The heme prostatic group from the bovine milk enzyme lactoperoxidase (LPO), termed heme l, is isolated through an approach that combines proteolytic hydrolysis and reverse-phase high performance liquid chromatographic separation of the resulting digest. Application of different proteases yields either a peptide-bound heme (with trypsin and chymotrypsin) or a peptide-free heme (with proteinase K). Both heme l and heme l- peptide species were investigated by paramagnetic $^1$H NMR spectroscopy, electrospray mass spectrometry, and peptide sequence analysis. Paramagnetic $^1$H NMR experiments on the low spin bis(cyano)-Fe(III)heme l complex conclusively define the heme l structure as a 1,5-bis(hydroxymethyl) derivative of heme b. The electrospray mass spectrum of heme l confirms the two-site hydroxyl functionalization on this heme. Paramagnetic $^1$H NMR spectra of the high spin bis(dimethyl sulfoxide)-Fe(III) complexes of the isolated heme species provide information regarding peptide content. Sequence analyses of peptides released from two heme l-peptide species by base hydrolysis suggest that heme-protein ester linkages in lactoperoxidase occur between the two hydroxyl groups of heme l and the carboxylic side chains of glutamate 275 and aspartate 125. These results confirm the earlier reported structural proposal (Rae, T. D., and Goff, H. M. (1996) J. Am. Chem. Soc. 118, 2103-2104).

Mammalian secretory fluids maintain antimicrobial activity as part of a nonspecific defense mechanism for the protection of mucosal surfaces against invading bacteria, parasites, and viruses (1). Among the agents that provide antimicrobial protection in these fluids are the products of peroxide-driven oxidation of halide or pseudohalide ions, catalyzed by a family of hemoprotein enzymes collectively known as peroxidases. These enzymes are key players in the antimicrobial defense of mucosal surfaces against invading bacteria, parasites, and viruses (1). Among the agents that provide antimicrobial protection in these fluids are the products of peroxide-driven oxidation of halide or pseudohalide ions, catalyzed by a family of peroxidase enzymes. The conclusions were supported by structural details provided by the x-ray analysis of human MPO (19) in which an additional unique methionine sulfonium linkage is seen (19, 20). Infrared vibrational measurements have also demonstrated the presence of ester groups attributed to heme-protein linkages in myeloperoxidase (MPO), the antimicrobial enzyme utilized in macrophage cells (11).

Several heme structures have been proposed for LPO. A covalent heme-protein linkage is suggested (12) by the fact that the heme is not removed from the protein by simple organic extraction methods (commonly used for extraction of hemes a and b (13)). Proposals for heme alcohol, amine, or thiol functional groups accounted for possible ester, amide, or disulfide heme-protein linkages (12, 14, 15). Yet another proposal concluded that the LPO heme is in fact only a heme b group that is unusually encapsulated by the surrounding protein (16).

Consensus has recently been reached about the general LPO heme structure and protein linkage. Isolation of the LPO heme species is achieved through proteolytic digestion of LPO followed by reverse-phase HPLC fractionation of the resulting digest mixture (17, 18). A dialcohol heme l structure (Fig. 1) is consistent with preliminary paramagnetic $^1$H NMR analysis (17) and mass spectra (17, 18). Concurrently, this same heme l structure was proposed to explain magnetic circular dichroism data obtained from studies of LPO in comparison with MPO and a simple heme b protein (myoglobin) (7). The conclusions were supported by structural details provided by the x-ray analysis of human MPO (19) in which an additional unique methionine sulfonium linkage is seen (19, 20). Irreversible vibrational measurements have also demonstrated the presence of ester groups attributed to heme-protein linkages in myeloperoxidase, lactoperoxidase, and eosinophil peroxidase (21).
the effects of the hydroxymethyl and ester functionalities on the electronic properties of the unique mammalian prosthetic group.

EXPERIMENTAL PROCEDURES

Materials—LPO was isolated from fresh, raw bovine (Guernsey) milk by the procedure of Goff and co-workers (8). Concentrations were determined by a 1.14 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1} extinction coefficient for the heme Soret band maximum at 412 nm. Proteinase K from Tritirachium album was a commercially supplied enzyme of Amresco. Trypsin (type III, non-TCPK treated) and chymotrypsin (type II) were purchased from Sigma.

Proteolytic Digestion—Two proteolytic schemes were used in order to obtain optimal quantities of either peptide-free LPO heme (trypsin and proteinase K) or peptide-bound LPO heme species (trypsin and chymotrypsin). Prior to the application of proteases in both schemes, LPO in a 100 mM phosphate buffer solution (pH 6.8) was concentrated to obtain optimal quantities of either peptide-free LPO heme (trypsin and proteinase K) or peptide-bound LPO heme species (trypsin and chymotrypsin). Prior to the application of proteases in both schemes, LPO was allowed to equilibrate with a pH of 8.0 for optimal protease activity. All proteolytic digestions were performed at room temperature.

A typical trypsin/chymotrypsin digestion utilized 80 mg of heat-treated LPO suspended in 10 ml of aqueous buffer (pH 8.0). The protein suspension was initially treated with 0.7 mg of trypsin for approximately 3 h until the LPO precipitate cleared. Both trypsin and chymotrypsin (0.7 mg each) were then added and allowed to hydrolyze the LPO protein for 18 h before another 0.7 mg of each was added. Aliquots of digest solution (20 \mu l) were taken during the digestion and run on reverse-phase HPLC (as described below, although on an analytical scale) to monitor the progression of proteolysis. Digestion was halted after 28 h by the addition of 20% trifluoroacetic acid solution to pH 2.4. This LPO digest solution was directly fractionated by semipreparative reverse-phase HPLC.

Isolation of LPO Heme Species—Prior to HPLC separation of the LPO digests, larger protein fragments can be separated from the desired smaller fragments by gel permeation chromatography (Sephadex G-25, H_2O mobile phase). The digest is separated into a larger molecular weight red heme fraction and a small molecular weight green heme fraction. Redigestion of the larger molecular weight fraction gives an additional quantity of smaller molecular weight heme species.

