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Mössbauer Investigations of Chloroperoxidase and Its Halide Complexes†

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ABSTRACT: The heme protein chloroperoxidase which is isolated from the mold *Caldariomyces fumago* has been investigated by Mössbauer spectroscopy. The heme iron of native chloroperoxidase is in a low-spin ferric state at low temperatures and undergoes a temperature-dependent spin transition to high-spin ferric around 200°K. The low-temperature Mössbauer spectra were simulated assuming that the heme iron resides in a ligand field potential of orthorhombic symmetry. The low-temperature Mössbauer spectra of the chloroperoxidase–Cl complex are quite similar to those of the native enzyme, suggesting that chloride does not bind as an axial ligand to the heme iron. The complexes of chloroperoxidase with iodide and fluoride are high-spin ferric at all temperatures. Both complexes reveal unusually large rhombic distortions at the heme iron. The ferrous form of chloroperoxidase is a high-spin species with an optical absorption spectrum and Mössbauer parameters which are almost identical with those of the reduced form of cytochrome P-450$_{\mathrm{cam}}$. Like the cytochromes of the P-450 type, reduced chloroperoxidase forms a stable complex with carbon monoxide characterized by a Soret band at unusually long wavelength (443 nm). The heme iron of this complex is found to be in a low-spin ferrous state. This investigation suggests close structural similarities between the active sites of chloroperoxidase and P-450$_{\mathrm{cam}}$.

Chloroperoxidase is a heme protein (mol wt ~ 42,000) which has been isolated from the mold *Caldariomyces fumago* (Morris and Hager, 1966). It catalyzes the chlorination reactions involved in the biosynthesis of caldariomycin (2,2-dichloro-1,3-cyclopentenedione). 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 such as pyrogallol and guaiacol (Thomas *et al.*, 1970). Moreover, it decomposes hydrogen peroxide to give molecular oxygen in a catalase-type reaction.

Chloroperoxidase is quite similar in many of its properties to other protoheme peroxidases such as horseradish peroxidase, Japanese radish peroxidase, and cytochrome *c* peroxidase. All are isolated as monomeric proteins having molecular weights in the range of 35,000–50,000 and exhibit similar optical spectra for the native and reduced forms, and for their cyanide and azide complexes. Although the physical properties of these peroxidases are quite similar, chloroperoxidase exhibits a basic catalytic difference in its ability to

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catalyze chlorination reactions at low pH's. Therefore, despite the gross similarities between the peroxidases, chloroperoxidase must differ significantly from the others in the vicinity of the active site. We have recently concerned ourselves with identifying the physical basis of this difference, and the resultant catalytic implications, in order to acquire a better understanding of peroxidative enzyme mechanisms.

Our success in isolating large quantities of chloroperoxidase grown on a ^{57}Fe-enriched medium, plus the existence of many spectrally observable, well-characterized complexes of chloroperoxidase with substrates and inhibitors, make this enzyme a suitable and promising candidate for Mössbauer investigations. Here we report experiments on native chloroperoxidase and some of its complexes. Of particular interest are the chloroperoxidase–halide complexes because of their suggested role as intermediates in the halogenation reaction. Furthermore, recent studies have brought out numerous similarities between the microsomal and bacterial cytochrome P-450's and chloroperoxidase. Mössbauer investigations further elucidate these similarities in terms of electronic structure at the heme iron.

Experimental Methods

Caldariomyces fumago was grown on a growth medium containing 90% enriched ^{57}Fe and chloroperoxidase was purified as reported (Morris and Hager, 1966). Under these conditions, more than 1% of the iron in the growth medium is incorporated into the heme prosthetic group of chloroperoxidase. These preparations had specific activities of greater than 2000 units/mg of protein in the standard chlorination assay and values of $R_z > 1.35$, indicating greater than 90% purity (Morris and Hager, 1966). Attempts to completely remove contaminating Mn^{2+} bound to the enzyme were unsuccessful; however, Mn^{2+} levels were reduced to less than 0.2 mol of Mn^{2+}/mol of enzyme. Two batches of enzyme have been prepared and Mössbauer spectra of both were taken.

The Mössbauer samples were prepared by dialysis *vs.* several changes of the designated buffer followed by concentration in an Amicon micro ultrafiltration cell using a PM-10 membrane. The samples contained 120 mg of protein in a volume of 0.7 ml in the first batch and 70 mg/0.7 ml in the second batch. The native enzyme sample was prepared by dialysis against 0.1 M NaP_i (pH3.0). The halide derivatives were made by dialysis against 0.1 M NaP_i (pH3.0) containing 0.1 M NaF, 1.0 M NaCl, or 0.05 M NaI. (Chloroperoxidase–halide complexes have recently been isolated using $^{36}Cl^-$ and $^{125}I^-$ and have been shown to contain 1 mol of tightly bound Cl^- and 2 of I^- per mol of enzyme.) The reduced chloroperoxidase sample contained 20 mg of protein in 0.7 ml of buffer and was dialyzed *vs.* 0.05 M NaP_i (pH 6.0), prior to reduction. Solid sodium dithionite was dissolved in degassed 10^{-3} M NaOH and a twofold molar excess was added under argon to the sample after flushing with argon for 15 min. The sample was immediately frozen in liquid nitrogen. The reduced carbon monoxide compound was made by adding a twofold molar excess of dithionite to the enzyme in an anaerobic cuvet which had been evacuated and flushed with carbon monoxide several times. The reduced carbon monoxide derivative was then transferred under argon to a Mössbauer cell and immediately frozen in liquid nitrogen.

Reagent grade sodium iodide, chloride, and fluoride were obtained from J. T. Baker as was the sodium dithionite. The carbon monoxide was obtained from Union Carbide. ^{57}Fe-enriched Fe_2O_3 was obtained from the Nuclear Division of Oak Ridge National Laboratories and converted to $FeCl_3$ before addition to the growth medium. All other chemicals were reagent grade.

TABLE I: Positions of Soret Peaks for Chloroperoxidase Complexes.

Complex	λ_{max} (nm) 296°K	77°K
Native chloroperoxidase	396	424
Chloroperoxidase–F	408	410
Chloroperoxidase–Cl	412	430
Chloroperoxidase–Br	396	424
Chloroperoxidase–I	390	400
Reduced chloroperoxidase	406	
Reduced chloroperoxidase + CO	443	

Room-temperature optical spectra were recorded on a Cary 15 using a light path of 1 cm and a protein concentration of 0.4 mg/ml. Saturating levels of the various halide ions were determined by measuring the difference spectrum between enzyme with halide ion and without at several different concentrations of halide ion. The spectral changes observed were then extrapolated to infinite halide concentration and a concentration giving at least 95% conversion was used for recording the absolute spectra. Concentrations which give at least 95% conversion are 0.1 M F^-, 1.0 M Cl^-, 1.0 M Br^-, and 0.05 M I^-.

Low temperature (77°K) spectra were recorded on a Cary 14 using the technique described by Wilson and Hager (1965). The samples were dissolved in 50% glycerol and spectra were taken after devitrification. The cell path length was 1 mm.

The Mössbauer spectrometer was of the constant acceleration type. Data for positive and negative acceleration were stored separately in two halves of a multichannel analyzer and then folded. A 60-mCi source of ^{57}Co in copper was used which gave a minimum observable line width of 0.25 mm/sec. The system was calibrated with a metallic iron absorber; all isomer shifts δ are listed relative to this standard. A Janis variable-temperature cryostat was used for most of the measurements. The samples were inserted into the tail section from the top and the γ rays from the ^{57}Co(Cu) source passed horizontally through the sample *via* two pairs of mylar windows.

