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# Kinetics and Thermodynamics of Halide and Nitrite Oxidation by Mammalian Heme Peroxidases

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The human heme peroxidases myeloperoxidase (MPO), eosinophil peroxidase (EPO) and lactoperoxidase (LPO) are able to oxidise (pseudo)halides and nitrite to reactive species that participate in host defence against foreign microorganisms as well as in immunomodulation and tissue degradation in certain pathologies. The heme in EPO and LPO is covalently linked to the apoprotein by two ester bonds, whereas in MPO it is additionally linked by a unique sulfonium ion bond to a methionine residue. As a consequence, the prosthetic group in MPO is significantly distorted from a planar conformation. These structural differences are reflected by distinct spectral and redox properties as well as reactivities toward chloride, bromide, iodide, thiocyanate and nitrite, which function as endogenous two- and one-electron donors for these enzymes in vivo. Standard reduction potentials at pH 7 have been determined for all redox couples involved in the halogenation and peroxidase cycle of MPO and LPO and partially of EPO. A detailed thermodynamic analysis of the formation of reactive halide species by MPO and EPO was also performed. Thus, for the first time, a comprehensive analysis of reactions catalysed by human heme peroxidases is presented that allows a better understanding of their role in physiological and pathophysiological processes.


## Heme Peroxidases

Peroxidases are iron-containing enzymes that have been used in a great number of analytical and technical applications[1] since they are able to catalyze one- and two-electron oxidation reactions of inorganic and organic compounds with hydrogen peroxide. Physiologically, these enzymes are not only responsible for protection against harmful side-effects of oxygen metabolism but are also involved in a variety of biosynthetic and degradative functions related to the consumption of hydrogen peroxide. Today we know, as result of gene and genome sequencing and increasing data deposited in protein data banks, of two heme-containing peroxidase superfamilies, namely the superfamily of plant, fungal and bacterial peroxidases[2] and the superfamily of animal peroxidases.[3] The physiological importance of these metalloenzymes is underlined by the fact that higher organisms often contain a high number of genes encoding peroxidases. For example, in *Arabidopsis thaliana*, the first plant for which the whole genome has been sequenced, 73 full-length genes encoding classical plant peroxidases[4] and several genes encoding so-called ascorbate peroxidases[5] have been identified in the genome. Moreover, the diversity in structure and function can be further increased by variations in gene transcription and translation resulting in the fact that one single gene can encode several multiple forms of a protein or enzyme.

In the human genome there are only four genes encoding for homologous heme peroxidases known as myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO) and thyroid peroxidase (TPO), which are members of the animal peroxidase superfamily. MPO, EPO and LPO are functionally homologous enzymes closely involved in host defence. MPO is secreted at inflammatory sites from stimulated polymorphonuclear leukocytes and also monocytes,[6] while EPO is released from activated eosinophils.[7] LPO is found in mucosal surfaces and exocrine secretions such as milk, tears and saliva.[8] In contrast, TPO is involved in the biosynthesis of the thyroid gland hormones thyroxine and triiodothyronine.[9]

Closely related with the physiological role is the nature of the substrates oxidized by human peroxidases. These enzymes prefer small anionic molecules as electron donors, such as halides (chloride, bromide, iodide), thiocyanate and nitrite. The corresponding oxidation products, for example

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hypohalous acids or nitrogen dioxide, are responsible for killing microorganisms[6,10] or in iodination of tyrosine residues of thyroglobulin in hormone syntheses.[9] However, since many of these reaction products are (strong) halogenating and nitrating oxidants, they also contribute to tissue injury in certain inflammatory diseases.[11] Moreover, certain products of the halogenating activity of peroxidases are known to function as immunomodulators such as taurine chloramine[12] and hypothiocyanite.[13]

These functional features are closely related with the peculiar structural and redox properties of these heme proteins. Recently, the standard reduction potentials of the redox couple compound I/ferric enzyme for MPO, EPO and LPO, as well as compound II/ferric enzyme and compound I/compound II for MPO and LPO have been determined.[14–16] With this knowledge, it is now possible to relate much better data on (pseudo)halide and nitrite oxidation to the structural and functional properties of perox-

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idases. This review summarises the present knowledge on halide and nitrite oxidation by MPO, EPO and LPO from the viewpoint of biophysical chemistry and compares their halogenation and nitration activity with respect to the modification of functional sites in proteins.

## Active Site of Mammalian Peroxidases

Among the mammalian peroxidases only the 3D structure of MPO is known. The structure of human MPO at 2.3 Å resolution was obtained and this structure has been refined at 1.8 Å, using X-ray data recorded at –180 °C.[17] Each half of dimeric MPO contains one iron, which is present as covalently bound heme. Besides differences in the overall structure, the mode of heme binding in animal peroxidases is the most significant structural difference to plant-type peroxidases, which have the heme not covalently attached to the protein. In MPO and other animal peroxidases, the methyl groups on pyrrole rings A and C of the protoporphyrin IX derivative are modified to allow formation of ester linkages with the carboxyl groups of Glu242 and Asp94. In addition, the β-carbon of the vinyl group on pyrrole ring A in MPO forms a covalent bond with the sulfur atom of Met243, giving rise to a sulfonium ion linkage (Figure 1, A).[17] The heme porphyrin ring is considerably distorted from planarity. Although pyrrole rings B and D are nearly coplanar, ring A and, to a lesser extent, ring C are tilted toward the distal side, resulting in a bow-shaped structure of the heme.

