*250,* 4451.
Pearson, P. H., and Bridger, W. A. (1975b), *J. Biol. Chem. 250,* 8524.
Pfeifer, M. (1962), *Z. Naturforsch. A 17,* 279.
Pfeifer, H., Michel, D., Sames, D., and Sprinz, H. (1966), *Mol. Phys. 11,* 591.
Ramaley, R. F., Bridger, W. A., Moyer, R. W., and Boyer, P. D. (1967), *J. Biol. Chem. 242,* 4287.
Reuben, J., and Cohn, M. (1970), *J. Biol. Chem. 245,* 6539.
Reuben, J., Reed, G. H., and Cohn, M. (1970), *J. Chem. Phys. 52,* 1617.
Rubinstein, M., Baram, A., and Luz, Z. (1971), *Mol. Phys. 20,* 67.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Solomon, I. (1955), *Phys. Rev. 99,* 559.
Solomon, I., and Bloembergen, N. (1956), *J. Chem. Phys. 25,* 261.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37,* 307.
Zalkin, A., Forrester, J. D., and Templeton, D. H. (1964), *Inorg. Chem. 3,* 529.
Zetter, M. S., Grant, M. W., Wood, E. J., Dodgen, H. W., and Hunt, J. P. (1972), *Inorg. Chem. 11,* 2701.

# Oxidation–Reduction Potential Measurements on Chloroperoxidase and Its Complexes†

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ABSTRACT: The oxidation–reduction potential of chloroperoxidase, an enzyme which catalyzes peroxidative chlorination, bromination, and iodination reactions, has been investigated. In addition to catalyzing biological halogenation reactions, chloroperoxidase is unusual in that the carbon monoxide complex of ferrous chloroperoxidase shows the typical long wavelength Soret absorption associated with P-450 hemoproteins. The pH dependence of the chloroperoxidase oxidation–reduction potential shows a discontinuity around pH 4.7. Similarly, measurements of the affinity of ferrous chloroperoxidase for carbon monoxide monitored both by spectroscopic and potentiometric titration exhibit a discontinuity in the pH 4.7 region. Oxidation–reduction potential measurements on chloroperoxidase in a CO atmosphere also show a discontinuous pH profile. These results suggest that ferrous chloroperoxidase undergoes reversible modification at low pH and that these changes are reflected in the oxidation–reduction potential. The oxidation–reduction potential of chloroperoxidase at pH 6.9 is −140 mV, close to that measured for cytochrome P-450$_{cam}$ in the presence of substrate. The oxidation–reduction potential of chloroperoxidase at pH 2.7, the pH optimum for enzymatic chlorination, is +150 mV. The oxidation–reduction potentials of the halide complexes of chloroperoxidase (chloride, bromide, and iodide) are essentially identical with the potential measurements on the native enzyme. These observations suggest that, although halide anions bind to the enzyme, they probably do not bind as an axial ligand to the heme ferric iron.

Chloroperoxidase is a unique hemoprotein which catalyzes the chlorination reactions involved in the biosynthesis of caldariomycin (Morris and Hager, 1966). In the presence of suitable halogen donors and acceptors, chloroperoxidase catalyzes the peroxidatic formation of a variety of halometabolites (Thomas et al., 1970a). Chloroperoxidase also catalyzes the oxidation of hydrogen donors such as guaiacol and ascorbate (Thomas et al., 1970a) and exhibits high catalase activity for hydrogen peroxide decomposition with oxygen evolution (Thomas et al., 1970a). Although chloroperoxidase is similar to plant and animal peroxidases in many of its catalytic functions, recent studies have drawn attention to numerous similarities between bacterial and microsomal P-450 type hemoproteins and chloroperoxidase. The physicochemical properties of chloroperoxidase, studied by optical, EPR,[1] and Mössbauer spectroscopy, are very similar to those of P-450$_{cam}$ in both the ferric and ferrous states (Champion et al., 1973, 1975; Hollenberg and Hager, 1973; Chiang et al., 1975). These similarities suggest that chloroperoxidase and P-450$_{cam}$ share a common heme environment.

The oxidation–reduction potentials of several isozymes of horseradish peroxidase and turnip peroxidase have been measured (Harbury, 1957; Yamada et al., 1975; Ricard et al., 1972). Here we report the oxidation–reduction potentials of the ferric–ferrous couple of native and liganded states of chloroperoxidase.

## Materials and Methods

*Enzyme Preparations.* Chloroperoxidase was isolated from *Caldariomyces fumago* and purified as reported previously (Morris and Hager, 1966). The preparations used in this study had $R_z$ values of 1.35 to 1.4. Horseradish peroxidase was ob-

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[1] Abbreviations used: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; M.V., methyl viologen.

tained from Sigma Chemical Company and was further purified according to the method of Shannon et al. (1966). Among the several isoenzymes of horseradish peroxidase obtained by this procedure the C isozyme was primarily used. Only those preparations of the C isozyme having $R_z$ values higher than 3.4 were used. Spinach ferredoxin reductase was prepared by the procedure described by Shin (1971).

*Materials.* Phenazine methosulfate and indigotetrasulfonate were purchased from Aldrich Chemical Company, Inc. Methyl viologen and anthraquinone-1,5-disulfonate were purchased from K & K Laboratories. Nile blue was obtained from Matheson Coleman and Bell, indigodisulfonate from J. T. Baker Chemical Co., phenosafranine from Mann Research Laboratory, Inc., and brilliant arizarin blue from Hartman-Leddon Co. NADPH was purchased from Sigma Chemical Co. Indigodisulfonate and anthraquinone-1,5-disulfonate were recrystallized from alcohol before use.

