Spectral and Kinetic Studies on the Formation of Eosinophil Peroxidase Compound I and Its Reaction with Halides and Thiocyanate†

Paul Georg Furtmüller, Ursula Burner, Günther Regelsberger, and Christian Obinger*

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

Received August 25, 2000; Revised Manuscript Received October 16, 2000

ABSTRACT: Compound I of peroxidases takes part in both the peroxidation and the halogenation reaction. This study for the first time presents transient kinetic measurements of the formation of compound I of human eosinophil peroxidase (EPO) and its reaction with halides and thiocyanate, using the sequential-mixing stopped-flow technique. Addition of 1 equiv of hydrogen peroxide to native EPO leads to complete formation of compound I. At pH 7 and 15 °C, the apparent second-order rate constant is $(4.3 \pm 0.4) \times 10^7$ M^{-1} s^{-1}. The rate for compound I formation by hypochlorous acid is $(5.6 \pm 0.7) \times 10^7$ M^{-1} s^{-1}. EPO compound I is unstable and decays to a stable intermediate with a compound II-like spectrum. At pH 7, the two-electron reduction of compound I to the native enzyme by thiocyanate has a second-order rate constant of $(1.0 \pm 0.5) \times 10^8$ M^{-1} s^{-1}. Iodide [$(9.3 \pm 0.7) \times 10^7$ M^{-1} s^{-1}] is shown to be a better electron donor than bromide [$(1.9 \pm 0.1) \times 10^7$ M^{-1} s^{-1}], whereas chloride oxidation by EPO compound I is extremely slow [$(3.1 \pm 0.3) \times 10^3$ M^{-1} s^{-1}]. The pH dependence studies suggest that a protonated form of compound I is more competent in oxidizing the anions. The results are discussed in comparison with those of the homologous peroxidases myeloperoxidase and lactoperoxidase and with respect to the role of EPO in host defense and tissue injury.

Eosinophils are a specialized form of white blood cells that play a unique role in host defense mechanisms. They are recruited against a variety of cancers as well as to mediate extracellular destruction of helminthic parasites and other large invading metazoan pathogens (*1*−*3*). But increased levels of circulating and tissue eosinophils are also implicated in promoting cellular injury during allergic inflammatory disorders (*1*−*6*). For example, eosinophils are one of the major effector cells in the inflammation associated with asthma. They accumulate and are activated within the airways, and are considered to be responsible for the majority of tissue damage in asthma (*6*). By contrast, the function of another type of white blood cells, namely, neutrophils, is to kill relatively small ingested microbes within the confines of a phagocytic vacuole. Neutrophils are also implicated in promoting tissue damage in numerous inflammatory diseases, and they are also frequently found in the airways of individuals with asthma, often in association with exacerbations of the disease.

However, the biochemical mechanisms used by eosinophils and neutrophils in vivo to perform these functions are not clearly understood. Both neutrophils and eosinophils utilize a heme peroxidase in combination with hydrogen peroxide. When stimulated, neutrophils and eosinophils undergo a marked respiratory burst in which oxygen is reduced to superoxide (*7*) and hydrogen peroxide (*8*). The heme enzymes eosinophil peroxidase (EPO)[1] and myeloperoxidase (MPO), which are present in abundance in specific granules of eosinophils [EPO is up to 25% of the granule protein (*9*) in eosinophils] and neutrophils, respectively, use the hydrogen peroxide to catalyze the production of hypohalous acids. EPO has been shown to oxidize bromide to hypobromous acid, and thiocyanate to hypothiocyanite (*10, 11*). Under physiological conditions, thiocyanate and chloride are equally preferred substrates of myeloperoxidase (*12, 13*), with the latter being converted to hypochlorous acid (*14*). Hypochlorous acid and hypobromous acid are highly cytotoxic and are thought to be of central importance in immune surveillance and host defense (*7*). They rapidly react with most biological molecules, cross-linking and fragmenting proteins, as well as inactivating enzymes and cytokines (*7*). In addition to catalyzing the production of hypohalous acids, human peroxidases oxidize numerous phenols and anilines (AH_2) to reactive free radicals which can promote lipid peroxidation (*15*), deplete cellular thiols (*16*), cross-link proteins (*17*), and inactivate enzymes (*18*).

The mechanism of reaction of EPO and MPO seems to be similar (*19*). Halide oxidation starts by reaction of the ferric enzyme with H_2O_2 to form compound I, which contains 2 oxidizing equiv more than the resting enzyme (reaction 1). Halides (X^-) reduce compound I directly to native enzyme by a two-electron process (reaction 2). Thereby, hypohalous acids are formed (HOX). Alternatively, com-

† This work was supported by the Austrian Science Foundation (FWP-Project P14187-MOB).

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

[1] Abbreviations: EPO, eosinophil peroxidase; MPO, myeloperoxidase; LPO, lactoperoxidase; HVA, homovanillic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; EPR, electron paramagnetic resonance.

10.1021/bi0020271 CCC: $19.00
Published on Web 11/22/2000

pound I is reduced to native enzyme via compound II by two successive one-electron reductions (reactions 3 and 4), releasing free radicals ($^{\bullet}$AH).

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

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

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

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

EPO and MPO share 61% identical amino acid residues (*20*), and an even higher homology can be found among the active site related residues. Together with lactoperoxidase and thyroid peroxidase, EPO and MPO are members of the mammalian peroxidase superfamily. Nevertheless, EPO significantly differs from MPO in its physical and chemical properties. EPO is a monomer ($\alpha\beta$) of 70 kDa, whereas MPO is a disulfide-linked dimer [$(\alpha\beta)_2$] of 145 kDa (*21*−*23*). Structurally, MPO is the best characterized of the mammalian peroxidases. A 1.8 Å crystal structure has been obtained that allowed assignment of two ester linkages and one sulfonium ion linkage between the heme group and the apoprotein (*24*). Biochemical evidence was presented that in EPO there is one ester linkage to the heavy subunit whereas a second ester linkage to the light chain is formed autocatalytically requiring hydrogen peroxide (*25*). These differences in heme linkage between MPO and EPO could be a major factor that contributes to the differences in optical properties as well as substrate specificity (*21*−*23*, *26*).

