The Effect of Chloride on the Redox and EPR Properties of Myeloperoxidase*

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Myeloperoxidase was purified from human polymorphonuclear leukocytes and the effect of chloride upon the EPR and potentiometric properties was studied. The redox titration between the ferrous and ferric states of the enzyme yielded a 1 Nernst plots between pH 9 and 4, with clear isosbestic points in the optical spectra during the redox change. The midpoint potential (E_m) between the ferric and ferrous forms of the enzyme exhibited a pH-dependent change between pH 4 and 9, and the effect of added chloride ion indicated that Cl^- competed with OH^- for a binding site on the enzyme. Interestingly, the pH dependence of the E_m indicated that the overall redox reactions of the enzyme was: ferric myeloperoxidase + 2e^- + 1H^+ = ferrous myeloperoxidase.

Myeloperoxidase exhibited a rhombic high spin EPR signal which exhibited reduced rhombicity upon the binding of chloride.

Our results strongly suggest that chloride binds to the sixth coordination position of the chlorin iron in myeloperoxidase by replacing the water which is the sixth ligand in the resting state. It is also concluded that the two iron centers are identical and that there is no interaction between them.

Myeloperoxidase (EC 1.11.1.7) is one of the essential components of the antimicrobial systems of polymorphonuclear neutrophils (Klebanoff and Clark, 1978). One of the unique properties of myeloperoxidase is its activity to catalyze the formation of hypochlorous acid from hydrogen peroxide and chloride ion (Harrison and Schultz, 1976). With its ability to cause rapid degradation of various biological compounds, hypochlorous acid may be the ultimate toxin generated by myeloperoxidase.

Since the discovery of the enzyme by Agner (1941), myeloperoxidases have been purified from various mammalian sources (Schultz, 1980). Contradictory results on both the size and heterogeneity of the purified enzyme, however, have plagued the characterization of enzyme structure. However, the recent work by Andersen et al. (1982) has shown that myeloperoxidase is a single molecular species, which consists of two heavy (M_r ~ 55,000) and two light (M_r ~ 15,000) subunits. They have also demonstrated that heterogeneity of the purified enzyme is a result of proteolytic degradation (Atkin et al., 1982) and stated that studies of heme and iron in myeloperoxidase must utilize enzyme isolated by methods designed to minimize proteolytic degradation. Each heavy chain has one iron-containing prosthetic group, which is covalently linked to the polypeptide chain. The structure of the prosthetic group has been proposed to be a derivative of an iron formyl-porphyrin (Harrison and Schultz, 1978; Odajima, 1980) because of the similarities between the optical absorption spectrum of the enzyme under denaturing conditions and that of heme a, and because of the chemical reactivity of myeloperoxidase and its isolated heme-type chromophore toward various carbonyl reagents. However, recent magnetic circular dichroism (Eglinton et al., 1982) and resonance Raman scattering (Ikeda-Saito et al., 1984; Sibbett and Hurst, 1984) data have indicated that the prosthetic group of the enzyme is an iron chlorin. Uncertainly has also existed with respect to the equivalence of the two iron centers. Although the enzyme can be separated into identical dimers (Andrews and Krinsky, 1981), there have been reports which favor inequivalence of the iron centers (Agner, 1958; Harrison and Schultz, 1978). Anerobic titration and EPR data have led Odajima (1980) to report that one of the iron centers is a non-heme iron. EPR spectra of resting enzyme reported by Weaver and Bakkenist (1980), Schultz (1980), and Eglinton et al. (1982) showed two kinds of rhombic high spin signals: a major component with g_1 = 6.8 and g_2 = 5 and a minor one with g_1 = 6.4 and g_2 = 5.5. This might indicate that these preparations of myeloperoxidase contained more than two molecular species, or that the electronic structure of the iron centers was a mixture of at least two high spin states. Weaver and Bakkenist also reported that the EPR spectrum of the enzyme-chloride complex was very similar to that of the resting enzyme, despite the early report by Stelmaszyńska and Żeligowsky (1974) on chloride-induced changes in the optical absorption spectrum of resting myeloperoxidase.

We have initiated a study of the spectroscopic properties of myeloperoxidase in order to gain insight into the structure-function relationship of the enzyme. Myeloperoxidase was prepared with care to minimize proteolytic degradation (Andersen et al., 1982) so that spectroscopic data could be obtained on a homogeneous preparation. This paper reports the effect of chloride binding on the EPR and redox properties of myeloperoxidase.

