Electron Paramagnetic Resonance Spectroscopy of Lactoperoxidase Complexes: Clarification of Hyperfine Splitting for the NO Adduct of Lactoperoxidase†

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ABSTRACT: Electron paramagnetic resonance (EPR) studies of the nitrosyl adduct of ferrous lactoperoxidase (LPO) confirm that the fifth axial ligand in LPO is bound to the iron via a nitrogen atom. Complete reduction of the ferric LPO sample is required in order to observe the nine-line hyperfine splitting in the ferrous LPO/NO EPR spectrum. The ferrous LPO/NO complex does not exhibit a pH or buffer system dependence when examined by EPR. Interconversion of the ferrous LPO/NO complex and the ferric LPO/NO_2^- complex is achieved by addition of the appropriate oxidizing or reducing agent. Characterization of the low-spin LPO/NO_2^- complex by EPR and visible spectroscopy is reported. The pH dependence of the EPR spectra of ferric LPO and ferric LPO/CN^- suggests that a high-spin anisotropic LPO complex is formed at high pH and an acid–alkaline transition of the protein conformation near the heme site does occur in LPO/CN^-. The effect of tris(hydroxymethyl)aminomethane buffer on the LPO EPR spectrum is also examined.

Lactoperoxidase (LPO)[1] is a glycoprotein of 78 kDa found in mammalian milk, saliva, and tears. The enzyme catalyzes the peroxidation of endogenous thiocyanate to the antimicrobial hypothiocyanite ion (Hamon & Klebanoff, 1973). As such, LPO serves as a component of the biological defense system(s) of mammals. The prosthetic group of LPO is a protoheme IX residue that is deeply buried in a crevice of the protein molecule (Sievers, 1979).

We have utilized EPR spectroscopy in order to probe the electronic and molecular environment of the heme site of native LPO and its anion complexes as a function of pH. The nature of the proximal ligand of the heme iron has been investigated via magnetic circular dichroism (Sievers et al., 1983), proton NMR spectroscopy (Shiro & Morishima, 1986), and EPR spectroscopy of the nitrosyl compound of ferrous LPO (Bolscher & Wever, 1984; Sievers et al., 1984; Yonetani et al., 1972). Electron paramagnetic resonance spectroscopy of the hemoprotein/NO complex is particularly valuable in determining whether or not the axial amino acid residue is bound to the iron through a nitrogen atom. Retention of an axial histidine residue in the ferrous hemoprotein/NO has invariably given rise to an EPR spectrum in which the g_z component of the spectrum is split into nine lines (Yonetani et al., 1972; Hori et al., 1981). However, the EPR studies of the ferrous LPO nitrosyl complex have yielded two conflicting sets of results, a three-line and a nine-line spectrum. Here we report the results of a study of the ferrous LPO/NO system undertaken to resolve and explain the differences in previously reported EPR spectra. The first descriptions of nitrite ion interaction with the enzyme and the conversion of the ferrous NO complex to the ferric nitrite complex are also offered.

Experimental Procedures

Lactoperoxidase was isolated from fresh raw skim cow's milk as described previously (Goff et al., 1985). After final chromatographic purification on Sephadex G-100, fractions with R_z values of 0.85–0.91 were combined. Samples were exchanged into 0.1 M phosphate buffer or 0.1 M Tris/acetate buffer in the pH range of 4.5–11.0 and concentrated in an Amicon ultrafiltration cell through the use of a PM-30 membrane. The buffer pH was adjusted with 1.5 M HCl or NaOH solution. At the extreme pH values, buffering action was not present. Under the conditions used, the acetate ion present in the Tris buffer did not form a complex with LPO detectable by optical spectral changes. The absorbance at 412 nm (114 cm^{-1} mM^{-1}) was employed for spectrophotometric determination of protein concentration. Concentrations for EPR samples ranged from 0.2 to 1.0 mM. Lyophilized LPO purchased from Sigma was also examined for purposes of comparsion with the freshly isolated protein.

Once in a septum-capped 4-mm EPR tube, the aqueous buffered protein solution was thoroughly equilibrated with nitrogen by periodic inversion of the nitrogen-filled tube. Reduction was achieved by addition of buffered anaerobic stock solutions of sodium dithionite or ascorbic acid. Conversion to the NO complex was effected by introduction of one atmosphere of nitric oxide above the reduced protein solution. The EPR tube was inverted once or twice, and the excess NO was flushed from the tube with nitrogen while the ferrous LPO/NO complex was being frozen in liquid nitrogen. The NO complex was also generated by reduction of the LPO/nitrite complex via the method of Yonetani and coworkers (Yonetani et al., 1972).

Lactoperoxidase anion complexes were generated by addition of excess of the desired reagent to protein samples that had been flushed with nitrogen. The salts were added either as solids or as stock solutions prepared in the appropriate buffer. Visible spectra of these complexes were recorded under anaerobic conditions, the same conditions utilized for EPR sample preparation.

