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Review

Reaction of haem containing proteins and enzymes with hydroperoxides: The radical view

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Abstract

The reaction between hydroperoxides and the haem group of proteins and enzymes is important for the function of many enzymes but has also been implicated in a number of pathological conditions where oxygen binding proteins interact with hydrogen peroxide or other peroxides. The haem group in the oxidized Fe^{3+} (ferric) state reacts with hydroperoxides with a formation of the Fe^{4+}=O (oxoferryl) haem state and a free radical primarily located on the π-system of the haem. The radical is then transferred to an amino acid residue of the protein and undergoes further transfer and transformation processes. The free radicals formed in this reaction are reviewed for a number of proteins and enzymes. Their previously published EPR spectra are analysed in a comparative way. The radicals directly detected in most systems are tyrosyl radicals and the peroxyl radicals formed on tryptophan and possibly cysteine. The locations of the radicals in the proteins have been reported as follows: Tyr133 in soybean leghaemoglobin; αTyr42, αTrp14, βTrp15, βCys93, (αTyr24−αHis20), all in the α- and β-subunits of human haemoglobin; Tyr103, Tyr151 and Trp14 in sperm whale myoglobin; Tyr103, Tyr146 and Trp14 in horse myoglobin; Trp14, Tyr103 and Cys110 in human Mb. The sequence of events leading to radical formation, transformation and transfer, both intra- and intermolecularly, is considered. The free radicals induced by peroxides in the enzymes are reviewed. Those include: lignin peroxidase, cytochrome *c* peroxidase, cytochrome *c* oxidase, turnip isoperoxidase 7, bovine catalase, two isoforms of prostaglandin H synthase, *Mycobacterium tuberculosis* and *Synechocystis* PCC6803 catalase-peroxidases.

Keywords: Haem; Heme; Peroxide; Tyrosine; Tryptophan; Cysteine; Radical

Abbreviations: AA, arachidonic acid; apo, prefix for the haem proteins that are forced to lose their haem group; BLC, bovine liver catalase; C*c*O, cytochrome *c* oxidase; C*c*P, cytochrome *c* peroxidase; CuOOH, cumene hydroperoxide $PhC(CH_3)_2OOH$; Cyt *c*, cytochrome *c*; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; EtOOH, ethyl hydroperoxide,C_2H_5OOH; FAD, flavin adenine dinucleotide; Hb, haemoglobin; HbA, human haemoglobin; HF, high frequency/high field (EPR spectroscopy); HH, horse heart (myoglobin); HRP, horseradish peroxidase; h.s., high spin; HSM, horse skeletal muscle; IS, 'inhibited singlet' (in PGHS); Lb, leghaemoglobin; l.s., low spin; Mb, myoglobin; met, prefix for the haem proteins in the oxidized Fe^{3+}state; MLC, *Micrococcus luteus* (*lysodeikticus*) catalase; MNP, 2-ethyl-2-nitrosopropane; NS, narrow singlet (a PGHS EPR signal); PAA, peroxyacetic acid $CH_3C(O)OOH$; PDB, Protein Data Bank; PG, prostaglandin; PGHS, prostaglandin H synthase; PMC, *Proteus mirabilis* catalase; PRRAD, Phenol Ring Rotation Angle Database; oxy, prefix for the haem proteins in the reduced and oxygenated state Fe^{2+}–O_2; SW, sperm whale; tBuOOH, *tert*-butyl hydroperoxide, C_4H_9OOH; TIP, turnip isoperoxidase; TRSSA, Tyrosyl Radical Spectra Simulation Algorithm; WD, wide doublet (a PGHS EPR signal); WS, wide singlet (in PGHS)

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1. Introduction

For a number of haem containing enzymes, reaction with hydroperoxides is a part of their main function. Catalases catalyse the dismutation of hydrogen peroxide (H_2O_2) into molecular oxygen and water. Peroxidases use peroxides as oxidants in the one electron oxidation of various substrates. Catalase-peroxidases, in accordance with their cumbersome name, can react with peroxides by both mechanisms. The normal function of many haem proteins and enzymes does not, however, involve interaction with hydroperoxides, but those proteins and enzymes would still enter the reaction if the haem is in the ferric form (Fe^{3+}) and if the haem's 6th coordination position is vacant and accessible for the hydroperoxide group R–O–O–H. As a result of such interaction, ferric haem is oxidized to the oxoferryl state (Fe^{4+}=O), and a free radical is formed either on the porphyrin moiety of the haem or on an amino acid residue. Extensive studies of these reactions started in early sixties in two general directions. Haemoglobin (Hb) and myoglobin (Mb) from various sources became the most popular subject proteins in such studies because these proteins provided the researchers with the models of the catalase and peroxidase action that would be more convenient for kinetic studies than the enzymes per se, since Hb and Mb react with H_2O_2 notably slower than catalases and peroxidases do. On the other hand, it soon became clear that these proteins, when mixed with peroxides, become very active pro-oxidants, and their role in promoting oxidation of many substrate molecules has been investigated in vitro. In recent years, the reaction of the haem proteins with peroxides has been implicated in a number of pathological conditions thus bringing a new biomedical significance to the old story.

2. Historical aspect: the "old story" from Kobert to Kelso King and Winfield

In 1900, Rudolf Kobert, at the time the Head of the Institute of Pharmacology and Physiological Chemistry, University of Rostock, reported a reaction between Hb and H_2O_2: The colour of solution of oxidized Hb (metHb) changed from brown to red on the addition of H_2O_2 [1]. Three diffused bands in the optical absorption spectrum were reported: 500–513 nm, 545–558 nm and 584–600 nm. Kobert attributed this change of colour to the formation of a complex between metHb and H_2O_2 which he called "Wasserstoffsuperoxydmethämoglobhin". The formation of such a complex was contested 24 years later by Haurowitz [2] who confirmed the optical changes observed by Kobert but attributed them to the acid-to-alkaline transition in metHb. This interpretation was, however, dismissed by Keilin and Hartree [3] who assigned the first band to the unbound acid metHb (from horse) and showed in pH controlled experiments that the protein remains in its acidic form even when the H_2O_2 induced spectral changes occur. They therefore concluded that the optical spectra changes observed are caused by the formation of an H_2O_2–metHb complex in a process similar to the reaction of metHb with KCN, H_2S, KF and NaN_3. However, it turned out that, in contrast to the complexes formed by these small molecules with metHb, the H_2O_2 complex with metMb can be reduced by ferrocyanide to yield normal metMb [4]. This finding allowed George and Irvine to suggest that the optical changes observed are not associated with a formation of an H_2O_2 adduct of metMb, but rather with a formation of a product of one electron oxidation of the met (ferric) haem. The intermediate compound must be one oxidizing equivalent higher than metMb, i.e. the iron atom should have effective oxidation number +4 (ferrylmyoglobin) [4,5]. Hydrogen peroxide is a two electron oxidant, so another oxidizing equivalent should be present. George and Irvine suggested the formation of a hydroxyl radical •OH, and indeed a radical was demonstrated to be formed during the formation of the complex as a flash was observed if luminol was present [4]. The free radical was soon detected directly by the newly available method of electron paramagnetic resonance (EPR) spectroscopy, in both Hb and Mb [6], and assigned to a porphyrin radical. Fig. 1 shows this very first reported EPR spectrum, recorded as an absorption line, of the radical formed in the metHb/H_2O_2 system. The authors, however, revised their assignment two years later [7]. They studied sperm whale (SW) Mb and showed that the free radical concentration was only ~9% of the ferryl haem and varied widely under conditions in which the amount of the ferryl form remained approximately constant, e.g. a pH increase from 6 to 10 resulted in an 80% decrease of the free radical concentration, while the ferryl haem was formed equally well at these pH values. The authors concluded that the radical cannot be associated with the optically detectable Fe^{4+}-haem and must be located on an amino acid of the protein (Hb or Mb) [7].

In 1963, Kelso King and Winfield published a milestone paper in the field [8]. They used optical and EPR spectroscopy and manometry to scrupulously study the reaction between horse metMb and H_2O_2 in a range of pH values. It was shown that the reaction is not a formation of the complex

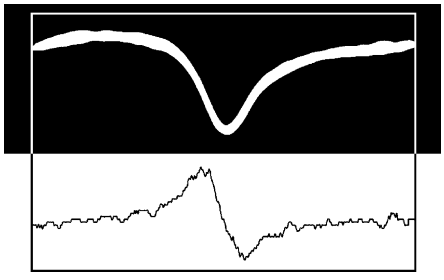


Fig. 1. The first reported EPR spectrum of the free radicals formed in a haem protein/peroxide system (metHb was mixed with H_2O_2 and rapidly frozen, the spectrum was measured at 20 K) [6]. This oscilloscopic image represents the EPR absorption curve of the radicals (on the top). This curve was digitized, its first derivative represents a spectrum view conventional for contemporary EPR spectrometers (the bottom race).

(the adduct) between the two but a process of two electron oxidation of Mb by H_2O_2 in which the transient product was characterised as Mb^{4+}—the form of the protein with the haem in the ferryl state and a free radical on the globin moiety:

$$Fe^{III} \quad R + H_2O_2 \rightarrow Fe^{IV}=O \quad R^{\bullet +} + H_2O \qquad (1)$$

The free radical was shown to be lost after the formation faster than the ferryl form was recovered back to the met state, and the regenerated met state was shown to be unidentical to the initial met state. The authors associated this modified met form with changes made to the amino acids where the radical was formed. They also showed that Mb could be re-oxidized by H_2O_2 at least 8 times before it loses its ability to react with H_2O_2. In a follow-up paper, Kelso King et al. studied the free radical formed in horse metMb, SW metMb and horse metHb, all treated with H_2O_2 [9]. In an attempt to attribute the free radical observed in the proteins to a particular amino acid, they compared the EPR spectra of the reaction mixture with those of various isolated amino acids and small peptides oxidized by UV irradiation in the presence of H_2O_2. A comparison of a number of EPR spectral features, such as EPR linewidth, saturation behaviour, EPR line asymmetry, ease of radical's formation and stability, brought the authors to the conclusion that the principal radical observed in Mb (the Mb^{4+}=O ferryl state with a radical on the globin) is most probably located on a tyrosine residue [9].

Contemporary methods of research reveal the reaction in much greater detail. Fig. 2 shows the low temperature EPR spectra of horse heart (HH) metMb before and after stoichiometric H_2O_2 addition, as well as the kinetic dependences during the reaction of the high spin (h.s.) met haem form, the ferryl haem form and the free radicals. The dependence of the ferryl haem formation is almost a mirror image of the ferric haem disappearance, indicating that the former is formed stoichiometrically from the latter. Although the total metMb concentration was 100 μM, the h.s. spin met form at zero time is only 91 μM indicating that 9 μM of metMb was initially in a low spin (l.s.) form [10]. The varying shape of the EPR spectra detected as a function of time is indicative of more than one radical species being observed. Consistent with other reports [8,9,11–13], the maximal observed concentration of the radicals is much smaller than maximal possible concentration (equal to the protein concentration). Also, the radical disappears from the EPR spectra considerably faster than the ferryl form is reduced back to the ferric state.

3. Oxidative capacity of the metHb/H_2O_2 and metMb/H_2O_2 systems; relevance to living systems

The addition of peroxide to metHb or metMb generates a chemically active medium in which many substrate molecules can be oxidized. The metHb+H_2O_2 mixture was

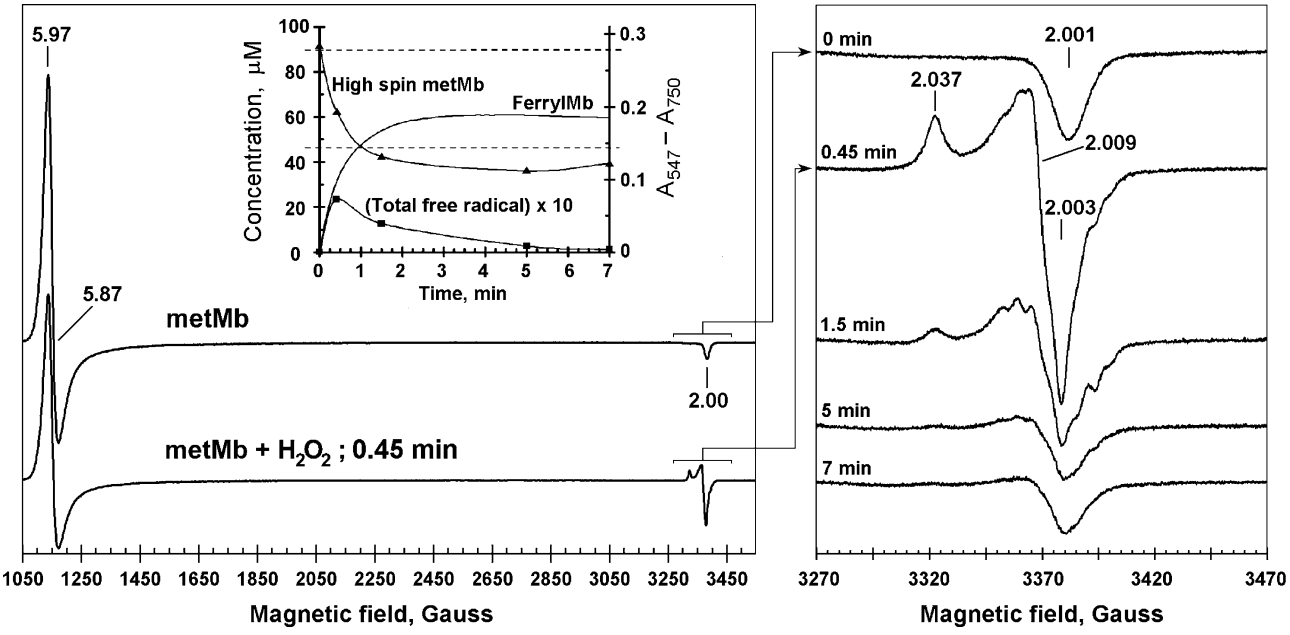


Fig. 2. The EPR spectra of 100 μM HH metMb before and different time after the addition of 100 μM H_2O_2 at pH 7. The first spectrum on the right (no H_2O_2 added yet) shows the *g*=2 component of the h.s. metMb form. In the inset, the kinetic dependences of the h.s. met haem form, ferryl haem form and total free radical are shown; the metMb and free radical dependences are related to the *Concentration* axis, whereas the ferrylMb dependence is related to the axis of optical absorbance difference at 547 and 750 nm. The dependence of the free radical is multiplied by 10 for illustrative purpose. The EPR measurements were performed at 10 K and the optical measurements—at 300 K.

shown to catalyse oxidation of hydroxy benzoic acid [14], hydroxylation of anilines [15–18], demethylation of aryl amines and ethers [18,19], oxidation of aryl hydrazines and olefins [20–23], epoxidation of styrene [24] and sulfoxidation of chlopromazine [25,26]. Both the metHb/H_2O_2 and metMb/H_2O_2 systems were demonstrated to initiate membrane lipid peroxidation [27]. The two sites of oxidation of a haem protein by peroxides, the ferryl haem ion and the protein-bound radical, were both implicated in the mechanism of such oxidative reactions.