**Experimental Procedures**

Myeloperoxidase was isolated from outdated leukopheresis preparations, which were supplied by the Penn-Jersey Blood Program of the American Red Cross, Philadelphia, PA. All the solutions used for preparation of the enzyme contained 1 mM phenylmethanesulfonyl fluoride and 1 μM peptatin A (Andersen et al., 1982), and the enzyme was prepared at 5 ºC. Residual erythrocytes in the leukopheric preparations were removed by the method of Rothstein et al. (1971).

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1 B. A. Averill, personal communication.
Red cell-free granulocytes were suspended in 0.1 M potassium phosphate, pH 7.8, containing 2% hexadecyltrimethylammonium bromide and homogenized by a Potter-Elvehjem homogenizer, which was chilled in ice. The homogenate was centrifuged at 30,000 × g for 10 min, and the supernatant was collected and diluted with twice its volume of cold distilled water. The solution containing the enzyme was loaded on a column of CM-Sepharose CL-6B previously equilibrated with 0.1 M phosphate buffer, pH 7.8, containing 0.002% hexadecyltrimethylammonium bromide. The column was washed with the same buffer until the eluent was free from absorbance at 280 nm. The dark green band containing the enzyme was eluted with 0.5 M phosphate buffer, pH 7.8. The reinitz zah (purity number) value (\( A_{400nm} / A_{280nm} \)) of the preparation was about 0.2. Ultrafiltration was employed to exchange the buffer system to 0.2 M phosphate buffer, pH 7.8. Solid ammonium sulfate was added to the solution containing the enzyme to 50% saturation, and the precipitate was collected by centrifugation. The precipitate was dissolved in 0.2 M phosphate buffer, pH 7.8. The measuring electrode was made of platinum (Radiometer K601) and the reference electrode was either a calomel electrode (HgCl₂, Radiometer K401) or a mercurous sulfate electrode (HgSO₄, Radiometer K601). The latter was used when a rigorously chloride-free solution was required. Assuming that the calomel electrode had an \( E_r \) of +247 mV, the mercurous sulfate electrode had an \( E_r \) of +604 mV under the conditions used here. Redox mediation between myeloperoxidase and the electrodes was provided by 40 \( \mu \)M of 2,3,5,6-tetramethyl phenylenedianime (\( E_{m7} = 275 \) mV), N-methylphenazonium methosulfate (\( E_{m7} = 85 \) mV), N-ethylphenazonium ethosulfate (\( E_{m7} = +65 \) mV), and 2-hydroxy-1,4-naphthoquinone (\( E_{m7} = -145 \) mV) (Prince et al., 1981). The buffer systems used were 0.1 M citrate-phosphate, pH 4–6, phosphate, pH 6–8 and glycine-NaOH, pH 8–10.

**RESULTS**

Fig. 1 illustrates a typical example of optical absorption spectra recorded during a redox titration of myeloperoxidase between the ferric and ferrous states. In this set of spectra, the spectrum of the enzyme at \(-90 \) mV was taken as a base-line and successive spectra during the oxidative titration were recorded. The appearance of isosbestic points during the change in valence states between the ferric and ferrous forms indicates that the two iron chlorins in the enzyme have the same optical properties. Fig. 2 shows the Nernst plots for the titrations at pH 4.3 and 7.9 in the presence and absence of 0.1 M NaCl. The points are experimental data and the curves are calculated by least squares fitting. In all the cases studied in the present investigation (between pH 4.2 and 9 in the presence and absence of 0.1 M NaCl), titration curves were described by \( n = 1 \) Nernst equations. At pH 7.9, the midpoint potential between ferric and ferrous forms (\( E_m \)) was independent of 0.1 M NaCl. At pH 4.3, the \( E_m \) value was 143 mV.
Addition of 0.1 M NaCl at pH 4.3 lowered the $E_m$ value to 21 mV, essentially to the same $E_m$ value observed at pH 7.0. Fig. 3 plots the $E_m$ as a function of pH in the presence and absence of 0.1 M NaCl. Above pH 7, the presence of 0.1 M NaCl suppressed the pH dependence. Surprisingly, the slope of the pH dependency was −30 mV/pH, indicating the binding of only a single proton per two electrons.