Visible Absorption Spectra

As seen in Table I, the Soret band of chloroperoxidase at room temperature exhibits a λ_{max} at 396 nm which is typical for high-spin ferric heme proteins. The addition of halide ions to the enzyme results in a shift of the Soret band characteristic for each halogen anion. At room temperature, the addition of F^- or Cl^- to chloroperoxidase results in shifts to longer wavelengths (408 and 412 nm, respectively), while I^- causes a shift to shorter wavelengths. Although Br^- has no significant effect on the position of the λ_{max}, it causes the band to broaden significantly and decreases the extinction coefficient at 396 nm. The spectrum of native chloroperoxidase taken at liquid nitrogen temperature (77°K) shows the Soret band shifted to 424 nm. As at room temperature, the addition of halide ions to chloroperoxidase results in characteristic shifts of the spectra at liquid nitrogen temperature.

The addition of dithionite to chloroperoxidase under anaerobic conditions results in the formation of a new spectral species having the λ_{max} of the Soret band at 406 nm. When the reduction is formed in an atmosphere of carbon monoxide (CO), the Soret band shifts to a much longer wavelength (443 nm) than usually observed with heme–CO derivatives. This type of spectral shift has been observed in only one class of proteins, the cytochrome P-450's, which are classified together on the basis of this characteristic. Both the reduced and the reduced-CO derivatives readily decompose to native ferric enzyme upon the addition of oxidizing equivalents.

Mössbauer Results

Native Chloroperoxidase and Its Chloride Complex. EXPERIMENTAL RESULTS. Native Chloroperoxidase. The Mössbauer spectra of native chloroperoxidase, pH 3, taken in parallel and transverse applied magnetic fields, are shown in Figure 1. Such broad spectra are commonly observed for heme proteins with iron in a low-spin ferric state ($S = {}^1/_2$). The small absorption peak around +5.3 mm/sec shows that in addition to the low-spin material the sample contains a high-spin ferric ($S = {}^5/_2$) component as a minority species. These observations are confirmed by electron paramagnetic resonance (epr) investigations[1] at 1.5°K.

At temperatures above 100°K the electron spin relaxation time is short compared to the nuclear precession time and the Mössbauer spectra consist of fairly well-resolved quadrupole doublets. The spectrum taken at 194°K (Figure 2) consists of two doublets, showing the presence of a low-spin and a high-spin component. The absorption of the high-spin material shows up in the small peak at about −0.4 mm/sec and as a shoulder at about +0.8 mm/sec. When the temperature is raised the relative intensities of the high-spin and low-spin components change drastically. The three spectra in Figure 2 show clearly that the sample undergoes a spin transition. The spectra taken at high temperatures were evaluated by least-squares fitting two quadrupole doublets to the data. The solid lines in Figure 2 are the results of the fitting procedure.[2] The uncertainties of the parameters obtained are quite large because both quadrupole doublets are asymmetric due to some residual magnetic hyperfine broadening. In evaluating the relative amounts of low-spin and high-spin material we have assumed that the recoilless fractions are the same for both spin states. In Figure 3 we have plotted the fraction of low-spin material as a function of temperature. Spectra taken at 80°K still show the presence of high-spin material, although magnetic hyperfine broadening does not allow quantitative evaluation of the data. As mentioned above, the Mössbauer spectra taken at 4.2°K also show the presence of high-spin material; this suggests that the transition to low spin is not complete even at very low temperatures. As can be seen from Figure 3 the quadrupole splitting of the low-spin component has a strong temperature dependence. The high-spin component, on the other hand, has a quadrupole splitting $|\Delta E_Q| = 1.16 \pm 0.05$ mm/sec which is independent of temperature within the experimental uncertainties.

It has been reported in the literature that for heme proteins the high-spin–low-spin transition may depend on the sample concentration, the freezing rate, on the buffer and on the pH of the buffer (Lang *et al.*, 1969). Preliminary experiments on these matters have indicated that for chloroperoxidase the rate of freezing does not influence the Mössbauer spectra; however, the concentration of the sample seems to affect the spin transition.[3] Therefore, we do not attempt here to analyze the high-temperature data in terms of thermodynamical parameters.

Chloroperoxidase–Chloride. The Mössbauer spectra of the chloroperoxidase–Cl complex measured at 4.2°K in transverse and parallel magnetic fields are shown in Figure 4. As in native chloroperoxidase the data show that the sample contains a majority component of low-spin ferric iron and a small amount of high-spin material. In Figure 4 we have plotted the experimental spectra[4] of native chloroperoxidase (circles) over the data of chloroperoxidase–Cl. The striking resemblance between these low-temperature Mössbauer spectra suggests close structural similarities between the iron environments of native chloroperoxidase and chloroperoxidase–Cl. At higher temperatures, however, the Mössbauer spectra of chloroperoxidase–Cl are quite distinct from those observed for the native enzyme. Up to 250°K only one quadrupole doublet, characteristic of a low-spin ferric species, is observed; a typical spectrum is shown in Figure 5. The decrease in the quadrupole splitting ΔE_Q as the temperature is raised is as pronounced as that of the native enzyme (see Figure 3). The small absorption peak at about −0.4 mm/sec (Figure 5) is possibly associated with the high-spin ferric material observed in the low-temperature Mössbauer and epr spectra. In contrast to the native enzyme the temperature dependence of the quadrupole splitting for chloroperoxidase–Cl does not seem to depend on the sample preparation.[3] Spectra taken on the chloride complexes of samples 1 and 2 gave reproduc-

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[1] Drs. W. E. Blumberg, J. Peisach, and P. Hollenberg have kindly provided us with the epr spectra of chloroperoxidase and many of its complexes. Although the epr investigations are not completed at this time, the preliminary results obtained from these spectra were very helpful to our Mössbauer studies. The presence of manganese in all samples makes the epr analysis around $g = 2$ difficult. As a result only two g values for each species were obtained with accuracy. For the evaluation of our Mössbauer data we used the following g values. Native chloroperoxidase: $g_1 = 2.63$, $g_2 = 2.26$, (the sample contained a minority high-spin ferric species giving signals at $g_1 = 7.44$ and $g_2 = 4.30$); chloroperoxidase–Cl: $g_1 = 2.66$, $g_2 = 2.27$ (signals of minority high-spin species at $g_1 = 7.62$, $g_2 = 4.05$); chloroperoxidase–F: $g_1 = 7.01$, $g_2 = 4.80$; chloroperoxidase–I: $g_1 = 7.48$, $g_2 = 4.17$.

[2] The line widths used in fitting the low-spin component remained approximately constant ($\Gamma = 0.48$ mm/sec) as the temperature was raised from 194°K to 245°K. The line widths for the high-spin absorption peaks varied from 0.68 to 0.42 mm per sec (peak at −0.4 mm/sec) and from 1.0 to 0.78 mm per sec (peak at +0.8 mm/sec) as the temperature was raised.

[3] We have compared two preparations of native chloroperoxidase. Sample 1 had a concentration of 170 mg/ml. After the spectra were taken, the sample was converted into the Cl^-, I^-, and F^- complexes. Later on, the sample was reconverted into the native form and Mössbauer spectra were taken again. The results agreed with the earlier measurements. A second batch was grown, purified and concentrated to 100 mg/ml (sample 2). The growing and purification procedure was the same as used to prepare sample 1. For sample 2, the high-spin:low-spin ratio and the quadrupole splitting of the low-spin component at a temperature T' were found to be identical with the values found for sample 1 at a temperature T, where $T' - T \approx 10$°K. (In our experiments the temperature is measured with a thermistor embedded in the frozen solution. The temperature was known to within 1°K, the stability over a period of 1 day was better than 0.1°K; thermal gradients across the sample were less than 0.2°K.)