All high performance liquid chromatographic separations were performed on a Varian Star HPLC system with a 9012 solvent delivery pump and 9050 variable wavelength UV-visible detector. LPO digest mixtures from both schemes were separated on a Vydac 218TP 10 mm \times 25 cm semipreparative C18 column with a 0.5–1.0-ml sample injection volume. A two-solvent (A = 0.1% trifluoroacetic acid in H_2O; B = 0.1% trifluoroacetic acid in CH_3CN) gradient mobile phase of 0–40% solvent B (0–60 min), followed by 40–100% solvent B (60–90 min) at a flow rate of 3 ml/min was applied to elute the proteolytic fragments. Detection of the heme-containing fractions eluting from the column was monitored by absorbance at 400 nm. Five major heme fractions, as numbered in Fig. 2, were collected from the trypsin/chymotrypsin LPO digest. The single major heme fraction obtained from the trypsin/proteinase K-digested LPO was collected along with two minor fractions as labeled in Fig. 2.

The combined trypsin and proteinase K digestion was performed on 40 mg of heat-treated LPO suspended in 10 ml of aqueous buffer (pH 8.0). The protein suspension was prehydrolyzed with 1.0 mg of trypsin for 1 h until the precipitate cleared, after which 1.0 mg of proteinase K was added. A 28-h proteolysis period was allowed before the addition of another 1.0 mg of proteinase K. Digestion was halted after a total time of 48 h by acidification of the digest solution to pH 2.4 with 20% trifluoroacetic acid. This LPO digest solution was directly fractionated by semipreparative reverse-phase HPLC.

FIG. 1. The heme structures utilized by peroxidase enzymes.

All peroxidases isolated from plant and fungal sources contain heme b. Note that the MPO heme m vinyl sulfonium ion linkage shown here from structural work (19) has been challenged with the suggestion of an \alpha-carbon sulfonium ion vinyl addition product (21).

FIG. 2. HPLC chromatograms for proteolytic digests of LPO.

Detection is by 400-nm absorbance. The time window displayed is 30–70 min, and the heme fractions collected are labeled. A, trypsin/chymotrypsin digest. B, trypsin/proteinase K digest.
the lyophilizate. The detector wavelength was alternatively set at 400 and 215 nm to examine the effective removal of contaminant peptides from the desired heme species. Heme fractions were collected and combined after repeated injections and then lyophilized and stored at −20 °C.

Hydrolysis and Isolation of Peptides from Heme-Peptide Species—

The twice-purified fractions designated TC-4 and TC-5 from the trypsin/chymotrypsin digestion of LPO were dissolved in 3.0 ml of 1.0 mm Na2CO3. After pH adjustment to 10.6 with dilute NaOH, the heme-peptide was allowed to hydrolyze for 20 h at room temperature. Hydrolysis was terminated by the addition of 5.0 μl of aqueous 20% trifluoroacetic acid (brining the solution to pH 2.3) before the solvent was removed under vacuum. The dried residue was dissolved in a minimal volume (~200 μl) of 0.1% trifluoroacetic acid in H2O and subjected to the same analytical column HPLC method used to purify the original heme-peptide species. Fractions eluting without 400-nm absorbance but detectable 215-nm absorbance were collected and lyophilized. Fractions were confirmed as peptides by 1H NMR in Me2SO-d6, further purification of peptide fractions prior to sequence analysis was accomplished on a Superdex Peptide HR 10/30 column from Amersham Pharmacia Biotech with an isotropic mobile phase of 1:3 CH3CN/H2O (0.1% trifluoroacetic acid) at a flow rate of 0.5 ml/min. Isolated and lyophilized peptides were dissolved in water and sequenced on a Procise 492 protein sequencer by Applied Biosystems with conversion of amino acids to their phenylthiohydantoin-derivatives.

NMR Spectroscopy—

Paramagnetic proton NMR spectra were recorded on a Bruker AMX-600 FT NMR spectrometer. Samples utilized for NMR spectroscopy consisted of semipreparatory HPLC heme fractions that were lyophilized and dissolved in deuterated solvents. No further purification of these samples was necessary to selectively assign paramagnetically influenced proton resonances of the LPO heme. Chemical shift values are referenced to the residual Me2SO signal at 2.5 ppm, calibrated in turn with tetramethylsilane.

Proton NMR spectra for the high spin bis(Me2SO)-Fe(III)heme complexes (30–50 μm) in Me2SO-d6 were obtained over a 120-kHz spectral width with 8000 data points. Scans (500,000–800,000) were acquired at a repetition rate of 20 s−1. Resulting free induction decay data were subjected to a 50-Hz exponential multiplication line-broadening factor before Fourier transformation. Nonselective T1 values for heme proton resonances were calculated from a standard inversion-recovery sequence. Curie plots and selected resonance saturation spectra, with 32,000 data points over a 120-kHz spectral width.

Mass Spectrometry—

After semipreparatory reverse-phase HPLC, LPO heme fractions were lyophilized and subsequently chromatographed on a Superex Peptide HR gel permeation column under the same conditions used to further purify peptides for sequence analysis. The desired heme species were taken up in 1:1 CH3OH/H2O with 0.5% CH3COOH to a concentration of 10–40 ng/μl and injected into an Autospect Electrospray mass spectrometer from Micromass. Data were acquired in positive ion mode. The same solvent mixture was used to deliver the sample. Molecular masses were either found for the singly charged K digestion species or determined for a series of multiply charged species on OPUS (version 3.3) processing software. Verification of the peptide-free heme molecular mass was accomplished by fast atom bombardment ionization (xenon, 8 kV) on a ZAB-HF mass spectrometer from VG Analytical in both glycerol and 3-nitrobenzyl alcohol matrices.

RESULTS

Isolation and Identification of LPO Heme Species—

Several LPO heme species are recovered from reverse-phase HPLC separation of the digests resulting from application of the two protease combinations. Protein removal by a combination of trypsin and chymotrypsin yields a number of major fragments with 400-nm absorbance (Fig. 2A). Digestion with trypsin followed by proteinase K yields one clearly dominant 400-nm absorbing species (Fig. 2B), although minor species are also present in the digest. Similar results were reported for proteinase K digestion of MPO (20), where one clearly dominant heme m species was recovered when proteolysis was applied after an autolytic cleavage of heme-protein bonds by high temperature acidic incubation (27). The same single heme species (PK-2) was obtained from LPO in this study whether or not the acid treatment conditions were applied prior to proteinase K digestion. However, the minor species had different retention times and were of slightly larger relative quantities when trypsin predigestion was used in place of the acid treatment procedure. The major heme species (PK-2) (~60–70% total heme in digest), along with the two largest of the minor species (PK-1 and -3) (~5–8% total heme in digest) were collected for structural analysis following HPLC separation of trypsin/proteinase K-digested LPO (40 mg).

