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Scavenging of oxygen radicals by heme peroxidases*/**

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The reactions of two heme peroxidases, horseradish peroxidase and lactoperoxidase and their compounds II (oxoferryl heme intermediates, Fe(IV)=O or ferric protein radical Fe(III)R$^{\cdot}$) and compounds III (resonance hybrids [Fe(III)-$O_2^{\cdot}$ ↔ Fe(II)-O_2] with superoxide radical anion generated enzymatically or radiolytically, and with hydroxyl radicals generated radiolytically, were investigated. It is suggested that only the protein radical form of compound II of lactoperoxidase reacts with superoxide, whereas compound II of horseradich peroxidase, which exists only in oxoferryl form, is unreactive towards superoxide. Compound III of the investigated peroxidases does not react with superoxide. The lactoperoxidase activity loss induced by hydroxyl radicals is closely related to the loss of the ability to form compound I (oxoferryl porphyrin π-cation radical, Fe(IV)=O(Por$^{+\cdot}$) or oxoferryl protein radical Fe(IV)=O(R$^{\cdot}$)). On the other hand, the modification of horseradish peroxidase induced by hydroxyl radicals has been reported to cause also restrictions in substrate binding (Gębicka, L. & Gębicki, J.L., 1996, *Biochimie* 78, 62–65). Nevertheless, it has been found that only a small fraction of hydroxyl radicals generated homogeneously does inactivate the enzymes.

The heme-containing peroxidases are known to catalyze one-electron oxidation of a wide range of substrates with concomitant reduction of peroxides [1]. Peroxides oxidize ferric peroxidase by a two electron process to the enzyme intermediate, compound I, described as an oxoferryl porphyrin π-cation radical, Fe(IV)=O(Por$^{+\cdot}$) (like in the case of horseradish peroxidase (HRP)) or oxoferryl protein radical, Fe(IV)=O(R$^{\cdot}$) where R$^{\cdot}$ is an amino-acid residue. It has been shown that in the case of lactoperoxidase (LPO) and thyroid peroxidase the primary π-cation radical form of compound I spontaneously transforms to the oxoferryl protein radical form [2, 3]. Compound I accepts one electron from a substrate yielding the corresponding substrate free radical and oxoferryl heme intermediate, Fe(IV)=O, or Fe(III)R$^{\cdot}$, both known as compound II [1, 4]. A subsequent one-electron reduction of compound II by a

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Abbreviations: HRP, horseradish peroxidase; HX, hypoxanthine; LPO, lactoperoxidase; MPO, myeloperoxidase; SOD, superoxide dismutase; XO, xanthine oxidase.

second substrate molecule leads to ferric peroxidase. Substrate radicals may dimerize, disproportionate, transfer electrons, or may be scavenged by molecular oxygen yielding peroxy radicals or superoxide radical anion.

Prooxidant action of heme peroxidases has been recently reviewed by Metodiewa & Dunford [5]. On the other hand, heme peroxidases may be regarded as antioxidant enzymes because they scavenge hydrogen peroxide. Peroxidases react also with superoxide radical anion to form compounds III (oxyperoxidases), which may be represented as resonance hybrids [Fe(III)-O_2^- ↔ Fe(II)O_2] [6]. Compounds I of peroxidases are also active towards superoxide [7–9].

Peroxidases, like other proteins, react effectively with an extremely reactive oxidant, the hydroxyl radical. We have found that $OH^\cdot$ radicals do not react directly with heme of HRP or LPO [10, 11] and that the $OH^\cdot$-induced activity loss of HRP is observed only when the $OH^\cdot$/HRP concentration ratio exceeds 5 [11].

In this paper we try to answer the question whether compound II and compound III are also able to scavenge the superoxide radical anion. Further results concerning peroxidase reactions with hydroxyl radicals are also presented. Two heme peroxidases, horseradish peroxidase and lactoperoxidase, a plant enzyme and a mammalian one, have been chosen for the studies.

