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Reaction of Lactoperoxidase Compound I with Halides and Thiocyanate[†]

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ABSTRACT: Lactoperoxidase (LPO) is found in mucosal surfaces and exocrine secretions, including milk, tears, and saliva, and has physiological significance in antimicrobial defense which involves (pseudo-) halide oxidation. This study for the first time presents transient kinetic measurements of the reactivity of its competent redox intermediate compound I with halides and thiocyanate, using the sequential stopped-flow technique. Compound I was produced with either H_2O_2 [$(1.1 \pm 0.1) \times 10^7$ M^{-1} s^{-1}] or hypochlorous acid [$(3.2 \pm 0.1) \times 10^7$ M^{-1} s^{-1}]. At pH 7 and 15 °C, the two-electron reduction of compound I to native LPO by bromide and iodide has a second-order rate constant of $(4.1 \pm 0.1) \times 10^4$ M^{-1} s^{-1} and $(1.2 \pm 0.04) \times 10^8$ M^{-1} s^{-1}, respectively. With thiocyanate the reaction is extremely fast (2.0×10^8 M^{-1} s^{-1}), whereas chloride cannot function as electron donor. The results are discussed with respect to known kinetic data of homologous mammalian peroxidases and to the physiological role of LPO in antimicrobial defense.

Together with myeloperoxidase (MPO), eosinophil peroxidase (EPO), and thyroid peroxidase (TPO), lactoperoxidase (LPO; E.C. 1.11.1.7) constitutes the mammalian peroxidase superfamily II, which is distinguished from the peroxidase superfamily I (enzymes from plants, fungi and bacteria) that the prosthetic heme group is covalently attached to the protein.

LPO has a single polypeptide chain and is a heme- and calcium-containing glycoprotein with one heme group per molecule. Until now, crystallographic data have not been available, but there is evidence that the heme in LPO is covalently attached to the protein via two ester linkages, namely, between the heme 1- and 5-methyl groups and Glu375 and Asp225, respectively. The cross-linking process is thought to occur autocatalytically, with the 5-hydroxymethyl bond formed before the 1-hydroxymethyl bond (*1*, *2*). Two ester linkages and a similar self-processing mechanism were also postulated for EPO (*3*) and TPO (*4*). By contrast, in myeloperoxidase—the only mammalian peroxidase for which a structure is available—three covalent links between the heme and the protein exist (*5*). In addition to the two ester linkages, a thioether sulfonium bond between the β-carbon of the 2-vinyl group and Met243 is present (*5*). Generally, the existence of covalently linked heme and the differences in heme linkage within the mammalian peroxidase superfamily are responsible for the optical properties of these proteins and could be a major factor in the observed differences in substrate specificity.

It is thought that the major physiological function of LPO, EPO, and MPO is to play a role in host defense mechanisms. EPO and MPO are present in specialized white blood cells (*6*, *7*), which are recruited against invading pathogens, whereas LPO is found in mucosal surfaces and exocrine secretions, including milk, tears, and saliva (*8*). The role of the peroxidases in antimicrobial processes seems to be the catalyzation of halide and thiocyanate oxidation, thereby producing cytotoxic hypohalous acids and hypothiocyanite.

The mechanism of halide oxidation is similar. It starts by reaction of the ferric enzyme with H_2O_2 to form compound I, which contains two oxidizing equivalents more than the resting enzyme (reaction 1). LPO also reacts rapidly with HOCl to form compound I (reaction 2). Halides (X^-) or thiocyanate reduce compound I directly to native enzyme by a two-electron process (reaction 3). Thereby, hypohalous acids are formed (HOX). Alternatively, substrates (AH_2) reduce compound I to native enzyme via compound II by two successive one-electron reductions (reactions 4 and 5), releasing free radicals (${}^{\bullet}AH$)

$$\text{native enzyme} + \text{HOOH} \rightarrow \text{compound I} + H_2O \quad (1)$$

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

$$\text{compound I} + X^- + H^+ \rightarrow \text{native enzyme} + \text{HOX} \quad (3)$$

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

$$\text{compound II} + AH_2 \rightarrow \text{native enzyme} + {}^{\bullet}AH + H_2O \quad (5)$$

The mammalian peroxidases differ in their ability to catalyze oxidation of halide ions. At neutral pH, only MPO

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[1] Abbreviations: LPO, lactoperoxidase; MPO, myeloperoxidase; EPO, eosinophil peroxidase; TPO, thyroid peroxidase; *E′°*, standard reduction potential.

