The bovine spleen green heme protein, a peroxidase which exhibits spectrophotometric properties similar to those of granulocyte myeloperoxidase, was purified using an improved method. The ligand affinity of the ferric enzyme was spectroscopically determined using chloride and cyanide as exogenous ligands. The pH dependence of the apparent dissociation constant of the enzyme-chloride complex showed the presence of a proton dissociable group with a pKₐ value of 4 on the enzyme; chloride binds to the enzyme when this group is protonated with a dissociation constant of 60 μM. The cyanide affinity of the enzyme is also regulated by the group with a pKₐ value of 4, but in this case cyanide binds to the unprotonated enzyme with a dissociation constant of 0.6 μM; only the protonated, uncharged form of cyanide reacts with the enzyme. Cyanide binding is competitively inhibited by chloride, and chloride binding is also competitively inhibited by cyanide. The EPR spectrum of the resting enzyme exhibited a rhombic high spin signal with g-values of 2.25, 2.32, and 1.97 with a low spin signal at g = 2.55, 2.32, and 1.82. Upon formation of the chloride complex, the spectrum was replaced with a new high spin EPR signal with g-values of 6.81, 5.04, and 1.95. The cyanide complex showed a low spin EPR signal with g-values of 2.83, 2.25, and 1.66. Examination of the enzymatic activity of the spleen green heme protein by following the chlorination of monochlorodimedon has indicated that the enzyme has the same chlorinating activity as myeloperoxidase; the spleen green peroxidase can catalyze the formation of hypochlorous acid from hydrogen peroxide and chloride ion.

Comparison of the present data with those of myeloperoxidase has led to the conclusion that the structure of the iron center and its vicinity in spleen green heme protein is very similar, if not identical, to that of myeloperoxidase. The spleen enzyme can thus be used as a model to study the active center, and its environment, in myeloperoxidase.

Recently, there have been several reports of unusual green hemeproteins from various sources (Jacob and Orme-Johnson, 1979; DeFilippi and Hultquist, 1978; Davis and Averill, 1981). One is a peroxidase found in bovine spleen (Davis and Averill, 1981) which has optical spectral properties very similar to those of myeloperoxidase (EC 1.11.1.7) of polymorphonuclear leukocytes. The resonance Raman (Babcock et al., 1985) and magnetic circular dichroism (Ikeda-Saito et al., 1985b) results indicate that the structure of the chromophore of the spleen peroxidase is identical to that of myeloperoxidase, probably an iron chlorin (Sibbett and Hurst, 1984; Ikeda-Saito et al., 1985a; Babcock, et al., 1985). However, the two enzymes are clearly distinct in their size; the spleen green hemeprotein consists of a single polypeptide chain (M, 57,000) with one prosthetic group per enzyme molecule (Davis and Averill, 1981), while myeloperoxidase is tetrameric (M, 140,000), consisting of two heavy chains (M, 55,000 with a single prosthetic group per chain) and two light chains (M, 15,000) (Andrews and Krinsky, 1981).

One of the important properties of myeloperoxidase is its ability to catalyze the formation of hypochlorous acid from hydrogen peroxide and chloride ion (Harrison and Schultz, 1976). In the catalytic cycle, myeloperoxidase first reacts with hydrogen peroxide to form compound I which has two oxidizing equivalents more than the resting enzyme. Compound I is converted to the resting ferric enzyme in a two-electron reduction by which chloride ion (the electron donor) is oxidized into a chloronium ion, and hypochlorous acid is produced (Harrison and Schultz, 1976). With its ability to cause rapid degradation of various biological compounds, hypochlorous acid is considered to be the pertinent bacteriocidal agent. Like other peroxidases, myeloperoxidase also forms compounds II and III upon reaction with excess amount of hydrogen peroxide (Odajima and Yamazaki, 1970; Harrison et al., 1980). As one of the essential components of the antimicrobial systems of polymorphonuclear neutrophils (Klebanoff, 1975), myeloperoxidase is an important protein. But studies of purified myeloperoxidase have remained limited to a small number of laboratories due to the difficulties of preparing the enzyme. With spectrophotometric properties similar to those of myeloperoxidase, the spleen green hemeprotein has the potential to serve as a model of myeloperoxidase. Before this can be addressed, however, a detailed comparison of the spleen green hemeprotein with myeloperoxidase is required. Since the substrate binding and redox reactions are processes which involve the heme moiety, the properties

* This investigation was supported by Research Grant AI-20463 from the National Institute of Allergy and Infectious Diseases. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Spleen Green Hemeprotein

