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The reaction of oxygen with radicals from oxidation of tryptophan and indole-3-acetic acid

Luis P. Candeias [a,*], Peter Wardman [a], Ronald P. Mason [b]

[a] *Gray Laboratory Cancer Research Trust, PO Box 100 Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, UK*

[b] *Laboratory of Pharmacology and Chemistry, NIEHS National Institutes of Health, Research Triangle Park, NC 27709, USA*

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Abstract

The oxidation of tryptophan and indole-3-acetic acid (IAA) by the dibromine radical anion or peroxidase from horseradish in aqueous solution was investigated and compared, especially with respect to the involvement of oxygen and superoxide. Using EPR with spin-trapping, the tryptophanyl radical, generated by either method was found to react with oxygen, although this reaction is too slow to be observed by pulse radiolysis ($k < 5 \times 10^6$ dm^3 mol^{-1} s^{-1}). No superoxide results from this reaction, thus excluding an electron-transfer mechanism and suggesting the formation of a tryptophan peroxyl radical, possibly in a reversible process. These observations imply that in proteins where the tryptophanyl radical exists as a stable species it must either have its reactivity modified by the protein environment or be inaccessible to oxygen. The related molecule IAA is oxidized by either peroxidase or $Br_2^{\cdot-}$ to a radical cation that decarboxylates to yield a skatolyl radical. The latter reacts with oxygen to give a peroxyl radical that does not release superoxide. However, $O_2^{\cdot-}$ is formed during the peroxidase-catalyzed oxidation of indoleacetic acid. This supports the hypothesis that the peroxidase can act in an oxidase cycle involving ferrous enzyme and compound III, with superoxide as a product. © 1997 Elsevier Science B.V.

Keywords: Tryptophan; Indole-3-acetic acid; Superoxide; Peroxidase; Radiolysis; EPR; Spin-trapping

1. Introduction

Tryptophan, tyrosine and cysteine are the most easily oxidizable aminoacids and in recent years the involvement of their radicals in biochemical processes has become increasingly evident. The reactivity of those radicals with oxygen is of obvious importance in the understanding of mechanisms of the reactions in which they are involved. Cysteinyl radicals are known to react rapidly with oxygen [1,2] whereas tyrosyl radicals do not appear to react ($k < 10^3$ dm^3 mol^{-1} s^{-1} [3]). Pulse radiolysis experiments suggested that tryptophanyl radicals are unreactive towards oxygen [4] but recent studies have provided evidence for the reaction of a tryptophanyl radical in myoglobin with oxygen [5,6].

L-Tryptophan (Trp) and the related substance indole-3-acetic acid(IAA) are substrates for heme peroxidases, such as the peroxidase from horseradish. The enzymatic oxidation of IAA follows an exceptionally complex mechanism that is not yet completely understood, despite some recent very detailed studies [7,8]. In particular, the origin of the superoxide radical detected by EPR with spin-trapping [9]

* Corresponding author.

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remains unclear. Moreover, superoxide does not appear to be formed on chemical oxidation of IAA [10], in contrast to the observations in the enzyme system. In the present study, we have compared the oxidation of IAA and Trp by peroxidase and by the dibromine radical anion ($Br_2^{\cdot -}$), focusing in particular on the reaction of oxygen with the intermediate radicals and on the formation of superoxide. As shown below, the results shed new light on the behavior of the tryptophanyl radical and on the mechanism of peroxidase catalyzed oxidation of indoles.

2. Experimental section

Trp, IAA and α-phenyl-*N*-*tert*-butylnitrone (PBN) were purchased from Sigma and used as received. 5,5-Dimethyl-1-pyrroline *N*-oxide (Sigma) was purified over charcoal and stored as aqueous solution under nitrogen at -20°C, as described elsewhere [11]. Horseradish peroxidase was also from Sigma (type VI-A). Trp labeled with ^{13}C at C(3) (isotope purity 95–99%) was prepared by Cambridge Isotope Labs (Andover MD). Potassium bromide, phosphate and acetate buffers and other reagents were from BDH/Merck and of AnalaR purity. Oxygen-free nitrous oxide and a mixture of nitrous oxide-oxygen (20% v/v) were obtained from British Oxygen Company. All solutions were freshly prepared using water purified with a Millipore Milli-Q system. For the radiolysis experiments the reagents were dissolved directly in the buffers to the desired concentration. For the experiments on peroxidase catalysis, stock solutions in ethanol of IAA (0.1 mol dm^{-3}) and PBN (0.01 mol dm^{-3}) were used.

