Reaction of Myeloperoxidase Compound I with Chloride, Bromide, Iodide, and Thiocyanate

Paul Georg Furtmüller, Ursula Burner, and Christian Obinger*

Institute of Chemistry, University of Agricultural Sciences, Muthgasse 18, A-1190 Vienna, Austria

Received August 5, 1998; Revised Manuscript Received September 23, 1998

ABSTRACT: Myeloperoxidase plays a fundamental role in oxidant production by neutrophils. The enzyme uses hydrogen peroxide to oxidize chloride (Cl^-), bromide (Br^-), iodide (I^-), and the pseudohalide thiocyanate (SCN^-) to their respective hypohalous acids. This study for the first time presents transient kinetic measurements of the oxidation of these halides and thiocyanate by the myeloperoxidase intermediate compound I, using the sequential mixing stopped-flow technique. At pH 7 and 15 °C, the two-electron reduction of compound I to the native enzyme by Cl^- has a second-order rate constant of $(2.5 \pm 0.3) \times 10^4$ M^{-1} s^{-1}, whereas reduction of compound I by SCN^- has a second-order rate constant of $(9.6 \pm 0.5) \times 10^6$ M^{-1} s^{-1}. Iodide [$(7.2 \pm 0.7) \times 10^6$ M^{-1} s^{-1}] is shown to be a better electron donor for compound I than Br^- [$(1.1 \pm 0.1) \times 10^6$ M^{-1} s^{-1}]. The pH dependence studies suggest that compound I reduction by (pseudo-)halides is controlled by a residue with a pK_a of about 4.6. The protonation of this group is necessary for optimum (pseudo-)halide anion oxidation. These transient kinetic results are underlined by steady-state spectral and kinetic investigations. SCN^- is shown to be most effective in shifting the system myeloperoxidase/hydrogen peroxide from the peroxidatic cycle to the halogenation cycle, whereas iodide is shown to be more effective than bromide which in turn is much more effective than chloride. Decreasing pH increases the rate of this transition. Our results show that thiocyanate is an important substrate of myeloperoxidase in most environments and that hypothiocyanate is likely to contribute to leukocyte antimicrobial activity.

Myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) is present in high concentrations in the granules of polymorphonuclear leucocytes and monocytes (*1*). It is released during phagocytosis and is believed to have a role in the killing of microorganisms and inactivation of viruses (*1*). Myeloperoxidase (MPO) catalyzes the oxidation of halide ions and the pseudo-halide thiocyanate by H_2O_2, producing oxidizing and halogenating agents. Oxidation of a halide (X^-) yields the halogen (X_2), hypohalous acid (HOX), or hypohalite ion (OX^-). In aqueous media, products of halide oxidation are in rapid equilibrium, and the principal form is a HOX/OX^- mixture. These agents have antimicrobial and antiviral activity but may also damage host tissues and contribute to inflammatory tissue injury (*2*).

Halide oxidation starts by reaction of ferric-MPO with H_2O_2 to form compound I, which contains 2 oxidizing equiv more than the resting enzyme. Halides reduce compound I directly to native enzyme by a two-electron process. During turnover, some compound I is also converted to compound II because of the presence of exogenous (AH_2) or endogenous electron donors (*3*). Compound II is believed to be inactive in (pseudo-)halide oxidation.

$$\text{native enzyme} + \text{HOOH} \rightarrow \text{compound I} + \text{HOH} \quad (1)$$

$$\text{compound I} + X^- + H^+ \rightarrow \text{native enzyme} + \text{HOX} \quad (2)$$

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + {}^{\bullet}AH \quad (3)$$

$$\text{compound II} + AH_2 \rightarrow \text{native enzyme} + {}^{\bullet}AH \quad (4)$$

Because of its high concentrations, chloride is assumed to be the physiological substrate for myeloperoxidase (MPO). Its plasma concentrations are 100−140 mM (*4*), in contrast to 20−100 μM bromide, 0.1−0.6 μM iodide, and 20−120 μM thiocyanate (*5*). The evidence that hypochlorous acid is produced in the phagosome is mostly indirect (*1*, *2*). Though the actual phagosomal concentrations of chloride are unknown, there is no doubt that appreciable amounts of hypochlorous acid are discharged into the extracellular space surrounding neutrophils. Depending on the stimulus, it has been shown that hypochlorous acid accounts for 20−70% of the hydrogen peroxide liberated by these inflammatory cells (*6*, *7*).

Recently, van Dalen et al. (*8*) showed that thiocyanate is by far the most preferred substrate for myeloperoxidase. They showed that at plasma concentrations of chloride and thiocyanate, up to half of the H_2O_2 used by MPO is converted into hypothiocyanite. Moreover, other substrates compete well with chloride, particularly when chloride levels are low,

* To whom correspondence should be addressed. Telephone: +43-1-36006-6073. Fax: +43-1-36006-6059. Email: cobinger@edv2.boku.ac.at.

10.1021/bi9818772 CCC: $15.00 © 1998 American Chemical Society
Published on Web 12/03/1998

such as in secreted fluids. Thomas and Fishman (*9*) investigated the oxidants produced under conditions similar to those of saliva, where thiocyanate is present at 1−5 mM, and showed that hypothiocyanite is the dominant oxidant formed.

Chlorination is known to show a very complex kinetic pattern depending on both the ratio of H_2O_2/chloride and the pH (*10*). Moreover, chloride not only acts as a substrate for MPO but also behaves as a competitive inhibitor of H_2O_2 (*11*−*13*). With these limitations, steady-state kinetic analysis of MPO is complicated. One unknown element in this overall kinetic puzzle is the knowledge how efficiently halides and thiocyanate are oxidized by the redox intermediate compound I. So far, several problems made it difficult to determine actual rates of these reactions. Compound I of human myeloperoxidase is very unstable and spontaneously decays to its one-electron reduction product, compound II. Moreover, an excess of H_2O_2 is required to achieve the full formation of compound I, which makes the investigation of myeloperoxidase compound I much more difficult than those of other peroxidases.