MATERIALS AND METHODS

HRP (type VI) (EC 1.11.1.7) with the purity number RZ = A_{403}/A_{280} (a measure of hemin content using the aromatic amino acid content as reference [12]) of 3.0, LPO (EC 1.11.1.7) with RZ = A_{412}/A_{280} of 0.8, XO (grade I) (EC 1.1.3.22) from butter milk, cytochrome *c* from horse heart and SOD (lyophilized powder) (EC 1.15.1.1) from bovine erythrocytes were obtained from Sigma.

Compound II of LPO stable for 15 min (i.e. no decay can be detected within this time period) was produced by addition of a 10-fold excess of H_2O_2 to LPO at pH 7.0 (phosphate buffer). Compound II of HRP stable for 3 min at pH 7.0 was formed by mixing equimolar amounts of HRP, ferrocyanide and H_2O_2. Compound III was produced by mixing peroxidases with a 100-fold (LPO) or 250-fold (HRP) excess of H_2O_2. Both compounds III were stable for about 10 min.

Superoxide radical anion was generated: (i) by the HX/XO method [13], at a rate of 0.08–0.8 μmol dm^{-3} s^{-1} as measured by the rate of cytochrome *c* reduction, (ii) by pulse irradiation of O_2-saturated aqueous solutions of the enzymes in the presence of t-butanol to scavenge $OH^\cdot$ radicals as described by us earlier [14]. About 10 μmol dm^{-3} of $O_2^{\cdot-}$ was produced in the pulse.

Hydroxyl radicals were generated by pulse irradiation of N_2O-saturated aqueous solution of the enzymes, where all e_{aq}^- were converted to $OH^\cdot$. About 20 μmol dm^{-3} of $OH^\cdot$ was produced in the pulse.

Pulse radiolysis experiments were performed with a linear electron accelerator, at the Institute of Applied Radiation Chemistry. Pulses of 17 ns delivering a dose of about 40 Gy were applied.

Kinetic measurements were performed on the DX-17 MV (Applied Photophysics) stopped-flow spectrofluorimeter (millisecond and second observations) and on the Hewlett-Packard 8452A diode-array spectrophotometer (conventional time-scale).

Enzyme activity was assayed using 2,2'-azino-bis[3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS) [15].

All experiments were carried out at ambient temperature and at pH 7.0 (phosphate buffer). Water from Milli Q (Millipore) was used throughout.

RESULTS

Reactions of compounds II with superoxide

We attempted to check whether the investigated peroxidases (Per) possess "superoxide peroxidase activity", i.e. if the following reaction cycle is possible:

$$\text{Per} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + O_2^{\cdot-} \rightarrow \text{Compound II} + O_2 \quad (2)$$

$$\text{Compound II} + O_2^{\cdot-} \rightarrow \text{Per (?)} \quad (3)$$

We have found that the absorption spectrum of the LPO compound II does not change in the

presence of HX/XO and that the rate of decay of the HRP compound II (which is not stable at pH = 7.0) does not change in the presence of HX/XO (not shown). This means that the investigated compounds II of HRP and LPO do not transform back to native enzyme in the presence of HX/XO. When superoxide was generated by the pulse radiolysis method in the presence of the LPO compound II, formation of an unstable species, decaying within seconds after the pulse, was observed (Fig. 1). In the presence of 10 μmol dm^{-3} of SOD the formation of this species was inhibited. In the pulse radiolysis experiments with HRP, compound II was formed in reactions (1) and (2) following the first pulse [7] (H_2O_2 came from water radiolysis). After the second pulse we obtained a system containing O_2^- and H_2O_2 in the presence of compound II of HRP. No changes in the spectrum of compound II of HRP after the second pulse were observed. This means that compound II of HRP does not react with superoxide.

Reactions of compounds III with superoxide

It has been suggested that compounds III of some peroxidases may react with superoxide [16, 17]. However, we did not observe any changes in the absorption spectra of compounds III of HRP and LPO after O_2^- generation by the pulse radiolysis or HX/XO methods (Fig. 2). The rate of compound III decay was the same in the presence and in the absence of the HX/XO system. The above results strongly indicate that superoxide is unreactive towards compounds III of HRP and LPO.

