Mössbauer Investigations of High-Spin Ferrous Heme Proteins. II. Chloroperoxidase, Horseradish Peroxidase, and Hemoglobin†

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ABSTRACT: Reduced samples of chloroperoxidase, horseradish peroxidase, and deoxyhemoglobin were studied by Mössbauer spectroscopy in strong magnetic fields. The intricate paramagnetic spectra of chloroperoxidase were evaluated in detail in the framework of a spin Hamiltonian pertinent to high-spin ferrous iron. The studies strongly suggest that, in their reduced states, chloroperoxidase from *Caldariomyces fumago* and cytochrome P-450 from *Pseudomonas putida* have similar, if not identical ligand structures of the heme iron. The spectral similarities of these two proteins, noted in an earlier Mössbauer investigation, are further explored and substantiated. Reduced horseradish peroxidase and deoxyhemoglobin, on the other hand, show high-field Mössbauer spectra that differ considerably from each other and, in particular, from those of the P-450 type, suggesting a different ligand arrangement of the heme iron for each case.

Studies on the biosynthesis of caldariomycin (2,2-dichloro-1,3-cyclopentenedione) have led to the isolation of chloroperoxidase from *Caldariomyces fumago* (Morris and Hager, 1966). The purified enzyme is a monomer having a molecular weight of approximately 42,000 and contains one ferriprotoporphyrin IX per molecule. There are at least three basic types of catalytic reactions that can be performed by chloroperoxidase. In the presence of hydrogen peroxide and a suitable halogen donor (I^-, Br^-, or Cl^-, but not F^-), the enzyme catalyzes the peroxidative formation of a carbon–halogen bond with a suitable halogen acceptor. In addition to the halogenation reaction, chloroperoxidase also catalyzes the peroxidative oxidation of classical peroxidase substrates (Thomas et al., 1970). Moreover, it decomposes hydrogen peroxide to give molecular oxygen in a catalase-type reaction. These three reactions are outlined schematically in eq 1. The halogen acceptor (AH) in eq 1a can be, for example, a β-keto acid or an aromatic ether (X^- = halogen donor). The classical peroxidase substrate (BH_2) in eq 1b might be pyrogallol, guaiacol, or ascorbate (Thomas et al., 1970).

$$AH + X^- + H_2O_2 + H^+ \xrightarrow{\text{chloroperoxidase}} AX + 2H_2O \quad (1a)$$

$$BH_2 + H_2O_2 \xrightarrow{\text{chloroperoxidase}} B + 2H_2O \quad (1b)$$

$$2H_2O_2 \xrightarrow{\text{chloroperoxidase}} O_2 + 2H_2O \quad (1c)$$

Chloroperoxidase exhibits some rather unusual physical characteristics that were previously thought to be particular to cytochromes of the P-450 type (Champion et al., 1973; Hollenberg and Hager, 1973a). The most striking resemblance is the appearance of a Soret band at the unusually long wavelength of 443 nm in the carbon monoxide complex of the reduced protein. Many other spectral properties relate the active site structure of cytochrome P-450 to that of chloroperoxidase. The preceding paper (Champion et al., 1975a) presented a detailed analysis of the Mössbauer spectra of reduced cytochrome P-450 measured in strong magnetic fields; in this paper, we show that the Mössbauer spectra of reduced chloroperoxidase are almost identical with those of cytochrome P-450, but quite different from those of other high-spin ferrous heme proteins such as deoxyhemoglobin and reduced horseradish peroxidase. The deeper exploration of this similarity is the main goal of this paper.

Native chloroperoxidase contains heme iron in the ferric state. During catalysis the enzyme–substrate complex undergoes a sequence of oxidation and reduction steps that possibly bring the iron atom into the Fe^{4+} valence state (Dolphin et al., 1971), but so far as we know, never into the Fe^{2+} state. It is the reduced state (Fe^{2+}, $S = 2$) of chloroperoxidase that we will discuss in this paper, however, so that we can compare the results with those obtained for cytochrome P-450.

Chloroperoxidase can be reduced with dithionite to a stable, homogeneous high-spin ferrous compound and it regains its biological activity upon reoxidation. As will be shown under Results, the low-temperature Mössbauer spectra of reduced chloroperoxidase taken in strong magnetic fields show well-resolved hyperfine splittings. On the basis of the spin Hamiltonian formalism developed in Part I (Champion et al., 1975a) we have attempted to simulate these spectra and have derived a set of parameters that characterize the electronic ground state of the iron in great detail.

For comparison we also discuss the high-field Mössbauer spectra of deoxyhemoglobin and reduced horseradish peroxidase. Of all these high-spin ferrous heme proteins the structure is known for hemoglobin only. Specifically, in deoxyhemoglobin the iron is 0.75 Å above the heme plane coordinated to the four pyrrole nitrogens and the nitrogen

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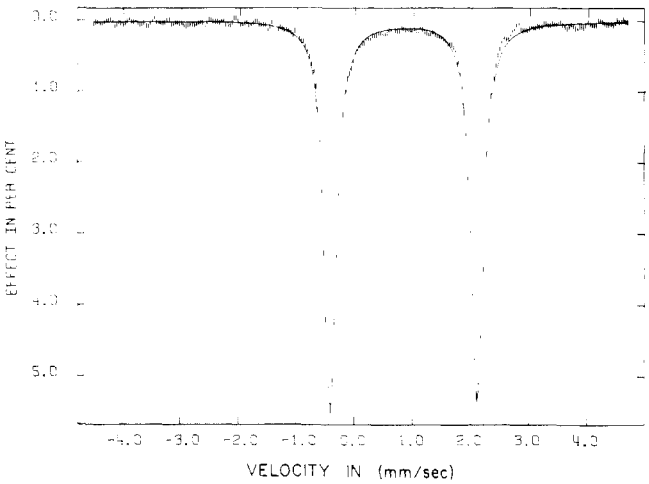

FIGURE 1: The Mössbauer spectrum of reduced chloroperoxidase taken in zero magnetic field at 4.2 K. The solid line is the result of a least-squares fit to the data. The quadrupole splitting was found to be 2.50 ± 0.03 mm/sec while the isomeric shift was 0.85 ± 0.02 mm/sec with respect to iron metal.

of the proximal histidine (Perutz, 1970). Horseradish peroxidase catalyzes the reactions outlined in eq 1b and c but not, under normal conditions, the halogenation reaction, eq 1a. There is recent evidence, however, that horseradish peroxidase can use $NaClO_2$ as both the oxidant and halogen donor for the peroxidative chlorination of monochlorodimedone (Hollenberg et al., 1974). Horseradish peroxidase can be reduced with dithionite to a stable high-spin ferrous state, suitable for study by the Mössbauer spectroscopic methods discussed here.