Unlike the two acidic residues, Met243 has no obvious equivalent in EPO, LPO or TPO,[18] although there is biochemical and biophysical evidence for a heme linkage through esters in EPO,[19] LPO[20] and TPO.[21] The uncommon manner of heme binding is responsible for the peculiar spectroscopic features[18] and redox properties of mammalian peroxidases. MPO has the largest red shift of the maximum of the Soret band in the visible spectrum among peroxidases and the highest values for standard reduction potentials of redox couples where compound I is involved. The MPO-specific sulfonium ion linkage could have the following roles: first, it serves as an electron-withdrawing substituent due to its positive charge, and, second, together with its neighbouring residue Glu242 (Figure 1, A), it appears to be responsible for the lower symmetry of the heme group and distortion from the planar conformation. The spectral properties of EPO, LPO and TPO are very similar, thereby underlining that they share an identical way of heme binding by two ester bonds.[18] All other active site residues, i.e. the proximal histidine (His336) and the heme distal site residues histidine (His95 in MPO), arginine (Arg239) and glutamine (Gln91; see Figure 1) are highly conserved in mammalian peroxidases. Moreover, the overall structure of the four representative proteins seems to be similar. There are only a small number of insertions/deletions and modelling suggests that almost all the regions corresponding to helices in MPO are well conserved in the other members of this protein superfamily.[18]

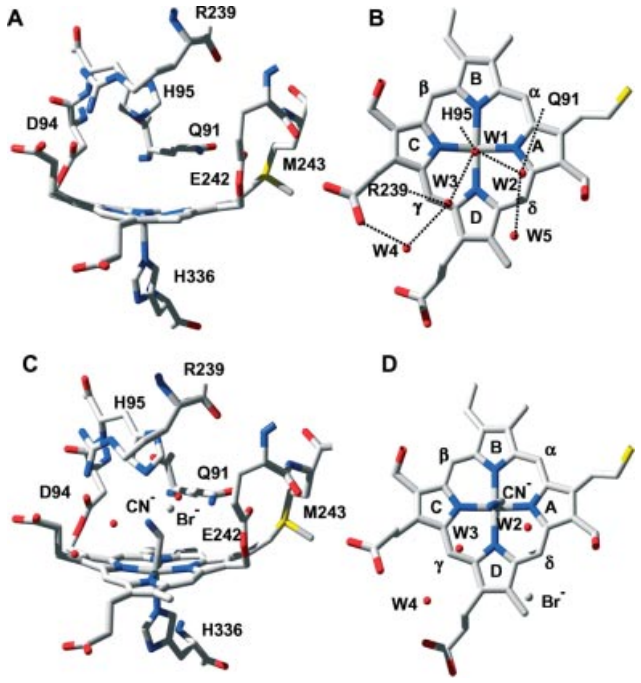


Figure 1. (A) The non-planar porphyrin ring in MPO and its covalent attachments to the protein through two ester bonds (Asp94 and Glu242) and one sulfonium linkage (Met243). The catalytic residues His95, Arg239 and Gln91 are also shown, the latter of which is important in halide binding. (B) The locations of the five water molecules in the distal heme cavity of ferric high-spin MPO. This figure was constructed using the coordinates deposited in the Protein Data Bank (accession code 1CXP). (C) The structure of the myeloperoxidase-cyanide-bromide double complex (MPO complexed with cyanide and bromide). The view in (D) is approximately normal to the heme plane. Please note that in the MPO–bromide complex the halide binds at W2, which is positioned above the δ-methine bridge carbon (i.e. the potential electron donation site of both nitrite and halides) between pyrrole rings A and D. This figure was constructed using the coordinates deposited in the Protein Data Bank (accession code 1D7W).

## Nitrite and Halide Binding

Cyanide and nitrite bind very tightly to the active site of mammalian peroxidases and convert the protein into a low-spin species. The pH dependency of cyanide binding resembles that of $H_2O_2$ in compound I formation (see below), since both require the deprotonated form of a group with a p$K_a$ of 4.0–4.3, which seems to be the distal histidine.[22–24] Following deprotonation of HCN by the distal histidine, the cyanide anion binds to the heme iron to produce an $S = 1/2$ low-spin complex. The crystal structure of the MPO–cyanide complex[25] shows that the orientation of the cyanide ion is determined by the formation of three hydrogen bonds between its nitrogen atom and the distal histidine as well as water molecules W2 and W3 (see C and D in Figure 1). Water W1 is displaced by cyanide and the heme iron moves about 0.2 Å into the plane of the heme during the transition of high-spin MPO to its low-spin form. No significant protein conformational changes are associated with cyanide binding.[25]

Similar to cyanide, nitrite binding converts ferric high-spin to low-spin MPO.[24,26] The structure of the MPO–

  

$NO_2^-$ complex is unknown. However, because of the large spectral changes, one would expect that nitrite also binds directly to $Fe^{III}$ through the nitrogen atom [Equation (1)], with $PorFe^{III}$–$NO_2^-$ being the peroxidase nitrite complex.

$$PorFe^{III} + NO_2^- \leftrightarrows PorFe^{III}–NO_2^- \quad (1)$$

The pH dependency of nitrite binding resembles that of halide binding to MPO (see below). In contrast to cyanide binding, nitrite and halide binding is favoured at acidic pH and needs protonation of the distal histidine. This is shown by a significant decrease of the dissociation constant of bound nitrite upon decreasing the pH from 7.0 (2.3 mM) to 5.0 (31 μM).[24] LPO shows a much reduced affinity toward nitrite, with a dissociation constant of about 45 mM at neutral pH.[28] From the pH dependence of $NO_2^-$[24] and chloride binding,[27] the p$K_a$ value of the distal histidine was found to be around 4.3.