Here we present for the first time a comparative transient-state kinetic analysis and pH dependence of the reaction of compound I with halides and thiocyanate. Sequential stopped-flow spectroscopy was used to circumvent the problems which so far prevented analysis of this reaction. We show that thiocyanate is by far the best electron donor for compound I. Iodide is shown to be a more effective electron donor to compound I than bromide which in turn is much more effective than chloride. There is a dramatic increase in reaction rate upon decreasing the pH from 8 to 4. Our pH studies suggest that reaction of compound I with (pseudo-)-halides is controlled by a residue in the enzyme with a pK_a at 4.6. The physiological consequences are discussed.

EXPERIMENTAL PROCEDURES

Materials. Myeloperoxidase was purified from human neutrophils to a purity index (A_{430}/A_{280}) of at least 0.86 as described by Kettle and Winterbourn (*14*). Its concentration was calculated using $\epsilon_{430} = 91\ 000\ M^{-1}\ cm^{-1}$ per heme (*15*). Hydrogen peroxide, obtained from a 30% solution from Sigma Chemical Co., was diluted and the concentration determined by absorbance measurement at 240 nm where the extinction coefficient is 39.4 $M^{-1}\ cm^{-1}$ (*16*). The other chemicals were also purchased from Sigma Chemical Co. at the highest grade available.

SDS−PAGE was performed in the denaturating reducing system ($T = 12\%$, $C = 2.67\%$) described by Laemmli (*17*) using high molecular weight silver stain markers (Sigma) for subunit molecular weight determination.

MALDI-TOF-MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) was carried out on a DYNAMO MALDI-TOF-MS (Thermo BioAnalysis, Santa Fe, NM) with a sinapinic acid matrix. Spectra were recorded in the dynamic extraction mode (setting 0.1) and calibrated externally using BSA. The protein sample was mixed with a 1% solution of the matrix in 70% acetonitrile. One microliter of this mixture was deposited to a probe, air-dried, and inserted into the mass spectrometer to acquire a spectrum.

Transient-State Experiments. The sequential stopped-flow apparatus (Model SX-18MV) and the associated computer system were from Applied Photophysics (U.K.). For a total of 100 μL/shot into a flow cell with 1 cm light path, the fastest time for mixing two solutions and recording the first data point was of the order of 1.5 ms. Because of the inherent instability of MPO compound I (*18*, *19*), sequential stopped-flow (multimixing) analysis was used for determination of rates of the reaction of compound I with halides and thiocyanate. Consequently, we started to look at the reaction of MPO with hydrogen peroxide at 430 nm in the conventional stopped-flow mode; 430 nm is the absorbance maximum (Soret band) of both the native enzyme and the redox intermediate compound I, which—compared with the native enzyme—is characterized by a 50% hypochromicity in the Soret band. In a typical experiment, one syringe contained 2 μM MPO in 100 mM phosphate buffer (pH 7.0) while the other contained various concentrations of H_2O_2. The bimolecular rate constant of compound I formation was derived from a plot of k_{obs} versus varying peroxide concentrations (not shown). It was calculated to be $(1.4 \pm 0.2) \times 10^7\ M^{-1}\ s^{-1}$ at 15 °C (the temperature used in the pre-steady-state experiments), which is in accordance with the literature (*18*, *19*). With 1.0 μM heme in the cuvette, the minimum hydrogen peroxide concentration required for complete formation of compound I was 10 μM. With these concentrations, compound I is formed completely within 20 ms and is stable for at least 40 ms.

Rapid spectral scans of MPO and its intermediates during reaction of MPO with H_2O_2 were conducted by taking time-dependent spectra from single-wavelength shots. The spectral region from 400 to 480 nm was scanned at 2 nm intervals for 1000 ms. The spectra generated from the rapid scans were reconstructed using the GLint application software from Applied Photophysics.

For determination of actual rates of the reaction of MPO compound I with halides and thiocyanate, MPO (4 μM heme) was premixed with 40 μM H_2O_2 in the aging loop for 20 ms (100 mM phosphate buffer, pH 7.0). Finally compound I was allowed to react with varying concentrations of (pseudo-)halides, the final concentrations of which were at least 10 times in excess of the enzyme to ensure first-order kinetics. The reactions were followed at both 430 and 456 nm. At 456 nm, compound II formation is monitored. Compound II formation is known to occur upon oxidation of H_2O_2 by compound I (*19*) or the presence of endogenous electron donors and represents an undesirable side-reaction in our investigation. At least three determinations (1000−2000 data points) of k_{obs} were performed for each substrate concentration, and the mean value was used in the calculation of the second-order rate constants, which were calculated from the slope of the line defined by a plot of k_{obs} versus substrate concentration.

To determine the pH dependence of the reduction of compound I to the native enzyme, the sequential stopped-flow experiments were performed at different pH values from 4−8.0. In a typical pH-jump experiment, 4 μM MPO in 5 mM phosphate buffer (pH 7.0) was premixed with 40 μM H_2O_2 in distilled water. After a delay time of 20 ms, compound I was allowed to react with varying concentrations of (pseudo-)halides in 200 mM phosphate/citrate buffer (pH 4−8). A dramatic increase of reaction rates at acidic conditions made it necessary to perform all stopped-flow investigations at 15 °C.

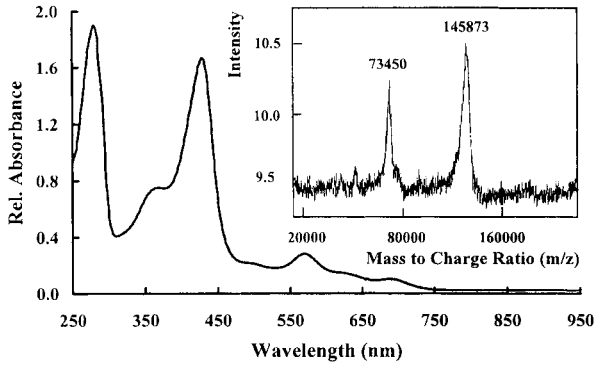

FIGURE 1: Spectrum of native myeloperoxidase (15 μM) in 5 mM phosphate buffer (pH 7.4). The enzyme used in these studies had RZ values (A_{430}/A_{280}) of at least 0.86. The inset shows the corresponding MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) mass spectrum.

