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Broad-Line Nuclear Magnetic Resonance Studies of Chloroperoxidase[†]

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ABSTRACT: Chloroperoxidase, a heme glycoprotein isolated from the mold *Caldariomyces fumago,* was studied by NMR relaxation techniques. Interaction of the chloride ion substrate with the enzyme may be analyzed as consisting of at least three contributions: a weak interaction with the iron atom, nonspecific anion–protein interactions, and a specific interaction generated at low pH. The data indicate that a specific interaction, which develops in parallel with enzyme activity at low pH, does not occur at the iron atom first coordination sphere site. The results are summarized in terms of an enzymatic mechanism not involving chloride ion coordination to the iron atom.

Heme proteins constitute a major class of important metalloenzymes. Many of these enzymes catalyze the oxidation or reduction of various substrates presumably with a corresponding change in the electronic nature of the iron atom of the heme prosthetic group. The electronic processes in these reactions are very rapid and the intermediates short-lived, making direct study of the reaction mechanisms difficult. However, information about the arrangement of the ligands at the fifth and sixth coordination positions of the iron atom can lead indirectly to important conclusions about the catalytic process. Nuclear magnetic resonance relaxation techniques have provided a sensitive and efficient means for investigating metals such as iron in metalloenzymes (Wuthrich, 1970; Mildvan and Cohn, 1970; Ellis et al., 1969, Dweck, 1973).

Chloroperoxidase has a molecular weight of 42 000 and one ferriprotoporphryn IX moiety per monomeric species. This enzyme utilizes hydrogen peroxide to catalyze the oxidation of chloride, bromide, and iodide ions to an enzyme-bound halonium species (Morris and Hager, 1966). Halogenation of various substrates occurs at activated positions such as α to a carbonyl or ortho–para on an aromatic ring. Previous evidence suggests the mechanism is ionic (Brown and Hager, 1967). A possible reaction pathway could involve peroxide and halide ions as ligands at the fifth and sixth ligand position of the iron atom (Hager et al., 1970). Electron transfer could then proceed through the iron to give the oxidized halogen, which could then react with the organic substrate. Recent evidence on the nature of the adduct between hydrogen peroxide and iron, i.e., compound I, indicates that the peroxide does coordinate to the iron atom (Hager et al., 1972); however, the location of a binding site for the halide ion remains uncertain. There are two possibilities: (a) The halide ion could coordinate to the iron atom at a sixth coordination position. (b) The halide ion could

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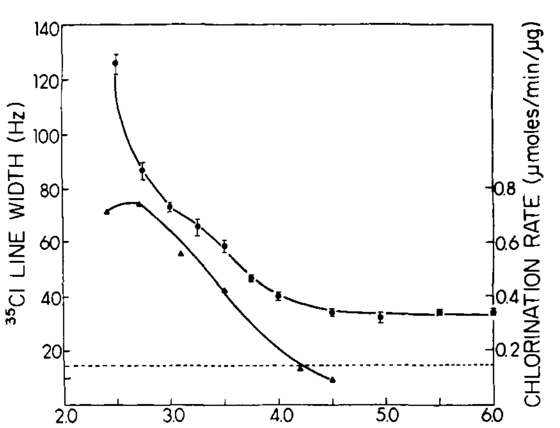

FIGURE 1: (▲) Enzymic activity as a function of pH using the standard chlorination assay; (●) ^{35}Cl NMR line width in a solution containing 0.5 M KCl, 3 × 10^{-4} M CPO, and 0.1 M phosphate buffer as a function of pH; (- - -) ^{35}Cl NMR line width in the absence of enzyme.

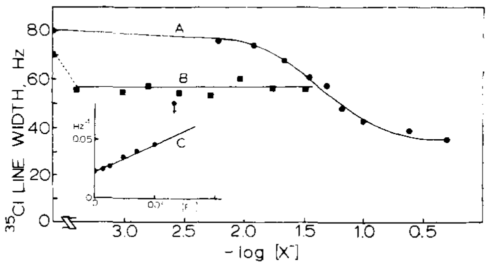

FIGURE 2: (A) ^{35}Cl line width as a function of sodium fluoride concentration. The concentrations were CPO, 3 × 10^{-4} M; KCl, 0.5 M; and phosphate buffer, 0.1 M. The pH was maintained at 3.0 by addition of phosphoric acid and at higher fluoride concentrations with hydrochloric acid. (B) ^{35}Cl NMR line width as a function of sodium azide concentration. Concentrations were: CPO, 3 × 10^{-4} M; KCl, 0.5 M; and phosphate buffer, 0.1 M. The pH was maintained at 3.0 with additions of phosphoric acid. (C) Reciprocal ^{35}Cl NMR line broadening vs. sodium fluoride concentration.

bind in the vicinity of the iron atom either to the protein residue or a position on the heme moiety. The following study was undertaken because it is possible to distinguish between these possibilities by observing the nuclear magnetic resonance relaxation times of the water protons and the ^{35}Cl ions in chloroperoxidase solutions.

