Spectral and Kinetic Studies on the Formation of Myeloperoxidase Compounds I and II: Roles of Hydrogen Peroxide and Superoxide†

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ABSTRACT: The conversion of myeloperoxidase to compounds I and II in the presence of H_2O_2 has been reinvestigated in order to explain the abnormal stoichiometry of compound I formation and the fast spontaneous decay of compound I to compound II. Rapid-scan studies show that at least a 20-fold excess of H_2O_2 is required to obtain a good spectrum of relatively pure compound I; a further increase in H_2O_2 concentration causes compound I to be reduced to compound II, which is a very stable intermediate. Compound I formation is reversible, with an apparent second-order forward rate constant of $(1.8 \pm 0.1) \times 10^7$ M^{-1} s^{-1} and a reverse rate constant of 58 ± 4 s^{-1}, giving a constant of 3.2 μM for the dissociation of compound I to native enzyme and H_2O. This reversibility is one factor that can explain the large excess of H_2O_2 required to form compound I. The apparent second-order rate constant for compound II formation from compound I and H_2O_2 is $(8.2 \pm 0.2) \times 10^4$ M^{-1} s^{-1}. We confirm pH dependence studies, which suggest that the formation of compounds I and II is controlled by a residue in the enzyme with a pK_a of about 4.0. Excess H_2O_2 is also converted to O_2 via catalase activity of the enzyme. However, we do not consider this a dominant pathway because it fails to account for the fast spontaneous reduction of compound I to compound II. The time courses for both the decay of compound I and the formation of compound II are biphasic. Biphasic kinetics was explained in terms of two possible routes of the reduction of compound I to compound II: a fast reaction with H_2O_2 and a slow reaction with O_2^- as reducing agent. Increasing the concentration of H_2O_2, lowering the pH from neutral to acidic values, or adding SOD to the system results in the convergence of the two phases to a monophasic process. Superoxide was detected as a product in the myeloperoxidase/H_2O_2 system by the tetranitromethane reduction test. The possibility of an autoreduction pathway for the conversion of compound I to compound II involving the protein moiety and impurities in enzyme preparation is also discussed. Compound II possibly reacts with generated O_2^- to form native enzyme, but only to a very limited extent. We exclude heme inequivalence as a possible explanation for the abnormal stoichiometry of compound I formation from cyanide binding studies on the dimeric enzyme and its monomer, which indicate that the two heme prosthetic groups of the enzyme have the same reactivity.

Myeloperoxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7), an enzyme found in leukocytes, catalyzes the formation of HOCl from H_2O_2 and Cl^-. It is believed to play a role in the antimicrobial activity of these cells (Harrison & Schultz, 1976; Klebanoff & Clark, 1978). It has a heterodimeric structure consisting of two heavy and two light subunits, with a heme prosthetic group associated with each heavy subunit (Andrews & Krinsky, 1981; Olsen & Little, 1984). Recent X-ray crystallographic studies also confirm the presence of a single disulfide bond between the heavy subunits (Zeng & Fenna, 1992). Peroxidases undergo a normal catalytic cycle in the presence of a hydroperoxide, ROOH, and an electron donor, AH_2 (George, 1952; Chance, 1952):

$$\text{native enzyme} + \text{ROOH} \rightarrow \text{compound I} + \text{ROH}$$

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + {}^{\bullet}AH$$

$$\text{compound II} + AH_2 \rightarrow \text{native enzyme} + {}^{\bullet}AH + H_2O$$

The spectral properties of some peroxidases and their enzyme intermediates have been reviewed extensively (Dunford & Stillman, 1976).

The reaction of myeloperoxidase with H_2O_2 initially results in the formation of compound I, which oxidizes Cl^- to HOCl and is reduced directly to the native enzyme. During turnover, some compound I is also converted to compound II because of the presence of exogenous or endogenous electron donors (Harrison & Shultz, 1976; Odajima & Yamazaki, 1970). Previous studies have revealed the unique behavior of myeloperoxidase in the presence of H_2O_2. Horseradish peroxidase, frequently taken as an example of many other peroxidases, forms relatively stable compound I with a half-life of 15–20 min after the addition of equimolar H_2O_2 (Dunford & Nadezhdin, 1982). Compound I of canine myeloperoxidase, however, is very unstable and spontaneously decays to its one-electron reduction product, compound II (Harrison et al., 1980). Moreover, a large excess of H_2O_2 is required to achieve the full formation of compound I, although the stoichiometries reported up to now are not in agreement (Odajima & Yamazaki, 1970; Harrison et al., 1980; Agner, 1963; Bolscher & Wever, 1984).

In this article, we report the optical spectra of compounds I and II of bovine spleen myeloperoxidase. Of particular importance is the spectrum of relatively pure compound I, which has eluded previous detection. Transient-state kinetic analyses and pH dependence studies were performed to deduce the mechanism of formation of these intermediates using H_2O_2 as substrate. We explored the possible reasons behind the unique behavior of myeloperoxidase in its reaction with H_2O_2. We show experimental evidence for the reversibility of

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compound I formation and its spontaneous decay to compound II and rectify a previously reported dissociation constant for compound I. Evidence is also presented that supports a scheme where compound I is reduced by both H_2O_2 and O_2^- to compound II. We also investigated the effect of enzyme purity on the stoichiometry of the formation of these oxidized intermediates. That the two heme prosthetic groups of the enzyme have the same reactivity was demonstrated by cyanide binding rates to the dimeric holoenzyme as compared to its monomeric form.