Five major heme fractions (TC-1 to -5) were collected from the trypsin/chymotrypsin LPO digest (80 mg). These five species were consistently the dominant heme products from trypsin/chymotrypsin proteolysis of LPO with variable digest times and protease concentrations. Relative quantities of the five species varied with digest conditions (TC-1, -2, -3, and -5 were obtained as approximately 3–10% each of the total heme in the
The production of TC-2 heme species.

Lactoperoxidase (LPO) heme fractions TC-4 and TC-5 upon incubation in an alkaline aqueous environment. The earliest eluting heme fractions derived from base-hydrolyzed TC-4 and TC-5 showed production of TC-2 species with 215-nm absorbance upon treatment with aqueous solutions of Na₂CO₃. The second 215-nm absorbance species derived from base treatment of TC-4 (marked with an X in Fig. 3C) displayed no proton resonance signals to identify it as a peptide and is probably a heme degradation product.

Amino acid sequences determined for these two isolated peptides were as follows: TC-4P (and TC-5P1), Ala-Ser-Glu-Gln-Ile-Leu; TC-5P2, Gly-Gln-Ile-Val-Glu-Thr-Glu-Leu. Both sequences correspond to regions found in the total amino acid sequence of bovine LPO (2, 3). The hexapeptide originates from amino acids Ala₂⁷³–Leu₂⁷⁸, and the 16-amino acid peptide with Gly₁²¹¹–Leu₁³₃⁶.

Paramagnetic ¹H NMR Spectra—Despite the reported similarity in pyridine hemochrome spectra (16), the paramagnetic ¹H NMR spectra of the LPO heme and common heme b reveal clear differences. The most obvious distinction between the hemes in both high spin and low spin iron(III) spectra is the detection of only two methyl signals from the LPO heme (Figs. 4 and 5) in the spectral region where the four methyl resonances are detected for heme b (Fig. 4). In turn, an extra pair of methylene signals are seen for the LPO heme. All other signals for the LPO heme parallel those of heme b (22), differing only in respective shift values.

High Spin Fe(III) LPO Heme Complexes—¹H NMR spectra for the high spin bis(Me₂SO) complexes of the eight LPO heme species collected from proteolytic digests (PK-1 to -3, TC-1 to -5) reflect the differences in attached peptide content. Four distinct resonance shift patterns are displayed by the eight heme species isolated, as represented in Fig. 4 by the spectra for LPO heme species PK-2, TC-4, TC-5, and PK-3. Other isolated LPO heme species have resonance shift patterns similar to those shown. In addition to a similar chromatographic elution time, the spectrums of TC-2 species are virtually identical to that of PK-2 (Fig. 4A). However, resonances in the spectra for both TC-1 and PK-1 have chemical shift values nearly identical to those of TC-4 (Fig. 4B), despite significantly different chromatographic retention properties. Likewise, the shift patterns for both TC-3 and TC-5 (Fig. 4C) are similar. With regard to chemical shift pattern, PK-3 is unique among the eight heme species (Fig. 4D) investigated. Differences in shift pattern for the four LPO heme forms are most evident in the methylene region, where both shift and spread of resonances are indicative of structural variability in peptide attachment to the heme (see below). Heme methyl resonance shifts are also affected by peptide content, although the spread of the two signals varies little among the four heme forms.

The line widths of the resonances vary significantly for all eight heme species in the high spin form. The well resolved methyl signals most clearly demonstrate this feature (Table I). In general, those heme species with similar shift patterns have methyl resonance line widths markedly different from those with another shift pattern. In one exception to this generalization, the methyl resonance line widths for PK-3 fall in proximity to those exhibited by PK-1, TC-1, and TC-4 species. The narrowest methyl resonance signals are seen for PK-2 and TC-2, which are approximately half as wide as those seen for TC-3 and TC-5. Taking into account both the shift pattern and resonance line widths listed in Table I, six distinct structures are identified by the high spin ¹H NMR spectra of the eight species collected from both digestion schemes. PK-2 and TC-2 are the same heme species, as are possibly PK-1 and TC-1.

Low Spin Fe(III) Heme l—The low spin bis(cyano) complex of LPO heme l. As is the case for the trypsin/chymotrypsin whole digest, although TC-4 (~15–20% of the total heme in the digest) was in all cases the largest fraction. One of the major heme fractions from the trypsin/chymotrypsin digestion of LPO (TC-2) was found to elute with the same retention time as the major heme fraction resulting from proteinase K digestion (PK-2). The quantity of this second major fraction from the trypsin/chymotrypsin digest (TC-2) was increased significantly relative to the other major species when the digest mixture was briefly exposed to alkaline conditions prior to HPLC separation (data not shown).

Electronic spectra for the major isolated heme species show a decrease in absorptivity below 300 nm, thus suggesting loss of much of the native protein content. The Rₑ value (Åₘₜₑₛён/Åₘₜₑₜₖ for heme to protein absorbance), was increased severalfold from native LPO in all species. The shift in Soret band position from 412 nm for the native LPO enzyme to 400 nm for the isolated heme species is consistent with the replacement of the protein histidine imidazole ligand with a solvent ligand.

As is the case for the trypsin/chymotrypsin whole digest mixture, exposure of the TC-4 isolated heme fraction to alkaline aqueous solutions yields a heme species with retention time equal to that of TC-2 and PK-2 (Fig. 3D). Base treatment of species TC-5 gave not only an apparent TC-2 species but another 400-nm species with a retention time corresponding to TC-4 (Fig. 3B). With the interconversion of these heme fractions, additional species without 400-nm absorbance, but with significant 215-nm absorbance were produced. TC-4 and TC-5 each produced two species with 215-nm absorbance upon treatment with aqueous solutions of Na₂CO₃. The earliest eluting species derived from TC-4 and TC-5, designated as TC-4P and TC-5P1 in Fig. 3, A and C, respectively, had identical retention times.

¹H NMR spectra of TC-4P and TC-5P1 were also identical and displayed five clearly resolved amide doublet resonances (8) of 7.85, 8.06, 8.09, 8.17, and 8.56 ppm, which identify them as the same hexapeptide. TC-5P2 likewise gave a spectrum identifying it as a peptide but with several overlapping signals in the amide region (7.5–9 ppm). The second 215-nm absorbing species derived from base treatment of TC-4 (marked with an X in Fig. 3C) displayed no proton resonance signals to identify it as a peptide, and is probably a heme degradation product.