In contrast to MPO and mainly because of its limited availability, EPO is the least studied human peroxidase. But in order to fully understand its physiological role in host defense and inflammatory disorders, a comprehensive biochemical investigation of its reactive redox intermediates is necessary. Here, for the first time a multi-mixing stopped-flow study is presented yielding spectral and kinetic data of the redox intermediates compound I and compound II. Actual bimolecular rate constants of compound I formation and its reactivity toward halides and thiocyanate are published. Differences to MPO as well as the physiological relevance are discussed.

EXPERIMENTAL PROCEDURES

Materials. Eosinophil peroxidase was purified from human white blood cells to a purity index (A_{413}/A_{280}) of at least 1.1 as described by Olson and Little (*23*). Its concentration was calculated using $\epsilon_{413} = 110\,000\ M^{-1}\ cm^{-1}$ (*22*). Hydrogen peroxide, obtained as a 30% solution from Sigma Chemical Co., was diluted and the concentration determined by the absorbance measurement at 240 nm where the extinction coefficient is 39.4 $M^{-1}\ cm^{-1}$ (*27*). Hypochlorous acid was purchased from Fluka. Stock solutions were prepared freshly half-daily in 5 mM NaOH and stored in the dark. The HOCl concentration was determined spectrophotometrically shortly before the experiments ($\epsilon = 350\ M^{-1}\ cm^{-1}$ at 292 nm in 5 mM NaOH) (*28*). The other chemicals were also purchased from Sigma Chemical Co. at the highest grade available.

Methods. SDS−PAGE was performed in the denaturating reducing system (T = 12%, C = 2.67%) described by Laemmli (*29*) using molecular weight Coomassie 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.

The formation of compound I was monitored at 413 nm (absorbance maximum of EPO compound I) and at 408 nm, the isosbestic point between compound I and compound II in the conventional stopped-flow mode. In a typical experiment, one reservoir contained 1 μM eosinophil peroxidase in 100 mM phosphate buffer, pH 7, and the other at least a 10-fold excess of hydrogen peroxide in 100 mM phosphate buffer or HOCl in 5 mM NaOH. Pseudo-first-order rate constants, k_{obs}, were evaluated using an exponential curve-fit program and then plotted against H_2O_2 concentration.

A good spectrum of compound I of human EPO was obtained with equimolar concentrations of hydrogen peroxide. However, compound I of EPO was not stable. Therefore, for determination of actual rates of the reaction of EPO compound I with halides and thiocyanate, the sequential-mixing stopped-flow technique had to be used. Typically EPO (2 μM) was premixed with 2 μM H_2O_2 in the aging loop for 100 ms (100 mM phosphate buffer, pH 7). 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 course of the reaction was followed by monitoring the absorbance change at 413 nm, the wavelength of maximum absorbance of both ferric EPO and compound I. The kinetic traces were fitted using the single-exponential equation of the Applied Photophysics software, and from the slopes of the linear plots of the k_{obs} values versus substrate concentration, the apparent second-order rate constants were obtained by linear square regression analysis. At least 3 determinations (2000 data points) of k_{obs} were performed for each substrate concentration, and the mean value was used in the calculation.

To determine the pH dependence of the reduction of compound I to the native enzyme, the sequential stopped-flow experiments were performed at different pHs from 4 to 9. In a typical pH jump experiment, 2 μM EPO in 5 mM phosphate buffer (pH 7) was premixed with 2 μM H_2O_2 in distilled water. After a delay time of 100 ms, compound I was allowed to react with varying concentrations of (pseudo-)halides in 200 mM phosphate/citrate buffer (pH 4−5), 200 mM phosphate buffer (pH 5−8), or carbonate buffer (pH 9). The resulting pH was controlled at the outlet. The high reaction rates made it necessary to perform all stopped-flow investigations at 15 °C.

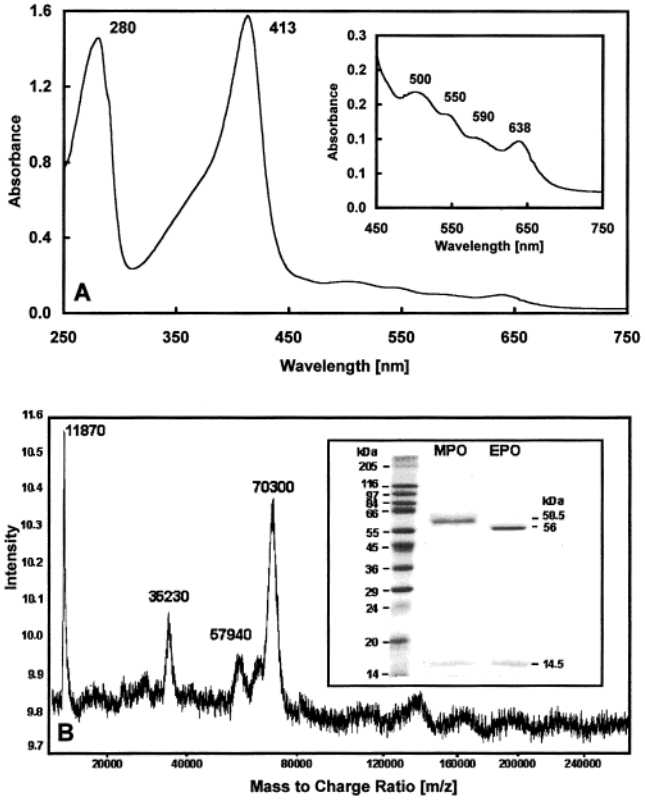

FIGURE 1: (A) Spectrum of native eosinophil peroxidase (14.3 μM) in 5 mM phosphate buffer (pH 7). The enzyme used in these studies had RZ values (A_{413}/A_{280}) of at least 1.1. (B) Corresponding MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrum of this enzyme preparation. The inset shows SDS−polyacrylamide gel electrophoresis of this preparation (5 μg of EPO) in comparison with 5 μg of MPO. Electrophoresis was performed on boiled reduced samples. The first lane shows the migration of standard proteins.