*Methods.* The oxidation–reduction potential measurements of the ferric–ferrous couple of chloroperoxidase were monitored by a micro-combination electrode obtained from Metrohm (EA 234). The electrode potentials were measured with a platinum electrode against an Ag/AgCl half cell which was filled with saturated KCl and AgCl. The measured potentials were referred to the normal hydrogen electrode using the value of Clark (1960; $E_m$ = 197 mV) for the Ag/AgCl couple. The electrode was connected to a Radiometer TTTlb pH/mV meter equipped with an external meter, sensitive to 0.5 mV. The electrode was repeatedly tested against indigodisulfonate. It was also tested by measuring the known potentials of FAD and the ferricyanide/ferrocyanide couple. The oxidation–reduction titration vessel was similar to that reported by Reinhammer (1972). The titration vessel had a 10-mm light path and was fitted with both a standard taper joint for inserting the combination electrode and a rubber gasket for the injection of titrant. For potential measurements, the apparatus minus the electrode was deoxygenated by repetitive evacuation and flushing with argon or carbon monoxide. The flushing gases were prepurified by bubbling through towers containing solutions of vanadium sulfate. After evacuation and flushing, a positive gas pressure was applied and the combination electrode was inserted into the titration vessel. In order to obtain homogeneous addition, the solution was continually stirred by a small magnetic bar. Since the ferric–ferrous couple of chloroperoxidase is electromotively sluggish, all of the potentiometric titrations were carried out in the presence of an electromotively active dye as mediator. Indigodisulfonate, indigotetrasulfonate, anthraquinone-1,5-disulfonate, phenazine methosulfate, nile blue, and brilliant arizarine blue were used as mediators. The concentration of mediator was approximately 5–10% of the hemoprotein concentration on a molar basis. Sodium dithionite, the semiquinone of methyl viologen, and NADPH were used as reductants. In the NADPH titration, spinach ferredoxin reductase, in catalytic amounts, was added to the system. Sodium dithionite solutions were prepared by dissolving granular sodium dithionite in 1 mM disodium phosphate buffer, previously deaerated by repeated evacuation and flushing with prepurified argon, in the anaerobic flask designed by Burleigh et al. (1969). The semiquinone of methyl viologen was prepared by reducing methyl viologen with hydrogen using platinized asbestos or palladium oxide as catalyst in an unbuffered solution at pH 11.0. Following reduction, the methyl viologen solution was thoroughly evacuated and flushed with prepurified argon to remove excess hydrogen gas. All titrants were added from a 50-$\mu$l Hamilton gas-tight microsyringe.

The equilibrium of the reaction of ferrous chloroperoxidase with carbon monoxide was determined by the method described by Kertesz et al. (1965). In this procedure a ferric chloroperoxidase solution (5 to 5.3 $\mu$M), from which oxygen had been removed by bubbling with prepurified argon gas or by enzymatic uptake (ascorbate in the presence of ascorbate oxidase), was placed in a 1-cm optical cuvette (5.75 ml total volume) closed by a long glass stopper having a small hole in the center. The trapped air bubble in the closed cuvette was carefully removed and the ferric chloroperoxidase solutions were then reduced with sodium dithionite or reduced methyl viologen. The ferrous form was then titrated with an aliquot of a 1.47 mM carbon monoxide solution (water equilibrated with 1 atm of CO at 0 °C). The reaction mixture was stirred with a small magnetic bar. When the reaction between ferrous chloroperoxidase and CO reached equilibrium, spectra were recorded.

For the spectroscopic measurements of the chloroperoxidase–CO complex under a CO atmosphere, the anaerobic cuvette was degassed and flushed with a 9.7% CO–90.3% nitrogen gas mixture. This was followed by the anaerobic addition of a five- to tenfold excess of the semiquinone of methyl viologen. Since reduced methyl viologen has almost no absorption in the 440–450 nm range, the addition of an excess of reduced methyl viologen did not interfere with the measurement of the CO–ferrous chloroperoxidase complex at 446 nm.

The chloride, bromide, and fluoride complexes of chloroperoxidase were prepared by dialyzing solutions of the enzyme against 1 M potassium chloride, 1 M potassium bromide, and 1 M potassium fluoride, respectively. In order to avoid the formation of molecular iodine, the iodide complex was prepared by the addition of potassium iodide to a final concentration of 0.15 M. The pH of all solutions was measured directly with a glass electrode without correction.

Optical spectra were recorded on a Cary 15 or Beckman Acta III spectrophotometer. Circular dichroism spectra were measured on a JASCO 40A spectrometer. The CD spectra of ferrous chloroperoxidase and its carbon monoxide complex were measured in an anaerobic cuvette.

## Results

*Oxidation–Reduction Potentials of the Ferric–Ferrous Couple of Chloroperoxidase.* Preliminary experiments showed that mediators were required for the potentiometric titration of chloroperoxidase. Even in the presence of single additions or mixtures of mediators, the measured potentials were unsteady at low and high states of reduction, indicating that equilibrium between reductant and enzyme was difficult to reach. However, in the central portion of the titration curves, stable potentials could be recorded within a few minutes. The spectroscopic results for the anaerobic titration of ferric chloroperoxidase with the semiquinone of methyl viologen are shown in Figure 1. During the titration, the absorption maximum of ferric chloroperoxidase at 513 nm decreases and the absorption of ferrous chloroperoxidase around 550 nm increases. During the reductive titration, isosbestic points appear at 490, 530, and 590 nm.