MATERIALS AND METHODS

Bovine spleen myeloperoxidase was isolated and purified using a combination of published procedures with minor modifications (Davis & Averill, 1981, 1984; Ikeda-Saito, 1985). Macerated beef spleens were suspended in 0.25 M KCl at 4 °C and then homogenized in a Waring blender at maximum speed for 2 min. The pH of the homogenate was adjusted to 3.5 with 6 M HCl. The acidic homogenate was stirred at room temperature for 5 h and then centrifuged at 8000 rpm in a JA-10 rotor with a Beckman Model J2-21 centrifuge for 15 min. The supernatant was filtered through glass wool; citric acid was added to the filtrate to a final concentration of 0.1 M, followed by pH adjustment to 5.5 with 12% NaOH. The solution was then treated with precycled Whatman P11 cellulose phosphate fibrous cation exchanger and stirred for 1 h. The cation exchanger was washed with water and then resuspended with stirring in 2 M KCl for another hour followed by filtration. The filtrate was centrifuged at 10 000 rpm for 10 min. The supernatant was concentrated using an Amicon ultrafiltration apparatus and an XM-50 membrane. It was then diluted to an ionic strength of 0.1 M, which was measured using a Yellow Springs Instrument Model 31 conductivity bridge and a KCl standard curve. The crude enzyme solution was then loaded to a CM-Sepharose CL-6B (Pharmacia) column preequilibrated with 0.1 M phosphate (pH 6.0). The column was eluted using a linear ionic strength pH gradient apparatus with 0.1 M phosphate buffer (pH 6.0) as the starting buffer and 1.0 M phosphate (pH 7.0) as the limiting buffer. Further purification was performed by gel filtration using a Sephacryl S-200 (Pharmacia) column. Fractions with RZ values (A_{430}/A_{280}) of 0.80 or higher were collected and dialyzed against deionized water overnight. The concentration of the enzyme solution was determined spectrophotometrically using $\epsilon_{430} = 178\ mM^{-1}\ cm^{-1}$ (Agner, 1958). The activity was measured using the guaiacol assay (Maehly & Chance, 1954).

Cu/Zn superoxide dismutase (SOD) from bovine erythrocytes was purchased from Sigma. The concentration of the dissolved enzyme was determined spectrophotometrically using $\epsilon_{265} = 15.9\ mM^{-1}\ cm^{-1}$ (Briggs & Fee, 1978). Bovine serum albumin (BSA) was also obtained from Sigma.

Hydrogen peroxide, obtained as a 30% solution from BDH Chemicals, was standardized using the oxidation of iodide ion to I_3^- by horseradish peroxidase (Cotton & Dunford, 1973). Stock buffer solutions were prepared using deionized water from the Milli-Q system (Millipore). Citrate buffers were used between pH 3.0 and 5.5 and phosphate buffers between pH 6.0 and 8.0. A Fisher Accumet Model 25 digital pH meter was used for pH measurements.

Oxygen evolution was measured using a Yellow Springs Instrument Model 5331 oxygen probe. A 3.0-mL solution containing 2.5 μM myeloperoxidase in 0.1 M phosphate buffer at pH 6.6 was flushed with nitrogen for 10 min. H_2O_2 (25–75 μM) was then injected into the mixture, and oxygen evolution was monitored at 30 °C.

Stopped-flow and rapid-scan experiments were performed using a Photal (formerly Union Giken) rapid reaction analyzer (Model RA 601) equipped with a 1-cm observation cell thermostated at 25.0 ± 0.5 °C. Rate constants from the experimental traces were determined by nonlinear least-squares analysis carried out by an Alps Electrical Co. Model No. CP 10A computer interfaced to the apparatus. Rapid-scan absorption spectra were measured by means of a multichannel photodiode array and memorized in the digital computer system. Spectral regions of 96 nm were scanned centered at about 430 nm. Myeloperoxidase (2.0 μM) in 0.2 M phosphate buffer (pH 7.1) was mixed with 40 μM H_2O_2 (concentrations after mixing: 1.0 μM myeloperoxidase and 20 μM H_2O_2; ionic strength, 0.1 M due entirely to the buffer). For recording the spectrum of native enzyme, the peroxide solution was replaced with water.

The formation of compound I was monitored at 429 nm, the isosbestic point between compound I and compound II. In a typical experiment, one reservoir contained 1.0 μM myeloperoxidase in 0.2 M ionic strength phosphate buffer at pH 6.6, and the other contained at least an 8-fold excess of hydrogen peroxide in aqueous solution. Pseudo-first-order rate constants, k_{obs}, were evaluated using an exponential curve-fit program and then plotted against H_2O_2 concentration. The same experiment was performed at different pH's from 3.0 to 8.0.

The time courses of the conversion of compound I to compound II were followed by monitoring both the decay of compound I and the formation of compound II. One reservoir contained 0.8 μM myeloperoxidase in 0.1 M buffer of the desired pH value; the other contained H_2O_2 of different concentrations (20–250-fold excess of the enzyme) in the same buffer. The decay of compound I was observed at 442 nm, the isosbestic point between native enzyme and compound II, and the formation of compound II was observed at 456 nm, the isosbestic point between native enzyme and compound I. To study the effect of pH on the conversion of compound I to compound II, the time courses were evaluated at different pH values between 3.5 and 8.0.

The reduction of compound I to compound II was also conducted in the presence of superoxide dismutase in order to probe the involvement of superoxide in the reaction. SOD was replaced with bovine serum albumin in some experiments to identify the possible role of the protein moiety of SOD in the conversion of compound I to compound II.

The formation of O_2^- was further detected using the reduction of tetranitromethane (Sigma) to trinitromethane anion (McCord & Fridovich, 1969). The latter absorbs strongly at 350 nm ($\epsilon = 1.50 \times 10^4\ M^{-1}\ cm^{-1}$) (Henglein et al., 1959). Since solutions of tetranitromethane tend to deteriorate on standing, freshly prepared stock solutions in ethanol were used. Reactions were performed at 25 ± 0.5 °C in a total volume of 2.5 mL. The reaction mixture contained phosphate at pH 6.6 and ionic strength 0.05 M, 100 μM EDTA, 100 μM tetranitromethane, and myeloperoxidase. The reaction was initiated by the addition of H_2O_2, and absorbance changes at 350 nm were monitored.