It should be noted that at no time during these LPO complex preparations were the solutions allowed to come in contact with stainless steel needles. Teflon canulas were employed throughout for introduction of gases and solutions into EPR tubes.

All the X-band EPR spectra were obtained on a Varian Model E-104A EPR spectrometer equipped with a 6-kG magnet and an Air Products helium cryostat system. The EPR

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[1] Abbreviations: LPO, lactoperoxidase; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane.

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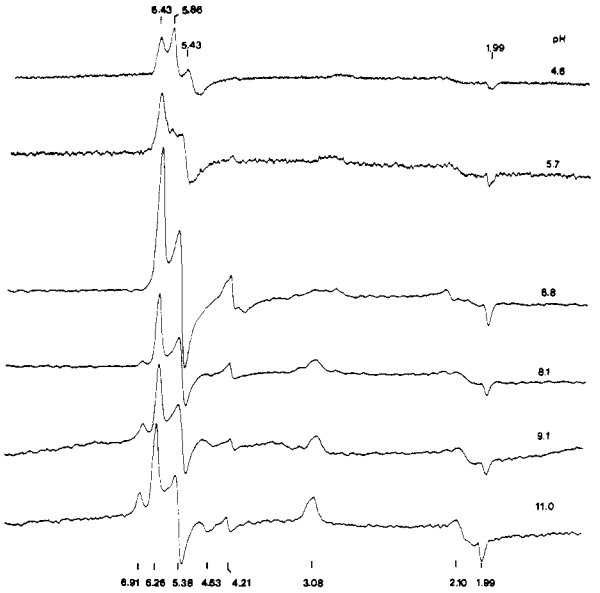

FIGURE 1: EPR spectra of ferric LPO in 0.1 M Tris/acetate buffer observed at the indicated pH values. At pH 4.6 and 5.7 the protein concentration was 0.2 mM. In all other spectra the protein concentration was 0.5 mM. EPR conditions were as follows: temperature, 6.0 K; frequency 9.190 GHz; power, 2 mW; modulation amplitude, 10 G at 100 kHz; sweep width, 4 kG.

signals were referenced to solid pitch for which $g = 2.002$. Spectra of protein/NO complexes were run at 70 K; data for anion complexes were collected at 6.0 K.

RESULTS

Lactoperoxidase. The pH dependencies of the EPR spectra of native LPO, the ferric LPO/CN^- complex, and the ferrous LPO/NO complex were examined. Three changes were observed in the pH titration of the native protein in phosphate buffer. At pH 5.0 four resolved peaks with g values of 6.43 and 1.99 and g markers[2] 5.86 and 5.43 were observed. Raising the pH from 5.0 to 6.8 resulted in the disappearance of the g marker 5.86 peak and a shift of other low-field signals from $g = 6.43$ to $g = 6.26$ and from g marker 5.43 to g marker 5.38; subsequent increases in pH did not cause further shifting of these features. However, at pH values greater than 6.8, features appeared on either side of the major $g = 6.26$ and 5.38 signals (g markers 6.91 and 4.63) accompanied by a small but detectable decrease in the signal amplitude at $g = 6.26$ and $g = 5.38$. When the same study was repeated in Tris buffer over the same pH range, the behavior was unchanged with two notable exceptions. In the Tris-buffered protein solutions, the g marker 6.91 and 4.63 features on either side of the major peaks were better defined and of greater intensity than those observed in the phosphate-buffered protein solutions. Formation of the species responsible for the new features observed at high pH was reversible upon lowering the pH. Second, at pH 8.1 and above, features appeared in the spectrum at $g = 3.08$, 2.10 (see Figure 1), and 1.50 (obtained from the absorption spectrum). Their signal amplitude increased as the pH was raised. Absorption EPR spectra obtained with the rapid-passage method indicated that at least one low-spin species was present at all pH values examined. A $g = 4.21$ signal, due presumably to a rhombic iron(III) impurity, is present in all spectra taken at intermediate pH values. At pH 8.1, the EPR spectrum obtained from the Sigma protein was identical with that of the fresh LPO.

[2] The term g marker is used to designate the position of a feature whose actual g value cannot be acertained by visual examination of the EPR spectrum.