All reactions were also investigated using the diode-array detector (Applied Photophysics PD.1) attached to the stopped-flow machine. Typically, in these experiments the EPO concentration in the optical cell was 2 μM. The first spectrum could be monitored 1.3 ms after mixing. Normal data sets were also analyzed using the Pro-K simulation program from Applied Photophysics which allows the synthesis of artificial sets of time-dependent spectra as well as spectral analysis of enzyme intermediates.

RESULTS

Enzyme Purity. The eosinophil peroxidase used in these studies had a purity index (A_{413}/A_{280}) of at least 1.1, which indicates highly purified enzyme preparations. Figure 1A shows the spectrum of a 14.3 μM EPO stock solution used in this work. There is no peak or shoulder at 430 nm, which would indicate the presence of myeloperoxidase (*23*). Myeloperoxidase is known to oxidize halides and thiocyanate (*13*) and could copurify with EPO. Both SDS−PAGE (inset to Figure 1B) and MALDI-TOF spectroscopy (Figure 1) underlined the high purity of the enzyme preparations. SDS−PAGE under reducing conditions gave two bands with molecular masses of 56 ± 1 and 14.5 ± 0.5 kDa, in contrast to the two or three bands of purified MPO at 58 ± 1, 38 ± 1, and 15 ± 0.5 kDa (*23*). The mass spectrum (Figure 1B) unequivocally shows that there is only one protein present. The peak at 70.3 kDa very well fits with the molecular mass

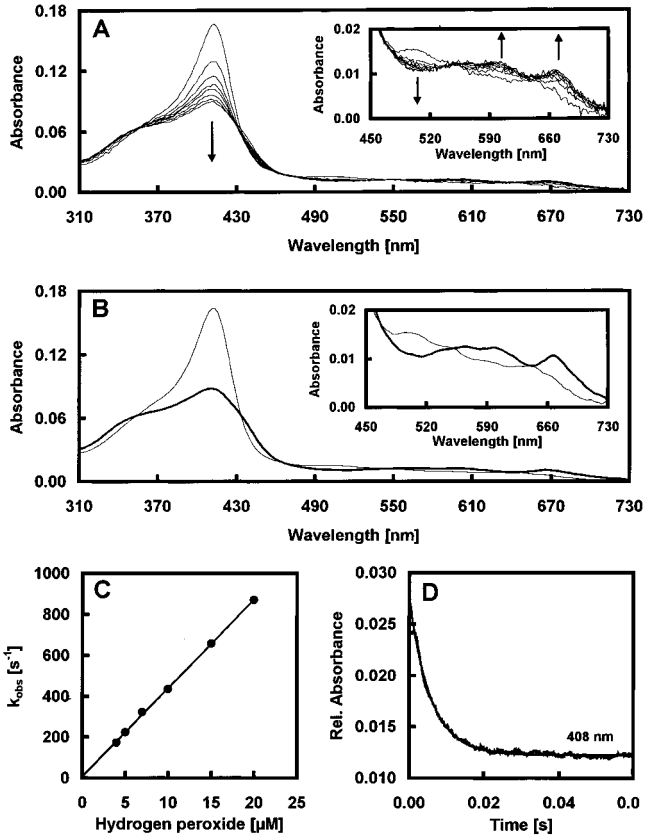

FIGURE 2: (A) Spectral changes upon mixing of 2 μM ferric eosinophil peroxidase (EPO) with 2 μM hydrogen peroxide. The first spectrum was taken 1.3 ms after mixing, subsequent spectra at 9, 16.6, 24.3, 32, 47.4, 70.4, and 117 ms. Arrows show the direction of absorbance changes with time. Conditions: 100 mM phosphate buffer, pH 7, and 15 °C. (B) Calculated spectra of ferric EPO and compound I using the Pro-K simulation program from Applied Photophysics. (C) Pseudo-first-order rate constants for compound I formation plotted against hydrogen peroxide concentration. Final enzyme concentration: 0.5 μM in 100 mM phosphate buffer, pH 7. (D) Typical time trace and fit of the reaction of native EPO with 4 μM H_2O_2. The reaction was monitored at 408 nm, the isosbestic point between compound I and compound II.

of the monomeric enzyme [$(M + H)^+$, singly charged species], whereas the peak at 35.2 kDa represents another *m*/*z* value of the same analyte species [$(M + 2\ H)^{2+}$, doubly charged species]. The peaks at 57.9 and 11.9 kDa derived from the heavy chain and the light chain, respectively, giving the EPO monomer with the expected size of about 70 kDa, whereas MPO is a disulfide-linked dimer of about 145 kDa (*21*−*23*).