Halides do not bind directly to the heme iron. The structure of the MPO–bromide complex[25] demonstrates that bromide binds at the position of water molecule W2 (Figure 1, B), which is hydrogen bonded to the amide nitrogen of Gln91. Bromide binding is accompanied by small shifts of the side-chains of His95 and the heme-ester-linked Glu242 as well as of water molecules W1 and W5. Possible electrostatic interactions occur between the bromide and water molecules W1 and W5, the Nε of His95 and the amide nitrogen of Gln 91.[25] The closest heme atom to the bromide is the δ-methylene bridge carbon between pyrrole rings A and D (Figure 1). Thus, halides are typical high-spin ligands in contrast to the low-spin ligands cyanide or nitrite. As a consequence, spectral changes upon halide binding are relatively small.[18]

## The Redox Couple Compound I/Native Enzyme

Hydrogen peroxide is known to convert resting ferric heme peroxidases ($PorFe^{III}$) into compound I ($^{\cdot+}PorFe^{IV}$=O),[29] which is a porphyrin π-cation radical with an oxygen atom coupled by a double bond to iron(IV) [Equation (2)].[30]

$$PorFe^{III} + H_2O_2 \rightarrow {}^{\cdot+}PorFe^{IV}=O + H_2O \quad (2)$$

In this redox reaction, the reduction of hydrogen peroxide to water is coupled with the oxidation of ferric heme. The oxidation and reduction steps of this redox reaction consist of the two half reactions (both written as reduction process) given in Equations (3) and (4).

$$^{\cdot+}PorFe^{IV}=O + 2e^- + 2H^+ \rightarrow PorFe^{III} + H_2O \quad (3)$$

$$H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O \quad (4)$$

As the absorption of the Soret band of heme peroxidases shows a marked hypochromicity upon formation of compound I, it is possible to monitor this fast conversion by stopped-flow spectroscopy. By monitoring the resulting absorbance change at the wavelength for which the extinction coefficient of native enzyme and corresponding compound I are known, and taking into account the standard reduction potential, $E'°$, of 1.32 V at pH 7 for the redox couple $H_2O_2/2H_2O$,[31] equilibrium concentrations of all reactants and standard reduction potentials of the couples compound I/ferric enzyme have been determined for human heme peroxidases using the Nernst equation [Equation (5)] (Table 1). An alternative approach to generate compound I of peroxidases is the use of peroxyacetic acid, which is reduced to acetate and water. The redox couple peroxyacetic acid/acetate, $H_2O$ has a standard reduction potential of 1.14 V at pH 7.[16]

$$E' = E'^{o} + (RT/nF) \ln(a_{ox}/a_{red}) \quad (5)$$

Table 1. Standard reduction potentials ($E'°$) at pH 7 of all redox couples of the peroxidase cycle of human heme peroxidases.

| Redox couple | MPO | EPO | LPO |
|---|---|---|---|
| Compound I/native enzyme | 1.16 V[14] | 1.10 V[14] | 1.09 V[16] |
| Compound I/compound II | 1.35 V[37] | n.d.[a] | 1.14 V[16] |
| Compound II/native enzyme | 0.97 V[37] | n.d.[a] | 1.04 V[16] |

[a] n. d.: not determined.

In Equation (5) $E'$ is the reduction potential of any redox couple and $E'^{o}$ is the standard reduction potential that is referred to a concentration of 1 M or a pressure of 101.3 kPa in the case of gases. The gas constant $R$ is equal to 8.31 $JK^{-1}mol^{-1}$. The temperature $T$ is conventionally set to 298 K and $n$ represents the number of electrons transferred in a single reaction step by the redox couple. The Faraday constant $F$ is 96485 $A{\cdot}s\,mol^{-1}$ and $a_{ox}$ and $a_{red}$ are the activities of the oxidised and reduced species, respectively. This method has been applied since a direct electrochemical determination of the reduction potential of the compound I/native enzyme couple is not possible due to the very high instability of compound I.

The highest $E'°$ value for the compound I/ferric enzyme couple was found to be 1.16 ± 0.01 V for MPO[14] followed by EPO and LPO, with $E'°$ values of 1.10 ± 0.01 V[14] and 1.09 ± 0.01 V,[16] respectively. These differences are apparently caused by the mode of heme-to-protein linkages described above: only in MPO, in addition to the two ester linkages that are also present in EPO and LPO, is there the unique sulfonium ion linkage at Met243 (see A and C in Figure 1), which, besides its electron-withdrawing properties, will provoke an additional distortion of the prosthetic group with the consequence of a pronounced out-of-plane structure of the iron in the resting state.[17] This uncommon way of heme binding is also responsible for the red-shift of the Soret band in MPO (430 nm), EPO (413 nm) and LPO (412 nm) compared to proteins with a more coplanar heme such as ferric hemoglobin and horseradish peroxidase (HRP).[18] The critical role of Met243 in the heme–protein interaction is underlined by site-directed mutagenesis studies. Replacement of Met243 in mutant MPO strongly affects the physical properties, substrate binding, substrate specificity and substrate oxidation of the corresponding mutants.[32] In general, Met243 variants exhibit spectral and

  

catalytic properties similar to LPO and EPO, the heme of which is linked only by two ester bonds to the apoprotein.

## The Redox Couples Compound I/Compound II and Compound II/Ferric Enzyme

Compound I of heme peroxidases can be reduced by two consecutive one-electron steps to the ferric enzyme via the intermediate complex compound II [$PorFe^{IV}=O$], whereby numerous small molecular substrates, including aromatic amino acids, nitrite, indole derivatives and others, are oxidised by abstracting one electron.[24,33,34] For example, the generally accepted mechanism of nitrite oxidation involves the intermediates compound I and compound II [Equations (6) and (7)].

$$^{\cdot+}PorFe^{IV}=O + NO_2^- \rightarrow PorFe^{IV}=O + {}^{\cdot}NO_2 \quad (6)$$

$$PorFe^{IV}=O + NO_2^- + 2H^+ \rightarrow PorFe^{III} + {}^{\cdot}NO_2 + H_2O \quad (7)$$

These reactions, together with the oxidation of the ferric enzyme by hydrogen peroxide [Equation (2)], form the peroxidase cycle.