Pulse radiolysis was performed using a 6 MeV, magnetron driven, linear accelerator delivering electron pulses of ca. 0.5 μs [12]. The dose delivered by each pulse was monitored by a toroid detector calibrated using aerated thiocyanate aqueous solutions (10 mmol dm^{-3}) and assuming $G \times \varepsilon = 2.59 \times 10^{-4}$ m^2 J^{-1} at 475 nm [13]. Steady-state radiolysis used γ-radiation from a ^{60}Co source with an activity of ca. 1 kCi. The solutions were irradiated in gas-tight syringes at a dose-rate of ca. 6.5 Gy min^{-1}, calibrated using the Fricke dosimeter [14]. The solutions for pulse or steady-state radiolysis were saturated with the appropriate gas by ca. 30 min before irradiation. All experiments were performed at room temperature (22 ± 2°C).

The irradiation of aqueous solutions of potassium bromide (0.1 mol dm^{-3}) saturated with N_2O produces the dibromine radical anion in a yield of 0.7 μmol J^{-1} in < 0.5 μs [15–18].

$$^{\cdot}OH + 2Br^- \rightarrow Br_2^{\cdot -} + OH^- . \quad (1)$$

In solutions saturated with a mixture of $N_2O + O_2$ (20% v/v) the oxygen concentration (250 μmol dm^{-3}) does not interfere significantly with the formation and decay of $Br_2^{\cdot -}$ but may react with secondary organic radicals.

X-Band EPR spectra were recorded with a Bruker EMX spectrometer equipped with a TM resonator and operated at a modulation frequency of 100 MHz. The samples were in a 15 mm wide suprasil flat cell. In the steady-state radiolysis experiments, the solutions were aspired into the cell and the spectra recorded < 15 min after the end of the irradiation. In the enzymatic oxidation experiments, the reagents were mixed and the solution was aspirated into the cell, so that the spectra were recorded ca. 2 min after mixing. The EPR spectra were analyzed using software available over the World Wide Web [19].

The analysis of irradiated solutions for carbon dioxide was done by converting CO_2 to carbonate by addition of degassed aqueous sodium hydroxide followed by high performance ion chromatography (HPIC) as described previously [20].

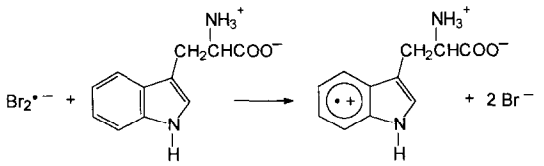
3. Results

3.1. Oxidation of IAA and Trp by $Br_2^{\cdot -}$

The oxidation of indoles by the dibromine radical anion ($Br_2^{\cdot -}$) has been extensively studied [4,10,21–26]. In acidic solution, the primary products of these reactions are the respective radical cations. At higher pH values, deprotonation from N(1), with pK_a values 5.1 and 4.3 for the IAA and Trp radical cations respectively, yields indolyl radicals [21,25]. Pulse radiolysis and product analysis experiments [10] showed that the IAA radical cation decays by scis-

sion of the side chain carbon-carbon bond to yield carbon dioxide and the skatolyl radical. The later reacts rapidly with oxygen to give a peroxyl radical.

In this study, we have generated the radical cation of Trp by pulse radiolysis, using the reaction with $Br_2^{\bullet -}$ at pH 3:



Its formation and decay were monitored by the absorbance at 560 nm. The radical cation decayed by strictly second order kinetics and the reciprocal of the first half-life was found to increase linearly with the radiation dose (ca. 2–30 Gy), i.e. with the initial radical concentration (ca. 1–20 μmol dm^{-3}, not shown). From the slope of the straight line, we estimated the rate of bimolecular decay of the tryptophanyl radical: $2k = (1.4 \pm 0.1) \times 10^8$ dm^3 mol^{-1} s^{-1} at ionic strength $I \approx 0.1$ mol dm^{-3}. Moreover, the intercept of this plot, i.e. the reciprocal of the first half-life extrapolated to zero dose, was zero (within the experimental error), from which we estimate that any first order component in the decay of the radical cation has a rate constant $k < 500$ s^{-1}. Identical experiments were performed with solutions at pH 7.4. Under these conditions the radical cation deprotonated rapidly to yield the indolyl radical which was monitored by the absorbance at 520 nm. The subsequent decay kinetics were similar to those described for the experiments at pH 3; the rate of bimolecular decay was estimated as $2k = (5.2 \pm 0.2) \times 10^8$ dm^3 mol^{-1} s^{-1} with no first-order contribution to the decay ($k < 100$ s^{-1}). This value is in agreement with those previously determined under similar conditions [21,27].