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is capable to oxidize chloride at a reasonable rate (*9*), and it is assumed that chloride and thiocyanate are competing substrates in vivo (*10*). EPO can oxidize chloride only at acidic pH (*11*), and at normal plasma concentrations, bromide and thiocyanate function as substrates (*12*). For LPO it has been shown that it has barely detectable activity with bromide at neutral pH but oxidizes iodide and thiocyanate, the latter being thought to be the physiological substrate (*8*, *13*, *14*).

Halide oxidation is governed by reaction 3. From both EPO and MPO, apparent second-order rate constants for the reaction between compound I and halides have been determined (*9*, *11*), and recently, the standard reduction potential of the redox couple compound I/native enzyme has been determined for both EPO and MPO (*15*). Here, for the first time, a multimixing stopped-flow study is presented yielding kinetic data of LPO compound I reactivity with halides and thiocyanate. The obtained apparent second-order rate constants are discussed with respect to those known of EPO and MPO and the occurrence of halides and thiocyanate in secreted fluids.

EXPERIMENTAL PROCEDURES

Materials. Lactoperoxidase from bovine milk was purchased as a lyophilized powder (Sigma Chemical Co. type L-8257, purity index $A_{412}/A_{280} = 0.9$). Enzyme concentration was determined by using the extinction coefficient 112 000 M^{-1} cm^{-1} at 412 nm (*16*). Hydrogen peroxide, obtained as 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} (*17*). Hypochlorous acid was obtained from Fluka.

The HOCl stock solutions were prepared in 5 mM NaOH and stored in the dark. The HOCl concentration was determined spectrophotometrically ($\epsilon = 350$ M^{-1} cm^{-1} at 292 nm in 5 mM NaOH) shortly before the experiments (*18*). Peroxide and HOCl stock solutions were prepared freshly half-daily. Other chemicals were purchased from Sigma Chemical Co. at the highest grade available.

Transient State Experiments. The sequential stopped-flow apparatus (model SX-18MV) and the associated computer system were from Applied Photophysics (UK). 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 about 1.2 ms.

Conventional stopped-flow analysis was used to monitor the reaction of the native ferric enzyme with H_2O_2 and HOCl at 412 nm (maximum of ferric LPO) and at 431 nm (maximum of LPO compound II). In a typical experiment, one syringe contained 2 μM lactoperoxidase in 200 mM phosphate buffer pH 7, and the other syringe various concentrations of hydrogen peroxide in 5 mM phosphate buffer or hypochlorous acid in 5 mM NaOH. The pH was controlled at the outlet. At least three determinations (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. The measurements were performed at 15 °C in order to guarantee similar conditions as in experiments previously performed with MPO and EPO (*9*, *11*).

Similar to eosinophil peroxidase (*11*), compound I of lactoperoxidase is unstable. Consequently, for determination of the actual rates of the reaction of lactoperoxidase compound I with halide and thiocyanate, the multimixing stopped-flow technique had to be used. 4 μM LPO in 200 mM phosphate buffer pH 7 was premixed with 16 μM HOCl in 5 mM NaOH in the aging loop for 100 ms (HOCl was diluted in 5 mM NaOH). Finally, compound I was allowed to react with varying concentrations of halide and thiocyanate. The reaction was followed by monitoring the absorbance change at 412 nm. 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 least-squares regression analysis.

In addition, all conventional and multimixing reactions were analyzed by using the diode-array detector (Applied Photophysics PD.1) attached to the stopped-flow machine and by 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.