Compound I Formation. 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 EPO was already obtained when hydrogen peroxide in equimolar concentration was added (Figure 2A). This is similar to lactoperoxidase (*30*) and in contrast to human myeloperoxidase, where an at least 10-fold excess of H_2O_2 is needed (*13*). The transition of ferric EPO (with distinct peaks at 413, 500, and 638 nm) to compound I involved broadening and a decrease in the extinction coefficient at 413 nm from 110 to 51.7 $mM^{-1}\ cm^{-1}$ as well as a decrease at 500 nm and the formation of two new peaks at 600 and 670 nm. Isosbestic points, derived from both normal data sets (Figure 2A) and computer analysis using the Pro-K simulation program (Figure 2B),

between ferric EPO and compound I were determined to be at 355, 433, 468, and 548 nm. Under conditions of excess H_2O_2, the pseudo-first-order rate constant, k_{obs}, was obtained from the exponential time course (Figure 2D). In Figure 2C, these k_{obs} values are plotted against H_2O_2 concentration. The forward second-order rate constant (k_{1f}), obtained from the slope of the plot, was calculated to be $(4.3 \pm 0.4) \times 10^7$ M^{-1} s^{-1} (pH 7 and 15 °C). The finite intercept of this plot was (7.3 ± 0.9) s^{-1}. Assuming that this intercept represents the reverse reaction (k_{1b}), the ratio of k_{1b}/k_{1f} gives a value for the dissociation constant of EPO compound I to native enzyme and H_2O_2 of 0.17 μM, which is about 20 times smaller than that of MPO (*31*).

We have also investigated the reaction between ferric EPO and hypochlorous acid, which is known to oxidize ferric myeloperoxidase to compound I (*32*, *33*). When HOCl was mixed with EPO in the conventional stopped-flow mode, the resulting spectra were identical to those obtained with H_2O_2. The corresponding time traces were monophasic, and an apparent second-order rate constant of $(5.6 \pm 0.7) \times 10^7$ M^{-1} s^{-1} at pH 7 and 15 °C could be calculated.

Stability of Compound I and Spectrum of Compound II. In contrast to the model enzyme horseradish peroxidase (*34*) and similar to lactoperoxidase (*35*) and myeloperoxidase (*13*, *31*) compound I of EPO was unstable. Figure 3A shows the decay of EPO compound I formed with hydrogen peroxide in equimolar concentrations within the first 5 s. A similar spectral transition occurred when compound I was formed with HOCl (not shown). With both H_2O_2 and HOCl, the resulting intermediate was stable for more than 30 s. Its Soret band was red-shifted to 433 nm, and two distinct peaks at 536 and 565 nm were formed (Figure 3A). The corresponding time-trace at 433 nm is shown in Figure 3C. The resulting spectrum was very similar to that described for lactoperoxidase compound II (maxima at 430, 535, and 567 nm) (*36*). Since no exogenous electron donor was present in this experiment, the reaction of compound I with homovanillic acid (HVA), a typical one-electron donor, was also investigated. Because of the instability of EPO compound I, the sequential-mixing technique had to be used. Compound I was formed with equimolar H_2O_2, and after a delay time of 100 ms, 20 μM HVA was added. Figure 3B indicates similar spectral transitions as in the absence of a one-electron donor, indicating that compound II or an intermediate with identical spectral features was formed spontaneously upon addition of 1 equiv of H_2O_2 to native EPO. Isosbestic points between compound I and compound II were determined to be at 408, 473, and 586 nm, and the maximum yield of absorbance of compound II at 433 nm was about 83% that of native EPO at 413 nm, corresponding to an extinction coefficient ϵ_{433} of 91 mM^{-1} cm^{-1}. The isosbestic points in the Soret region between compound II and ferric EPO were at 424, 465, 523, and 598 nm.

Compound II formation strongly depended on the HVA concentration (Figure 3C), and an apparent bimolecular rate constant for compound I reduction by HVA was calculated to be $(2.3 \pm 0.2) \times 10^5$ M^{-1} s^{-1} at pH 7 and 15 °C. At higher HVA concentrations, reduction of compound II interfered and was also monitored at 433 nm (Figure 3C, curves 3 and 4). Thus, for exact rate calculation of compound I reduction by HVA, the time traces at 424 nm, the isosbestic point between compound II and native EPO, had to be analyzed.

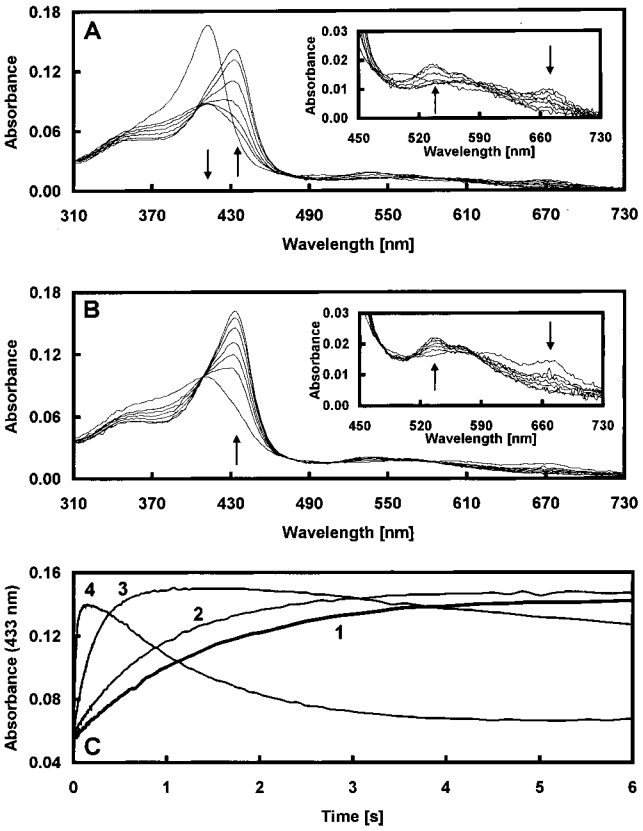

FIGURE 3: (A) Spectral changes upon addition of 2 μM hydrogen peroxide to 2 μM eosinophil peroxidase in the conventional stopped-flow mode. The first spectrum was recorded at 1.3 ms, subsequent spectra at 0.16, 0.33, 0.66, 1.3, 2.7, and 5.5 s. Reaction conditions: 100 mM phosphate buffer, pH 7, and 15 °C. (B) Spectral changes upon addition of 20 μM homovanillic acid to 2 μM compound I in the sequential-mixing stopped-flow mode (see Experimental Procedures). The first spectrum was recorded at 1.3 ms, subsequent spectra at 93, 155, 227, 355, 547, and 1088 ms. Reaction conditions as in (A). (C) Kinetics of transition of compound I to compound II in the absence and presence of electron donors. Conditions: 2 μM EPO and 2 μM H_2O_2 were premixed in 100 mM phosphate buffer, pH 7, and 15 °C. After a delay time of 100 ms, buffer (1), 2 μM HVA (2), 20 μM HVA (3), or 200 μM HVA was added (4). Time traces were monitored at 433 nm, the Soret maximum of EPO compound II.