To determine the effect of enzyme purity on the stoichiometry of compound II formation, fractions from the Sephacryl S-200 gel filtration with different RZ values were titrated with H_2O_2. Microliters of 1 mM H_2O_2 were added successively to 2 μM enzyme, and the spectra were scanned each time using a Beckman Model DU 650 spectrophotometer.

To investigate the possibility of cooperativity or different reactivity of the two heme prosthetic groups in the native

enzyme, cyanide binding behavior was compared between the dimeric enzyme and its monomeric form, hemi-myeloperoxidase. The monomer was prepared by reductive alkylation of the native enzyme under nondenaturing conditions, as described by Andrews and Krinsky (1981). Potassium cyanide (Fisher Scientific Co.) was reagent grade and was used without further purification. Aqueous solutions were freshly prepared. All experiments were carried out at 25 ± 0.5 °C and an ionic strength of 0.05 M due to the contribution of the buffer. Optical absorption spectra were obtained with the Beckman spectrophotometer. Stopped-flow measurements were made using the Photal instrument at 430 nm. One reservoir contained 2 μM enzyme (on a per heme basis) in phosphate buffer (pH 6.5), and the other reservoir contained at least a 20-fold excess of cyanide in aqueous solution. Pseudo-first-order rate constants were evaluated by an exponential curve-fit program, and the second-order rate constant was obtained from the slope of the plot of k_{obs} against cyanide concentration.

RESULTS

Formation of Compound I of the Holoenzyme. Peroxidase compound I typically is readily distinguished from the native form of the enzyme by its reduced absorbance in the Soret region. A good spectrum of compound I of bovine spleen myeloperoxidase is obtained only when a 20-fold excess of H_2O_2 is used. The rapid-scan spectra in Figure 1A were taken 4–7 ms after the flow had stopped. The formation of compound I from ferric myeloperoxidase exhibited an isosbestic point at 456 nm and involved broadening and a decrease in the extinction coefficient at 430 nm from 178 for the native enzyme to 88 mM^{-1} cm^{-1} for compound I. A smaller excess of H_2O_2 (2–10-fold) led to incomplete formation of compound I, whereas a larger excess (40-fold or more) led to the fast formation of compound II, which exhibits a Soret maximum at 455 nm with an extinction coefficient of about 160 mM^{-1} cm^{-1} (Figure 1B).

Under conditions of a large excess of H_2O_2, the pseudo-first-order rate constant, k_{1obs}, was obtained from the exponential time course. The first-order kinetics is described by:

$$-d[MPO]/dt = k_{1obs}[MPO] \quad (1)$$

where

$$k_{1obs} = k_{1f}[H_2O_2] + k_{1b} \quad (2)$$

The pseudo-first-order rate constant, k_{1obs}, measured at pH 6.5 is plotted against H_2O_2 concentration in Figure 2. The inset shows a typical stopped-flow trace for compound I formation displaying monophasic exponential character. The forward second-order rate constant, k_{1f}, obtained from the slope of the plot is (1.8 ± 0.1) × 10^7 $M^{-1}s^{-1}$. An interesting feature of this plot is a finite intercept, 58 ± 4 s^{-1}, which represents a reverse rate constant, k_{1b}. This is evidence that compound I formation is reversible. The ratio of the rate constants, k_{1b}/k_{1f}, gives a value for the dissociation constant of compound I to native enzyme and H_2O_2 of 3.2 μM. The pH dependence of k_{1f} is shown in Figure 3. The values of k_{1b} were pH-independent within experimental error.

Reduction of Compound I of the Holoenzyme by H_2O_2. The conversion of compound I to compound II was followed by both the disappearance of compound I and the appearance of compound II. The corresponding time courses are shown in Figure 4A,B. The rate constants obtained in the two cases are the same within experimental error. We chose to present the results of compound II formation, which have smaller

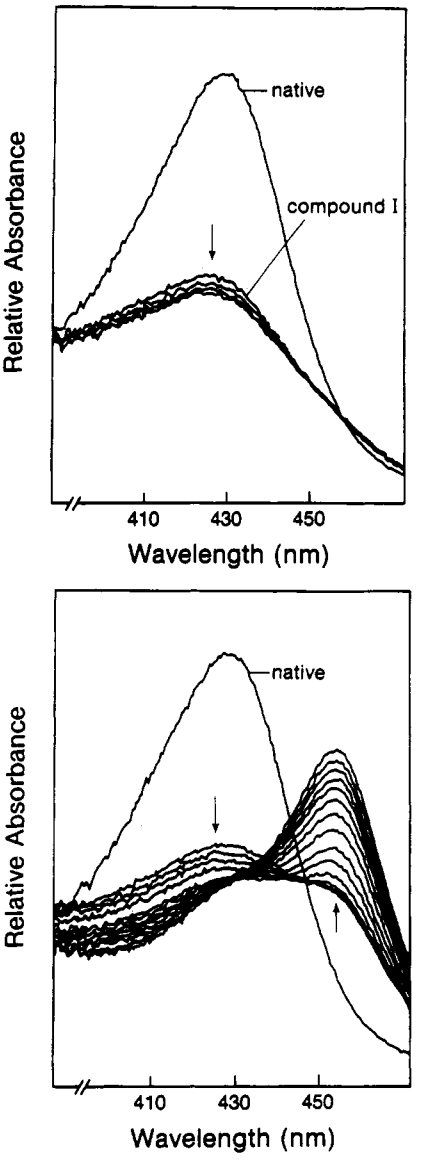

FIGURE 1: (A, top) Rapid-scan spectra of native myeloperoxidase and after adding 20-fold excess H_2O_2. The spectra were taken 4–7 ms after the flow stopped. Final enzyme concentration: 1.0 μM in 0.1 M phosphate buffer, pH 7.1. (B) Rapid-scan spectra of the reaction of myeloperoxidase at 40-fold excess H_2O_2. Same enzyme concentration in buffer as in A. The first scan was taken 3 ms after the flow stopped, and the subsequent scans were at 1-ms intervals. The arrows show the direction of absorbance changes with time.

experimental errors, mainly because the absorbance change accompanying compound II formation at 456 nm is larger than that for compound I disappearance at 442 nm. The apparent second-order rate constant for compound II formation, k_2, obtained from the linear plot of k_{2obs} against H_2O_2 concentration, is (8.1 ± 0.1) × 10^4 $M^{-1}s^{-1}$ at pH 7.1. The dependence of k_2 on pH is similar to the k_{1f}–pH profile (data not shown).