The role of the ferryl haem iron in the promotion of lipid peroxidation has been positively established [28–33]. Since the radical decays faster than the ferryl haem iron (Fig. 2, inset), there is a phase of the reaction when all radicals are reduced but the ferryl haem is still present. This radical free, ferryl-only state of the haem proteins can initiate lipid peroxidation, and it has been shown that this process has a strong pH dependence, being much more efficient at acidic pH values [31,32]. To explain this effect, it has been suggested that at low pH the ferryl species is protonated to form the state Fe^{4+}–OH^- with a pK value of ~3.5 (2) [32]. The instability of the protonated ferryl species enables this species to be considered as possessing a radical-like nature, since it is electronically equivalent to the ferric haem and a radical on either the hydroxyl ligand (3), the porphyrin (4) or the globin (5).

$$Fe^{4+} - O^{2-} + H^+ \rightarrow Fe^{4+} - OH^- \quad (2)$$

$$Fe^{4+} - OH^- \equiv Fe^{3+} - {\bullet}OH \quad (3)$$

$$Fe^{4+} - OH^- \equiv Fe^{3+} - OH^- por^{\bullet +} \quad (4)$$

$$Fe^{4+} - OH^- \equiv Fe^{3+} - OH^- protein^{\bullet +} \quad (5)$$

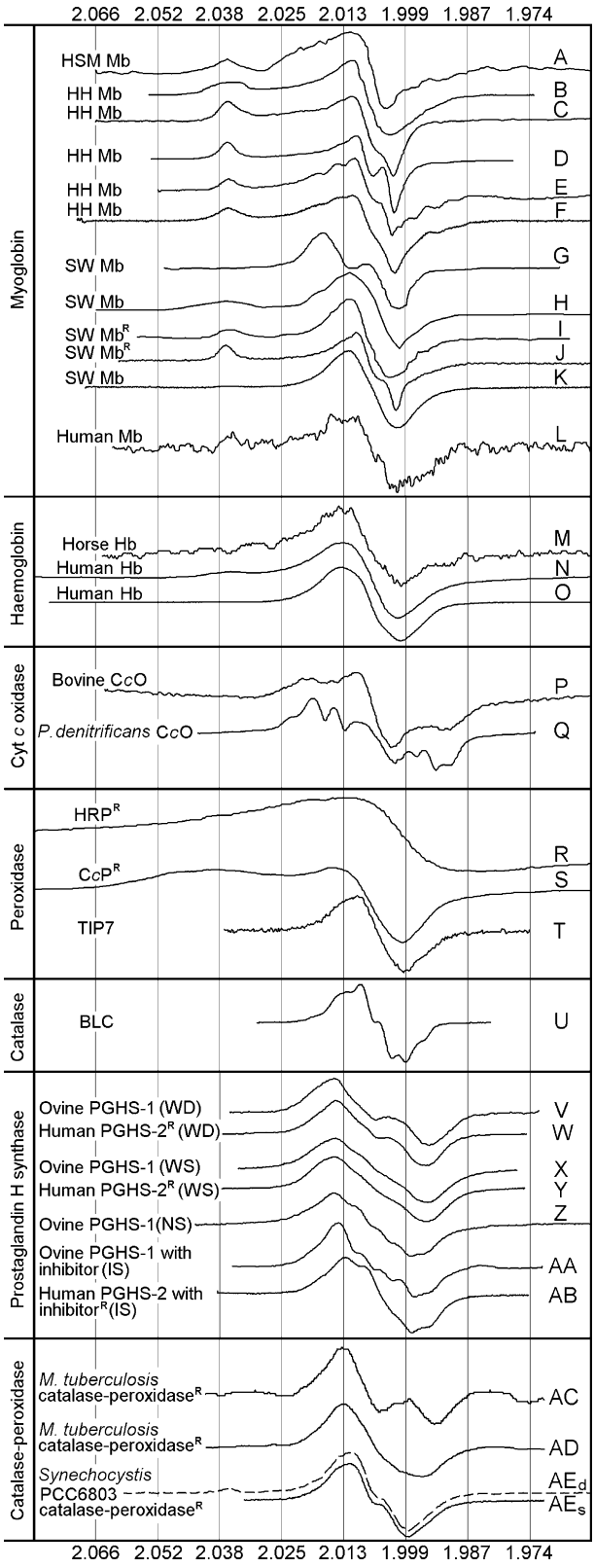
The pH profiles of Mb-induced oxidation of low density lipoproteins (LDL) [31], isoprostane formation from LDL [34] and lipid hydroperoxide consumption by Mb and by free haemin [32] were found to be similar to the pH dependence of the rate constant for ferryl auto-reduction, itself dependent on the protonation state of the oxyferryl form [32].

The protein bound free radical is also thought to be responsible for substrate oxidation. Indeed, the total yield of radicals, quite different in such protein systems as human metHb/H_2O_2, SW metMb/H_2O_2 and horse metMb/H_2O_2 even when measured under identical haem and H_2O_2 concentration conditions and at the same time after initiation of the reaction [35], correlates with accordingly different efficiencies of these systems to oxidize styrene [24]. The yield of styrene oxidation products [24] as well as the yield of the radicals [35] were greatest for the Hb/H_2O_2 system[1], followed by SW Mb/H_2O_2 and then by horse Mb/H_2O_2. Oxygen atoms from O_2, either initially present in the medium or formed from H_2O_2 decomposition, were implicated in the formation of the oxidation products of styrene [24]. Also, substrate oxidation is associated with radical reduction, as this has been demonstrated for such substrates as glutathione, styrene, arachidonic acid and linoleic acid [36] and for albumin and a number of antioxidants [37]. The simplest explanation of these effects is that the radical directly reacts with the substrates. There are, however, alternative possibilities that the substrates react with a precursor of the observed radical, or that the antioxidants studied simply depleted molecular oxygen thus preventing the observed peroxyl radical formation (Section 5.1).

It is certainly interesting if the oxidation reaction promoted by the haem proteins in vitro can have a biological relevance. The free radicals that can be detected in frozen samples of whole animal and human blood are the very same radicals that are formed when H_2O_2 reacts with purified metHb [13,38]. It has been suggested that H_2O_2 intrinsically formed in blood from the disproportionation reaction of $^{\bullet}O_2^-$, formed in turn during Hb autoxidation, reacts with metHb to form the radical observed in blood. It has been demonstrated that the radical is located on a tyrosine residue of Hb [39]. This reaction, shown to take place in blood ex vivo, is likely to be a characteristic of normal blood in vivo.

The detection of a protein containing a ferryl haem in vivo is difficult. This haem form could be induced by peroxides and detected in biological tissues ex vivo using the addition of sodium sulfide [40]. The latter reacts with the ferryl haem form to give a distinctive spectral band with absorbance maximum at 617 nm [41,42]. Unfortunately, both the low stability of the sulfur–haem complex and the toxicity of sulfide make this method unsuitable for identifying the ferryl haem states in vivo. There is, however, an alternative method. The reaction of metMb (or metHb) with peroxides yields a stable product—the form of the protein in which the haem is covalently bound to an amino acid residue [43,44]. The pH dependence for the formation of the Mb cross-linked forms is remarkably similar to the pH dependences of Mb-induced oxidation of substrates, indicating a common feature in the mechanism, possibly the protonation of the oxoferryl haem. The specific optical characteristics [44,45] of the cross-linked forms of Mb or Hb (Mb-X or Hb-X) has allowed their detection in two disease states. One is rhabdomyolysis, a condition where damaged muscle releases Mb to the blood stream leading to renal failure. The urine of patients suffering from rhabdomyolysis is characterised by a high concentration of Mb-X [45]. The other condition is associated with a brain haemorrhage, caused by trauma, aneurysm or alveolar malformations, that can result in bleeding into the CSF (cerebrospinal fluid) filled spaces surrounding the brain (also known as the subarachnoid

[1] Bovine Hb was used in the styrene oxidation experiments [24] and human Hb was used in the free radical study [35].



space). About one third of patients who survive such subarachnoid haemorrhage develop delayed vasospasm causing ischemia. It has been found that a large fraction of the Hb recovered from the spinal fluid of such patients is in the cross-linked Hb-X form [45]. These two conditions are associated with oxidative stress, since isoprostanes, the vasoactive molecules which are the most reliable markers of oxidative stress in vivo [46], have been found in high concentration in patients suffering from both rhabdomyolysis [34] and vasospasm following subarachnoid haemorrhage [47,48]. The finding of the cross-linked haem forms in both pathologies is highly suggestive that the haem proteins have been cycled through the ferryl haem form thus possibly causing the lipid oxidation and isoprostane formation. This suggests an important role of the haem proteins in the mechanism of oxidative stress caused by rhabdomyolysis and subarachnoid haemorrhage.

The role of free iron in promoting the oxidative reactions in vivo via the Fenton chemistry has been suggested long ago and is considered overwhelmingly accepted. The understanding of the important role that the haem proteins might play in similar reactions is just emerging. The recent review that covers these issues can be recommended [45].

4. An overview of the reported EPR spectra of different haem proteins reacting with different peroxides

The pattern of radical formation and decay is strongly dependent on the nature of the haem protein and peroxide used, their concentrations, the pH value at which the reaction is conducted and on the method of radical detection (low temperature or room temperature EPR). Understandably, the reported EPR spectra represent a wide range of different lineshapes. Fig. 3 shows the low

Fig. 3. The low temperature X-band EPR spectra of the radicals formed in different haem proteins (in the ferric state) under peroxide treatment. All spectra were taken from the literature and scanned, digitized, stretched to the same scale of magnetic field and moved relative to each other horizontally to common *g*-factor values. The absolute magnetic field axis is individual for different spectra due to different microwave frequency at which the spectra were measured, therefore it is not shown. Instead, the *g*-values, calculated for the gridlines drawn at a 20 G interval, are indicated. The details of the samples used to record the spectra are collected in Table 1. The abbreviations used for the first time: HSM, horse skeletal muscle; C*c*O, cytochrome *c* oxidase; HRP, horseradish peroxidase; C*c*P, cytochrome *c* peroxidase; TIP7, turnip isoperoxidase 7; BLC, bovine liver catalase; PGHS, prostaglandin H synthase. For PGHS, the signals referred to in the literature as 'wide doublet' (WD), 'wide singlet' (WS), 'narrow singlet' (NS) and 'inhibited singlet' (IS) are indicated. Superscript (^{R}) indicates recombinant protein.

Table 1
Details of the protein/enzyme samples used to register the low temperature EPR spectra shown in Fig. 3[a]

EPR spectrum	Protein	[protein] (μM)	Peroxide	[peroxide] (μM)	pH[b]	$T_{reaction}$ (°C)	Reaction time (min)[c]	$T_{detection}$ (K)	Reference
Myoglobin									
A	Horse skeletal muscle Mb	200	H_2O_2	345	8.5	23	30/60	78	[11]
B	Horse heart Mb	740	H_2O_2	1029	6.0	0	0.6	173	[9]
C	Horse heart Mb	95	H_2O_2	119	7.4	room	5/60	100	[12]
D	Horse heart Mb	532	H_2O_2	400	7.4	X	2	77	[36]
E	Horse heart Mb	640	H_2O_2	710	7.4	19	1	77	[37]
F	Horse heart Mb	100	H_2O_2	100	7.6	room	26/60	10	[35]
G	Sperm whale Mb	870	H_2O_2	1209	8.0	0	1.4	173	[9]
H	Sperm whale Mb	X	H_2O_2	4X	7.4	0	2	77	[54]
I	Sperm whale Mb[d]	300	H_2O_2	450	X[e]	X	0	X	[97]
J	Sperm whale Mb[d]	50	H_2O_2	45	7.4	X	6/60	77	[64]
K	Sperm whale Mb	100	H_2O_2	100	7.6	room	23/60	10	[35]
L	Human Mb	~1000	H_2O_2	~1200	7.4	X	0	77	[65]
Haemoglobin									
M[f]	Horse Hb	X	H_2O_2	X	X	X	0	20	[6]
N	Human Hb	400	H_2O_2	4500	7.4	room	30/60	10	[163]
O	Human Hb	100[g]	H_2O_2	100	8.0	room	29/60	10	[35]
Cytochrome c oxidase									
P	Bovine *Cc*O	14	H_2O_2	500	6.0	room	3	45	[134]
Q[h]	*P. denitrificans Cc*O	200	H_2O_2	200	6.0	4	0	80	[164]
Peroxidase									
R	Horseradish peroxidase[d]	~400	H_2O_2	≥400	7.0	25	1	5	[107]
S	*Cc*P peroxidase form yeast[d]	1250	H_2O_2	1250	6.0	0	1	4	[122]
T	Turnip isoperoxidase 7	325[i]	H_2O_2	650[i]	7.7	0	30/60	30	[111]
Catalase									
U	Bovine liver catalase (BLC)	176	PAA	12353	7.5/5.6	0	15/60	86	[112]
Prostagland in H synthase									
V	Ovine PGHS-1	18.8[g]	EtOOH	376	7.2	−15	3/60	11	[144]
W	Human PGHS-2[d]	11.2	EtOOH	56	7.2	24	0.005/60	96	[147]
X	Ovine PGHS-1	213	EtOOH	1065	7.4	0	15/60	115	[145]
Y	Human PGHS-2[d]	X	EtOOH	15X	7.2	4	10/60	113	[149]
Z	Ovine PGHS-1	50	AA	5000	X	−12	110/60	77	[72]
AA	Ovine PGHS-1 pre-treated with indomethacin	15[j]	EtOOH	30[j]	7	0	2/60	X[k]	[146]
AB	Human PGHS-2[d] pre-treated with nimesulide	X	EtOOH	15X	7.2	4	10/60	113	[149]
Catalase–peroxidase									
AC	*M. tuberculosis* catalase-peroxidase, KatG[d]	50[g]	PAA	150	7.2/X	25	0.25/60	77	[160]
AD	*M. tuberculosis* catalase-peroxidase, KatG[d]	50[g]	PAA	150	7.2/X	25	10/60	77	[160]
AE_{solid}	*Synechocystis* PCC6803 catalase-peroxidase[d]	1000[l]	PAA	10000[l]	8.0/4.5	0	10/60	60	[115]
AE_{dashed}	*Synechocystis* PCC6803 catalase-peroxidase[d]	1000[l]	PAA	10000[l]	8.0/4.5	0	10/60	4	[115]

temperature X-band[2] EPR spectra of the free radicals reported in the literature for various haem proteins and enzymes reacting with various peroxides (details of the reaction in each case are given in Table 1). Low temperature EPR spectroscopy is characterised by a high signal/noise ratio. Also, the free radicals, once frozen and stabilized in the water matrix, become substantially long-lived species, so that a set of samples frozen at various time after mixture