From MPO, it is known that compound I can be reduced to compound II by hydrogen peroxide forming the superoxide radical (*31*). In the case of EPO, addition of increasing concentrations of H_2O_2 (0−1 mM) to compound I had no effect on the rate of its decay (6−7 s^{-1}) at pH 7.

Reaction of Halides and Thiocyanate with Compound I. As has been outlined above, EPO compound I was not stable. To study its reactivity with halides and thiocyanate, we premixed native EPO with 1 equiv of H_2O_2 in the aging loop for 100 ms. During this aging time, compound I formation took place, and before its decay, it was mixed with varying concentrations of halides and thiocyanate. Figure 4A shows the direct two-electron reduction of compound I to native EPO with identical isosbestic points observed for compound I formation with hydrogen peroxide (compare with Figure 2A). Typical kinetic traces displayed single-exponential character (Figure 4C). In Figure 4B, the apparent second-order rate constant for the reaction between compound I and

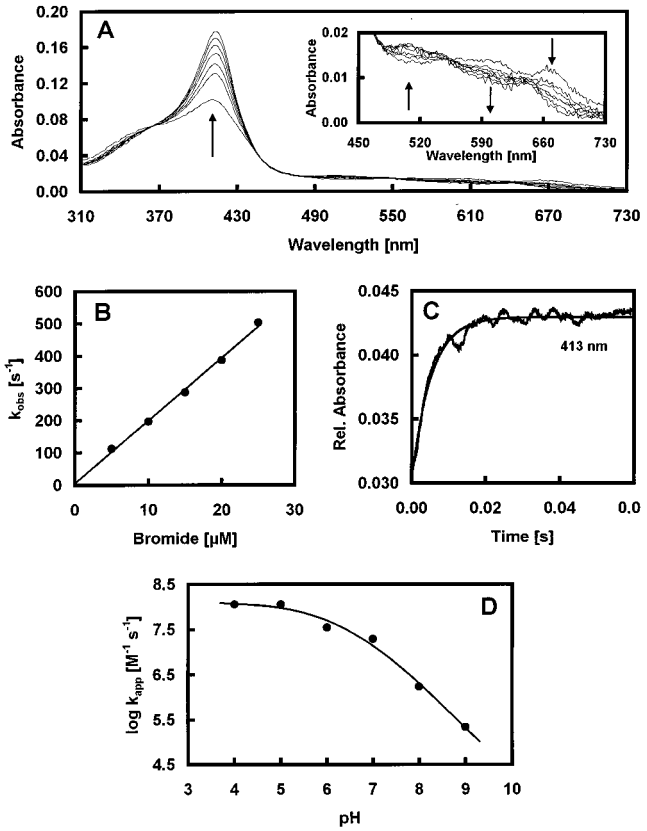

FIGURE 4: (A) Spectral changes upon addition of 2 μM bromide to 2 μM EPO compound I in the sequential-mixing stopped-flow mode. The first spectrum was recorded at 1.3 ms, subsequent spectra at 19.2, 37, 73, 109, 183, and 500 ms. Conditions: 100 mM phosphate buffer, pH 7, and 15 °C. (B) Pseudo-first-order rate constants for compound I reduction by bromide. (C) Typical time trace and fit of the reaction of compound I with 10 μM bromide followed at 413 nm. Final conditions were 0.5 μM EPO and 0.5 μM H_2O_2, 100 mM phosphate buffer, pH 7, and 15 °C. (D) pH dependence of the apparent second-order rate constant of the reaction between compound I and bromide. Final concentrations were 0.5 μM EPO, 0.5 μM H_2O_2, in phosphate/citrate buffer (pH 4−5), phosphate buffer (pH 5−8), and carbonate buffer (pH 9). For details, see Experimental Procedures.

Table 1: Apparent Second-Order Rate Constant for the Reactions of Eosinophil Peroxidase (EPO) Compound I with Thiocyanate, Chloride, Bromide, and Iodide, Respectively, at pH 7 or pH 5[a]

	k_{app} (M^{-1} s^{-1})	
(pseudo-)halides	pH 7.0	pH 5.0
chloride	$(3.1 \pm 0.3) \times 10^3$	$(2.6 \pm 0.7) \times 10^4$
bromide	$(1.9 \pm 0.1) \times 10^7$	$(1.1 \pm 0.2) \times 10^8$
iodide	$(9.3 \pm 0.7) \times 10^7$	nd[b]
thiocyanate	$(1.0 \pm 0.5) \times 10^8$	nd[b]

[a] The second-order rate constants were calculated from the slope of plots of halide or thiocyanate concentration versus the pseudo-first-order rate constant. Reaction was followed at 413 nm and 15 °C using the sequential-mixing stopped-flow technique. Final concentrations were 0.5 μM EPO and 0.5 μM H_2O_2 in 100 mM phosphate buffer. [b] nd, not detectable (too fast).