The Mössbauer spectra of deoxyhemoglobin[1] and reduced horseradish peroxidase lack much of the informative detail that is evident in the spectra of P-450 and chloroperoxidase. For this and other[2] reasons, no attempt is made to simulate the spectra using the multiparameter spin Hamiltonian described in Part I (Champion et al., 1975a). We do, however, wish to present the data for comparative purposes and discuss the spectra from a qualitative standpoint.

Experimental Methods

^{57}Fe-enriched chloroperoxidase was isolated from *Caldariomyces fumago* grown on a medium containing 90% enriched ^{57}Fe and purified as reported (Champion et al., 1973). The preparation had specific activities of greater than 2000 units/mg of protein in the standard chlorination assay and values of Rz were 1.34, indicating about 90% purity. The heme content was determined from the pyridine hemochrome using ϵ_{557} 3.3 × 10^4 M^{-1} cm^{-1}. It agreed within 5% with the value derived from the standard chlorination assay. The reduction of chloroperoxidase was carried out under vacuum. After degassing the enzyme solution (0.7 ml) in a Mössbauer sample cell, a twofold molar excess of crystalline $Na_2S_2O_4$ (J. T. Baker) was added and the sample was then frozen in liquid nitrogen.

Partially purified horseradish peroxidase obtained from Sigma Chemical Co. (Type III, Rz > 1.0) was purified as previously described (Hollenberg et al., 1974). Only fraction C was used for further modification with the Rz value greater than 3.4. The heme in the purified enzyme was removed as described by Teale (1959). The apoprotein solution was dialyzed against 0.1 *M* sodium phosphate at pH 7, then a 1.5 molar excess of ^{57}Fe-enriched heme was added. The reconstituted horseradish peroxidase was purified to a Rz value greater than 3.4.

The Mössbauer samples were prepared by dialysis vs. several changes of buffer followed by concentration to 1 m*M* in an Amico microultrafiltration cell using a PM-10 membrane. The samples contained about 40 mg of enzyme in 1 ml of solution. The reduction of horseradish peroxidase was done with a setup similar to that reported by Barleigh et al. (1969). The cell was evacuated and flushed with argon at least four times, then a twofold molar excess of $Na_2S_2O_4$ was added to the enzyme solution (0.7 ml) in the Mössbauer cell. The reduced enzyme was immediately frozen in liquid N_2.

The sickle cell hemoglobin samples were provided by Dr. Joel Groves of Columbia University. These samples were unenriched and highly concentrated. The 2% natural abundance of ^{57}Fe in these samples was sufficient for the observation of the Mössbauer resonance.

The Mössbauer apparatus has been described in the preceding paper (Champion et al., 1975a). All Doppler velocities shown in the figures are referred to metallic iron.

Results

Chloroperoxidase. Mössbauer spectra of reduced chloroperoxidase have been reported previously (Champion et al. 1973). Since these experiments were carried out in the absence of magnetic fields, only the magnitude and temperature dependence of the quadrupole splitting were obtained. In Figure 1, we display a typical quadrupole doublet obtained for reduced chloroperoxidase. The sharpness of the lines and the absence of absorption in the region 0–1 mm/sec imply that the sample is pure and homogeneous. The temperature dependence of the quadrupole splitting is given in Figure 2 of the preceding paper (Champion et al., 1975a). Notice the resemblance between the temperature independent quadrupole splittings of chloroperoxidase and P-450, while the quadrupole splittings of deoxyhemoglobin and horseradish peroxidase show strong variation with temperature.

We have studied reduced chloroperoxidase in strong magnetic fields as described in the previous paper. The intricate paramagnetic Mössbauer spectra, rich in information about the electronic structure of the iron site, are displayed in Figure 2. When fields of 8.6 kG (upper), 17 kG (middle), and 25 kG (lower) are applied to the sample, the concomitant variation of the internal magnetic field at the iron nucleus causes changes in the splitting of the Mössbauer spectra.

As with P-450, the complex interplay of the magnetic hyperfine splitting and the quadrupole interaction yields interesting and detailed high-field Mössbauer spectra. The amazing thing is that, with so many electronic and nuclear parameters involved, there is so much similarity between the spectra of P-450 and chloroperoxidase. The same type of outer wings are present in the 25-kG spectrum, along

[1] Deoxyhemoglobin was first studied with Mössbauer spectroscopy by Lang and Marshall, 1966. We have repeated their high magnetic field experiments using several different kinds of hemoglobin samples, each giving almost identical Mössbauer spectra. The spectra displayed here are from sickle cell hemoglobin provided by Dr. Joel Groves, Columbia University.

[2] Hemoglobin and horseradish peroxidase have temperature dependent quadrupole splittings that make application of the spin Hamiltonian formalism highly questionable in these compounds (see Champion et al., 1975a or Champion, 1975).