It has been previously reported [4] that the tryptophanyl radical has very low reactivity with oxygen. We have re-examined this question by comparing the decay of the radical after low-dose pulses (1.2 Gy, 0.8 μmol dm^{-3} initial concentration) in oxygen free solution ([O_2] < 1 μmol dm^{-3}) and in 20% oxygen ([O_2] = 250 μmol dm^{-3}). The presence of oxygen had only a small effect on the first half-life (Fig. 1): it decreased from 2.6 ms to 1.9 ms. From this difference, an upper limit on the rate of reaction of the tryptophanyl radical with oxygen can be set at $k_{O_2} \leq 4 \times 10^6$ dm^3 mol^{-1} s^{-1}. At pH 3, the first half-life of the tryptophan radical cation decreased from 2.8 ms to 2.4 ms on going from oxygen-free solution to 20% oxygen, from which we estimate that the rate of reaction of the radical cation with oxygen is $\leq 5 \times 10^6$ dm^3 mol^{-1} s^{-1}.

Experiments reported previously [10] have shown that carbon dioxide is released on oxidation of IAA by $Br_2^{\bullet -}$, even in neutral solution. The CO_2 was suggested to be derived from the radical cation that is in equilibrium with the indolyl radical under conditions of long radical life-times, such as prevailing in steady-state irradiation at low dose-rates. In contrast, with Trp no evidence for carbon dioxide formation could be found after irradiation (75 Gy) at pH 7.4; under the same conditions the irradiation of IAA solutions yielded ca. 30 μmol dm^{-3} CO_2 readily detected by HPIC as carbonate, after addition of NaOH.

The free radical intermediates of the oxidation of IAA and Trp by $Br_2^{\bullet -}$ were also investigated by EPR with spin-trapping by DMPO. Solutions of IAA (pH = 7.4) containing DMPO and potassium bromide were saturated with N_2O and irradiated. The EPR

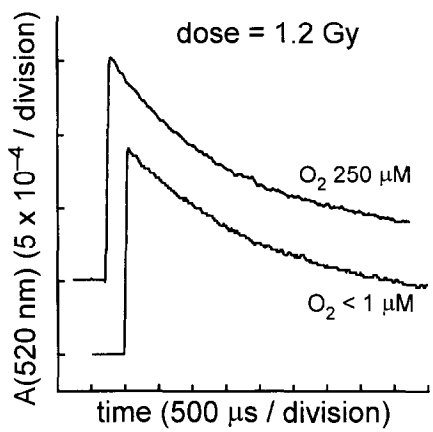
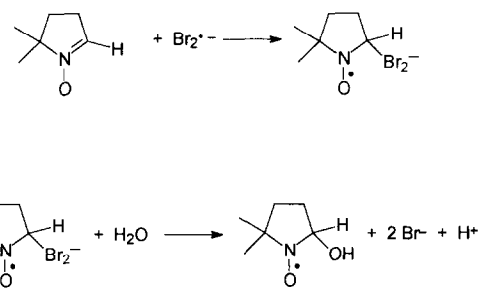


Fig. 1. Transient absorption at 520 nm of the tryptophanyl radical observed by pulse radiolysis of solutions of Trp (200 μmol dm^{-3}) and KBr (100 mmol dm^{-3}) in phosphate buffer (10 mmol dm^{-3}) at pH 7.4. The solutions were saturated with oxygen-free N_2O ([O_2] < 1 μM) or with N_2O with 20% oxygen ([O_2] = 250 μM).