Measurement of Hydrogen Peroxide Utilization by Lactoperoxidase. The activity of LPO in the presence of halides was measured by continuously monitoring hydrogen peroxide concentration polarographically with a platinum electrode covered with a hydrophilic membrane and fitted to the Amperometric Biosensor Detector 3001 (Universal Sensors, Inc., U.S.A.). The applied electrode potential at pH 7 was 650 mV, and the H_2O_2 electrode filling solution was prepared freshly twice a day. The electrode was calibrated against known concentrations of hydrogen peroxide. All reactions were performed at 25 °C and started by the addition of lactoperoxidase.

RESULTS

The bovine lactoperoxidase used in these studies had a purity index (A_{412}/A_{280}) of at least 0.9 (Figure 1E), which indicates highly purified enzyme preparation (*8*).

Compound I Formation. Compound I is the only redox intermediate which takes part in the halogenation (reactions 1 and 3) and the peroxidatic (reactions 1, 4 and 5) cycle. It is distinguished from the ferric form of the enzyme by its reduced absorbance in the Soret region. A good formation of compound I of LPO was already obtained when a 1.5-fold excess of H_2O_2 was added. With equimolar concentration of H_2O_2, about 95% of native LPO were transformed to compound I. The calculated rate of $(1.1 \pm 0.1) \times 10^7$ M^{-1} s^{-1} at pH 7 and 15 °C as well as its spectral features (not shown) fit well with those kinetic and spectral data already published (*19*, *20*).

Alternatively, compound I could be made by using hypochlorous acid as oxidant according to reaction 2. When HOCl was mixed with LPO in the conventional stopped-flow mode, the resulting spectra were identical to those obtained with H_2O_2 (Figure 1A). The transition involved peak broadening at 412 nm and the formation of two new peaks at 608 and 662 nm (Figure 1E). The corresponding time traces were monophasic (Figure 1D), and an apparent second-order rate constant of $(3.2 \pm 0.1) \times 10^7$ M^{-1} s^{-1} at pH 7