The effect of chloride binding was also studied by EPR measurements and the results are shown in Fig. 4. The x-band spectrum of myeloperoxidase exhibits a rhombic high spin EPR signal together with a low spin signal, which seems to be saturated at the experiment conditions, with $g$ values of $g_1 = 2.57$, $g_2 = 2.32$, and $g_3 = 1.83$. The presence of this low spin signal was also reported by Bakkenist et al. (1978). In the spectrum of the chloride complex, a new high spin signal appears with decreased rhombicity. The low spin signal also disappears. EPR spectra of the enzyme were also measured at neutral and alkaline pH. Above pH 10, a decrease in the signal intensity of the high spin signal and the appearance of a new low spin signal at $g = 2.89, 2.21$, and 1.69 were noticed.

**DISCUSSION**

First we shall discuss the equivalence of the two iron centers of myeloperoxidase. The following results obtained from the present experiments on a homogeneous preparation of myeloperoxidase establish the equivalence and independence of the two iron centers, and completely rule out the possible presence of non-heme iron as one of the iron centers of the enzyme. 1) The redox titration curves were described by $n = 1$ Nernst equations, with sharp isosbestic points in the absorption spectra during the redox titrations, indicating that the two iron centers have the same oxidation-reduction midpoint potential, and similar absorption spectra. 2) Sharp high spin EPR lines for both the resting and chloride form of the enzyme are indicative of the presence of only one high spin species in the preparation, and provide no evidence for a magnetic interaction between the two iron centers. 3) If we omit ammonium sulfate fractionation in the preparative procedure, we have an enzyme preparation with a reinheit zahl value of about 0.5. This preparation, which exhibits a light absorption spectrum in the visible and Soret regions similar to that of the highly purified enzyme, gives an EPR spectrum similar to that of the highly purified enzyme except for the presence of a large $g = 4.3$ signal, the line shape of which was very similar to the one reported by Odajima (1980) as a non-heme iron center of myeloperoxidase. We conclude that the $g = 4.3$ signal claimed by Odajima (1980) to be one of the iron centers of the enzyme originates from contamination of iron(s) or iron-protein complex(es) in his preparation of myeloperoxidase. It is noteworthy that the intensity of this $g = 4.3$ signal decreased considerably upon the addition of 0.1 M NaCl or 0.002% hexadecyltrimethylammonium bromide, with the appearance of a rhombic high spin signal centered at $g = 6$. This observation warns that the small intensity of $g = 4.3$ EPR signal does not necessarily guarantee the purity of a...
preparation of myeloperoxidase. EPR spectra of human myeloperoxidase reported previously (Schultz, 1980; Wever and Bakkenist, 1980; Eglington et al., 1982) exhibited the presence of minor high spin component(s). We did not observe minor components in the high spin region of our EPR data of the resting enzyme. The absence of such a minor component suggests that our enzyme, prepared with an adequate use of protease inhibitors, is more homogeneous than those used in previous reports.

One might claim that the low spin signal observed in the resting enzyme could come from a denatured form of myeloperoxidase. We believe that the presence of the low spin form is a characteristic of the enzyme at cryogenic temperature for the following reasons. 1) The low spin form is converted into a high spin state by the addition of chloride, since there is no low spin signal detected in the chloride complex spectrum. 2) The optical absorption spectrum at 77 K shows the presence of a low spin state (Wever and Plat, 1981). 3) This low spin EPR signal was observed also by others (Bakkenist et al., 1978; Eglington et al., 1982). Thermal mixing of the low spin and high spin states, as seen in other ferric hemoproteins, is highly likely.

The appearance of another low spin signal \( g_1 = 2.89, g_2 = 2.21 \), and \( g_3 = 1.69 \) at high pH confirms the previous report by Bakkenist et al. (1978). They proposed that the ligation of \( \mathrm{OH}^- \) gives rise to a transition from high spin to low spin state at alkaline pH as in metmyoglobin hydroxide. However, these \( g \) values seem to correspond to a species where the sixth ligand of the iron is an imino-nitrogen (type H according to the classification of Blumberg and Peisach (1971) rather than to a hydroxide complex. Such compounds, involving the binding of an endogenous amino acid as the sixth ligand of the iron, are called hemichromes. They have been observed in the isolated chain of ferric hemoglobin or the mutant hemoglobins (Hyafil et al., 1976), in which the distal histidine occupies the sixth coordination of the heme iron. Furthermore, the \( g \) values of this low spin species are similar to those of cytochrome \( b_5 \) at pH 12 (Ikeda et al., 1974). It is tempting to speculate that the low spin form at alkaline pH is a hemichrome in which both the 5th and 6th coordination positions are occupied by histidyl residues. Resonance Raman data (Ikeda-Saito et al., 1984) indicate that the resting enzyme is a six-coordinated high spin state with histidine as the fifth ligand and a water molecule as a possible sixth ligand. We propose that there are two proximal and distal histidine residues in myeloperoxidase and in other peroxidases, myoglobins, and hemoglobins.