To confirm that MPO catalyzes (pseudo-)halide oxidation via two-electron reduction of compound I, we have also investigated the reaction between compound II and the (pseudo-)halides in the sequential mixing mode. Compound II was prepared by mixing 4 μM MPO with 200 μM H_2O_2 in the aging loop (100 mM phosphate buffer, pH 7.0). Finally after 20 s, MPO compound II was allowed to react with 100 μM (pseudo-)halides. The decrease in absorbance at 456 nm was followed by monitoring compound II disappearance.

Steady-State Experiments. To evaluate the steady-state spectra during (pseudo-)halide oxidation, MPO was incubated with 100 μM (pseudo-)halides and hydrogen peroxide. MPO (500 nM) was dissolved in 100 mM phosphate buffer (pH 7.0 and 5.0) containing 200 mM glucose. Reaction was started by addition of glucose oxidase at 25 °C. To guarantee a constant flux of 5 μM H_2O_2 per minute, 0.0131 unit of GOD/mL at pH 7.0 and at 0.0124 unit of GOD/mL at pH 5.0 were necessary [unit definition: 1 unit of glucose oxidase (GOD) oxidizes 1 μmol of β-D-glucose to D-gluconic acid and H_2O_2 at pH 5.1 at 35 °C]. Upon incubation with hydrogen peroxide, the enzyme shifted to compound II. After 30 s, 100 μM (pseudo-)halides was added, and the spectral conversions were monitored. Routine absorbance measurements were made on a Zeiss Specord S-10 diode-array spectrophotometer equipped with a stirred thermally jacketed cuvette holder (25 °C).

RESULTS

Enzyme Purity. The myeloperoxidase used in these studies had RZ values (A_{430}/A_{280}) of at least 0.86, which indicated highly purified enzyme preparations. Figure 1 shows the spectrum of a 15 μM MPO stock solution used in this work. There was no shoulder at 412 nm, which would indicate the presence of eosinophil peroxidase (*20*). Eosinophil peroxidase is known to oxidize thiocyanate to hypothiocyanate (*20*) and could copurify with MPO. Both SDS−PAGE (data not shown) and MALDI-TOF-spectroscopy (inset to Figure 1) demonstrated the high purity of the enzyme preparations. SDS−PAGE under reducing conditions gave three bands with molecular masses of 59, 38, and 15 kDa (data not shown), as reported by others (*8*, *21*, *22*), whereas the mass spectrum (inset to Figure 1) unequivocally showed that there was only one protein present. The peak at 145.8 kDa very well fitted with the molecular mass of the dimeric enzyme

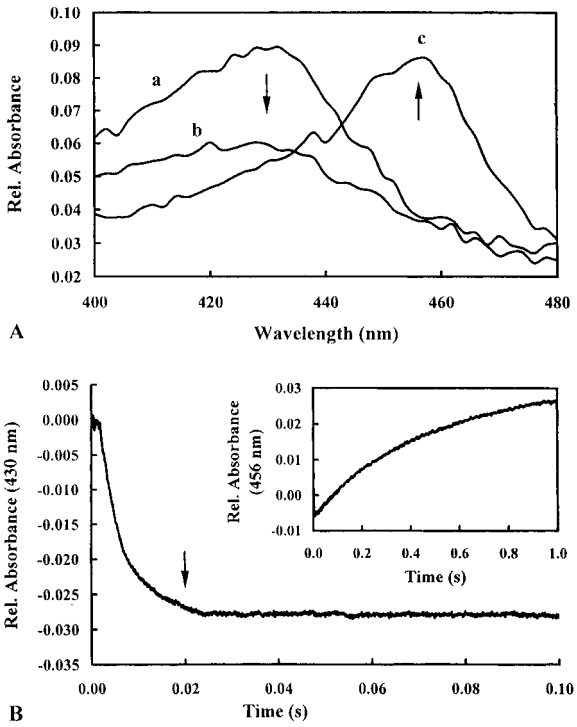

FIGURE 2: (A) Rapid-scan spectra of native myeloperoxidase (a) and after adding 10-fold excess H_2O_2. The spectra were taken (b) 20 ms and (c) 1000 ms after the flow stopped. Arrows show the direction of absorbance changes with time. Final enzyme concentration: 1 μM in 100 mM phosphate buffer, pH 7.0. (B) Typical time traces of this reaction followed at 430 and 456 nm (inset) showing compound I formation followed by slower conversion of compound I to compound II. The arrow indicates the delay time used in the sequential mixing stopped-flow experiments.

[$(M+H)^+$, singly charged species], whereas the peak at 73.5 kDa represented another *m*/*z* value of the same analyte species [$(M+H)^{2+}$, doubly charged species].

Compound I Formation. Myeloperoxidase compound I is distinguished from the native form of the enzyme by its reduced absorbance in the Soret region. In contrast to horseradish peroxidase, which forms a relatively stable compound I with equimolar H_2O_2 concentrations (*23*), compound I of human myeloperoxidase is very unstable and spontaneously decays to its one-electron reduction product, compound II. Figure 2A shows the spectra of the relevant enzyme intermediates formed upon addition of a 10-fold excess of hydrogen peroxide to the native enzyme. The spectra were generated from a sequence of kinetic records collected at a series of monochromator wavelengths between 400 and 480 nm at 2 nm intervals. In Figure 2A, the points in time at which the slices across these records were taken are at 0 ms (spectrum a, representing native enzyme), 20 ms (spectrum b, compound I), and 1000 ms (spectrum c, compound II) after the flow had stopped. Figure 2B shows typical stopped-flow traces at 430 and 456 nm (inset to Figure 2B). The conversion of native MPO to compound I was followed at 430 nm and displayed a monophasic exponential character. Compound I formation was followed by a much slower increase in absorbance at 456 nm (Soret maximum of compound II) as a result of the reaction between compound I and H_2O_2 (*19*).