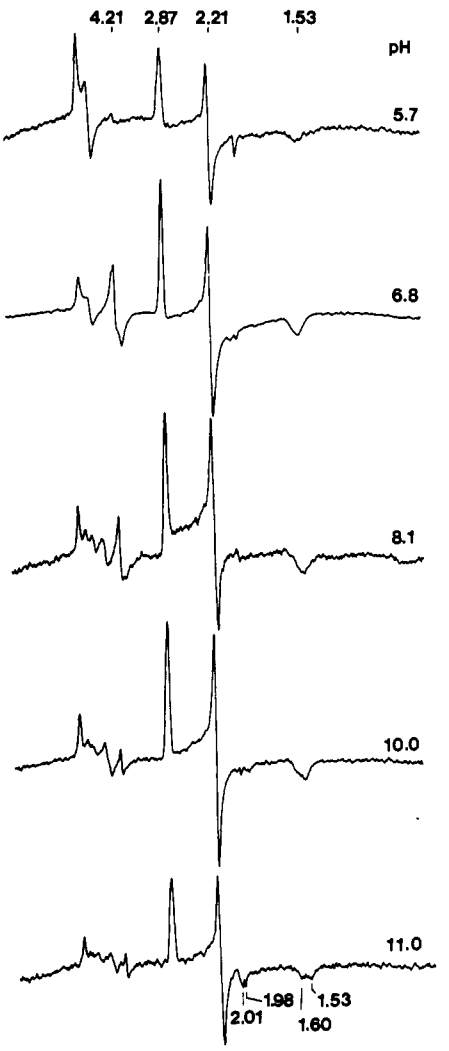

FIGURE 2: EPR spectra observed for 0.51 mM ferric LPO in 0.1 M Tris/acetate buffer incubated with a 10-fold excess of KCN at the indicated pH values. EPR parameters were as follows: temperature, 5.6 K; frequency, 9.189 GHz; power, 3 mW; modulation amplitude, 10 G at 100 kHz; sweep width, 4 kG.

Lactoperoxidase/Cyanide. Upon reaction with cyanide ion, the majority of the LPO was converted to the low-spin cyanide complex whose g values (2.87, 2.21, 1.53) remained constant over the pH range 5.7–11.0 (see Figure 2). These g values are in good agreement with those reported previously for the cyanide complex of LPO (2.930, 2.239, 1.567) (Bolscher et al., 1984). However, features corresponding to high-spin species persist in the presence of a 10-fold excess of cyanide ion. Even after 1.5 h of reaction at room temperature, the LPO was not completely converted to the low-spin cyanide complex.

Nitric Oxide Complexes of Lactoperoxidase. No pH dependence was observed in the EPR spectra of the ferrous LPO/NO derivative between pH 6.0 and pH 10.7. EPR spectra of nitric oxide derivatives prepared in sodium phosphate buffer were identical with those of the NO derivatives prepared in Tris/acetate buffer. All samples that were reduced with excess dithionite ion yielded low-spin spectra in which the g_z component was split into nine lines with hyperfine splitting parameters of 19.5 and 7.7 G (see Figure 3D). Hyperfine splitting of the g_y component was also observed; the five lines were split by 8 G. The g values and splitting parameters measured for the various preparations are summarized in Table

Table I: Electron Paramagnetic Resonance Parameters for Ferrous Lactoperoxidase/NO

LPO complex	conditions	g_x	g_z	g_y	A_I (G)	A_{II} (G)	ref
LPO/^{14}NO	0.1 M phosphate buffer, pH 7.5, ascorbate, 30 K	2.087	2.004	1.962	20.9	7.3	Bolscher & Wever, 1984
LPO/^{15}NO	0.1 M phosphate buffer, pH 7.5, ascorbate, 30 K	2.088	2.004	1.963	27.5	7.3	Bolscher & Wever, 1984
LPO/^{14}NO	0.1 M Tris-HCl, pH 8.0, sodium dithionite, 70 K	2.09	2.01	1.97	17.5	n.o.[a]	Sievers et al., 1984
LPO/^{14}NO	buffer not indicated, sodium dithionite, 77 K	2.070	2.004	1.958	16	n.o.[a]	Yonetani et al., 1972
LPO/^{15}NO	buffer not indicated, sodium dithionite, 77 K	2.070	2.004	1.958	25	n.o.[a]	Yonetani et al., 1972
LPO/^{14}NO	0.1 M phosphate buffer, pH 6.0, sodium dithionite, 70 K	2.081	2.001	1.958	19	8	this work
LPO/^{14}NO	0.1 M phosphate buffer, pH 6.8, sodium dithionite, 70 K	2.081	2.008	1.958	19	8	this work
LPO/^{14}NO	0.1 M phosphate buffer, pH 10, sodium dithionite, 70 K	2.082	2.002	1.957	19	8	this work
LPO/^{14}NO	0.1 M Tris/acetate, pH 8.0, sodium dithionite, 70 K	2.073	2.004	1.957	19.5	7.7	this work
LPO/^{15}NO	0.1 M phosphate buffer, pH 6.8, sodium dithionite, 70 K	2.079	2.008	1.965	28.0	7.5	this work

[a] n.o. indicates that hyperfine splitting was not observed.