11689

associated with the heme, such as exogenous ligand binding and EPR spectra, will be excellent probes for judging to what extent the active centers of these two enzymes are similar. It is also of interest to test whether the spleen peroxidase is capable of catalyzing the peroxidation of chloride. To this end, I have purified the spleen green hemeprotein and compared its ligand binding, enzymatic, and EPR spectral properties with those of myeloperoxidase. Since chloride and cyanide have been used to study the ligand binding properties of myeloperoxidase, as high spin and low spin ligands, respectively (Stelmaszynska and Zgliczynski, 1974; Harrison, 1976; Bakkenist et al., 1980; Bolscher and Wever, 1984a), they were also used here as exogenous ligands. Enzymatic properties were studied by steady-state kinetic measurements of the formation of hypochlorous acid, as followed by the chlorination of monochlorodimedon, again as reported for myeloperoxidase (Bakkenist et al., 1980). Here, I report that the spleen green hemeprotein has ligand binding, enzymatic properties, and EPR spectra, very similar to those of myeloperoxidase.

EXPERIMENTAL PROCEDURES

The green hemeprotein was extracted from bovine spleens obtained from the local abattoir and partially purified by batch absorption to a Sephacryl column according to the method of Davis and Averill (1981). The partially purified enzyme was then diluted to 0.1 M KCl, pH 5.5, and loaded to a column of CM-Sepharose CL-6B previously equilibrated with 0.1 M phosphate buffer, pH 6.0. The column was eluted with a linear gradient of 0.1 M phosphate buffer, pH 6 (starting buffer), and 0.6 M phosphate buffer, pH 7 (limiting buffer). The green colored fractions with $A_{400}/A_{280}$ values larger than 0.4 were pooled, and concentrated by ultrafiltration (Amicon Centrifo CF-25). The enzyme fraction was gel-filtered on a column of Sephacryl S-200, equilibrated with 0.2 M phosphate buffer, pH 7.0. Fractions with $A_{400}/A_{280}$ values larger than 0.83 were pooled and concentrated as above. The enzyme fraction was pure as judged by sodium dodecyl sulfate-polyacrylamide electrophoresis, and the rechromatography on an ion exchange column, which was used in the original procedure to remove purple acid phosphatase contamination (Davis and Averill, 1981), was unnecessary. The $A_{400}/A_{280}$ value of the preparations used in this study was 0.84. Myeloperoxidase was prepared from out-dated leukopheretic preparations (supplied from the Penn-Jersey Blood Program of the American Red Cross, Philadelphia, PA) as described previously (Ikeda-Saito and Prince, 1985).

Optical spectra were recorded with a HITACHI 557 spectrophotometer, which was interfaced to an IBM Personal Computer for storing optical data. All the measurements were performed at 20 °C unless specified. Ligand binding measurements were performed by addition of a known amount of ligand solutions (sodium chloride or potassium cyanide) into the enzyme solution followed by optical measurements at the Soret peak. Fractional saturation was calculated from the absorbance difference induced by ligand binding. Ligand affinity was expressed in terms of dissociation constant. All the ligand equilibrium data in the present study followed $n = 1$ titration curve, and the equilibrium constants, $K_{app}$, were obtained by analyzing the experimental data using the equation,

$$Y = \frac{K_{app}[X]}{1 + [X]}$$

where $Y$ and $[X]$ are fractional saturation and the ligand concentration, respectively, by least squares fitting. All the calculations were done on an IBM Personal Computer.

EPR spectra were recorded by a Varian E-109 spectrometer operating at 9.326 GHz with 100-kHz field modulation. Measurements were carried out at 10 K by using an Air-Products liquid helium flow cryostat. Microwave frequency was measured with an EIP model 545 frequency counter, and magnetic field was calibrated with various standards of known g-values.

The rate of hypochlorous acid formation was measured by following the chlorination of monochlorodimedon, using the technique described by Hager et al. (1966) for chloroperoxidase, that has been used for myeloperoxidase (Bakkenist et al., 1980). The concentration of hydrogen peroxide was determined by the methods of Cotton and Dunford (1973). Monochlorodimedon was purchased from Sigma. Chlorination of monochlorodimedon was spectrophotometrically monitored at 290 nm at 20 °C. The experimental conditions were chosen to be close to those used for myeloperoxidase by Bakkenist et al. (1980) so that the results on the spleen green hemeprotein can be easily compared with those of myeloperoxidase.

Buffers used were 0.2 M citrate-phosphate, pH 3–6, 0.2 M potassium phosphate, pH 6–8.