It is worth noting in Figure 4A,B that the reduction of compound I to compound II appears biphasic when a modest excess (20-fold) of H_2O_2 is used. The trace becomes monophasic at higher H_2O_2 concentrations and lower pH (data not shown). Moreover, the addition of SOD also renders monophasic exponential character to the time course. Replacment of SOD with bovine serum albumin increases the rate, but does not cause the convergence of the two phases to a single phase (Figure 5).

To explore further the involvement of O_2^- in the reduction of compound I to compound II, we used tetranitromethane

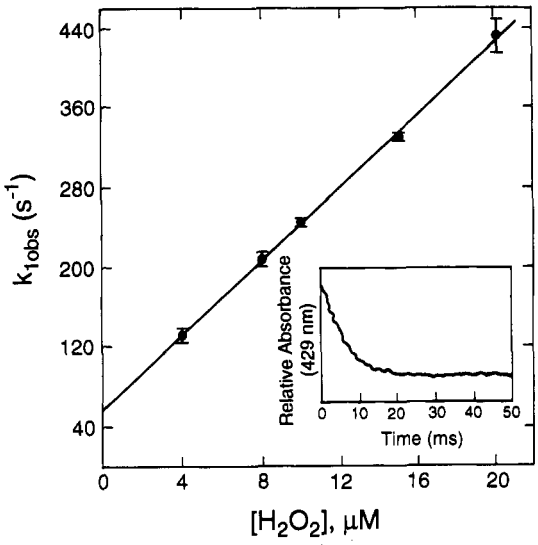

FIGURE 2: Pseudo-first-order rate constants for compound I formation plotted against $[H_2O_2]$. The inset shows a typical trace of the reaction followed at 429 nm. Final enzyme concentration: 0.5 μM enzyme in 0.1 M phosphate buffer, pH 6.6. The forward and reverse rate constants were calculated from the slope and intercept, respectively.

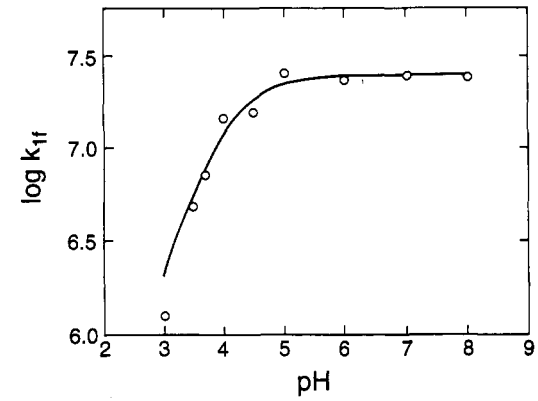

FIGURE 3: pH dependence of the apparent second-order rate constant of compound I formation. Same conditions as in Figure 2, except that the pH was varied from 3.0 to 8.0; therefore, different buffers were used. The curve was computed on the basis of best-fit parameters obtained from a nonlinear least-squares analysis of the data points using eq 4 in the text.

reduction. Tetranitromethane is effectively reduced by O_2^- to the nitroform anion, which has a strong absorbance at 350 nm. We employed this reaction when attempts to determine O_2^- by the reduction of ferric cytochrome *c* did not give detectable results. Figure 6 shows that O_2^- is produced in the myeloperoxidase–H_2O_2 system, and the amount generated is dependent on the concentrations of both enzyme and H_2O_2.

Effect of Enzyme Purity on the H_2O_2 Requirement. Compared to compound I, compound II is very stable. Figure 7 shows the Soret–visible spectral scan of the spontaneous decay of compound II to native enzyme, with isosbestic points at 442, 586, and 670 nm. Since it is more convenient to work with compound II, we decided to look into the effect of enzyme purity on the amount of H_2O_2 required to produce this oxidized intermediate of the enzyme. Figure 8 shows that it takes more H_2O_2 to completely form compound II for an enzyme fraction of RZ 0.81 compared to a fraction of RZ 0.85. The amount of H_2O_2 required also varied when enzyme fractions (with RZ from 0.56 to 0.82) were obtained from different steps in the enzyme purification procedure (data not shown).

Cyanide Binding to Hemi- and Holo-myeloperoxidase. Cyanide binding to myeloperoxidase is accompanied by a gradual shift in the Soret peak to 454 nm (Figure 9). Both the native enzyme and hemi-myeloperoxidase exhibited the same spectral changes as cyanide was added successively. Under pseudo-first-order conditions, the observed rate constant increases linearly with the concentration of cyanide and intersects at the origin. The slope yields the apparent second-order rate constant for cyanide binding. The values obtained, $(2.5 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for native myeloperoxidase and $(2.6 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for hemi-myeloperoxidase, are similar. These results suggest that the two subunits of the enzyme have the same binding affinity for cyanide.

DISCUSSION

Considerable effort has been made to explore the mechanism behind the unique behavior of myeloperoxidase in its reaction with H_2O_2 (Bolscher & Wever, 1984; Kettle et al., 1988; Iwamoto et al., 1987; Hoogland et al., 1988). Of the oxidized intermediates of myeloperoxidase, compound I is the only species that takes part in both the peroxidatic cycle and the chlorinating reaction (Harrison, 1982). Despite its significant role in these reactions, some mechanistic details about compound I remain unsolved. Among these are the abnormal stoichiometry for compound I formation and the spontaneous decay of compound I upon its formation, which make the investigation of myeloperoxidase compound I much more difficult than those of other peroxidases.