Reaction of Halides and Thiocyanate with Compound I. To guarantee pre-steady-state conditions and to minimize

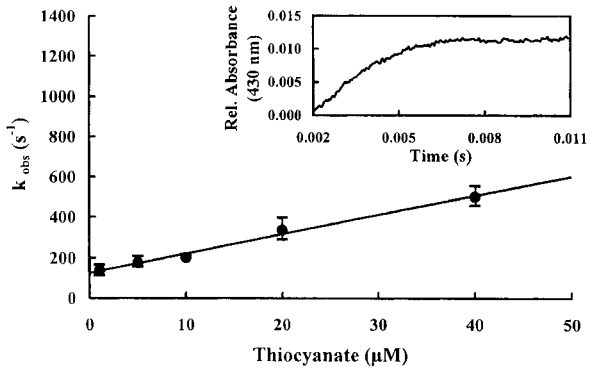

FIGURE 3: Pseudo-first-order rate constants for compound I reduction by thiocyanate. The inset shows a typical trace of the reaction followed at 430 nm using the sequential mixing mode. Final concentrations were 1 μM MPO, 10 μM H_2O_2 in phosphate buffer (100 mM, pH 7.0). The second-order rate constant was calculated from the slope. The standard deviations are indicated.

Table 1: Apparent Second-Order Rate Constants for the Reactions of Myeloperoxidase (MPO) Compound I with Thiocyanate, Chloride, Bromide, and Iodide, Respectively, at pH 7.0 and 5.0[a]

(pseudo-)halides	pH 7.0	pH 5.0
chloride	$(2.5 \pm 0.3) \times 10^4$	$(3.9 \pm 0.4) \times 10^6$
bromide	$(1.1 \pm 0.1) \times 10^6$	$(3.0 \pm 0.2) \times 10^7$
iodide	$(7.2 \pm 0.7) \times 10^6$	$(6.3 \pm 0.7) \times 10^7$
thiocyanate	$(9.6 \pm 0.5) \times 10^6$	$(7.6 \pm 0.6) \times 10^7$

[a] The second-order rate constants were calculated from the slope of plots of halide or thiocyanate concentration versus pseudo-first-order rate constants. Reaction was followed at 430 nm and 15 °C using the sequential mixing stopped-flow technique. Final concentrations were 1 μM MPO and 10 μM H_2O_2 in 100 mM phosphate buffer.

compound II formation, we determined the minimum hydrogen peroxide concentration which was necessary in order to get a maximum decrease in absorbance at 430 nm. It is worth noting that with highly purified MPO (RZ > 0.86) it took less H_2O_2 to completely form compound I. With our preparations (RZ > 0.86), a 2−8-fold excess of hydrogen peroxide led to incomplete formation of compound I, whereas a larger excess than 10-fold did not improve the absorbance decrease at 430 nm, but brought a stronger interference with compound II formation. Consequently, we premixed a 10-fold excess of H_2O_2 with MPO in the aging loop for 20 ms. During this aging time, compound I formation took place, and before its decay, it was mixed (arrow in Figure 2B) with varying concentrations of halides and thiocyanate, the final concentrations of which were at least 10 times in excess of the enzyme to ensure pseudo-first-order kinetics. The reaction was followed at both 430 nm (monitoring formation of native enzyme) and 456 nm (monitoring compound I reduction to compound II by H_2O_2).

Typical kinetic traces for compound I reduction by halides and thiocyanate displayed single-exponential character (see inset to Figure 3). In Figure 3, the apparent second-order rate constant for the reaction between compound I and thiocyanate was obtained from the slope of the linear plot of pseudo-first-order rate constants, k_{obs}, against thiocyanate concentration. Similar plots were obtained with chloride, bromide, and iodide, respectively. As Table 1 summarizes, thiocyanate was by far the best electron donor for compound I [$(9.6 \pm 0.5) \times 10^6$ M^{-1} s^{-1}] at pH 7.0 and 15 °C. Iodide was a more effective electron donor to compound I [$(7.2 \pm 0.7) \times 10^6$ M^{-1} s^{-1}] than bromide [$(1.1 \pm 0.1) \times 10^6$ M^{-1} s^{-1}], which in turn was much more effective than chloride [$(2.5 \pm 0.3) \times 10^4$ M^{-1} s^{-1}]. With chloride, at least 2 mM of substrate was necessary in order to get the typical exponential increase of absorbance at 430 nm. This was consistent with the fact that at pH 7.0 (25 °C) the rate constant of (one-electron) reduction of compound I to compound II by hydrogen peroxide is $(8.2 + 0.2) \times 10^4$ M^{-1} s^{-1} (*19*) which is 3−4 times faster than (two-electron) reduction of compound I to native MPO by chloride. All plots showed a similar relatively large finite intercept at pH 7.0 (135 ± 15 s^{-1}), which could represent both reversible reaction between compound I and (pseudo-)halides and interference of compound I reduction by H_2O_2 to compound II. Moreover, the oxidation products (e.g., HOCl) could undergo a rapid reaction with the native MPO to give compound I, which would has been ostensibly the reverse of the halide oxidation reaction (*3*, *24*) and thus contributed to these large intercepts.