RESULTS

Fig. 1 illustrates the optical spectra of the spleen green hemeprotein and its chloride and cyanide complexes. The resting state exhibits major peaks at 624, 569, and 428 (Soret) nm with small peaks around 686 and 500 nm. These did not change between pH 3 and 8. Addition of chloride slightly changes the spectrum; the chloride complex shows the Soret peak at 434 nm and major peaks in the visible region at 623 and 574 nm with small peaks at 500 and 687 nm. Upon binding of cyanide, a new spectrum appears with peaks at 633 and 454 (Soret) nm. These spectral shapes, and the position of the peaks of the resting and chloride and cyanide complexes, agree well with those reported for myeloperoxidase (Ager, 1941; Wever and Plat, 1981; Bolscher and Wever, 1984a). Although the features of the optical spectra of ferric cyanide, ferrous, ferrous cyanide, and ferrous carbon monoxide forms of the present preparation of the spleen green hemeprotein agree very well with those reported by Davis and Averill (1981), there is a discrepancy in the peak positions of the resting ferric enzyme. The Soret and major peaks in the visible region of their resting enzyme were reported as 434 and 574 nm, which correspond to the peaks of the chloride complex in the present study.

The chloride affinity of the spleen green hemeprotein was determined between pH 3 and 9 at 20 °C. An isosbestic point was observed at 432 nm during the titration of the enzyme with chloride, and the chloride equilibrium curves were expressed by $n = 1$ titration curves. These indicate that there is only one binding process. Fig. 2 (data set A) plots the apparent dissociation constant for the chloride complex between pH 3 and 7 at 20 °C. Between pH 7 and 5, the slope of the pH dependence of the dissociation constant was unity, showing an involvement of a proton in the reaction of the enzyme with chloride. Such a pH dependence can be expressed by assuming that either a protonated form of the enzyme or the protonated form of the ligand undergoes the reaction. Since the pK value of HCl does not fall in the pH range of interest, it can be concluded that the pK must be on the enzyme and that only the protonated form of the enzyme binds chloride. The follow-

![Fig. 1. Light absorption spectra of the spleen green hemeprotein and its chloride and cyanide complexes in 0.1 M citrate-phosphate buffer, pH 4.0, at 20 °C. Enzyme concentration was 9.35 μM.](image)
The pH dependences of the apparent dissociation constant of the chloride complex (data set A, ■■■■), and that of the cyanide complex (data set B, ●●●●) at 20 °C. Symbols are experimental data points, and the line for data set A was drawn by Equation 1 with pKₐ of 3.96 and Kᶜ of 60 μM, and that for data set B was drawn using Equation 2 with pKₐ of 3.9 and Kᶜ of 0.6 μM.

The cyanide affinity of the spleen green hemeprotein was studied between pH 5 and 8.3 (Fig. 2, data set B). An isosbestic point was observed at 442 nm during the titration of the enzyme with cyanide. Above pH 8.5, the cyanide complex of the enzyme was unstable and exhibited a time-dependent change in its absorption spectrum. This change seemed to correspond to the decomposition of the cyanide complex to the resting enzyme, and it was not feasible to determine the dissociation constants above pH 8.5. The equilibrium curves were described as n = 1 titration curves as was the case for chloride binding. In contrast to the chloride complex formation, cyanide affinity is essentially independent of pH between pH 5 and 8.3, but declines below pH 5. The pH independence of the apparent cyanide affinity on pH between pH 5 and 8.3 is indicative of binding of the protonated form of cyanide (HCN) to the enzyme, since the pKₐ of HCN is known to be around 9.3 at 20 °C. The decrease in the apparent affinity below pH 5 indicates that cyanide binds only to the unprotonated form of the enzyme. The following scheme can express the enzyme cyanide reaction.

\[
\begin{align*}
E + H^+ & \underset{K_a}{\overset{K_{CN}}{\rightleftharpoons}} E\cdot H^+ \\
E\cdot H^+ & \underset{K_{CN}}{\overset{K_a}{\rightleftharpoons}} E\cdot HCN
\end{align*}
\]

**Scheme B**

where \( K_a \) is a dissociation constant for the enzyme-cyanide complex, and other symbols hold the same meaning as in Scheme A. Then, the apparent dissociation constant, \( K_{app} \), is expressed as

\[
K_{app} = K_a(1 + [H^+] / K_a)
\]

(2)

where \( K_a \) is an ionization constant of proton dissociable group of the enzyme and \( K_{CN} \) is a dissociation constant for the enzyme cyanide complex. The pKₐ value of 3.95 and \( K_{CN} \) of 0.6 μM fit the experimental data, as seen in Fig. 2 (data set B). Although the enzyme exhibits the opposite pH effect on the cyanide and chloride affinities, it seems likely that the same ionizable group with a pK value of 4 may influence both the chloride and cyanide ligand affinities.