bromide was obtained from the slope of the plot of pseudo-first-order rate constants, k_{obs}, against bromide concentration. Similar plots were observed with thiocyanate, iodide, and chloride, respectively. As Table 1 summarizes, thiocyanate was the best electron donor for compound I [$(1.0 \pm 0.5) \times 10^8$ M^{-1} s^{-1}] at pH 7 and 15 °C. The finite intercept of this plot was at (5.1 ± 1.1) s^{-1}. Iodide was a more effective electron donor to compound I [$(9.3 \pm 0.7) \times 10^7$ M^{-1} s^{-1}] than bromide [$(1.9 \pm 0.1) \times 10^7$ M^{-1} s^{-1}]. Chloride is an extremely bad electron donor for EPO compound I [$(3.1 \pm 0.3) \times 10^3$ M^{-1} s^{-1}]. In contrast to similar experiments with MPO (*13*), with EPO this two-electron transition could easily be followed. The full theoretical re-increase of the absorbance amplitude at 413 nm could be followed, and the intercepts of the plots k_{obs} versus halide concentration (which indicate side reactions) were relatively small (ranging from 5 s^{-1} with bromide to 18 s^{-1} with chloride). With MPO the system is more complicated since (i) an excess of H_2O_2 is necessary for compound I formation which favors cycling of the enzyme (*13*) and (ii) compound II formation is mediated by H_2O_2 (*31*).

Figure 4D shows the pH dependence of the apparent second-order rate constants (k_{app}) for the reaction of compound I with bromide. A similar pH dependence was seen for k_{app} for the reaction with chloride (not shown), whereas the reaction with iodide and thiocyanate was too fast to follow at acidic pH; most of the absorbance amplitude was lost in the dead time (1.5 ms) of the stopped-flow machine. Similar to our previous findings with MPO (*13*), these results suggest the existence of an acid−base group which, when protonated, favored reaction between compound I and these anions. 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 −1 (*37*).

DISCUSSION

Compound I of human peroxidases is the only redox intermediate that takes part in both the peroxidatic cycle and the halogenation reaction (*38*). Despite its significant role in these reactions, the formation and reactivity of eosinophil peroxidase compound I has not been investigated so far. Considerable effort has been made to explore the mechanism of compound I formation of myeloperoxidase and lactoperoxidase (LPO) (reviewed in *19*). We obtained an apparent second-order rate constant for EPO compound I formation of $(4.3 \pm 0.4) \times 10^7$ M^{-1} s^{-1} at 15 °C, which is significantly higher than the rates published for MPO (1.8×10^7 M^{-1} s^{-1}) (*31*) and for LPO (1.2×10^7 M^{-1} s^{-1}) (*36*); the latter reactions were followed at 25 °C. The kinetics of formation and decay of EPO compound I were similar to LPO and different than MPO. Upon addition of 1 equiv of hydrogen peroxide to native EPO, compound I was completely formed and subsequently decayed to a species with spectral features similar to compound II. On the contrary, MPO exhibits an abnormal stoichiometry of compound I formation, and its formation is thought to be reversible, which was indicated by a relatively high intercept in the plot of k_{obs} versus H_2O_2 concentration (*31*). With all three peroxidases, at first the native enzyme reacts with H_2O_2 or HOCl to give a ferryl/porphyrin radical cation compound I intermediate (*19*). In the case of LPO, this species is thought to convert rapidly to a ferryl/protein radical species (*30*, *39*, *40*). Evidence for the existence of such a protein radical was given recently by EPR spin trapping (*41*). This species has similar UV−Vis spectral features as compound II, which is a fairly well-defined intermediate with an Fe^{IV}=O species in which iron is one oxidation state above its resting state. Our findings demonstrate that EPO could behave similarly. EPO compound I decays to an intermediate with spectral features

nearly identical to pure compound II (which in this work was formed by addition of homovanillic acid to compound I). Transformation of the ferryl/porphyrin radical cation EPO compound I to an intermediate that retains the ferryl species but not the porphyrin radical cation would suggest that electron transfer from an amino acid residue quenches the porphyrin radical cation. The resulting intermediate would still retain 2 oxidizing equiv, as shown with LPO where it oxidizes two molecules of one-electron reducing substrates (*42*−*44*). It has to be investigated whether this transformation occurs with EPO.

In the presence of halides and thiocyanate, the decay of compound I is negligible. With the exception of chloride, our experiments have shown that these two-electron donors are very effective in reducing EPO compound I to its native form. There is some evidence that EPO is also capable of generating chlorinating species in the absence of alternative electron donors (*45*−*47*). However, regarding the plasma concentrations of halides and thiocyanate, which are 100−140 mM for chloride (*48*), 20−100 μM for bromide, 0.1−0.6 μM for iodide, and 20−120 μM for thiocyanate (*49*), our results unequivocally demonstrate that chloride oxidation by EPO in vivo is meaningless. Thiocyanate is a more than 30 000-fold better electron donor than Cl^- [about 400-fold with MPO (*13*)], and bromide is still more than 6000-fold better than Cl^- [about 45-fold with MPO (*13*)]. These findings fit well with several in vitro observations, namely, that (i) even when the chloride concentration is more than 10 000-fold greater than the [Br^-], EPO-mediated incorporation of bromide and not of chloride into tyrosyl residues of proteins occurs (*45*); (ii) with 100 mM Cl^- and 2−100 μM Br^-, the exposure of EPO, L-tyrosine, and H_2O_2 yields only two new products, which were identified to be 3-bromotyrosine and 3,5-dibromotyrosine (*50, 51*); and, finally, (iii) exposure of EPO, L-tyrosine, and H_2O_2 with nitrite in the presence of 100 mM chloride yielded formation of only 3-nitrotyrosine (*52*).