spectra of the irradiated solutions show the presence of three different radicals (Fig. 2). Their hyperfine couplings and relative concentrations are summarized in Table 1. Two of these radicals were formed also in similar solutions that contained no IAA, suggesting that they result from reactions not involving the indole. On the basis of their hyperfine coupling constants, they can be identified as the hydroxyl radical ($^{\cdot}OH$) and hydrogen atom ($H^{\cdot}$) adducts of DMPO. The latter (DMPO-H) is obviously formed by the reaction of DMPO with the $H^{\cdot}$ generated by the water radiolysis. In fact, $H^{\cdot}$ reacts with DMPO at nearly diffusion-limited rate, but its rate of reaction with bromide is ca. three orders of magnitude lower [28]. The $^{\cdot}OH$ adduct of DMPO (DMPO-OH) may arise from the competition of DMPO with bromide for the hydroxyl radical formed by irradiation. From the rates of reaction of $^{\cdot}OH$ with Br^- and DMPO, it is estimated that $\approx 3\%$ of this radical will react directly with DMPO. Alternatively, the addition of another radical followed by nucleophilic substitution by water may also yield DMPO-OH. To test this hypothesis, the reaction of $Br_2^{\cdot-}$ with DMPO was investigated by pulse radiolysis of nitrous oxide-saturated solutions of potassium bromide (0.1 mol dm^{-3}) in phosphate buffer at pH 7.0 and containing DMPO (5–25 mmol dm^{-3}). Under these conditions the absorbance at 360 nm showed a fast increase followed by an exponential decay with rate that increased with increasing DMPO concentration, showing that the $Br_2^{\cdot-}$ formed by irradiation reacted with DMPO. The reaction resulted in a stable species with maximum absorbance at 270 nm, identical to that observed on reaction of DMPO with the hydroxyl radical [29]. From the observed rate of decay of absorbance at 360 nm or the increase at 270 nm the rate of reaction of $Br_2^{\cdot-}$ with DMPO is estimated as $\approx 10^7$ dm^3 mol^{-1} s^{-1}. The similarity of the absorption and EPR spectra obtained on reaction of DMPO with $Br_2^{\cdot-}$ with those of the DMPO-OH adduct suggest that the oxidation of DMPO by $Br_2^{\cdot-}$ is followed by the fast reaction of water with the $Br_2^{\cdot-}$ adduct ($k > 2 \times 10^5$ s^{-1}):



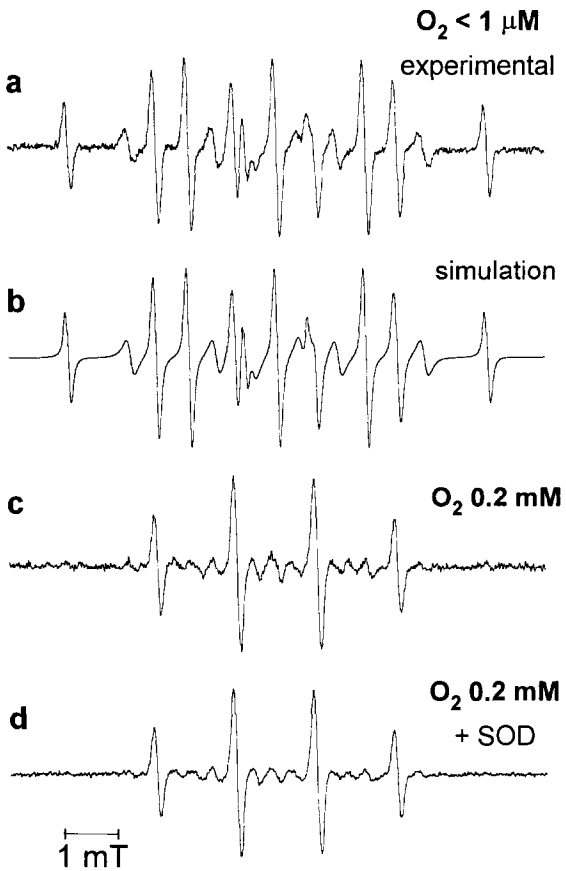


Fig. 2. EPR spectra observed on oxidation of IAA by $Br_2^{\cdot-}$ in the presence of DMPO (conditions described in Table 1). (a) In oxygen free solution; (b) simulation (see Table 1); (c) in the presence of oxygen; (d) in the presence of oxygen and SOD. Spectrometer conditions: microwave power 20 mW; gain 2×10^5; modulation amplitude 0.1 mT; time constant 42 ms; sweep rate 0.23 mT s^{-1}; the spectra are the average of five scans.

In addition to DMPO-H and DMPO-OH, a third species was detected in the EPR spectra of irradiated solution of bromide and IAA. This species, which was formed only in the presence of IAA, has the large hydrogen hyperfine coupling constant typical of a DMPO adduct of a carbon-centred radical.

