.0	404.5	1.0
					491	0.059
			541	0.107		
HRP						
	633	0.026	363	0.326	613	0.054
	403	1.0	422.5	1.0	404.5	1.0
	498	0.115			491	0.059
			540.5	0.125		
	643	0.035			613	0.054

<sup>a</sup> Absorption peaks normalized to Soret band as 100%.

band of native LPO is at 626 nm in comparison with 633 nm for human Mb (high spin, 6-coordinate) and 643 nm for HRP (high spin, 5-coordinate). The Soret band of LPO is at 412 nm in contrast to 409.5 nm for Mb and 403 nm for HRP. Upon formation of the Fe<sup>3+</sup>-CN<sup>-</sup> adducts, the spectra of all the proteins are altered, the high spin marker band disappears from the visible region, and the Soret bands are red shifted. For HRP and Mb the spectral features of the cyanide adducts are not identical although they are very closely similar to one another. This is as expected given identical heme moieties and identical 6th ligands, with the only difference between the Fe<sup>3+</sup>-CN<sup>-</sup> adducts of HRP and Mb being their respective 5th ligands. In contrast, the absorption spectrum of Fe<sup>3+</sup>-CN<sup>-</sup>-LPO is markedly red shifted from the cyano adducts of HRP and Mb. Again, in the case of the fluoro adducts, we see a close similarity in the spectral properties of Fe<sup>3+</sup>-F<sup>-</sup>-HRP and -Mb, with the spectral features of the LPO complex being distinctly different. Considering the identity of the 6th ligands for these three proteins and the common assumption that LPO has a 5th ligand identical to either Mb or HRP, these data do not appear to support a common heme moiety for the three proteins.

For MPO, the recent elucidation of the novel heme m structure (Fig. 1C) (9, 19) permits analysis of the observed features in the electronic absorption spectra. Presumably the atypical spectral properties of MPO are derived from both the unusual 2-substituent and the 1,5-ester connections to the protein. One would also expect the lowered symmetry of heme m, arising from constraints placed on the porphyrin macrocycle by tethering to the protein at the 1-, 2-, and 5-positions of the heme, to affect structural and consequently spectral properties. As reported by Fenna *et al.* (9), the high degree of sequence homol-

ogy between mammalian peroxidases (LPO, TPO, SPO, and intestinal peroxidase (7, 10)) does not include the Met residue necessary to form the novel 2-substituent link. However, because the sequence homology between MPO and LPO includes the Glu and Asp residues needed for the 1- and 5-ester linkages, Fenna and colleagues (9) suggested that the heme of LPO might be at least partially analogous to that of MPO.

### Magnetic Circular Dichroism Spectra

**Formate-Mb**—For LPO, Sievers *et al.* (35) proposed a high spin, 6-coordinate iron PPIX (heme b) moiety with an N-His 5th ligand and a carboxylate 6th ligand. Testing this hypothesis by binding a carboxylate ligand such as formate to Mb is an obvious starting place for evaluation of structural hypotheses for the heme moiety of LPO. Fig. 2 presents MCD spectra of native ferric LPO and the formate adduct of ferric human Mb. As is clearly evident, the MCD spectrum of LPO is similar to, but red shifted from, that of formate-Mb. These data demonstrate that formate ligation of Mb is insufficient to mimic the LPO heme moiety and its axial ligation. They also reveal that the peripheral substituents on the LPO prosthetic group are more electron withdrawing than can be mimicked by ligation of an electron-withdrawing substituent to Mb (iron PPIX, Fig. 1A).

