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Redox Intermediates of Plant and Mammalian Peroxidases: A Comparative Transient-Kinetic Study of Their Reactivity Toward Indole Derivatives

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A comparative study on the reactivity of five indole derivatives (tryptamine, *N*-acetyltryptamine, tryptophan, melatonin, and serotonin), with the redox intermediates compound I (k_2) and compound II (k_3) of the plant enzyme horseradish peroxidase (HRP) and the two mammalian enzymes lactoperoxidase (LPO) and myeloperoxidase (MPO), was performed using the sequential-mixing stopped-flow technique. The calculated bimolecular rate constants (k_2, k_3) revealed substantial differences regarding the oxidizability of the substrates by redox intermediates at pH 7.0 and 25°C. With HRP it was shown that k_2 and k_3 are mainly determined by the reduction potential (E°′) of the substrate with k_2 being 7–45 times higher than k_3. Compound I of mammalian peroxidases was a much better oxidant than HRP compound I with the consequence that the influence of the indole structure on k_2 of LPO and MPO was small varying by a factor of only 88 and 38, respectively, which is in strong contrast to a factor of 160,000 determined for k_2 of HRP. Interestingly, the k_3 values for all three enzymes were very similar. Oxidation of substrates by mammalian peroxidase compound II is stronly constrained by the nature of the substrate. The k_3 values for the five indoles varied by a factor of 3,570 (LPO) and 200,000 (MPO), suggesting that the reduction potential of compound II of mammalian peroxidase is less positive than that of compound I, which is in contrast to the plant enzyme.

Key Words: **myeloperoxidase; lactoperoxidase; horseradish peroxidase; compound I; compound II; transient-state kinetics; indole oxidation; serotonin; melatonin; tryptophan; reduction potential.**

Peroxidases are widespread in nature and are found in animals, plants, fungi, and bacteria. In 1855 Schönbein made one of the first observations of a peroxidase-catalyzed reaction, when he noted the appearance of a blue color upon the addition of hydrogen peroxide to animal tissue samples treated with guaicum (1). The name peroxidase was officially introduced by Linossier in 1898 (2), when he demonstrated that certain organic compounds could be oxidized using an oxidase-free preparation of white blood cells. The following general mechanism (Reactions 1–3) was proposed for a peroxidase reaction (3, 4), with ROOH being a peroxide (e.g., hydrogen peroxide, H_2O_2) and ROH the corresponding two-electron reduction product (e.g., water, H_2O). Compound I and compound II are reactive redox intermediates which are responsible for the one-electron oxidation of substrates (AH_2) to their corresponding radicals ($AH^{\bullet}$).

$$\text{peroxidase} + \text{ROOH} \leftrightarrows \text{compound I} + \text{ROH} \quad \text{Reaction 1}$$

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH^{\bullet} \quad \text{Reaction 2}$$

$$\text{compound II} + AH_2 \rightarrow \text{peroxidase} + AH^{\bullet} + H_2O \quad \text{Reaction 3}$$

Today—based on sequence homologies and X-ray crystal structures—two peroxidase superfamilies are distinguished, the superfamily I containing plant, fungal, and bacterial peroxidases (5) and superfamily II containing animal and human peroxidases (6). Superfamily I is grouped in three classes which exhibit low

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overall amino acid identity but the same helical fold (5, 7) and have a heme *b* as their prosthetic group. Horseradish peroxidase (HRP),[2] a classical secretory plant peroxidase, is the most intensively studied example of superfamily I.

The primary and tertiary structure of mammalian peroxidases as well as the nature of their heme group differ greatly from those of superfamily I (6, 8, 9). Superfamily II includes myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), thyroid peroxidase (TPO), and prostaglandin H synthase. There is a much higher degree of amino acid conservation among animal peroxidases than among plant, fungal, and bacterial peroxidases (e.g., MPO shares 61% amino acid identity with LPO) (10). An important feature of peroxidases from superfamily II is the covalent link of the heme group to the protein that contributes to the differences in optical properties as well as in substrate specificity between the two peroxidase superfamilies (11–13). 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 (14). Biochemical evidence was presented that in LPO and 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 (15, 16). MPO, EPO, and LPO play a role in the antimicrobial system. MPO and EPO belong to the most abundant proteins of neutrophils and eosinophils, respectively, which are our primary defenders against invading pathogens and among the major effector cells in numerous inflammatory pathologies (17). LPO is found in secretion fluids such as milk, saliva and tears (18). Besides being involved in the peroxidase cycle (reactions 1–3) compound I of animal peroxidases is known to oxidize (pseudo-)halides (bromide, thiocyanate and chloride) via a single two-electron reaction to produce the respective cytotoxic hypohalous acids and regenerate the native enzyme according to reaction 4, with X^- being the halide and XOH the corresponding hypohalous acid.

$$\text{Compound I} + X^- \leftrightarrows \text{peroxidase} + XOH \quad \text{Reaction 4}$$

Generally, in the peroxidase cycle (reactions 1–3), the rate of reaction of compound I and compound II with the substrate depends on the nature of the electron donor (e.g., substitution pattern, reduction potential of the $AH^{\cdot}/AH_2$ couple) and the nature of the peroxidase (reduction potentials of the redox couples compound I/compound II and compound II/ferric peroxidase). It was demonstrated that HRP reduction of compound I to compound II requires electron and proton donation to the protein (19) and that principally the rates of reduction correlate with the reduction potentials of the substrates (20, 21). As with compound I, the rates of reduction of compound II correlate with the oxidizability of the substrate (22), but compound II is less reactive and thus accumulates during steady-state. Yamazaki and coworkers have demonstrated that the reduction potentials (E°′) of compounds I and II of HRP are—despite the different reactivities of the two compounds—very similar in acidic and neutral solutions, namely about 0.90 V, respectively (23, 24). The difference in reactivity between HRP compound I and II have been interpreted qualitatively in terms of the relative simplicity of the reactions. In compound II the electron is fed to the ferryl group at the center of the porphyrin in a reaction accompanied by two proton additions to the ferryl oxygen atom, one from the protein and the other from the substrate or solvent followed by loss of water (reaction 3) (22). By contrast compound I reduction is a simpler chemical process at the heme edge involving electron donation to the porphyrin radical cation (21). The different reactivities of the two enzyme states of HRP have also been ascribed by application of the Marcus equation explaining that the lower reactivity of compound II may be due to the longer electron-transfer distance (25). Recent investigations have indicated that these concepts are not fully applicable to human peroxidases. MPO compound II reduction is much more constrained by the nature of the substrates than HRP compound II reduction, whereas compound I in MPO is a very strong oxidant (26–29). The one-electron reduction potentials for compounds I and II from superfamily II are unknown.