^{35}Cl NMR has provided a sensitive method for the study of chloride ion interactions with macromolecules. The ^{35}Cl NMR line width may be determined by competing relaxation mechanisms; however, in most cases studied, the quadrupole mechanism appears to dominate. A discussion of quadrupole relaxation as it affects this work has been presented (Stengle and Baldeschwieler, 1966; Marshall, 1970; Collins et al., 1973; Bull, 1973). The use of paramagnetic metal ions such as the iron(III) ion in heme to effect changes in proton relaxation times that reflect changes at macromolecular metal binding sites is a powerful approach which has been extensively reviewed and will not be discussed further here (Mildvan and Cohn, 1970; Dweck, 1973).

Experimental Section

Chloroperoxidase was prepared as previously described (Morris and Hager, 1966). Enzyme activity was measured by the standard chlorination assay and the samples used in this study had specific activities of 2000 units per mg and values of R_z greater than 1.30, indicating a purity of 90% or greater (Hager et al., 1966). All inorganic salts used to prepare the buffers and halide solutions were reagent grade. The enzyme samples were brought to the correct pH and halide concentrations by dialysis in tubing boiled with 1 mM EDTA[1] and washed four times with water. Metal ion analyses were made on a Perkin-Elmer Model 303 atomic absorption apparatus at two enzyme concentrations. Quenching by the enzyme was negligible. The CPO samples used in this study contained 0.25 mol of manganese per mol of enzyme. Optical measurements were made on a Coleman Hitachi Model 165 spectrophotometer employing 1-cm silica cells.

^{35}Cl NMR measurements were made on a modified Varian DP-60 NMR spectrometer as previously described (Bryant et al., 1972).

Proton T_1 measurements were made employing a V-4311 rf unit using the adiabatic rapid passage method (Varian Associates, 1960). These measurements were made on 0.5-ml samples contained in nonspinning 5-mm o.d. tubes at the magnet gap temperature of 27 ± 2 °C. The recovery of magnetization was followed directly on a Midwestern Instruments LCR2 oscillographic recorder and T_1 was determined by a least-squares fit to the data for each measurement. The error for three or more separate measurements is between 5 and 10%.

Results

The interactions of chloride ion with chloroperoxidase may be detected directly as a broadening of the chlorine NMR spectrum as shown in Figure 1. The chloride ion line width increases dramatically at lower pH values as the enzymic activity increases. The line broadening due to the enzyme approaches a limiting value of approximately 21 Hz at pH values above 4.5. The chlorine line width continues to increase after the enzyme activity has plateaued at pH of approximately 2.8. The chloride ion line width is linear in enzyme concentration.

Steady-state kinetic studies indicate that fluoride ion inhibits the chlorination reaction catalyzed by chloroperoxidase. Although the kinetic studies are complex, the inhibition results indicate that fluoride ion is competitive with both hydrogen peroxide and chloride ion (Hager et al., 1966). The competitive nature of the fluoride inhibition may be investigated directly by observing the chloride ion line width as a function of fluoride ion concentration at pH 3.0 as shown in Figure 2. The decrease in ^{35}Cl line width may be used to estimate K_F if the binding constant for chloride ion, K_{Cl}, is known. A plot of the reciprocal of the line broadening vs. fluoride concentration is linear up to 0.05 M as shown in the insert of Figure 2. If a chlorine binding constant of 5 M^{-1} is assumed (Thomas et al., 1970), the value of the fluoride ion binding constant derived from the NMR data is 0.14 M. These values agree well with previous reports (Thomas et al., 1970).