**Ferric-Cyano Adducts**—Comparison of the Fe<sup>3+</sup>-CN<sup>-</sup> adducts of LPO, Mb, HRP, and MPO provides another approach to structural evaluation of the LPO heme moiety. If the prosthetic group of LPO is iron PPIX (17) and LPO has an N-His or N-His<sup>-</sup> 5th ligand, then Fe<sup>3+</sup>-CN<sup>-</sup>-LPO should be spectrally analogous to the Fe<sup>3+</sup>-CN<sup>-</sup> adduct of Mb or HRP. Alternatively, if LPO has the m heme moiety like MPO then it should be spectrally analogous to Fe<sup>3+</sup>-CN<sup>-</sup>-MPO. Fig. 3 presents MCD spectra of Fe<sup>3+</sup>-CN<sup>-</sup> complexes of LPO, Mb, HRP, and MPO (*inset*).

The first interesting observation is that the MCD spectra of the Fe<sup>3+</sup>-CN<sup>-</sup> adducts of Mb and HRP, while very similar in shape, intensity, and band position, are not identical. There is a small blue shift for the HRP complex relative to the Mb complex, concurring with the slight blue shift of the absorption spectral bands of the HRP-cyanide adduct relative to the Mb-cyanide adduct. Given the clear-cut identity of the heme moieties as well as the 5th and 6th ligands for cyano-HRP and cyano-Mb, it is intriguing to speculate that the spectral differences noted here are derived from the key structural difference between the two proteins, the protonation state of the 5th ligand. New results for our laboratory, supported by x-ray structural analysis (32), reveal the notable sensitivity of CD spectroscopy to the presence/absence of a hydrogen bond to heme-bound H<sub>2</sub>O. We have also shown that MCD spectroscopy is sensitive not only to the presence of axially bound H<sub>2</sub>O but also to the presence of hydrogen bonding between the heme-

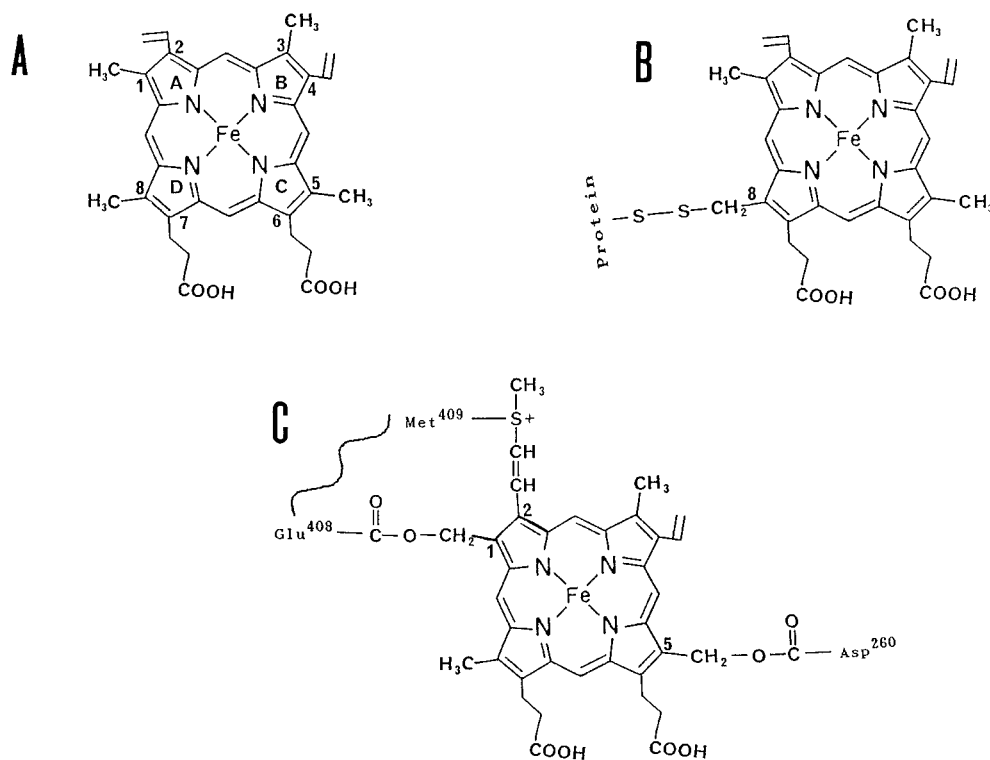


FIG. 1. **Proposed heme structures for lactoperoxidase.** A, iron protoporphyrin IX (heme b) (17); B, 8-thiomethylene-substituted heme b (18); C, the m heme of myeloperoxidase (9, 19).