[2] Class of EPR spectrometers operating at 9 GHz microwave frequency.

is a convenient system for kinetic studies. However, freezing of the reaction mixture can sometimes affect the radicals' structure and kinetics. This is due to the freezing process itself, when the molecules diluted in water are driven across the sample and brought to a non-uniform concentration by the liquid/solid phase separation surface that moves stochastically over the sample's volume during the first seconds of freezing. Although this is not a major problem in most cases, it should be kept in mind when analysing the experiments involving frozen samples.

Free from this drawback is stopped-flow or continuous flow EPR spectroscopy, which allows for the direct detection of radicals in liquid solutions. Due to the short lifetime of the radicals, the EPR spectra should be recorded within a reasonably short time, in a stopped-flow EPR experiment. The continuous flow experiments, on the other hand, need a significant amount of protein to be put through the continuous flow system, which is not always possible, particularly with enzymes that are difficult to purify, or which are expensive if commercially available. The sensitivity of the liquid phase EPR (the signal/noise ratio) is generally lower than in the low temperature experiments. All these possibly explain why there are fewer works reporting the liquid phase EPR spectra of the radicals, and why those are mainly performed on the most easily available proteins—Mb and Hb (Fig. 4). The details of the samples used to record the real time EPR spectra shown in Fig. 4 are presented in Table 2.

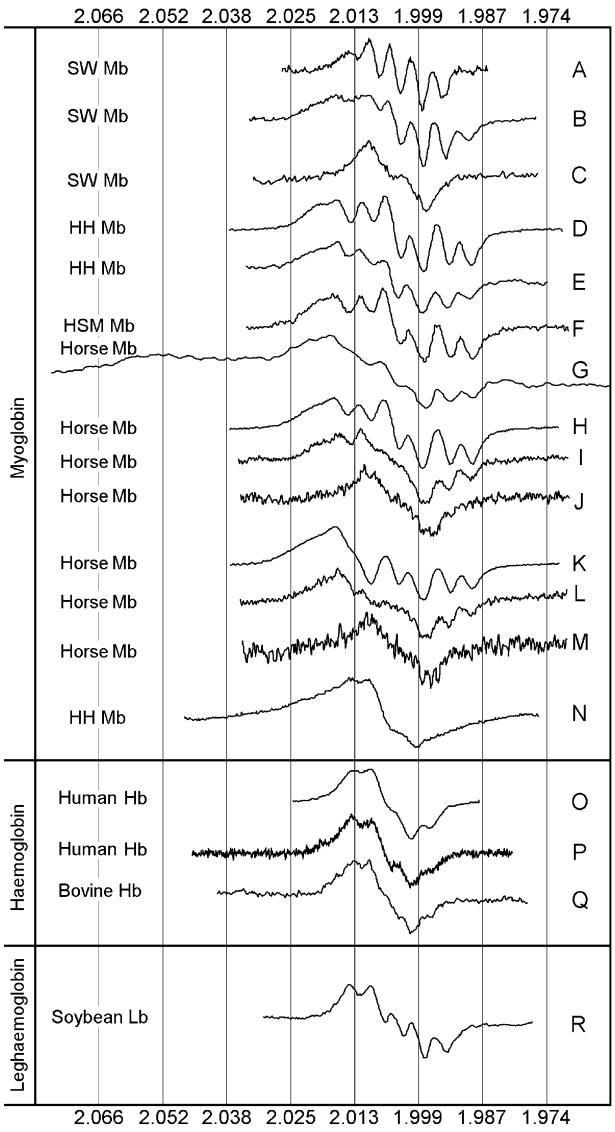


Fig. 4. The liquid phase X-band EPR spectra of the radicals formed in different haem proteins (in the ferric state) under peroxide treatment. All spectra were taken from the literature and plotted on a common axis as described in Fig. 3 legend. The gridlines are drawn at a 20 G interval. Details of the samples used to record the spectra are collected in Table 2. The abbreviation used for the first time: Lb, leghaemoglobin.

5. Free radicals in non-enzymes: Mb, Lb, Hb

5.1. Peroxyl radicals

There is one certainty in the dramatic lineshape variability of the EPR spectra of myoglobins and haemoglobins treated with peroxides: all low temperature spectra (Fig. 3, A–O) have the central feature at $g \approx 2.00$, and practically all spectra show a component of variable relative intensity, distinctly separated from the main feature, at $g \approx 2.03$. The latter g=2.03 component originates from the EPR signal of the

Notes to Table 1:
[a] X as an entry in the table indicates that the parameter was not specified in the original paper.
[b] In the cases of peroxyacetic acid (PAA) used as a peroxide (spectra U, AE), the pH values of protein solution before/after the treatment are indicated.
[c] Reaction time '0 min' indicates 'immediate', according to the authors, freezing after peroxide addition; style '30/60' means 30 s or 30/60=0.5 min.
[d] Recombinant protein.
[e] A 50 mM potassium phosphate buffer was used.
[f] This trace is a first derivative of the actually reported spectrum (see Fig. 1).
[g] The *haem* concentration is indicated.
[h] This trace is a result of subtraction of the spectrum of untreated C*c*O from that of the peroxide treated enzyme, at the same pH and temperature.
[i] This value is correct if equal volumes of protein and peroxide were mixed, like in a similar study by the same author performed in the same year [122].
[j] This value is correct if equal volumes of protein and ethyl hydroperoxide were mixed.
[k] Liquid He temperature range.
[l] This value is correct if equal volumes of protein and PAA were mixed (see i and j).

Table 2
Details of the protein/enzyme samples used to register the liquid phase EPR spectra shown in Fig. 4[a]

EPR spectrum	Protein	[protein] (μM)	Peroxide	[peroxide] (μM)	pH	T (°C)[b]	Reaction time (min)[c]	Radical's half-life time (s)	Reference
Myoglobin									
A	Sperm whale Mb	200	EtOOH	200	9.5	room	1.3/60[d]	X	[80]
B	Sperm whale Mb	200	EtOOH	200	7.0	room	0.63/60[d]	X	[80]
C	Sperm whale Mb	200	EtOOH	200	7.0	room	Later time[e]	X	[80]
D	Horse heart Mb	200	EtOOH	200	8.0	room	1.7/60[d]	X	[80]
E	Horse heart Mb	1000	H_2O_2	1000	7.4	22	0[f]	30	[58]
F	Horse skeletal muscle Mb	250	H_2O_2	2500	7.4	room	X[d]	X	[85]
G	Horse Mb	160	H_2O_2	200	7.4	20	0[f]	30	[165]
H	Horse Mb (N_2-saturated)	500	H_2O_2 (N_2-saturated)	500	X	room	3/60[d]	X	[81]
I	Horse Mb (N_2-saturated)	500	H_2O_2 (N_2-saturated)	500	X	room	50/60[d]	X	[81]
J	Horse Mb (N_2-saturated)	500	H_2O_2 (N_2-saturated)	500	X	room	400/60[d]	X	[81]
K	Horse Mb (O_2-saturated)	500	H_2O_2 (O_2-saturated)	500	X	room	3/60[d]	X	[81]
L	Horse Mb (O_2-saturated)	500	H_2O_2 (O_2-saturated)	500	X	room	50/60[d]	X	[81]
M	Horse Mb (O_2-saturated)	500	H_2O_2 (O_2-saturated)	500	X	room	400/60[d]	X	[81]
N	Horse heart Mb (incubated in N_2)	3000	tBuOOH (incubated in N_2)	12000	7.4	room	X[f]	X	[103]
Haemoglobin									
O	Human Hb	103	H_2O_2	25000	X	17	X[d]	X	[14]
P	Human Hb	710[g]	H_2O_2	7100	8.0	room	10/60[f]	15	[39]
Q	Bovine Hb	375	H_2O_2	1500	7.4	22	0[f]	50	[79]
Leghaemoglobin									
R	Soybean Lb	250	H_2O_2	250	7.4	room	0[f]	40	[76]

[a] X as an entry in the table indicates that the parameter was not specified in the original paper.
[b] In contrast to Table 1, only one temperature is indicated in this table since it is always the case in the liquid phase that $T_{reaction}=T_{detection}$.
[c] Reaction time '0 min' indicates 'immediate', according to the authors, registration after mixing; style '1.3/60' means 1.3 s or 1.3/60=0.0217 min.
[d] Continuous flow method of detection was used.
[e] The spectrum was detected by the continuous flow method after the flow was stopped.
[f] Stopped flow method of detection was used.
[g] The *haem* concentration is indicated.

peroxyl radical ROO• [35]. The lineshape of the peroxyl radical EPR spectrum by itself is well known from low temperature EPR spectroscopic studies of chemical systems [49–53]. It consists of the g=2.03 component and two other components, often poorly resolved at the g-factor region of ~2.005. The radical is thus characterised by an almost axial symmetry with $g_{||}$=2.03 and $g_{\perp} \approx 2.005$. Spectra C, D (HH Mb) and J (SW Mb) in Fig. 3 represent the cases of practically pure peroxyl radical EPR spectra. The fact that other Mb and Hb spectra have a different lineshape of the central feature at g=2.00 indicates the presence of non-peroxyl radicals admixed with the peroxyl radicals.

Peroxyl radicals are formed when molecular oxygen reacts with a radical. This reaction in the Mb(Hb)/H_2O_2 systems was first suggested as early as 1967 when the measurement of O_2 evolution and consumption in the system allowed the authors to conclude that "oxygenation of free radicals occurs to some extend" [9]. Indeed, the EPR spectrum reported in that work clearly shows the 2.03 component (Fig. 3, B) which we know now originates from the peroxyl type radicals. But that fact was not known at the time, and the 2.03 component in spectrum B (Fig. 3) was assigned to a sulfur radical [9] or, as in another work (spectrum A in Fig. 3), to the radical intermediates •OH or •OOH [11]. It wasn't until 1989 that the line at $g \approx 2.03$ in the spectrum of metMb treated with H_2O_2 (Fig. 3, H) was attributed to a peroxyl radical for the first time [54]. However, the definitive proof of the peroxyl radical formation in the haem protein/peroxide systems has come from the spin trapping and isotopic labelling studies.

Spin trap adducts of radicals are more stable than the primary radicals, although the interpretation of their EPR spectra is more difficult and is not always free from ambiguities. A technique to spin trap a peroxyl radical in hematin/peroxide system with 5,5-dimethyl-l-pyrroline *N*-oxide (DMPO) was suggested in 1983 [55]. Peroxyl radicals (as well as alkoxyl radicals) were reported to be spin trapped by DMPO in a number of systems containing *tert*-butyl or

cumene hydroperoxides (tBuOOH and CuOOH) and several haem proteins including Hb and Mb [56]. It had been suggested that the trapped radical is a peroxyl radical derived from molecular oxygen that had reacted with a radical located on a tyrosine (Tyr) residue (tyrosyl radical) [57,58]. This interpretation was contested in [59]. The authors showed that the spectrum of the trapped species is exactly the same for anoxic conditions and when the solutions were oxygenated with either $^{16}O_2$ or $^{17}O_2$. Thus the radical in the metMb/peroxide system that can be trapped by DMPO cannot be a peroxyl radical [59]. It was later shown that DMPO traps a tyrosyl radical at position 103 in the SW metMb/H_2O_2 system (Section 5.2.2.3) [60]. The same group of authors later demonstrated that a peroxyl radical was indeed formed in the HH metMb/H_2O_2 system. A low temperature EPR spectrum typical for a peroxyl radical (Fig. 3, D) was observed. The spectrum was dependent on the presence of O_2 and showed additional hyperfine splitting when molecular oxygen isotopically labelled with ^{17}O was used [36]. But this peroxyl radical was not the radical that can be trapped by DMPO. Employment of another spin trap, 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS), in combination with protease treatment of the protein reacted with peroxide was used to demonstrate that the spin trap adduct of the protein radical is likely to be identical to the adduct of a radical formed on free tryptophan (Trp) [61]. Although a conflicting report showed that it is a tyrosyl radical that is spin trapped under these conditions [62], the controversy was resolved when the type of the radical trapped was shown to be dependent on the molar excess of H_2O_2 [63]. The simulation of the detected EPR lineshape, that employed the hyperfine splitting constants consistent with Trp structure, has shown that the most likely site of O_2 addition to Trp is atom C3 of the indole ring [61].