Table 1
DMPO adducts formed on oxidation of indoles by $Br_2^{\cdot-}$ in the presence of DMPO[a]

	Oxygen concentration	Hyperfine coupling constants (mT)	assignment[b]	relative concentration (% of total)
DMPO only	< 1 μmol dm^{-3}	$a_N = 1.50$ $a_H = 1.47$	DMPO-OH	72%
		$a_N = 1.65$ $a_H(2\times) = 2.26$	DMPO-H	28%
IAA	< 1 μmol dm^{-3}	$a_N = 1.51$ $a_H = 1.47$	DMPO-OH	20%
		$a_N = 1.65$ $a_H(2\times) = 2.26$	DMPO-H	48%
		$a_N = 1.60$ $a_H = 2.28$	DMPO-Sk DMPO-OH	32%
	0.2 mmol dm^{-3}	$a_N = 1.51$ $a_H = 1.47$	DMPO-OH DMPO-OH	≈ 100% ≈ 100%
Trp	< 1 μmol dm^{-3}	$a_N = 1.66$ $a_H(2\times) = 2.26$	DMPO-H	58%
		$a_N = 1.55$ $a_H = 2.27$	DMPO-Trp	42%
	0.2 mmol dm^{-3}	$a_N = 1.65$ $a_H(2\times) = 2.26$	DMPO-H	71%
		$a_N = 1.54$ $a_H = 2.31$	DMPO-Trp	29%

[a]Conditions: IAA or Trp 0.2 mmol dm^{-3}, DMPO 0.01 mol dm^{-3}, KBr 0.1 mol dm^{-3} saturated with N_2O or $N_2O + O_2$ (20% v/v) in phosphate buffer (10 mmol dm^{-3}) at pH 7.4.
[b]Key: DMPO-H = H -adduct; DMPO-OH = $^{\cdot}$OH adduct; DMPO-Sk = Skatolyl radical adduct; DMPO-Trp = C-centered tryptophanyl radical adduct.

The effect of oxygen was tested by experiments in similar solutions but which were saturated with a mixture of N_2O and oxygen (80:20 v/v). Under these conditions, the EPR spectrum was dominated by the DMPO-OH adduct, with other features being too weak to be identified. In order to test whether DMPO-OH originates from the decay of the superoxide adduct, the experiment was repeated with identical solutions but containing superoxide dismutase (1 mg/mL). The presence of this enzyme did not affect the intensity of the DMPO-OH signal. For comparison, a 70-fold lower concentration of the same enzyme sample completely inhibited the reduction of ferric cytochrome *c* by xanthine (0.1 mmol dm^{-3}) and xanthine oxidase (40 μg/mL), a well known superoxide generating system.

Analogous spin-trapping experiments were also performed with tryptophan. The oxidation by $Br_2^{\cdot-}$ in oxygen-free solution gave an EPR spectrum attributable to two DMPO adducts: the DMPO-H adduct and a second radical with the characteristics of a carbon-centred radical adduct (Table 1). Oxygen partially inhibited both signals, but did not cause appearence of any detectable new signals (Fig. 3).

Additional EPR experiments were performed using the spin-trap PBN. Solutions of PBN and potassium bromide in phosphate buffer at pH 7.4 were saturated with N_2O and irradiated. Complex EPR spectra were observed, in which the predominant species (ca. 60%) was identified as the H-atom adduct of PBN, on the basis of its hyperfine coupling constants ($a_N = 1.67$ mT, $a_H(2\times) = 1.06$ mT). The spectra also included a small contribution from a species with a complex spectrum. In similar solutions containing Trp, the EPR spectra were much simpler to interpret (Fig. 4); the dominant component (79%) was a six line spectrum with $a_N = 1.59$ G and $a_H = 0.40$ mT. This spectrum, which is not observed

in the absence of Trp, must arise from the adduct of a tryptophanyl radical to PBN. The remaining signal (21%) exactly matched the spectrum of the H-adduct of PBN. In solutions irradiated in the presence of oxygen, the tryptophanyl radical adduct vanished, leaving only a weak and complex spectrum whose components could not be identified. From this result, it can be concluded that oxygen efficiently competed with PBN for the tryptophanyl radical.

Recently, the addition of tryptophanyl radicals to oxygen or spin traps through the C(3) position has been suggested [5,6]. We used Trp isotopically labeled at that position with ^{13}C to test this hypothesis. The oxidation of the isotopically labeled compound by $Br_2^{\bullet -}$ in the presence of PBN was carried out and the EPR spectrum recorded, as described for isotopically natural Trp. A 6-line spectrum was observed, identical to that seen with the unlabelled compound.