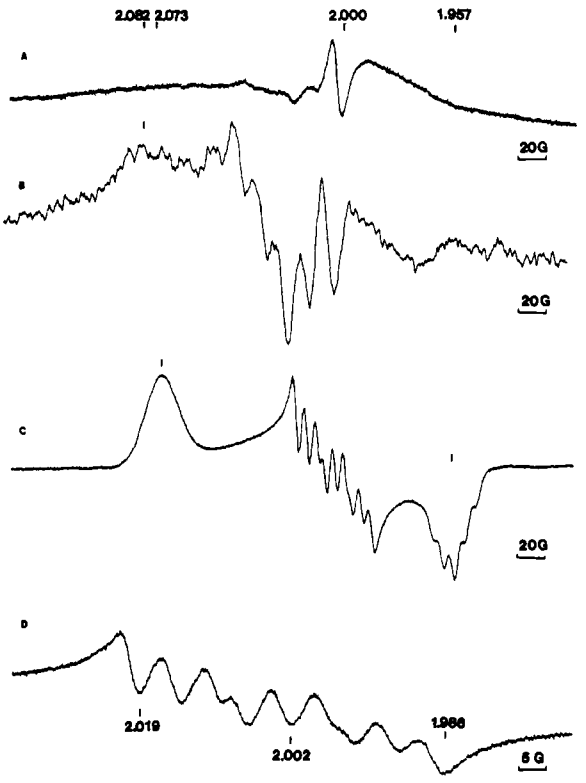

FIGURE 3: (A) EPR spectrum obtained for a 0.85 mM LPO/NO complex prepared with NO(g) and less than 1 equiv of sodium dithionite in 0.1 M Tris/acetate, pH 8.0. (B) EPR spectrum obtained with approximately 5 equiv of dithionite ion and all other conditions the same as those for spectrum A. (C) EPR spectrum of the LPO/NO complex in the presence of approximately 25 equiv of dithionite ion. All other conditions were the same as in spectra A and B. EPR parameters for spectra A–C were as follows: temperature, 70.0 K; frequency, 9.192 GHz; power, 5 mW; modulation amplitude, 2 G at 100 kHz; sweep width, 400 G; receiver gain, 1.25×10^4 (A) 1.25×10^5 (B), and 5×10^3 (C). (D) Expanded g_z region of the completely reduced LPO/NO complex in 0.1 M Tris/acetate, pH 8.0. EPR parameters for spectrum D were the same as those for spectra A–C with the exception of a 100-G sweep width.

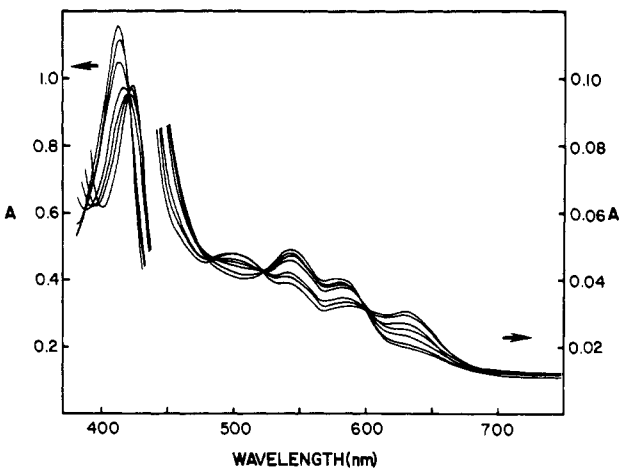

FIGURE 4: Visible absorption spectra observed for the titration of native ferric LPO with nitrite ion. Protein concentration was 10.1 μM in 0.1 M phosphate buffer, pH 6.8, at 25 °C. The nitrite to LPO ratios were 0, 430, 1300, 3025, 4750, 6470, and 15 100. The association constant for the LPO/nitrite complex was estimated to be 14.2 L/mol at 25 °C from the data obtained at 412 nm. Lack of isosbesticity at lower wavelengths was due to the intense absorbance of nitrite ion at 355 nm.

I. The two weak EPR spectra that lack defined hyperfine splitting in Figure 3A,B were generated when insufficient reducing agent was used. When 5 equiv or less of dithionite ion was added, the EPR spectrum due to ferric LPO was still observed at 5.8 K (spectra not shown), and the species observed in the EPR spectra obtained at 70 K are believed to be minor species on the basis of signal to noise and receiver gain comparisons to those of the final LPO/NO spectrum. However, subsequent treatment with excess dithionite ion elicited conversion to ferrous LPO/NO, which produced the nine-line spectrum shown in Figure 3C,D.

Since the ferric nitrite complex could be used as a precursor to the ferrous LPO/NO complex, it was characterized by visible and EPR spectroscopies. Titration of native LPO with sodium nitrite was monitored by optical spectroscopy (see Figure 4). This series of spectra contains isosbestic points at 420, 523, and 600 nm. The Soret band was shifted from 413 nm for native LPO to 424 nm in the LPO/NO_2^- complex. Upon addition of a 2-fold excess of dithionite ion (on the basis of the nitrite ion concentration), the Soret band shifted to 433 nm, corresponding to the ferrous LPO/NO complex.