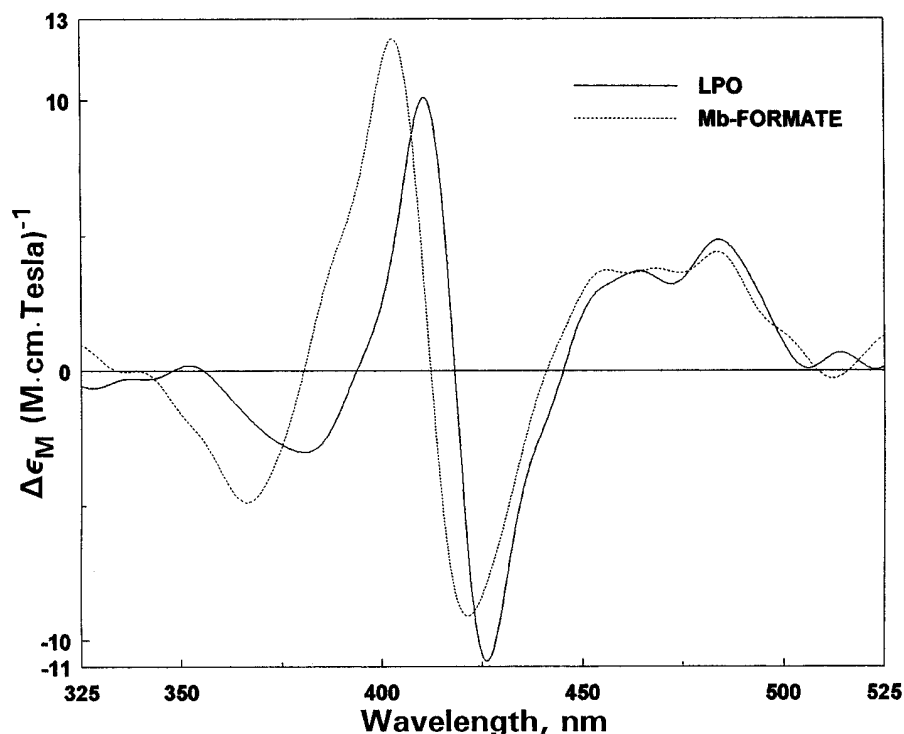


FIG. 2. **MCD spectra of native LPO and Mb-formate.** Solid line, native LPO (20.7  $\mu\text{M}$ ); dotted line, Mb-formate (9.73  $\mu\text{M}$  Mb, 0.1 M formate). Experimental conditions are as described under "Materials and Methods."

bound water and a distal residue, once again supported by x-ray structural analysis (16).<sup>2</sup> This work is the first report of MCD sensitivity to a hydrogen bond interaction at the 5th ligand of a heme protein.

The MCD spectrum for the  $\text{Fe}^{3+}\text{-CN}^-$  adduct of LPO, while similar in overall shape to the analogous spectra of Mb and HRP, is red shifted by  $\sim 8\text{--}10$  nm for all features. These include, for example, the Soret peak of LPO at 423.5 nm (413.5 nm for HRP, 415.5 nm for Mb), the crossover (MCD zero point)

of LPO at 431 nm (421 and 423 nm for HRP and Mb), and the Soret trough of LPO at 438.5 nm (428.5 and 430 nm for HRP and Mb). These data indicate that the heme of LPO is not identical with the heme b moiety of HRP and Mb. Furthermore, they indicate that the LPO heme group differs from heme b by the presence of one or more electron-withdrawing substituents in addition to the two vinyl moieties of heme b (Fig. 1A).