Thiocyanate was shown to be the best electron donor for compound I, followed by iodide and bromide. The relative rates of compound I reduction were similar to MPO ($SCN^- > I^- > Br^- \gg Cl^-$); however, EPO compound I reduction by SCN^-, I^-, or Br^- was about 10 times more effective than that of MPO (*13*). Apparently, this is caused by a greater affinity of these anions to their binding site in EPO compared to MPO and not by a higher oxidation capacity of EPO compound I compared with that of MPO, because—in constrast to MPO—EPO compound I cannot oxidize hydrogen peroxide to superoxide and only poorly oxidizes chloride. The reduction potential for MPO compound I was determined to be 1.1 V (*53*); thus, it is reasonable to assume that that of EPO compound I is less positive. Nevertheless, our kinetic results unequivocally demonstrate that EPO is more effective than MPO in metabolizing both hydrogen peroxide and SCN^-, I^-, or Br^-. This is also underlined by recent findings that at plasma levels of halides and physiologically plausible levels of H_2O_2, EPO, but not MPO, promotes aromatic bromination of L-tyrosine (*50*).

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 proposed for MPO (*13*). It is likely that the distal histidine, upon protonation, is presumably the preferred substrate binding site (*13, 24*).

Though iodide is an excellent electron donor for EPO compound I, and although the EPO/H_2O_2/I^- system has pronounced toxicity for bacteria (*54*), parasites (*55*), and mammalian cells (*56*), this typically requires iodide concentrations far above physiological levels. Therefore, the physiological two-electron donors thiocyanate and bromide are most likely to be oxidized by EPO compound I in vivo. Whether SCN^- or Br^- (the plasma concentration of both is comparable) is the preferred substrate for EPO cannot be answered easily, since eosinophils perform their biological functions in a large variety of tissues and fluids whose concentrations of halides and SCN^- (and of other so far unknown effective one-electron donors of compound I) differ significantly from plasma. Principally, thiocyanate is 5 times more effective in this reaction. Normal plasma levels of SCN^- are below 70 μM (*57*), but higher levels may be observed in secreted fluids or following excessive tobacco smoking or after eating certain vegetables (e.g., cabbage) (*57*). On the contrary, depletion of serum thiocyanate, which occurs in chronic hypereosinophilic states (*58*), should lead to increased generation of the more cytotoxic hypobromous acid (HOBr). Hypothiocyanite is much less toxic than HOBr. HOSCN is a weak oxidant that is bacteriostatic by reacting with critical intracellular sulfhydryl groups and nicotinamide nucleotides (*59*). HOBr (similar to HOCl) is a more potent, surface-acting bleaching oxidant that is rapidly bactericidal by destroying surface membrane integrity (*7*). Consequently, depletion of serum SCN^- would greatly amplify the toxicity of EPO bound to host cells by permitting the utilization of bromide in its place. The advantage of using SCN^- is that it is relatively innocuous for mammalian host cells but it is toxic for helminthic parasites (*2*). However, when thiocyanate is depleted (in acute disorders), the formation of HOBr should dominate the formation of HOSCN and then also harms normal host tissue and contributes to oxidative injury.

REFERENCES

1. Gleich, G. J., Ottesen, E. A., Leiferman, K. M., and Ackerman, S. J. (1989) *Int. Arch. Allergy Appl. Immunol. 88*, 59−62.
2. Spry, C. J. F. (1988) *Eosinophils: A Comprehensive Review and Guide to the Medical Literature*, Oxford University Press, Oxford, U.K.
3. Wardlaw, A. J. (1984) *Postgrad. Med. J. 70*, 536−552.
4. Gleich, G. J., and Kay, A. B. (1994) *Eosinophils in Allergy and Inflammation*, Marcel Dekker, New York.
5. Horwitz, R. J., and Busse, W. W. (1995) *Clin. Chest. Med. 16*, 583−602.
6. Bousquet, J., Chanez, P., Lacoste, J. Y., Barneon, G., Ghavanian, N., Enander, I., Venge, P., Ahlstedt, S., Simony-Lafontaine, J., Godard, P., and Michel, F. (1990) *N. Engl. J. Med. 233*, 1033−1039.
7. Klebanoff, S. J. (1999) in *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J. I., and Snyderman, R., Eds.) pp 721−768, Lippincott Williams & Wilkins, Philadelphia.
8. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) *Blood 92*, 3007−3017.
9. Carlson, M. G. C., Peterson, C. G. B., and Venge, P. (1985) *J. Immunol. 134*, 1875−1879.
10. Weiss, S. J., Test, S. T., Eckmann, C. M., Roos, D., and Regiani, S. (1986) *Science 234*, 200−203.
11. Slungaard, A., and Mahoney, J. R. (1991) *J. Biol. Chem. 266*, 4903−4910.
12. Van Dalen, C. J., Whitehouse, M., Winterbourn, C. C., and Kettle, A. J. (1997) *Biochem. J. 327*, 487−492.