In order to get further insights in the differences of redox intermediate reactivity from the two superfamilies, the following multimixing stopped-flow study was performed. Indole derivatives were selected because (i) their reduction potentials are known and (ii) indole structures are present in a great number of important biological compounds, e.g., in the plant growth hormone indoleacetic acid, in melatonin (*N*-acetyl-5-methoxytryptamine, a hormone involved in several physiological functions associated with circadian and seasonal rhythms (30)), in tryptophan, or in serotonin (5-hydroxytryptamine, a serum vasoconstrictor and neurotransmitter (31)). Bimolecular rate constants of the reaction of compounds I and II of three representative peroxidases (HRP, LPO, and MPO) with indole derivatives at pH 7.0 and 25°C were determined under identical con-

[2] Abbreviations used: MPO, myeloperoxidase; LPO, lactoperoxidase; HRP, horseradish peroxidase; E°′, standard reduction potential at pH 7.0.

ditions. Significant differences between the two enzyme superfamilies are demonstrated as well as one important characteristic of animal and human peroxidases, namely that the oxidizability of the electron donor by compound I and compound II can vary by several orders of magnitude, which not only suggests a less positive E°′ of compound II compared to compound I but is especially in case of MPO indicative of structural changes occurring during transition of compound I to compound II and thereby altering the substrate binding mode.

MATERIALS AND METHODS

Materials

Horseradish peroxidase (Sigma Chemical Co. type VI-A, purity index $A_{403}/A_{280} = 3.0$) and lactoperoxidase from bovine milk (Sigma Chemical Co. type L-8257, purity index $A_{412}/A_{280} = 0.9$) were purchased as a lyophilised powder. Enzyme concentrations were determined by using the following extinction coefficients: $\epsilon_{403\ nm}$ (HRP) = 102 $mM^{-1}\ cm^{-1}$ (32) and $\epsilon_{412\ nm}$ (LPO) = 112 $mM^{-1}\ cm^{-1}$ (33). Highly purified myeloperoxidase of a purity index (A_{430}/A_{280}) of a least 0.85 was purchased from Planta Natural Products (http://www.myeloperoxidase.com). Its concentration was calculated using $\epsilon_{430} = 91\ mM^{-1}\ cm^{-1}$ (34). All other chemicals were also purchased from Sigma Chemical Co. at the highest grade available.

Hydrogen peroxide, obtained from a 30% solution was diluted and the concentration determined by absorbance measurement at 240 nm where the extinction coefficient is 39.4 $M^{-1}\ cm^{-1}$ (35). Indole stock solutions were prepared fresh daily in dimethylformamide (DMF) and stored in dark flasks. Dilution was performed with 100 mM phosphate buffer, pH 7.0, to a final DMF concentration of 2% ($^{v}/_{v}$) in all assays.

Methods

Transient-state kinetics. The multimixing stopped-flow measurements were performed with the Applied Photophysics (UK) instrument SX-18MV. When 100 μl was shot into a flow cell having a 1-cm light path, the fastest time for mixing two solutions and recording the first data point was 1.3 ms. Kinetics were followed both at single wavelength and by using a diode-array detector. At least three determinations (2000 data points) of pseudo-first-order rate constants (k_{obs}) were performed for each substrate concentration (pH 7.0, 25°C) 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 allow calculation of pseudo-first-order rates, the concentrations of substrates were at least 10 times in excess of the enzyme.

Horseradish peroxidase. HRP forms a compound I that is very stable. With the protein preparation used in this work a stoichiometric amount of hydrogen peroxide completely formed compound I (bold spectrum in Fig. 1A), which was stable for minutes. In a typical experiment, 4 μM HRP was premixed with 4 μM H_2O_2 in the ageing loop for 1200 ms and was then allowed to react with varying concentrations of indoles in 200 mM phosphate buffer, pH 7.0. Reactions were followed at 420 nm (Soret maximum of HRP compound II) and at 412 nm, the isosbestic point between compound II and the ferric enzyme. HRP compound II (bold spectrum in Fig. 2A) is stable at pH > 9 (21). As a consequence, its reactivity was tested by using the pH jump technique (21): 4 μM HRP wase premixed with 4 μM H_2O_2 in 20 mM Tris buffer, pH 9.8, and after a delay time of 30 s, compound II was allowed to react with varying concentrations of indoles in 500 mM Tris/HCl buffer, pH 6.5. The resulting pH 7 was controlled at the outlet and reaction was followed at 420 nm (disappearance of compound II).

Lactoperoxidase. Similar to HRP, LPO compound I (Fig. 1C) can be formed with stoichiometric amounts of H_2O_2, but because of its instability the delay time was set to 100 ms. In a typical experiment 4 μM LPO was mixed with 4 μM H_2O_2 and after 100 ms the substrates in 200 mM phosphate buffer, pH 7.0, were added. Compound II formation was followed at 430 nm (Soret maximum of compound II) or at 422 nm, the isosbestic point between LPO compound II and the ferric enzyme. Compound II of LPO (bold spectrum in Fig. 2C) is formed spontaneously from compound I (36) and is stable for minutes (assay as in compound I formation with the exception that the delay time is set to 2 s before the substrates were added). Alternatively, with good substrates compound II formation and reduction could be followed in one measurement. The resulting biphasic curves at 430 nm showed the initial formation of compound II and then its subsequent reaction with the indoles causing an exponential decrease in absorbance.