Examination of the EPR spectrum of the ferric LPO/NO_2^- complex revealed that the complex is low spin (g values of 2.53, 2.32, and 1.81; Figure 5). Its rhombicity parameter (V/Δ = 1.11) (Palmer, 1983) is considerably larger than that for LPO/CN^- (V/Δ = 0.53) and LPO/N_3^- (V/Δ = 0.50) (Bolscher et al., 1984).

Discussion

Native Lactoperoxidase. The pH dependence of EPR spectra for native ferric LPO indicates that two rhombic high-spin species are present below pH 6.0 (g_x markers 6.43 and 5.86, signal amplitude increases significantly as pH decreases). A reversible spectrophotometric change has been noted for LPO between pH 3 and pH 5 (Morrison et al., 1970), and subsequently, Kimura and Yamazaki (1978) have reported a pK_a = 3.5 for ferric LPO. The species with g_x marker 5.86 may correspond to the acid form of LPO predominant below pH 3.5. The values g_x = 6.43, g_y = 5.43, and g_z = 1.99 are in good agreement with those reported for native LPO at pH 7.0 (Sievers et al., 1983) and are very similar to those for ferric

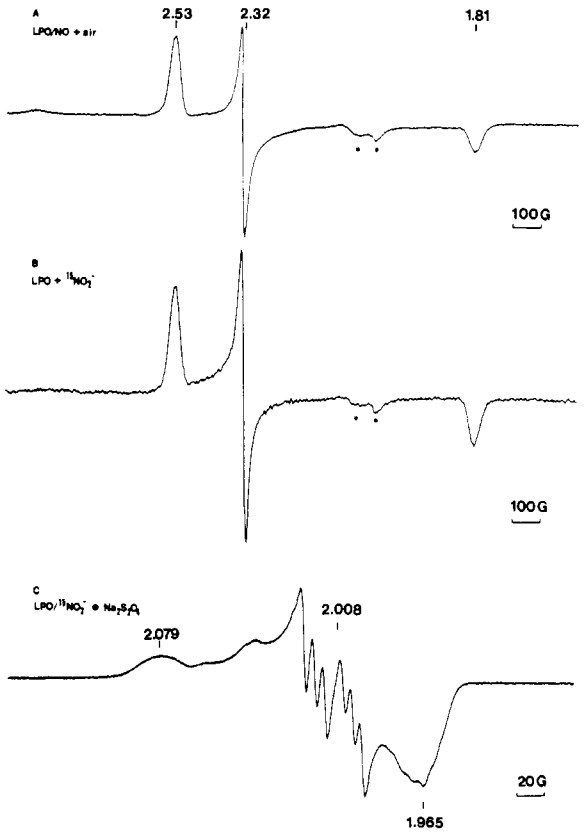

FIGURE 5: (A) EPR spectrum observed for a ferrous LPO/NO sample (from Figure 3C) in 0.1 M Tris/acetate, pH 8.0, after incubation at 4 °C for 3 days. (B) EPR spectrum obtained for a 0.40 mM ferric LPO sample reacted with 0.055 M $Na^{15}NO_2$ in 0.1 M phosphate, pH 6.8. EPR parameters for spectra A and B were as follows: temperature, 6.0 K; frequency, 9.194 GHz; power, 1.5 mW; modulation amplitude, 10 G at 100 kHz; sweep width, 2000 G. (C) EPR spectrum observed upon dithionite reduction of the sample in (B). EPR conditions were as follows: temperature, 70.7 K; frequency, 9.190 GHz; power, 5 mW; modulation amplitude, 2 G at 100 kHz; sweep width 400 G. Asterisks indicate signals due to high-spin species.

eosinophil peroxidase at pH 7.2 (Bolscher et al., 1984). However, native LPO above neutral pH exhibits one prominent high-spin species (g_x = 6.26, g_y = 5.38, and g_z = 1.99). As the pH is raised above pH 8.0, at least two high-spin species and at least one low-spin species become evident as judged by multiple EPR signals (see Figure 1). The features at g markers 6.91 and 4.63 suggest that the new high-pH, high-spin form is more rhombic in nature than the high-spin species found at neutral pH values. This high-pH form is probably the result of subtle conformational changes due to pH alteration of protein hydrogen bonding or a new high-spin hydroxide complex. The pK_a values corresponding to ionizable groups of LPO have been reported at 6.3 and 7.6 on the basis of the pH dependence of the LPO/CN^- association rate (Dolman et al., 1968). The changes observed in the EPR spectra of native LPO with increasing pH (from 5.0 to 6.8; from 6.8 to 8.1) parallel these pK_a values.