13. Furtmüller, P. G., Burner, U., and Obinger, C. (1998) *Biochemistry 37*, 17923−17930.
14. Kettle, A. J., and Winterbourn, C. C. (1997) *Redox Rep. 3*, 3−15.
15. Savenkova, M. L., Mueller, D. M., and Heinecke, J. W. (1994) *J. Biol. Chem. 269*, 20394−20400.
16. Goldman, R., Claycamp, G. H., Sweetland, M. A., Sedlov, A. V., Tyurin, V. A., Kisin, E. R., Tyurina, Y. Y., Ritov, V. B., Wenger, S. L., Grant, S. G., and Kagan, V. E. (1999) *Free Rad. Biol. Med. 27*, 1050−1063.
17. Heinecke, J. W., Li, W., Francis, G. A., and Goldstein, J. A. (1991) *J. Clin. Invest. 91*, 2866−2872.
18. Divi, R. L., and Doerge, D. R. (2000) *Biochemistry 39*, 9668−9674.
19. Dunford, H. B. (1999) *Heme Peroxidases*, Wiley-VCH, New York.
20. Sakamaki, K., Tomonaga, M., Tsukui, K., and Nagata, S. (1989) *J. Biol. Chem. 264*, 16828−16836.
21. Andrews, P. C., and Krinsky, N. I. (1981) *J. Biol. Chem. 256*, 4211−4218.
22. Bolscher, B. G. J. N., Plat H., and Wever, R. (1984) *Biochim. Biophys. Acta 784*, 177−186.
23. Olson, R. L., and Little, C. (1983) *Biochem. J. 209*, 781−787.
24. Fiedler, T. J., Davey, C. A., and Fenna, R. E. (2000) *J. Biol. Chem. 275*, 11964−11971.
25. Oxvig, C., Thomsen, A. R., Overgaard, M. T., Sorensen, E. S., Hojrup, P., Bjerrum, M. J., Gleich, G. J., and Sottrup-Jensen, L. (1999) *J. Biol. Chem. 274*, 16953−16958.
26. Kooter, I. M., Moguilevsky, N., Bollen, A., Sijtsema, N. M., Otto, C., and Wever, R. (1997) *J. Biol. Inorg. Chem. 2*, 191−197.
27. Nelson, D. P., and Kiesow, L. A. (1972) *Anal. Biochem. 49*, 474−478.
28. Morris, C. (1966) *J. Phys. Chem. 70*, 3798−3805.
29. Laemmli, U. K. (1970) *Nature 227*, 680−685.
30. Kimura, S., and Yamazaki, I. (1979) *Arch. Biochem. Biophys. 198*, 580−588.
31. Marquez, L. A., Huang, J. T., and Dunford, H. B. (1994) *Biochemistry 33*, 1447−1454.
32. Floris, R., and Wever R. (1992) *Eur. J. Biochem. 207*, 697−702.
33. Furtmüller, P. G., Burner, U., Jantschko, W., Regelsberger, G., and Obinger, C. (2000) *Redox Rep. 5*, 171−176.
34. 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.
35. Kimura, S., and Yamazaki, I. (1979) *Arch. Biochem. Biophys. 198*, 580−588.
36. Kohler, H., Taurog, A., and Dunford, H. B. (1988) *Arch. Biochem. Biophys. 264*, 438−449.
37. Wever, R., Kast, W. M., Kasinoedin, J. H., and Boelens, R. (1982) *Biochim. Biophys. Acta 709*, 212−219.
38. Harrison, J. E. (1982) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., and Morrison, M., Eds.) pp 717−732, Pergamon Press, Oxford, U.K.
39. Hu, S., and Kincaid, J. R. (1991) *J. Am. Chem. Soc. 113*, 7189−7194.
40. Taurog, A., Dorris, M., and Doerge, D. R. (1994) *Arch. Biochem. Biophys. 315*, 82−89.
41. Lardinois, O. M., Medzihradszky, and Ortiz de Montellano, P. R. (1999*) J. Biol. Chem. 274*, 35441−35448.
42. Monzani, E., Gatti, A. L., Profumo, A., Casella, L., and Gulotti, M. (1997) *Biochemistry 36*, 1918−1926.
43. Courtin, F., Deme, D., Virion, A., Michot, J.-L., Pommier, J., and Nunez, J. (1982*) Eur. J. Biochem. 124*, 603−609.
44. Courtin, F., Michot, J.-L., Virion, A., Pommier, J., and Deme, D. (1984) *Biochem. Biophys. Res. Commun. 121*, 463−470.
45. Mayeno, A. N., Curran, A. J., Roberts, R. L., and Foote, C. S. (1989) *J. Biol. Chem. 264*, 5660−5668.
46. Jorg, A., Pasquier, J. M., and Klebanoff, S. J. (1982) *Biochim. Biophys. Acta 701*, 185−189.
47. Thomas, E. L., Bozeman, P. M., Jefferson, M. M., and King, C. C. (1995) *J. Biol. Chem. 270*, 2906−2913.
48. Oka, S., Sibazaki, Y., and Tahara, S. (1981) *Anal. Chem. 53*, 588−593.
49. Wood, J. L. (1975) in *Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives* (Newman, A. A., Ed.) pp 156−221, Academic Press, Orlando.
50. Wu, W., Chen, Y., d'Avignon, A., and Hazen, S. L. (1999) *Biochemistry 38*, 3538−3548.
51. Wu, W., Samoszuk, M. K., Comhair, S. A. A., Thomassen, M. J., Farver, C. F., Dweik, R. A., Kavuru, M. S., Erzurum, S. C., and Hazen, S. L. (2000) *J. Clin. Invest. 105*, 1455−1463.
52. Wu, W., Chen, Y., and Hazen, S. L. (1999) *J. Biol. Chem. 274*, 25933−25944.
53. Hurst, J. K. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., and Grisham, M. B., Eds.) pp 37−62, CRC Press, Inc., Boca Raton, FL.
54. Jong, E. C., Henderson, W. R., and Klebanoff, S. J. (1980) *J. Immunol. 124*, 1949−1953.
55. Jong, E. C., Chi, E. Y., and Klebanoff, S. J. (1984) *Am. J. Trop. Med. Hyg. 33*, 104−115.
56. Nathan, C. F., and Klebanoff, S. J. (1982) *J. Exp. Med. 155*, 1291−1308.
57. Teitz, N. W. (1994) in *Teitz Textbook of Clinical Chemistry* (Burtis, C. A., and Aswood, E. R., Eds.) 2nd ed., p 2216, W. B. Saunders Co., Philadelphia, PA.
58. Bass, D. A., Grover, W. H., Leweis, J. C., Szejda, P., DeChatelet, L. R., and McCall, C. E. (1980) *J. Clin. Invest. 66*, 1265−1273.
59. Thomas, E. L. (1975) in *The Lactoperoxidase System, Chemistry and Biological Significance* (Pruitt, K. M., and Tenovou, J. O., Eds.) pp 31−53, Marcel Dekker, New York.

BI0020271