Myeloperoxidase. The conditions of MPO compound I formation were described recently (37). Typically, 4 μM MPO was premixed with 40 μM H_2O_2 and after a delay time of 20 ms, compound I was allowed to react with varying concentrations of indole derivatives in 200 mM phosphate buffer, pH 7.0. The reactions were followed at the Soret maximum of compound II (456 nm) and at 442 nm, the isosbestic point between compound II and the ferric protein. Reduction of compound II was measured as described in (38). In a typical experiment 4 μM MPO was premixed with 40 μM H_2O_2 and 3.6 μM homovanillic acid and after a delay time of 40 s, compound II was allowed to react with varying concentrations of indoles in 100 mM phosphate buffer, pH 7.0. Reactions were followed at 456 nm (disappearance of compound II) and 430 nm (formation of ferric MPO). Alternatively, with good substrates compound II formation and reduction could be followed in one measurement as described above with LPO. With slow substrates reduction of compound II was measured as described by Kettle and Candaeis (28). In this case 500 nM MPO was premixed with 35 μM H_2O_2 (70-fold excess). After 35 s, 10 μg/ml catalase was added to remove H_2O_2 and finally compound II was allowed to react with the substrates in 100 mM phosphate buffer, pH 7.0. These reactions were studied using a conventional spectrophotometer (Zeiss Specord S-10).

RESULTS AND DISCUSSION

In this comparative study the reactivity of five indole derivatives (tryptamine, *N*-acetyltryptamine, tryptophan, melatonin, and serotonin) with the redox intermediates compound I and II of HRP, LPO, and MPO was investigated. The sequential-mixing stopped flow technique had to be used because of the instability of the redox intermediates. The spectral features of the peroxidases, the amount of hydrogen peroxide and the time necessary for complete compound I formation strongly depended on the nature of the peroxidase (21). The Soret maximum of HRP is at 403 nm, whereas that of LPO and MPO is at 412 and 430 nm, respectively, reflecting the differences in the nature of the prosthetic group between the two peroxidase superfamilies and also within superfamily II.

Compound I reduction. Generally, the spectra of compound I of the three peroxidases were distinguished from those of the ferric enzyme by a hypochro-

micity of the Soret band of 40–50% (Figs. 1A, 1C, and 1E). Upon mixing with an indole, a one-electron reduction of compound I to compound II occurred indicated by a red-shift of the Soret band to 418 nm (HRP), 430 nm (LPO), and 456 nm (MPO). The absorbance changes monitored either at the Soret maximum of compound II or at the isosbestic point between compound II and the ferric enzyme (412 nm (HRP), 422 nm (LPO), and 442 nm (MPO)) could be fitted to a single-exponential function (inset to Figs. 1B, 1D, and 1F). The obtained pseudo-first-order rate constants were linearly dependent on the concentration of the electron donor and allowed the calculation of the second-order rate constants from the corresponding plots (Figs. 1B, 1D, and 1F). With all indoles the intercept was close to zero for both HRP and LPO. In case of MPO the intercept was finite (Fig. 1F) because compound I reduction was also mediated by the excess of hydrogen peroxide necessary for complete compound I formation (47). In Figs. 1 and 2 the reactions with tryptamine as electron donor are shown.

Compound II reduction. Similarly, compound II was reduced in a one-electron process. Clear isosbestic points indicate the direct conversion of compound II back to the ferric resting enzyme (Figs. 2A, 2C, and 2E). With tryptamine the conversion was relatively slow but still exhibited a typical single-exponential behavior (insets to Figs. 2B, 2D, and 2F). In case of MPO the enzyme cycled because an excess of hydrogen peroxide was necessary for compound II formation (inset to Fig. 2F). A small steady-state phase was observed which, after depletion of hydrogen peroxide, was followed by an exponential decrease which strongly depended on the substrate concentration (Fig. 2F). With the exception of serotonin, this steady-state phase was seen with all indole derivatives. Thus, MPO compound II reduction was also followed by conventional spectrophotometry as described by Kettle and Candaeis (28). Compound II was formed by adding an excess of H_2O_2 to ferric MPO. After addition of catalase to get rid of hydrogen peroxide, the substrates were added and the resulting time trace was fitted to a single-exponential curve. Similar results as with the stopped-flow method were obtained within experimental error. With all peroxidases the plots of pseudo-first-order rate constants against the substrate concentration showed very small intercepts indicating that there were no side reactions present.

Indole structure and reactivity with peroxidases. The bimolecular rate constants obtained from these experiments are summarized in Table II and show substantial differences regarding both the oxidizability of the indoles and their reactivity towards the peroxidase redox intermediates. The structures of the indole derivatives (Table I) differ in both the substitution at the indole ring (unsubstituted, 5-methoxy-, 5-hydroxy-) as well as in the nature of the side chain. It has been shown by Harriman (42) that in neutral solution redox potentials measured for substituted indoles and phenols are clearly related to Hammett coefficients for the substituents and that the side chain is of minor importance in the redox chemistry of these substrates. Generally, the findings of the present work confirm that this is also the fact when indole oxidation is mediated by peroxidase redox intermediates. Similar conclusions were drawn in a recent paper about the dependence of rates of reaction of various derivatives of indoleacetic acids with horseradish peroxidase compound I (20). Table II clearly indicates that both the one-electron reduction potentials ($E°'$) of the indole derivatives as well as the rate constants k_2 and k_3 were influenced mainly by the substitution of the indole ring and not by the nature of the side chain. The electron donating hydroxy group in serotonin is responsible for its reduction potential $E°'$ of 0.65 V at pH 7 (40) and for the fact that serotonin is by far the best electron donor used in this kinetic study. By contrast, tryptophan and tryptamine differ in the nature of the side chain but have an identical reduction potential ($E°' = 1.015$ V) and exhibit a similar reactivity.