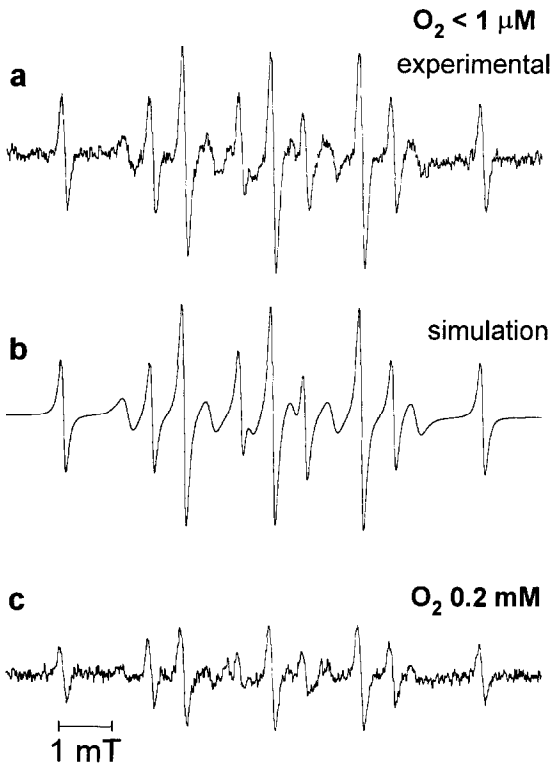


Fig. 3. EPR spectra observed on oxidation of Trp by $Br_2^{\bullet -}$ in the presence of DMPO (conditions described in Table 1). (a) In oxygen free solution; (b) simulation (see Table 1); (c) in the presence of oxygen. The spectrometer conditions were the same as in Fig. 2.

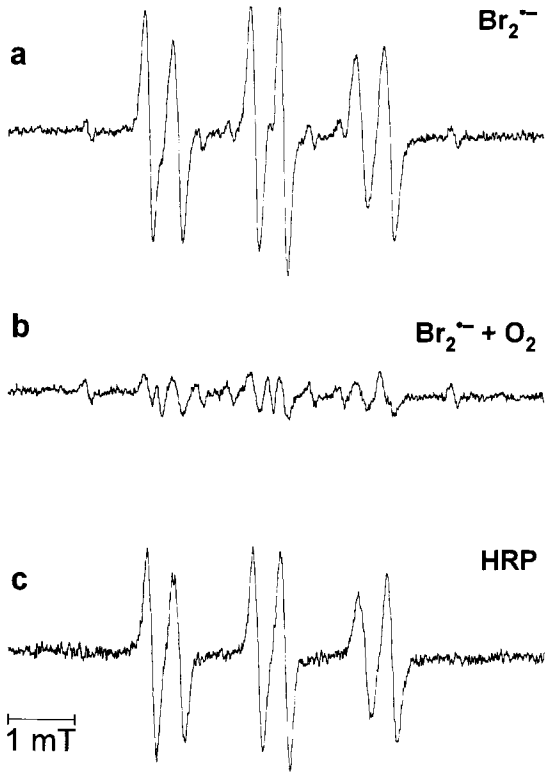


Fig. 4. EPR spectra observed on oxidation of Trp by $Br_2^{\bullet -}$ in the presence of PBN. (a) In oxygen free solution and (b) in the presence of oxygen (0.2 mol dm^{-3}). (c) EPR spectrum recorded on oxidation of Trp by HRP and H_2O_2 in the presence of PBN. Spectrometer conditions: microwave power 50 mW; gain 2×10^5; modulation amplitude 0.1 mT; time constant 20 ms; sweep rate 0.38 mT s^{-1}; the spectra are the average of 20 scans.

Since ^{13}C has spin 1/2, addition of the tryptophanyl radical to the nitrone through this position would have caused additional line splitting. This was not the case, which suggests that the addition of the tryptophanyl radical to PBN is through a position other than C(3).

3.2. Oxidation of IAA and Trp by HRP

The oxidation of IAA by horseradish peroxidase has been previously studied using, among other techniques, EPR with spin-trapping [9,30]. Our results using DMPO confirm and expand previous observations. Both at pH 4.5 and 7.4, the spectra recorded immediately (i.e. 2 min) after initiating the reaction show contributions from three DMPO adducts: the superoxide (DMPO-OOH), hydroxyl (DMPO-OH)

and skatolyl radical adducts. After ca. 12 min the DMPO-OOH signal vanishes as the enzymatic reaction slows down and DMPO-OOH decays to DMPO-OH. Simultaneously, the skatolyl radical adduct accumulates to become the dominant component of the spectrum. The oxidation of IAA by HRP has the peculiarity of not requiring hydrogen peroxide [7,8,31,32]. In an experiment at pH 7.4, we tested the effect of omitting hydrogen peroxide from the reaction mixture. In this case, the contribution of the skatolyl radical adduct to the spectrum was markedly smaller, reflecting the slower oxidation of IAA under these conditions (see below). However, the superoxide and hydroxyl radical adducts were still observed with no discernible change of intensity.