[4] We must point out that the low temperature Mössbauer spectra of samples 1 and 2 (see footnote 3) were slightly different in the region −2.0 to +2.0 mm per sec. The resonance absorption in this region also changed somewhat when the magnitude of the applied magnetic field was varied from 360 G to 1.3 kG. This might suggest that electron spin relaxation of the high-spin minority species is affecting the middle of the spectra (see section on Fluoride and Iodide complexes). All spectra in Figure 4 were taken with sample 2.

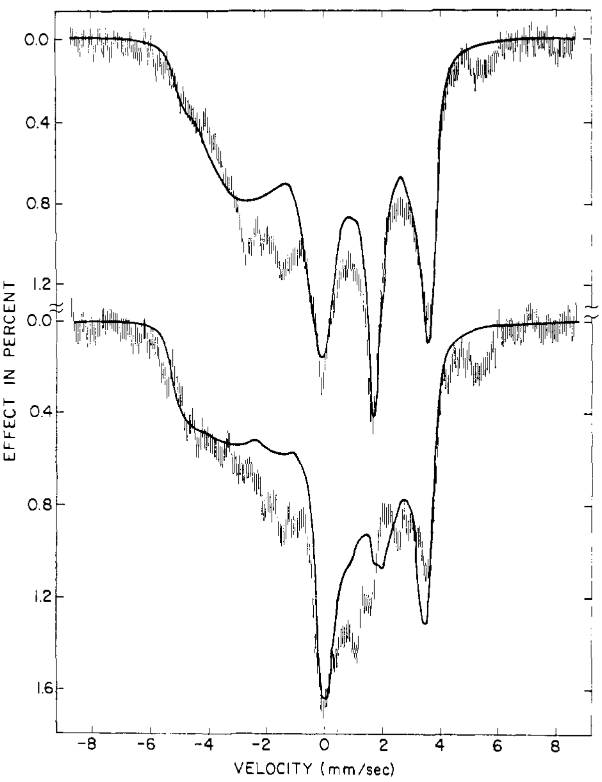

FIGURE 1: Mössbauer spectra of native chloroperoxidase, pH 3, taken at 4.2°K in 1300-G external magnetic fields, applied transverse (upper spectrum) and parallel (lower spectrum) to the observed γ rays. The solid lines are computed spectra using the parameters quoted in Table II. The absorption peak at about +5.3 mm/sec shows the presence of some high-spin material (approximately 5–10% of total Fe present). No attempt was made to superimpose calculated high-spin spectra.

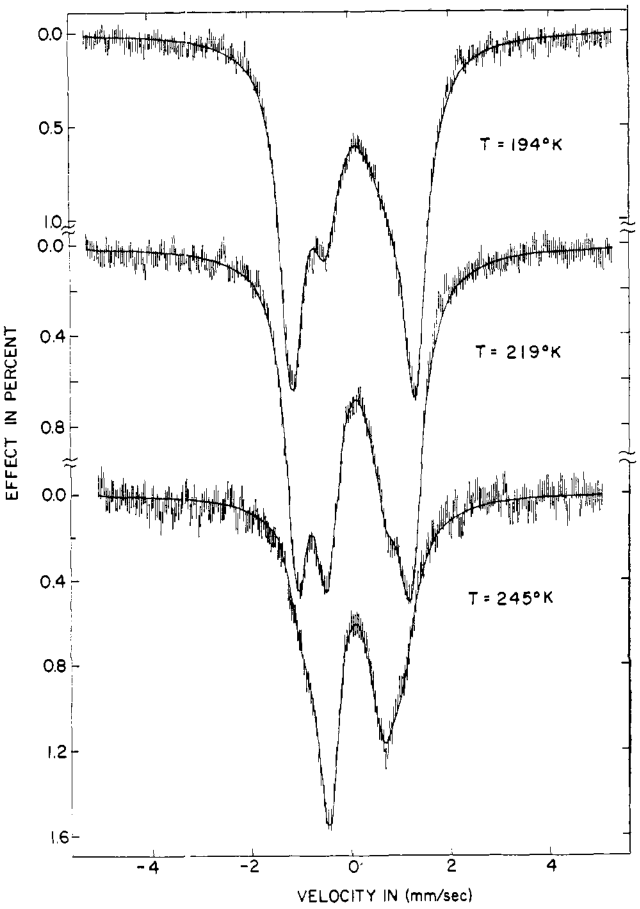

FIGURE 2: Mössbauer spectra of native chloroperoxidase taken at 194°K, 219°K, and 245°K. The spectra show that native chloroperoxidase undergoes a spin transition from low spin to high spin as the temperature is raised. The solid lines are the results of fitting two quadrupole doublets to the data.

ible results for the low-spin material. However, the intensity of the peak at −0.4 mm/sec was weaker for sample 2.

ANALYSIS. To evaluate the low temperature data we adopt a simple model describing the low-spin ($S = {}^1/_2$) ferric ion. We restrict ourselves to a ligand field potential of orthorhombic symmetry, with tetragonal and rhombic distortions along the cubic axes. Treating the problem in lower symmetry requires more parameters than can be determined reliably from the data obtained so far. Furthermore, the reproducibility problem mentioned in footnote 4 does not warrant elaborate attempts to fit the spectra.

The theory of the low-spin ferric ion has been described extensively in the literature (Griffith, 1957; Harris, 1966; Oosterhuis and Lang, 1969); we confine ourselves here to a brief description of the method. The cubic component of the ligand field potential is assumed to be strong enough so that only the ${}^2T_{2g}(t_{2g}{}^5)$ ground term needs to be considered. Working in the complementary representation, the one-particle (hole) Hamiltonian perturbing the sixfold degenerate ground state can be written as

$$\mathcal{H} = -\frac{14}{3}\sqrt{\frac{\pi}{5}}\Delta Y_2{}^0 - \frac{7}{2}\sqrt{\frac{2\pi}{15}}\, V(Y_2{}^2 + Y_2{}^{-2}) - \lambda \mathbf{l}\cdot\mathbf{s} \quad (1)$$

The tetragonal distortion parameter, Δ, and the rhombic parameter, V, are defined as those used by Lang and Oosterhuis (1969); the last term describes the spin–orbit coupling. Upon choosing a suitable basis, the Hamiltonian matrix can be factorized into two 3×3 submatrices which depend on parameters Δ/λ and V/λ. The result of a diagonalization procedure yields three Kramers doublets; associated with the doublets are the state vectors $|\psi_\pm{}^i\rangle$, $i = 1, 2, 3$.

The g values of each doublet are evaluated by calculating

$$\langle\psi_\pm{}^i|(k\mathbf{l} + 2\mathbf{s})\cdot\mathbf{H}|\psi_\pm{}^i\rangle = \langle\psi_\pm{}^i|\mathbf{S}'\cdot\tilde{g}^i\cdot\mathbf{H}|\psi_\pm{}^i\rangle \quad (2)$$

Equation 2 introduces the (isotropic) orbital reduction factor k and defines an effective spin $\mathbf{S}'$ and a g tensor for each doublet.