The oxidation–reduction potential of chloroperoxidase was obtained by plotting the electrode potential against percent reduction as measured by the decrease in absorbance at 513 nm. A series of potentiometric titrations was carried out in the pH range 3.0 to 6.9. Similar results were obtained in potentiometric titrations using either dithionite or reduced methyl viologen as reductants. At all pH values, the oxidation–re-

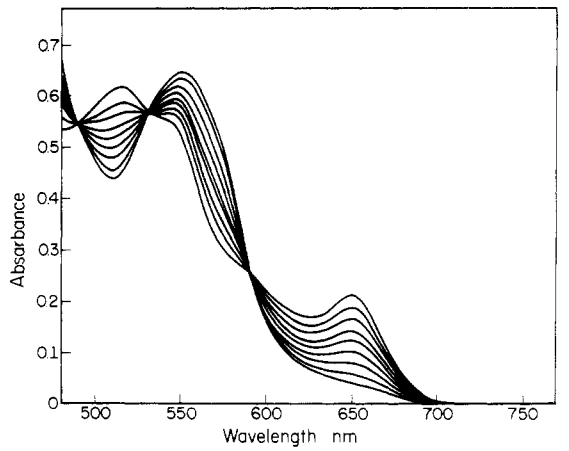


FIGURE 1: The reduction of chloroperoxidase by methyl viologen semiquinone. The anaerobic reduction of chloroperoxidase with the semiquinone of methyl viologen was carried out in 0.1 M phosphate buffer, pH 3.7. The absorption spectrum of the native ferric chloroperoxidase shows an absorption maximum at 513 nm and a minimum at 550 nm. Increasing amounts of reductant convert the enzyme to the ferrous state which shows a minimum at 513 nm and an absorption maximum at 550 nm. The concentration of chloroperoxidase was 45 $\mu$M. See Materials and Methods for other experimental details.

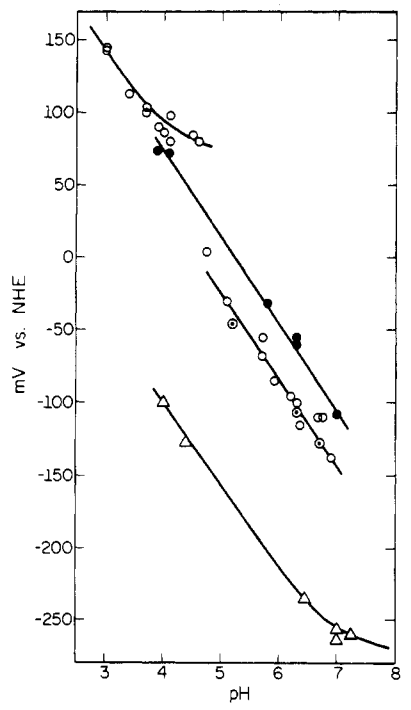


FIGURE 2: The effect of pH on the oxidation-reduction potentials of chloroperoxidase, horseradish peroxidase, and indigodisulfonate. The experimental conditions were the same as those described in Table I and in Materials and Methods. The measured potential expressed in mV relative to the normal hydrogen electrode is plotted as a function of pH for chloroperoxidase (○—○), horseradish peroxidase (△—△), and indigodisulfonate (●—●). Points labeled ⊙ indicate overlapping points for duplicate experiments with chloroperoxidase.

duction equilibrium curves showed the typical behavior for a one electron oxidation-reduction titration according to eq 1:

$$E_h = E_m + \frac{RT}{nF} \ln \frac{(Ox)}{(Red)} \quad (1)$$

where $E_h$ is the electrode potential relative to the normal hydrogen electrode, and $E_m$ is the midpoint potential. These data confirm a previous report (Chiang et al., 1975) that chloroperoxidase accepts only one electron ($n$ = 1 in eq 1) during the reductive titration of the native resting ferric form to the ferrous enzyme species.

TABLE I: Oxidation-Reduction Potential Measurements on Chloroperoxidase.[a]

| pH | Buffer[b] | Mediator[c] | Titrant | $E_m$ (mV) |
|---|---|---|---|---|
| 6.9 | Phosphate | A + B | NADPH | −138 |
| 6.75 | Phosphate | A + B | Dithionite | −110 |
| 6.7 | Phosphate | C + B | Dithionite | −110 |
| 6.7 | Phosphate | A + B | Dithionite | −127 |
| 6.7 | Phosphate | A + B | M.V. semiquinone | −127 |
| 6.35 | Phosphate | A + B | Dithionite | −115 |
| 6.3 | Phosphate | A + B | Dithionite | −100 |
| 6.3 | Phosphate | C + B | Dithionite | −105 |
| 6.3 | Phosphate | C + B | Dithionite | −105 |
| 6.2 | Phosphate | A + B | Dithionite | −95 |
| 5.85 | Phosphate | C + B | Dithionite | −85 |
| 5.7 | Phosphate | A + B + C | Dithionite | −55 |
| 5.7 | Phosphate | A + D | Dithionite | −68 |
| 5.2 | Phosphate | B + C | Dithionite | −46 |
| 5.2 | Phosphate | C + D | Dithionite | −46 |
| 5.1 | Citrate-phosphate | A + B + E | M.V. semiquinone | −30 |
| 4.75 | Acetate | A + B | Dithionite | +4 |
| 4.6 | Acetate | A + B | Dithionite | +80 |
| 4.5 | Citrate-phosphate | A + B | Dithionite | +85 |
| 4.1 | Acetate | A + B | Dithionite | +98 |
| 4.1 | Acetate | A + B | Oxygen | +80 |
| 4.0 | Citrate | A + E | M.V. semiquinone | +86 |
| 3.9 | Citrate | A + E + F | M.V. semiquinone | +90 |
| 3.7 | Phosphate | A + E | M.V. semiquinone | +100 |
| 3.7 | Phosphate | A + B | Dithionite | +100 |
| 3.4 | Phosphate | A + E + F | M.V. semiquinone | +114 |
| 3.0 | Phosphate | A + B | Dithionite | +143 |
| 3.0 | Citrate-phosphate | A + E | M.V. semiquinone | +144 |

[a] The experimental details for determining the oxidation-reduction potential of chloroperoxidase have been described in Materials and Methods. The concentration of chloroperoxidase in all experiments was in the range of 40 to 50 $\mu$M. [b] The buffers used were: 0.1 M sodium phosphate (pH 5.2 to 6.9), 50 mM sodium citrate-phosphate (pH 5.1, 4.5, and 3.0), 0.1 M sodium acetate (pH 4.75, 4.6, and 4.1), 40 mM sodium citrate (pH 4.0 and 3.9), and 0.1 M sodium phosphate (from pH 3.7 to 3.0). [c] Mediators: (A) indigodisulfonate; (B) anthraquinone-1,5-disulfonate; (C) nile blue; (D) brilliant arizarin blue; (E) phenazine methosulfate; (F) indigotetrasulfonate.