When Trp replaced IAA in analogous experiments, rather different results were observed. At pH 7.4 very weak, unidentifiable EPR signals were observed, even after 15 min incubation. At pH 4.5, clear EPR signals were observed, and assigned to the 2-oxo derivative of DMPO (DMPO-Ox). No signals attributable to DMPO adducts of tryptophanyl derived radicals were detected. We therefore used PBN to trap the tryptophanyl radicals. After ca. 10 min incubation of a solution of Trp, H_2O_2 and HRP in phosphate buffer at pH 7.4, a 6-line spectrum was observed. The measured hyperfine coupling constants ($a_N = 1.59$ mT, $a_H = 0.40$ mT) agree with those measured for the PBN adduct of the tryptophanyl radical formed on oxidation by $Br_2^{\cdot-}$, suggesting that the same radical is generated in the two systems. In a control experiment, no EPR signals were detected on incubation of PBN with HRP and H_2O_2.

Experiments were also performed with ^{13}C-labeled tryptophan. It was found that the isotopic substitution at position C(3) did not affect the EPR spectra of the PBN-adducts formed on HRP-catalyzed oxidation of tryptophan at pH 7.4. This is consistent with the observations made on $Br_2^{\cdot-}$ oxidation and confirms that the addition of the tryptophanyl radical to PBN does not take place through position C(3).

4. Discussion

The pulse radiolysis results presented here and elsewhere [10] demonstrate the different behavior of the IAA and Trp radical cations. Whereas the former undergoes rapid scission of the C-C bond in the side chain to yield carbon dioxide ($k = 1.8 \times 10^4$ s^{-1} [10]) the latter decays by second order reactions without detectable formation of CO_2. On the basis of studies with a series of indoleacetic acids and related compounds [33] we suggest that the different behavior is due to the stabilizing effect of the delocalized π-electron system of the indole moiety at the α-position to the radical site. In tryptophan, the radical resulting from decarboxylation would be at the β-position to the indole group; the consequent loss of stabilization results in total absence of decarboxylation.

The hydroxyl adduct of DMPO was an important component to the EPR spectrum detected on oxidation of IAA by $Br_2^{\cdot-}$ in the presence of DMPO. The superoxide adduct of DMPO is known to decay spontaneously into DMPO-OH [11]. However, this mechanism can not account for the formation of DMPO-OH in the oxygen-free solutions. Moreover, the signal was not affected by superoxide dismutase, showing that superoxide is not formed on *chemical* oxidation of IAA. This supports previous conclusions based on the absence of reduction of nitroblue tetrazolium or ferric cytochrome *c* on oxidation of IAA in the presence of oxygen [10].

The experiments on the peroxidase-catalyzed oxidation of the indoles demonstrated the different behavior of the radicals from IAA and Trp. The oxidation of IAA by HRP has been the subject of a detailed study [9], which we were able to confirm. In particular, we obtained clear evidence for the formation of superoxide. From the comparison with the results from the oxidation by $Br_2^{\cdot-}$, it is evident that $O_2^{\cdot-}$ does not result from the breakdown of the skatole peroxyl radical. It must therefore be formed by reactions involving peroxidase intermediates. Smith et al. [31] suggested the involvement of ferrous peroxidase and compound III in an oxidase cycle competing with the compound I-compound II cycle (Fig. 5). According to the proposed reaction scheme, superoxide is a product of the reaction of compound III with IAA. Compound III has been suggested to result from reactions involving ternary complexes of peroxidase, IAA and oxygen [32] or reduction of ferric peroxidase by IAA [31]. Although our results do not allow a distinction between these