First, we sought the MPO/H_2O_2 ratio that would yield complete formation of compound I. Previously, the spectrum of compound I of canine myeloperoxidase was reported using a 40-fold excess of H_2O_2 (Harrison et al., 1980). We found that a 20-fold excess of H_2O_2 is sufficient for forming a relatively more stable compound I of beef spleen myeloperoxidase (Figure 1A). Compound I formation is accompanied by a broadening and diminution in the Soret absorption, which have also been observed for other peroxidases (Chance, 1951). An earlier investigation suggested that the addition of an equimolar amount of H_2O_2 to myeloperoxidase yields compound I (Odajima & Yamazaki, 1970), although the very slight decrease in the Soret absorption that it showed clearly indicated that compound I formation is incomplete.

Why would such a large excess of H_2O_2 be required to form compound I? A natural assumption is that the enzyme preparation had catalase impurities. However, this explanation was excluded since catalase is an acidic enzyme (p*I* = 5.5), and therefore it would have been removed during the two cation-exchange chromatographic steps in the isolation procedure. Iwamoto et al. (1987) ascribed the abnormal stoichiometry to the "true catalase activity" of myeloperoxidase. Compound I is presumed to react with H_2O_2 and to be recycled to the ferric state with concomitant evolution of oxygen. Although we also detected O_2 formation under the same conditions that these workers used it does not appear that the catalase reaction is a dominant pathway for the following reasons. Our rapid-scan spectral results (Figure 1) show that compound I does not recycle back to native enzyme. Moreover, this mechanism gives no account for the rapid spontaneous reduction of compound I to compound II, which is obviously one of the important factors contributing to the abnormal stoichiometry of compound I formation. We found that as the ratio of $[H_2O_2]/[MPO]$ is increased, more complete formation of compound I is observed; also, the decay of compound I to compound II becomes faster. If the catalase reaction were a dominant pathway, which is estimated to be even faster than compound I formation (Iwamoto et al., 1987),

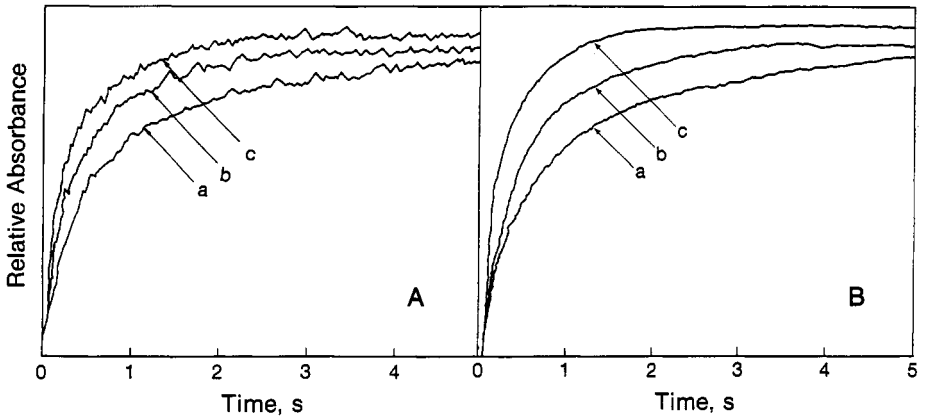

FIGURE 4: Time courses for (A) compound I disappearance (at 442 nm) and (B) compound II formation (at 456 nm) at different concentrations of H_2O_2: (a) 20-fold excess; (b) 50-fold excess; and (c) 100-fold excess over the enzyme concentration. Final concentration of enzyme: 0.4 μM in 0.1 M (pH 7.0) phosphate buffer. Absorbance scale: 1 cm corresponds to 0.0015 absorbance units in A and 0.0035 units in B.

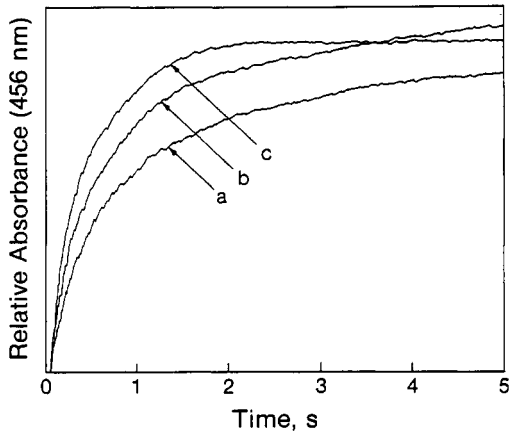

FIGURE 5: Effects of SOD and BSA on the conversion of compound I to compound II, as reflected in the time courses of compound II formation. Absorbance changes were followed at 456 nm. One reservoir contained 16 μM H_2O_2. The other contained (a) 0.8 μM myeloperoxidase, (b) a mixture of 0.8 μM myeloperoxidase and 10 μM BSA, and (c) a mixture of 0.8 μM myeloperoxidase and 20 μM SOD. All reactions were carried out in 0.1 M (pH 7.0) phosphate buffer at 25 °C. Absorbance scale: 1 cm corresponds to 0.003 absorbance units.