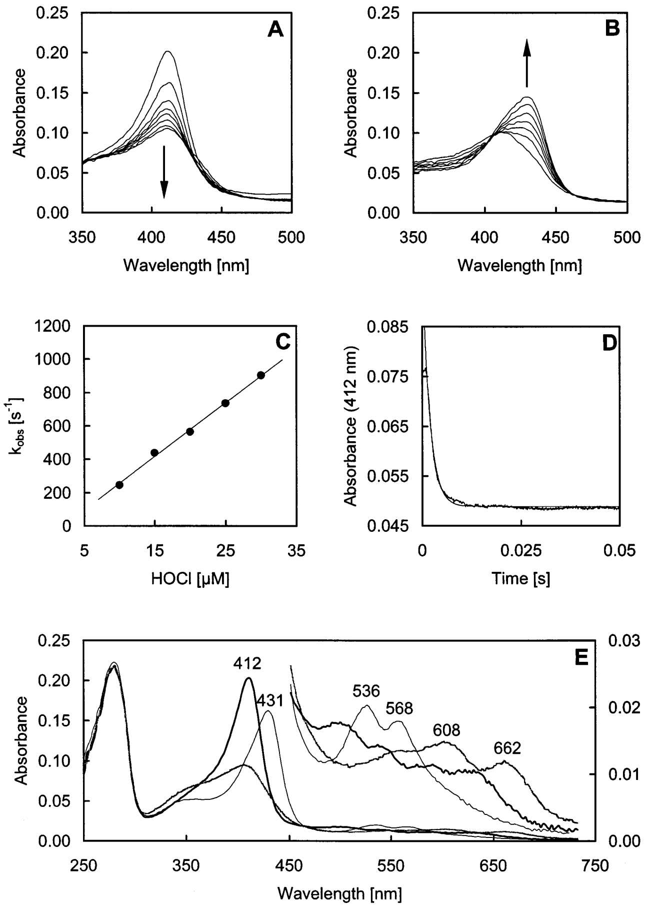

FIGURE 1: (A) Spectral changes of 2 μM LPO upon addition of 10 μM hypochlorous acid in the conventional stopped-flow mode. The first spectrum shows the native enzyme. The second spectrum was recorded at 1.3 ms after mixing, subsequent spectra at 3.8, 6.4, 9, 14, 24, and 40 ms showing compound I formation. Conditions: 100 mM phosphate buffer, pH 7, and 15 °C. (B) Continuation of panel A. Spectra were taken at 60, 157, 262, 378, 578, 1010, and 4060 ms and showing compound I decay to a compound II-like species. Arrows indicate direction of absorbance change with time. (C) Pseudo-first-order rate constant for compound I formation plotted against the hypochlorous acid concentration. The second-order rate constant was calculated from the slope. Final enzyme concentration: 1 μM LPO in 100 mM phosphate buffer, pH 7. (D) Typical time trace and single-exponential fit of the reaction of LPO with 20 μM hypochlorous acid at 15 °C. The reaction was followed at 412 nm. (E) Spectra of native lactoperoxidase (bold line), compound I (gray line) and a compound II-like species (thin line). For spectral analysis the Pro-K simulations program from Applied Photophysics was used to calculate these redox intermediates using a set of spectral data from the reaction between 2 μM LPO compound I and 4 μM hydrogen peroxide in 100 mM phosphate buffer, pH 7.

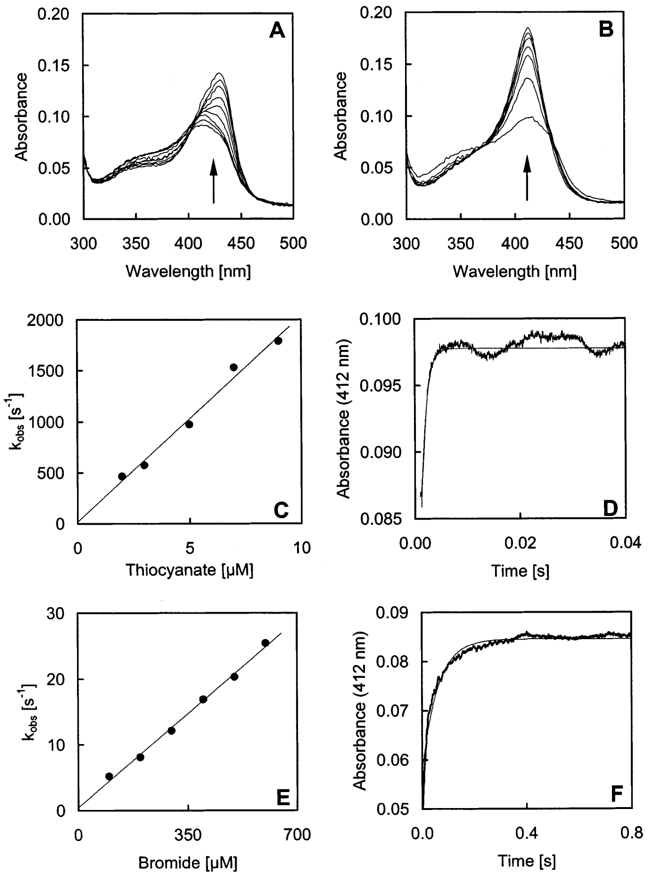

FIGURE 2: (A) Spectral changes upon addition of 10 mM chloride to lactoperoxidase compound I (2 μM) in the sequential mixing mode. The first spectrum indicate compound I, the origin of the reaction, the second spectrum was recorded 1.3 ms after mixing, subsequent spectra at 6.4, 14.8, 109, 283, 434, 764, and 1100 ms. The reaction was carried out in 100 mM phosphate buffer, pH 7, at 15 °C. Compound I was preformed with HOCl as described in Experimental Procedures. (B) Spectral changes upon addition of 4 μM thiocyanate to 2 μM LPO compound I. The first spectrum shows compound I, the second spectrum was taken 1.3 ms after mixing, and subsequent spectra were taken at 3.8, 6.4, 16.6, 183, and 1010 ms. (C) k_{obs} values plotted against the thiocyanate concentration. Conditions are the same as those in panel A. (D) Typical time trace of the reaction between preformed compound I (1 μM) and 5 μM thiocyanate at pH 7. The reaction was followed at 412 nm in 100 mM phosphate buffer, pH 7 (15 °C). Compound I was formed by mixing 2 μM lactoperoxidase with 8 μM hypochlorous acid and waiting for 100 ms. (E) Pseudo-first-order rate constants for compound I reduction by bromide. (F) Typical time trace of the reaction between preformed compound I (1 μM) and 500 μM bromide at pH 7. Conditions are the same as those in panel D.

and 15 °C could be calculated from the plot of k_{obs} versus HOCl concentration (Figure 1C).