An addition of a large excess of chloride to the cyanide complex of the enzyme in the low pH region changes the absorption spectrum of the cyanide form to the chloride form, as reported for myeloperoxidase (Zgliczynski and Stelmazska, 1979). This indicates that the enzyme-bound cyanide is released from the enzyme when chloride binds to the enzyme. The effect of cyanide concentration on the chloride affinity of the enzyme was determined at pH 4.6 (Fig. 3). The equilibrium curves were expressed by \( n = 1 \) Hill plots, and the optical absorption spectra during the chloride titration gave clear isosbestic points. These indicate that cyanide, chloride, and the enzyme are in a rapid equilibrium. Since equilibrium data show that cyanide and chloride bind only to the unprotonated and protonated form of the enzyme, respectively, chloride equilibrium in the presence of cyanide and cyanide equilibrium in the presence of chloride can be expressed by combination of the Schemes A and B (Scheme C).

\[
E\cdot HCN \underset{K_{CN}}{\overset{K_a}{\rightleftharpoons}} E + H^+ \underset{K_a}{\overset{K_{CN}}{\rightleftharpoons}} E\cdot H^+ \overset{K_{CN}}{\underset{K_a}{\rightleftharpoons}} E\cdot H^+ \overset{K_{CN}}{\underset{K_a}{\rightleftharpoons}} E\cdot HCN
\]

**Scheme C**

Then, the apparent chloride equilibrium constant is given as

\[
K_{app} = K_{Cl} \left( 1 + \frac{K_a}{[H^+]} \right) \left( 1 + \frac{[HCN]}{K_{CN}} \right)
\]

(3)

where symbols hold the same meaning as in Equations 1 and 2. The curve in Fig. 3 was drawn by using the values of \( pK_a = 3.9, K_{CN} = 0.6 \) μM, and \( K_{Cl} = 60 \) μM, as independently determined in chloride and cyanide titrations described above.

**Fig. 3. Cyanide concentration dependence of the apparent dissociation constant of enzyme-chloride complex at pH 4.6, 20 °C. The symbols are experimental data points, and the curve was drawn by using Equation 3 with \( pK_a = 3.95, K_{Cl} = 60 \) μM, and \( K_{CN} = 0.6 \) μM.**
The dissociation constant of the enzyme-cyanide complex at pH 4.6 and 7.1, 20 °C. The symbols are experimental data points, and the curves were drawn using Equation 4 with the $pK_a = 3.95$, $K_1 = 60 \mu M$, and $K_{CN^-} = 0.6 \mu M$.

The set of equilibrium constants fits the experimental data well. Cyanide competitively inhibits the binding of chloride to the enzyme.

I have also measured the chloride concentration dependence of the apparent cyanide equilibrium constants at pH 4.6 and 7.1 (Fig. 4). The Hill plots for cyanide equilibria also showed slopes values of unity, and clear isosbestic points were observed during the cyanide titration. Both at pH 4.6 and 7.1, an increase in chloride concentration decreases the cyanide affinity of the enzyme. From Scheme C the apparent cyanide dissociation constant is written as

$$K_{app} = K_C \left(1 + \frac{[H^+]}{K_a} \left(1 + \frac{[Cl^-]}{K_{Cl^-}}\right)\right)$$  \hspace{1cm} (4)

Fig. 4 also plots the curves calculated by using the same equilibrium constants as used above. Again, as is the case of the effect of cyanide on the chloride affinity, the equilibrium constants derived by the independent cyanide and chloride equilibria and Equation 4 can adequately explain the effect of chloride on the cyanide affinity of the enzyme. Cyanide competitively inhibits cyanide binding to the enzyme.

I have also studied the effect of cyanide and chloride on the EPR spectral properties of the enzyme. The *spectrum A* in Fig. 5 is an EPR spectrum of the resting enzyme (enzyme concentration was 150 $\mu M$ in 0.1 M phosphate buffer, pH 8). This spectrum shows that the enzyme is in a mixture of high spin ($g = 6.65, 5.28, \text{and } 1.97$) and low spin ($g = 2.55, 2.32, \text{and } 1.81$) states at 10 K. Addition of 0.17 $\mu M$ chloride to the resting enzyme increased the rhombicity in the high spin EPR signal ($g = 6.81, 5.04, \text{and } 1.95$), and the low spin signal observed in the resting state is diminished, as seen in *spectrum B*. Addition of 130 $\mu M$ KCN to this chloride complex reduces the intensity of the high spin signal and a new low spin signal appears at $g_1 = 2.83, g_2 = 2.25, \text{and } g_3 = 1.66$ (*spectrum C*). Further addition of cyanide to a concentration of 1 $mM$ eventually converts all the high spin EPR signal to the low spin one. Addition of cyanide to the resting enzyme also gave the low spin EPR spectrum identical to *spectrum D*. In our previous report on myeloperoxidase (Ikeda-Saito and Prince, 1985), where the EPR measurements were carried out in 0.1 M citrate-phosphate buffer, pH 4.0, the rhombicity of the high spin EPR signal was reduced upon chloride binding. Thus, I have also measured the EPR spectra of the green hemeprotein in 0.1 M citrate-phosphate buffer, pH 4, as well as those of myeloperoxidase of human granulocytes in 0.1 M phosphate buffer, pH 8.0. The general features of the EPR spectrum of the spleen green hemeprotein at pH 4 are the same as those at pH 8: a rhombic high spin signal and a low spin signal at $g = 2.55, 2.32, \text{and } 1.81$, and the low spin signal is absent in the chloride complex. This was also the case for myeloperoxidase at pH 8. In Table I, the low field $g$-values of the high spin EPR signal are listed together with those of myeloperoxidase.