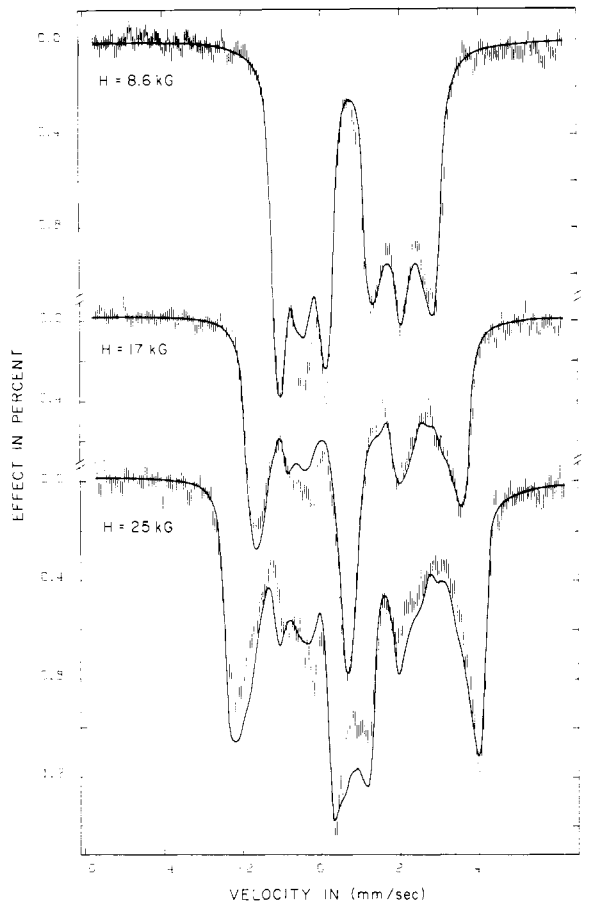

FIGURE 2: The Mössbauer spectra of reduced chloroperoxidase in various parallel applied magnetic fields at 4.2 K. The field strength is listed beside each spectrum. The solid lines are the result of computer simulations (fast relaxation limit) using the following parameters: $D = 20$ K, $E/D = 0.15$, $g_x = 2.24$, $g_y = 2.32$, $g_z = 2.0$, $\Delta E_Q = +2.5$ mm/sec, $\eta = 1.0$, $\mathbf{A}_x/\mathbf{g}_g\beta_n = -260$ kG, $\mathbf{A}_y/\mathbf{g}_g\beta_n = -120$ kG, $\mathbf{A}_z/\mathbf{g}_g\beta_n = -150$ kG. The Euler angles that rotate the zero-field splitting tensor into the principal-axes frame of the electric field gradient tensor were $\alpha = 70°$, $\beta = 70°$, and $\gamma = 0°$.

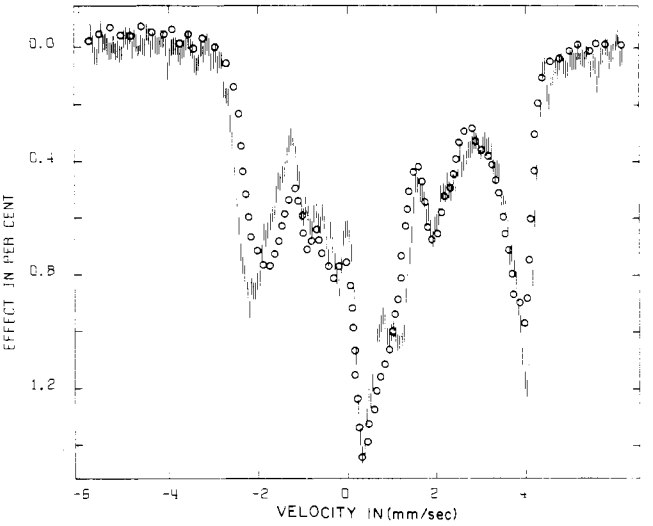

FIGURE 3: An overlay of the high-field Mössbauer data of P-450 (Figure 5, paper I) and chloroperoxidase (Figure 2). The velocity scales of the two experiments were matched and the P-450 data were plotted using small circles. The similarity is striking, especially considering the very different biological functions of these two proteins.

with indications of a field gradient rotated so that its major component is in the x–y plane of the zero-field splitting tensor (see Champion, 1975, for details). A more direct comparison is made in Figure 3, where the two 25-kG spectra are displayed on the same velocity scale. The circles represent the experimental spectrum of P-450. There are some minor differences, but the overall similarity between these two spectra strongly suggests that P-450 and chloroperoxidase have a common active site structure (e.g., the same axial ligand).

In the preceding paper (Champion et al., 1975a), we described two methods that could be used to obtain fairly good estimates of the zero-field splitting parameters (D and E/D) of P-450. When dealing with chloroperoxidase, however, magnetic susceptibility measurements are ruled out because of the presence of paramagnetic manganese ions that are difficult (if not impossible) to separate from the enzyme. Thus we must again utilize low-field Mössbauer spectroscopy as a method of determining the values of D and E/D. Figure 4 shows a set of Mössbauer spectra of chloroperoxidase taken with a 6.6-kG magnetic field applied transverse to the transmitted γ radiation. The sample temperature of each spectrum is indicated on the left-hand side of the figure. The very slight change in the width of the absorption features upon lowering of the temperature from 4.2 to 1.6 K indicates that the range of D and E/D is very similar to that found for P-450. Figure 5 shows the Mössbauer spectrum of chloroperoxidase measured at 180 K in a magnetic field of 45 kG parallel to the γ-rays. The spin polarization induces an internal magnetic field at the iron nucleus that opposes the externally applied field and, as a consequence, the experimental splitting is somewhat smaller than that expected for a diamagnetic compound in 45 kG. Notice that chloroperoxidase differs slightly from P-450 (Figure 6 of the preceding paper (Champion et al., 1975a)) in the left absorption peak, but that otherwise their high-field, high-temperature spectra are almost identical. A close study of the chloroperoxidase spectrum shows that, as with P-450, large values of the asymmetry parameter η are necessary for adequate fits to the data ($0.6 \leq \eta \leq 1.0$).

Using these values of η and values of D and E/D compatible with Figure 4, the remaining parameters, in particular the magnetic hyperfine tensor $\tilde{\mathbf{A}}$ and the orientation of the electric-field gradient, were adjusted to obtain simulations of the low-temperature, strong-field spectra of Figure 2. The best parameter set is listed in the legend of Figure 2; it was used to generate the solid lines through the data points in Figures 2, 4, and 5.