Heme methyl resonance shifts are also affected by peptide content, although the spread of the two signals varies little among the four heme forms.

The line widths of the resonances vary significantly for all eight heme species in the high spin form. The well resolved methyl signals most clearly demonstrate this feature (Table I). In general, those heme species with similar shift patterns have methyl resonance line widths markedly different from those with another shift pattern. In one exception to this generalization, the methyl resonance line widths for PK-3 fall in proximity to those exhibited by TC-1, PK-1, and TC-4 species. The narrowest methyl resonance signals are seen for PK-2 and TC-2, which are approximately half as wide as those seen for TC-3 and TC-5. Taking into account both the shift pattern and resonance line widths listed in Table I, six distinct structures are identified by the high spin ¹H NMR spectra of the eight species collected from both digestion schemes. PK-2 and TC-2 are the same heme species, as are possibly PK-1 and TC-1.

Low Spin Fe(III) Heme l—The low spin bis(cyano) complex of LPO heme l.
the LPO heme is more amenable to detailed structural analysis by ¹H NMR spectroscopy. The sensitivity for proton resonance detection is greatly increased in the low spin form due to the considerably sharper lines. However, only the peptide-free form of the LPO heme was accessible to ¹H NMR experiments in the low spin bis(cyano) form. Upon the addition of excess KCN to an isolated LPO heme-peptide (TC-4 or TC-5), a mixture of species is initially seen, indicated by the appearance of more than one pair of methyl signals (data not shown). This mixture converts to a single species over a period of hours to give a spectrum identical to that of the peptide-free heme species (heme l). Buffering the sample solution at pH 8.0 to counter potential base hydrolysis from added CN⁻ had no effect in limiting the conversion of heme l-peptides to heme l. The bis(CN)-Fe(III) complex of the peptide-free LPO heme (heme l) gives the ¹H NMR spectrum shown in Fig. 5A. The paramagnetic shift of the heme proton signals allows for resolution of most of the expected heme substituent proton resonances from impurity and residual solvent signals. Application of an inversion-recovery sequence with a 𝑡{-relaxation delay of 400 ms permits the resolution of all heme l proton signals at 25 °C (Fig. 5B). Spin-spin splittings of signals from protons on heme propionate and vinyl groups are also distinguishable. Based on the features of the simple one-dimensional spectrum alone (relative resonance intensities, signal splitting, and chemical shift in comparison with those of heme b), signals can be assigned to heme methyl, meso, α- and β-vinyl, and α- and β-propionate substituent protons. All six signals from the two vinyl groups are readily apparent in the heme l low spin spectrum, discounting the type of vinyl group functionalization known for heme c. Resonance assignments and 𝑇1 relaxation times for the protons of heme l in the low spin ferric bis(cyano) complex form are listed in Table II.

The paramagnetic shift pattern for the proton signals of the LPO heme in its low spin iron(III) form is significantly perturbed by the hydroxyl functionalization at two methyl positions. In comparison with the low spin spectrum of heme b, the two methyl signals are shifted further downfield (a and b), whereas the two methylene signals with alcohol functionalities are shifted far upfield from the methyl proton region. Inspection of the other downfield-shifted signals reveals that the spread between the pair of α-propionates (e and g) and α-vinyl signals (c and d) is significantly increased for heme l, clearly indicating that this type of substitution on the two methyls of the LPO heme causes a greater asymmetry in the unpaired electron density on the porphyrin ring. The pattern of paramagnetic shifts for the signals of the LPO heme in its low spin form also suggests that the functionalized methyl positions are located on pyrroles with one of the vinyl groups and one of the propionates. Conclusive positioning of these groups, however, is established only by NOE experiments.

Due to the large line widths and decreased sensitivity for protons on paramagnetic molecules, two-dimensional NMR spectra are typically quite difficult to obtain (28). However, the relatively sharp signals of heme l in the low spin form permitted the magnitude COSY spectrum at elevated temperature (63 °C) shown in Fig. 5C. While providing little information about the overall structure of the heme, the COSY map relates the signals for protons at the α-position with β-positions of the vinyl and propionate groups. Identification of coupled signals arising from the same vinyl or propionate group complements the interpretation of NOE results.

Lack of scalar couplings for porphyrin ring substituents dictates that the ring position assignments be made through NOE correlations between adjacent substituent groups. Detection of NOE signals is problematic for paramagnetic molecules, not only due to the decreased sensitivity for the signals but also due to the dependence of the effective NOE on the nuclear...
Increased relaxation rates for paramagnetically influenced nuclei results in very small NOE signal intensities. However, with the use of a viscous solvent (in this case a Me$_2$SO-$d_6$/D$_2$O mixture at low temperatures), NOE enhancement is achieved through an increase in the rotational correlation time of the molecule, and thus an increase in cross-relaxation between nearby protons (30, 31). To further enhance the possibility of NOE detection, the resonances selected for saturation were those with the smallest $T_1$ values of expected NOE pairs so that resulting NOEs could be detected on resonances with the slowest relaxation rates. The steady-state NOE difference experiment is also preferred for its greater sensitivity over NOE correlation spectroscopy (32).

Table III summarizes the results of steady-state NOE difference experiments performed on the bis(CN)-Fe(III)heme $l$ complex. NOE signals are negative due to the slow rotation of the heme molecule in viscous solvents (emulating a large molecule). To resolve both the irradiated nucleus and the nuclei that experience NOE transfer, various temperatures and solvent compositions were required (Table III). Cross-relaxation from the meso protons ($i, j, k, r$) to their adjacent pyrrole substituents was found to be much more efficient than between substituents on the same pyrrole. Detection of NOE signals for $\alpha$-vinyl protons upon saturation of the protons on the adjacent pyrrole substituent groups is particularly difficult, due to poor cross-relaxation and inherently small signals for the single, multiply split $\alpha$-vinyl proton signals. The trans-$\beta$-vinyl proton NOE signal is more apparent ($n$ and $o$) in these cases.