The addition of azide strongly inhibits chloroperoxidase with a K_I of 2 × 10^{-6} M measured at pH 3. The chlorine line width as a function of azide concentration is shown in Figure 2. Over the concentration range studied, azide shows a quite different effect on the ^{35}Cl broadening compared with fluoride. Comparing the binding constants for azide and fluoride, it would be expected that azide would be much more efficient than fluoride in eliminating the ^{35}Cl line broadening if both ions bind at the same site and compete with chloride. Since azide is known to bind as an axial ligand to heme iron in several hemoproteins, these results suggest that the major chloride (and

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; CPO, chloroperoxidase.

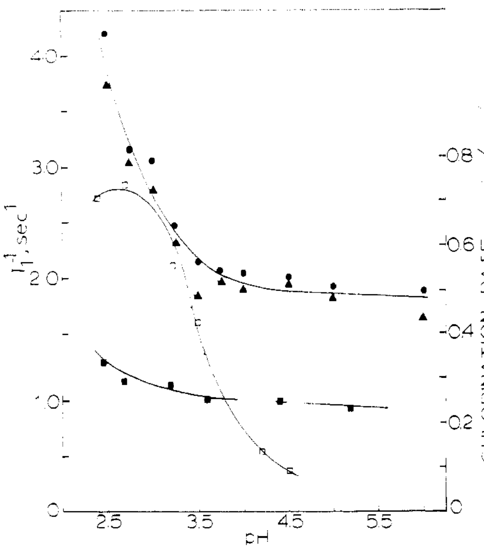

FIGURE 3: Proton relaxation rate $(T_1)^{-1}$ of CPO (●), CPO plus 0.5 M KCl (▲), and CPO, 0.5 M KCl, and 13 equiv of azide (■) as a function of pH. The concentration of CPO is 7.7×10^{-4} M. Also the rate of chlorination by CPO as measured by the standard assay procedure is plotted vs. pH (□).

the competitive fluoride) binding site is *not* at the heme iron.

An independent method of investigating the nature of the anion interactions with chloroperoxidase is to study the proton relaxation rate of the water protons. Graphs of proton relaxation rates of chloroperoxidase solutions are shown as a function of pH in Figure 3. Within experimental error the addition of chloride ion does not change the solvent proton relaxation time. When the same solutions were measured after the addition of 13 equiv of sodium azide ion, which is sufficient to block coordination of water at the iron atom, the whole relaxation curve is shifted to longer relaxation times.

Discussion

The data of Figure 1 show that chloroperoxidase exhibits at least two types of chloride ion–enzyme interaction. In the pH region above pH 4.5, ^{35}Cl NMR line broadening caused by the enzyme is relatively constant and most probably is the result of nonspecific interactions between chloride ion and the enzyme. There is ample evidence that protein molecules may bind chloride ions in a relatively nonspecific manner in the absence of metal ions. For example, the chloride ion interaction with metal-free bovine serum albumin and horseradish peroxidase may be observed directly by ^{35}Cl NMR (Bryant, 1969; Magnuson and Magnuson, 1972; Ellis et al., 1969). The nonspecific chloride association is not directly responsible for enzyme activity because an additional strong chloride interaction is observed with decreasing pH which closely parallels the development of enzyme activity.

Examination of Figure 3 indicates that the proton relaxation rate increases significantly with decreasing pH in a manner parallel to the development of enzyme activity. Because the relaxation is so efficient, the proton binding sites must be close to the iron atom. There are at least two possibilities: (1) The proton site closest to the iron atom is a water molecule directly coordinated to the metal. (2) The porphyrin moiety or a protein group protonated at low pH is sufficiently close to the iron to cause efficient proton relaxation. The second alternative is attractive because the creation of additional positive charge generates a potential binding site for an anion such as a halide ion which is very close to, but not necessarily coordinated to, the active iron site in the enzyme (Hager et al., 1970). The increase in proton relaxation rate with decreasing pH in solutions of chloroperoxidase containing chloride ion is consistent with the model that the chloride binding site is generated by a protonation on or near the heme ring.

The interaction of chloride ion with the active site of chloroperoxidase probably does not involve coordination of chloride ion to the iron atom directly. This hypothesis is supported by the azide experiment shown in Figure 2. Except for the initial 15-Hz drop, the chloride line width is independent of azide over the range shown. Throughout this concentration range, the enzyme is completely inhibited by azide; however, the ^{35}Cl line width decrease is small, indicating that the azide ion may displace only a fraction of the bound chloride ion from enzyme sites available at low pH. The equilibrium constants for the association of chloride and azide ion with iron(III) are approximately 5 and 10^5 M^{-1}, respectively (Sillen and Martell, 1964), so that a weak chloride ion–iron complex is expected in the absence of a stronger iron ligand, but such an association should be quantitatively eliminated by the addition of azide ion.