At pH values above pH 7.2, chloroperoxidase is irreversibly converted to a denatured species, which has a cytochrome $b$ type spectrum. The ferric form of the alkaline denatured species has absorption maxima at 418 and 540 nm. The denatured ferrous form of the enzyme has absorption peaks at 425, 528, and 558 nm and at 421, 540, and 568 nm for the ferrous-carbon monoxide complex (Hollenberg and Hager, 1973). Unfortunately, the denatured species was too unstable for accurate oxidation-reduction potential measurements.

The midpoint potentials ($E_m$) of chloroperoxidase, measured under a variety of conditions, are summarized in Table I. A plot of the midpoint potentials of chloroperoxidase as a function of pH is shown in Figure 2. Data obtained with horseradish peroxidase and indigodisulfonate are also shown in Figure 2.

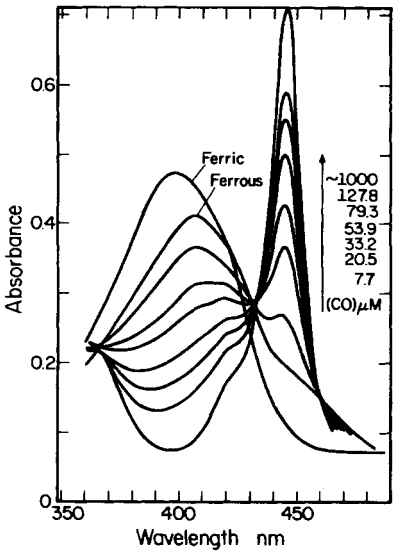


FIGURE 3: The effect of carbon monoxide concentration on the formation of the ferrous chloroperoxidase-carbon monoxide complex. The extent of the formation of the carbon monoxide-ferrous chloroperoxidase complex at 7.7, 20.5, 33.2, 53.9, 79.3, 127.8, and 1000 $\mu$M concentrations of carbon monoxide was measured by the increase in the 446-nm absorption peak. The concentration of chloroperoxidase was 5 $\mu$M. Further experimental details are given in Materials and Methods.

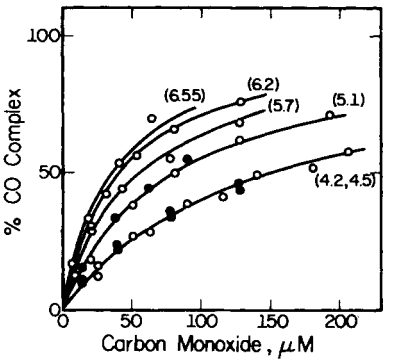


FIGURE 4: The effect of carbon monoxide concentration on complex formation. The amount of complex formed (expressed as a percent of total complex) at pH values of 4.2, 4.5, 5.1, 5.7, 6.2, and 6.55 is plotted as a function of the concentration of carbon monoxide. The open circles (O—O) show the reaction of carbon monoxide with ferrous chloroperoxidase formed by reduction of the ferric enzyme with dithionite. The closed circles (●—●) show the reaction of carbon monoxide with ferrous chloroperoxidase formed by reduction of the native enzyme with methyl viologen semiquinone. The concentration of chloroperoxidase was 5 $\mu$M. The curves are theoretical lines corresponding to dissociation constants ($K_d$) of 35, 42, 55, 80, and 150 $\mu$M for the complex.

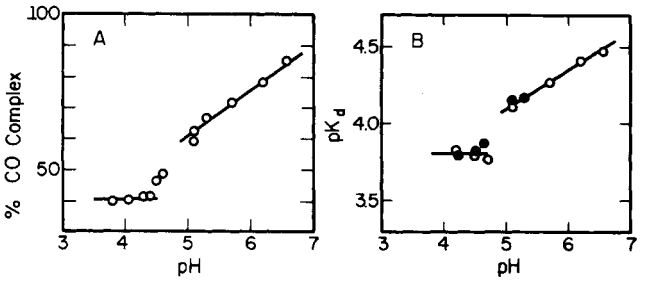


FIGURE 5: The effect of pH on the formation of the carbon monoxide-ferrous chloroperoxidase complex. In A, the amount of complex formed (expressed as a percent of total complex) is plotted as a function of pH. The concentration of ferrous chloroperoxidase was 100 $\mu$M in all cases and the reactions were carried out in a 9.7% CO-90.3% $N_2$ atmosphere. Ferrous chloroperoxidase was prepared by reduction of the native enzyme with the semiquinone of methyl viologen. The various buffers used were: 0.1 M sodium phosphate buffer, pH 5.3 to 6.7; 50 mM sodium citrate-phosphate buffer, pH 5.1; 4.0 mM sodium citrate buffer, pH 3.8 to 4.6; and 0.1 M sodium acetate buffer, pH 4.4. In B, the effect of pH on the log of the reciprocal of the dissociation constant ($pK_d = \log(1/K_d)$) of the carbon monoxide-chloroperoxidase is shown. The experimental points were calculated from data obtained in Figure 4 and similar experiments. Ferrous chloroperoxidase was prepared by reduction using either dithionite (O—O) or methyl viologen semiquinone (●—●).