**FIG. 5.** The paramagnetic $^1$H NMR (600 MHz) spectra for the low spin bis(CN)-Fe(III)heme $l$ complex in 50:50 Me$_2$SO-$d_6$/D$_2$O with 0.01% trifluoroacetic acid-$d_1$. A, standard one-dimensional spectrum at 298 K. B, spectrum with inversion-recovery sequence, $\tau = 400$ ms at 298 K. C, selected region of the magnitude COSY spectrum at 336 K showing cross-peaks between $\alpha$- and $\beta$-protons of heme vinyl and propionate substituents. Peak labels are defined in Table II (reported chemical shifts in Table II are not those for signals in the COSY spectrum due to temperature difference). Chemical shifts are referenced to the residual Me$_2$SO signal at 2.5 ppm.

**TABLE I**

| Sample | $\delta$-8-CH$_3$ ppm | LW$^*$ 8-CH$_3$ Hz | $\delta$-3-CH$_3$ ppm | LW$^*$ 3-CH$_3$ Hz | Peptides |
|--------|------------------------|---------------------|------------------------|---------------------|----------|
| PK-1   | 69.80                  | 214                 | 63.43                  | 191                 | 1        |
| PK-2   | 67.59                  | 188                 | 61.80                  | 153                 | 0        |
| PK-3   | 65.46                  | 236                 | 59.68                  | 275                 | 1        |
| TC-1   | 69.77                  | 206                 | 63.34                  | 198                 | ND (1)$^b$ |
| TC-2   | 67.58                  | 168                 | 61.60                  | 160                 | 0        |
| TC-3   | 68.07                  | 320                 | 61.88                  | 320                 | ND (2)   |
| TC-4   | 69.67                  | 252                 | 63.33                  | 236                 | 1        |
| TC-5   | 67.68                  | 359                 | 61.80                  | 336                 | 2        |

$^a$ LW, line width at half-height of resonance ($\pm 7$ Hz).
$^b$ ND, not determined. The number in parentheses is the expected number of peptides attached based on correlations.
of the functionalized methylene is also unreliable due to significant on another was not feasible. Detection of an NOE to the resonances examined. Hence, saturation of one signal and detection of the substituent groups on heme l. groups must be located on the same pyrrole, however, on the bis(cyano)-heme l complex (Fig. 6) illustrates that, whereas most of the resonances follow Curie behavior (data not shown) demonstrates that all of the proton resonances follow Curie behavior in the high spin form of the heme. Electrospray Mass Spectra—The molecular weights of five LPO heme species isolated by proteolytic digestion (PK-1 to -3, -4 and -5) were determined by positive ion electrospray mass spectrometry (Table IV). For heme species PK-2, a pair of singly charged ions were detected at masses of 648 and 680. A heme b standard likewise gave two masses representing approximately a 1:1 mixture of the free heme complex and a monomethanol adduct. The mass of the adduct-free PK-2 species is therefore concluded to be 648, with the 680 mass representing the heme with a ligated methanol molecule. The methanol adduct assumption was confirmed by an electrospray ionization spectrum of heme b in CH3CN/H2O, where the second ion peak had a mass 41 units greater than the adduct-free heme. Mass spectra of the other LPO heme species displayed peaks for multiply charged states. The mass signals of heme species could in most cases be distinguished from signals of peptide impurities by the ~5% intensity 57Fe isotope peak in the monocation state. No methanol adduct signals were detected for any of the peptide-bound heme l species studied.

**Heme l of Lactoperoxidase**

**TABLE II**

Proton resonance assignments and T1 relaxation times for the bis(CN)-Fe(III) heme l complex

The complex was in 50/50 Me2SO-d6/D2O, 0.01% trifluoroacetic acid-d4, 298 K.

| Peak label | Chemical shift* (ppm) | Assignment | T1* (ms) |
|------------|-----------------------|------------|----------|
| a          | 20.52                 | 8-Methyl   | 187      |
| b          | 18.68                 | 3-Methyl   | 204      |
| c          | 13.05                 | 4α-Vinyl   | 327      |
| d          | 10.20                 | 2α-Vinyl   | 468      |
| e          | 10.02                 | 7α-Propyl  | 244      |
| f          | 5.53                  | 5-Hydroxymethyl | 291 |
| g          | 5.18                  | 6α-Propyl  | 297      |
| h          | 3.92                  | 1-Hydroxymethyl | 308 |
| i          | 1.75                  | β-Meso     | 86       |
| j          | 1.52                  | δ-Meso     | 65       |
| k          | 1.41                  | γ-Meso     | 137      |
| l          | 0.41                  | 7β-Propyl  | 270      |
| m          | -0.28                 | 6β-Propyl  | 301      |
| n          | -0.77                 | 2β-Vinyl (trans) | 372 |
| o          | -1.09                 | 4β-Vinyl (trans) | 298 |
| p          | -1.22                 | 2β-Vinyl (cis) | 491 |
| q          | -2.04                 | 4β-Vinyl (cis) | 444 |
| r          | -2.58                 | α-Meso     | 78       |

* Referenced to residual Me2SO solvent signal at 2.5 ppm.  
* Nonselective.

**TABLE III**

Summary of detected NOE correlations for bis(CN)-Fe(III) heme l complex

| Irradiated resonance | Detected NOE resonance | Label | Assignment | NOE intensity conditions | % |
|----------------------|------------------------|-------|------------|-------------------------|---|
| a 8-Methyl e 7α-Propyl |                       | -2.0  | C          |                         |   |
| b 8-Methyl l 7β-Propyl |                       | -1.6  | C          |                         |   |
| c 4α-Vinyl (trans) |                        | -4.8  | A          |                         |   |
| d 4α-Vinyl |                        | -3.0  | A          |                         |   |
| e 2α-Vinyl (trans) |                       | -3.2  | A          |                         |   |
| f 2α-Vinyl |                        | -2.5  | A          |                         |   |
| g 5-Hydroxymethyl |                        | -7.2  | B          |                         |   |
| h 1-Hydroxymethyl |                        | -9.3  | A          |                         |   |
| i 8-Methyl |                        | -4.2  | A          |                         |   |
| j γ-Meso |                        | -7.8  | A          |                         |   |
| k γ-Meso |                        | -6.5  | B          |                         |   |
| l 2α-Vinyl (cis) |                       | -18.4 | A          |                         |   |
| m 3-Methyl |                        | -5.3  | A          |                         |   |

* Experimental conditions were as follows: A, 50:50 D2O/Me2SO-d6 (0.01% trifluoroacetic acid-d4), 273 K; B, 20:80 D2O/Me2SO-d6, 268 K; C, 20:80 D2O/Me2SO-d6, 278 K; D, 20:80 D2O/Me2SO-d6, 273 K.