Hemoglobin and Horseradish Peroxidase. The Mössbauer spectra of the two heme proteins that we have dealt with in the previous sections appear to be distinctly different from those of hemoglobin and horseradish peroxidase. A first indication of a difference is seen in Figure 2 of the preceding paper (Champion et al., 1975a), which shows the quadrupole splitting as a function of temperature. The strong temperature dependence of the quadrupole splitting observed in both hemoglobin and horseradish peroxidase (and several other heme proteins) means that the spin Hamiltonian formalism described in paper I (Champion et al., 1975a) is probably not directly applicable. The high-field, low-temperature Mössbauer spectra of these proteins (Figures 8 and 10) differ markedly from those of cytochrome P-450 and chloroperoxidase. They are broad and unresolved, but show definite evidence of paramagnetic hyperfine interaction. The spectra are not completely under-

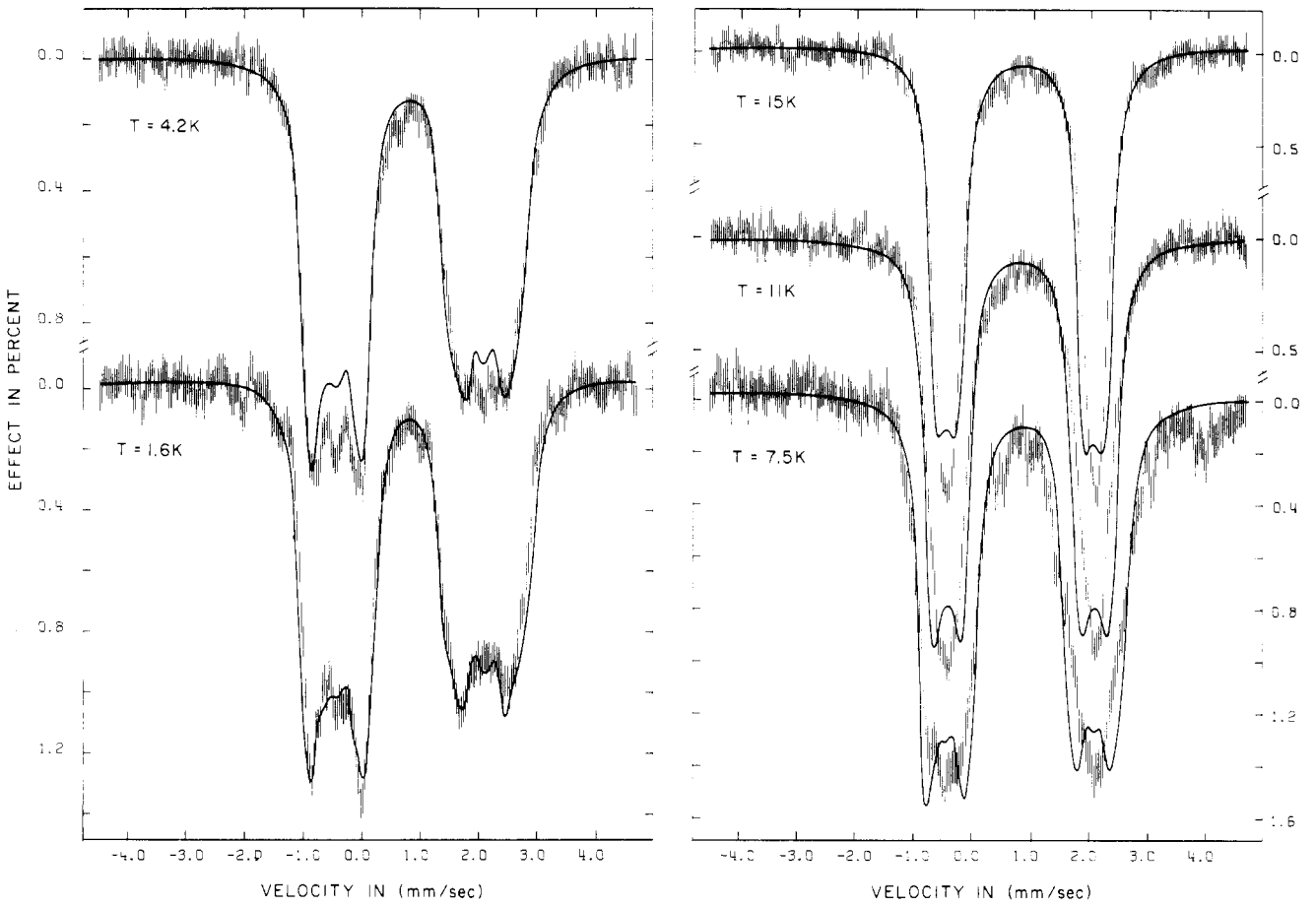

FIGURE 4: Low-field Mössbauer spectra of reduced chloroperoxidase taken at various sample temperatures. The 6.6-kG applied magnetic field was directed transverse to the incident γ-rays. The nearly equal widths of the left and right absorptions at 4.2 K indicate an electric field gradient tensor which has its z axis close to the electronic x-y plane. The solid lines through the data points are the result of computer simulations (fast relaxation limit) using the same parameters as quoted in Figure 2.

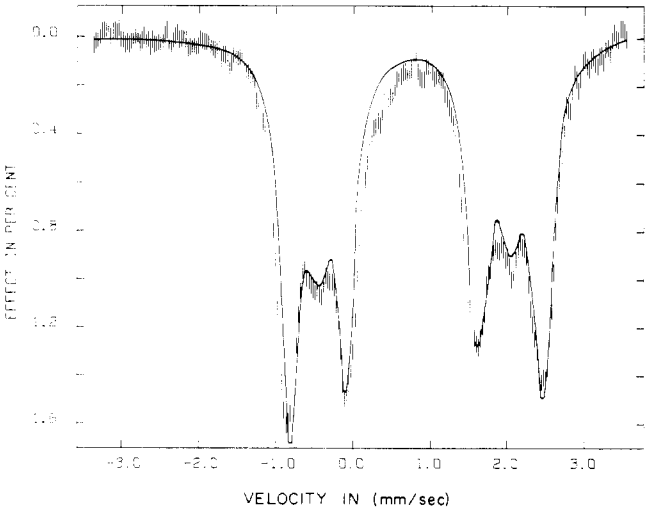

FIGURE 5: Mössbauer spectrum of reduced chloroperoxidase taken at 180 K in a 45-kG magnetic field applied parallel to the γ-ray direction. The solid curve is a computer simulation using the same parameters as quoted in the caption of Figure 2.