To test the suggestion that the peroxyl radical is located on a Trp residue, a recombinant SW Mb was used to prepare site directed mutants in which two Trp residues of this protein, Trp7 and Trp14, were either labelled with ^{13}C (I=1/2) at the indole ring atom C3 or replaced singly or doubly with phenylalanine [64]. The EPR spectrum of the peroxyl radical, characteristic of the wild type SW metMb treated with H_2O_2 (Fig. 3, J), showed a hyperfine splitting on a nucleus with I=1/2, thus confirming that the peroxyl radical in this protein is formed when O_2 is attached to a Trp radical at atom C3, and the experiment with phenylalanine variants unequivocally pointed to Trp14 as the site of the peroxyl radical [64].

The peroxyl radical in HH Mb has identical spectroscopic characteristics with the peroxyl radical in SW Mb, both radicals also showing similar kinetic behaviour [35]. It has been therefore assumed that the HH Mb peroxyl radical is located on the same Trp14 as in the SW protein. The low temperature spectrum of the human metMb/H_2O_2 system (Fig. 3, L), that can be detected immediately after peroxide addition, also shows a peroxyl radical signal. This signal has also been attributed to the Trp14 peroxyl radical on the basis of similar g-factors in the HH and human Mb peroxyl radical spectra [65]. The human metHb/H_2O_2 system, when studied by the low temperature EPR spectroscopy, is characterised by two types of peroxyl radicals with slightly different spectroscopic characteristics ($g_{||}$=2.035 for ROO•-I and $g_{||}$=2.032 for ROO•-II) and markedly different kinetic and pH dependences [35]. The time dependences for ROO•-I and ROO•-II in the human metHb/H_2O_2 system, as well as the dependences for peroxyl radical in the HH and SW metMb/H_2O_2 systems, determined in the pH range of 6–8, are reported in Fig. 4 of the paper [35]. Both the g-factors and kinetic behaviour of ROO•-I matched those of the peroxyl radical observed in two myoglobins, therefore this isoform of human Hb (HbA) peroxyl radical was assigned to the same Trp site (Trp14 in the α-subunit and/or Trp15 in the β-subunit). The site of the other isoform (ROO•-II) was speculated to be on Trp37 of the β-subunit of HbA, the residue missing in the α-subunit and in other myoglobins [35]. However, in light of the studies of the human Mb/H_2O_2 system [65,66], the ROO•-II isoform can be re-assigned. A cysteine (Cys110) radical has been identified in human Mb with a kinetic behaviour similar to that of ROO•-II in human Hb. A cysteine radical can react with molecular oxygen to form peroxyl radical (CysSOO•) with typical peroxyl radical EPR signal [67,68]. Therefore it is possible, as suggested in [69], that the EPR line at g=2.036 that appears after the decay of Trp14-OO• in the human Mb/H_2O_2 system originates from a cysteine peroxyl radical located on Cys110. Similarly, a possibility exists that ROO•-II in the metHbA/H_2O_2 system originates from a cysteine peroxyl radical formed on one of these residues of HbA (Cys104 in two α- and Cys93 and Cys112 in two β-subunits). Recently, a radical on Cys93 of the β-subunit has been spin trapped with DMPO [70].

5.2. Non-peroxyl radicals

The radicals that can be directly detected in myoglobins and haemoglobins treated with peroxides, and which are not peroxyl radicals, are almost exclusively reported to be tyrosyl radicals. In fact, there is only one report, to the best of our knowledge, of a direct detection of a cysteinyl radical in the human Mb/H_2O_2 system [65], which we believe could actually be a peroxyl radical formed after the cysteinyl radical had reacted with O_2. It is unlikely to be a fixed rule, but practically all lineshapes of the non-peroxyl radicals directly detected so far at room and low temperatures are found to be from different tyrosyl radicals.

5.2.1. Tyrosyl radical EPR spectra, general information

The room temperature EPR spectra presented in Fig. 4 can be generally described as typical EPR signals of carbon centred radicals with effective g-factor at ≈2.005, overall linewidth in the range of 18–38 G, and with protein-specific hyperfine structures. The low temperature spectra of the

same protein systems (Fig. 3) contain the signals from the same radicals but the hyperfine structure of the signals might be slightly different or, due to apparent broadening of components, can be effectively lost.

The tyrosyl radical in proteins is characterised by EPR signals of extremely variable lineshape [71]. This variability is traditionally associated with the ability of the phenol group in tyrosine to rotate around the C_β–C1 bond (Fig. 5). The possibility to explain different EPR lineshapes of tyrosyl radicals in proteins by implying different rotation angle of the phenoxyl group in the radicals has been first demonstrated for prostaglandin H synthase in [72]. The EPR spectrum of a tyrosyl radical is defined by the hyperfine interaction of the unpaired electron mostly delocalised over the ring's π -system with the four ring protons (the oxygen proton is lost in the radical) and with the β-protons of the methylene group. The hyperfine interaction with the β-protons depends on the rotation angle θ (Fig. 5) according to the semi-empirical McConnell relation [73]:

$$A_{\mathrm{iso}}^{\beta} = \rho_{C1}\left(B' + B''\cos^2\theta\right) \tag{6}$$

where A_{iso}^{β} is the isotropic hyperfine splitting constant for a methylene β-proton; ρ_{C1} is the spin density on atom C1; B' and B'' are constants, B' being commonly neglected in practical applications and B'' being equal to 58 G [74]. Thus, tyrosyl radicals with different rotational conformations of the phenoxyl ring will have EPR spectra exhibiting different hyperfine structures. The A_{iso}^{β}-values for both β-protons can be determined via a simulation of the experimental X-band EPR spectrum. The two unknowns, ρ_{C1} and θ , can then be found from a system of two Eq. (6), written for the two protons:

$$\left.\begin{aligned} A_{\mathrm{iso}}^{\beta 1} &= \rho_{C1}B''\cos^2\theta \\ A_{\mathrm{iso}}^{\beta 2} &= \rho_{C1}B''\cos^2(\theta - 120^\circ) \end{aligned}\right\} \tag{7}$$

It is important to emphasise that system (7) always has *four* solutions (see Fig. 4 in [75]): a pair of θ-values for one value of ρ_{C1} and another pair of angles θ for the second value of ρ_{C1}. Each pair of θ-values is symmetrical with respect to either -30° or 60°, depending on the value of ρ_{C1}, so that $(\theta_1+\theta_2)/2=-30^\circ$ and $(\theta_3+\theta_4)/2=60^\circ$ (note that these θ-values are not for the pair of methylene protons; θ has been defined for one particular methylene proton $H_{\beta 1}$ (Fig. 5), the angle for the other proton $H_{\beta 2}$ is always $\theta-120^\circ$) [75]. Only one of two possible ρ_{C1}-values will be a characteristic of the tyrosyl radical, the spectrum of which has been simulated, therefore the other value (and two θ-values associated with it) should be discarded. Knowing the value of θ for a tyrosyl radical is important for the identification of the responsible tyrosine residue, because this angle can be directly measured for any tyrosine, if the 3-dimensional structure of the protein is known. One simple, though important consideration which seems to be repeatedly absent in publications reporting the simulated spectra of tyrosyl radicals is the following. Simulation alone does not allow knowing which of the two remaining θ-values, obtained as solutions of system (7), is actually the angle in the radical, since both angles define exactly the same pair of values for $A_{\mathrm{iso}}^{\beta 1}$ and $A_{\mathrm{iso}}^{\beta 2}$. Therefore, in looking in a 3-dimensional structure of a protein for the tyrosine residue that would have the rotational conformation consistent with the experimentally observed spectrum, one should compare the rotational conformation of the tyrosines with both (not just one) θ-angles found as solutions of system (7).

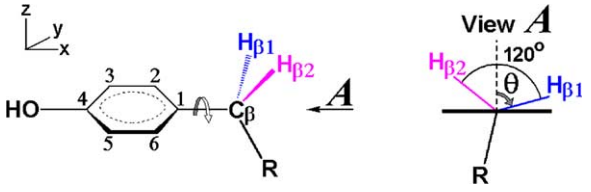


Fig. 5. The ring in tyrosine can rotate around the C_β–C1 bond. The rotation angle can be defined in a number of ways. Here the rotation angle θ is defined for view A. It is the angle for proton β1; it is positive when measured clockwise from the upward perpendicular to the ring plane when the C_β–R bond is below the horizontally oriented ring plane. Angle θ is defined on a 180° interval (−90°–+90°) and covers the whole 360° range of the ring rotation.

Recently, an algorithm has been suggested for computer simulation of tyrosyl radical EPR spectra [71]. The semi-empirical formulae used in this algorithm, termed in this review Tyrosyl Radical Spectra Simulation Algorithm (TRSSA), allow the calculation of all the parameters necessary for simulation of a tyrosyl radical EPR spectrum, when only two input variables are used: the spin density on atom C1 of a tyrosyl radical (ρ_{C1}), and the rotation angle of the phenoxyl ring θ (Fig. 5). TRSSA has been justified by the simulation of all known EPR lineshapes of tyrosyl radicals (both X-band and high frequency (HF) spectra of 7 radicals, plus X-band spectra only of other 4 radicals) [71]. Therefore, if a spectrum cannot be simulated by employing TRSSA (and that is pretty easy to find out having only two degrees of freedom in varying the simulation parameters), the radical responsible for the spectrum is unlikely to be on a tyrosine residue. According to TRSSA, 18 of the 30 simulation parameters are constants for all known tyrosyl radicals. In contrast, the other 12 parameters are very sensitive to the two input variables, so that a slight variation in ρ_{C1} or θ results in a noticeable change of the simulated lineshape. Thus, if a simulation performed with TRSSA results in a lineshape which is very close to the experimental spectrum, the method yields highly accurate values of the spin density ρ_{C1} (0.002–0.004) and the rotation angle θ (2–3°). Once the two possible rotation angles in the radical are determined, all tyrosine residues in the protein can be screened for the same or close rotation angle of the phenol ring by using the on-line database of rotational angles of phenol ring in different tyrosine residues in different proteins (Phenol Ring Rotation Angle database, PRRAD, http://privatewww.essex.ac.uk/~svist/lev1/tyrdb/home.shtml). It is possible with the use of the

database to rank all tyrosine residues by the closeness of their rotation angles to the value found from the EPR spectrum simulation. Examples of the application of TRSSA and PRRAD are presented below (Sections 5.2.2.1, 5.2.2.2, 6.4.3 and 6.4.4).

5.2.2. Tyrosyl radicals in haemoglobins and myoglobins

5.2.2.1. Soybean leghaemoglobin. Leghaemoglobin (Lb) is an oxygen-transporting monomeric haem protein found in the root nodules of leguminous plants. It has some essential similarity in sequence and structure with mammalian myoglobins. MetLb, like other haem proteins in the oxidized form, reacts with H_2O_2 with formation of a globin radical. The room temperature EPR spectrum of the radical consists of five components (Fig. 4, R) that were attributed to the hyperfine interaction of an unpaired electron with the protons in a tyrosyl radical [76]. Tyr133 has been suggested to be the site of the radical as the tyrosine most close to the haem [76]. Indeed, this spectrum has been successfully simulated using TRSSA, and the rotation angle θ was found to be either $45\pm3^\circ$ or $75\pm3^\circ$ [71]. There are three tyrosines in Lb in positions 25, 30 and 133 [77]. The analysis of the angle of phenol ring rotation in these tyrosines, performed with the use of PRRAD, is summarised in Table 3. The table also shows how close all these angles are to either 45° or 75°.

Clearly, Tyr133 is characterised by the value of the ring rotation angle most close to one of the two predicted values (45°). Thus Tyr133 is most likely to be site of the radical in the metLb/H_2O_2 system.

5.2.2.2. Mammalian haemoglobin. The yield of globin based free radicals in the metHb/H_2O_2 system is probably the highest among all other haem proteins [35,39]. Because of this fact, the non-peroxyl radicals are easily detectable in Hb in both liquid phase (Fig. 4, O, P, Q) and low temperature experiments (Fig. 3, M, N, O). The lineshape with a specific hyperfine structure of the room temperature spectra (Fig. 4, O, P, Q) is very close to that of the tyrosyl radical in Photosystem II [78]. When the same radical is studied in the frozen state, the hyperfine structure of the EPR spectrum is effectively lost [39], and a singlet-like signal is detected (Fig. 3, M, N, O). The simulation of the liquid phase spectrum of the metHbA/H_2O_2 system using TRSSA [71] supports the previous assignment to a tyrosyl radical [79]. The simulation also provides us with two possible values of rotation angle θ of the phenoxyl group in the radical: it should be either $53\pm2^\circ$ or $67\pm2^\circ$ [71]. Table 4 shows that when the residues are ranked by the closeness of the angle averaged over identical subunits to either 53° or 67°, Tyr42 in the α-subunits seems to be the most probable candidate residue to host the radical (with θ=53°) in the metHbA/H_2O_2 system. The αTyr42 residue has been suggested before to be the site of the radical formation in the bovine metHb/H_2O_2 system on the basis of its proximity to both haem groups in the α- and β-subunits [79].

Table 3
Rotation angle θ of the phenol ring in Tyr25, Tyr30 and Tyr133 of two chains (A and B) of soybean Lb[a]

Tyrosine	θ°	|θ−45|°	|θ−75|°
B Tyr133	51.38	**6.4**	23.6
A Tyr133	52.72	**7.7**	22.3
A Tyr30	29.14	15.9	45.9
B Tyr30	28.25	16.8	46.8
B Tyr25	14.27	30.7	60.7
A Tyr25	2.64	42.4	72.4

[a] Protein Data Bank (PDB, http://www.rcsb.org/pdb/) structure file 1BIN [77] was explored. The table is sorted by ascending |θ−45|°.