Reactions of peroxidases with hydroxyl radicals

Unlike superoxide, hydroxyl radicals are known to react very unspecifically with amino acids located on the surface of protein. We have found earlier that hydroxyl radicals, produced homogeneously by means of pulse radiolysis, partially inactivate HRP only when they are generated in excess with respect to the enzyme [11]. We have observed that the HRP and LPO transients formed in the reaction with $OH^{\cdot}$ radicals gave different absorption spectra [11, 18]. Now we try to check whether there are also differences in the $OH^{\cdot}$-induced activity loss profile between these two heme peroxidases. In competition with SCN^- at 5×10^{-4} mol dm^{-3}, the rate constant for the reaction of $OH^{\cdot}$ with LPO was found to be 2×10^{10} mol^{-1} dm^3 s^{-1}. The rate constant for the reaction of $OH^{\cdot}$ with HRP determined earlier, by the same method, equals 1.1×10^{11} mol^{-1} dm^3 s^{-1} [11]. The measurements of $OH^{\cdot}$-induced activity loss of LPO and the yield of the reaction of $OH^{\cdot}$-treated LPO with H_2O_2 were done 24 h after irradiation, i.e. after possible repair processes had been finished. The rate constant of this reaction was determined by the stopped-flow method to be 1.4×10^7 mol^{-1} dm^3 s^{-1}. This value remained the same after LPO treatment with $OH^{\cdot}$ radical. However, the reaction yield, expressed as a [compound I]/[LPO] ratio decreased with increasing $OH^{\cdot}$ radical concentration (Fig. 3). It is

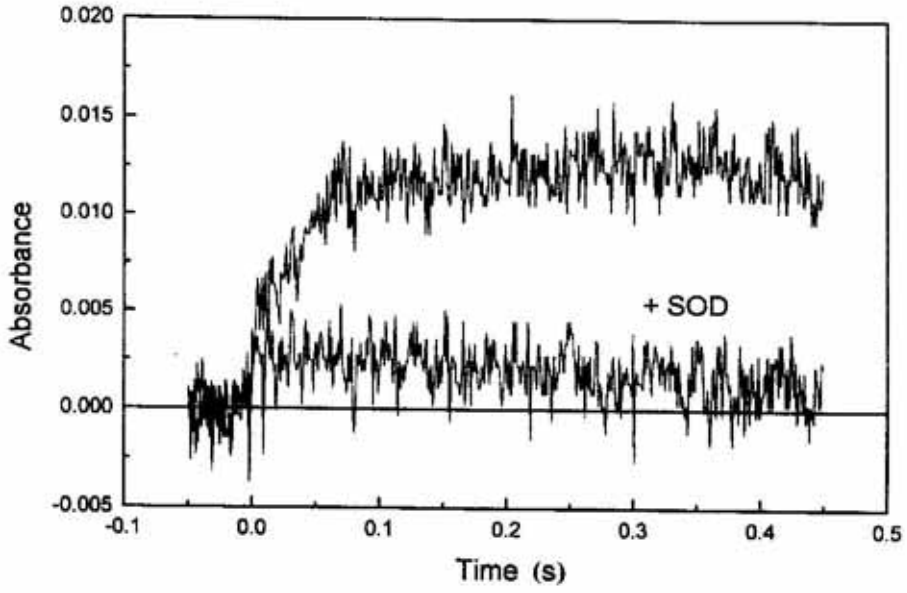

Fig. 1. Absorption changes observed at 430 nm after pulse irradiation to O_2-saturated 2 μmol dm^{-3} of LPO compound II solution, in the presence of 0.1 mol dm^{-3} of t-butanol.