Both enzymes exhibit a change in their high spin EPR signal in the same manner upon chloride binding: a decrease in rhombicity at pH 4 and an increase in rhombicity at pH 7. Davis and Averill (1981) also reported the EPR spectrum of the spleen green hemeprotein. However, there is a discrepancy in the EPR spectrum of the resting enzyme. Their spectrum exhibited high spin EPR signal at $g = 6.81, 4.99, \text{and } 1.85$ without low spin signal. Comparison of their spectrum with those studied here reveals that their spectrum resembles that of the chloride complex rather than that of the resting state. Such a discrepancy is also seen in their optical spectra, as described above.

The optical absorption spectra of compounds I, II, and III of myeloperoxidase have been reported (Odajima and Yamazaki, 1970; Harrison et al., 1980, Bolscher and Wever, 1984a). Compound I of myeloperoxidase spontaneously decays to compound II with a half-time of about 0.1 s (Harrison et al., 1980), and it is not feasible to record its optical spectrum with
a conventional spectrophotometer. Compounds II and III, however, are known to be stable enough to record their spectra. Great similarities in the spectroscopic properties of ferrous and ferric derivatives of the spleen green hemeprotein and those of myeloperoxidase have prompted me to compare the absorption spectra of compounds II and III of the spleen enzyme with those of myeloperoxidase. Fig. 6 shows the light absorption spectra of the spleen green hemeprotein (8 μM) in the absence (spectrum A) and presence (spectrum B) of a 50-fold excess and a 150-fold excess (spectrum C) of hydrogen peroxide at pH 7.0, 5°C. These ratios are the same as those used for formation of compounds II and III of myeloperoxidase (Bolscher and Wever, 1984a; Odajima and Yamazaki, 1970). Spectrum B shows absorption bands at 626 and 454 (Soret) nm, and spectrum C has absorption bands at 624, 570, and 449 (Soret) nm. As expected from the similarities in the optical properties between these two enzymes, spectra B and C are quite similar to those of compounds II and III of myeloperoxidase, respectively. Spectrum B was instantaneously changed back to spectrum A by reaction with stoichiometric amount of ascorbic acid, as reported for compound II of myeloperoxidase (Bolscher et al., 1984b). When the spleen enzyme (8 μM) was titrated with hydrogen peroxide to 0.5 mM, spectrum A was converted to spectrum B with a set of isosbestic points at 666, 586, 497, and 442 nm. Further titration with hydrogen peroxide to 2 mM converted spectrum B to spectrum C, with a set of isosbestic points at 636, 604, 574, 514, and 446 nm. Spectrum C was also formed by oxygenation of the reduced enzyme. Such features are analogous to the observations reported by Odajima and Yamazaki (1970) for the formation of compounds II and III of myeloperoxidase. Addition of 8 μM ferrocyanide to the sample of spectrum B slowly converted the spectrum to that of the resting enzyme (spectrum A). When 16 μM ferrous cytochrome c was added to the sample of spectrum B, the absorbance at 550 nm decreased slowly due to the oxidation of cytochrome c, and spectrum B was converted to spectrum A. The absorbance change at 550 nm after correcting for the absorbance difference between spectra A and B corresponded to the oxidation of about 8 μM cytochrome c, indicating that 1 mol of ferrous cytochrome c was oxidized, and 1 mol of the enzyme was reduced back to the resting state. The stable compound of spectrum B thus has one oxidizing equivalent more than the resting enzyme. The light absorption spectra B and C of Fig. 6 are thus assigned to those of compounds II and III of the spleen green hemeprotein.