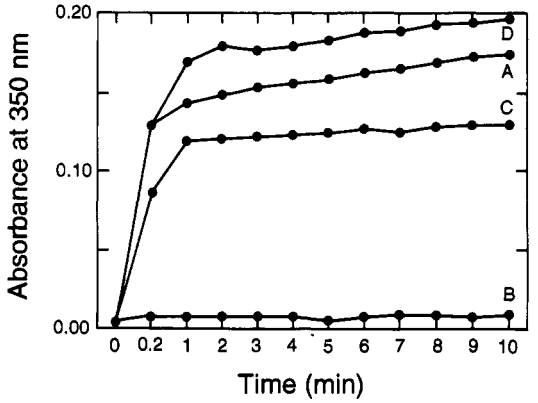

FIGURE 6: Detection of superoxide by the reduction of tetranitromethane. The reaction mixture generally contained phosphate at pH 6.6 and an ionic strength of 0.05 M, 100 μM EDTA, and 100 μM tetranitromethane plus the following: (A) O_2^- generating system of dihydroxyfumarate (50 μM DHF + 1 μM H_2O_2); (B) 100 μM H_2O_2; (C) 1.0 μM myeloperoxidase and 100 μM H_2O_2; and (D) 1.5 μM myeloperoxidase and 150 μM H_2O_2.

then there would be a rapidly established steady state at which the ratio of [compound I]/[MPO] would be constant, independent of the initial concentration of H_2O_2. One would predict that varying the initial concentration of H_2O_2 would not have much of an effect on the yield of compound I. This is not consistent with our present findings.

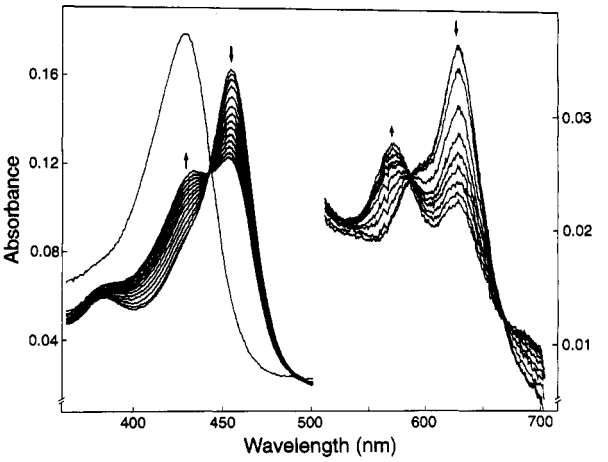

FIGURE 7: Spontaneous decay of compound II. Arrows show the direction of the change of absorbance with time. Compound II was formed by adding 17 μM H_2O_2 to 1.0 μM myeloperoxidase in 0.1 M phosphate buffer (pH 7.1). The total scan time is approximately 62 min.

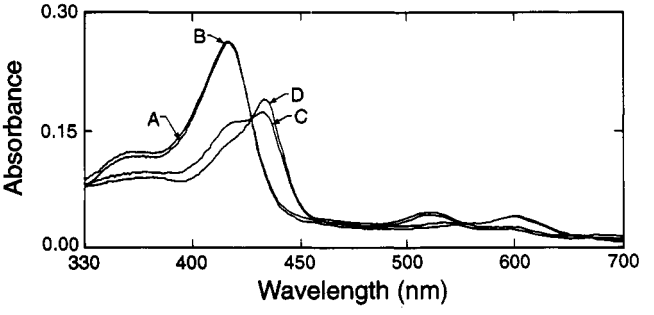

FIGURE 8: Effect of enzyme purity on the amount of H_2O_2 required to prepare compound II. Scans A and B are for the 1.5 μM native enzyme with RZ 0.81 and 0.85, respectively. Scans C and D were recorded after 20 equiv of H_2O_2 was added to A and B, respectively.

Pseudo-first-order conditions applied to compound I formation yield k_{obs} values that increase linearly with H_2O_2 concentration (Figure 2). The apparent second-order rate constant we obtained, $(1.8 \pm 0.1) \times 10^7$ M^{-1} s^{-1}, is comparable to values previously reported: $(1.0 \pm 0.2) \times 10^7$ M^{-1} s^{-1} for rat chloroma peroxidase (Newton et al., 1965); 2.0×10^7 M^{-1} s^{-1} for canine pus myeloperoxidase (Harrison, 1976); and 2.3×10^7 M^{-1} s^{-1} for human granulocyte myeloperoxidase (Bolscher & Wever, 1984). The rate of myeloperoxidase compound I formation is also the same as that for compound

I formation of horseradish peroxidase (Hewson & Dunford, 1975).

That compound I formation is reversible is indicated by a finite intercept in the plot of k_{obs} vs H_2O_2. This reversibility can also provide an explanation for the large excess of H_2O_2 required for compound I formation. The reversibility of compound I formation, however, is not readily accepted by some researchers because the formation of compound I of other peroxidases is generally believed to be irreversible. This issue was discussed in a recent review that invoked redox potentials to support the reversibility of myeloperoxidase compound I formation (Hurst, 1990). Moreover, a reversible compound I formation can also explain why it is difficult to record the spectra of relatively pure compound I. With too little H_2O_2, the equilibrium reaction will not go far to the right; hence compound I formation will be incomplete. On the other hand, this incompletely formed compound I is subject to further reaction with H_2O_2. Therefore, the spectra we recorded at the two extremes are those of a mixture of either compound I and native enzyme or compounds I and II. For intermediate cases, all three species are present simultaneously. Therefore, at no time could we observe absolutely pure compound I. In the case of a large excess of H_2O_2, however, the equilibrium lies much further to the right, so that compound I is almost fully formed before it is reduced further to compound II by H_2O_2, enabling us to record the spectrum of relatively pure compound I.

Although compound I of myeloperoxidase is not a Michaelis–Menten type complex, the assumption of a reversible process for its formation yields a dissociation constant, K_{diss}, of 3.2 μM from the ratio of the reverse and forward rate constants. The value was reported to be 1.7 μM in a previous paper, on the basis of a Scatchard plot (Bolscher & Wever, 1984). However, the authors of that paper interpreted the intercept of the ordinate as $1/K_{diss}$, while in fact it should be $2/K_{diss}$ for the two-site binding that occurs in myeloperoxidase. Therefore, the correct value for K_{diss} based on their data should be 3.4 μM. This correction is also necessary for self-consistency in interpreting their Scatchard plot: the K_{diss} derived from the slope of the plot is close to 3.4 μM.