Table 4
Ranking of tyrosine residues in human Hb by closeness of the phenol ring rotation angle, averaged over identical chains, to either 53° or 67°[a]

Tyrosine	$\theta_{average}$°	$\lvert\theta_{average}-53\rvert$°	$\theta_{average}-67\rvert$°
Tyr42(α)	56.5	**3.5**	10.5
Tyr130(β)	45.2	7.8	21.8
Tyr35(β)	44.2	8.8	22.8
Tyr145(β)	85.9	32.9	18.9
Tyr24(α)	−38.0	91.0	105.0
Tyr140(α)	−81.9	134.9	148.9

[a] PDB structure file 1HGB [166] was explored. The table is sorted by ascending $|\theta_{average}-53|$°.

Recently, a combination of analytical methods including mass-spectrometry and immuno-spin trapping has been used to identify the free radical products of the reaction of human metHb with H_2O_2 [70]. A radical on Tyr42 of the α-subunit has been found. Also the radical on Cys93 of the β-subunit has been spin trapped by DMPO. A new radical species that is a shared radical between neighbouring Tyr24 and His20 of the α-subunit has also been reported [70].

5.2.2.3. Sperm whale myoglobin. The low temperature EPR spectra of the SW metMb/H_2O_2 system (Fig. 3, G–K) are usually[3] characterised by a superposition of the peroxyl radical signal from Trp14-OO• [64] and a singlet with spectroscopic characteristics and kinetic behaviour similar to the singlet in HbA, assigned to a tyrosyl radical [39]. Like in HbA, the low temperature singlet in SW Mb becomes a resolved five-component spectrum of the same overall width when the liquid phase EPR method is used to study SW metMb mixed with ethyl hydroperoxide (EtOOH) (Fig. 4, A). The five component spectrum has been assigned to a tyrosyl radical located on Tyr151 [80]. A simulation of this quintet signal as a tyrosyl radical EPR spectrum has been performed for a rotation angle of the phenoxyl ring that was consistent with the rotational conformation of Tyr151 of SW Mb [39]. The formation of a phenoxyl radical Tyr151• in such a system has been demonstrated by selective mutation

[3] Spectrum G in Fig. 3 is notably different from the other SW metMb spectra (H–K) probably because of a rather high protein concentration used and a high temperature of detection (see Table 1). The most puzzling is the position of the maximum peak of doublet G at g~2.02: it doesn't fit to any signal detected in other systems.

of tyrosines in recombinant SW Mb and use of the spin trap 2-methyl-2-nitrosopropane (MNP) [60].

Tyr151• radical is not the only tyrosyl radical formed in SW metMb under peroxide treatment. There are three tyrosine residues in SW Mb, numbered 103, 146 and 151. In addition to the ability of MNP to trap the Tyr151• radical, DMPO, MNP and DBNBS were all shown to be able to trap specifically the Tyr103• radical [60,62]. Tyr103 in both HH and SW myoglobins has a rather unusual rotational conformation of the phenol ring. The ring is practically in the R-C_β-C1 plane, the situation corresponding to angle θ being close to −30° (see Fig. 5). The EPR spectrum of a Tyr103• radical consists of seven lines and has an overall width of ~38 G [71,81]. This septet EPR signal, overlaid with the Tyr151• quintet, was observed under certain conditions in the room temperature SW metMb/EtOOH spectra (Fig. 4, B) [80], also indicating that both Tyr151• and Tyr103• are formed in this system.

At a later time of reaction of SW metMb with EtOOH, a radical with a doublet EPR signal, also typical for tyrosyl radicals, is formed (Fig. 4, C). This radical might originate from Tyr146• (Section 5.2.2.4) formed in an intermolecular reaction (Section 5.3.3).

5.2.2.4. Horse myoglobin. There are two tyrosines (Tyr103 and Tyr146) in HH Mb, the third tyrosine of SW Mb being replaced by Phe151. The liquid phase EPR spectra recorded immediately after mixing of horse metMb with peroxides are characterised by a strong septet (Fig. 4, D–H, K) from the Tyr103• phenoxyl radical, better detectable at pH 8 rather than at lower values [80]. The initial yield of Tyr103• radical is at least one order of magnitude higher in HH Mb than in SW Mb (see Fig. 1 in [80]). The assignment of the septet to the Tyr103• radical was first made on the basis of its simulation employing an isotropic lineshape method (less accurate) [81] and then confirmed by the simulation performed with TRSSA [71]. This assignment of the major spectroscopic feature of the HH metMb/peroxide spectra is in accordance with the finding that the Tyr103• radical can be spin trapped in this system by MNP [82] or by DBNBS [62] as has been demonstrated by the use of mass spectrometry alone [82], or in combination with EPR spectroscopy and electrophoretic purification [62].

The lineshape of the room temperature spectra of oxygen- and nitrogen-saturated reaction mixtures are different at the first time point (Fig. 4, H and K) due to the presence of the peroxyl radical (Trp14-OO•) signal in the oxygenated system [81]. The HH metMb/H_2O_2 system is the only biological system in which a peroxyl radical has been detected directly in the liquid phase. The spectra measured at the earliest time point can be deconvoluted into individual signals, and those signals can be used to measure the kinetic dependences of the species (Fig. 6). The pure lineshape of the peroxyl radicals signal extracted from the liquid phase spectra (Fig. 6, C) corresponds well to the difference spectrum obtained by the subtraction of the spectra of oxygen- and nitrogen-saturated HH metMb/H_2O_2 systems (without use of variable coefficient) [81]. The degree of g-tensor anisotropy ($g_{||}$=2.023; $g_\perp$=2.013) (Fig. 6, C) is significantly less than in the frozen state (g_1=2.0366; g_2=2.0082; g_3=2.0028) [35] although the average g-factor is practically the same: $g_{\text{average, 20 °C}}$=(2.023+2×2.013)/3=2.016; $g_{\text{average, 10 K}}$=(2.0366+2.0082+2.0028)/3=2.0159. The fact that the room temperature ROO• signal is not completely isotropic indicates that the radical is bound to the protein molecule that tumbles slowly in the solution (partial immobilisation). The N_2 saturated HH metMb/H_2O_2 system was not completely peroxyl radical free (Fig. 6, B). The same conclusion can be made when analysing the spectra published earlier for this system [81]. This effect is likely to be due to a residual O_2 concentration in the solution, although another possibility is that a small amount of O_2 is evolving during the reaction of metMb with H_2O_2 [8].

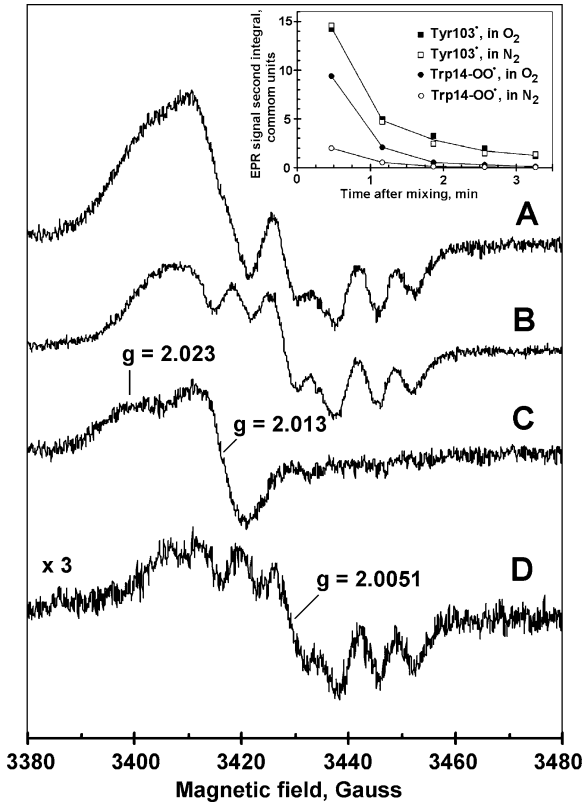


Fig. 6. The EPR signals of the Trp14 peroxyl radical and Tyr103 phenoxyl radical in the HH metMb/H_2O_2 system at room temperature (20 °C). (A) The EPR spectrum taken 30 s after mixing of 0.75 mM HH metMb and 0.75 mM H_2O_2 at pH 8 and oxygenated conditions. (B) The same as panel A, but solutions were nitrogen saturated. (C) The peroxyl radical signal obtained as the difference spectrum C=A−1.026×B (the coefficient of 1.026 was chosen to eliminate the septet components from the difference spectrum). (D) The same as panel B, but the spectrum was measured 70 s after mixing and multiplied by 3 for illustrative purpose. Inset, the kinetic dependences of the Trp14-OO• and Tyr103• radicals.

As the reaction progresses, the peroxyl radical decays and the septet is transformed to a doublet-like signal (Fig. 4, J and M) attributable to a tyrosyl radical spectrum [81]. A similar signal was also detected at later time points during the reaction of SW metMb with EtOOH (Fig. 4, C) [80]. One possibility to explain this result would be the formation of a tyrosyl radical on the other available tyrosine, Tyr146, during the time of Tyr103• decay.[4] Tyr146 in HH Mb has a θ angle of -57.5° (entry 1WLA in PRRAD) which means that one of the methylene protons is located only 2.5° away from the perpendicular to the ring's plane (see Fig. 5 and second Eq. in (7), $-57.5^\circ - 120^\circ \approx -180^\circ$). Such rotational conformation is typical for the tyrosyl radicals in the *E. coli* and mammalian ribonucleotide reductases, known to have doublet-like EPR spectra [71]. A different possibility to explain the double-like signal (Fig. 4, J and M) has been favoured in [81]. The authors concluded that the septet-to-doublet transformation is associated with a conformational change in the same Tyr103• radical during the reaction, since no radicals on Tyr146 could be spin trapped [60]. These two interpretations are considered below in Section 5.3.3.

The low temperature EPR spectra of the HH metMb/H_2O_2 system are dominated by the peroxyl radical signal (Fig. 3, A–F). Under certain conditions a multi-component structure can be seen on both sides of the perpendicular component of the peroxyl radical signal (Fig. 2, spectrum '1.5 min'; Fig. 3, E). This structure was thought to originate from the same septet signal as the one detectable at room temperature [37] and later proved to be the signal of Tyr103• [71,81]. The conjecture that the same septet is present in both liquid phase and low temperature spectra might have been wrong since two examples are known of tyrosyl radicals that have signals with resolved hyperfine structure in the liquid phase but which are turning into unresolved (or poorly resolved) singlets once the protein sample is frozen. These examples are: the HbA tyrosyl radical (probably αTyr42•) (Fig. 4, O, P, vs. Fig. 3, N, O) and the SW Mb radical on Tyr151 (Fig. 4, A, vs. Fig. 3, K).

By using the procedure of spectral subtraction with variable coefficient [39], it is possible to obtain a pure lineshape of the low temperature multi-component EPR signal (Fig. 7). Two sets of low temperature EPR spectra of the HH metMb/H_2O_2 system at pH 6 and pH 7 have been recorded for various time points after peroxide addition (spectra A–D and E–F, respectively). These spectra have been used in the spectral subtraction procedure as follows.

First, the pure lineshape of the peroxyl radical signals at pH 6 and pH 7 were composed (the EPR spectra are given in a bold font, style (**pH 6, 0.37 min**) designates the spectrum

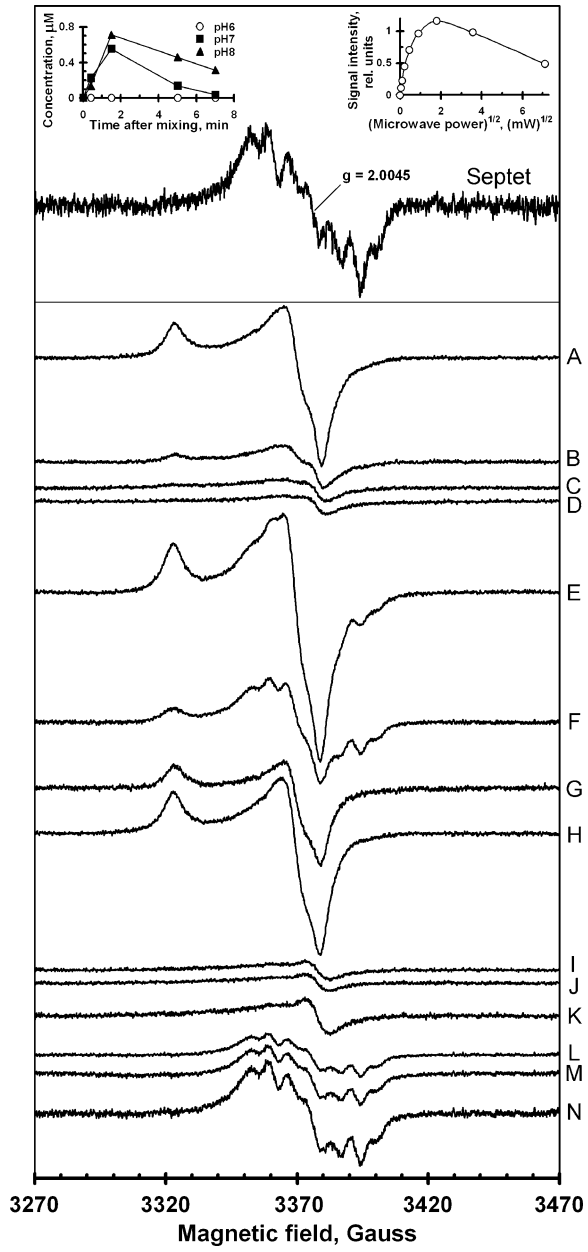


Fig. 7. Top: the seven component EPR signal responsible for the multi-comoponent structure in the low temperature spectra of HH metMb/H_2O_2. The insets show the kinetic dependences of the radical responsible for the low temperature septet at three pH values and the microwave power saturation curve for the septet measured in the 1.5 min sample of the pH 8 series. Below: the EPR spectra (A–N) used to obtain the septet in the procedure of spectral subtraction with variable coefficient: A=(pH 6, 0.37 min); B=(pH 6, 1.50 min); C=(pH 6, 5.00 min); D=(pH 6, 7.00 min); E=(pH 7, 0.42 min); F=(pH 7, 1.50 min); G=A−1.38 B; H=E−0.73 F; I=C−0.08 G; J=D−0.06 G; K=I+J; L=E−1.245 H; M=F−0.324 H; N=L+M; Septet=2(N−1.38 K). The details of the spectral subtraction are given in the text.

of the sample frozen 0.37 min after initiation of the reaction at pH 6:

G=(**pH 6, 0.37 min**)−1.38 (**pH 6, 1.5 min**)=**A**−1.38 **B** (a pure ROO• signal at pH 6);

[4] This signal in SW Mb, if it is indeed from a tyrosyl radical, should also be attributed to Tyr146, since the radicals on other two tyrosines in this protein, Tyr103 and Tyr151, have different EPR signals: the septet and the quintet respectively.