Monochlorodimedon has been used to study the chlorinating activity of myeloperoxidase (Harrison and Schultz, 1976; Bakkenist et al., 1980), and Fig. 7 examines this reaction for the spleen green hemeprotein. Incubation of the spleen enzyme with hydrogen peroxide, chloride, and monochlorodimedon results in a decrease in absorbance at 290 nm, due to the formation of dichlorodimedon (Hager et al., 1966) as observed for myeloperoxidase (Bakkenist et al., 1980). Fig. 7A shows the initial rate of this reaction at pH 4.5 with 0.1 mM chloride at different hydrogen peroxide concentrations. The rate depends on the sequence of adding substrates and enzyme to the cuvette. If the reaction was started by the addition of chloride to a cuvette that had contained hydrogen peroxide and the enzyme for 30 s (solid circles of Fig. 7A), the rate was slower than that obtained by starting the reaction by either...
FIG. 7. A, chlorination of monochlorodimedon catalyzed by the spleen green hemeprotein. Measurements were carried out in 0.2 M citrate-phosphate buffer (2 ml), pH 4.5, in the presence of 0.1 M chloride at 20 °C. Open circles are data points for the reactions initiated by the enzyme or hydrogen peroxide, and closed circles are by chloride. The enzyme concentration was 23 nM. B, Lineweaver-Burk plot for the chlorination of monochlorodimedon at various chloride concentrations. Experimental conditions are the same as those for Fig. 7A. The reactions were started by the addition of the enzyme. NaCl concentration was 0.2 M (O—O), 0.1 M (■—■); 0.05 M (▲—▲), and 0.025 M (▼—▼).

hydrogen peroxide or the enzyme (open circles of Fig. 7A). This indicates that hydrogen peroxide in the absence of chloride inactivates the enzyme as reported for myeloperoxidase (Bakkenist et al., 1980; Harrison, 1976). Reaction of the enzyme with excess hydrogen peroxide in the absence of chloride rapidly converts the enzyme into compound II. Compound II of myeloperoxidase does not function in hypochlorous acid formation (Harrison and Schultz, 1976), and this also seems to be the case for the spleen green peroxidase. The rate of the chlorination reaction was proportional to the amount of enzyme present and independent of monochlorodimedon concentration from 5 to 50 μM. Fig. 7B illustrates the Lineweaver-Burk plots of the chlorination reaction at various NaCl concentrations at pH 4.5. The reactions were

2 In the plots, "absorbance change at 290 nm/min" was used as the rate of the reaction, so that the reaction rates can be directly compared with those reported by Bakkenist et al., (1980) who used the same y axis unit.
started by addition of enzyme into a cuvette containing monochlorodimedonation, hydrogen peroxide, and chloride. As is the case for myeloperoxidase, it is evident that chloride is not only a substrate for the spleen green peroxidase, but also behaves as a competitive inhibitor with respect to hydrogen peroxide. The chlorination rates are about 80% of those catalyzed by myeloperoxidase. The effects of hydrogen peroxide and chloride concentrations on the chlorination rates for the spleen green hemeprotein are analogous to those for myeloperoxidase (Bakkenist et al., 1980), and Fig. 7B and the Lineweaver-Burk plots reported for myeloperoxidase (Fig. 6 of Bakkenist et al., 1980) are similar. The procedure used by Bakkenist et al. (1980) yielded an estimated Michaelis constant for hydrogen peroxide of approximately 0.05 ± 0.02 mM at zero chloride concentration for the spleen enzyme, which seems to be in agreement with 0.11 mM reported for myeloperoxidase under the same conditions (Bakkenist et al., 1980). It is concluded that the spleen green hemeprotein catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ion as myeloperoxidase.

**DISCUSSION**

The present study confirms the original report of Davis and Averill (1981) in two aspects: (i) the presence of a green peroxidase in spleen with optical spectral properties similar to those of myeloperoxidase of polymorphonuclear leukocytes, and (ii) the effectiveness of the acid extraction method and the batch absorption to and elution from cellulose phosphate. In this paper, I present an improved method of preparation and a further characterization of the enzyme, together with a detailed comparison of its ligand binding character with that of myeloperoxidase.

The major modification in the preparative methods is the use of CM-Sepharose CL-6B column chromatography with a pH and ionic strength gradient, in place of the ionic strength use of CM-Sepharose CL-GB column chromatography with a procedure. The enzyme preparation showed the same high purity to those of myeloperoxidase of polymorphonuclear leukocytes, and a further characterization of the enzyme, together with a conventional purity index chromato-
spectrum of the cyanide complex of the spleen green heme-protein exhibits a symmetrical g signal, as seen in the spectrum of myeloperoxidase-cyanide reported by Bolscher et al. (1984a), but differs from that reported by Eglington et al. (1982), which exhibits a shoulder at the lower fields side of the \( g \) signal. Bolscher and Weyer (1984a) reported the presence of a single set of isosbestic points in the spectra of myeloperoxidase and its cyanide complex. The present data on the cyanide complex of the spleen green heme-protein agree well with these recent data on the cyanide complex of myeloperoxidase.