The pH dependence of k_{1f} (Figure 3) suggests the existence of an acid-base group which, when protonated, prevents the reaction between myeloperoxidase and H_2O_2. Such a group cannot come from the substrate, H_2O_2, because its pK_a is around 12. Therefore, the only mechanism that will account for the pH dependence is:

$$\begin{array}{ccc} \text{MPO} + \text{H}_2\text{O}_2 & \underset{k_{1b}}{\overset{k_{1f}}{\rightleftharpoons}} & \text{compound I} \\ \text{H}^+ \Big\| K_1 & & \\ \text{MPO–H}^+ & & \end{array} \qquad (3)$$

which leads to eq 4:

$$k_{1f} = \frac{k_{1,int}}{1 + [H^+]/K_1} \qquad (4)$$

where $k_{1,int}$ is the intrinsic or pH-independent second-order rate constant. A nonlinear least-squares analysis was performed to fit the data points in Figure 3 to eq 4. The fitting yields a pK_1 value of 4.0 ± 0.1 for the acid–base group in the enzyme and a value of (2.4 ± 0.1) × 10^7 M^{-1} s^{-1} for $k_{1,int}$.

The pH dependence of the formation rate of compound I has been studied for lactoperoxidase (Maguire et al., 1971), turnip peroxidase (Job et al., 1978), intestinal peroxidase

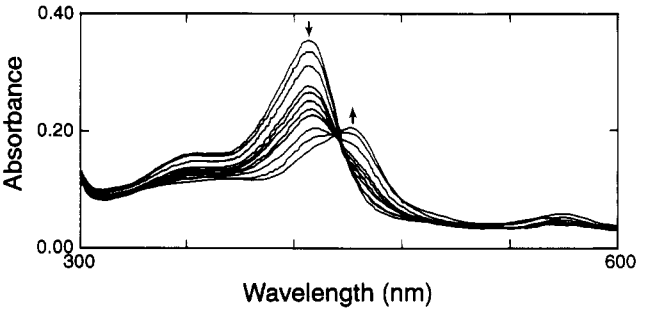

FIGURE 9: Spectral changes for cyanide binding to myeloperoxidase. The same patterns were observed when hemi-myeloperoxidase was titrated with the same amounts of cyanide. 5 μM cyanide was added successively to 4 μM enzyme (on a per heme basis) in 0.1 M ionic strength phosphate buffer at pH 6.6. The arrows show the direction of absorbance changes with time.

(Kimura & Yamazaki, 1979), horseradish peroxidase (Araiso & Dunford, 1980), human myeloperoxidase (Bolscher & Wever, 1984), bromoperoxidase (Manthey & Hager, 1985) and lignin peroxidase (Glenn & Gold, 1985). These studies indicate that peroxidases possess a distal ionizable group, the protonation state of which controls the rate of compound I formation. The pK value of the group has been reported to be in the range 3.0–5.3. The pK value of 4.0 obtained here suggests that compound I formation of myeloperoxidase is similar to that of other peroxidases, in which an electron push–pull mechanism may be involved (Dunford, 1990): a deprotonated proximal group provides the push, while a distal histidine provides the pull in the heterolytic cleavage of H_2O_2. Thus, His 52 in cytochrome c peroxidase, His 42 in horseradish peroxidase (Poulos, 1987), and His 47 in lignin peroxidase (Edwards et al., 1993) have been proposed to be the distal residue. Recently, X-ray crystallographic studies revealed that His 95 and Arg 239 are the amino acid residues likely to participate directly in the catalytic mechanism of myeloperoxidase (Zeng & Fenna, 1992). That histidine is present in the active site of the enzyme was further confirmed by the effect of diethyl pyrocarbonate on the enzymatic activity of mature and recombinant myeloperoxidase (Jacquet et al., 1991).

The second-order rate constant, k_2, for the reduction of compound I by H_2O_2 is also pH-dependent, and its pH profile appears similar to that for compound I formation. On the basis of the same reasoning for compound I formation, a mechanism for the reaction of compound I with H_2O_2 is proposed:

$$\begin{array}{ccc} \text{MPO–I} + \text{H}_2\text{O}_2 & \xrightarrow{k_2} & \text{MPO–II} + \text{O}_2^- \\ \text{H}^+ \Big\| K_2 & & \\ \text{MPO–I–H}^+ & & \end{array} \qquad (5)$$

A k_2–pH relationship similar to that in eq 4 is obtained and expressed:

$$k_2 = \frac{k_{2,int}}{1 + [H^+]/K_2} \qquad (6)$$

where $k_{2,int}$ represents the intrinsic second-order rate constant for compound I reacting with H_2O_2. The parameters obtained from the curve fit are pK_2 = 4.6 ± 0.1 and $k_{2,int}$ = (8.2 ± 0.2) × 10^4 M^{-1} s^{-1}.

Superoxide is generated from the one-electron oxidation of H_2O_2 by compound I to compound II. Cytochrome c added to the MPO/H_2O_2 system was found by other workers to be

reduced, presumably by superoxide generated therein (Hoogland et al., 1988). Although we were unable to demonstrate the generation of O_2^- by the reduction of ferric cytochrome *c*, reduction of tetranitromethane clearly showed that O_2^- is indeed produced in the system. Moreover, increasing the concentration of myeloperoxidase and/or H_2O_2 increases the amount of O_2^- produced (Figure 6). It is unlikely that O_2^- stays as a mere spectator in the conversion of compound I to compound II. Its role as a reducing agent needs to be considered as well.

An interesting feature associated with the time courses for the conversion of compound I to compound II is their biphasic nature, which becomes monophasic when (i) H_2O_2 of higher concentration is used, (ii) the pH is lowered from neutral to acidic values, or (iii) superoxide dismutase is present. Apparently, the reduction of compound I to compound II is dependent not only upon H_2O_2 but also upon O_2^- generated from the oxidation of H_2O_2. Hydrogen peroxide directly and rapidly reduces compound I. Superoxide, on the other hand, once generated from H_2O_2 oxidized by compound I, can rapidly bind to compound I and then slowly convert compound I to compound II, causing a slower reaction. Thus, a biphasic time course is observed. However, a high concentration of H_2O_2 favors the fast reduction of compound I by H_2O_2. Therefore, a convergence of the time courses to one faster phase should be observed with increasing H_2O_2 concentration. This is confirmed by the results shown in Figure 4A,B.