Horseradish peroxidase reactivity. Comparing the calculated rate constants (Table II was completed by literature data regarding the oxidation of tyrosine by peroxidases) it becomes obvious that compound I of peroxidases from superfamily II is a better oxidant compared with HRP compound I. Oxidation of tryptophan and tryptamine by HRP compound I is extremely slow. By contrast, serotonin is the best electron donor for both HRP intermediates, followed by tyrosine, melatonin and *N*-acetyltryptamine. These data reflect the dependence of the reaction rates on the reduction potentials of both the substrates and the redox intermediates. In the present study reduction of HRP compound II is 7–45 times slower than compound I reduction. In HRP both compounds were reported to have nearly the same $E°'$ values (23, 24), which has been interpreted in terms of a higher mobility of a porphyrin π-electron than an iron valence electron (25). The reduction potential of HRP compound I restricts the possibility of a molecule to be oxidized by HRP. The reaction with tryptamine and tryptophan is extremely slow at pH 7, which could be interpreted that the $E°'$ values of tryptamine and tryptophan of 1.015 V (39) mark the thermodynamic limit of an electron donor to mediate the transition of HRP compound I to compound II and of compound II to the ferric enzyme. This is only true if the corresponding equilibrium constant determined from equilibrium concentrations of all reactants involved in compound I or compound II reduction by tryptamine or tryptophan would be about 1. In the