It has been reported that the binding of chloride induces a change in the optical absorption (Stelmaszynska and Zgliyczynski, 1974; Wever and Plat, 1981) and resonance Raman (Ikeda-Saito, et al., 1984) spectra of myeloperoxidase, but Wever and Bakkenist (1980) reported that the EPR spectrum of the chloride complex of myeloperoxidase is very similar to that of the resting enzyme. In contrast to their results, we have found that the binding of chloride reduces the rhombicity of the high spin EPR signal. In other words, chloride binding changes the crystal field parameters of the iron in myeloperoxidase. Such a change probably reflects a binding of chloride at very close proximity to the prosthetic group, most likely a binding to the chlorin iron. Binding of another halide, fluoride, which is known to coordinate to the iron of myeloperoxidase (Wever and Bakkenist, 1980), also induced a reduction of rhombicity in the high spin EPR spectrum (Wever and Bakkenist, 1980). Considering the pH and chloride concentration in phagosomes (pH 4 and 0.1 M NaCl) (Page, 1961; Jense and Bainton, 1973) myeloperoxidase in vivo must be ligated with chloride. In halogenation reactions catalyzed by myeloperoxidase at acidic pH, chloride competitively inhibits with respect to hydrogen peroxide (Bakkenist et al., 1978; Andrews and Krinsky, 1982). This inhibitory action could be explained if the 6th coordination position of the chlorin iron is the binding site for both hydrogen peroxide and chloride.

The midpoint potential of the resting enzyme seems to agree with the previous report at pH 7.0 and 8.6 by Harrison and Schultz (1978). We have extended their measurements to a wider range of pH and in the presence of chloride. The pH dependence of the \( E_m \) shows a slope (\( E_m/pH \)) of about \(-30\) mV. At face value, this corresponds to the binding of one \( \mathrm{H}^+ \), or the release of one \( \mathrm{OH}^- \), per two electrons bound to the enzyme, which has two redox centers. Alternately, there might be a series of ionizable “groups” on the enzyme, each having a different pK on the ferric and ferrous states. The addition of chloride ion eliminates the pH dependence of the \( E_m \) at acid pH. Fig. 3 shows that 0.1 M NaCl eliminates the pH dependence of the \( E_m \) at pH more acidic than pH 7.5. However, pH 7.5 is only an apparent pK on the chloride-bound ferric enzyme, for lower chloride concentrations show progressively more acid-apparent pK values. The simplest explanation of Fig. 3 is that chloride and hydroxide compete in binding to the resting enzyme, although this need not be at the same binding site. Indeed, while as discussed above it seems likely that chloride does bind very near to the iron, the binding of only one hydroxide per two equivalent but noninteracting iron atoms makes it likely that the hydroxide is bound elsewhere, and competes with chloride via long-range, protein-mediated interactions. Since the effect of 0.1 M NaCl is equivalent to pH 7.5 in the absence of chloride, where \( [\mathrm{OH}^-] = 3.2 \times 10^{-4} \) (Fig. 3), we may infer that myeloperoxidase has a higher affinity for hydroxide by 10\(^6\) than for chloride.

It should be borne in mind that the \( E_m \) values reported here are equilibrium values, and include any slow “conformational” changes that may occur subsequent to the redox reaction itself. For example, cytochrome \( c_5 \) from Rhodopseudomonas sphaeroides exhibits an apparent pK on the oxidized form at pH 8. However, this may more properly be ascribed to a combination of a pK beyond pH 11, and a slow conformational change of the ferric cytochrome (Prince and Bashford, 1979).

Thus, the data presented here for myeloperoxidase do not necessarily indicate that chloride and hydroxide compete for binding sites on conformationally identical molecules.

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Chloride Binding to Myeloperoxidase