With the electronic state vectors $|\psi_\pm{}^i\rangle$ known, the low-temperature Mössbauer spectra may be calculated from a spin Hamiltonian for the lowest Kramers doublet. In an external magnetic field, $\mathbf{H}$, the pertinent Hamiltonian is given by

$$\mathcal{H} = \beta_e\mathbf{S}'\cdot\tilde{g}\cdot\mathbf{H} + \mathbf{S}'\cdot\tilde{A}\cdot\mathbf{I} - g_n\beta_n\mathbf{H}\cdot\mathbf{I} + H_Q \quad (3)$$

It is necessary to include the electronic Zeeman interaction when describing the Mössbauer spectra, since the applied magnetic field determines the quantization axis of the electron spin. The expectation value $\langle\mathbf{S}'\rangle$, in turn, determines the internal magnetic field experienced by the nucleus *via* the magnetic hyperfine tensor $\tilde{A}$. The interaction of the nuclear magnetic moment $g_n\beta_n\mathbf{I}$ with the applied magnetic field, $\mathbf{H}$, is

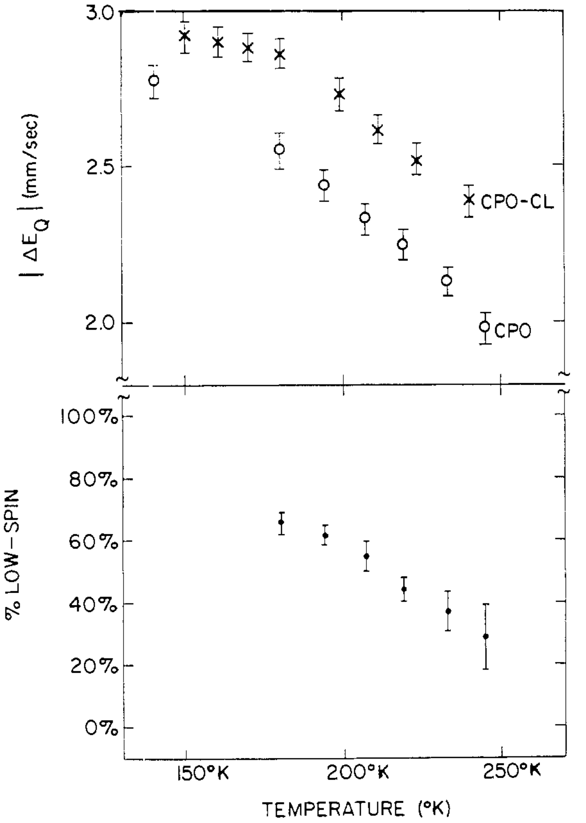

FIGURE 3: The fraction of low-spin ferric material in native chloroperoxidase is plotted as a function of temperature. The upper part of the figure shows the temperature dependence of the quadrupole splitting for the low-spin component of native chloroperoxidase (circles) and the chloride complex (crosses).

small for fields used in our measurements. The last term describes the electric quadrupole interaction

$$\mathcal{H}_Q = \frac{eQV_{zz}}{4I(2I-1)}[3I_z^2 - I(I+1) + \eta(I_x^2 - I_y^2)] \quad (4)$$

where $\eta = (V_{xx} - V_{yy})/V_{zz}$ is the asymmetry parameter and V_{xx}, V_{yy}, and V_{zz} are the principal axes components of the electric field gradient tensor (EFG tensor);

$$\Delta E_Q = \frac{eQV_{zz}}{2}\sqrt{1 + \frac{\eta^2}{3}}$$

is the quadrupole splitting which would be observed in the absence of magnetic interactions. The three tensors appearing in eq 3 can be expressed in terms of k and the coefficients of the basis states spanning $|\psi_\pm{}^1\rangle$, the state vectors of the lowest Kramers doublet; these coefficients depend on Δ/λ and V/λ. In orthorhombic symmetry the three tensors have a common principal axis system.

In our evaluation procedure a computer program was used to search for those values of Δ/λ, V/λ, and k giving the correct experimental g values. (Since one g value is unresolved Δ/λ, V/λ and k are not determined uniquely.) For each set of Δ/λ, V/λ, and k satisfying this condition we calculated the A tensor and the electric field gradient tensor. These parameters in turn were used to calculate the Mössbauer spectra for a polycrystalline sample. The computer program used in calculating the Mössbauer spectra is described elsewhere (Münck *et al.*, 1972, 1973).

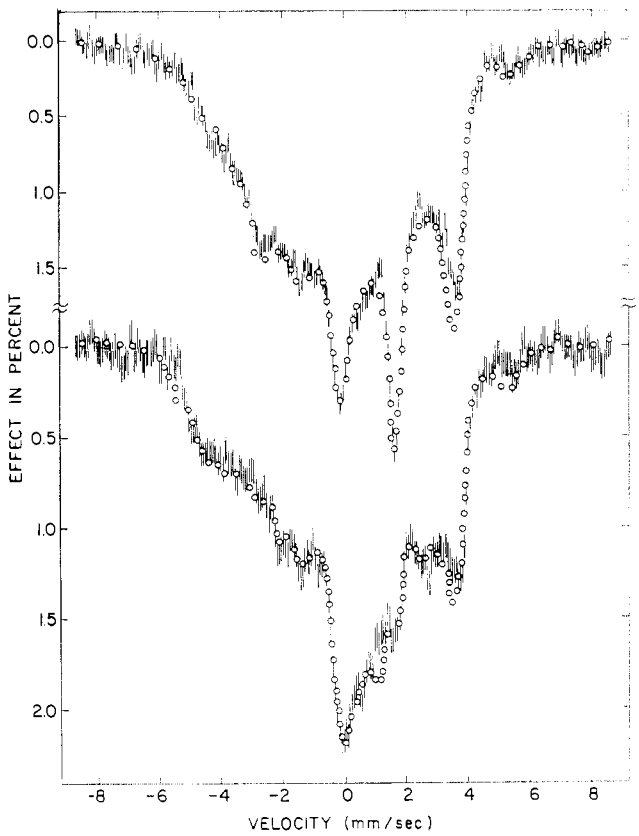

FIGURE 4: Mössbauer spectra of chloroperoxidase–Cl taken at 4.2°K in applied magnetic fields (upper spectrum: 1300G transverse field; lower spectrum: 1300G parallel field). To show the similarity of the low-temperature spectra of native chloroperoxidase and chloroperoxidase–Cl the experimental spectra of native chloroperoxidase (circles) are plotted over the chloroperoxidase–Cl data.

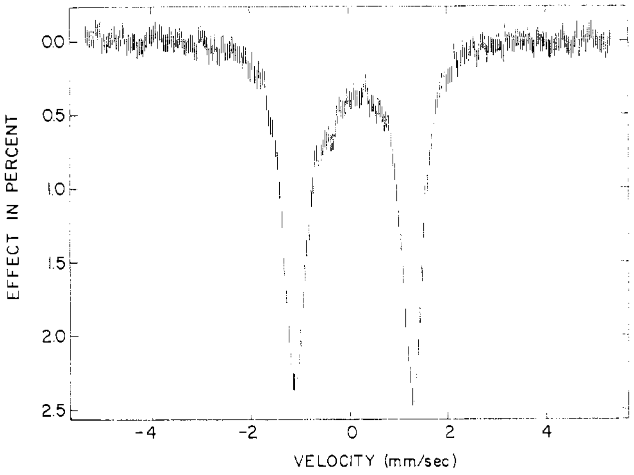

FIGURE 5: Typical Mössbauer spectrum of chloroperoxidase–Cl at higher temperatures. The spectrum shown was measured at 223°K. The small peak at −0.4 mm/sec shows the presence of some high-spin ferric material (possibly the same high-spin material as observed at 4.2°K).