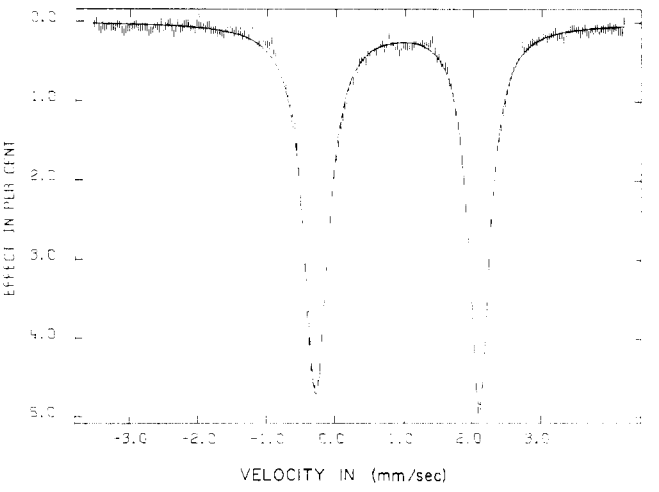

FIGURE 6: Mössbauer spectrum of sickle cell deoxyhemoglobin taken at 4.2 K in zero magnetic field. The quadrupole splitting was found to be 2.38 ± 0.03 mm/sec and the isomer shift relative to iron metal at 300 K was 0.91 ± 0.02 mm/sec. The widths (FWHM) of the left and right line are 0.42 and 0.37 mm/sec, respectively.

stood but they nevertheless allow us to draw some qualitative conclusions.

Figure 6 shows the Mössbauer spectrum of sickle cell hemoglobin taken in zero applied magnetic field at 4.2 K. The solid line is the result of fitting two independent Lorentzian line shapes to the data. The quadrupole splitting, isomer shift, and line widths are all typical for deoxyhemoglobin. These parameters are quoted in the figure caption. The absence of an absorption peak between 0 and 1.0 mm/sec indicates that we have a clean sample uncontaminated by low-spin hemochromes.

In a magnetic field of 8.6 kG applied parallel to the γ-ray

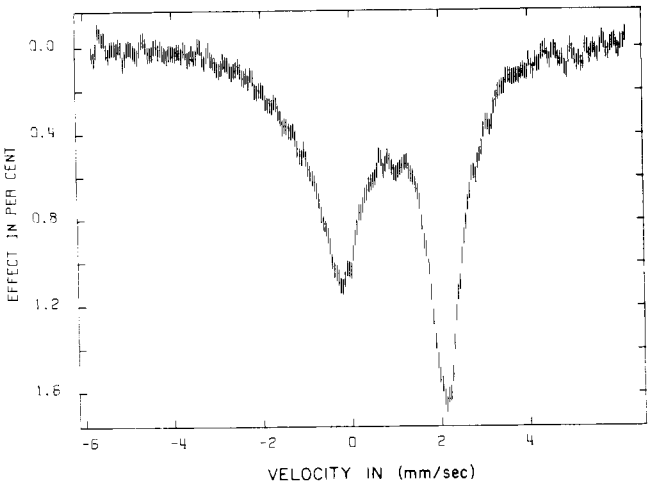

FIGURE 7: Mössbauer spectrum of sickle cell deoxyhemoglobin in a 8.6-kG magnetic field, applied parallel to the transmitted γ-rays.

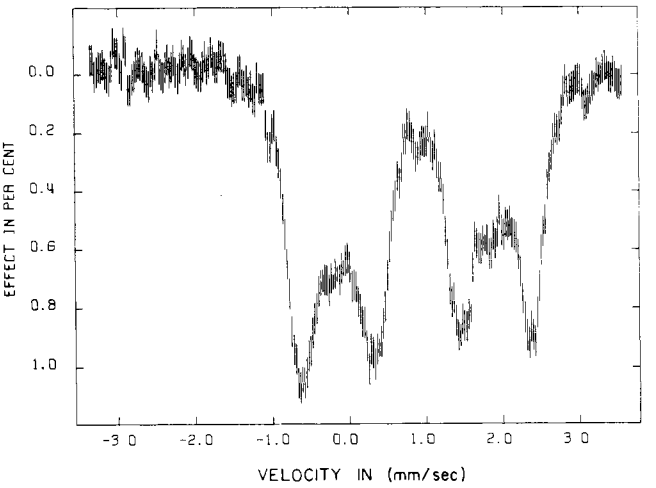

FIGURE 9: Mössbauer spectrum of sickle cell deoxyhemoglobin taken at 180 K in a parallel magnetic field of 45 kG.

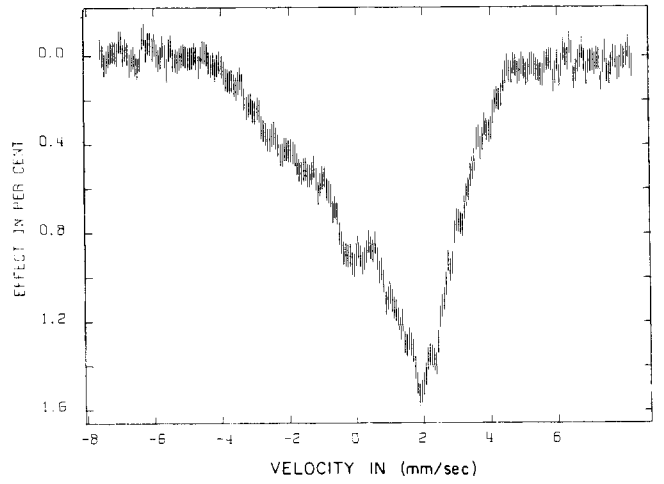

FIGURE 8: Mössbauer spectrum of sickle cell deoxyhemoglobin taken at 4.2 K in a magnetic field of 25 kG applied parallel to the γ-rays.