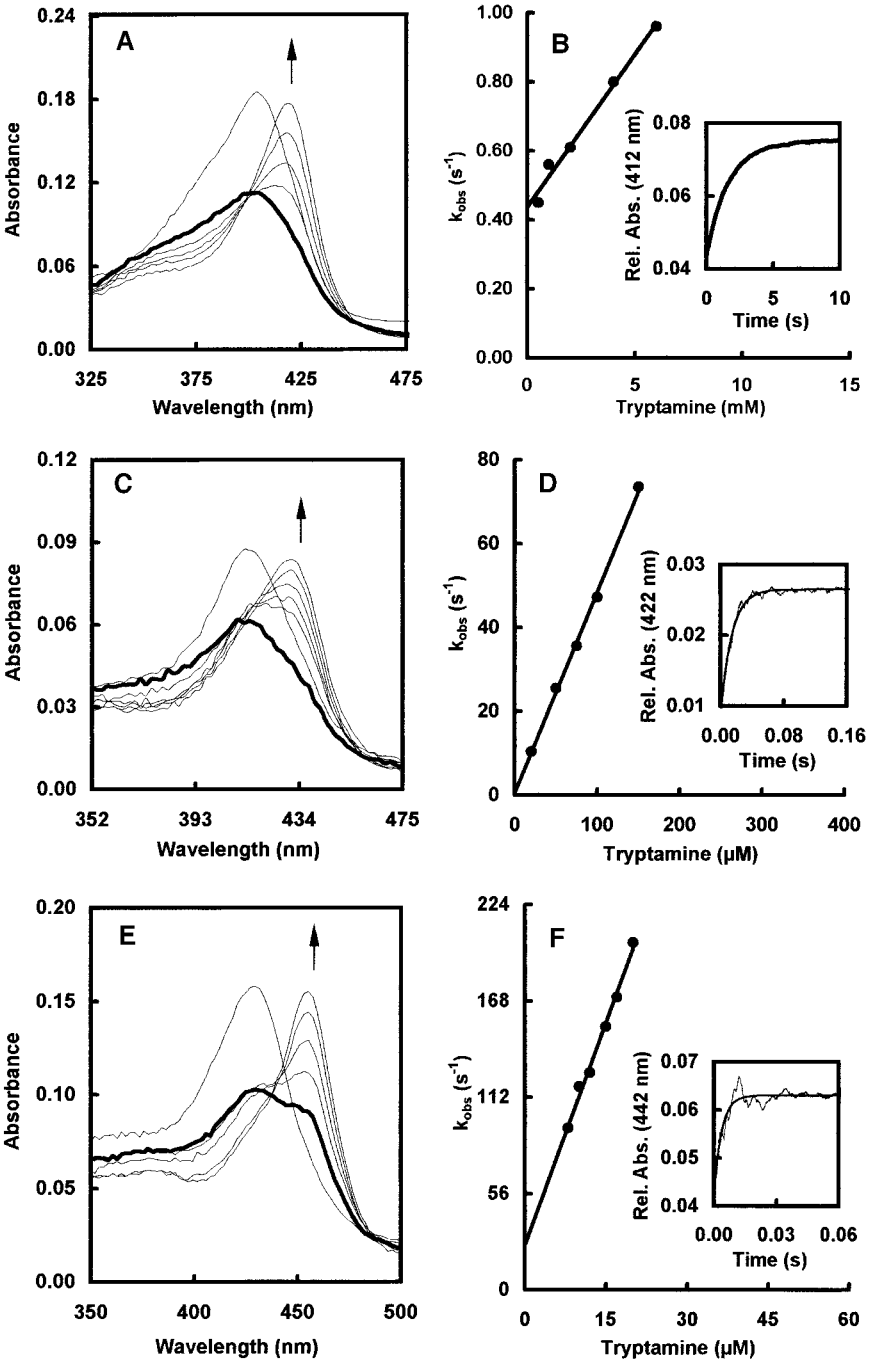


FIG. 1. Reaction of compound I of horseradish peroxidase (HRP), lactoperoxidase (LPO), and myeloperoxidase (MPO) with tryptamine at pH 7.0 and 25°C. Experiment was performed in the sequential-mixing stopped-flow mode. For details in formation of compound I see Materials and Methods. In A, C, and E, the corresponding spectra of the ferric proteins is also shown. (A) Spectral changes upon reaction of HRP compound I with tryptamine. HRP compound I (2 μM) reacts with 2 mM tryptamine. First (bold) spectrum (compound I) was recorded 1.3 ms after mixing, subsequent spectra at 265, 544, 987, and 1807 ms. (B) Pseudo-first-order rate constants for HRP compound I reduction plotted against tryptamine concentration. The inset shows a typical time trace and fit of HRP compound II formation followed at 412 nm (1 μM HRP, 2 mM tryptamine in 100 mM phosphate buffer, pH 7.0). (C) Spectral changes upon reaction of LPO compound I with tryptamine. LPO compound I (1 μM) reacts with 150 μM tryptamine. First (bold) spectrum (compound I) was recorded 1.3 ms after mixing, subsequent spectra at 13, 14, 27, 51, and 200 ms. (D) Pseudo-first-order rate constants for LPO compound I reduction plotted against tryptamine concentration. The inset shows a typical time trace and fit of LPO compound II formation followed at 422 nm (1 μM LPO, 150 μM tryptamine in 100 mM phosphate buffer, pH 7.0). (E) Spectral changes upon reaction of MPO compound I with tryptamine. 2 μM MPO compound I reacts with 10 μM tryptamine. First (bold) spectrum (compound I) was recorded 1.3 ms after mixing, subsequent spectra at 3.8, 6.4, 17, and 42 ms. (F) Pseudo-first-order rate constants for MPO compound I reduction plotted against tryptamine concentration. The inset shows a typical time trace and fit of MPO compound II formation followed at 442 nm (500 nM MPO, 10 μM tryptamine in 100 mM phosphate buffer, pH 7.0). In (A), (C), and (E) the corresponding spectrum of the protein in the resting (ferric) state is included. Arrows indicate spectral changes.

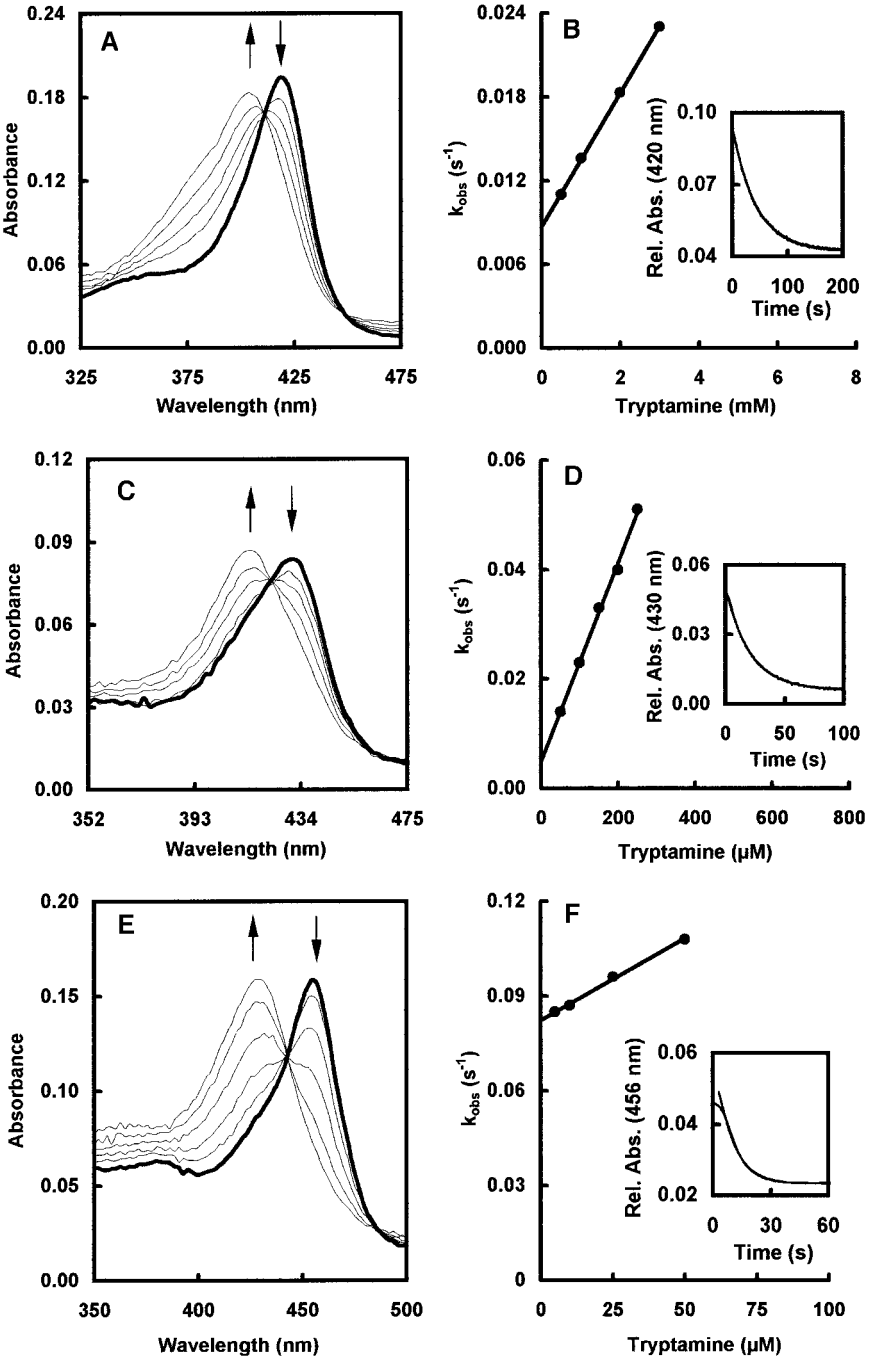
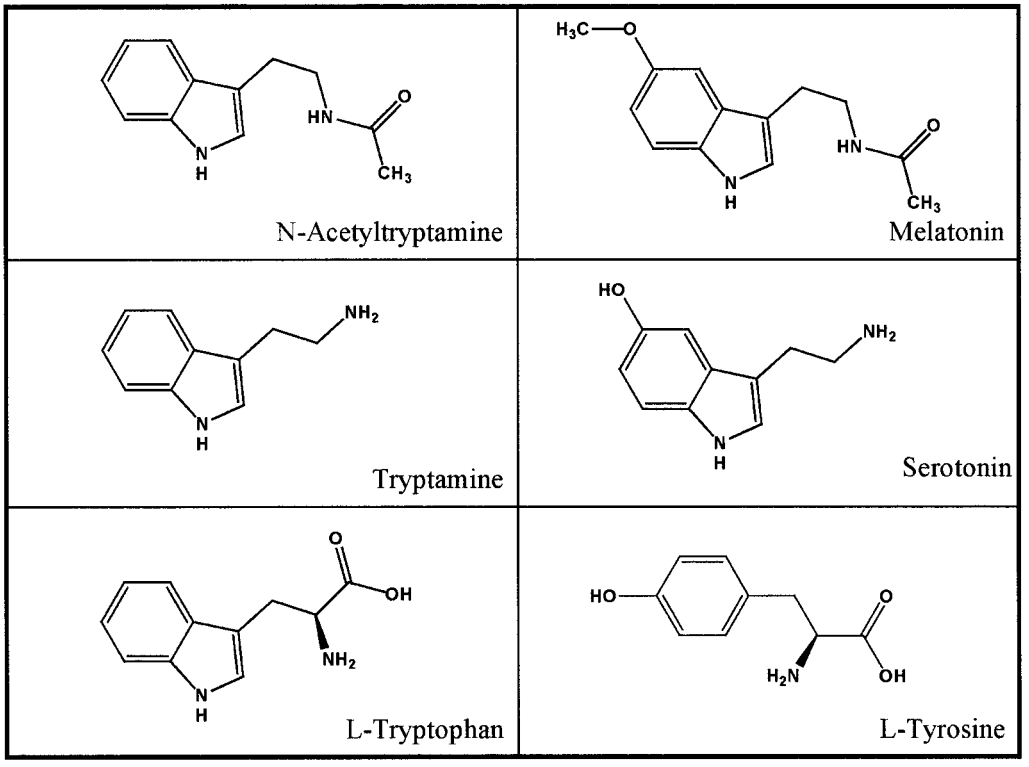