The solid lines in Figure 1 are the best results of the calculations. The parameters used were calculated strictly within the model described above. To allow for some lattice contribution to the EFG tensor and for the Sternheimer factor, different scaling factors were used for the A tensor and the

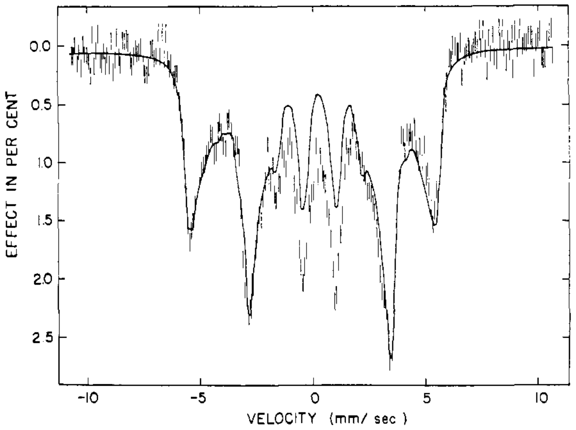

FIGURE 6: Mössbauer spectrum of the iodide complex of chloroperoxidase taken at 1.5°K in a transverse magnetic field of 1300 G. The solid line is a computed spectrum using the parameters quoted in Table II. The line width used in the calculations was 0.4 mm/sec. The strong intensities of the inner lines are due to relaxation.

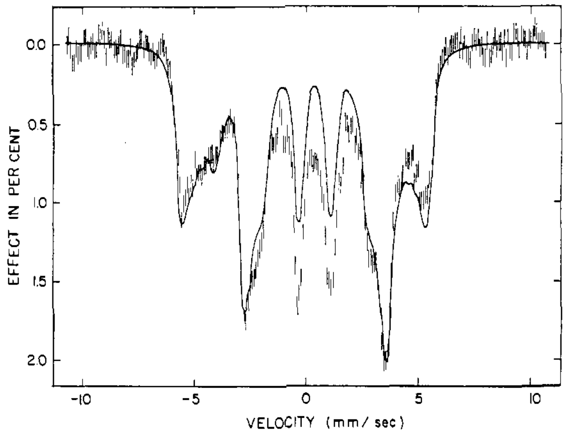

FIGURE 7: Mössbauer spectrum of the fluoride complex of chloroperoxidase taken at 4.2°K in a transverse field of 1300 G. The solid line is a computed spectrum using the parameters quoted in Table II. The line width used in the calculations was 0.4 mm/sec.

EFG tensor. We have to point our that parameters could be found giving better fits to the data taken in transverse magnetic field. However, bad fits resulted when these parameters were cross-checked by calculating the parallel field spectra. Obviously, to guard against gross errors in the interpretation, Mössbauer spectra for low-spin ferric compounds should be taken in both parallel and transverse magnetic fields. Considering that slight changes in the Mössbauer spectra were observed for different preparations and keeping in mind that the sample contains some high-spin material the calculated spectra represent the data quite well.

The following parameters were used to generate the spectra shown in Figure 1: $g_x = 1.84$, $g_y = 2.26$, $g_z = 2.63$, $k = 1.15$, $\Delta/\lambda = 5.58$, $V/\lambda = -3.58$, $A_x/g_n\beta_n = -490$ kG, $A_y/g_n\beta_n = +79$ kG, $A_z/g_n\beta_n = +284$ kG, $\Delta E_Q = +2.9$ mm/sec and $\eta = -2.9$. The line width (full width at half-maximum) was 0.35 mm/sec.

Since no single crystal data for chloroperoxidase are available, the directions x, y, and z are not defined with respect to the heme prosthetic group; the coordinate system used is therefore arbitrary.[5] The results imply that the electron hole resides in an almost pure orbital of d_{yz} symmetry (square of the expansion coefficient = 0.98). The EFG tensor has nearly axial symmetry around the smallest component of the g tensor, *i.e.*, $V_{yy} \approx V_{zz} \approx -(V_{xx}/2)$ and $V_{xx} < 0$. In the calculations the orbital reduction factor, k, was varied (an experimental determination of g_x would determine k in our model) between 0.7 and 1.3. The calculated Mössbauer spectra are not very sensitive to small variations of k, but decidedly unsatisfactory fits resulted for $k < 0.9$. The value of $k = 1.15$ which we used in the final simulation is, at best, only an estimate of some "average k." Griffith (1971) has discussed mechanisms which can give rise to an orbital reduction factor $k > 1$.

Our simple model accounts fairly well for the low-temperature data. It can be checked further at higher temperatures by calculating the temperature dependence of ΔE_Q; this is accomplished by taking a thermal average of the components of the EFG tensors of the three Kramers doublets. The energy differences between the Kramers doublets needed for the Boltzmann factors contain the spin-orbit coupling constant, λ, as a scaling factor. The calculations show that the theory fails to account for the large decrease in ΔE_Q at higher temperatures for both native chloroperoxidase and its chloride complex. Even for $\lambda = 200\ \text{cm}^{-1}$, which is rather low compared to $\lambda \simeq 340\ \text{cm}^{-1}$ used to fit the g values of another low-spin ferric heme protein (Salmeen and Palmer, 1968), the theory predicts a drop in ΔE_Q of only 2% from 4.2°K to 240°K, in contrast to the experimentally observed decrease of 30%. The failure of our simple model to predict the correct temperature dependence of ΔE_Q might be attributed to neglected contributions from excited states outside the $t_{2g}{}^5$ configuration. Griffith (1971) points out that such contributions can be substantial. Additionally, variations in the ligand field parameters caused by conformational changes of the protein might take place between 4.2°K and 250°K. It is interesting to note that other peroxidases (Lang *et al.*, 1969; Maeda, 1968) show a similar (unexplained) temperature dependence of ΔE_Q. Magnetic susceptibility data and measurements of the optical absorption spectra in this temperature range are clearly necessary.

Chloroperoxidase–Fluoride and Chloroperoxidase–Iodide. The low-temperature Mössbauer spectra of the chloroperoxidase–iodide and chloroperoxidase–fluoride derivatives are shown in Figures 6, 7, and 8. The spectra display a complex magnetic hyperfine interaction with an overall splitting of about 11 mm/sec, characteristic for heme iron in a high-spin ferric state. We observed that the intensity of the two innermost lines could be decreased by cooling the sample from 4.2°K to 1.5°K or by increasing the applied magnetic field from 360 G to 2.5 kG. This indicates that even at 4.2°K electron-spin relaxation is still affecting the spectra.

At 225°K the Mössbauer spectra for both complexes consist of broad and asymmetric quadrupole doublets, as they are commonly found in high-spin ferric heme proteins. For the quadrupole splitting we found: $\Delta E_Q = +(1.00 \pm 0.05)$ mm/sec for chloroperoxidase–F and $\Delta E_Q = +(0.65 \pm 0.07)$ mm/sec for chloroperoxidase–I. The sign of ΔE_Q is positive for both complexes, in agreement with results for other high-spin ferric heme compounds (Lang, 1970).