The oxidation of nitrite to nitrogen dioxide [$E'°({}^{\cdot}NO_2/NO_2^-) = 1.04$ V at pH 7][35] as well as of tyrosine to tyrosyl radical [$E'°$(tyrosyl radical/tyrosine) = 0.94 V at pH 7][36] has been used to convert compound II of MPO or LPO to the ferric enzyme and to calculate the standard reduction potentials of the redox couple compound II/ferric enzyme of these peroxidases (Table 1). Finally, the standard reduction potentials of the couple compound I/compound II have been calculated from the two other experimentally determined potentials of the peroxidase cycle (Table 1). Experimental details and calculations are described elsewhere.[15,16,37]

Significant differences were observed in the oxidation of indole derivatives by compound I and II of MPO and LPO. The rates of reduction of compound II to ferric peroxidase were similar, which is reflected by the fact that the standard reduction potentials of the redox couple compound II/ferric enzyme for MPO and LPO differ by only 0.07 V.[16,37] Higher differences, however, were found in one-electron substrate oxidation for reactions catalysed by compound I. The highest standard reduction potential of couple compound I/compound II ($1.35 \pm 0.01$ V at pH 7) was obtained for MPO.[37] The potential for this couple for LPO ($1.14 \pm 0.02$ V) is considerably lower,[16] and the corresponding potential for EPO is still unknown. As EPO exhibits more similarities to LPO than to MPO in its redox properties, it is assumed that the couple compound I/compound II of EPO has a similar $E'°$ value to LPO.

Thus, compound I of MPO is able to oxidise substrates with a reduction potential around 1.2 V and higher with sufficient rates,[38] thus underlining its extraordinary role in substrate oxidation during bacterial killing. A partial reduction of compound I of MPO to compound II is even observed in the presence of 250 mM hydrogen carbonate.[39] The redox couple $CO_3^{\cdot-}/HCO_3^-$ has a standard reduction potential at pH 7 equal to 1.78 V.[40] Assuming a very low concentration for the unstable carbonate radical, it follows from the Nernst equation [Equation (5)] that compound I of MPO is readily able to oxidise high concentrations of hydrogen carbonate.

The formation of isomeric compound I, where the radical function is transferred from the porphyrin ring to the protein moiety, is still under discussion.[18,41] The conversion from porphyryl to protein radical has been shown in MPO[42,43] and LPO[43] in the absence of good one- or two-electron donors. Apparently, this conversion can be neglected when considering the composition of the medium at inflammatory sites containing a high number of substrates being oxidised by peroxidases.

## Nitrite Oxidation by Mammalian Peroxidases

The oxidation of nitrite by its reaction with either compound I or compound II requires the formation of nitrogen dioxide [Equations (6) and (7)].[44] As has been demonstrated above, both compound I and compound II are able to perform the one-electron reaction of nitrite. Some of the rate constants for these reactions have been determined for mammalian peroxidases. Table 2 also includes, for comparative purpose, data for the plant model enzyme HRP.[44,45] Brück et al.[46] have reported that LPO compound I reacts with nitrite in a two-electron process to produce nitrate and the resting enzyme directly due to the lack of observation of the spectral features of compound II when compound I was mixed with nitrite in a stopped-flow apparatus. However, this could be due to the high rate of LPO compound II reduction by nitrite, which would prevent accumulation of compound II. Normally, the compound II spectrum dominates in a peroxidase cycle that consists of Equations (2), (6) and (7). In fact, with a sequential-mixing stopped-flow apparatus with millisecond mixing time, we have been able to observe the transient formation of LPO compound II (unpublished results) when compound I was mixed with nitrite. Unfortunately, the reaction rate constant for the reduction of EPO compound I by nitrite has not been obtained yet, although it is assumed to be much higher than that of compound II.[44k]

Table 2. Second-order rate constants for the reduction of the peroxidase intermediates by nitrite.

| Enzyme | Intermediate | pH | $k$ ($M^{-1}s^{-1}$) |
|---|---|---|---|
| LPO | compound I | 7.2 | $2.2 \times 10^7$ [46] |
| | compound II | 7.2 | $3.5 \times 10^5$ [46] |
| | compound II | 7.0 | $6.8 \times 10^4$ [16] |
| MPO | compound I | 7.0 | $(2.2 \pm 0.2) \times 10^6$ [24] |
| | compound II | 7.0 | $(5.5 \pm 0.1) \times 10^2$ [24] |
| | compound I | 5.0 | $(1.1 \pm 0.2) \times 10^7$ [24] |
| | compound II | 5.0 | $(8.9 \pm 0.6) \times 10^4$ [24] |
| EPO | compound II | 7.4 | $(5.6 \pm 0.2) \times 10^3$ [44k] |
| HRP | compound I | 6.93 | $6.7 \times 10^2$ [45a] |
| | compound II | 7.5 | $(6.6 \pm 0.4)$[44e] |
| | compound II | 7.0 | 13.3[45b] |

Generally, reduction of compound I [Equation (6)] is faster than that of compound II [Equation (7)], and this is