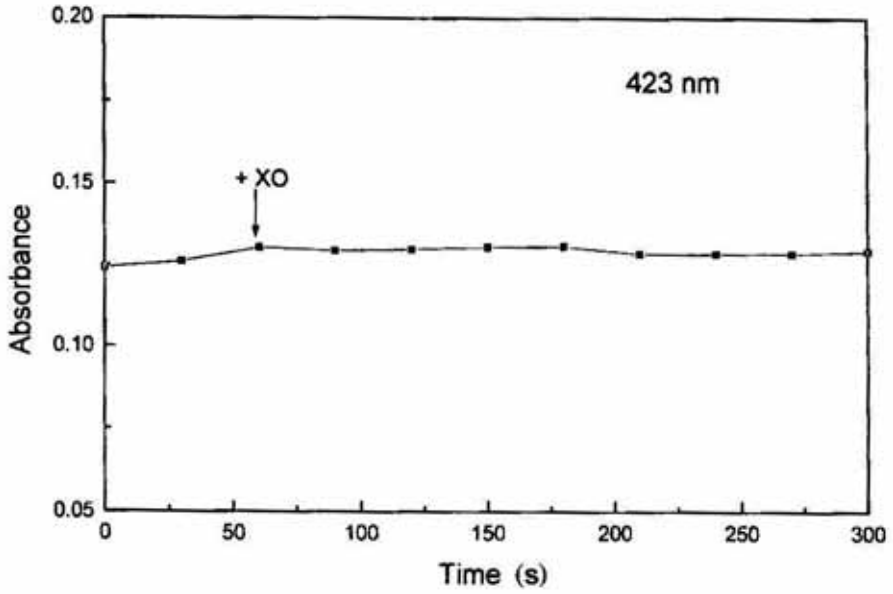

Fig. 2. Absorbance at 423 nm (λ_{max} for compound III of LPO) versus time profile in 1.5 μmol dm^{-3} of compound III of LPO + 0.1 mmol dm^{-3} HX.
The arrow indicates injection of XO solution. No influence of the generated $O_2^{\cdot}$ can be seen. The same was observed for the HRP compound III.

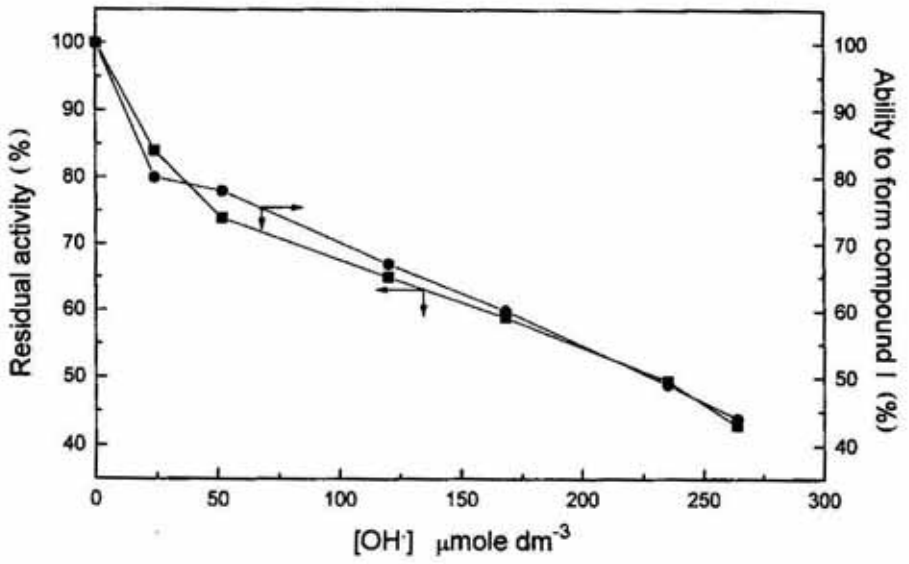

Fig. 3. Hydroxyl radicals-induced loss of LPO activity (■) and loss of its ability to form compound I (●). (6.4 μmol dm^{-3} LPO in N_2O-saturated solution).

also seen in Fig. 3 that the activity loss induced by $OH^{\cdot}$ radicals parallels the loss of the enzyme ability to form compound I.