In ferric heme proteins the ^{6}S free ion ground state is split into three Kramers doublets. Owing to the dominant ^{6}S char-

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[5] For some of the low-spin ferric heme proteins epr data on single crystals are available. For these proteins, the largest component of the g tensor, g_z, was found in a direction close to the normal of the heme group (Peisach *et al.*, 1971a; Helcké *et al.*, 1968).

TABLE II: Parameters Used in the Simulations of the Low-Temperature Mössbauer Spectra.

Compound	Valence	Spin	g_x	g_y	g_z	$A_x/g_n\beta_n$ (kG)	$A_y/g_n\beta_n$ (kG)	$A_z/g_n\beta_n$ (kG)	ΔE_Q (mm/sec)	η
Chloroperoxidase	Fe^{3+}	$S = {}^1/_2$	1.84	2.26[a]	2.63[a]	−490	+79	+284	+2.9	−2.9
Chloroperoxidase–F[b]	Fe^{3+}	$S = {}^5/_2$	4.80[a]	7.01[a]	1.94	−480	−700	−194	+1.0	0.1
Chloroperoxidase–I[b]	Fe^{3+}	$S = {}^5/_2$	4.17[a]	7.48[a]	1.76	−389	−698	−164	+0.65	0.1

[a] Experimental g values determined by W. E. Blumberg and J. Peisach (see footnote 1). [b] The g and A values of these high-spin compounds refer to the lowest Kramers doublet. The A values are referred to the nuclear ground state (g_n = 1805).

acter of the ground state, orbital contributions to the electron Zeeman interaction and the magnetic hyperfine interaction are negligible. In this approximation the electron Zeeman term is isotropic ($g\beta\mathbf{H}\cdot\mathbf{S}$) and the magnetic hyperfine interaction contains only the isotropic Fermi contact term ($A_0\mathbf{S}\cdot\mathbf{I}$). The pertinent electronic and nuclear Hamiltonian is

$$\mathcal{H} = D\left(S_z^2 - \frac{S(S+1)}{3} + \frac{E}{D}(S_x^2 - S_y^2)\right) + g\beta\mathbf{H}\cdot\mathbf{S} + A_0\mathbf{S}\cdot\mathbf{I} + \mathcal{H}_Q - g_n\beta_n\mathbf{H}\cdot\mathbf{I} \quad (5)$$

For heme proteins the zero field splitting, described by D and E in eq 5, is large enough ($D \approx$ 10°K − 20°K; see Lang (1970)) so that at 4.2°K essentially only the lowest Kramers doublet contributes to the Mössbauer spectrum. As long as mixing of the doublets by the applied magnetic field can be neglected, it is convenient to describe each doublet by an effective spin ($S' = {}^1/_2$) Hamiltonian which has the form of eq 3. For each Kramers doublet the components of the g and the A tensor are proportional (Wickman *et al.*, 1966). Thus, if the principal axes values of the g tensor are known, the magnetic hyperfine tensor is determined to within a scaling factor.

The quadrupole splitting of high-spin ferric compounds is generally independent of temperature and we have therefore used the ΔE_Q values at 225°K in fitting the low-temperature data. Since for both complexes two principal axes values of the g tensor are known from epr (the third can be calculated using eq 5), the low-temperature Mössbauer spectra can be computed straightforwardly. The parameters used are quoted in Table II. Except for the middle part of the spectra, where relaxation effects show up, the calculated curves fit the data quite well.

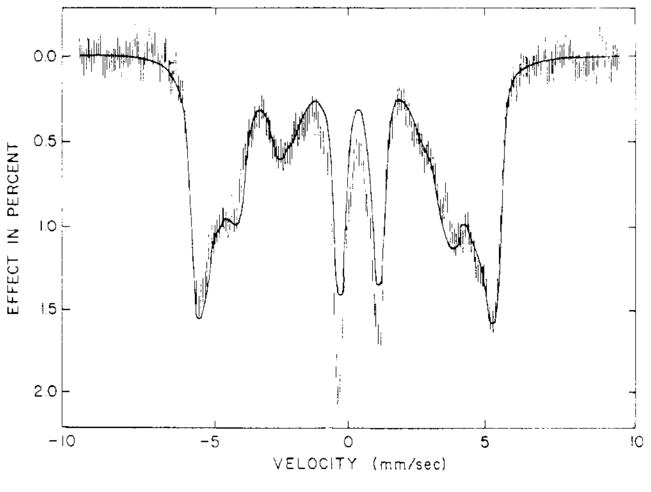

FIGURE 8: Spectrum of the fluoride complex of chloroperoxidase taken at 4.2°K in a 2500 G parallel magnetic field. The computed spectrum (solid line) was calculated with the parameters quoted in Table II.

The internal magnetic field H_{sat} = −(500 ± 10) kG of chloroperoxidase–F ($H_{sat} = -5A_0/2g_g\beta_n$, g_g = 0.1805) falls into the range (480 kG − 530 kG) commonly found for high-spin ferric hemes (Lang, 1970). The lower field found for the iodine complex, H_{sat} = −(460 ± 10) kG, indicates a larger degree of electron delocalization at the heme iron. Recently we found H_{sat} = −450 kG for cytochrome P-450$_{cam}$ (Sharrock *et al.*, 1973).

The simulations of the Mössbauer spectra require A_x and A_y to differ by at least 200 kG. This fact, or alternatively the anisotropic g tensor, clearly reveals large rhombic distortions at the heme iron, not observed before for high-spin heme–halide complexes. In terms of the zero-field-splitting parameters defined in eq 5 the rhombic distortions can be expressed as E/D = 0.05 for chloroperoxidase–F and E/D = 0.07 for chloroperoxidase–I. These values for E/D follow from the epr investigation by W. E. Blumberg and J. Peisach (see footnote 1).

Finally, we should note that there is no conclusive evidence that the halides are coordinated to the iron. This information could be obtained from epr by observing fluorine hyperfine structure around g = 2. We have observed significant differences between the chloroperoxidase–F and chloroperoxidase–I Mössbauer spectra taken in zero magnetic field; this might suggest the presence of transferred hyperfine interactions between the halide and the iron. A detailed analysis of these rather complex spectra is necessary, however, before definite conclusions can be drawn.

Reduced Chloroperoxidase. The Mössbauer spectrum of dithionite reduced chloroperoxidase taken at 4.2°K is shown in Figure 9. The spectrum shows a well-defined quadrupole doublet. The isomeric shift and the magnitude of the quadrupole splitting ΔE_Q are in the range typically found for iron in a high-spin ferrous state. Magnetic hyperfine structure is generally not observed in high-spin ferrous compounds unless strong external magnetic fields are applied. The slight asymmetry observed in the spectrum indicates that a small amount of the sample material is still ferric. The solid line in Figure 9 is the result of fitting two lines of Lorentzian shape to the data. For the spectrum measured at 4.2°K we find $|\Delta E_Q|$ = (2.48 ± 0.02) mm/sec and δ = +(0.85 ± 0.02) mm/sec (with respect to iron metal). The line width (full width at half-maximum) used to fit the spectrum was 0.29 mm/sec. A spectrum taken at 200°K revealed that ΔE_Q is essentially independent of temperature; $|\Delta E_Q|$ (200°K) = (2.43 ± 0.02) mm/sec. The

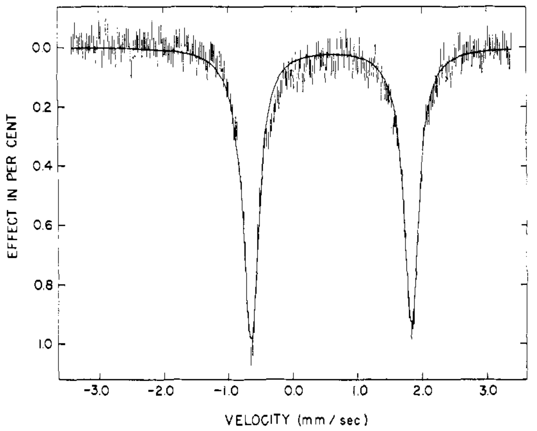

FIGURE 9: Mössbauer spectrum of reduced chloroperoxidase taken at 4.2°K.