  

reflected by the higher redox potential for the compound I/compound II couple with respect to the compound II/native enzyme couple (Tables 1 and 2). Nevertheless, the same trend occurs with HRP (Table 2), which has a standard reduction potential of about 0.9 V for both redox intermediates.[47] This suggests that, besides the thermodynamic frame, kinetic aspects have to be taken into account. Considering Equations (6) and (7), according to the Marcus theory, there are significant differences in complexity. In compound I reduction the porphyrin cation radical has to be quenched; the rigid and highly delocalised aromatic skeleton of the macrocycle facilitates the electron transfer. In the case of MPO, it has been demonstrated that the δ-meso carbon of the porphyrin π-cation radical of compound I is most likely the electron donation site in compound I since it is located adjacent to the positively charged sulfonium ion linkage to the vinyl group attached to pyrrole ring A (Figure 1).[18,48] By contrast, reduction of the oxoiron(IV) species in compound II requires a more pronounced rearrangement; in fact, Equation (7) requires two protons for the formation of a water molecule from the oxoiron(IV) group. Furthermore, formation of the native enzyme includes a low-spin to high-spin shift that restores the out-of-plane disposition of $Fe^{III}$. The need for protons in compound II reduction by nitrite is also shown by the effect of pH on the reaction rates determined in the case of MPO (Table 2). Both reductions of compound I and compound II are controlled by an amino acid residue with a p$K_a$ value of $4.3 \pm 0.3$,[24] most likely the distal histidine, and the rates are higher at acidic pH. Reduction of compound I is approximately fivefold higher at pH 5 with respect to pH 7, whereas for compound II the ratio of the reaction rate constants at the two pH values increases by a factor of 160, thus indicating a much larger dependence on the proton concentration in the latter reaction.

## Redox Chemistry of (Pseudo)halide Oxidation by Peroxidases

Human heme peroxidases are unique among human enzymes in their ability to oxidise (pseudo)halides to (pseudo)hypohalous acids with concomitant reduction of compound I in a two-electron step to the ferric enzyme form. The overall reaction is given in Equation (8)

$$^{\cdot+}PorFe^{IV}{=}O + X^- + H^+ \rightarrow PorFe^{III} + HOX \quad (8)$$

where X denotes $Cl^-$, $Br^-$, $I^-$ or $SCN^-$. This redox process can be divided into two half-reactions, where the reduction of compound I [Equation (3)] is coupled with the (pseudo)halide oxidation ([Equation (9)], written as a reduction process.

$$XOH + 2e^- + H^+ \rightarrow X^- + H_2O \quad (9)$$

Standard reduction potentials for the redox couple XOH/$X^-$, $H_2O$ are given for pH 0 and pH 7 in Table 3. At pH 7, HOCl, HOBr and HOI exist predominantly in the undissociated form according to their p$K_a$ values (see also Table 3). Standard reduction potentials for HOCl, HOBr and HOI were originally described at pH 0. The reduction potentials of these couples decrease between pH 0 and pH 7 by about 0.03 V with increasing pH unit, as two electrons and one proton are involved in the corresponding half-reaction. Thus, the standard reduction potentials of the couples HOCl/$Cl^-$, $H_2O$, HOBr/$Br^-$, $H_2O$, and HOI/$I^-$, $H_2O$ are 1.28, 1.13 and 0.77 V, respectively, at pH 7. A further data set exists in the literature for these standard reduction potentials at pH 7;[31,49] they are about 0.20 V lower than those indicated in Table 3. These data were also originally determined from standard values at pH 0 by using, unfortunately, a wrong coefficient to consider the pH dependence.

Table 3. Standard reduction potentials [V] for two-electron oxidation of (pseudo)halides and p$K_a$ values of (pseudo)hypohalous acids.

| $X^-$ | p$K_a$ (HOX) | $E'°$(HOX/$X^-$, $H_2O$) pH 0 | pH 7 | $E'°$($X_2$/2 $X^-$) |
|---|---|---|---|---|
| $Cl^-$ | 7.53[50] | 1.49[51] | 1.28 | 1.39[52] |
| $Br^-$ | 8.8[53] | 1.33[54] | 1.13 | 1.09[55] |
| $I^-$ | 10.0[56] | 0.99[57] | 0.78 | 0.54[58] |
| $SCN^-$ | 5.3[59] | 0.82[a] | 0.56[a] | 0.72[a] |

[a] This work.

The standard reduction potential for the couple HOSCN/$SCN^-$, $H_2O$ can be calculated from the equilibrium constant of the following redox reaction [Equation (10)].

$$H_2O_2 + SCN^- \rightarrow OSCN^- + H_2O \quad (10)$$

An equilibrium constant of $3.7 \times 10^3$ $M^{-1}$ has been obtained for this reaction.[60] However, a critical re-evaluation yielded a totally different value of $5.65 \times 10^{25}$ $M^{-1}$.[61] With the latter value and $E'° = 1.32$ V at pH 7 for the couple $H_2O_2$/2 $H_2O$,[31] the unknown standard reduction potential for the couple $OSCN^-$/$SCN^-$, $H_2O$ can be determined to be 0.56 V at pH 7. With the p$K_a$ value of HOSCN (5.3),[59] a standard value of 0.82 V is obtained for this redox couple at pH 0.