The MCD spectrum of  $\text{Fe}^{3+}\text{-CN}^-$ -LPO is also distinct from that of  $\text{Fe}^{3+}\text{-CN}^-$ -MPO (Fig. 3, *inset*) (12). The latter has a

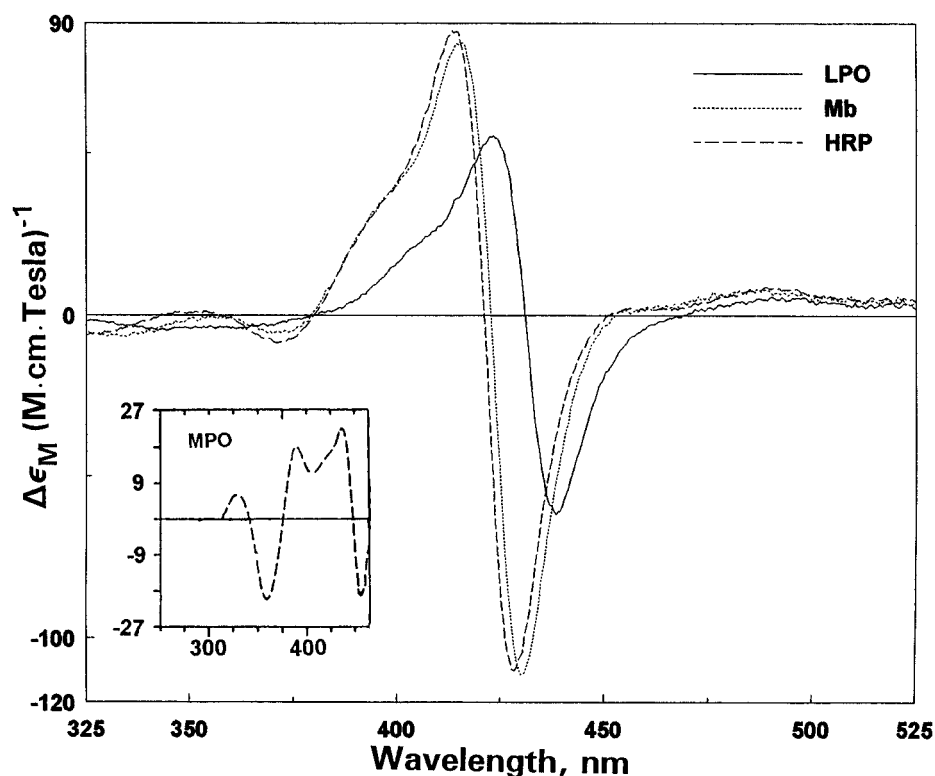


FIG. 3. MCD spectra of ferric-cyanide adducts of LPO, Mb, HRP, and MPO. Solid line, LPO (15  $\mu\text{M}$ , 200-fold  $\text{CN}^-$ ); dotted line, Mb (8.70-fold  $\mu\text{M}$ , 50  $\times$   $\text{CN}^-$ ); dashed line, HRP (18.4  $\mu\text{M}$ , 100  $\times$   $\text{CN}^-$ ). Experimental conditions are as described under "Materials and Methods." The inset is the ferric-cyano adduct of MPO, adapted from Ref. 12.