The temperature dependence of the heme l proton resonances shows an unusual feature in the low-spin complex form. A Curie plot of the bis(cyano)-heme l complex (Fig. 6) illustrates that, whereas most of the resonances follow Curie behavior (reduction in the paramagnetic shift at higher temperatures) in the temperature range studied (280–336 K), four resonances show anti-Curie behavior (d, f, g, and h). These anti-Curie resonances are those belonging to the two functionalized methylenes as well as the α-position protons of groups on the two pyrroles with these methylenes. The y intercepts of these traces also show that the methyl proton resonances (a and b) do not match their expected diamagnetic values. Some process other than the paramagnetic behavior of the heme is also being affected by temperature changes, and this process is most influential at the site of the functionalized methylenes. In contrast, the Curie plot of the bis(Me2SO)-Fe(III) heme l complex (data not shown) demonstrates that all of the proton resonances follow Curie behavior in the high spin form of the heme.

**Isolation of LPO Heme Species**—The use of proteolytic enzymes to obtain structurally identifiable heme derivatives from LPO holds two distinct advantages over chemical extraction procedures: (i) modification of the heme in the process of removal from the protein is much less likely; (ii) information regarding the covalent linkage between heme and protein is retained. Through the application of protease enzymes and HPLC separation of the resulting digests, isolation of the LPO heme in both a peptide-free form, and a series of peptide-bound forms was achieved.

**DISCUSSION**

Structural and Physical Properties of Heme l—The structure of the peptide-free LPO heme prosthetic group (heme l) was characterized by paramagnetic 1H NMR spectroscopy and electrospray ionization mass spectrometry. The molecular mass of heme l (PK-2) determined by electrospray mass spectrometry is 32 mass units greater (648) than that of heme b (616). An identical heme l mass was found by fast atom bombardment mass spectrometry with both glycerol and 3-nitrobenzyl alcohol matrices. Given the two-site functionalization of the LPO heme in comparison with heme b (from NMR data), the difference in mass is attributed to the oxygen atoms of two hydroxyl groups. Thus, it is concluded that heme l is a bis(hydroxymethyl) derivative of heme b, and heme-protein linkage through ester, rather than amide or disulfide bonds, is implicated.

Proton NMR spectroscopy of the low spin, cyanide-bridged heme l complex permits detailed structural characterization. The standard one-dimensional 1H NMR spectrum for bis(cyano)-heme l clearly reveals the functionalization of two methyl sites relative to the common heme b structure. Narrower proton resonance line widths and longer relaxation times for protons of the low spin heme l complex allow execution of NMR experiments that are commonly applied to diamagnetic compounds. Steady-state NOE difference and COSY experiments provide essential structural assignments for the low spin bis(cyano)-heme l complex. With the combined data from
these two experiments, the hydroxymethylene groups of heme l can conclusively be placed at the 1- and 5-positions of the porphyrin ring. All other resonances can likewise be assigned to their respective positions (Table II).

The greatly reduced downfield paramagnetic shifts and significantly longer relaxation times for protons of the two functionalized methylene groups (relative to those expected for heme methyl groups) reflect the presence of the more strongly electron-withdrawing hydroxyl groups at these sites. For bis(cyano) low spin complexes of ferric heme derivatives (33) and analogous synthetic iron-porphyrin models (34), it has been observed that strongly electron-withdrawing groups placed on selective pyrrole positions cause a decrease in the paramagnetic influence for substituent groups on the same pyrrole. Furthermore, since the total unpaired electron spin density transferred to the porphyrin is relatively constant (34), a decrease in density at one pyrrole, and corresponding degree of the paramagnetic influence, results in an increase at the others.

The 1H NMR spectrum for the low spin heme l complex demonstrates this in-plane asymmetry effect. Resonances for the hydroxymethylene substituent protons, as well as the \( \alpha \)-position protons of substituents on the same pyrrole, display smaller paramagnetic shifts and longer \( T_1 \) relaxation times (Table II) than the same type of functional groups on the other two pyrroles. Likewise, relative to the analogous groups on

| Heme species | \( m/z \) | Assignment | Relative intensity \(^a\) |
|--------------|--------|-----------|------------------|
| PK-2         | 648    | Heme l (+1) | 89  |
|              | 680    | Heme l (+1), \( \text{CH}_3\text{OH} \text{ adduct} \) | 100 |
| TC-4         | 1289   | Heme l-monopeptide (+1) | 100 |
|              | 645    | Heme l-monopeptide (+2) | 58 |
| TC-5         | 1535   | Heme l-dipeptide (+2) | 14 |
|              | 1024   | Heme l-dipeptide (+3) | 59 |
|              | 768    | Heme l-dipeptide (+4) | 22 |
| PK-3         | 1900   | Heme l-monopeptide (+1) | 14 |
|              | 951    | Heme l-monopeptide (+2) | 100 |
| PK-1         | 992    | Heme l-monopeptide (+1) | 5.5 |

\(^a\) Percentage of peak intensity relative to the spectrum base peak intensity (±2%).

*Fig. 6. Curie plot for the low-spin bis(CN)-Fe(III)heme l complex in 50:50 \( \text{Me}_2\text{SO-d}_6/\text{D}_2\text{O} \) with 0.01% trifluoroacetic acid-\( d_1 \). Sample temperatures were calibrated with the methanol thermometer method of Van Geet (23). Resonance shifts are referenced to the residual \( \text{Me}_2\text{SO} \) signal at 2.5 ppm. Peak labels are defined in Table II.*

**Table IV**

Electrospray mass spectrometry data for isolated LPO heme species

**FIG. 6. Curie plot for the low-spin bis(CN)-Fe(III)heme l complex in 50:50 Me\(_2\)SO-d\(_6\)/D\(_2\)O with 0.01% trifluoroacetic acid-d\(_1\).** Sample temperatures were calibrated with the methanol thermometer method of Van Geet (23). Resonance shifts are referenced to the residual Me\(_2\)SO signal at 2.5 ppm. Peak labels are defined in Table II.
heme b, the proton resonances for the groups on pyrroles other
than the two with hydroxymethylene groups show an increase
in paramagnetic shift. The unusual Curie behavior displayed by
proton resonances of the LPO heme in the low spin complex
form can also be explained by this in-plane asymmetry effect.
A change in hydrogen bonding strength of the hydroxymethylene
groups at various temperatures may impose a change in elec-
tron-withdrawing capacity of these groups and thereby induce
the anti-Curie behavior of the paramagnetic resonances.