Thus, the ease of oxidation of (pseudo)halide ions is the following: $SCN^- > I^- > Br^- > Cl^-$ (Table 3). In conversion of compound I to the ferric enzyme form by (pseudo)halide ions studied by rapid scan spectroscopy, the same order of sequence for the interacting (pseudo)halide has been obtained (Table 4). Only MPO compound I is able to react with chloride at pH 7.0 at reasonable rates.[42,62] It has been suggested that the experimental data for the interaction of chloride with compound I can be fitted best by a mechanism that includes a compound I–chloride complex.[62b] At low chloride concentration, the formation of the compound I–chloride complex is rate limiting, whereas at high chloride concentration its transition to ferric MPO and release of hypochlorous acid (HOCl) is rate controlling.[62b]

  

Table 4. Apparent second-order rate constants for the reactions between compound I of MPO, EPO and LPO with (pseudo)halide at pH 7 and 15 °C.

| | MPO[42] $\times 10^4$ ($M^{-1} s^{-1}$) | EPO[65] $\times 10^4$ ($M^{-1} s^{-1}$) | LPO[66] $\times 10^4$ ($M^{-1} s^{-1}$) |
|---|---|---|---|
| Chloride | 2.5 | 0.31 | – |
| Bromide | 110 | 1900 | 4.1 |
| Iodide | 720 | 9300 | 12000 |
| Thiocyanate | 960 | 10000 | 20000 |

At acidic pH compound I reduction is accelerated significantly, which is also reflected by an increase in the steady-state chlorination activity of MPO.[63] At pH 5 EPO compound I is also an oxidant of chloride (Table 4). At acidic pH, chloride binds to a protonated amino acid close to the ferryl oxygen since there is a halide-dependent change in the EPR spectrum at acidic pH.[64] Supported by the crystal structure of the MPO–bromide but not the MPO–cyanide–bromide double complex,[25] which can be regarded as a model of the compound I–halide complex, it is likely that the distal histidine, upon protonation, allows such direct interaction between chloride (but not bromide or iodide) and ferryl oxygen. In the native enzyme, bromide replaces water molecule W2, which is hydrogen bonded to the amide of Gln91 in the proximity of the Nε atom of His95 (Figure 1, B), but in the cyanide complex W2 is not displaced and bromide binds in place of W5, thereby preventing a direct H-bond interaction with protonated His95 (Figure 1, C and D).[25] Maybe the W2 site is inaccessible for larger anions like bromide or iodide when cyanide is bound to the iron. Although no crystallographic studies are available for chloride binding in the distal cavity of MPO, it is possible that the W2 position could accommodate this ion equally well in both native MPO and its cyanide complex, since chloride (1.81 Å) is significantly smaller than bromide (1.96 Å) or iodide (2.2 Å). The steric hindrance of $Br^-$ and $I^-$ to bind at the W2 position seems to be reflected by the fact that with increasing radii of the anionic substrates the increase of the rate of compound I reduction by the halide is less pronounced (Table 4).

It is important to note that EPO compound I is a much better oxidant of bromide, iodide and thiocyanate than MPO (Table 4),[65] which suggests some differences in substrate access and binding sites. LPO has been shown to have barely detectable activity with bromide at neutral pH but oxidizes iodide and thiocyanate very efficiently (Table 4).[66] Thus, in addition to redox thermodynamic properties, other factors such as anion size, anion binding and topological effects determine the effect of pH and substrate specificity of compound I reduction directly to ferric peroxidase.[18]

It has also been proposed that MPO is able to oxidise chloride to chlorine, bromide to bromine or to form the interhalogen bromine chloride (BrCl). The formation of $Cl_2$ and BrCl in myeloperoxidase-driven reactions and neutrophil suspensions has been reported in several publications.[67] In these cases, the reduction of compound I to the ferric enzyme [Equation (8)] would be coupled with halide oxidation by one of the following half-reactions [Equations (11), (12) and (13), all written as reduction process].

$$Cl_2 + 2e^- \rightarrow 2Cl^- \quad (11)$$

$$Br_2 + 2e^- \rightarrow 2Br^- \quad (12)$$

$$BrCl + 2e^- \rightarrow Br^- + Cl^- \quad (13)$$

The standard reduction potentials of the couples $Cl_2/2Cl^-$, $Br_2/2Br^-$ and $I_2/2I^-$ are given in Table 3. They are independent of pH. The redox couple $BrCl/Br^-$, $Cl^-$ has a standard reduction potential of 1.21 V.[68] Whether other halogenated oxidation products, including interhalogens, can result from (pseudo)halide oxidation by peroxidases is purely speculative. Due to the low iodide concentration (<1 μM[69]) in blood and most tissues (with the exception of the thyroid gland), iodide oxidation by MPO, EPO and LPO is of little importance. The redox chemistry of $SCN^-$ oxidation by peroxidases is rather complex. In addition to the formation of hypothiocyanite ($OSCN^-$), further oxidation products such as cyanate ($OCN^-$) and $(SCN)_2$ have been reported.[61,70] Hypothiocyanous acid (HOSCN) and $SCN^-$ are the hydrolysis products of $(SCN)_2$ with a hydrolysis constant of $5.7 \times 10^{-4}$ M².[61] From this constant and the values for the standard reduction potential of the couple $HOSCN/SCN^-$, $H_2O$ (Table 3), the unknown potential for the couple $(SCN)_2/2SCN^-$ (0.72 V) can be calculated.

In general, it follows from thermodynamic rules that the oxidation of a (pseudo)halide by compound I of peroxidases is only possible if the actual reduction potentials for (pseudo)halide oxidation [$E'(HOX/X^-, H_2O)$ or $E'(X_2/2X^-)$] are lower than the reduction potential for the reduction of compound I $E'$[compound I/PorFe$^{III}$] [Equations (14) and (15)].

$$E'(HOX/X^-, H_2O) < E'[\text{compound I/PorFe}^{III}] \quad (14)$$

$$E'(X_2/2X^-) < E'[\text{compound I/PorFe}^{III}] \quad (15)$$

The reduction potential of any redox couple depends on reactant concentrations and pH value and can be determined by means of the Nernst equation [Equation (5)]. As the redox couples involved in halide oxidation by peroxidases possess different dependencies on pH, certain pH threshold values depending on halide concentration exist for a given peroxidase − only below this pH threshold value is the halide oxidation thermodynamically favourable.[68] The reduction potential of the couple compound I/ferric enzyme declines by –0.059 V per increasing pH unit at neutral and slightly acidic pH values. The potential of the couple $HOX/X^-$, $H_2O$ is diminished by –0.0295 V per pH unit if the pH is lower than the p$K_a$ value of the corresponding hypohalous acid. At pH values higher than the p$K_a$ value, the slope will change by –0.059 V per pH unit. The redox couple $X_2/2X^-$ is independent of pH.