The thermodynamic properties of chloride binding to the spleen green heme-protein could be compared with those of ligand binding to other hemeproteins with a similar coordination structure. Resonance Raman data (Babcock et al., 1985) indicated that the resting enzyme is a six-coordinated high spin state with a water molecule as the sixth axial ligand. The presence of a proximal histidine residue in myeloperoxidase has been suggested (Ikeda-Saito et al., 1984; Bolscher and Weyer, 1984b). The resonance Raman, EPR, and magnetic circular dichroism spectra of myeloperoxidase and the spleen green heme-protein indicate that the coordination structure of the iron centers in these two enzymes are similar, and the presence of a proximal histidine residue in the spleen enzyme, as in myeloperoxidase, is reasonably assumed. The spleen enzyme seems to have a water-iron-histidine axial coordination structure, as methemoglobin. The heat of chloride binding to the enzyme falls in the range of values reported for ligand binding to methemoglobin. For example, the values of heat of fluoride and thiocyanate ligation to methemoglobin were \(-2.9\) and \(-7.5\) kcal/mol, respectively (Anusium et al., 1968). This might suggest that chloride binds to the iron in the enzyme as fluoride and thiocyanate bind to the heme iron in methemoglobin, by replacing a bound water molecule.

Cyanide is a common low spin exogenous ligand for a variety of hemeproteins, and there is no doubt that its binding site is the sixth coordination position of the heme iron. Although chloride is known to bind at the axial coordination position of the iron in hemin-chloride, cyanide is not a usual heme ligand in hemeproteins. Myeloperoxidase and chloroperoxidase belong to a small clan which exhibits chloride induced changes in their optical absorption spectra. Changes in the optical, EPR, and resonance Raman spectroscopic properties have been considered as evidence supporting a proposal of coordination of chloride at the sixth coordination position of the iron in myeloperoxidase. In chloroperoxidase, NMR (Krejcarek et al., 1976) and Mössbauer (Champion et al., 1973) studies concluded that chloride does not coordinate to the heme iron, although recent ligand equilibrium measurements have suggested that it does (Dawson et al., 1984).

The data presented in this paper clearly show that cyanide and chloride compete in binding to the spleen green heme-protein. The steady-state enzyme kinetics study has also shown that chloride behaves as a competitive inhibitor with respect to hydrogen peroxide. The straightforward interpretation of the competition between chloride and typical heme ligands, such as cyanide and hydrogen peroxide, might be the direct competition at the same binding site. Such interpretations have been made for myeloperoxidase and chloroperoxidase (Zgliczynski and Stelmaszynska, 1979; Weaver and Bakkenist, 1980; Bolscher and Weyer, 1984a; Dawson et al., 1984). It should be borne in mind, however, that the competition in binding by chloride and cyanide does not necessarily imply competitive binding to the same binding site on the enzyme. It is still possible that the inhibitory action of chloride against cyanide or hydrogen peroxide binding to the enzyme may reflect the binding of chloride at a different site which secondarily interacts with the heme ligand binding site in an anticooperative manner. Such a possibility might seem remote when considering the large body of data favoring direct binding of chloride to the heme iron, but more structural and spectroscopic characterization of the chloride complex are necessary to definitely determine the chloride-binding site in myeloperoxidase and the spleen green heme-protein.

The present enzyme kinetics results suggest similarities in the catalytic properties of the spleen green heme-protein and myeloperoxidase. However, Davis and Averill (1981) reported that the spleen enzyme could not catalyze the peroxidation of ascorbic acid, which is a good substrate of myeloperoxidase (Ager, 1941), and described the possible difference in the structure of the substrate-binding sites between the two peroxidases. Ascorbic acid is known as a good reducing agent for compound II of various peroxidases including myeloperoxidase (Bolscher et al., 1984b): compound II is reduced to the native enzyme upon reaction with ascorbic acid. In my experiments, rapid conversion of compound II to the resting state by ascorbic acid was also observed for the spleen green heme-protein, indicating that the spleen enzyme can utilize ascorbic acid as a substrate. A preliminary test of the steady-state kinetics shows that peroxidation of ascorbic acid is catalyzed by the spleen enzyme, in contrast to the original report of Davis and Averill (1981). Although the origin of this discrepancy is not clear, the present results are indicative of the similarities in catalytic properties between the spleen green heme-protein and myeloperoxidase.

Together with the results obtained by resonance Raman and magnetic circular dichroism, the present results strongly indicate that the structure of the prosthetic group, and its vicinity, in the spleen green heme-protein is identical to that of myeloperoxidase. It is concluded that the spleen enzyme can be used as a model for the study of the active center of myeloperoxidase of granulocytes, although the relationship between the spleen green heme-protein and myeloperoxidase is not clear as pointed out by Davis and Averill (1981). There are several practical advantages to the use of the spleen green heme-protein over myeloperoxidase. The source is easily obtained at the local abattoir in large quantity, preparation is simple, and the smaller molecular size may be of great advantage in the eventual determination of the molecular structure.

Acknowledgments—I thank Drs. T. Vonetani and T. Ohnishi for their facilities, Dr. R. C. Prince for his comments, and the members of the Penn-Jersey Blood Program of the American Red Cross for their supply of out-dated granulocyte concentrate.