The potentials measured for horseradish peroxidase and indigodisulfonate were in good agreement with those reported previously (Harbury, 1957; Preisler et al., 1959), and did not show any discontinuity in their pH profiles. However, as shown in Figure 2, the pH dependency of the midpoint potential of the ferric-ferrous chloroperoxidase couple showed a sharp discontinuity around pH 4.7. In the pH regions both above and below pH 4.7, the slope of the straight line plot, $-\Delta mV/pH$, was equal to 59 mV per pH unit. This slope is in agreement with the theoretical value (59 mV/pH at 25 °C) for a one electron transfer process. The pH titration curve in the acidic region indicates the presence of an ionizing group having an approximate p$K$ of 4.3. At pH 6.9 the oxidation-reduction potential of chloroperoxidase was approximately −138 mV. At low pH values (pH 3.0) the measured potential was +144 mV.

*Affinity for Carbon Monoxide and Oxidation-Reduction Potentials in a Carbon Monoxide Atmosphere.* The absorption spectrum of the carbon monoxide complex of ferrous chloroperoxidase is quite similar to the characteristic spectrum of the carbon monoxide complexes of P-450 type hemoproteins (Hollenberg and Hager, 1973). During the titration of ferrous chloroperoxidase with carbon monoxide, isosbestic points appear at 365, 432, and 468 nm, as shown in Figure 3. Ferrous chloroperoxidase exhibits an unusually broad absorption band in the 450 nm region (Figure 4). This broad 450-nm absorption band is independent of the procedure used to prepare the ferrous enzyme. The broad 450-nm band appears when the enzyme is chemically or enzymatically reduced. The addition of carbon monoxide to ferrous chloroperoxidase produces a strong absorption band centered at 446 nm (Figure 3). The equilibrium binding curves for the formation of the carbon monoxide-ferrous chloroperoxidase complex at various pH values are shown in Figure 4. The binding curves are consistent with a model for a simple equilibrium process having a 1:1 stoichiometry. As shown in Figures 5A and 5B, between pH 5.1 and 6.6 the affinity of ferrous chloroperoxidase for carbon monoxide shows a linear dependence on pH, while at pH values below pH 4.6 the binding is independent of pH. In the pH range around pH 4.7, the affinity is decreased by one-half over a span of a 0.5 pH unit change. The unusual result prompted us to measure the oxidation-reduction potential of chloroperoxidase in the presence of 1 mM carbon monoxide. The midpoint potential of the chloroperoxidase-CO complex would be expected to be dependent upon the affinity of chloroperoxidase for carbon monoxide at the various pH values tested and upon the concentration of carbon monoxide. These dependencies can be calculated according to the equation (eq 2) described by Clark (1960);

$$E_h = E_m + \frac{RT}{nF} \ln \frac{K_{ox}}{K_{red}} + \frac{RT}{nF} \ln \frac{K_{red} + (CO)}{K_{ox} + (CO)} \qquad (2)$$

where $K_{ox}$ and $K_{red}$ are the dissociation constants for carbon monoxide binding to ferric and ferrous chloroperoxidase, respectively. Table II summarizes the results of the potential

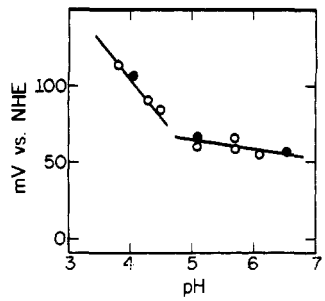


FIGURE 6: The pH-dependent potentiometric titration of the ferrous-ferric couple of chloroperoxidase in the presence of carbon monoxide. The oxidation-reduction potential (expressed as mV relative to the normal hydrogen electrode) was measured under a 1 mM CO atmosphere at pH 3.8, 4.1, 4.3 , 4.5, 5.7, 5.1, 6.55, and 6.1. Open circles (O—O) refer to enzyme reduced with dithionite, and closed circles (●—●) refer to enzyme reduced with methyl viologen semiquinone.

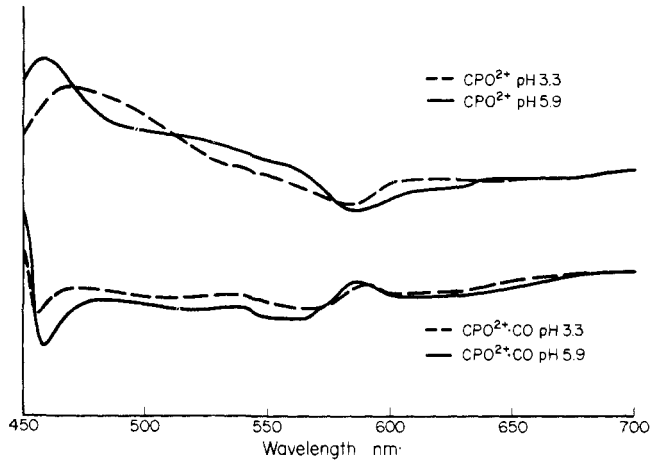


FIGURE 7: Circular dichroic spectra of ferrous chloroperoxidase and its carbon monoxide complex. The upper curves record the CD spectrum of 45 $\mu$M ferrous chloroperoxidase in 0.1 M sodium phosphate at pH 3.3 (- - -) and pH 5.9 (—). The lower two curves record the CD spectrum of the carbon monoxide-ferrous chloroperoxidase complex at pH 3.3 (- - -) and pH 5.9 (—). Ferrous chloroperoxidase was prepared by dithionite reduction of the native enzyme. The enzyme-CO complex was formed under 1 atmosphere of carbon monoxide.

measurements on the ferrous chloroperoxidase-carbon monoxide complex. The experimentally determined midpoint potentials of the carbon monoxide complex of ferrous chloroperoxidase as a function of pH are illustrated in Figure 6 where titrations were carried out in the presence of 1 mM carbon monoxide. As shown in Figure 6, at pH values above pH 5, the oxidation-reduction potentials of the ferrous-CO complex were considerably increased (100-150 mV) compared with those in the absence of CO. At pH values above pH 5, the slope of plots of $E_m$ vs. pH was approximately 10 mV. At pH values below pH 4.7, the redox potentials measured in a carbon monoxide atmosphere were approximately 15 mV greater than those measured under an argon atmosphere, and the slope of the plot of $E_m$ vs. pH was the theoretical 59 mV per pH unit.