A selected example for the calculation of pH threshold values is presented for the formation of HOCl and HOBr by MPO and EPO assuming equimolar concentrations for compound I and the ferric enzyme form as well as concen-

  

trations of $10^{-6}$ M for HOCl, $10^{-1}$ M for $Cl^-$, $10^{-6}$ M for HOBr and $10^{-4}$ M for $Br^-$ (Figure 2). Under these conditions, clear pH thresholds are found for the formation of HOCl and HOBr by EPO at pH 5.75 and 7.6, respectively. The formation of HOBr by MPO would be possible at all pH values indicated in Figure 2, while the formation of HOCl is unlikely at pH values higher than 7.5. Both reduction potentials of both couples are nearly identical in this pH range. It is necessary to point out that these considerations are influenced by uncertainties about the knowledge of the real concentration values of all reactants.

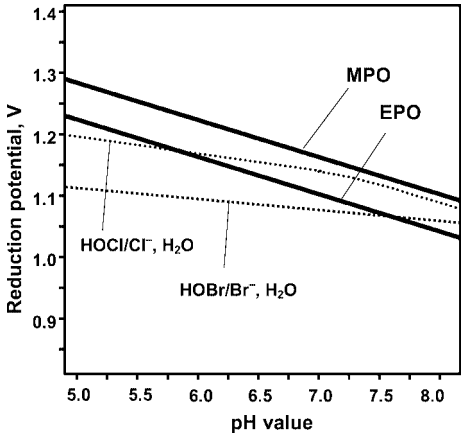


Figure 2. Selected example for the determination of pH threshold values below which the formation of HOBr or HOCl would be thermodynamically possible by the MPO (or EPO)-hydrogen peroxide-halide system. Reduction potentials of the redox couples compound I/ferric enzyme are given for MPO and EPO as a function of pH (solid lines) using equimolar concentrations of compound I and the ferric enzyme form. The reduction potentials of the couples $HOCl/Cl^-$, $H_2O$ and $HOBr/Br^-$, $H_2O$ (dotted lines) are indicated in the same pH range for $10^{-6}$ M HOCl, $10^{-1}$ M $Cl^-$, $10^{-6}$ M HOBr and $10^{-4}$ M $Br^-$.

Calculated pH thresholds for the chloride and bromide oxidation by MPO and EPO have been compared with experimentally determined pH threshold values for the formation of chloro- and bromohydrins from unsaturated phosphatidylcholines by these peroxidases at varying halide concentrations. This analysis revealed that chlorine, bromine and bromine chloride are the favoured chloride and bromide oxidation products by MPO, whereas hypochlorous and hypobromous acid will primarily be formed by EPO.[68]

The inability to induce MPO effects at 0.1 M $Cl^-$ and neutral pH values and the existence of certain pH threshold values by lowering the pH was also evidenced in other investigations on lipids, such as the formation of lysophospholipids from highly unsaturated phosphatidylcholines[71] or the accumulation of diene conjugates in low-density lipoproteins.[72] In contrast, when using taurine or monochlorodimedon as detection system, the chlorinating activity of MPO has been reported at neutral pH values up to pH 8.[73] Taurine is known to bind to MPO and be modified by the MPO–$H_2O_2$–$Cl^-$ system by so-called enzyme-bound hypochlorous acid but not by free HOCl, as revealed by kinetic studies at low pH.[74] Similarly, the chlorination of monochlorodimedon by chloroperoxidase occurs clearly by enzyme-bound HOCl.[75] Thus, the mechanism of chlorination of taurine and monochlorodimedon by MPO at neutral pH values remains unknown from the viewpoint of redox chemistry.

For MPO and chloride, it has been shown that chloride forms a reversible complex with compound I, the absorbance of which is in between those of compound I and native MPO.[62b] At pH 7 it was impossible to reconstitute the ferric enzyme form completely from compound I of MPO by adding chloride or bromide in the stopped-flow experiment.[42,74] Apparently, only the complex between compound I and $Cl^-$ or $Br^-$ was formed without inducing the halide oxidation; only at pH 5 did a complete reconstitution of ferric MPO from compound I take place.[28,76] How the addition of taurine facilitates the reconstitution of ferric MPO in halide oxidation at neutral pH values remains unknown.

## Protein Modification by Peroxidase Products

Reactive halide and nitrogen species produced by heme peroxidase contribute to the modification of functional sites of proteins and other biomolecules. Hypochlorous acid, hypobromous acid and nitrogen dioxide in particular have been investigated for their ability to induce post-translational modifications in proteins. As a result of the action of hypohalous acid, most of all, oxidation of sulfhydryl and methionine residues as well as halogenation of aromatic amino acid residues have been reported.[77] Although the chlorination of tyrosine residues in protein by the MPO–$H_2O_2$–$Cl^-$ system has been reported,[78] the formation of 3-chlorotyrosine is an unfavourable reaction for HOCl.[77a] Only 2% of HOCl produced by MPO or neutrophils is used to convert tyrosine residues in albumin to chlorotyrosines.[78a] A substantial bromination of tyrosine residues of albumin is observed at physiological concentrations of chloride and bromide at a pH higher than 7.[79]