DISCUSSION

Reactions of compounds II with superoxide

Superoxide has previously been shown to react with compound II of MPO to give native MPO [19]. On the other hand, the reaction of compound II of HRP with radiolytically generated $O_2^{\cdot}$ has not been observed [7, 20]. Our results show that superoxide reacts with the LPO compound II (Fig.1). As it has been postulated that the oxoferryl and ferric/protein radical (only small fraction) forms of the LPO compound II exist at pH 7.0 [2], we suggest that superoxide reacts only with the protein radical form of LPO compound II to give an intermediate which decays to an unknown product, possibly to native enzyme:

$$Fe(III)R^{\cdot} + O_2^{\cdot} \rightarrow Fe(III)RO_2^{\cdot} \text{ (unstable)} \rightarrow \text{products} \quad (4)$$

Note that the absorption spectra of $Fe(III)R^{\cdot}$ and native enzyme were found to be hardly distinguishable [2].

Although the radical site on protein in LPO compounds I and II has not been identified yet, it has been suggested that aromatic amino

acids, tryptophan or tyrosine, are involved [4]. Tryptophan as well as tyrosine radicals are known to be unreactive with oxygen but do react with superoxide anion [21, 22]. It seems, however, that superoxide does not react with the ferryl form of compound II. As the HRP compound II exists exclusively as an oxoferryl species, no reaction with $O_2^{\cdot-}$ is observed.

Reactions of compounds III with superoxide

Compound III of peroxidases does not participate in the normal peroxidative cycle. It can be formed *via* three reaction paths: from the native enzyme and superoxide, from compound II and H_2O_2, and from ferrous enzyme and molecular oxygen. Compound III tends to revert spontaneously to native enzyme [6]. For some peroxidases, a release of superoxide during the decay of compound III has been reported [17, 23]. It has also been suggested that the released free superoxide reacts further with the remaining compound III to give native enzyme and hydrogen peroxide [17], like in case of oxyhemoglobin [24]. On the other hand, it has been reported that the oxy form of diacetyldeuteroperoxidase is reduced by e_{aq}^- to compound I of this enzyme [25]. The once proposed possibility of one-electron reduction of compound III of MPO to compound I by superoxide [16], was questioned by other authors [19]. Our findings show that compounds III of HRP and LPO are unreactive towards $O_2^{\cdot-}$.

Reactions of peroxidases with hydroxyl radicals

We have shown here that the activity loss of LPO induced by hydroxyl radicals is closely related to the loss of its ability to form compound I. On the other hand, we have earlier found that the activity loss of HRP is connected with such enzyme modifications which cause both the interference with substrate binding and partial blocking of the channel used by peroxide [11]. Heme in HRP is less deeply buried in the heme crevice than it is in LPO [26]. It seems that some of aromatic amino acids in the vicinity of the heme in HRP, which take part in substrate binding, may be modified by $OH^{\cdot}$. We have suggested in the previous work that absorption changes at 440 nm observed after reaction of HRP with $OH^{\cdot}$ may be due to modification of the apoprotein in a manner that slightly influences heme absorption [11].

We have calculated inactivation yields, i.e. moles of inactivated enzyme per 1 J of radiation energy absorbed (G_{in}) and have found values of 0.096 and 0.070 μmol J^{-1} for HRP and LPO, respectively. Taking into account that the yield of $OH^{\cdot}$ generation in N_2O-saturated solution is 0.54 μmol J^{-1} [27], less than 2% of $OH^{\cdot}$ radicals generated radiolytically is involved in inactivation of HRP and LPO.

Our data concerning two heme peroxidases, HRP and LPO, suggest that only compound II existing in a protein radical form is able to react with superoxide. The oxoferryl form of compound II and compound III are unreactive towards superoxide. Although the investigated peroxidases react effectively with $OH^{\cdot}$, only a small fraction of $OH^{\cdot}$ radicals, generated homogeneously, inactivates the enzymes.

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