Compound I of LPO is not stable (*20*). Figure 1B shows the decay of LPO compound I formed with HOCl within the first 4 s. A similar transition occurred when compound I was formed with H_2O_2. With both HOCl and H_2O_2, the rate of transition is (2 ± 0.5) s^{-1}, and the resulting intermediate was stable for more than 20 s. It exhibited spectral features such as LPO compound II (*21*), showing a red-shifted Soret band (431 nm) and two distinct peaks at 536 and 568 nm, respectively (Figure 1E).

Reaction of Halides and Thiocyanate with Compound I. In these experiments, native LPO was premixed with either a 2-fold excess of hydrogen peroxide or a 4-fold of HOCl in the aging loop for 100 ms. With both oxidants the same results were obtained within experimental error. During the aging time, compound I formation took place, and before its decay, it was mixed with varying concentrations of halides and thiocyanate. With the exception of chloride, a direct two-electron reduction of compound I to native LPO was observed (Figure 2B). With chloride, the observed spectral transition was similar to that in the absence of an electron donor. Even in the presence of 10 mM chloride, the compound II-like species appeared at a rate of about 2 s^{-1} (Figure 2A), indicating that chloride could not function as electron donor to compound I. However, in the presence of the other (pseudo)halides this transition is kinetically irrelevant. Here, an increase of absorbance at 412 nm was

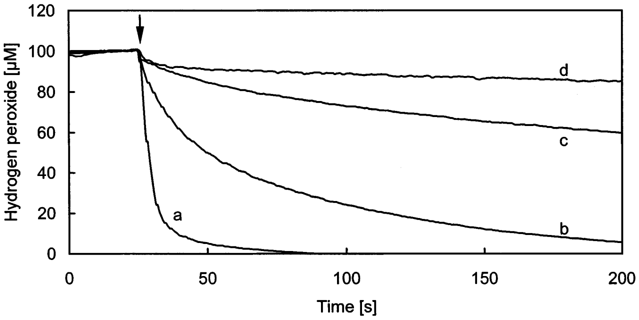

FIGURE 3: Polarographical determination of hydrogen peroxide depletion in the system lactoperoxidase/H_2O_2/halide. Reactions were started by adding 500 nM lactoperoxidase to 100 μM H_2O_2 and various halide concentrations: a, 50 μM thiocyanate; b, 200 μM bromide; c, 100 μM bromide; d, 10 mM chloride in 100 mM phosphate buffer, pH 7, containing 100 μM EDTA. Data are the means of at least triplicate experiments. Values were corrected for the loss of hydrogen peroxide in the absence of halides.

observed upon addition of SCN^-, I^-, or Br^- (Figure 2B). Figure 2D,F shows typical time traces displaying single-exponential character. In case of thiocyanate, the reaction was extremely fast ,and it was not possible to guarantee a 10-fold excess of substrate because at higher thiocyanate concentrations most of the absorbance amplitude was lost in the dead time (1.2 ms) of the stopped-flow machine. This can clearly be seen in Figure 2D, which shows the time trace of the reaction between 1 μM LPO compound I and 5 μM thiocyanate followed at 412 nm. Nevertheless, by plotting these obtained k_{obs} values against thiocyanate concentration, an apparent bimolecular rate of 2×10^8 M^{-1} s^{-1} was obtained (Figure 2C). With bromide the pre-steady state condition could easily be kept (Figure 2E,F), and a rate of $(4.1 \pm 0.1) \times 10^4$ M^{-1} s^{-1} was calculated at pH 7 and 15 °C. For iodide the calculated rate under these conditions was $(1.2 \pm 0.04) \times 10^8$ M^{-1} s^{-1}.