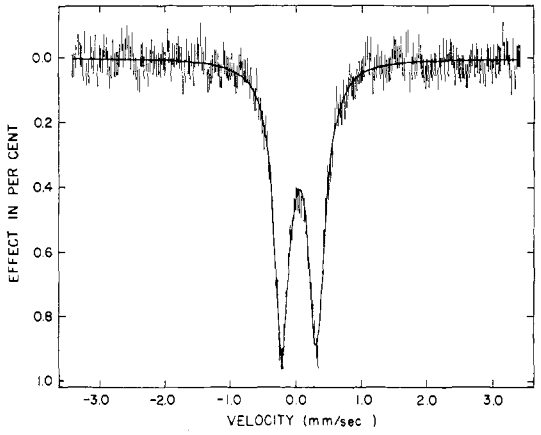

FIGURE 10: Mössbauer spectrum of the reduced chloroperoxidase–CO measured at 4.2°K.

Mössbauer parameters of reduced chloroperoxidase and reduced P-450$_{\mathrm{cam}}$ (Sharrock *et al.*, 1973) are almost identical. The temperature dependence of ΔE_Q for reduced chloroperoxidase and reduced P-450$_{\mathrm{cam}}$ differs quite markedly from that of deoxyhemoglobin (Eicher and Trautwein, 1969) and Japanese radish peroxidase a (Maeda, 1968). (The latter enzymes show an appreciable decrease of ΔE_Q at higher temperatures.)

Reduced Chloroperoxidase Complexed with CO. The optical spectra and the Mössbauer data on reduced chloroperoxidase and cytochrome P-450$_{\mathrm{cam}}$ (Sharrock *et al.*, 1973) suggest close similarities between the active sites of the two proteins. Therefore it seemed interesting to extend this comparison to the carbon monoxide complexes of both enzymes. We found that reduced chloroperoxidase–CO has a Soret band (Table I) at unusually long wavelength, to the present time thought to be characteristic of cytochromes of the P-450 type.

The Mössbauer spectra of chloroperoxidase–CO, taken at 4.2°K, 133°K, and 200°K, exhibit a sharp quadrupole doublet. The isomeric shift, δ, and the temperature-independent quadrupole splitting are typical for a low-spin ferrous (S = 0) compound. The spectrum shown in Figure 10 was taken at 4.2°K. From a least-squares fit to data taken at 4.2°K we obtained $|\Delta E_Q| = (0.52 \pm 0.04)$ mm/sec and $\delta = (+0.29 \pm 0.02)$ mm/sec (with reference to iron metal). The value for ΔE_Q is somewhat larger than those found for other heme–CO complexes: $|\Delta E_Q| = 0.32$ mm/sec for P-450$_{\mathrm{cam}}$–CO (Sharrock *et al.*, 1973); $|\Delta E_Q| = 0.36$ mm/sec for hemoglobin–CO (Lang and Marshall, 1966); $|\Delta E_Q| = 0.21$ mm/sec for Japanese radish peroxidase *a*–CO (Maeda, 1968).

Discussion

For an understanding of the mechanisms involved in heme protein catalysis, a knowledge of the environment of the heme, the state of the iron, and the effect of the substrates on these properties is critical. The evidence presented in this paper indicates that halide anions, which are substrates for chloroperoxidase halogenation reactions, can form complexes with the enzyme and cause significant changes in the heme environment and the spin state of the iron.

Analysis of the optical spectra by the method of Brill and Williams (1961) indicates that the native enzyme is predominantly high-spin at room temperature. The addition of halide anions to the enzyme results in the formation of complexes having more (chloroperoxidase–I), the same (chloroperoxidase–Br), and less (chloroperoxidase–F and chloroperoxidase–Cl) high-spin character than the native enzyme. The dramatic effects exhibited by the halide anions on the Soret band of chloroperoxidase suggest that they must be binding close to the heme site.

Hopefully, the information obtained from the spectroscopic investigations of chloroperoxidase described here will aid in determining the chemical structure of the active site. In the following paragraphs, we discuss the spectra of chloroperoxidase and some of its complexes without attempting to suggest the axial ligands of the native protein.

Our Mössbauer studies have shown clearly that the heme iron of the native enzyme undergoes a spin transition from predominantly low spin to predominantly high spin as the temperature is raised from 4.2°K to 245°K. Over this same temperature range, the quadrupole splitting of the low-spin component drops from 2.9 to 2.0 mm per sec. This behavior of $|\Delta E_Q|$ is not compatible with the model of an orthorhombic crystal field which successfully describes the Mössbauer spectra taken at 4.2°K. The difficulty in calculating the temperature dependence of ΔE_Q could be caused by neglecting contributions from excited states outside of the $t_{2g}{}^5$ configuration. Alternatively, the strong temperature dependence of $|\Delta E_Q|$ may indicate a change in geometry and therefore in the crystal field parameters of the heme iron as the temperature is raised.

Blumberg and Peisach (1971) have classified the low-spin ferric form of native chloroperoxidase as a type-O compound. Their classification is based on the two resolved g values and, hence, must assume an orbital reduction factor $k = 1.0$. From an analysis of the low-temperature Mössbauer data we obtained an estimate of $k \cong 1.15$ and of the third component of the g tensor, $g_x \cong 1.84$. The crystal field parameters we found using these estimates would place native chloroperoxidase near the boundary between type-O and type-P compounds in the truth diagrams of Blumberg and Peisach.

Of the three chloroperoxidase–halide complexes studied, chloroperoxidase–Cl is unique because, at 4.2°K, it exhibits Mössbauer spectra which are almost identical with the spectra of the native enzyme. Additionally, the optical spectrum of chloroperoxidase–Cl at room temperature indicates more low-spin character ($\lambda_{\max}$ 412 nm) than is found for any of the other ferric complexes studied. Although the quadrupole splitting of chloroperoxidase–Cl exhibits a temperature dependence similar to that of native chloroperoxidase, this complex does not undergo a spin transition to high spin in the observed temperature range. However, the shift of the Soret band of chloroperoxidase–Cl from 430 nm at 77°K to 412 nm at 296°K indicates some increase in high-spin character as the temperature is raised. The similarities in the *g* values and the low-temperature Mössbauer spectra of chloroperoxidase and chloroperoxidase–Cl suggest that the axial ligands of the heme iron in the two proteins are the same, *i.e.*, that Cl is not an axial ligand. The data show, however, that Cl binding causes sufficient structural changes at the heme group to affect the optical spectrum and to cause slight shifts in the *g* values away from those of the native enzyme. The absence of a spin transition in the chloroperoxidase–Cl complex at temperatures below 245°K does not contradict this interpretation, since the structural changes associated with Cl binding might shift the low-spin–high-spin transition to higher temperatures as suggested by the optical data.