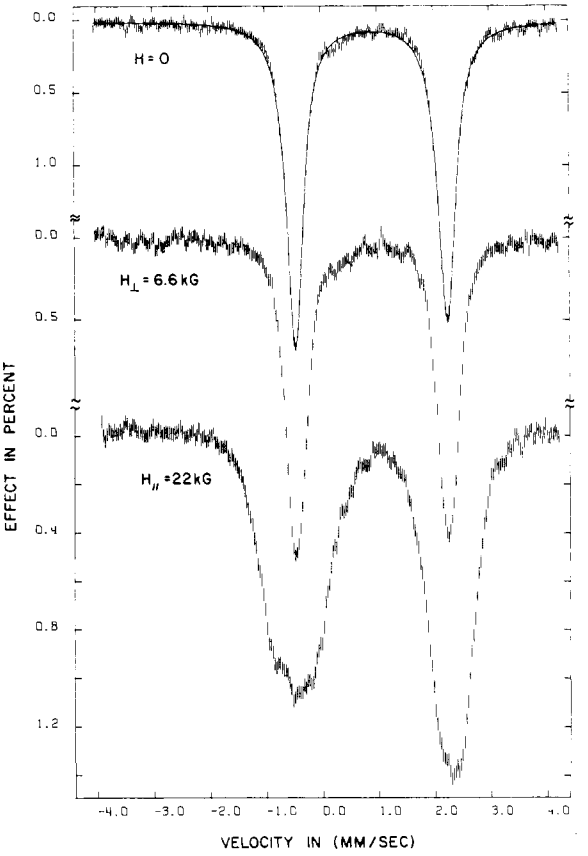

FIGURE 10: (Top) Mössbauer spectrum of reduced horseradish peroxidase taken at 4.2 K in zero field. The solid line is a least-squares fit to the data. The quadrupole splitting was found to be 2.70 ± 0.03 mm/sec and the isomeric shift 0.90 ± 0.03 mm/sec with respect to iron metal. The widths (FWHM) of the left and right line were 0.36 and 0.38 mm/sec, respectively. (Middle) Mössbauer spectrum of the same sample at 4.2 K but in a 6.6-kG transverse magnetic field. (Bottom) Same as above, but with a 22-KG magnetic field applied parallel to the γ-ray direction.

direction, the spectrum of Figure 7 results. Notice that the magnetic hyperfine interaction is beginning to broaden the lines in a rather nondescript fashion. The relative intensities of the left and right lines in Figure 7 are, however, in marked contrast to the spectra shown in Figure 4 of paper I (Champion et al., 1975) and Figure 2. A magnetic field of 25 kG yields the spectrum shown in Figure 8. This spectrum is very similar to the one obtained for reduced red blood cells (Lang and Marshall, 1966). It is rather featureless and not conducive to multiparameter fits utilizing the assumptions that were successful in the previous section.

We have also performed experiments at high temperatures in magnetic fields in an effort to determine η, the asymmetry parameter. The decrease of the quadrupole splitting with temperature means that higher orbitals are being populated. The electric field gradient tensor at 180 K is, therefore, a thermal average that does not reflect the properties of only the ground state orbital. For this reason we display in Figure 9 the data from this experiment without a (possibly misleading) simulated curve. We should remark that whatever the combination of orbital levels populated at 180 K, the resultant field gradient has a large asymmetry parameter η like chloroperoxidase and P-450.

The spectra of horseradish peroxidase are displayed in Figures 10 and 11. The zero-field spectrum was again fitted with two Lorentzian lines; the parameters are quoted in the caption of Figure 10. As with hemoglobin, the absorption lines are broad compared to those of P-450 and chloroperoxidase. This is possibly due to the existence of a set of horseradish peroxidase conformers that have slightly different quadrupole parameters. The existence of these conform-

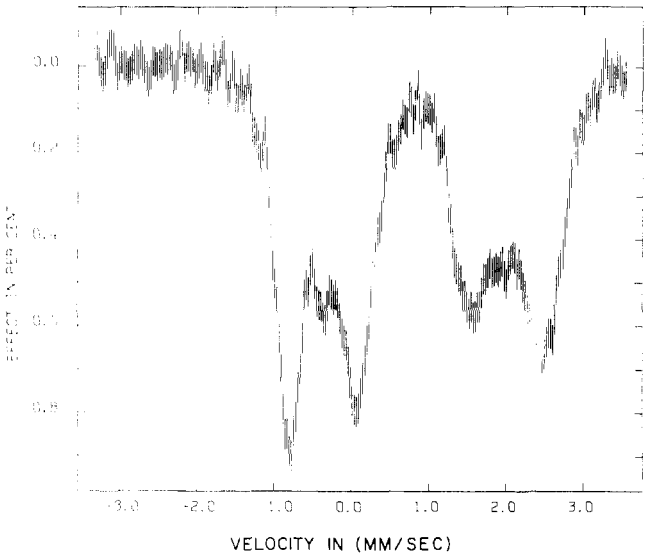

FIGURE 11: The Mössbauer spectrum of reduced horseradish peroxidase taken at 170 K in a parallel applied magnetic field of 45 kG. The triplet on the left side indicates a positive value for V_{zz} and a rather small η (see text).

ers has been indicated by electron paramagnetic resonance investigations of the native protein (R. Chiang and P. M. Champion, unpublished results).

Notice in Figure 10 that horseradish peroxidase, upon application of magnetic fields, displays a behavior different from hemoglobin, chloroperoxidase, or P-450. The very weak magnetic hyperfine interaction is indicative of either a very large zero-field splitting[3] ($D > 30$ K) or very small values for the transverse components of $\tilde{\mathbf{A}}$ ($|\mathbf{A}_\perp/\mathbf{g}_n\beta_n| \simeq 50$ kG) or a combination of both. An experiment with the sample at 1.6 K in 22 kG (not displayed here) showed no change in the magnetic splitting, indicating that horseradish peroxidase does not have excited state spin levels that are thermally populated at 4.2 K.

The high-temperature spectrum displayed in Figure 11 should, like Figure 9, be interpreted with caution. Since horseradish peroxidase has a temperature dependent quadrupole splitting, this spectrum contains information about the entire set of orbitals populated at 170 K. The clear triplet structure on the left side, however, indicates a positive quadrupole coupling constant ($V_{zz} > 0$) and a rather small η compared to the other proteins.

Discussion

We have successfully parametrized the Mössbauer data of chloroperoxidase following the procedure outlined for cytochrome P-450 in the preceding paper (Champion et al., 1975a). In view of the similarity of their high-field spectra, Figure 3, it is not surprising that the relevant parameters are almost identical for both proteins. They show that a crystal field of triclinic symmetry is required in order to explain the data. Here we do not reiterate the physical arguments leading to these conclusions but rather explore the implications they might have on the ligand configuration of the heme iron. The analysis presented is largely based on plausibility arguments, but since the heme ligands are neither known for chloroperoxidase nor for cytochrome P-450, some speculation may be permitted. Essentially all of the physical arguments concerning the low symmetry of the iron atom in P-450 apply to chloroperoxidase as well. Moreover, the model calculations discussed by Champion (1975) can be applied to both P-450 and chloroperoxidase.