FIG. 2. Reaction of compound II of horseradish peroxidase (HRP), lactoperoxidase (LPO), and myeloperoxidase (MPO) with tryptamine at pH 7.0 and 25°C. Experiment was performed in the sequential-mixing stopped-flow mode. For details in formation of compound II see Materials and Methods. (A) Spectral changes upon reaction of HRP compound II with tryptamine. HRP compound II (2 μM) reacts with 2 mM tryptamine. First (bold) spectrum (compound II) was recorded 1.3 ms after mixing, subsequent spectra at 21.2, 27.5, 36.3, and 50 s. (B) Pseudo-first-order rate constants for HRP compound II reduction plotted against tryptamine concentration. The inset shows a typical time trace and fit of HRP compound II reduction followed at 420 nm (1 μM HRP, 2 mM tryptamine in 100 mM phosphate buffer, pH 7.0). (C) Spectral changes upon reaction of LPO compound II with tryptamine. 1 μM LPO compound I reacts with 150 μM tryptamine. First (bold) spectrum (compound II) was recorded 1.3 ms after mixing, subsequent spectra at 9, 14.6, 23.7, and 41.2 s. (D) Pseudo-first-order rate constants for LPO compound II reduction plotted against tryptamine concentration. The inset shows a typical time trace and fit of LPO compound II reduction followed at 430 nm (1 μM LPO, 150 μM tryptamine in 100 mM phosphate buffer, pH 7.0). (E) Spectral changes upon reaction of MPO compound II with tryptamine. MPO compound I (2 μM) was mixed with 10 μM tryptamine. First (bold) spectrum (compound II) was recorded 1.3 ms after mixing, subsequent spectra at 4.1, 4.7, 5.4, 6.6, and 9.3 s. (F) Pseudo-first-order rate constants for MPO compound II reduction plotted against tryptamine concentration. The inset shows a typical time trace and fit of MPO compound II reduction followed at 456 nm (500 nM MPO, 10 μM tryptamine in 100 mM phosphate buffer, pH 7.0).

TABLE I

Structures of Indole Derivatives and Tyrosine



actual system the concentration of the redox intermediates is in the micromolar region and that of the tryptamine in the millimolar region (Figs. 1A, 1B, 2A, and 2B). Assuming a ratio of 10^{-1} for the couples compound I/compound II and compound II/ferric HRP and of about 10^{-3} (or lower) for the couple tryptamine radical/tryptamine, then an equilibrium constant of about 10^{-2} would result. With $E°' = 1.015$ V for the tryptamine radical/tryptamine couple (35) and an estimated equilibrium constant of 10^{-2}, a $E°'$ value for the compound I/compound II and compound II/ferric HRP couple of about 0.90 V would result by use of the Nernst equation. Clearly, this value is roughly estimated but fits well with the experimentally determined values for the compound I/compound II and compound II/ferric HRP couples (23, 24).

Lactoperoxidase and myeloperoxidase reactivity. As Table II shows, the rates of LPO and MPO compound I reduction by the various indoles were higher in comparison to HRP and the calculated k_2 values for the five indoles varied only by a factor of 88 and 38, respectively, in contrast to a factor of 159,000 in case of HRP compound I. This was indicative of a more positive reduction potential for the mammalian peroxidase compound I/compound II couple. Generally, the more positive the corresponding $E°'$ value was, the smaller was the influence of the nature of the substrate on k_2. This was best demonstrated with MPO. Its compound I is extremely reactive which is best underlined by the well known fact that MPO is the only peroxidase of superfamily II which oxidizes chloride at a substantial rate (11) and is also capable of the one-electron oxidation of hydrogen peroxide to superoxide (48, 49). So far, little is known about the redox chemistry of human peroxidase intermediates. The reduction potential ($E°'$) for the ferric/ferrous couple was calculated to be -190 mV for lactoperoxidase (50) and $+25$ mV for MPO (51, 52). Recently, for MPO and eosinophil peroxidase (which exhibits similar spectral and kinetic features as LPO) the standard reduction potentials of the couple compound I/native enzyme have been determined to be 1.16 and 1.10 V, respectively (53). But the one-electron reduction potentials of MPO and LPO compound I and compound II are unknown. Regarding the fact that LPO and MPO compound I oxidize the indole derivatives very efficiently, with k_2 (MPO) being by average 10 times faster than k_2 (LPO), the $E°'$ value of the LPO and MPO compound I/compound II couple should be $\geqslant 1.015$ V, with $E°'$ (MPO) being more positive than $E°'$ (LPO). This conclusion is valid since the concentration of all reactants is in the micromolar region and thus the equilibrium constant is close to 1. It is worth noting that these suggested ranges for the $E°'$ values reflect the known differences in the $E°'$ values of