To confirm that MPO catalyzes hypohalite production via two-electron oxidation of (pseudo-)halides by compound I, we also investigated their reaction with compound II using the sequential mixing stopped-flow technique. The conversion of preformed compound II to the native enzyme was followed by both the disappearance of compound II (456 nm) and the appearance of the native enzyme (430 nm). With chloride, bromide, and iodide, no direct reduction of compound II (1 μM) to ferric MPO, which would be characterized by an exponential absorbance decrease at 456 nm, was seen. Addition of iodide and bromide led to a linear decrease in absorbance at 456 nm and a corresponding increase at 430 nm (not shown). Because a 50-fold excess of H_2O_2 to native enzyme was used for compound II preparation, this spectral conversion indicated a shift from the peroxidation cycle to the halogenation cycle. The peroxidation cycle was maintained by hydrogen peroxide and superoxide, which is known to be produced during compound I reduction to compound II by H_2O_2 (*19*). In contrast to bromide and iodide, addition of 10−200 μM thiocyanate to preformed compound II induced an absorbance decrease at 456 nm within 4−6 s, similar to the kinetics known from compound II reduction by poor one-electron donors. Consequently, at the moment we cannot exclude compound II reduction by thiocyanate (which would occur at about 10^2 M^{-1} s^{-1}), because such slow rates make it difficult to maintain pre-steady-state conditions.

pH Dependence. Figure 4 shows the pH dependence of the apparent second-order rate constants (k_{app}) for the reaction of compound I with chloride. A similar pH dependence was seen for k_{app} for the reaction of compound I with bromide, iodide, and thiocyanate, respectively (not shown). The pH dependence suggested the existence of an acid−base group which, when protonated, favored reaction between compound I and the substrates. Such a group could not come from the substrates, because HCl, HBr, and HI are strong acids and the pK_a value of HSCN is −2 (*25*). Thus, both the halides and thiocyanate were completely in the anionic form in the pH range investigated, and the only mechanism that would account for the pH dependence was

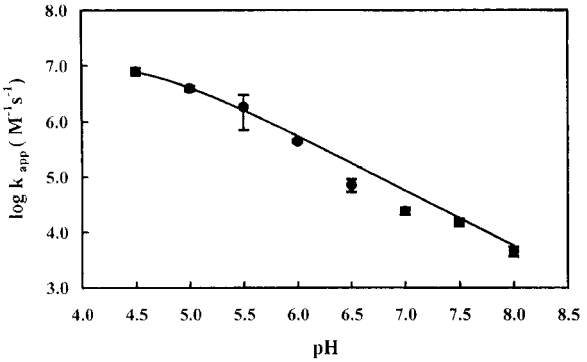

FIGURE 4: pH dependence of the apparent second-order rate constant of the reaction between compound I and chloride. Final concentrations were 1 μM MPO, 10 μM H_2O_2 in phosphate/citrate buffer (100 mM, pH 4−8). The curve was computed on the basis of best-fit parameters obtained from nonlinear least-squares analysis of the data points using eq 7 in the text.

$$\text{compound I} + H^+ \leftrightarrow \text{compound I}{-}H^+ \quad (5)$$

$$\text{compound I}{-}H^+ + X^- \rightarrow \text{native enzyme} + \text{HOX} \quad (6)$$

which led to eq 7:

$$k_{app} = (k_{int} \times [H^+])/(K_a + [H^+]) \quad (7)$$

where k_{int} is the intrinsic or pH-independent second-order rate constant of the reaction between compound I and chloride. A nonlinear least-squares analysis was performed to fit the data points in Figure 4 to eq 7. The fitting yielded a pK_a value of 4.6 ± 0.05 for the acid−base group in the enzyme and a value of (1.4 ± 0.08) × 10^7 M^{-1} s^{-1} for k_{int}. The pH-independent values calculated for bromide, iodide, and thiocyanate are (6.1 ± 0.1) × 10^7, (2.4 ± 0.15) × 10^8, and (2.6 ± 0.1) × 10^8 M^{-1} s^{-1}, respectively. The pK_a value calculated from these plots is in the range 4.5−4.9 within experimental error (not shown). Since the pH at the end of phagocytosis has been reported to fall in the range from 4.5 to 5.0 (*26*, *27*), we have summarized corresponding bimolecular rate constants at pH 5.0 in Table 1. A value of (3.9 ± 1.9) × 10^6 M^{-1} s^{-1} was obtained for compound I reduction by chloride, which is 150 times faster than at pH 7.0. Similarly, compound I mediated oxidation of bromide, iodide, and thiocyanate is increased at pH 5.0.

Steady-State Experiments. We were also interested in the enzyme form which dominated during (pseudo-)halide oxidation. Hydrogen peroxide was generated with the glucose/glucose oxidase system at a constant flux of 5 μM H_2O_2 per minute (thus guaranteeing H_2O_2 generation during a period of at least 40 min). Reaction was started by addition of glucose oxidase to MPO (500 nM) and glucose (200 mM) in air-saturated phosphate buffer (100 mM, pH 7.0). In the absence of (pseudo-)halides, the enzyme was converted to compound II within 10−20 s. Upon addition of 100 μM bromide, iodide, or thiocyanate, the dominating steady-state MPO intermediate changed from compound II (maximum at 456 and 625 nm) to ferric MPO (maximum at 430 nm). With chloride, no shift was detectable. Figure 5A demonstrates the spectral conversion upon addition of iodide to preformed compound II. With thiocyanate and bromide, similar spectral conversions occurred but at different time scales. In Figure 5B, the corresponding time traces at 456 nm, showing compound II disappearance, are compared. Thiocyanate was established to be very efficient in removing compound II as the dominating steady-state species, whereas with chloride the spontaneous reduction of compound I to compound II by H_2O_2 seemed to dominate over compound I reduction to native MPO by chloride. This was consistent with our stopped-flow studies showing chloride to be the worst electron donor for compound I.