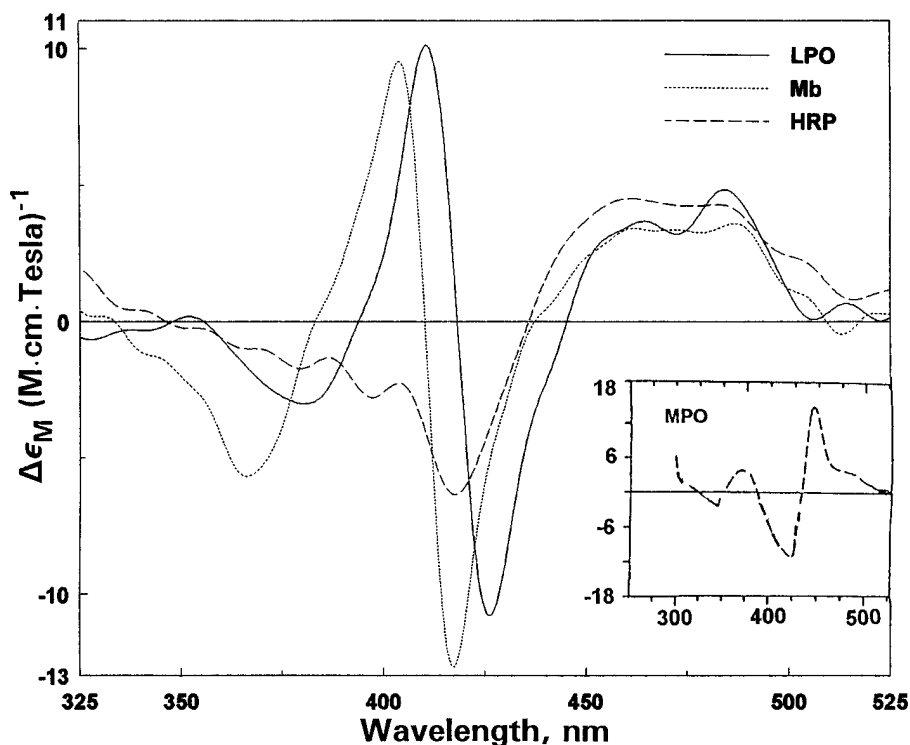


FIG. 4. MCD spectra of native ferric LPO, Mb, HRP, and MPO. Solid line, LPO (20.7  $\mu\text{M}$ ); dotted line, human Mb (10.9  $\mu\text{M}$ ); dashed line, HRP (19.93  $\mu\text{M}$ ). Experimental conditions are as described under "Materials and Methods." The inset is the native ferric form of MPO, adapted from Ref. 12.

resolved positive band slightly below 400 nm, a stronger positive peak at  $\sim 430$  nm, a crossover at  $\sim 440$  nm, and a trough at  $\sim 447$  nm. Given the newly reported structure of heme m of MPO (Fig. 1C), the peripheral substituents of this heme must play a dominant role in the MCD spectrum, because MPO has also been shown to have a histidine 5th ligand (9). It is evident that the  $\text{Fe}^{3+}\text{-CN}^-$  adduct of LPO is distinct from that of

$\text{Fe}^{3+}\text{-CN}^-$  adducts of either heme b or heme m systems.

**Native Ferric Proteins**—MCD spectra of the native ferric forms of LPO, Mb, HRP, and MPO are presented in Fig. 4. MCD spectra of LPO (solid line) and Mb (dotted line) both display the band pattern and intensity expected for high spin, 6-coordinate ferric heme systems (16, 27–31, 34, 47, 48). In contrast, the spectrum of native ferric HRP at pH 7 (dashed

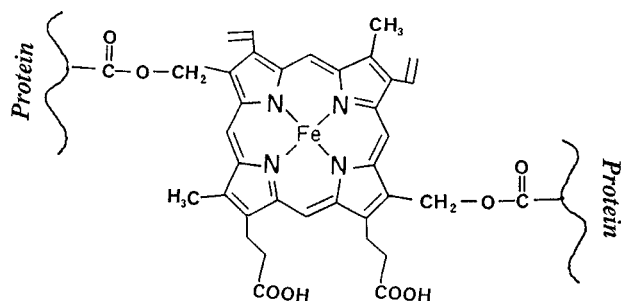


FIG. 5. New structure proposal for the heme l moiety of LPO.

line) has the weak intensity and pattern expected for a high spin, 5-coordinate heme system (27, 34, 47). However, although the MCD spectrum of ferric LPO is that of a 6-coordinate high spin heme, it is  $\sim 8$  nm red shifted from that of native Mb. As shown above (Fig. 2), this red shift is unlikely to originate from a formate (carboxylate) 6th ligand.