Nitration of proteins is often observed during pathophysiological situations when oxidative and nitrative stress conditions are simultaneously produced and, in particular, both nitrite and hydrogen peroxide concentrations in cells increase.[80] The MPO–$H_2O_2$–nitrite system causes both nitration and nitrosylation of selected tyrosine and tryptophan residues in proteins, for example in human serum albumin.[79b] The most commonly detected marker of biological nitration is 3-nitrotyrosine, and different mechanisms, which are probably simultaneously active, have been proposed for its formation in vitro.[80e]

Nitrogen dioxide is derived from the one-electron oxidation of nitrite by compound I and compound II of heme peroxidases (see above). Dimerisation of two $^{\cdot}NO_2$ species in a reversible reaction generates $N_2O_4$, which forms symmetrical ($O_2N$–$NO_2$) and asymmetrical (ON–$NO_3$) tautomers that are able to produce nitrated and nitrosated tryptophan derivatives.[81]

Formation of 3-nitrotyrosine is also thought to occur by coupling of nitrogen dioxide with the phenoxy radical

  

[Equation (16)], which is also produced by one-electron oxidation of tyrosine residues in proteins mediated by the peroxidase intermediates compound I and compound II.

$$^{\cdot}NO_2 + Ph–O^{\cdot} \rightarrow O_2N–Ph–OH \quad (16)$$

Since the Tyr$^{\cdot}$/Tyr standard reduction potential at pH 7 is 0.94 V,[36] the phenoxy radical can be generated by oxidation of the phenol by both nitrogen dioxide or the peroxidase intermediates compound I and compound II.

Even though the dominant mechanism for nitration by peroxidases in the presence of nitrite and hydrogen peroxide considers the formation of nitrogen dioxide by the classical enzyme intermediates, several data show that another active species with chemical properties similar to those of peroxynitrite can be generated by mammalian peroxidases. This alternative species has been proposed for LPO based on kinetic analysis and product distribution in nitration/oxidation experiments of *p*-cyanophenol, phenylacetic acid[44d,44e] and tryptophan derivatives[44f] as mechanistic probes. For the enzymatic reactions, the importance of this second nitrating species becomes dominant as the nitrite concentration in solution increases. The generation of an oxidant species with the chemical properties of peroxynitrite by MPO and EPO in the presence of nitrite has been proposed in vivo, through the use of EPO- and MPO-knockout mice, from the detection of hydroxylated targets.[44h] It has been shown that while the major reacting nitrogen species generated by peroxidase catalysis is nitrogen dioxide, a further minor, but not negligible, contribution produces products typical of $ONOO^-$ reactivity.

The formation of the enzyme nitrating species with peroxynitrite reactivity can be explained by considering that, after coordination of nitrite to the iron, the nitrite adduct can undergo oxidation by hydrogen peroxide according to the following reaction [Equation (17)][44d–44f]

$$PorFe^{III}–NO_2^- + H_2O_2 \rightarrow PorFe^{III}–N(O)OO^- + H_2O \quad (17)$$

where the $PorFe^{III}–N(O)OO^-$ species is assumed to be an iron-bound peroxynitrite coordinated through the nitrogen donor atom. This assumption is based on its chemical properties and on the similarities between the spectral features of $PorFe^{III}–NO_2^-$ and the intermediate formed as a transient species upon treatment of LPO with hydrogen peroxide in the presence of an excess of nitrite.[44e]

The characterisation of this intermediate is complicated by its high reactivity, which causes a quick degradation by reaction with nearby amino acid residues of the protein. In addition, the lifetime of the heme-bound peroxynitrite may be reduced through the fast conversion to nitrate. In fact, the catalytic effect of heme complexes in the dismutation of (O-bound) peroxynitrite is well known,[82] and a similar activity can also be exerted with the N-bound species. For these reasons, the redox properties of the $PorFe^{III}–N(O)OO^-$ intermediate are not known. The species generated by LPO shares several reactivity features with free peroxynitrite, such as a certain preference to produce *N*-nitrosotryptophan rather than *N*-nitrotryptophan, and the capability of reacting with aromatic residues to give both nitration and hydroxylation of the ring.[44f] The latter activity indicates a strong oxidising property for $PorFe^{III}–N(O)OO^-$, although the relative relevance of the one-electron (radical) reactions with respect to the two-electron oxidations promoted by this species is still unclear. The lack of effect of $CO_2$ on the observed reactivity of the $PorFe^{III}–N(O)OO^-$ intermediate[44e] indicates that the reactivity is not, or is only partly, due to free peroxynitrite generated by the dissociation of the adduct; thus, the reactions observed occur within the protein active site and are due to the protein iron-bound peroxynitrite.

It has to be mentioned that MPO and EPO are strong cationic proteins with p$K_a$ values around 10. LPO, however, has a p$K_a$ value of about 7.5. The formation of complexes with acidic proteins is known for MPO. The binding and uptake of MPO to endothelial cells is mediated by MPO–albumin complexes.[83] After endocytosis by the endothelial, MPO becomes associated with fibronectin-rich fibrils.[84] Other proteins known to interact with myeloperoxidase are apoprotein A-1, ceruloplasmin and antitrypsin,[85] although the physiological meaning of these findings is unclear. Regarding the antimicrobial role of these enzymes, however, the combination of adhesion to pathogens with the biochemical capacity to generate strong oxidants seems to be a general strategy in unspecific immune defence that developed very early in evolution.

## Conclusion

Here we have demonstrated how the functional features of human peroxidases to use small anionic one- and two-electron donors are closely related with their peculiar structural and redox properties and how the corresponding products contribute to the modification of functional sites of proteins and other biomolecules. This knowledge will help in the rational design of new potent and specific inhibitors that will dampen inflammation without precipitating infectious diseases.

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