Presumably, O_2^- binds to an amino acid somewhat distant from the ferryl oxygen and initiates a protein-mediated electron transfer to the iron center of compound I, much like the scheme proposed for halide binding to the myeloperoxidase–cyanide complex (Lee et al., 1991). The binding does not lead to a direct contact between superoxide and the ferryl oxygen. Hence, no significant spectral changes can be achieved to prove the existence of the compound I–O_2^- complex.

The proposed binding between O_2^- and compound I may also be weakened by the protonation of O_2^-, which occurs at around pH 4.8. Moreover, the pH–rate profile for compound II formation indicates that the rate of reduction of compound I by H_2O_2 to form O_2^- is lower at pH values below 5.0. With O_2^- being produced at such a slow rate, the reduction of compound I by O_2^- becomes insignificant. Thus, a monophasic time course for compound I reduction is also observed at acidic pH.

The interference of O_2^- in the reduction of compound I by H_2O_2 can be suppressed by adding SOD to the system, and this is demonstrated by the results shown in Figure 5. The requirement of a relatively high concentration of SOD prompted us to consider the possible role of the protein moiety of SOD as a reducing substrate for compound I. While both SOD and bovine serum albumin promote the reduction of compound I, there is a notable difference between their effects: SOD can turn the biphasic time course into a monophasic one whereas BSA cannot. We attribute this difference to the removal of O_2^- by SOD but not by BSA. However, we still have to consider why BSA causes the rate to be faster. Once compound I has formed, the possibility of its autoreduction cannot be neglected. With a reduction potential sufficiently high to oxidize chloride, compound I of myeloperoxidase should be able to oxidize many other species as well. The possible electron donors responsible for the autoreduction of compound I may include one or several residues in the protein moiety of the enzyme that facilitate the reduction of compound I through an intramolecular electron transfer. Thus, BSA can speed up the reduction of compound I to compound II, presumably through the involvement of its residue(s) as reducing substrate(s) for compound I. Another possible source of electron donors are the impurities in the samples. We found that even a slight difference in the RZ values of the enzyme preparation used could affect the amount of H_2O_2 required to prepare the oxidized intermediates of myeloperoxidase. Thus, the different stoichiometries reported for the reaction of myeloperoxidase with H_2O_2 may also be due to differences in the enzyme preparation.

It is expected that oxygen would be produced as a result of the reduction of compound I to compound II by O_2^-, although at a much lower level because the amount of O_2^- generated would depend on the initial amount of enzyme and the rate of recycling of compound II to native enzyme.

The reduction of compound II by O_2^-, which was previously proposed for lactoperoxidase (Kohler & Jenzer, 1989), is neglected in this study because the return of compound II to native enzyme is very slow. Evidence can be found in the decay spectra of compound II to native enzyme (Figure 7). We do not deny previously presented spectral evidence for the reduction of compound II by O_2^- to native enzyme, but it must be noted that those studies were performed under conditions where an O_2^-- generating system was added to the MPO/H_2O_2 system (Kettle & Winterbourn, 1988, 1989). The slow reduction of compound II to native enzyme by O_2^- should not be cause for alarm, as compound II is inactive in the generation of HOCl. There are other reducing substrates in the leukocyte that are capable of recycling compound II to native enzyme at considerably faster rates. Ascorbic acid is one example (Marquez & Dunford, 1990).

There is ample data in the literature on O_2^- reactions with native heme enzymes to form compound III that are inhibited by SOD, which are related to our present studies on compound I reduction, in that they provide other examples of heme enzyme inhibition by O_2^- (Odajima & Yamazaki, 1972; Kono & Fridovich, 1982; Shimizu et al., 1984).

One other possibility explored to explain the unique behavior of myeloperoxidase in its reaction with H_2O_2 is a difference in the reactivity of the enzyme subunits. The biphasic time courses could have been caused by a different reactivity of the two hemes, or it could be that the hemes exert a certain degree of negative cooperativity, such that when one heme is bound the rate of binding to the other is reduced. This possibility was considered in view of a recent finding that distinct chromatographic forms of human hemi-myeloperoxidase have been obtained by cleaving the dimeric enzyme (Taylor et al., 1990).

Cyanide binding has been used to investigate the binding site of heme proteins (Yoshikawa et al., 1985). To investigate whether functional inequivalence exists between the subunits of myeloperoxidase, we used reductive alkylation to break the enzyme into its monomer units (Andrews & Krinsky, 1981), and we performed cyanide binding studies on the dimer and monomer. Titration of both monomer and dimer resulted in the same spectral patterns. Moreover, the second-order rate constants were very similar. We therefore conclude that the subunits have the same reactivity. These results confirm identical activities reported for native and hemi-myeloperoxidase (Andrews et al., 1984).

In conclusion, we have investigated the formation of myeloperoxidase compound I and its conversion to compound II in the presence of H_2O_2. At least a 20-fold excess of H_2O_2 was used to obtain relatively pure compound I. pH dependence studies on both compound I and compound II formation support the presence of a distal histidine residue in the active

site of the enzyme. The time courses for the conversion of compound I to compound II are biphasic and dependent on H_2O_2 concentration. We found that this is not due to a different reactivity of the enzyme subunits. Using sufficient H_2O_2, lowering the pH of the reaction mixture or adding SOD can convert the biphasic reactions to monophasic ones. We explain the unique behavior of myeloperoxidase in its reaction with H_2O_2 as due to (i) the reversibility of compound I formation, (ii) the role of both H_2O_2 and O_2^- as reducing substrates of compound I, and (iii) the importance of enzyme purity in the stoichiometry of formation of the enzyme's oxidized intermediates.

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