*CD Measurements of Chloroperoxidase.* In addition to the differential pH behavior revealed by the ligand binding studies, the CD spectra of ferrous chloroperoxidase shows a significant difference at acidic and neutral pH values (Figure 7). In the 470 nm range the CD spectra of ferrous chloroperoxidase were different from those reported by Peterson (1971) for ferrous P-450$_{cam}$. Chloroperoxidase exhibits a positive CD band at this

TABLE II: Oxidation-Reduction Potential Measurements on the Carbon Monoxide-Chloroperoxidase Complex.

| pH | Buffer[b] | Mediator[c] | Titrant | $E_m$ (mV) |
|---|---|---|---|---|
| 6.55 | Phosphate | A + C | M.V. semiquinone | 56 |
| 6.1 | Phosphate | A + C | Dithionite | 53 |
| 5.7 | Phosphate | A + C | Dithionite | |
| 58 | | | | |
| 5.7 | Phosphate | A + C | Dithionite | 65 |
| 5.15 | Citrate-phosphate | A + C | M.V. semiquinone | 66 |
| 5.1 | Citrate-phosphate | A + C | Dithionite | 60 |
| 4.5 | Citrate | A + B | Dithionite | 84 |
| 4.3 | Citrate | A + B + C | Dithionite | 90 |
| 4.1 | Citrate | A + B + C | M.V. semiquinone | 106 |
| 3.8 | Citrate | A + B | Dithionite | 112 |

[a] See Materials and Methods and Table I for experimental details. [b] The buffers used were: 0.1 M sodium phosphate (pH 6.55 to 5.7), 50 mM sodium citrate-phosphate (pH 5.15 and 5.1), and 40 mM sodium citrate (from pH 4.5 to 3.8). [c] Mediators: (A) phenazine methosulfate; (B) indigodisulfonate; (C) indigotetrasulfonate.

wavelength which might be related to the unusual broad optical absorption of ferrous chloroperoxidase around 450 nm (Figure 3). In the case of the carbon monoxide complex, the CD spectrum is essentially independent of pH and very similar to that reported for P-450$_{cam}$ (Peterson, 1971).

*Oxidation-Reduction Potentials to Halide Complexes of Chloroperoxidase.* As shown in Figure 8, chloroperoxidase forms complexes with substrates such as chloride, bromide, and iodide (Thomas et al., 1970b) which have characteristic Soret absorption bands. Figure 9 plots the results of oxidation-reduction potential measurements on the chloroperoxidase-halide complexes. The potentials of the chloride and bromide complexes of chloroperoxidase were decreased 30 to 40 mV compared with those determined for the native enzyme. However, most of this difference can be accounted for in terms of a change in potential in response to changes in ionic strength. Native chloroperoxidase also showed a 30 to 40 mV decrease in oxidation-reduction potential in going from a low ionic strength environment to the high ionic strength environment required for halide complex formation. The fluoride-chloroperoxidase complex showed a small decrease in oxidation-reduction potential (~15 mV) while the iodide complex was equivalent to native enzyme. As shown in Figure 9, the oxidation-reduction process with the chloroperoxidase-halide complexes is monoelectronic regardless of the halogen anion used for formation of the complex. Judging from the spectral properties of the ferrous enzyme in the presence of chloride, bromide, iodide, and fluoride, it seems unlikely that these halides bind to the ferrous enzyme (see Figure 8).

## Discussion

In order to gain further information on the heme environment of chloroperoxidase, it was deemed important to measure the oxidation-reduction potential of the ferrous-ferric couple of chloroperoxidase under a variety of conditions. Although chloroperoxidase is similar in functional properties to plant and animal peroxidases, the optical, EPR, and Mössbauer properties of the enzyme are much more similar to those of P-450$_{cam}$ in both the ferric and ferrous states (Hollenberg and Hager, 1973; Champion et al., 1973, 1975b). These compar-

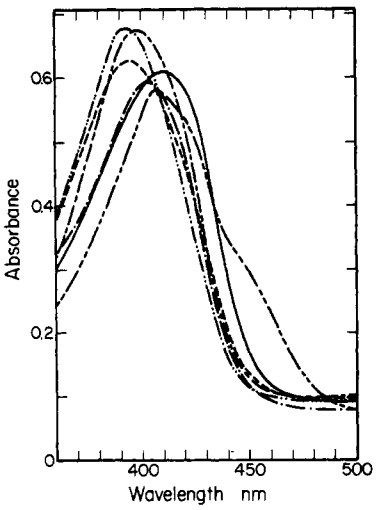


FIGURE 8: Soret absorption of the halide complexes of chloroperoxidase. The Soret absorption spectra of native ferric chloroperoxidase (— · —); the chloride complex, native enzyme in 1 M potassium chloride (—); bromide complex, native enzyme in 1 M potassium bromide (- - -); iodide complex, native enzyme in 0.15 M potassium iodide (—··—); and fluoride complex, native enzyme in 1 M potassium fluoride (—·—). The absorption spectrum of ferrous chloroperoxidase was recorded on an enzyme preparation reduced with methyl viologen semiquinone in the presence of 1 M potassium chloride (— · · —). The concentration of chloroperoxidase was 7.5 $\mu$M. All spectra were recorded at pH 3.0 in the presence of 0.1 M sodium phosphate buffer.