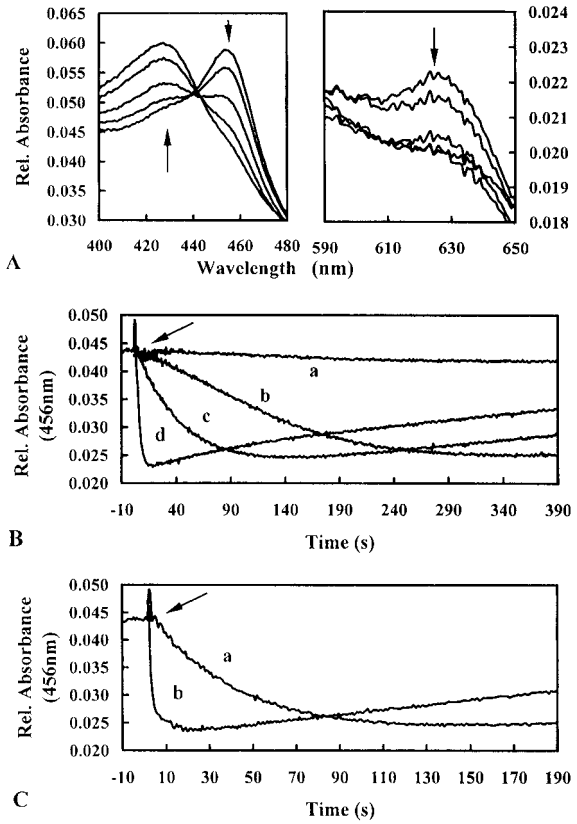

FIGURE 5: (A) Spectral conversion upon addition of 100 μM iodide to compound II (500 nM). Spectra were taken between 0 s (compound II) and 100 s (ferric enzyme) after addition of iodide. Compound II was generated by addition of glucose oxidase to MPO (500 nM), generating a constant flux of 5 μM H_2O_2 per minute in 100 mM phosphate buffer (pH 7.0) containing 200 mM glucose. (B) A comparison of typical time traces at 456 nm showing compound II disappearance upon addition of chloride (a), bromide (b), iodide (c), and thiocyanate (d), respectively. Same conditions as in (A). The arrow indicates addition of (pseudo-)halides 30 s after starting incubation of native MPO with H_2O_2. (C) Effect of pH on the conversion of compound II to native enzyme by iodide at pH 7.0 (a) and pH 5.0 (b), respectively. Same conditions as in (B).

Similar experiments were also performed at pH 5. Disappearance of compound II was accelerated at acidic pH values. In Figure 5C, the corresponding kinetic traces at 456 nm for the reaction with iodide at pH 5 and pH 7 are shown. That was in accordance with our pre-steady-state pH studies. Decreasing the pH favored electron donation to compound I by (pseudo-)halides whereas reduction of compound I by H_2O_2 became more and more unlikely (*19*). Consequently, the shift from the peroxidatic cycle to the halogenation cycle was more efficient at pH 5 rather than pH 7.

DISCUSSION

Myeloperoxidase-catalyzed H_2O_2-dependent oxidation of halide ions or SCN^- to yield halogens or other oxidizing

agents is important for its biological in vivo functions. Myeloperoxidase can catalyze the oxidation of Cl^-, Br^-, I^-, and SCN^-, but not of F^-. The reduction potential of 1.1 V for the MPO redox intermediate compound I (*10*) enables MPO to perform this two-electron oxidation, whereas the redox intermediate compound II is outside of the halogenation cycle. Compound I is a ferryl π-cation radical in which the iron has a formal oxidation state of +5 (*28*).

Considerable effort has been made to explore the ability of myeloperoxidase to discriminate in favor of a particular (pseudo-)halide (*8, 9*). The oxidation of halides and SCN^- shows an overall complex kinetic pattern. Steady-state studies have revealed that there is no fixed optimum pH for MPO-catalyzed (pseudo-)halide oxidation. In chlorination reactions, chloride not only acts as a substrate for MPO but also behaves as a competitive inhibitor of H_2O_2. Reversible binding of halogenides to native enzyme has been documented in several reports (*11*−*13*). The binding is pH-dependent. While hydrogen peroxide reacts with the native enzyme when the distal histidine is unprotonated (*19*), chloride appears to bind its protonated form to make an inhibitory chloride complex. Consequently, upon increasing pH, the affinity of Cl^- for the enzyme decreases. Moreover, it is a unique feature of MPO compound I that it is capable of oxidizing hydrogen peroxide to superoxide with the simultaneous formation of compound II (*18*), which is outside the chlorination pathway. Consequently, it depends on both the relative halide and H_2O_2 concentrations and the pH at which reaction predominates.

So far little was known about the direct reaction of compound I with halides. Kinetic parameters were extracted from steady-state data. For the reaction of compound I with thiocyanate (pH 7, 25 °C), bimolecular rate constants have been published to be 1.1×10^5 M^{-1} s^{-1} (*25*) and 3.3×10^5 M^{-1} s^{-1} (*8*), respectively. But these values are considerably less than the rate constants which were obtained by our pre-steady-state stopped-flow measurements [$(9.6 \pm 0.5) \times 10^6$ M^{-1} s^{-1} at pH 7.0 and 15 °C]. The reason for this discrepancy is the known fact that MPO activity decreases rapidly during the first seconds (*29*) with the consequence of grossly underestimating the real activity in classical steady-state experiments compared with that in the first milliseconds. Similarly, the values of k_{Cl^-} reported in the literature are considerably less than the rate constant obtained by our pre-steady-state measurements [$(2.5 \pm 0.3) \times 10^4$ M^{-1} s^{-1} at pH 7.0 and 15 °C]. There is only one paper in the literature reporting a value of the rate constant for compound I reduction by chloride, namely, $(4.7 \pm 0.1) \times 10^6$ M^{-1} s^{-1} (*30*). Whether this discrepancy between their results and our data derives from either the enzyme preparation or the experimental conditions (different temperature and method of preparation of compound I) cannot be answered presently. Nevertheless, our steady-state data demonstrate unequivocally that even at a large excess of chloride the rate-limiting step in enzyme turnover is reduction of compound II. In our system, the partitioning between the halogenation cycle and the peroxidation cycle depends on the relative rates at which the halides and H_2O_2 reduce compound I. Using bromide, iodide, and thiocyanate, we demonstrated that MPO exists mainly in the ferric form. Since reduction of compound I with hydrogen peroxide is known to occur with $(8.2 \pm 0.1) \times 10^4$ M^{-1} s^{-1} at 25 °C and pH 7 (*18*, *19*) and MPO exists mainly as compound II during chloride oxidation even at an excess of chloride, the value of the rate constant for compound I reduction by chloride should be of the same order of magnitude as that for compound I reduction by hydrogen peroxide.