H=(**pH 7, 0.42 min**)−0.73 (**pH 7, 1.5 min**)=**E**−0. 73 **F** (a pure ROO• signal at pH 7).

Second, the pure ROO• signal at pH 6 (G) was subtracted from two spectra at pH 6:

I=(**pH 6, 5 min**)−0.08 **G**=**C**−0.08 **G** (a pH 6 spectrum at 5 min, free from a ROO• signal);
J=(**pH 6, 7 min**)−0.06 **G**=**D**−0.06 **G** (a pH 6 spectrum at 7 min, free from a ROO• signal).

Third, a similar ROO• signal elimination was performed for two pH 7 spectra (but using this time the pH 7 ROO• pure lineshape, **H**):

L=(**pH 7, 0.42 min**)−1.245 **H**=**E**−1.245 **H** (a pH 7 spectrum at 0.42 min, free from a ROO• signal);
M=(**pH 7, 1.5 min**)−0.324 **H**=**F**−0.324 **H** (a pH 7 spectrum at 1.5 min, free from a ROO• signal).

Finally, the two pH 6 spectra (**I** and **J**) and two pH 7 spectra (**L** and **M**) were respectively averaged (effectively added without division by 2). The two resultant spectra are free from the ROO• signals and contain the septet in different relative amounts: There is a substantial amount of septet in the pH 7 average (**N**=**L**+**M**) and the input of the septet is next to nothing in the pH 6 average (**K**=**I**+**J**). These two average spectra were subtracted, and the variable coefficient was chosen (1.38) to minimise the non-septet signals in the difference spectrum:

Septet=**N**−1.38 **K** (the spectrum shown on the top of Fig. 7 with a magnification factor 2).

A comparison of the septet extracted from the low temperature spectra of the HH metMb/H_2O_2 system (Fig. 7) with the liquid phase septet detected in this system at room temperature (Fig. 6, D) suggests that it is the same EPR signal (Fig. 8). Thus, radical Tyr103• is detectable at both room and low temperature in HH Mb, and unlike Tyr151• in SW Mb and the tyrosyl radical in HbA (probably αTyr42•), the hyperfine structure is preserved in the low temperature spectra.

It has been reported that a septet EPR signal is also a characteristic of the bovine oxyhaemoglobin/H_2O_2 system at room temperature [83]. This is however an unfortunate mistake, as the reported spectrum is exactly the same, including the noise trace, as the spectrum already reported before for the horse metMb/H_2O_2 system in two publications [84,85].

5.2.2.5. Human myoglobin. The low temperature EPR spectrum of human Mb (Fig. 3, L) detected in the sample frozen immediately after mixing with H_2O_2 comprises a signal from the peroxyl radical (presumably Trp14-OO•) and a central EPR line from a different radical. Upon the incubation of the reaction mixture, the peroxyl radical disappeared, but a new feature with a component at g=2.036 (i.e. the same as $g_{||}$ of the peroxyl radical) emerged with a concomitant increase and broadening of the central line [65]. The secondary radical with g=2.036 was attributed to the formation of a thiyl radical on the only cysteine residue in human Mb, Cys110 (there are no cysteines in SW or HH myoglobins). The broadening of the central line was interpreted as the formation of a tyrosyl radical [65].

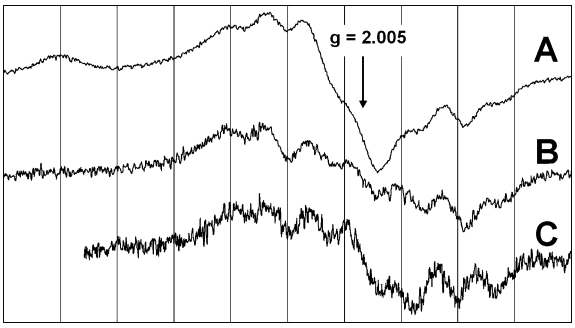


Fig. 8. A comparison of the septet EPR signals detected in the HH metMb/H_2O_2 system at 10 K and at room temperature. (A) The EPR spectrum measured at 10 K of the sample of 100 μM HH metMb, pH 7, frozen 1.5 min after the addition of 100 μM H_2O_2—the components of the septet are clearly seen. (B) The pure lineshape of the septet (the same as in Fig. 7); C, the septet directly measured at room temperature (signal D of Fig. 6). The distance between the gridlines is 10 G.

Variable molar excess of DMPO was used to successfully spin-trap two different radicals identified by the hyperfine splitting of the spin-adducts as a tyrosyl radical (presumably on **Tyr103**) and a thiyl radical on Cys110 [65,66]. The results of radical trapping with MNP were interpreted as consistent with presence of a tyrosyl radical in the human Mb/H_2O_2 system [66]. The presence of Tyr103 in the protein was essential for the formation of the thiyl radical on Cys110 [66]. The cysteine thiyl radical was shown to be responsible for the formation of the Mb homodimers through establishing of a disulfide bond between two Mb molecules [65,66]. The experiments that involved the Tyr103Phe and Cys110Ala variants of the wild type protein have allowed the authors to suggest that the radical they assigned to the Tyr103 phenoxyl radical is a precursor of Cys110•, and that the reduction of the tyrosyl radical by Cys110 occurs in an intermolecular process [66] (Section 5.3.3).

5.3. Radical formation and transformation

5.3.1. Wasserstoffsuperoxydmethämoglobhin (before a radical is formed)

Before the electron transfer occurs from the haem group to the peroxide, a complex between the two must be formed. It is the complex Kobert thought he had been dealing with in his study of the reaction between haemoglobin and hydrogen peroxide (Wasserstoffsuperoxydmethämoglobhin) [1]. This complex, we understand now, must be very short-lived

and difficult to detect. However, in the reaction of H_2O_2 with mutated SW metMb in which the distal histidine was replaced with a glutamine or a valine, effectively giving more room for an external ligand and changing the polarity of the side chain of the 64th residue, it was possible to freeze–quench a paramagnetic complex which was interpreted as an l.s. ferric haem with a H_2O_2 molecule as the distal ligand [86]. Although this was a provisional interpretation, it can be strongly supported by the works from a different field. The low temperature EPR studies of the products induced by ionizing radiation in oxyHb and oxyMb have revealed the formation of electron adducts to oxyHb which then undergo protonation [87–91]:

$$Fe^{2+} - O_2 + e^- \rightarrow Fe^{3+} - O_2^{2-} \quad (8)$$

$$Fe^{3+} - O_2^{2-} + H^+ \rightarrow Fe^{3+} - HO_2^- \quad (9)$$

The products of both reactions (8) and (9) are paramagnetic and have been characterised [87–89]. It is clear that these products are equivalent to doubly (8) or singly (9) deprotonated H_2O_2 molecule complexed with the haem in the ferric state. A comparison of the *g*-factors of complexes (8) and (9) with those of the l.s. forms reported in the His64Gln and His64Val variants of SW metMb reacting with H_2O_2 [86] certainly points to the singly deprotonated H_2O_2 as the distal ligand in metMb.

5.3.2. The primary radical and intramolecular radical transfer

The π-cation porphyrin radical [Fe^{4+}=O por$^{\bullet+}$] (Compound I) has a significantly shorter life-time in the oxygen binding proteins than in enzymes (Section 6.1) and is therefore more difficult to detect. This formal 5+ oxidation state of haem in Mb has been first characterised optically using flash photolysis [92] and then detected in SW and HH metmyoglobins reacting with H_2O_2 by stopped flow rapid-scan spectrophotometry in the ~100 ms range of the reaction time [93]. The distal histidine (His64) has been suggested as the residue critical for the low stability of Compound I in myoglobins since its replacement in SW Mb by a variety of different amino acid residues such as alanine, leucine, serine [94] and aspartic acid [95] allowed the detection of Compound I over a much longer period (~1 s range), when the mutant proteins were reacting with *m*-chloroperbenzoic acid [94,95]. The site of the primary detectable radical has not been recognized in full certainty in any of the proteins studied. Tyrosine, tryptophan and cysteine are the most likely residues to form the radical as they have the lowest one-electron oxidation potential of all the amino acid residues normally incorporated into proteins [69]. Fig. 9 shows the position of these residues in the oxygen binding proteins for which the reaction with peroxides has been studied.

Many agree that the site of the primary radical in myoglobins might be Trp14. The peroxyl radical on Trp14 is the earliest detectable species in the HH metMb/H_2O_2 system; also it disappears faster than the other radicals [96]. In contrast, at the early stage of metHbA reaction with H_2O_2, a tyrosyl radical is formed at the highest concentration and disappears faster than the peroxyl radical [35,39]. This means the tyrosyl radical is likely to be the primary radical in Hb. In the SW metMb/EtOOH system, a mixture of tyrosyl radicals on Tyr151 and Tyr103 is detected as early as ~1 s after the initiation of the reaction (Fig. 4, A and B). At later time of reaction (~1 min), Tyr151• becomes the major component [39]. If Tyr103 is replaced with phenylalanine, the yield of Tyr151• is decreased by almost an order of magnitude [39]. These results mark Tyr103 as the primary site of radical formation with respect to Tyr151 [39]. However, a radical on Tyr151 apparently can still be formed without the participation of Tyr103 as the Mb dimers cross-linked via Tyr151 can be detected in the protein variant lacking Tyr103 [97]. When all three tyrosine residues, Tyr103, Tyr151 and Tyr146, are replaced with phenylalanine in SW Mb, a reaction with H_2O_2 still results in a peroxyl radical formation (as can be concluded from the typical peroxyl radical spectra published in [97], even though the radical has not been recognized as such), which is probably the same radical as in the wild type protein, i.e. Trp14-OO•. This result means that a tryptophanyl radical on Trp14 can be formed without any participation of tyrosine residues.

The primary radical formed by the subtraction of an electron from a neutral Trp or Tyr residue is a cation radical. It must be deprotonated in order to become either a neutral tyrosyl radical, detectable in the direct EPR experiments, or the neutral tryptophanyl radical that reacts with O_2 to form a peroxyl radical. It is possible that the deprotonation of the residues takes place simultaneously with the electron transfer (concerted or proton-coupled electron transfer [98]). This mechanism is likely to be important in the situations where the oxidant is located in close proximity to the group to be oxidized and where the oxidant is accepting the proton as well as the electron. In the case of a relatively long distance transfer, for example between the haem and a residue a few angstroms away, a proton-coupled electron transfer must involve a third molecule or group that would accept the proton. Unless such an H^+−accepting group is present in close proximity to a tyrosine or a tryptophan, it is likely that the deprotonation takes place after the cation radical has been formed. The charged cation radical can be very fast transferred to another tryptophan or tyrosine residue. The process of such radical transfer must be slowed down (or completely halted) if at some stage of this residue-to-residue cation radical transfer a radical in the chain happens to get deprotonated. (A neutral radical is likely to be a less potent oxidant towards neighbouring residues than a positively charged radical.) The chains of amino acid residues that are capable of 'conducting' a free radical state over long distances have been identified in ribonucleotide reductases and DNA photolyases [99–101]. Photolyase repairs UV-induced cyclobutane pyrimidine dimers in DNA by using photoinduced electron transfer. The enzyme contains a non-