**Structural and Physical Properties of Heme l-Peptides**—The
various heme l-peptides isolated from LPO digestes were inves-
tigated by electrospray ionization mass spectrometry, peptide
analysis, and paramagnetic \(^{1}H\) NMR spectroscopy of the high
spin bis(Me\(_2\)SO) complexes. A series of hemes with varying
peptide content provides conclusions about the nature of the
heme-protein linkage in LPO.

The major heme species from proteinase K digestion (PK-2)
of LPO is the peptide-free heme prosthetic group, heme l.
Proteinase K reportedly catalyzes hydrolysis of ester as well as
peptide amide bonds (35), and with the NMR and mass spec-
trometry data that indicate ester bonds between heme and
protein in LPO, it follows that the single major heme species
produced by the proteinase K digestion would be peptide-free.
The heme species with identical properties isolated in smaller
quantities from the trypsin/chymotrypsin LPO digest (TC-2) is
also peptide-free heme l.

Other isolated LPO heme species differ in the number and
length of peptide(s) tethered to the heme l base structure.
Differences in chromatographic retention properties of these
heme species must reflect variability in attached peptide con-
tent, since variability in heme structure would be inconsistent
with the relatively similar visible absorption and \(^{1}H\) NMR
spectra among these species. Conversion of heme l-peptide
species (TC-4 and TC-5) to TC-2 upon exposure to alkaline
conditions therefore indicates the loss of peptide content from
the heme l group. In addition, the appearance of a TC-4 species
upon base treatment of TC-5 and the release of an identical
peptide from both of these species suggests the following re-
lated series.

```
| Reaction 1 |
|-----------------------------|
| TC-5 base | TC-4 base | TC-2 (PK-2) |
| Peptide (TC-5P2) | Peptide (TC-4P) |
```

The sequence identity of the two peptides in this series
confirms the protein sequence sites of heme covalent attach-
ment in LPO. The sequence sites responsible for the two heme-
protein ester bonds in human MPO, as determined by x-ray
crystallographic analysis (19), occur within regions highly con-
served among mammalian peroxidase enzymes (2, 3). This
finding, along with the similarity in the \(^{1}H\) NMR spectra of the
MPO and LPO active sites (9), leads to the presumption of a
common covalent linkage in both enzymes. The Ala\(^{273}\)Leu\(^{278}\)
peptide (TC-4P) corresponds to the region of glutamate ester
linkage, and the Gly\(^{121}\)Leu\(^{126}\) peptide (TC-5P2) corresponds
to the region of aspartate ester linkage found in human MPO.
These sequences thus identify heme-protein covalent linkage in
LPO through ester bonds to the carboxylic side chains of
Glu\(^{275}\) and Asp\(^{125}\) (Fig. 1).

Although the low spin complexes of peptide-bound LPO heme
species are inaccessible to \(^{1}H\) NMR due to cyanide-promoted
ester bond hydrolysis, the high spin complexes yield key infor-
mation about peptide content. The four chemical shift patterns
seen for the bis(Me\(_2\)SO) high spin complexes of the eight LPO
heme species collected after proteolytic digestion (Fig. 4 and
Table I) give a measure of peptide occupation on the heme (how
many peptides and where bound). Varying lengths of the pep-
tide(s) bound to the heme are unlikely to cause significant
alterations in the paramagnetic shift of heme proton reso-
nances, as the structural inequivalence is too far removed from
the heme. Identical resonance shift patterns observed for heme
species with significantly different chromatographic retention
properties also argue against any significant influence of pep-
tide length on heme paramagnetic shifts. Structural dissimilar-
ity in the LPO heme species having different shift patterns must therefore involve the heme-peptide ester bonds.

Variable hydrolysis of the two heme-protein ester bonds dur-
ing proteolytic digestion of the LPO protein should result in
four possible combinations of heme types: a bispeptide heme,
two monopeptide hemes, and a peptide-free heme. The four
distinct resonance shift patterns among the eight heme species
isolated reflect these four possible heme types. Peptide analy-
yses reveal a bispeptide heme (TC-5) and one of the two possible
monopeptide heme types (TC-4), as well as the peptide-free
heme (PK-2, TC-2). Sequence homology between LPO and MPO
in the regions of heme-protein ester bond sites suggests that
the single peptide of heme species TC-4 is attached to the
1-hydroxymethyl site. Similarity in chemical shift patterns for
both TC-1 and PK-1 to that of TC-4 imply that these two heme
species also have single peptides bound at the 1-hydroxymethyl
position. The significantly different chemical shift pattern for
PK-3 is interpreted to indicate a single peptide attached at the
5-hydroxymethyl position for this heme species. Along with the
experimentally determined peptide content for four of the eight
heme species, Table I lists the expected peptide occupation of
the other four isolated heme species based on this high spin
paramagnetic shift pattern correlation.

Determination of peptide occupation on heme l, along with
the identification of specific sequence sites involved in ester
linkage to heme l in LPO allows for the structural determina-
tion of heme l-peptides by electrospray ionization mass spec-
trometry. Molecular masses determined for TC-4 and TC-5
heme l-peptide species are in agreement with the peptide com-
position determined by peptide sequence analysis. Subtraction
of the calculated masses for the peptide(s) obtained from both
TC-4 and TC-5 (TC-4P = 659; TC-5P2 = 1798) gives the pre-
dicted peptide-free heme l mass, confirming both the peptide
analyses and the mass of the peptide-free LPO heme (heme l)
itself. PK-3, the 1900-Da Asp\(^{125}\) to 5-hydroxymethyl-linked
heme l species, is thus determined to be the Ile\(^{123}\)GLU\(^{133}\)
11-amino acid heme l monopeptide, and the 992 mass of PK-1
identifies it (and possibly TC-1) as the 1-hydroxymethyl-linked
Ser\(^{274}\) to Glu\(^{276}\) triamino acid heme l monopeptide.