REFERENCES

Ager, K. (1941) *Acta Physiol. Scand.* 2, Suppl. 8, 1-62
Andrews, P. C., and Krinsky, N. I. (1981) *J. Biol. Chem.* 256, 4211-4218
Anusium, A. C., Beetlestone, J. G., and Irvine, D. H. (1968) *J. Chem. Soc. (Lond.), A*, 1337-1340
Babcock, G. T., Ingle, R. T., Oertling, W. A., Davis, J. S., Averill, B. A., Hulse, C. L., Stufkens, D. J., Bolscher, B. G. J. M., and Weyer, R. (1985) *Biochim. Biophys. Acta* 828, 58-66
Bakkenist, A. R. J., De Boer, J. E. G., Plat, H., and Weyer, R. (1980) *Biochim. Biophys. Acta* 613, 327-348
Blum, H., Chance, B., Gunson, D. E., and Lichtfield, W. J. (1978) *FEBS Lett.* 66, 37-40
Bolscher, B. G. J. M., and Weyer, R. (1984a) *Biochim. Biophys. Acta* 788, 1-10
Bolscher, B. G. J. M., and Weyer, R. (1984b) *Biochim. Biophys. Acta* 791, 75-81
Bolscher, B. G. J. M., Plat, H., and Weyer, R. (1984a) *Biochim. Biophys. Acta* 784, 177-186
Bolscher, B. G. J. M., Zoutberg, G. R., Cuperus, R. A., and Weyer, R. 

*Spleen Green Hemeprotein* 11695
Spleen Green Hemeprotein

(1984b) Biochim. Biophys. Acta 784, 189–191
Champion, P. M., Munk, E., Debrunner, P. G., Hollenberg, P. F., and Hager, L. P. (1973) Biochemistry 12, 426–435
Cotton, M. L., and Dunford, H. B. (1973) Can. J. Biochem. 51, 582–587
Davis, J. C., and Averill, B. A. (1981) J. Biol. Chem. 256, 5992–5996
Dawson, J. H., Sono, M., and Hager, L. H. (1984) Fed. Proc. 43, 1560
DeFilippi, L. J., and Hultquist, D. E. (1978) J. Biol. Chem. 253, 2946–2953
Eglinton, D. G., Barber, D., Thomson, A. J., Greenwood, C., and Segal, A. W. (1982) Biochim. Biophys. Acta 703, 187–195
Hager, L. P., Morris, D. R., Brown, F. S., and Eberwein, H. (1966) J. Biol. Chem. 241, 1769–1777
Harrison, J. E. (1976) in Cancer Enzymology (Shultz, J., and Ahmad, F., eds) pp. 305–316, Academic Press, New York
Harrison, J. E., and Schultz, J. (1976) J. Biol. Chem. 25, 1371–1374
Harrison, J. E., Araiso, T., Palacic, M., and Dunford, H. B. (1980) Biochem. Biophys. Res. Commun. 94, 34–40
Ikeda-Saito, M., and Prince, R. C. (1985) J. Biol. Chem. 260, 8301–8305
Ikeda-Saito, M., Prince, R., Argade, P. V., and Rousseau, D. L. (1984) Fed. Proc. 43, 1961
Ikeda-Saito, M., Argade, P. V., and Rousseau, D. L. (1985a) FEBS Lett. 184, 52–55
Ikeda-Saito, M., Sono, M., and Dawson, J. H. (1985b) Biochim. Biophys. Acta 784, 189–191
Ikeda-Saito, M., Sono, M., and Dawson, J. H. (1985c) Biochem. J. 241, 1769–1777
Klebanoff, S. J. (1975) Semin. Hematol. 12, 117–142
Krejcarek, G. E., Bryant, R. G., Smith, R. J., and Hager, L. P. (1976) Biochemistry 15, 2508–2511
Makino, R., and Yamazaki, I. (1973) Arch. Biochem. Biophys. 157, 356–368
Odajima, T. (1980) J. Biochem. (Tokyo) 87, 379–391
Odajima, T., and Yamazaki, I. (1970) Biochim. Biophys. Acta 206, 71–77
Sibbett, S. S., and Hurst, J. K. (1984) Biochemistry 23, 3007–3013
Segal, A. W. (1982) Biochim. Biophys. Acta 703, 187–195
Stelmaszynska, S. J., and Zgliczynski, J. M. (1974) Eur. J. Biochem. 45, 305–312
Wever, R., and Bakkenist, A. R. J. (1980) Biochim. Biophys. Acta 612, 178–184
Wever, R., and Plat, H. (1981) Biochim. Biophys. Acta 661, 235–239
Zgliczynski, J. M., and Stelmaszynska, S. J. (1979) Biochim. Biophys. Acta 567, 309–314