Our results show that thiocyanate is by far the best electron donor for compound I (k_{SCN^-}:k_{Cl^-} = 385:1), followed by iodide (k_{I^-}:k_{Cl^-} = 290:1) and bromide (k_{Br^-}:k_{Cl^-} = 44:1), respectively. In the neutral region, chloride is demonstrated to be less effective in electron donating to compound I even than H_2O_2. These results are confirmed by the work of van Dalen et al. (*8*), who extracted specificity constants from steady-state data. They showed that the specificity constant for thiocyanate is 730 times that for chloride, whereas the value for bromide is 60 times that for chloride.

Our pH studies suggest that a protonated form of compound I is more competent in oxidizing the (pseudo-)halide anions. Presumably, the halides and SCN^- bind to an amino acid near the iron center of compound I, much like the scheme for halide binding to the myeloperoxidase−cyanide complex proposed by Lee et al. (*31*). These authors presented electron paramagnetic resonance (EPR) spectral evidence of halide interactions with the low-spin cyanide complex of MPO. Cyanide complexes of peroxidases contain a six-coordinated, low-spin iron center similar to compound I in peroxidases [S = 1, ferryl iron with an oxygen atom (Fe(IV)═O) as the axial sixth ligand]. The authors demonstrated halide-dependent spectral changes at the acidic pH. The electronic structure of the low-spin ferric iron in cyanide complex appeared to be modulated by halide binding to a protonated amino acid in the distal heme cavity. Their data very well fit with our findings which also suggest halide interaction with the ferryl oxygen in compound I during enzyme catalysis to form hypohalous acid. Based on these data and results of steady-state enzyme kinetic studies (*32*), it is reasonable to assume that halide binding to compound I follows a similar way as to the MPO−CN complex. Only at acidic pH there seems to be a direct interaction between halide substrates and ferryl oxygen. The Poulos−Kraut mechanism for cytochrome *c* peroxidase compound I formation (*33*), based on X-ray crystal structure, utilizes distal histidine and arginine residues for heterolytic cleavage of hydrogen peroxide. Based on sequence homology, results of enzyme kinetic studies, and X-ray crystal structure (*34*), it appears likely that such a bonding network is also present in MPO compound I formation. At acidic pH, the halides bind to a protonated amino acid, which has to be very close to the ferryl oxygen since there is a halide-dependent change in the EPR spectrum at the acidic pH (*31*). It is likely that the distal histidine, upon protonation, allows such direct interaction between halide substrates and ferryl oxygen, and is presumably the substrate binding site. This conclusion is also confirmed by the studies of Bolscher and Wever (*18*), who investigated the effects of chloride and thiocyanate on the binding of cyanide to the native enzyme. Their conclusion was that hydrogen peroxide, cyanide, chloride, and thiocyanate bind at the same site on the enzyme.

Our findings, that SCN^- is by far the best electron donor for compound I at both neutral and acidic conditions, are physiologically relevant. Although Cl^- may be present in substantial concentrations, thiocyanate is likely to be a major substrate of myeloperoxidase in most environments. More-

over, its concentrations in secreted fluids are much higher than in blood plasma or the extracellular space of the tissues, whereas Cl^- concentrations are much lower. In these environments SCN^- appears to be the major physiological substrate. Only in plasma, where the concentration of Cl^- is at least 1000 times higher than that of SCN^-, Cl^- should be oxidized at least 2.5 times faster than SCN^-. Our findings are underlined by the work of van Dalen et al. (*8*), who showed that the rate of H_2O_2 loss catalyzed by MPO in the presence of 100 μM chloride doubled when 100 μM thiocyanate was added, and was maximal with 1 mM thiocyanate. This indicates that at plasma concentrations of thiocyanate and chloride, MPO is far from saturated.

The conditions of the environment at the beginning of phagocytosis which are encountered by MPO seem to be almost optimal with respect to thiocyanate oxidation. During the course of phagocytosis, the pH of the phagosome changes. Initially, it increases to about pH 8, followed by a slow acidification to below pH 6 after 1 h (*26*, *27*). Assuming that the thiocyanate concentration within the phagocyte is similar to that in blood plasma, at neutral pH compound II formation can be neglected. The reaction rate of compound I reduction by SCN^- is about 200 times higher than the rate constant for compound I reduction by H_2O_2. Moreover, the concentration of thiocyanate is likely to exceed the peroxide concentration during phagocytosis. On the contrary, when chloride is present exclusively even at high concentrations, there is a progressive formation of compound II, which is an inactive form with regard to oxidation of halides. At lower pH values, both (pseudo-)halides and H^+ act as competitive inhibitor with respect to H_2O_2 (*18*, *25*). The overall kinetics of peroxidation of SCN^- to $OSCN^-$ show a sharp optimum between pH 5 and 7.5, the position of which is determined by the concentrations of both SCN^- and H_2O_2 (*25*). At pH values below these optima, enzyme activity decreases progressively. At higher H_2O_2 concentrations, the optima are shifted toward lower pH values. Since the activity of the NADPH-oxidase (that assembles in the plasma membrane of the neutrophil and is responsible for oxygen activation during phagocytosis) is optimal at pH 7.0−7.5 and declines at low pH values (*35*), it is resonable to assume that at the end of phagocytosis (i.e., acidic pH and low H_2O_2 concentrations) MPO activity decreases, thus preventing MPO from damaging the surrounding tissue.