A correlation is observed between the proton resonance line
widths of the high spin bis(Me\(_2\)SO)-heme complexes to the
effective size of the heme l-peptides (Tables I and IV). Those
hemes with two attached peptides have larger methyl reso-
nance line widths than those with one, which in turn have
greater line widths than the peptide-free heme. The effect of
peptide quantity on signal line widths for protons of the high
spin bis(Me\(_2\)SO)-heme complexes may be attributed to the ef-
flect of the overall molecular size on Curie spin relaxation. A
Curie relaxation contribution to \(T_2\) is expected for high spin
\((S = \frac{5}{2}) \) Fe(III)-heme complexes in a large applied magnetic
field (14 Tesla) (29). Curie relaxation is dependent on the
rotational correlation time (\(\tau_{c}\)) of the molecule, and factors
that affect the molecular rotation rate, such as the effective radius,
would consequently affect the Curie relaxation contribution to
nuclei 1/T\(_2\) relaxation rates and corresponding line widths.

**The Mammalian Peroxidase Prosthetic Group**—The struc-
nature of the lactoperoxidase heme prosthetic group (heme l) is conclusively identified as the iron complex of 1,5-bis(hydroxy-methyl)-3,8-dimethyl-2,4-divinylporphyrin-6,7-dipropionic acid, covalently bound to its protein matrix with ester bonds between the 1-hydroxymethylene and glutamate 275 side chain and the 5-hydroxymethylene and aspartate 125 side chain (Fig. 1). Given that the electronic spectra of all other mammalian peroxidases except MPO are very similar to that of LPO, it is likely that these enzymes contain a common heme l prosthetic group. Furthermore, the highly conserved sequences of mammalian peroxidase enzymes in the region of covalent linkage for LPO and MPO suggest similar ester bonds between heme and protein throughout this family of enzymes. Hence, after decades of effort, the direct elucidation of heme l (17, 18) and heme m (19, 20) structures as well as indirect spectral (7, 21) and modeling (36) correlations have resolved the controversy of a novel heme prosthetic group in mammalian peroxidases.

Acknowledgments—We thank Dr. Lynn Teesch and Diane Lamb for providing the mass spectral data and Dr. Elena Rus at the University of Iowa Protein Analysis Facility for determination of peptide sequences. We also thank Dr. John Tillotson and Sandy Wormer for assistance with lactoperoxidase preparations.

REFERENCES

1. Pruitt, K. M., and Tenovuo, J. O. (eds) (1985) Immunology Series, Vol. 27, Marcel Dekker Inc., New York, NY
2. Dull, T. J., Uyeda, C., Strosberg, A. D., Nedwin, G., and Seilhamer, J. J. (1990) *J. Biol. Chem.* 265, 3406–3412
3. Cals, M., Mailliart, P., Brignon, G., Anglade, P., and Dumas, B. R. (1991) *J. Immunol.* 147, 150–154
4. Carlstrom, A. (1969) *Eur. J. Biochem.* 19, 467–474
5. Kohler, H., Taurog, A., and Dunford, H. B. (1988) *Acta Chem. Scand.* 42, 435–439
6. Redman, L. W., and Tustanoff, E. R. (1984) *J. Immunology* 133, 479–482
7. Anderson, L. A., Byikas, S. A., and Wilsen, A. E. (1990) *J. Biol. Chem.* 265, 3406–3412
8. Goff, H. M., Gonzalez-Vergara, E., and Ales, D. C. (1985) *Biochem. Biophys. Res. Commun.* 133, 379–387
9. Dugad, L. B., La Mar, G. N., Lee, H. C., Ikeda-Saito, M., Booth, K. S., and Caughey, W. S. (1990) *J. Biol. Chem.* 265, 7173–7179
10. Everse, J., Everse, K. E., and Grisham, M. B. (eds) (1991) *Peroxidases in Chemistry and Biology*, Vol. I, CRC Press, Inc., Boca Raton, FL
11. Hurst, J. K. (1991) in *Peroxidases in Chemistry and Biology*, Vol. I (Everse, J., Everse, K. E., and Grisham, M. B., eds) pp. 37–62, CRC Press, Inc., Boca Raton, FL
12. Hallquist, D. E., and Morrison, M. (1963) *J. Biol. Chem.* 238, 2843–2846
13. Yonetani, T. (1967) *J. Biol. Chem.* 242, 5003–5013
14. Morell, D. B., and Clezy, P. S. (1983) *Biochim. Biophys. Acta* 71, 157–164
15. Nichol, A. W., Angel, L. A., Moon, T., and Clezy, P. S. (1987) *Biochem. J.* 247, 147–156
16. Sievers, G. (1979) *Biochim. Biophys. Acta* 589, 181–190
17. Rae, T. D., and Goff, H. M. (1996) *J. Am. Chem. Soc.* 118, 2103–2104
18. DePillis, G. D., Otaki, S., Kuo, J. M., Malby, D. A., and Ortiz de Montellano, P. R. (1997) *J. Biol. Chem.* 272, 8857–8860
19. Fenna, R., Zeng, J., and Davey, C. (1995) *Arch. Biochem. Biophys.* 316, 653–656
20. Taylor, K. L., Strobel, F., Yue, K. T., Ram, P., Pohl, J., Woods, A. S., and Kinkade, J. M. (1995) *Arch. Biochem. Biophys.* 316, 653–642
21. Koster, J. M., Pierik, A. J., Merks, M., Averill, B. A., Mogulievsky, N., Bellen, A., and Wever, R. (1997) *J. Am. Chem. Soc.* 119, 11542–11543
22. Budd, D. L., La Mar, G. N., Langry, K. C., Smith, K. M., and Nyyir-Mazhir, R. (1979) *J. Am. Chem. Soc.* 101, 6091–6096
23. Van Geet, A. L. (1970) *Anal. Chem.* 42, 679–680
24. Dugad, L. B., La Mar, G. N., and Unger, S. W. (1990) *J Am. Chem. Soc.* 112, 1386–1392
25. Bani, L., Piccioli, M., and Scouzafla, A. (1992) *Coord. Chem. Rev.* 120, 1–28
26. La Mar, G. N., and de Ropp, J. S. (1993) in *Biological Magnetic Resonance*, Vol. 12 (Berliner, L. J., and Reuben, J., eds) pp. 1–78, Plenum Press, New York
27. Taylor, K. L., Pohl, J., and Kinkade, J. M., Jr. (1992) *J. Biol. Chem.* 267, 25282–25288
28. de Ropp, J. S., and La Mar, G. N. (1991) *J. Am. Chem. Soc.* 113, 4348–4350
29. Bani, L. (1993) in *Biological Magnetic Resonance*, Vol. 12 (Berliner, L. J., and Reuben, J., eds) pp. 79–112, Plenum Press, New York