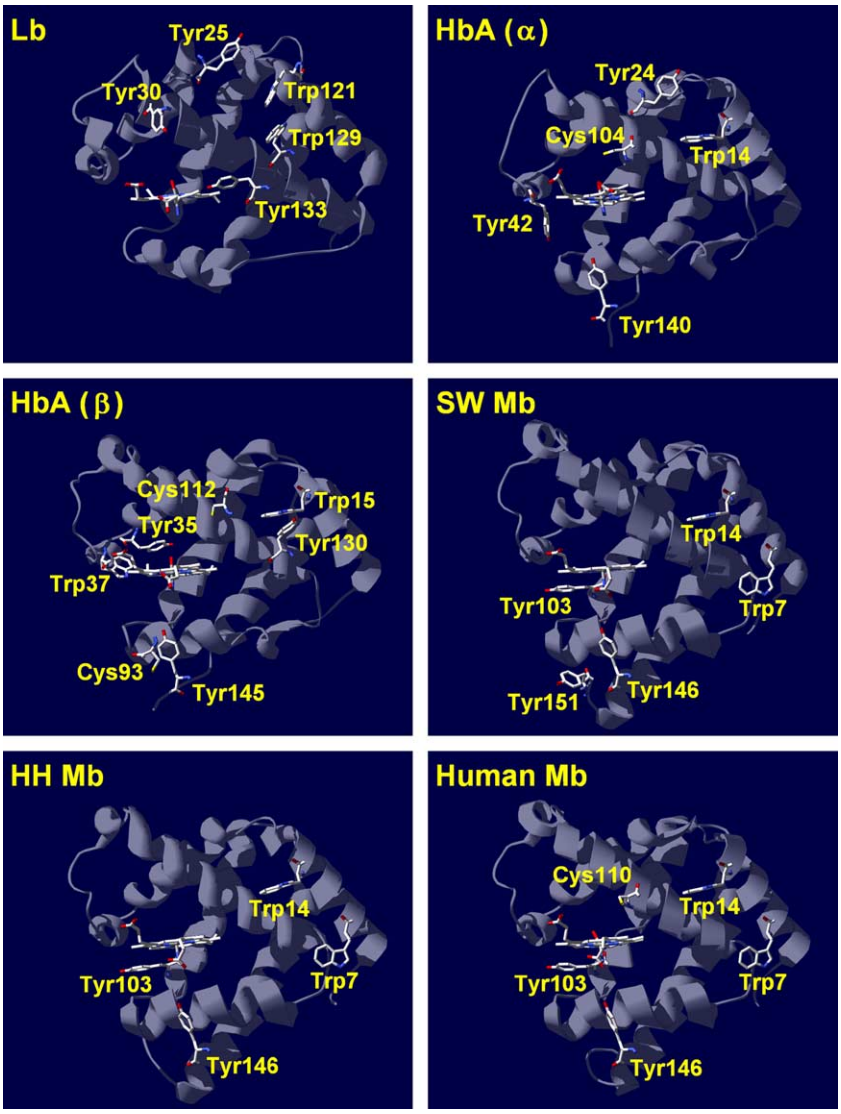


Fig. 9. Relative position of all tryptophan, tyrosine and cysteine residues that might be involved in the formation of free radicals in the oxygen binding proteins under peroxide treatment. All panels represent the same view of the three-dimensional structure. The plane of the haem is roughly perpendicular to the plane of the figure. The ribbon view of the proteins is placed behind the displayed residues. The PDB structure files used to generate this figures were: soybean Lb, 1BIN [77]; HbA, 1HGB [166]; SW Mb, 1BZ6 [168]; HH Mb, 1WLA [169]; human Mb, 2MM1 [170]. Note that 2MM1 is a structure file of a variant of human Mb in which Cys110 is replaced with Ala110, as the wild type structure is not available. Because of this Ala110 has been artificially 'mutated' back to Cys110 in this figure.

covalently bound flavin adenine dinucleotide (FAD) and can only function when the FAD is fully reduced ($FADH^-$). In the isolated photolyase, the FAD is usually in the semi-reduced radical state FADH•, but it can be easily reduced to the catalytically competent $FADH^-$ state by illumination with visible light in the presence of an exogenous electron donor, a process called photoactivation [100]. In the *E. coli* DNA photolyase, light excited FADH• is reduced to the active $FADH^-$ state by the electron transferred over three Trp residues: FADH•←Trp382←Trp359←Trp306. The last tryptophan Trp306 is solvent-accessible and its radical can be reduced by an exogenous electron donor. The electron transfer between the tryptophans occurs much faster (in 30 ps from the first Trp to the substrate) than the protons could dissociate from the cation radicals $Trp^{\bullet+}$ (in 300 ns at the site of the last Trp) [100]. Interestingly, in the absence of the terminal donor, a reverse electron transfer (with a lower rate) brings the flavin co-factor to the semireduced inactive state FADH• [101]. In DNA photolyase from *Anacystis nidulans*, a similar electron transfer involves a Trp and a Tyr residues: FADH•←Trp←Tyr [99].

These examples support the view that the primary radical in the haem protein/peroxide systems can be considered as a cation radical effectively delocalised over tyrosine and tryptophan residues. The cation radicals of different Trp and Tyr residues must have different pK_1 values, depending on the immediate molecular environment of the residue as well as on the residue's nature. Thus, at a given pH value,

the deprotonation of different cation radicals will take place with different rates, creating a split in the type and population of observed neutral radicals. The proportion of radicals of different types will thus depend of the pH value of the reaction (e.g. Fig. 4, A and B; Table 2).

Globin-bound radicals can also be formed as a result of the oxoferryl haem state autoreduction. The initial fast stage of this process in the SW metMb/H_2O_2 system has been shown to be protein concentration independent, indicating that the electron employed in the reduction is delivered intramolecularly [102]. Kinetic studies of the formation of Mb oligomers during oxoferryl autoreduction has pointed to Tyr151 as the source of the electron (i.e. the site of radical formation), although other residues might also be involved in this intramolecular radical transfer [102].

Although the peroxyl radical in HH and SW Mb (Trp14-OO•) was implicated in the oxidation of substrate molecules, thus suggesting an intermolecular mechanism of their decay, an indication exists that this radical can decay via both inter- and intramolecular electron transfer (Section 5.3.3).

5.3.3. Intermolecular radical transfer

For the human metMb/H_2O_2 system, an intermolecular electron transfer from Cys110 in one molecule to Tyr103• in another Mb molecule has been demonstrated in an elegant experiment, when the Mb disulfide homodimers (Section 5.2.2.5) were still formed in the mixture of two mutants of human Mb, either of which, when taken separately, was unable to produce the dimers, because one was lacking Tyr103 (Tyr103Phe) and the other did not have Cys110 (Cys110Ala) [66].

A similar idea was used to demonstrate an intermolecular radical transfer in the SW metMb/H_2O_2 system. Like in human Mb, this system can also form protein dimers (and trimers) but, as there is no cysteine in SW Mb, the dimers were shown to be formed via linking Tyr151 on one molecule with Tyr151 on another [97,102]. Thus, the formation of a tyrosyl radical on Tyr151 is the necessary condition for the formation of the oligomers. It has been demonstrated that a mixture of the Tyr151Phe mutant with apoMb, when treated with H_2O_2, generates an apoMb–apoMb dimer [102]. ApoMb is the protein that has been forced to lose the haem group and is therefore unable to react with H_2O_2 to produce free radicals and, consequently, dimers. The Tyr151Phe variant of SW Mb, despite the presence of an intact haem group, is not able to dimerize. Thus neither of the proteins is able to produce the dimers if it is incubated with H_2O_2 alone. The fact that the dimers were detected in the mixture of these proteins provides strong evidence of an intermolecular radical transfer [102].

The radical transfer processes have been studied also in the HH metMb/H_2O_2 system. Three radical species can be directly detected in this system at both low and room temperatures: a peroxyl radical, likely to be formed on Trp14 by analogy with SW Mb, a tyrosyl radical on Tyr103 unambiguously assigned to this residue by a unique θ angle (Sections 5.2.2.3 and 5.2.2.4), and a third radical that becomes visible in the room temperature spectra after the disappearance of the Tyr103• signal and can also be detected by low temperature spectroscopy (Table 5).

This third type of radical has the liquid phase EPR signal typical of tyrosyl radicals (Fig. 4, J and M). There are only two tyrosine residues in HH Mb, Tyr103 and Tyr146 (Fig. 9). The site-specific spin trapping experiments with SW Mb showed that no tyrosyl radical can be formed on Tyr146 if two other tyrosines are replaced with phenylalanine [60]. Based on this result, the possibility has been rejected that Tyr146 could be the site of the radical responsible for the doublet [81]. The only option left was to assign the doublet to the same Tyr103•, the change of spectral lineshape from the septet to the doublet being a consequence of a conformational change which the radical undergoes during the reaction [81]. It seems, however, possible that if Tyr146• can only be formed as a result of electron transfer from Tyr146 to Tyr103•, in the absence of Tyr103, no Tyr146• could be spin trapped. Also, if Tyr103• is the immediate precursor of Tyr14ć, one should not expect a measurable yield of the Tyr146• spin adducts if the spin trap is present in a significant excess with respect to the protein before the addition of peroxide. This would explain the result that only Tyr103• and no Tyr146• were spin trapped in the experiments involving peptide mapping (18.4 mM MNP in 200 μM Mb [82] and 2.5 mM DBNBS in 0.5 mM Mb [62]). Under such molar excess of the spin traps over the protein, Tyr10$\dot{3}$ would react more readily with the spin trap rather than with another protein molecule at the Tyr146 site.

A radical transfer from Tyr103 to Tyr146 was demonstrated in the low temperature study of the HH metMb/H_2O_2 system [96], when the same radical species that has the doublet signal in the liquid phase is characterised by a low temperature singlet. Accordingly, this radical has been interpreted as Tyr146•. The fact that Tyr146• is characterised by a liquid phase spectrum with a relatively well resolved hyperfine structure, but the low temperature spectrum of the same species is unresolved, makes this the third example of such behaviour of tyrosyl radicals, adding to the already documented cases for the tyrosyl radicals in the metHbA/H_2O_2 and SW metMb/H_2O_2 systems [39].

The radical transfer from Tyr103 to Tyr146 (or electron transfer from Tyr146 to Tyr103•) has been studied in an

Table 5
Three radical species detectable in the HH metMb/H_2O_2 system

Radical	Low temperature EPR signal	Room temperature EPR signal
Trp14-OO•	Fig. 2, '0.45 min'; Fig. 3, D	Fig. 6, C; also in [81]
Tyr103•	Fig. 7; Fig. 8, B	Fig. 4, H; Fig. 8, C
Either Tyr103• or Tyr146•	[39,96]	Fig. 4, J and M

experiment in which a variable concentration of HH apoMb was added to the native HH metMb prior to H_2O_2 addition [96]. As apoMb does not react with H_2O_2, a constant concentration of intact HH metMb and H_2O_2 ensured the formation of the same level of primary radicals while the total protein concentration was variable. It has been demonstrated that as the apoMb concentration increases, (1) the concentration of Tyr146• becomes greater, (2) the concentration of Tyr103• becomes lower and (3) their total concentration did not depend on the amount of apoMb added. This result clearly shows that a protein concentration dependent process of electron transfer from Tyr146 to Tyr103• takes place. The obvious interpretation of this result is that the transfer is intermolecular, from Tyr146 on one Mb molecule to Tyr103• on another. Such a process would represent the same pattern of radical propagation as the Tyr151• radical formation via an intermolecular electron transfer mechanism in SW Mb [102], or the thiyl radical formation on Cys110 of human Mb via an intermolecular electron transfer to Tyr103• on another Mb molecule [66]. Note that Tyr146 in HH Mb, both Tyr146 and 151 in SW Mb, and Cys110 in human Mb are all close to the protein surface and exposed to the solution, meaning they are accessible for inter-protein interactions (Fig. 9).

If the radical on Tyr146 is formed in HH Mb in an intermolecular reaction of electron transfer, could a radical on this residue be formed in the SW protein? Two types of tyrosyl radical have been identified in the SW metMb/peroxide system: Tyr151• (Fig. 4, A) and Tyr•103 (Fig. 4, spectrum B represents the septet signal from this radical admixed with spectrum A). In the same paper where these spectra have been reported, a third signal was presented for a later time of reaction of SW metMb with EtOOH—spectrum C (Fig. 4). This signal corresponds perfectly, both by the lineshape and the time of detection, to the doublet in HH Mb (Fig. 4, J and M), thus raising the possibility that Tyr146• is formed in both HH and SW myoglobins via an intermolecular electron transfer. In the first case the electron is transferred to Tyr103•. In the case of SW Mb, Tyr103• can possibly oxidize both Tyr151 and Tyr146 residues in the intermolecular reactions. These two tyrosines are close to each other and are both close to the protein surface, Tyr151 being completely solvent exposed (Fig. 9). The presence of Tyr103 has been shown to be critical for the formation of the major part of Tyr151• in SW Mb [39], and the radical itself has been shown to be formed in an intermolecular process [102]. A similar intermolecular mechanism can be responsible for the Tyr146• radical formation. In addition, it is likely that Tyr146• can also be formed as a result of an intramolecular electron transfer Tyr151•←Tyr146 as these residues are only about 7 Å apart.

The assignment of the singlet detected in the low temperature spectra of the HH metMb/H_2O_2 system to a Tyr146• has not been strongly supported spectroscopically, due to a low intensity of the signal [96]. For the same reason, the assignment of the liquid phase doublet (Fig. 4, J and M) to a tyrosyl radical [81] needs more support. Fortunately, such support can be found in the spectrum reported for the HH metMb/t-BuOOH system (Fig. 4, N) when the concentration of the protein was high (3 mM) [103]. An extrapolation of the results of the low temperature experiments involving HH apoMb [96] to a high protein concentration allows us to suggest that the concentration of the radical on Tyr103 should be very low, and the concentration of the radical of the 'third' type (Table 5) should be high (the peroxyl radicals are not expected in the N_2-saturated reaction mixture, see Table 2, N). Indeed, the reported liquid phase spectrum (Fig. 4, N) does not show the septet from Tyr103•, which is quite unexpected for an HH Mb system. Instead, the lineshape of the reported spectrum can be attributed, without reasonable doubts, to a tyrosyl radical (compare to the Hb spectra O, P and Q), and thus can only originate from the Tyr146• radical.

It has been suggested before that the effect of total protein concentration on the relative concentration of Tyr103• and Tyr146• does not necessarily imply an intermolecular electron transfer [96]. Alternatively, an intramolecular electron transfer from Tyr146 to Tyr103• was suggested to be triggered by an intermolecular event (e.g. a change in pK1 of tyrosine cation radicals caused by molecular impact) [96]. Considering the recent studies of the intermolecular radical transfer in human and SW Mb [66,102], when the experimental design did not allow such alternative interpretation, it becomes more likely that in the HH metMb/H_2O_2 system, too, the electron transfer from Tyr146 to Tyr103• occurs via an intermolecular mechanism.