Polarographic measurement of H_2O_2 loss in the system LPO/H_2O_2/halide. We also measured the ability of lactoperoxidase to catalyze the oxidation of (pseudo)halides by monitoring the loss of hydrogen peroxide polarographically (Figure 3). There is no activity at neutral pH even at high chloride concentrations. Thiocyanate was shown to be extremely effective in H_2O_2 reduction mediated by LPO, in contrast to bromide, which only at higher, nonphysiological concentrations of both bromide and LPO could mediate this reaction at a reasonable rate. These results are consistent with the stopped-flow data and indicate that thiocyanate should be an extremely powerful electron donor for LPO in vivo.

DISCUSSION

Despite its significant role in halogenation activity, the reactivity of LPO compound I with (pseudo)halides has not been investigated so far. There are investigations of the formation of LPO compound I using hydrogen peroxide, which could be confirmed in this study. The rate of compound I formation with LPO (1.1×10^7 M^{-1} s^{-1}) is slower compared to that with MPO (1.4×10^7 M^{-1} s^{-1}) (*22*) and EPO (4.3×10^7 M^{-1} s^{-1}) (*11*) under identical conditions (pH 7, 15 °C). Formation of LPO compound I with HOCl was determined to be $(3.2 \pm 0.1) \times 10^7$ M^{-1} s^{-1} compared with 2×10^8 M^{-1} s^{-1} for MPO (*23*) and 5.6×10^7 M^{-1} s^{-1} for EPO (*11*), respectively. With all mammalian peroxidases, the same ferryl/porphyrin radical cation compound I intermediate is formed upon addition of either H_2O_2 or HOCl.

Similar to MPO and EPO, this compound I is unstable and—in the absence of an exogenous electron donor—decays to an intermediate with a compound II ferryl-like spectrum with a red-shifted Soret band and distinct peaks in the visible region. Whereas with MPO this transition could be mediated by the one-electron oxidation of hydrogen peroxide forming compound II and superoxide (*23*−*25*), with both EPO and LPO, an internal electron donor (i.e., the protein moiety) could catalyze this transition. In case of MPO, a pure ferryl species (compound II) would be formed at higher H_2O_2 concentrations; in case of EPO and LPO, this reaction does not occur (*11*), and consequently a ferryl/protein radical species would result, which is still two oxidizing equivalents above the resting enzyme. Evidence for the existence of such a species was given by EPR spin trapping (*26*, *27*) and by the fact that it could oxidize two molecules of one-electron reducing substrates (*28*, *29*).

With the exception of chloride, the formation of this redox intermediate was negligible when halides or thiocyanate were present. With even 10 or 100 mM chloride, the kinetics of transition were similar to that in the absence of an exogenous electron donor, which unequivocally indicated that chloride cannot function as substrate for LPO compound I. This is in contrast to EPO (*11*) and MPO (*9*), the latter being the only mammalian peroxidase competent to oxidize chloride at a reasonable rate. Bromide oxidation by LPO compound I is also relatively slow compared to MPO and EPO, where the calculated rates were determined to be 27-fold and 460-fold higher (*9*, *11*). By contrast, iodide and thiocyanate are excellent two-electron donors of LPO compound I. The calculated rates were similar to those obtained for EPO (*11*) and about 1 magnitude higher than those for MPO (*9*). Table 1 summarizes these kinetic parameters. The relative rates of compound I reduction of MPO and EPO are similar ($SCN^- > I^- > Br^- \gg Cl^-$) (*9*, *11*), whereas with LPO chloride oxidation does not occur and bromide oxidation is relatively slow ($SCN^- > I^- \gg Br^-$). In each case it reflects the hierarchy of the reduction potential of the involved couple hypohalous acid/halide (Table 1).