Since the heme iron is coordinated to four porphyrin nitrogen atoms in all heme proteins, we expect variations in the Mössbauer spectra to reflect the character of the axial ligands. This means that a basic difference between the detailed paramagnetic Mössbauer spectra of two heme proteins is probably a good indication that they either have different axial ligands or a different structural arrangement of ligands. Conversely, if the detailed spectra are almost identical, they strongly suggest that the two proteins have the same axial ligands.[4] This is especially true when the heme iron is in the high-spin ferrous state since, under these conditions, it probably has only one axial ligand.[5]

In view of these arguments the near identity of the Mössbauer spectra of chloroperoxidase and cytochrome P-450 displayed in Figure 3 strongly suggests that, in the *reduced* state, these proteins have the same axial ligand. The basic differences between the spectra in Figure 3; and the spectra in Figures 8 and 10 make us believe that the axial ligand of P-450 and chloroperoxidase is not common to horseradish peroxidase[6] and hemoglobin. Pursuing this argument further, we have listed in Table I several unique physical similarities between cytochrome P-450 and chloroperoxidase that reinforce the idea of an identical axial ligand.

Aside from the Mössbauer spectra, the short wavelength (408 nm) Soret absorptions of the reduced proteins are of particular interest. In contrast, deoxyhemoglobin and reduced horseradish peroxidase have Soret bands at 430 and 437 nm, respectively (Mahler and Cordes, 1966). Additionally, we must recall that the carbon monoxide complexes of P-450 and chloroperoxidase have unusual optical spectra. The rhombic electron paramagnetic resonance (EPR) spectra of the high-spin ferric forms of these proteins (g = 8.0, 4.0, 1.8 for cytochrome P-450 (Tsai et al., 1970) and g = 7.4, 4.3, 1.9 for chloroperoxidase) are also quite unique since most heme proteins show only small deviations from

[3] We use the phrase "zero-field splitting" rather loosely here, since it is not clear whether the spin Hamiltonian discussed in Champion et al. (1975a) really applies to horseradish peroxidase.

[4] These arguments do not always apply to six-coordinated, low-spin heme complexes. The cytochromes c and b_5, for instance, have very similar Mössbauer parameters in their oxidized and reduced states (Münck, 1975).

[5] X-Ray diffraction experiments (Perutz, 1970) have shown that the high-spin ferrous heme iron in deoxyhemoglobin is displaced by 0.75 Å out of the heme plane toward a single axial ligand (histidine F8). This is quite understandable since the high-spin ferrous ion is rather large ($R \sim 2.18$ Å) and does not easily fit into the space provided in the heme plane (nitrogen to nitrogen distance ~ 4 Å). It is reasonable to assume that high-spin ferrous chloroperoxidase, cytochrome P-450, and horseradish peroxidase are also five coordinated.

[6] Yonetani et al. (1972) have studied the complex of nitric oxide with reduced horseradish peroxidase using electron paramagnetic resonance spectroscopy. On the basis of observed transferred hyperfine interactions of the unpaired electron with nitrogen nuclei it was concluded that the proximal ligand of horseradish peroxidase is a nitrogen-containing group. The Mössbauer spectra shown in Figure 8 and 10 might suggest that horseradish peroxidase and hemoglobin do not share the same ligand, namely the histidyl residue. One can argue, however, that constraints imposed by the protein environments could account for the differences. Measurements on hemoglobin and a suitable five-coordinated model complex (ferrous protoporphyrin IX complexed to 1-methylimidazole, see Münck, 1975) do not reveal any differences in the Mössbauer parameters, i.e., possible protein constraints are not reflected in the quadrupole splitting and isomeric shift of hemoglobin. Thus if the proximal ligand of horseradish peroxidase were to be a histidyl residue it would follow that the protein environment imposes a severe constraint on the binding of this ligand.

Table I: Comparison of Physical Measurements on the Various Charge and Spin States of Cytochrome P-450 and Chloroperoxidase (CPO).

	EPR Spectra
Fe^{3+}, $S = 5/2$	P-450: Strongly rhombic g values[a] (8.0, 4.0, 1.8)
	CPO: Strongly rhombic g values[b] (7.4, 4.3, 1.9)
	EPR Spectra
Fe^{3+}, $S = 1/2$	P-450: Truth table analysis of g values;[b] class P
	CPO: Third g value not resolved, but Mössbauer[b] analysis places CPO close to class P
	Mössbauer Spectra
Fe^{2+}, $S = 2$	P-450: Detailed and informative (see paper I); temperature independent ΔE_Q
	CPO: Detailed and informative, almost identical to spectra of P-450; temperature independent ΔE_Q
	Optical Spectra
	P-450: Soret maximum at short wavelength of 408 nm[a]
	CPO: Soret maximum at 406 nm[c]
	Optical Spectra of CO complex
Fe^{2+}, $S = 0$	P-450: Soret maximum at unusually long wavelength of 446 nm[a]
	CPO: Soret maximum at unusually long wavelength of 443 nm[c]

[a] See Gunsalus et al. (1974). [b] See Champion et al. (1973). [c] See Hollenberg and Hager (1973a).

the axially symmetric g value of 6.0. The low spin ferric forms of P-450 and chloroperoxidase, on the other hand, do not seem to be quite so similar, although chloroperoxidase can be placed near to the class P compounds[7] (Champion et al., 1973).

It has been suggested recently that reduced P-450 possesses a mercaptide anion (RS^-) as the ligand trans to CO in the P-450 carbon monoxide complex (Stern and Peisach, 1974) and we would like to briefly address this point.