The complexes of chloroperoxidase with fluoride and iodide are high-spin ferric over the entire temperature range studied with Mössbauer spectroscopy. In the classification scheme for high-spin ferric heme complexes used by Peisach *et al.* (1971b), chloroperoxidase–I and chloroperoxidase–F occupy extreme positions on the rhombicity scale (the *g* values quoted in footnote 1 imply 20% rhombicity for chloroperoxidase–I and 14% rhombicity for chloroperoxidase–F). Since halide complexes of other heme proteins have been found to display nearly tetragonal symmetry (Peisach *et al.*, 1971b; Lang *et al.*, 1969) and since large rhombic distortions are also observed for the high-spin component of native chloroperoxidase (minority species), it appears that these distortions may be the result of a specific fifth ligand or peripheral heme–protein interaction rather than of a heme–halide bond. Finally, the fact that the chloroperoxidase–I and chloroperoxidase–F complexes exhibit high-spin ferric Mössbauer spectra, in contrast to the low-spin spectra of the native enzyme, need not imply that fluoride and iodide coordinate as axial ligands to the heme iron. Considering that spin transitions in chloroperoxidase can be caused by raising the temperature, it is certainly possible that the binding of fluoride or iodide to sites other than the sixth heme ligand position could result in the observed Mössbauer spectra.

One of the goals of a Mössbauer investigation is to get clues about the chemical nature of the axial ligands which coordinate to the heme iron. Such information is generally based on analogies to heme complexes having a known chemical structure. For chloroperoxidase, few similarities with known model heme proteins have been found, and it is therefore rather difficult to deduce axial ligands. The present investigation, however, has brought out striking similarities between chloroperoxidase and cytochrome P-450$_{\mathrm{cam}}$, a heme protein for which, unfortunately, the ligand structure is also unknown. Reduced P-450$_{\mathrm{cam}}$ and reduced chloroperoxidase have almost identical optical absorption spectra. Additionally, both compounds have similar Mössbauer spectra typical of heme iron in a high-spin ferrous state; the observed isomeric shift and the temperature-independent quadrupole splitting are almost identical. The carbon monoxide complexes of both proteins show a Soret band at unusually long wavelength ($\lambda_{\max}$ 443 nm for chloroperoxidase–CO; $\lambda_{\max}$ 446 nm for P-450$_{\mathrm{cam}}$–CO). The Mössbauer spectra of these reduced-CO complexes consist of a quadrupole doublet characteristic of low-spin ferrous iron.

The ferric forms of chloroperoxidase and P-450$_{\mathrm{cam}}$ have similarities worthy of note also. The orthorhombic crystal field model used to calculate the low-spin ferric Mössbauer spectra indicates that chloroperoxidase is very near the class of P-type compounds (Blumberg and Peisach, 1971) which includes cytochrome P-450.[6] Also, the high-spin ferric form of camphor-complexed P-450$_{\mathrm{cam}}$ displays an unusually large rhombic distortion (Tsai *et al.*, 1970) very much like those found in native chloroperoxidase, chloroperoxidase–F, and chloroperoxidase–I.

In view of the numerous similarities between these two proteins, it seems likely that the chemical structure of chloroperoxidase in the vicinity of the heme iron is somehow related to that of P-450$_{\mathrm{cam}}$. One possibility is that the fifth heme ligand of both proteins is identical and that this axial ligand is responsible for the large rhombic distortions experienced by the heme irons of both proteins. The possibility of functional similarities between the two proteins is presently being explored. Recently we have observed that the addition of type-II substrates (aniline, *N*-phenylimidazole) causes similar shifts in the Soret bands of both chloroperoxidase and P-450$_{\mathrm{cam}}$.

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[6] According to Blumberg and Peisach (1971) all type-P compounds are likely to have a sulfhydryl group binding to the heme iron. It is interesting to note that chloroperoxidase has no such ligand, yet must be classified close to the P type. The only two cysteine residues in chloroperoxidase are known to form a disulfide bridge.

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Cations and Ribosome Structure. I. Effects on the 30S Subunit of Substituting Polyamines for Magnesium Ion[†]

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ABSTRACT: The polyamines putrescine ($NH_3^+(CH_2)_4NH_3^+$) and spermidine ($NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_3^+$) have been used to replace the Mg^{2+} normally associated with the 30S ribosomal subunit of *Escherichia coli*. By means of equilibrium dialysis, 30S particles, purified by sucrose gradient centrifugation, have been prepared with various levels of bound Mg^{2+}. Stoichiometric replacement of Mg^{2+} by either polyamine had no effect on the ability of the particles to synthesize polyphenylalanine as long as the level of bound Mg^{2+} was above a critical level (Mg^{2+}/RNA-P = 0.115). Further replacement resulted in gradual loss of activity until none remained when only polyamines were bound to the particles. This inactivation was accompanied by a concomitant conformational alteration in the particles which resulted in an increase in their sensitivity to attack by ribonuclease, a decrease in their sedimentation coefficients, and an increase in their reduced viscosities. The inactive preparations sedimented as a single boundary in the ultracentrifuge. Putrescine appeared to be somewhat less effective than spermidine in maintaining ribosome structure and function. Neither native structure nor function could be restored by dialysis against buffers containing Mg^{2+}. However, inactive particles, after isolation from sucrose gradients, could be fully reactivated by a brief treatment at 40° under specific ionic conditions, implying that inactivation was reversible and did not involve covalent modification or loss of essential material from the particles. These results suggest that a critical level of bound Mg^{2+} is necessary for the maintenance of the structure and function of the 30S ribosomal subunit.

Magnesium ion (Mg^{2+}) is a required growth factor for all cells. This ion is important for the activity of many enzymes (Wacker, 1969) and also appears to be necessary for the stability of ribosomes both *in vitro* and *in vivo*. McCarthy (1962) demonstrated that cells of *Escherichia coli*, when starved for Mg^{2+}, fail to maintain intact ribosomes. Tempest and his coworkers, while studying bacteria whose growth was limited by magnesium ion, demonstrated a definite stoichiometry between cellular Mg^{2+} and RNA which was independent of growth rate (Tempest and Strange, 1966; Tempest and Meers, 1968). This observation supported the hypothesis of a required interaction between Mg^{2+} and RNA. The removal of ribosomally associated Mg^{2+} *in vitro*, either through the use of chelating agents (Weller and Horowitz, 1964; Cammack and Wade, 1965; Gesteland, 1966a; Weller *et al.*, 1968; Miall and Walker, 1969; Eilam and Elson, 1971) or by displacement with high concentrations of monovalent cations (Elson, 1961; Spirin *et al.*, 1963; Maruta *et al.*, 1969; Spitnik-Elson and Atsmon, 1969; Ghysen *et al.*, 1970), results in disruption of the native structure. These observations suggest that one of the primary roles of intracellular Mg^{2+} is in the maintenance of ribosome structure and function.

The changes in ribosome structure observed upon the removal of magnesium ion have been interpreted as the formation of more asymmetric and distended structures. This interpretation has been based primarily on studies of the hydrodynamic properties of the resulting particles. Although the exact nature of the derived particles appears to be a complex function of ionic strength and magnesium levels, it seems from the above-mentioned studies that a number of intermediate conformational changes can exist during the unfolding of these particles. These structural changes appear to take place with no loss of ribosomal protein. During the early stages of unfolding, there is very little disruption of the structure of the rRNA as detected by optical means (Miall and Walker, 1969; Eilam and Elson, 1971). However, in the more highly unfolded particles, it is clear that there is extensive disruption of base pairing (Eilam and Elson, 1971). These changes in ribosome structure on removal of Mg^{2+} have been interpreted as resulting from electrostatic repulsion,

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