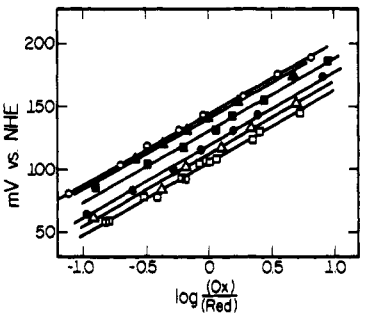


FIGURE 9: Potentiometric titration of halide complexes of chloroperoxidase. Experimental details for the titration are given in Materials and Methods. Control experiments for uncomplexed chloroperoxidase in low ionic strength buffers (○—○, 50 mM sodium citrate phosphate buffer, pH 3) and high ionic strength buffer (●—●, 0.3 M sodium citrate phosphate buffer, pH 3) are shown. The titration curves for the halide complexes of chloroperoxidase are: bromide complex (△—△), measured in 0.1 M sodium phosphate buffer, pH 3, and 1 M potassium bromide, ionic strength 1.1; iodide complex (▲—▲), measured in 0.1 M sodium phosphate buffer, pH 3, and 0.15 M potassium iodide, ionic strength 0.23; chloride complex (□—□), measured in 0.1 M sodium phosphate buffer, pH 3, and 1 M potassium chloride, ionic strength 1.1; and the fluoride complex (■—■), measured in 0.1 M sodium phosphate buffer, pH 3, and 0.1 M potassium fluoride, ionic strength 0.2. The reductant in all cases was the semiquinone of methyl viologen.

isons suggest that the heme environment in chloroperoxidase is very similar to the heme environment in the P-450 type hemoproteins. Since the oxidation-reduction potential of the iron porphyrin complex is very sensitive to the basicity of axial ligands (Falk and Perrin, 1961; Falk, 1964), it might reasonably be expected that P-450 hemoproteins and chloroperoxidase would have similar oxidation-reduction potentials. This expectation is supported by the results. The midpoint potential of chloroperoxidase at pH 6.9 was found to be −140 mV, very close to that measured for cytochrome P-450$_{cam}$ in the presence of substrate at pH 7.0 (Gunsalus et al., 1974). This result further suggests a functional similarity for P-450$_{cam}$ and chloroperoxidase, at least in the neutral pH range.

A more striking feature of the results reported here is the effect of pH on the oxidation-reduction potential of chloroperoxidase and the effect of pH on the reaction of ferrous chloroperoxidase with carbon monoxide. Chloroperoxidase shows a dramatic change in oxidation-reduction potential around pH 4.5. To our knowledge, no other protein shows such an abrupt change in its oxidation-reduction potential vs. pH profile. The discontinuity in the chloroperoxidase potential measurements raises the question of whether the ferric, ferrous, or possibly both forms of the enzyme are drastically modified by changes in hydrogen ion concentration. Some information suggests that hydrogen ion concentration does not exert a major effect on the ferric form of the enzyme. For example, the affinity of ferric chloroperoxidase for cyanide and azide does not exhibit a discontinuous pH profile and the CD spectra of ferric chloroperoxidase are almost pH independent. However, it should also be noted that the native ferric enzyme exhibits halogenating activity only at acidic pH values while it exhibits classical peroxidase activity over a broad pH range (Thomas et al., 1970b). On the other hand, the data on carbon monoxide binding to ferrous chloroperoxidase suggest an abrupt change in the reduced enzyme at pH values in the range pH 4.5 to 4.7. From pH 7 to 4.7 the binding of carbon monoxide to ferrous chloroperoxidase is characterized by a lowered affinity as the hydrogen ion concentration is increased. Below pH 4.7, the binding becomes pH independent. The dissociation constants for the chloroperoxidase-carbon monoxide complex estimated from the equilibrium studies reported in this paper are in good agreement with kinetic data obtained from stopped-flow experiments (unpublished observations). We interpret the carbon monoxide binding results in terms of the reversible equilibration of two pH-dependent forms of ferrous chloroperoxidase. This interpretation is also supported by the oxidation-reduction potential measurements carried out in the presence of carbon monoxide. As predicted from eq 2, the presence of carbon monoxide increased the midpoint chloroperoxidase potentials in the pH range above 4.7. The carbon monoxide adducts of microsomal cytochrome P-450 (Guengerich et al., 1975) and cytochrome oxidase (Lindsay, 1974) show a similar behavior. The carbon monoxide-chloroperoxidase complex shows an abnormal character with respect to the change in potential per pH unit. The slope of the straight line plot, $-\Delta mV/pH$, is approximately 10 mV per pH unit. The theoretical slope, assuming $K_{ox}$ in eq 2 to be constant, would be approximately 35 mV per pH unit. Below pH 4.7, the potentials measured in the presence of CO were only 15 mV greater than those on native chloroperoxidase and the slope, $-\Delta mV/pH$, was 60. This observation further supports the conclusion that carbon monoxide binding is independent of pH below 4.7. These results are qualitatively consistent with the results of the CO binding studies done under equilibrium conditions.