The proton relaxation data of Figure 3 provide further evidence for chloride binding to a site other than direct coordination to the heme iron. The addition of chloride ion to the enzyme solution does not alter the proton relaxation time significantly over the pH range studied. If chloride ion coordinated strongly at the iron atom, the reaction would displace a water molecule from the iron and result in a decrease in the proton relaxation rate similar to the reduction observed on the addition of sodium azide. The apparently systematic depression of the proton relaxation rate on the addition of chloride is on the order of the experimental errors of the T_1 measurement; however, a small depression is expected due to a small binding constant of chloride ion for iron(III).

The data of Figure 2 demonstrate that the chloride ion may be displaced from the acid-generated binding site by competition from fluoride ion. The line width at high fluoride concentration approaches the value observed in Figure 1 in the pH-independent region above 4.5. This result suggests that the fluoride ion competes effectively with chlorine for the acid-generated sites, but does not compete effectively for the less specific anion binding sites at low concentrations. Unlike chloride ion, fluoride ion has a high affinity for iron and it is quite possible that fluoride inhibition may result from either of two events: coordination at the peroxide site or at the chloride site (Hager et al., 1966).

The interaction of chloride ion with chloroperoxidase may be summarized by writing a relaxation equation including the several distinguishable contributions

$$\Delta\nu_{\mathrm{tot}} = P_{\mathrm{f}}\Delta\nu_{\mathrm{f}} + P_{\mathrm{E}}\Delta\nu_{\mathrm{E}} + P_{\mathrm{H}}\Delta\nu_{\mathrm{H}} + P_{\mathrm{Fe}}\Delta\nu_{\mathrm{Fe}} \qquad (1)$$

where $\Delta\nu_{\mathrm{f}}$ is the line width of a free solvated chloride ion, $\Delta\nu_{\mathrm{E}}$ is the average line width of a chloride ion bound nonspecifically to the enzyme, $\Delta\nu_{\mathrm{H}}$ is the line width of a chloride ion at an acid-generated site near the heme group, $\Delta\nu_{\mathrm{Fe}}$ is the chloride line width for chloride coordinated to the heme iron, and P_{f}, P_{E}, P_{H}, and P_{Fe} are the mole fractions of total chloride ion located at each site, respectively.

The distinction suggested by $\Delta\nu_{\mathrm{H}}$ and $\Delta\nu_{\mathrm{Fe}}$ is based upon the small decrease in ^{35}Cl line width in the azide experiment which is consistent with displacement of some chloride ion from the iron. It is also possible that changes in the spin state of iron on binding of azide could affect chlorine relaxation rates even if the chloride were not directly coordinated; however, recent measurements have demonstrated that chloride ion relaxation in solutions broadened significantly by ferric ion is dominated by the nuclear electric quadrupole relaxation mechanism (Rose and Bryant, 1976). In either case it is clear that there is a catalytically important interaction of chloride ion which need not involve the azide binding site identified with the iron.

Isolation of the third term in eq 1 is afforded by the fluoride ion data of Figure 2. Substitution of values for K_{Cl} and K_{F}, the binding constants for chloride and fluoride (Thomas et al., 1970), yields a value of the ^{35}Cl line width associated with the fluoride site of 10^5 Hz. This value is in the range where the chemical-exchange rate of chloride ion with the enzyme may contribute significantly to the relaxation (Man and Bryant, 1974). In the event that the exchange rate determines the line width for this site, the mean residence time for chloride would be 10^{-5} s.

In summary these experiments indicate that a mechanism for the chlorination reaction by CPO need not involve direct coordination of chloride ion to iron. The following pathway may be suggested. The enzyme bound heme iron accepts one or less protein donated ligands leaving one position to be occupied by water in the absence of other strong ligands. The addition of hydrogen peroxide displaces the water molecule to form compound I. At low pH a protonation occurs on or near the heme moiety thus generating an anion binding site in close proximity to the oxidizing agent, compound I. The halide may then undergo oxidation presumably by an outer sphere electron transfer with respect to the iron to generate a reactive chlorine species which may participate directly in the chlorination reaction (Taube and Gould, 1969; Castro and Davis, 1969).

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