TABLE II

Bimolecular Rate Constants of the Reactions between Compounds I (k_2) and II (k_3) of Horseradish Peroxidase (HRP), Lactoperoxidase (LPO), and Myeloperoxidase (MPO) with Indole Derivatives and Tyrosine at pH 7.0 and 25°C

Indole	$E^{\circ\prime}$ (V)	Horseradish peroxidase		Lactoperoxidase		Myeloperoxidase	
		k_2 (M^{-1} s^{-1})	k_3 (M^{-1} s^{-1})	k_2 (M^{-1} s^{-1})	k_3 (M^{-1} s^{-1})	k_2 (M^{-1} s^{-1})	k_3 (M^{-1} s^{-1})
Tryptamine	1.015 (39)	$(8.8 \pm 0.6) \times 10^1$	(4.8 ± 0.1)	$(4.8 \pm 0.1) \times 10^5$	$(1.8 \pm 0.1) \times 10^2$	$(8.6 \pm 0.4) \times 10^6$	$(5.2 \pm 0.2) \times 10^2$
Serotonin	0.65 (40)	$(8.1 \pm 0.4) \times 10^6$	$(1.2 \pm 0.1) \times 10^6$	$(2.0 \pm 0.1) \times 10^6$	$(3.0 \pm 0.1) \times 10^5$	$(1.7 \pm 0.1) \times 10^{7a}$	$(1.4 \pm 0.1) \times 10^{6a}$
N-acetyltryptamine	n.f.	$(3.8 \pm 0.2) \times 10^3$	$(2.1 \pm 0.1) \times 10^2$	$(1.5 \pm 0.1) \times 10^6$	$(4.4 \pm 0.2) \times 10^3$	$(1.3 \pm 0.1) \times 10^6$	$(1.3 \pm 0.1) \times 10^2$
Tryptophan	1.015 (39)	$(5.1 \pm 0.4) \times 10^1$	(2.0 ± 0.2)	$(2.4 \pm 0.1) \times 10^4$	$(8.4 \pm 0.3) \times 10^1$	$(4.5 \pm 0.1) \times 10^5$	(6.9 ± 1.3)
Melatonin	0.95 (41)	$(1.3 \pm 0.1) \times 10^4$	$(5.2 \pm 0.4) \times 10^2$	$(2.1 \pm 0.2) \times 10^6$	$(2.7 \pm 0.1) \times 10^3$	$(6.1 \pm 0.2) \times 10^{6b}$	$(9.6 \pm 0.3) \times 10^{2b}$
Tyrosine[c,d,e]	0.93 (42)	$(5 \pm 0.1) \times 10^4$	$(1.1 \pm 0.1) \times 10^3$	$(1.1 \pm 0.1) \times 10^5$	$(1.0 \pm 0.1) \times 10^4$	$(7.7 \pm 0.1) \times 10^5$	$(1.6 \pm 0.6) \times 10^4$

Note. The one-electron reduction potentials of the indole derivatives at pH 7 ($E^{\circ\prime}$) are included (with references in parenthesis). n.f., not found in the literature. For details concerning sequential stopped-flow experiments see Materials and Methods.

[a] Data from Ref. 43.
[b] Data from Ref. 29.
[c] HRP data from Ref. 44.
[d] LPO data from Ref. 45.
[e] MPO data from Ref. 46.

the ferric/ferrous couple of the three peroxidases, with HRP (−0.28 V (23, 24)) < LPO (−0.19 V (50)) < MPO (+0.025 V (51, 52)) and thus indicate some correlation between $E^{\circ\prime}$ (ferric/ferrous) and $E^{\circ\prime}$ (compound I/compound II). As will be discussed below this correlation does not exist with $E^{\circ\prime}$ (compound II/ferric peroxidase).

Regarding the calculated rates of the reactions between LPO and MPO compound II and the indole derivatives (k_3), the situation is completely different and more complex. First, with the exception of serotonin and tyrosine, k_3 was much smaller than k_2. Especially in case of MPO the differences were significant with k_2/k_3 ratios of 16,500 (tryptamine), 10,000 (*N*-acetyltryptamine), 65,000 (tryptophan), and 6,400 (melatonin). Second, interestingly the k_2/k_3 ratios of serotonin and tyrosine were similar to that observed with HRP. Third, the k_3 values of the five indoles varied by a factor of 3,570 (LPO) and 200,000 (MPO). And fourth, the calculated k_3 values of all three peroxidases were similar. These findings indicate that pure thermodynamic considerations cannot describe this phenomenon sufficiently. Regarding the slow reaction of compound II with tryptophan at pH 7, one could suggest that tryptophan oxidation mark the thermodynamic limit of an electron donor to mediate the transition of LPO or MPO compound II to the ferric enzyme. Similar considerations as performed above with HRP compound I and II reduction lead to the conclusion, that the $E^{\circ\prime}$ value for the (compound II/ferric peroxidase) couple of LPO and MPO should be similar to that of the corresponding HRP couple [i.e., $E^{\circ\prime}$ (MPO) ≈ $E^{\circ\prime}$ (LPO) ≈ $E^{\circ\prime}$ (HRP) ≈ 0.90 V]. This is in strong contrast to the more positive $E^{\circ\prime}$(compound I/compound II) values for LPO and MPO.