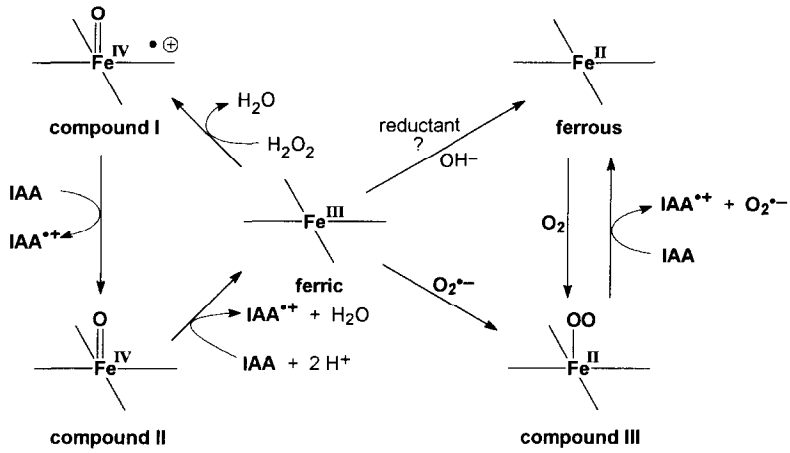


Fig. 5. Scheme of the oxidation of IAA by horseradish peroxidase, based on that suggested by Smith et al. [31] and supported by the results of the present study.

possibilities, they do support the formation of superoxide via this oxidase cycle. It is interesting to note that the shift of the enzyme from the compound I-compound II cycle to the compound III cycle is irreversible, i.e. there is no plausible mechanism to resume the former cycle. Moreover, the reaction of superoxide with ferric enzyme to yield compound III ($k = 6 \times 10^6$ dm^3 mol^{-1} s^{-1} at pH 5 [34]) continually recruits more enzyme to the oxidase cycle. Whether the compound III cycle is a peculiarity of the HRP-IAA system or a general phenomenon in heme peroxidase mechanisms remains an open question. Recently, a different reaction scheme was proposed by Krylov and Dunford [7,8] which does not account for the formation of superoxide. However, their scheme is applicable only to conditions of high enzyme/substrate ratio [31].

The EPR experiments on the oxidation of tryptophan by $Br_2^{\bullet-}$ showed the formation of DMPO or PBN adducts with the characteristics of an adduct through a carbon atom. Using PBN, we could find evidence for the formation of the same radical which could be trapped during the peroxidase-catalyzed oxidation, confirming that the radical in question results from the Trp oxidation. It was also shown that the tryptophanyl radical reacted with oxygen. However, the pulse radiolysis experiments put an upper limit on the rate of this reaction. The spin-trapping results can be explained by the competition between two slow reactions (too slow to be observed in the pulse radiolysis time-scale): the reaction of the tryptophanyl radical with oxygen with formation of a peroxyl radical and the addition of the radical to the spin-trap. The addition of oxygen to related radicals, such as hydroxycyclohexadienyl has been shown to be a reversible process [35]; a similar mechanism may apply to the tryptophanyl radical.

Recently, the tryptophanyl radical has been suggested to form adducts to oxygen (peroxyl radical) through C(3) [6], in line with the high spin-density at that position predicted by quantum chemical calculations [36]. Our experiments with isotopically labeled tryptophan showed no coupling of ^{13}C(3) with the unpaired electron in the nitroxide PBN-adduct, indicating that the tryptophanyl radical added through a position other than C(3). It appears that the bulky phenyl-*tert*-butylnitrone has regioselectivity different from that of the less voluminous oxygen.

It has been proposed on the basis of spin-trapping experiments [5,6] that a tryptophanyl radical is formed on reaction of metmyoglobin with hydrogen peroxide. However, the fact that the detected radical was quenched by oxygen seemed inconsistent with earlier pulse radiolysis observations [4]. Here, we have demonstrated that the tryptophanyl radical does react with oxygen, but this reaction is too slow to be monitored by pulse radiolysis ($k < 5 \times 10^6$ dm^3 mol^{-1} s^{-1}), giving credence to the suggestion that the amino acid radical site in myoglobin is at a tryptophan. The spin-trapping experiments on the

oxidation of tryptophan by $Br_2^{\cdot-}$ in the presence of oxygen reported here lead to the conclusion that superoxide is not formed., i.e. the tryptophan peroxyl radical does not release $O_2^{\cdot-}$. Assuming that the same mechanism applies to the tryptophanyl radical in myoglobin, a peroxyl radical maybe be formed, which may cause biological damage, possibly more efficiently than superoxide which should be readily scavenged by superoxide dismutase.

In recent years, it has been discovered that stable tryptophanyl radicals play a role in the catalytic cycle of some enzymes (e.g. cytochrome *c* peroxidase [37], DNA-photolyase [38]). Our results demonstrate that tryptophanyl radical reacts with oxygen, albeit slowly. Since in some proteins EPR spectra show the existence of a stable tryptophanyl radical, this implies that is must either be inaccessible to oxygen or have its reactivity modified by the protein environment.

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