The consequences of these findings are also important for the involvement of neutrophil oxidants in host defense and inflammation. The product of oxidation of thiocyanate is thought to be hypothiocyanate (*36*) or thiocyanogen, which rapidly hydrolyzes to hypothiocyanate (*37*). Hypothiocyanite is considerably less reactive than hypochlorous acid. It reacts mainly with thiols, forming disulfides, sulfenyl thiocyanates, and sulfenic acids (*38*). Thus, it is conceivable that thiocyanate may limit bacterial killing and neutrophil-mediated tissue damage by diverting H_2O_2 away from the production of hypochlorous acid.

REFERENCES

1. Klebanoff, S. J. (1991) in *Peroxidases in Chemistry in Biology* (Everse, J., Everse K. E., and Grisham, M. B., Eds.) pp 1−36, CRC Press, Inc., Boca Raton, FL.
2. Kettle, A. J., and Winterbourn, C. C. (1997) *Redox Rep. 3*, 3−15.
3. Harrison, J. E., and Schultz, J. (1976) *J. Biol. Chem. 251*, 1371−1374.
4. Oka, S., Sibazaki, Y., and Tahara, S. (1981) *Anal. Chem. 53*, 588−593.
5. Wood, J. L. (1975) in *Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives* (Newman, A. A., Ed.) pp 156−221, Academic Press, Orlando.
6. Weiss, S. J., Klein, R., Slivka, A., and Wei, M. (1982) *J. Clin. Invest. 70*, 598−607.
7. Foote, C. S., Goyne, T. E., and Lehler, R. I. (1983) *Nature 301*, 1371−1374.
8. van Dalen, C. J., Whitehouse, M. W., Winterbourn, C. C., and Kettle, A. J. (1997) *Biochem. J. 327*, 487−492.
9. Thomas, E. L., and Fishman, M. (1986) *J. Biol. Chem. 261*, 9694−9702.
10. Hurst, J. K. (1991) in *Peroxidases in Chemistry in Biology* (Everse, J., Everse K. E., and Grisham, M. B., Eds.) pp 37−62, CRC Press, Inc., Boca Raton, FL.
11. Bakkenist, A. R. J., De Boer, J. E. G., Plat, H., and Wever, R. (1980) *Biochim. Biophys. Acta 613*, 337−348.
12. Andrews, P. C., and Krinsky, N. I. (1982) *J. Biol. Chem. 257*, 13240−13245.
13. Ikeda-Saito, M. (1985) *J. Biol. Chem. 260*, 11688−11696.
14. Kettle, A. J., and Winterbourn, C. C. (1988) *Biochem. J. 252*, 529−536.
15. Odajima, T., and Yamazaki, I. (1970) *Biochim. Biophys. Acta 206*, 71−77.
16. Nelson, D. P., and Kiesow, L. A. (1972) *Anal. Biochem. 49*, 474−478.
17. Laemmli, U. K. (1970) *Nature 227*, 680−585.
18. Bolscher, B. G. J. N., and Wever, R. (1984) *Biochim. Biophys. Acta 788*, 1−10.
19. Marquez, L. A., Huang, J. T., and Dunford, H. B. (1994) *Biochemistry 33*, 1447−1454.
20. Slungaard, A., and Mahoney, J. R. (1991) *J. Biol. Chem. 266*, 4903−4910.
21. Pember, S. O., Shapira, R., and Kinkade, J. M. (1983) *Arch. Biochem. Biophys. 221*, 391−403.
22. Olsen, F. L., and Little, C. (1983) *Biochem. J. 209*, 781−787.
23. Dunford, H. B., and Nadezhdin, A. (1982) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., and Morrison, M., Eds.) pp 653−670, Pergamon Press, Oxford, U.K.
24. Hurst, J. K., and Barette, W. C. (1989) *Crit. Rev. Biochem. Mol. 24*, 271−238.
25. Wever, R., Kast, W. M., Kasinoedin, J. H., and Boelens, R. (1982) *Biochim. Biophys. Acta 709*, 212−219.
26. Cech, P., and Lehrer, R. I. (1984) *Blood 63*, 88−95.
27. Segal, A. W., Geisow, M., Garcia, R., Harper, A., and Miller, R. (1981) *Nature 290*, 406−409.
28. Dolphin, D., Forman, A., Borg, D. C., Fajer, B., and Felton, R. H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 614−618.
29. Zuurbier, K. W. M., Bakkenist, A. R. J., Wever, R., and Muijsers, A. O. (1990) *Biochim. Biophys. Acta 1037*, 140−146.
30. Marquez, L. A., and Dunford, H. B. (1995) *J. Biol. Chem. 270*, 30434−30440.
31. Lee, H. C., Booth, K. S., Caughey, W. S., and Ikedo-Saito, M. (1991) *Biochim. Biophys. Acta 1076*, 317−320.
32. Ikedo-Saito, M., Lee, C., Adachi, K., Eck, H. S., Prince, R. C., Booth, K. S., Caughey, W. S., and Kimura, S. (1989) *J. Biol. Chem. 264*, 4550−4563.
33. Poulos, T. L., and Kraut, J. (1980) *J. Biol. Chem. 255*, 8199−8205.
34. Davey, C. A., and Fenna, R. (1996) *Biochemistry 35*, 10967−10973.

35. Gabig, T. G., Bearman, S. I., and Babior, B. M. (1979) *Blood 53*, 1133−1139.
36. Aune, T. M., and Thomas, E. L. (1977) *Eur. J. Biochem. 80*, 209−214.
37. Thomas, E. L. (1981) *Biochemistry 20*, 3273−3280.
38. Thomas, E. L. (1985) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., and Tenovuo, J. V., Eds.) pp 31−53, Marcel Dekker Inc., New York.

BI9818772