For the native heme proteins, it is the MCD spectrum of MPO (Fig. 4, *inset*) that is anomalous. Note that its pattern of bands is reversed relative to those of LPO and Mb, with the most intense positive band at low energy and the most intense negative band at high energy. As previously discussed (11, 12), this reversed MCD pattern is the expected one for an iron chlorin or a formyl-substituted porphyrin. Given that neither of these is consistent with the new reports of the heme m structure of MPO (see Fig. 1C) (9, 19), it appears that this pattern is also representative of a very low symmetry heme with electron-withdrawing substituent(s). We suggest that three points of attachment between the heme and the protein may have a strong spectral affect, in analogy with cytochrome *c*, for which the two points of heme attachment to the protein lower the symmetry and affect the resonance Raman data (49).

Evaluation of the native ferric heme proteins therefore indicates that the heme moiety of LPO is distinct not only from the heme b moiety of Mb and HRP but also from the heme m group of MPO. The LPO heme is likely to have additional substituent(s) that are electron withdrawing and/or symmetry lowering (beyond the two vinyl groups of heme b), although less so than occurs for MPO where the peripheral substituents result in a total reversal of the MCD pattern.

From sequence information discussed by Fenna *et al.* (9), it appears that LPO is sufficiently related to MPO to result in retention of the two ester linkages to the heme (the 2-linkage is not possible). Given this information and NMR data demonstrating close similarity between MPO and LPO (22),<sup>3</sup> a structural hypothesis for the heme moiety of lactoperoxidase becomes possible.

#### CONCLUSIONS

The data presented in this work eliminate all three of the structures in Fig. 1 as the heme moiety of LPO. Iron PPIX (Fig. 1A) is eliminated by obvious spectral differences between native and cyano-forms of LPO in comparison with those of Mb and/or HRP. Previous suggestions of a formate-ligated heme b are also eliminated. Spectral differences between LPO and its various ligand complexes and the analogous Mb/HRP species are on the order of  $\sim 8$ –10 nm. We suggest that this spectral variance is too large to be accounted for by the simple modification of heme b that produces the thioethylene-modified heme b (Fig. 1B). The novel heme m group of MPO (Fig. 1C) can also

be eliminated as the prosthetic heme of LPO simply on the basis of clear-cut differences between the MCD data shown.

Our proposal for the structure of the prosthetic group of LPO is shown in Fig. 5. We suggest that the LPO heme moiety, which we call heme l, has vinyl moieties at the 2- and 4-positions as is the case for heme b. In addition, the heme l of LPO has ester groups at positions 1 and 5 like heme m of MPO. This agrees with NMR data for MPO and LPO where spectral similarities between the two mammalian peroxidases were observed (22). It is also consistent with reported sequence similarities between the two proteins (8, 9). The two substituents should be sufficient to induce the observed red shifting of the MCD data and to prevent ready removal of the LPO heme without causing the complete spectral changes observed for MPO. Finally, there is the intriguing evidence from Ikeda-Saito and colleagues (24), where the spectra of MPO following chemical modification (photoreduction) were surprisingly similar to those of LPO.

Mammalian peroxidases are fundamentally distinct from peroxidases from yeast (cytochrome *c* peroxidase), plant (horseradish and turnip peroxidases), or fungal (lignin and fungal peroxidases) species. The key difference is apparently simple; mammalian heme groups are non-extractable whereas yeast, plant, and fungal peroxidases all have extractable heme b prosthetic groups (1–3). In many cases failure of mammalian heme groups to respond to conventional chemical treatment has been attributed to factors such as unusual or constrained active-site environments that retain the heme as is the case for catalase (Ref. 34, and references therein). However, increasing evidence indicates that the origin of this non-extractability for the heme groups of the mammalian peroxidases rests in the important evolutionary distinction of covalent attachment of the heme group to the protein.

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<sup>3</sup> During revision of this manuscript, we were provided a preprint of the elegant NMR study by T. D. Rae and H. M. Goff (submitted for publication), in which a highly similar, if not identical, structure for the prosthetic heme of LPO is proposed.

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