At pH values from 4.7 to 7, the slope of a plot of $E_m$ values for chloroperoxidase vs. pH is about 59 mV per pH unit, indicating the involvement of one proton in the oxidation-reduction process and possibly indicating the presence of an ionization group in the ferrous form of the enzyme having a p$K$ above 7. Unfortunately, it was not possible to carry out titrations in this pH range because denaturation becomes an important process above pH 7. However, an ionizable group was detected in the pH region below pH 4.7 in the ferrous form of the enzyme. This ionizable group had an apparent p$K$ of

approximately 4.3. Such an ionization function might reasonably be assigned to a carboxylic acid group or possibly to an abnormal imidazole group of a histidine residue. Presumably this ionizable group could be situated as a fifth or sixth axial ligand of the heme-iron. If the unusual P-450 character of the chloroperoxidase-carbon monoxide complex is characterized by a specific heme iron ligand, it seems likely that the specific P-450 ligand remains bound through the entire pH titration range because the optical absorption properties of the carbon monoxide complex were independent of pH. A plausible model to explain the discontinuity in the oxidation-reduction potential of chloroperoxidase would be the pH-dependent exchange of one of the axial ligands of the heme prosthetic group. If this is indeed true, then the above arguments indicate that the ligand exchange should not involve the ligand which specifies the P-450 character of the enzyme. The net result of the ligand exchange hypothesis would be that carbon monoxide competes with two different heme ligands for binding to the heme iron, depending on the pH of the reaction. The chloroperoxidase-halide complexes are of particular interest because of their suggested role as intermediates in the halogenation reaction. On the basis of Mössbauer (Champion et al., 1973) and broad line NMR spectroscopy (Krejcarek et al., 1976), it has been suggested that chloride ion may not directly coordinate to the heme iron as a ligand. On the other hand the chloroperoxidase-iodide and -fluoride complexes exhibit a high spin ferric Mössbauer spectra over the entire temperature range studied. The high-spin character of these complexes would be consistent with fluoride and iodide coordinating as axial ligands to the heme-iron. However, these results do not demand that fluoride and iodide coordinate as axial ligands. As shown previously (Thomas et al., 1970b) the addition of halide ions to chloroperoxidase results in a shift of the Soret band characteristic for each halogen anion. If halide anions were directly binding to ferric chloroperoxidase as axial ligands, the oxidation-reduction potential of the halide complexes should be decreased (as predicted by the Nernst equation). Although the oxidation-reduction potentials of chloride and bromide complexes were significantly decreased by approximately 30 to 40 mV, the results of the experiments on the effect of ionic strength on oxidation-reduction potentials suggest that chloride and bromide ions decrease the potential via an ionic strength mechanism. When suitable corrections are made for ionic strength effects, the binding of chloride and bromide to ferric chloroperoxidase does not appear to affect the oxidation-reduction potential. The addition of fluoride causes a small decrease in the oxidation-reduction potential (~15 mV). This small change in the potential might be attributable to the affinity of fluoride for the heme iron. On the other hand, the binding of iodide does not cause significant differences in oxidation-reduction potentials. These results in sum total suggest that the substrate halides, such as chloride, bromide, and iodide, probably do not directly interact with the ferric heme-iron as axial ligands.

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References

Burleigh, B. D., Faust, G. P., and Williams, C. H. (1969), *Anal. Biochem. 27,* 536.

Champion, P. M., Chiang, R., Münck, E., Debrunner, P. G., and Hager, L. P. (1975), *Biochemistry 14,* 4159.

Champion, P. M., Münck, E., Debrunner, P. G., Hollenberg, P. F., and Hager, L. P. (1973), *Biochemistry 12,* 426.

Chiang, R., Makino, R., Spomer, W. E., and Hager, L. P. (1975), *Biochemistry 14,* 4166.

Clark, W. M. (1960), in Oxidation-Reduction Potentials of Organic Systems, Baltimore, Md., The Williams and Wilkins Company.

Falk, J. E. (1964), in Porphyrin and Metalloporphyrins, New York, N.Y., Elsevier.

Falk, J. E., and Perrin, D. D. (1961), in Haematin Enzymes, Falk, J. E., Lemberg, L., and Morton, R. K., Ed., Elmsford, N.Y., Pergamon Press.

Guengerich, F. P., Ballou, D. P. and Coon, M. J. (1975), *J. Biol. Chem. 250,* 7405.

Gunsalus, I. C., Meeks, J. R., Lipscomb, J. D., Debrunner, P. G., and Münck, E. (1974), in Molecular Mechanisms of Oxygen Activation, Hayaishi, O., Ed., New York, N.Y., Academic Press.

Harbury, H. A. (1957), *J. Biol. Chem. 225,* 1009.

Hollenberg, P. F., and Hager, L. P. (1973), *J. Biol. Chem. 248,* 2630.

Kertesz, D., Antonini, E., Brunori, M., Wyman, J., and Zito, R. (1965), *Biochemistry 4,* 2672.

Krejcarek, G. E., Bruant, R. G., Smith, R. J., and Hager, L. P. (1976), *Biochemistry* (accepted for publication).

Lindsay, J. G. (1974), *Arch. Biochem. Biophys. 163,* 705.

Morris, D. R., and Hager, L. P. (1966), *J. Biol. Chem. 241,* 1763.

Peterson, J. A. (1971), *Arch. Biochem. Biophys. 144,* 678.

Preisler, P. W., Hill, E. S., Loeffel, R. G., and Shaffer, P. A. (1959), *J. Am. Chem. Soc. 81,* 1991.

Reinhammer, B. R. M. (1972), *Biochim. Biophys. Acta 275,* 245.

Ricard, J., Mazza, G., and Williams, R. J. P. (1972), *Eur. J. Biochem. 28,* 566.

Shannon, L. M., Kau, E., and Lew, J. Y. (1966), *J. Biol. Chem. 241,* 2166.

Shin, M. (1971), *Methods Enzymol. 23,* 440.

Thomas, J. A., Morris, D. R., and Hager, L. P. (1970a), *J. Biol. Chem. 245,* 3129.

Thomas, J. A., Morris, D. R., and Hager, L. P. (1970b), *J. Biol. Chem. 245,* 3135.

Yamada, H., Makino, R., and Yamazaki, I. (1975), *Arch. Biochem. Biophys. 169,* 344.