In terms of amino acid content, chloroperoxidase is very different from P-450. In particular it appears to possess only two (Morris and Hager, 1966) cysteine residues compared to six (Tsai et al., 1971) for P-450. Furthermore, the two cysteines in chloroperoxidase have been shown to be linked in a disulfide bridge in both the oxidized and the reduced state (Chiang et al., 1975) and thus are not available to coordinate to the iron as an axial ligand. Therefore, since the fifth coordination positions of P-450 and chloroperoxidase appear to be occupied by the same ligand, it seems unlikely that cysteine can ligate either protein in the fifth position. Since cysteine is the only amino acid having a mercaptide anion we must come to a conclusion contrary to the one presented by Stern and Peisach (1974). The resolution of this conflicting evidence is certainly a very important problem that needs detailed investigation.

In addition to the strong physical evidence implying that cytochrome P-450 and chloroperoxidase have the same axial ligand, we have also uncovered some chemical analogies. The catalytic cycle of chloroperoxidase normally involves higher oxidation states of the enzyme, possibly the Fe^{4+} valence state (Dolphin et al., 1971). In this respect, it appears quite different from P-450. The similarity of the active sites (heme and its environment) of reduced chloroperoxidase and cytochrome P-450, on the other hand, motivated an investigation of chloroperoxidase catalyzed hydroxylation reactions. Preliminary results (Hollenberg and Hager, 1973b) have shown a catalytic hydroxylation of aromatic amines by chloroperoxidase. Detailed studies of the mechanisms for this unusual enzyme action by a peroxidase are underway (P. F. Hollenberg, private communication). On the other hand, Hrycay and O'Brian (1974) have shown that microsomal cytochrome P-450 can catalyze peroxidative oxidation. Additionally, the redox potential of the chloroperoxidase and reduced chloroperoxidase couple has shown unusually high potential at pH 6.8 ($E_0' \simeq -150$ mV) (R. Makino and R. Chiang, unpublished results), compared to that of horseradish peroxidase ($E_0' = -270$ mV). However, this potential is quite close to the redox potential of cytochrome P-450 complexed to camphor ($E_0' \simeq -170$ mV) (Gunsalus et al., 1974).

This leads us to a few final comments concerning future projects and prospects, centered around the techniques discussed in this work. Obviously there is a great need for chemically pure, five coordinated heme model compounds, as well as for additional high-spin ferrous heme proteins. Further efforts have to be made to parametrize the high-field Mössbauer spectra of hemoglobin and horseradish peroxidase. Measurements in stronger magnetic fields will improve the spectral resolution and facilitate the analyses. Finally, we must expand this type of measurement into other paramagnetic states that are EPR inactive. The obvious candidates are the compounds I and II, that occur in the catalytic cycle of peroxidative proteins such as chloroperoxidase and horseradish peroxidase (Thomas et al., 1970; Chance, 1952). There is no doubt that Mössbauer spectroscopy can be an enormous help in clearing up the mystery that has surrounded these compounds for over 20 years.

[7] Class P compounds refer to the notation of Blumberg and Peisach (1971) and are likely to have a sulfhydryl group binding to the heme iron. We feel that oxidized P-450 might have such a ligand in the sixth position, while chloroperoxidase does not.

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Chloroperoxidase: P-450 Type Absorption in the Absence of Sulfhydryl Groups†

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ABSTRACT: The oxidation state of the two half-cystine residues in the native ferric form of chloroperoxidase and in the reduced ferrous chloroperoxidase has been examined in order to evaluate the role of sulfhydryl groups as determinants of P-450 type spectra. Mössbauer and optical spectroscopy studies indicate that the ferrous forms of P-450cam and chloroperoxidase have very similar or identical heme environments. Model studies have suggested that sulfhydryl groups may function as axial ligands for developing P-450 character. However, chemical studies involving both sulfhydryl reagents and amperometric titrations show that neither the ferric nor the chemically produced ferrous forms of chloroperoxidase contain a sulfhydryl group. These results rule out the hypothesis that sulfhydryl groups are unique components for P-450 absorption characteristics. The optical and electron paramagnetic resonance (EPR) spectra of the nitric oxide complex of chloroperoxidase have been obtained and compared to those of myoglobin, hemoglobin, and cytochrome *c* and horseradish peroxidase. The EPR spectrum of the NO–ferrous chloroperoxidase complex, which is similar to that of cytochrome P-450cam, does not show the extra nitrogen hyperfine structure which appears to be characteristic of those hemoproteins which have a nitrogen atom as an axial heme ligand.

The absorption spectrum of the CO complex of ferrous chloroperoxidase is quite similar to the characteristic spectrum of the CO complexes of the P-450 type hemoproteins (Hollenberg and Hager, 1973). The unusual characteristic of the CO–ferrous complexes of the P-450 type is the extremely long wavelength Soret band. The Soret peak for the carbon monoxide complexes of ferrous P-450 hemoproteins occurs near 450 nm (Cooper et al., 1965; Lindenmayer and Smith, 1964; Murphy and West, 1970; Appleby, 1969) while the Soret peak for the carbon monoxide complexes of most other reduced hemoproteins is near 420 nm (Klingenberg, 1958).

There has been intense interest in the chemical nature of the heme ligands which give rise to the P-450 type absorption. Recent work has suggested that the ligands in P-450 type cytochromes are thiols. The typical low spin ferric electron paramagnetic resonance (EPR) signals exhibited by P-450 type hemoproteins were first observed by Mason et al. (1965) and Murakami and Mason (1967), who suggested that these signals could arise from the association of the heme iron with thiols. Subsequently, Bayer et al. (1969) prepared a series of Fe(III) hemoglobin and myoglobin thiol complexes and found that these complexes had a pattern of electron paramagnetic resonance EPR *g* values similar to those of cytochrome P-450. Jefcoate and Gaylor (1969) made a similar study of the isopropyl–thiol complex of Fe(III) myoglobin and showed that it too gave the characteristic P-450 *g* values. Blumberg and Peisach (1971a) have shown that the EPR spectra of low spin ferric cytochrome P-450 and cytochrome P-420 were similar to those obtained by the addition of thiols to ferric hemoglobin and myoglobin. Blumberg and Peisach (1971b) have extended their EPR studies to cover a wide variety of heme proteins and they have classified a large number of low spin ferric compounds according to their EPR behavior. From this classification, chloroperoxidase was assigned to the O-type class (Blumberg and Peisach, 1971a). Later information developed from Mössbauer data (Champion et al., 1973) placed chloroperoxidase near the boundary between type-O and type-P (type P = P-450) hemoproteins. In addition, re-

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