At least two spectrophotometrically distinct forms of LPO are present when the protein is reduced (Carlström, 1969a; Sievers, 1980). The form more stable at higher pH values is slowly converted to the low-pH form; the rate of conversion is increased at lower pH values. Addition of cyanide or carbon monoxide to LPO results in the disappearence of these multiple forms (Ohtaki et al., 1985; Carlström, 1969a; Kimura et al., 1981). Our EPR data indicate that multiple forms of LPO are also present in the ferric form of the protein (below pH 6.0 and above pH 8.0). Shiro and Morishima (1986) have reported that the optical and proton NMR spectra of native LPO are independent of pH between 7 and 12. This does not contradict the EPR-observed pH dependence since neither of the techniques used is as sensitive to the heme electronic environment as EPR. The resolved proton NMR spectrum of native LPO consists of very broad downfield features (Goff et al., 1985) so that minor species, particularly high-spin anisotropic species, observed in the high-pH EPR spectra (g markers 6.91 and 4.63) may easily go undetected.

There are two plausible explanations for the low-spin EPR signals (g = 3.08, 2.10, and 1.50) that appear in Tris buffer solutions (but not phosphate buffer) of LPO at elevated pH values. The first of these possibilities is that denaturation of the protein results in a six-coordinate heme with a histidine as the sixth ligand. This explanation is compatible with the fact that the low-spin features increase in intensity as the pH increases. At the highest pH values examined, a signal at g marker 2.10 becomes discernible. The average g values of 3.09, 2.22, and 1.48 have been reported as characteristic of low-spin bis(imidazole)–heme iron complexes (Peisach & Blumberg, 1973; Wever et al., 1980; Babcock et al., 1985). The g values of 3.10 and 1.50 and the g marker 2.10 observed for LPO are in good agreement with the average g values for low-spin bis(imidazole)–heme iron complexes. The g = 1.50 feature is either too broad or too weak to be detected in the derivative spectrum at the given concentration levels. The second explanation focuses on the interaction of Tris with LPO. A previously reported EPR spectrum of native LPO also contained a g = 3.11 signal of significant intensity and was obtained from a sample prepared in 0.1 M Tris-HCl (Sievers et al., 1984). Either Tris could act as a ligand for the heme iron, thereby generating a six-coordinate low spin species, or denaturation of the protein could be induced by the binding of the Tris at a site removed from the heme iron. The necessity of high-pH conditions for either of the above explanations probably lies in the required deprotonation of the Tris buffer (pK_a = 8.3) (Good et al., 1966) or the amino acid residue that acts as the sixth ligand for the heme center. The ability of Tris to alter the EPR spectrum of a protein species has previously been noted for cytochrome P-450 (Andersson & Dawson, 1984). Similarly, Sievers and co-workers (Sievers et al., 1983) noted the generation of two low-spin species (g = 3.17, 2.27, and 1.56; g = 2.94, 2.22, and 1.67) of LPO in the presence of HEPES buffer. Manthey and co-workers (Manthey et al., 1986) reported two sets of g values in low-spin components of native LPO in citrate buffer, which were in agreement with those reported by Sievers and co-workers (Sievers et al., 1983). These observations suggest that LPO may be very sensitive to buffers containing hydroxyl functional groups. The low-spin species appear to be present in greater relative concentrations in these buffers when compared to the native protein spectra acquired in phosphate buffer. However, it has been suggested that the low-spin components were due to freezing artifacts (Manthey et al., 1986). Assigning the low-spin species to freezing artifacts would also be in agreement with the lack of evidence for low-spin in species in the proton NMR data by Shiro and Morishima (1986) and Goff and co-workers (Goff et al., 1985).

Lactoperoxidase/Cyanide. No significant spectral changes are observed in the EPR spectra of LPO/CN^- below pH 10. Over the pH range examined, the EPR spectra of the LPO/CN^- samples contain high-spin iron(III) EPR signals that are similar to those found in the EPR spectra of the native ferric LPO at the corresponding pH values (see Figure 6). Evidence for the existence of two high-spin forms at and above

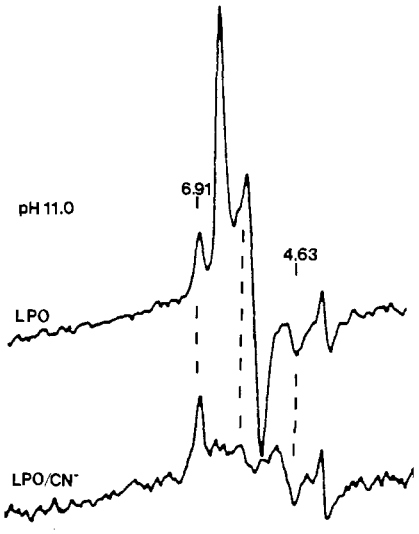

FIGURE 6: Comparison of low-field region of pH 11.0 EPR spectra of native LPO and LPO/CN^- from Figures 1 and 2A.