The other redox couple involved in (pseudo)halide oxidation is that of compound I/native enzyme. Recently, Arnhold et al. (*15*) demonstrated a measurement of the reduction potential of this couple for MPO and EPO by the use of stopped-flow analysis, which has overcome the problem of compound I instability. The determined reduction potential at pH 7.0 and 25 °C was (1.16 ± 0.01) V for MPO and (1.10 ± 0.01) V for EPO. The present data suggest that within the superfamily II peroxidases, the standard reduction potential (E'°) of the redox couple compound I/ferric enzyme follows E'° (MPO) > E'° (EPO) > E'° (LPO), thus reflecting the halide oxidation capacity of the corresponding enzymes. From TPO no presteady-state data are available at the moment, but taking in account investigations of bromination reactions using LPO and TPO under identical conditions (*13*), one could suggest that E'° (LPO) > E'° (TPO). In contrast to LPO, TPO could not catalyze bromination of tyrosine, thyroglobulin, and bovine serum albumin (*13*). Our both presteady-state and steady-state investigations confirmed that LPO is competent to oxidize bromide; however, this reaction is relatively slow at neutral pH.

Table 1: Apparent Second-Order Rate Constant of the Reactions between Compound I of Myeloperoxidase, Eosinophil Peroxidase, and Lactoperoxidase with (Pseudo)Halide at pH 7 and 15 °C[a]

compound I/ native enzyme	MPO	EPO	LPO	
	1.16V (*15*)	1.10 V (*15*)	?	HOX/X^-, H_2O
	$\times 10^4$ ($M^{-1}s^{-1}$)	$\times 10^4$ ($M^{-1}s^{-1}$)	$\times 10^4$ ($M^{-1}s^{-1}$)	
	(*9*)	(*11*)	(this paper)	
chloride	2.5	0.31	−	1.08 V (*20*)
bromide	110	1900	4.1	0.93 V (*20*)
iodide	720	9300	12000	0.57 V (*20*)
thiocyanate	960	10000	20000	−

[a] The two-electron reduction potential for the redox couple compound I/native enzyme and the redox couple HOX/X^-, H_2O at pH 7 are included.

Generally, the reduction potential of the couple compound I/ferric enzyme for the mammalian peroxidases define an important difference to the corresponding values known so far from peroxidases of superfamily I. The E'^{o} values of mammalian peroxidases are more positive (thus enabling these enzymes to oxidize bromide and even chloride) and correlate with compound I instability of these enzymes. For comparison, the corresponding standard reduction potential for horseradish peroxidase (HRP) was determined to be 884 mV at pH 7 (*30*), and HRP compound I is stable (*31*). A theoretical calculation yielded a value of 990 mV at pH 7 for the oxyferryl/ferric couple in an iron−porphyrin system and 870 mV for the couple oxyferrylhemoglobin/methemoglobin (*32*). The structural background for the more positive reduction potential in mammalian peroxidases is under discussion. Unfortunately, no crystal structure of both LPO and EPO is available. Several structural features could determine the observed differences between both peroxidase superfamilies and within superfamily II. One important feature is the mode of the heme link to the protein in superfamily II peroxidases (and variations in this heme linkage within the members), and a second feature is the imidazolate character of the proximal histidine. In superfamily I peroxidases, a hydrogen bond between a conserved aspartate and the proximal histidine exists (which gives the histidine a more imidazolate character and in consequence a less positive reduction potential). In mammalian peroxidases, an asparagine residue is found instead of aspartate.

Thiocyanate and iodide have been shown to be excellent electron donors for LPO compound I. However, thiocyanate (SCN^-) appears to be the physiological substrate for LPO because it is much more abundant than iodide and bromide oxidation is too slow. Normal plasma levels of thiocyanate have been shown to be 20−120 μM (*33*), and in secreted fluids, which contain LPO, the levels are even higher. So this work clearly underlines that the physiological role of LPO is to use H_2O_2 produced by host cells and/or microorganisms to produce hypothiocyanite (^-OSCN), which inhibits microbial metabolism and growth (*8*). HOSCN is a weak oxidizing agent that reacts primarily with sulfhydryl compounds converting them to sulfenyl thiocyanate or disulfide derivatives. On the other hand, it has also been suggested (*34*, *35*) that LPO could help to protect host cells against the toxicity of H_2O_2 by consuming hydrogen peroxide and producing hypothiocyanite as a less toxic oxidant. The observed high reactivity between LPO compound I and thiocyanate is compatible with both physiological roles.

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