Conclusion. The presented comparative stopped-flow study revealed substantial differences in the reactivity of the redox intermediates of the three peroxidases. The indole moiety was shown to function as one-electron donor for compounds I (k_2) and II (k_3) with a strong influence of the substituent but not the side chain on the reaction rates. There are only a few peroxidases where k_2 is similar to k_3. One interesting example is *Arthromyces ramosus* peroxidase (member of class II of superfamily I) exhibiting similar compound I and II reduction rates, which was explained by a more positive $E^{\circ\prime}$ value of compound II in comparison to compound I (54). The data obtained for HRP in this paper confirmed recent studies which have demonstrated that the k_2/k_3 ratio in case of HRP is about 10–100 and could be explained by both similar $E^{\circ\prime}$ values of the compound I/compound II and compound II/ferric HRP couples, an identical donor binding site but a longer electron transfer distance in compound II reduction (21–25). Based on the indole reactivity and the published $E^{\circ\prime}$ values for the substrates, we roughly

estimated an $E°'$ value for both HRP compound I and compound II to be about 0.90 V. By contrast, mammalian peroxidase compound I was shown to be a much stronger oxidant than HRP compound I. The k_2 values of MPO also exceeded that of LPO. Interestingly, the k_3 values of all three peroxidases were similar.

A convincing explanation for the higher k_2/k_3 ratios in LPO and MPO is not available at the moment. Principally, several items could be mentioned. First, the mode of interaction between the electron donor and the protein. The heme location and the active site of HRP and MPO are known to be substantially different (21). Structures of the corresponding enzymes with substrate analogues have been published (55–57). HRP oxidation of aromatic substrates has been proposed to take place at the heme edge, in the vicinity of the δ-meso carbon and the 8-methyl group on pyrrole ring D. In a recent paper (56) it has been suggested that the binding of ferulic acid (an excellent substrate for HRP with $k_2 = 4.8 \times 10^7\ M^{-1}\ s^{-1}$ and $k_3 = 1.3 \times 10^7\ M^{-1}\ s^{-1}$ (56)) is likely to be identical in compound I and compound II and that the association of the reducing substrate with the enzyme is relatively weak allowing the formed radicals to leave the active site rapidly (56). For MPO so far only the structure of the complex between the ferric protein and salicylhydroxamic acid was published, indicating that there are no significant conformational differences between the active site regions of the complex and the native enzyme (with the exception that three water molecules from the distal heme cavity were displaced by binding of salicylhydroxamic acid). In any case, if the overall topology of the actual substrate binding site as well as the substrate channel is identical for the aromatic electron donor which reduces either compound I and compound II, the active site topology should not be responsible for the high k_2/k_3 ratios measured in LPO and MPO mediated indole oxidation.

Second, indoles are known to bind to peroxidases (58, 59). Though the investigated indole derivatives could have different affinities to the binding site which could influence the apparent overall second-order rate constant, this would not explain the observed differences in k_2 and k_3, assuming the existence of an identical donor binding site in both compound I and compound II. In our experimental approach the reactions were started with the preformed redox intermediate, thus occupancy of the binding site can be excluded. Similarly, differences in the hydrophobicity of the substrates could influence the affinity but cannot explain the high k_2/k_3 ratio in either LPO or MPO. Since the calculated k_2 values varied by a factor of only 38 in MPO, we assume that there were no significant differences in the affinity of the investigated indoles to the binding site, which means that the calculated apparent second order rate constants mainly reflect rates of electron transfer. Third, because this study focused on transient kinetics an influence of produced radical products on compound II reduction by either LPO or MPO can be excluded. This does not mean that the produced indole oxidation products will not act as inhibitors in the overall reaction. In a recent study of transient and steady-state kinetics of the oxidation of benzoic acid hydrazides by MPO (60), it was clearly demonstrated that direct reaction of the parent molecules with either compound I or II was not influenced by the fact that benzoic acid hydrazides radicals are excellent inhibitors of MPO (60). It can also be excluded that protein radicals are involved in the described reactions. Formation of protein radicals has been detected in LPO and MPO (61, 62) but they formed relatively slow upon mixing the protein with H_2O_2 in the absence of an electron donor. In the present study electron donors were present and the rates of the monitored reactions were several orders of magnitude higher.

In accordance with the thermodynamic explanation of the k_2/k_3 ratios observed with HRP, one could conclude that with mammalian peroxidases the $E°'$ values of compound I reduction are more positive (with $E°'$ (MPO) $>$ $E°'$ (LPO)) than the corresponding $E°'$ values for compound II reduction. Regarding the calculated k_3 values summarized in Table II one could estimate that $E°'$ of the (compound II/ferric peroxidase) couple of MPO and LPO is similar to that of HRP (about 0.90 V). Generally, these findings suggest that in contrast to the $E°'$ values of the compound I/compound II couple of the three peroxidases there was no correlation between the $E°'$ values of the compound II/ferric couple with those of the ferric/ferrous couple. Since reduction potentials are controlled by several factors (e.g., basicity of pyrrol nitrogen of porphyrin and of the proximal ligand, electrostatic interaction, etc.) and since, especially in case of MPO, oxidation of substrates at compound II seemed to be much more constrained by the structure of the electron donor, we suppose that at least one of these factors changed during conversion of MPO or LPO compound I to compound II. Reactions 2 and 3 have been postulated for peroxidases of superfamily I. It is known for HRP that reduction of compound I to compound II and of compound II to the ferric enzyme involves uptake of an electron as well as a proton and that at neutral pH the charge state of the heme remains essentially the same in the ferric, compound I and compound II states (63, 64). However, less is known about proton balance in LPO and MPO catalysis and if the net charge varies at the redox site during enzyme turnover. Thus, this work

is suggestive for further research that could be important for the design of substrate analogues and specific inhibitors for myeloperoxidase, which is thought to contribute to numerous inflammatory pathologies (17).

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