pH 8.1 is obtained from the shifting and intensity changes of the EPR signals in the $g = 6$ region. Upon addition of cyanide to a LPO sample at pH 8.1 or above, the most intense feature in the low-field region is a feature also observed at g marker 6.91 in native LPO at high pH. The amplitude of the peaks that are predominant in the native LPO spectra at all pH values above pH 8.1 decreases in the corresponding LPO/CN^- spectra as the pH increases. In the same spectra the amplitude of the features at g markers 6.91 and 4.63 increases with increasing pH. The cause of this behavior may be differing affinities of the two high-spin forms for cyanide ion and/or hydroxide ion.

Paul and co-workers (Paul et al., 1980) and Carlström (1969b) have reported the existence of at least five LPO isozymes. The presence of isozymes in the EPR samples could account for the multiple high-spin forms observed in the LPO/CN^- samples. These isozymes may be altered in such a manner that their affinity for cyanide ion is very low relative to the predominant form of ferric LPO. Physiochemical and structural differences among the LPO isoenzymes remain to be defined, and the isoenzyme profile of our preparations has not been determined.

The third and most likely explanation for appearance of residual high-spin iron(III) signals is the generation of a hydroxide complex at high pH. Formation of hydroxide complexes is observed under high-pH conditions for a number of proteins (Morishima et al., 1977). These hydroxide complexes are typically low-spin heme complexes. However, such a low-spin complex is not observed at high pH for the native ferric protein. The possibility does exist that the hydroxide complex of LPO is a high-spin complex, possibly the more rhombic species that appears in the spectra of native LPO at high pH. If this is the case, the necessity of hydroxide ion displacement for the formation of a low-spin cyanide complex would explain the difference in reactivity of the two high-spin forms of the ferric protein (native LPO and LPO/OH^-) toward cyanide at high pH.

Evidence for a second low-spin ferric LPO complex can be seen in the LPO/CN^- pH 11.0 spectrum (see Figure 2) in which the high-field feature appears to be split into two peaks ($g = 1.54$ for LPO/CN^- and $g = 1.60$ for LPO/?). Recently, Shiro and Morishima (1986) reported the pH dependence of LPO/CN^- monitored by proton NMR spectroscopy. They observed a 1 ppm upfield shift of the heme methyl peak at 20 ppm for pH values above 6.0. A more drastic change was noted at pH 10–11; the 20 ppm methyl peak decreased in intensity, and a new peak grew in at 18.8 ppm. The NMR spectral change of LPO/CN^- at high pH (Shiro & Morishima, 1986) is comparable to that of horseradish peroxidase/cyanide (Morishima et al., 1977) and has been rationalized in terms of a protein conformational change in the vicinity of the heme attributable to the acid–alkaline transition for a low-spin cyanide complex. This alkaline form of LPO/CN^- with the 18.8 ppm methyl resonance appears in the same pH range where the $g = 1.60$ peak in the EPR spectrum becomes distiquishable. Thus, it is likely that the new EPR signal corresponds to the alkaline conformation of the LPO/CN^- complex.

Nitrogen Oxide Complexes of Lactoperoxidase. The nitrosyl complex of ferrous LPO has been characterized via EPR spectroscopy by a number of workers (Bolscher & Wever, 1984; Sievers et al., 1984; Yonetani et al., 1972). In the presence of excess dithionite ion, we observe a nine-line hyperfine structure in the EPR spectrum of LPO/NO. The EPR spectra reported here are consistent with assignment of the fifth axial ligand in native ferric LPO as being bound through a nitrogen atom. This is in agreement with magnetic circular dichroism data (Sievers et al., 1983). However, two of the previous EPR studies have reported spectra (three line) that appeared to indicate that the iron–histidine bond was broken in the ferrous LPO/NO complex (Sievers et al., 1984; Yonetani et al., 1972). Here we attempt to explain the reasons for the inconsistencies in the observed spectra.

One explanation that has been offered for observation of nine-line and three-line spectra was pH and/or buffer effects. Our results clearly indicate that this is not the case. The nine-line spectrum is reproducible regardless of pH in the range from pH 6.0 to pH 10.7. Our experiments in sodium phosphate and Tris/acetate buffers all yield EPR spectra with identical nine-line hyperfine splitting patterns.

The choice of reducing agent has been suggested as the cause of the differences. Bolscher and Wever (1984) reported a nine-line EPR spectrum generated by use of ascorbate. Dithionite ion was the reductant employed by Sievers and co-workers (Sievers et al., 1984); they were unable to generate the nine-line hyperfine spectrum. They did, however, report the observation of a low-spin spectrum ($g = 2.54$, 2.33, and 1.81). Our data show that dithionite is not problematic. The nine-line hyperfine split EPR spectrum is generated in either buffer system with dithionite ion as the reductant. However, our ferrous LPO/NO complex was converted to a species with the low-spin spectrum by thawing the sample and allowing it to incubate for several days at 4 °C. During this time the sample is not kept strictly anaerobic. Conversion of the NO ligand to NO_2^- and the Fe(II) to Fe(III) by oxygen that slowly seeps into the system presumably serves to generate a low-spin Fe(III)/nitrite complex. This Fe(III)/nitrite complex has an EPR spectrum with g values identical with those reported by Sievers and co-workers (Sievers et al. 1984) for LPO/NO and by Bolscher and Wever (1984) for myeloperoxidase/NO (g values of 2.572, 2.320, and 1.810). An EPR spectrum identical with that observed for the incubated ferrous LPO/NO sample is obtained from a sample prepared by addition of sodium nitrite to native LPO (see Figure 5A,B). Thus, the previously reported low-spin complexes are complexes of nitrite ion, not nitric oxide. Addition of a reductant converts the Fe(III)/NO_2^- system to Fe(II)/NO, as verified by visible and EPR spectroscopies.

The possibility of a low-spin ferric NO complex being responsible for the low-spin spectrum is eliminated by the fact

that, as expected, ferric LPO mixed with nitric oxide is EPR silent at 70 K. The presence of excess free NO in the solution precluded examination by EPR at lower temperatures as the NO gives rise to a broad, intense signal at $g = 2$. The presence of isosbestic points in the visible spectrum (see Figure 4) of LPO upon titration with nitrite ion also discredits the possibility of subsequent formation of ferric LPO/NO. Conversion of the ferric chloroperoxidase/nitrite and cytochrome P-450/nitrite complexes to the ferric nitric oxide complex is observed spectrophotometrically above 50% ligand (nitrite ion) saturation of these proteins (Sono et al., 1986; Sono & Dawson, 1982). This type of behavior was not readily observed in the lactoperoxidase/nitrite system under the conditions examined.

The presence of the low-spin ferric LPO/NO_2^- complex in reported LPO/NO reaction mixtures suggests that incomplete reduction may be responsible for the differences in observed EPR spectra. Titration of ferric LPO and NO with dithionite shows that at low ratios of dithionite to protein a spectrum is obtained that resembles the reported three-line spectrum (see Figure 3B). As the dithionite ion concentration is increased, the nine-line hyperfine splitting is resolved (see Figure 3C). Excess dithionite eliminates any oxygen that has not been removed from the system prior to NO complex formation, and therefore, side reactions with oxygen are prevented. Once the LPO/NO complex is formed in the presence of excess reductant (effectively anaerobic conditions), the nine-line hyperfine splitting is readily observable. Both the g values and the hyperfine splitting parameters for ferrous LPO/NO are consistent with those reported by Bolscher and Wever (1984).

Although ferrous LPO/NO has a slightly different g_z value and hyperfine splitting constants than eosinophil peroxidase, a number of similarities are noteworthy. EPR spectra of both ferrous protein derivatives show hyperfine structure on the g_x and g_y absorptions, previously attributed to splitting from the pyrrole nitrogen nuclei (Bolscher & Wever, 1984). The g_y splitting is perturbed by the ^{14}NO to ^{15}NO substitution (compare Figures 3C and 5C), however, and the hyperfine splitting must reflect a significant component from the NO ligand. Generation of ferrous LPO/NO from ferric LPO causes a 20-nm shift in the Soret peak; a similar shift (19 nm) is obtained when ferrous eosinophil peroxidase/NO is prepared from the ferric form of the protein (412 nm to 431 nm) (Bolscher & Wever, 1984). An eosinophil peroxidase/nitrite complex has not been reported. However, on the basis of the similarity of the EPR spectra of ferric LPO/NO_2^- and the myeloperoxidase/nitrite complexes, eosinophil peroxidase/nitrite should have similar characteristics and be obtainable from the oxidation of the ferrous NO complex of the protein.

In conclusion, the EPR studies of ferrous LPO/NO confirm that the fifth axial ligand in LPO is bound to the iron center through a nitrogen atom. However, complete reduction of the ferric LPO is necessary if the nine-line hyperfine splitting pattern is to be observed. The ferrous LPO/NO complex and the LPO/NO_2^- complex can be easily interconverted by addition of the appropriate oxidizing or reducing agent. Although the EPR spectrum of the ferrous LPO/NO complex remains unchanged as a function of pH, ferric LPO and ferric LPO/CN^- species do show a pH dependence in their EPR spectra. The EPR data indicate that an acid–alkaline transition of the protein conformation in the heme vicinity does occur in LPO. Furthermore, ferric LPO undergoes a conformational change or hydroxo complex formation at high pH, which results in multiple high-spin complexes with notably different reactivities toward cyanide ion.

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