6. Free radicals formed in enzymes

6.1. Compound I

The primary intermediate of the two electron oxidation of enzymes by peroxides is the oxoferryl porphyrin π-cation radical [Fe^{4+}=O por•$^{+}$] (Compound I) that catalyses the oxidation of various substrates in peroxidases or that reacts with H_2O_2 in the case of catalases [104]. As a paramagnetic species (a radical associated with a ferryl iron), Compound I has an unusually wide, for a radical, EPR spectrum: it has a central asymmetrical feature with a peak-to-trough width of about 40 Gauss (G) and the wings on both sides spreading over hundreds of Gauss (Fig. 3, R). This unusual lineshape is caused by an exchange interaction (ferromagnetic or antiferromagnetic) between the ferryl iron and the porphyrin cation radical [105]. The strength of this exchange interaction is different in different proteins and might have a strong pH-dependence. Therefore the EPR spectrum lineshape of Compound I is variable in different proteins and might differ significantly for the same protein prepared at different pH values. Compound I has been reported for horseradish peroxidase (HRP) [106,107]; chloroperoxidase

[108]; ascorbate peroxidase [109]; lignin peroxidase [110]; turnip isoperoxidases 1, 3 and 7 (TIP) [111]; *Micrococcus luteus* (formerly *lysodeikticus*) catalase (MLC) [105]; bovine liver catalase (BLC) [112] *Proteus mirabilis* catalase (PMC) [112]; *E. coli* catalase-peroxidase [113]; *A. nidulans* catalase-peroxidase [114]; *Synechocystis* PCC6803 catalase-peroxidase [115].

Compound I can be stable, as in PMC [116], relatively long lived, as in MLC treated with peroxyacetic acid (PAA) [105], or unstable, as in BLC, where the porphyrin radical disappears over the 0.04–15 s range with formation of a Tyr radical [112] (Fig. 3, U).

Compound I in mitochondrial cytochrome ***c*** peroxidase (C*c*P) represents a special case: The radical observed in this compound (termed compound ES) is located not on the porphyrin π-system, but on a proximal side tryptophan (Trp191) [117,118] (Fig. 3, S). C*c*P catalyses the redox reaction with cytochrome *c* (Cyt *c*), the sole substrate of C*c*P. The tryptophan radical, still coupled in an exchange interaction with the oxoferryl haem iron [Fe^{4+}=O $Trp^{\bullet+}$] [119], is necessary for the electron transfer from Cyt *c* to the porphyrin, because Cyt *c* cannot be directly accessible to the C*c*P haem active centre [120]. If this Trp191 is replaced with Phe, C*c*P stabilizes a tyrosyl radical [121]. Also, in the Trp191Gly variant, a Tyr radical was observed [122]. If the homologous phenylalanine in HRP (Phe221) is replaced with a tryptophan, to mimic the situation in C*c*P, a tryptophan radical is observed instead of $por^{\bullet+}$ normally formed in the wild type enzyme [107]. In addition to Trp191•, a tyrosyl radical has also been reported in wild type C*c*P [122].

6.2. Lignin peroxidase

In lignin peroxidase, the solvent exposed Trp171, located ~11 Å away from the haem, has been shown to be crucial for the oxidation of the small molecular substrate veratryl alcohol [123]. This residue has a unique post-translational modification, the hydroxylated C_β atom, formed auto-catalytically during the first few turnover cycles [124,125]. The Trp171Phe and Trp171Ser variants of lignin peroxidase were found to be entirely inactive towards veratryl alcohol, and neither Compound I nor Compound II[5] of these mutants could be reduced by veratryl alcohol [126]. The mutants reacted normally with H_2O_2 to give a Compound I. However, the rapid phase (2.3 s^{-1}) of spontaneous Compound I reduction, typical of wild type lignin peroxidase, was absent in the Trp171 mutants, strongly suggesting that Trp171 is in the pathway of electron transfer from the substrate to the haem of Compound I and/or II [126]. If so, a transient free radical state should exist for Trp171. Indeed, spin trapping with MNP in combination with peptide mapping revealed an adduct formation at atom C6 of the indole ring of Trp171 [127]. However, no direct observation of this radical has been reported yet. It is worth noting that a different atom, C3 of the indole ring, has been identified as the site of spin traps and O_2 addition to the tryptophanyl radical in the SW metMb/H_2O_2 system [61,64].

6.3. Cytochrome c oxidase

The formation of a radical at the cross-linked Tyr244–His240 structure of the bovine cytochrome *c* oxidase (C*c*O) has been proposed as a possible source for one of the electrons required in the O–O bond cleavage process [128]. The evidence of the radical formation on this structure has been obtained by radioactive iodide labelling, followed by peptide mapping, of the radicals in the **P** intermediate of bovine C*c*O, as iodide is known to covalently label tyrosine radicals but not neutral tyrosines [129]. For **P** generated either by CO and oxygen or by H_2O_2, the only site labelled by iodide was found to be the Tyr244–His240 dimer [129].

In the bovine enzyme treated with H_2O_2, the spin trapping with MNP followed by non-specific protease treatment had allowed the identification of cysteine radicals [130]. However, when the cysteine residues were blocked with *N*-ethylmaleimide, one or more radicals on tyrosine residues had been found [130].

Two radical species have been observed directly by low temperature EPR and electron nuclear double resonance (ENDOR) spectroscopy in bovine C*c*O treated with H_2O_2 [131–134]. One of the radicals has been assigned to a porphyrin cation radical [133]. The other radical with a 45 G wide EPR signal was proposed to be a tryptophan radical, possibly on Trp126 [133]. However, a different interpretation can be given of the reported experimental EPR signal (Fig. 3, P). This signal can be simulated as a spectrum of the Tyr129• radical, i.e. of the tyrosine residue highly conserved in cytochrome *c* oxidases and homologous to Tyr167 in *Paracoccus denitrificans* C*c*O [75]. The latter tyrosine has been suggested as the site of the radical observed in the *P. denitrificans* enzyme under H_2O_2 treatment (Fig. 3, Q) [75]. Although a tentative assignment to a specific tyrosine it was, the simulation of the experimental spectrum of the radical definitely allowed the authors to exclude the possibility that the radical is located on the cross-linked motif Tyr280–His276, since the rotational conformation of Tyr280 is very different from either of the two possible conformations predicted from the simulation [75]. The assignment of the radical in the *P. denitrificans* enzyme to Tyr167 has been recently confirmed in the site-directed mutagenesis study [135].

The complexity of the phenomenology of free radical formation in C*c*O probably reflects the fact of a radical equilibrium established among several residues under peroxide treatment. At least it has been demonstrated that three types of residues, cysteine, tryptophan and tyrosine,

[5] Compound II is another name, more in use for enzymes, for the pure oxoferryl state of the haem, i.e. when the porphyrin π-cation radical is already dissipated.

participate in such equilibrium in bovine C*c*O treated with CuOOH. The radical spin-trapped in this system with MNP had showed a dramatic increase in concentration when either type of the residues was chemically modified [136].

6.4. Tyrosyl radicals

The electron transfer in the haem enzymes from a tyrosine residue to Compound I or/and II results in a tyrosyl radical formation. Sometimes these radicals are implicated in the enzyme's mechanism.

6.4.1. Catalase

Catalases are responsible for the disproportionation of H_2O_2 into water and molecular oxygen. BLC is unique among other catalases since, in addition to porphyrin π-cation radical, a protein radical located on a tyrosine (possibly on Tyr369 [71]) can be observed (Fig. 3, U) [112, 137]. The crystal structure of BLC shows a tightly bound NADPH cofactor which function is to protect the enzyme against inactivation by preventing Compound II formation. NADPH apparently does not reduce Compound II directly, but rather reduces its immediate precursor, an intermediate formed during the decay of Compound I to Compound II [138]. It has been suggested that the tyrosyl radical observed simultaneously with the oxoferryl moiety in BLC might be that intermediate [112]. However, as indicated in the paper, the observed tyrosyl radical might not be of great importance for the catalytic mechanism of the catalase, since it is being formed within a relatively long time interval of 0.04–15 s (with a concomitant disappearance of the porphyrin π-cation radical) while the enzyme shows a very high turnover on H_2O_2 [112].

6.4.2. Turnip isoperoxidases

A tyrosyl free radical (Fig. 3, T) is formed, and observed in the same time as the porphyrin π-cation radical, in one of the three isozymes studied of turnip peroxidase [111]. All three isoperoxidases 1, 3 and 7, in their reaction with H_2O_2, showed the formation of the Compound I [Fe^{4+}=O $por^{\bullet+}$] intermediate. The lineshape of the EPR spectrum of the porphyrin π-cation radical was characterised by a dramatic pH dependence for all isozymes indicating a strong pH-dependence of the exchange interaction between the oxoferryl moiety and the porphyrin π-cation radical in the pH range of 4.5–7.7. At the conditions when $por^{\bullet+}$ radical cannot be detected (T >30 K, microwave power <50 μW) a free radical was observed in isoperoxidase 7 (g_{iso}=2.0043) that was assigned by the g-factor anisotropy, and on the basis of similarity with the EPR spectrum of the BLC radical (compare spectra T and U in Fig. 3), to a Tyr radical [111]. The broad g_x-component of the high frequency (HF) EPR spectrum and its relatively low value (g_x=2.0066) were attributed to the radical being hydrogen-bonded, with the strength of the bond being distributed in the population of the radicals [111]. The formation of the Tyr radical in isoperoxidase 7 was related in [111] to a higher redox potential of Compound I of this isozyme than that of isozyme 1 [139].

6.4.3. Prostaglandin H synthase

Prostaglandin H synthase (PGHS) catalyses the oxidation of arachidonic acid to hydroperoxide PGG_2 (prostaglandin G2) and the reduction of the hydroperoxide to the alcohol PGH_2, a precursor of many physiologically important prostaglandins [140]. These two processes, associated with the enzyme's cyclooxygenase and peroxidase activities respectively, are mutually dependent: hydroperoxide PGG_2 reacts with the ferric haem resting state to generate PGH_2 and Compound I; the latter relaxes to a protein bound radical believed to be directly involved in the oxidation of arachidonic acid which then leads to hydroperoxide PGG_2 formation [141]. There are two known isoforms of PGHS that can be found in many mammalian tissues, PGHS-1 and PGHS-2. Each isozyme shows roughly 90% sequence identity in different species; PGHS-1 and PGHS-2 from the same species show about 60% sequence identity, the residues identified as essential for catalysis being conserved [142].

The resting, ferric form of PGHS-1 reacts with hydroperoxides to form Compound I [Fe^{4+}=O $por^{\bullet+}$] [143]. A fast internal electron transfer then follows resulting in formation of a protein bound free radical with a *wide doublet* EPR signal (Fig. 3, V), which is transformed rapidly to what is known as *wide singlet* (sometimes *broad singlet*) (Fig. 3, X) followed by a continuous narrowing of the singlet to the final lineshape of a *narrow singlet* with a poorly resolved hyperfine structure, a characteristic EPR spectrum of self-inhibited enzyme (Fig. 3, Z) [72,144]. An analysis of the lineshape of these three EPR signals has resulted in the conclusion that the wide singlet can be represented as a composition of the wide doublet and the narrow singlet, suggesting that there are only two, not three, free radical species [72]. This conclusion has been later supported [145,146].

A similar pattern of radical formation has been observed in PGHS-2 reacting with EtOOH, but the apparent transformation of the wide doublet (Fig. 3, W) into wide singlet (Fig. 3, Y) takes place over a much shorter time of 50 ms as compared with 50 s in the case of PGHS-1 [147]. No conversion of the wide singlet to a narrow singlet in PGHS-2 was reported [147], possibly because the time of observation was not long enough (≤80 ms).

Complexes of either isozyme with cyclooxygenase inhibitors, such as indomethacin or nimesulide, also react with hydroperoxides with the formation of a free radical. In PGHS-1 pre-incubated with indomethacin [144,146], the EPR signal of the radical (Fig. 3, AA) is similar to the self-inhibited narrow singlet in this isoform [72,142,145,148]

(Fig. 3, Z), although it is usually detected with a better resolved hyperfine structure than in the narrow singlet. In PGHS-2, the nimesulide-inhibited EPR signal formed under EtOOH treatment (Fig. 3, AB) has recently been assigned to a tyrosyl radical on Tyr504 [149]. We suggest here that the indomethacin-inhibited singlet in PGHS-1 treated with EtOOH (Fig. 3, AA) can also originate from Tyr504• in this isoform. The new method of simulation of tyrosyl radical spectra (TRSSA, see Section 5.2.1) recently used to simulate signal AA in Fig. 3 has yielded two possible values of angle θ (Fig. 5) of the rotational conformations of the phenoxyl ring, 39° or 81° (±2°) [71]. The second value of 81° is highly consistent with the PGHS-1 tyrosyl radical being also on Tyr504, as the rotation angle of the ring for this residue in PGHS-1 is 82.6° (see entry 1PGF [150] in PRRAD). The very good correspondence of the experimental and simulated spectra [71] and the closeness of the two θ angles raise the possibility that the mechanism of hydroperoxide reaction with PGHS complexed with inhibitors involves formation of a radical on Tyr504 in both isoforms, PGHS-1 and PGHS-2.

The wide doublet EPR signal (Fig. 3, V and W) was assigned, first tentatively, on the basis of the similarity with other systems, to a tyrosyl radical [151]. It has been shown later, using tyrosine nitration and site-directed mutagenesis, that (1) all hydroperoxide induced radicals in the active and inhibitor-bound PGHS are on tyrosine residues; (2) Tyr385 is essential for both cyclooxygenase catalysis and the formation of the radicals [152–155]. The replacement of Tyr385 with phenylalanine resulted in the formation of a different, but a tyrosyl, radical [149,153,154]. Tyr385 is located between the haem and the site of arachidonic acid binding, and the radical on this residue is thought to be capable of abstracting the 13-pro(S) hydrogen from arachidonate to initiate cyclooxygenase catalysis [156]. The radical responsible for the wide doublet is hydrogen bonded which is consistent with the immediate environment of Tyr385 [146]. Therefore, many agree that Tyr385 is the site of the radical responsible for the wide doublet.

The transformation of the wide doublet to the narrow singlet during cyclooxygenase catalysis (Fig. 3, V→Z) has been attributed to the phenoxyl ring rotation in the Tyr385 radical in PGHS-1 [146,148], but in the case of PGHS-2, a radical transfer from Tyr385 to Tyr504 (i.e. to the same residue where the radical in the enzyme–inhibitor complex is formed) has been suggested [149]. A third way might also be suggested to explain the change in the EPR spectrum of a tyrosyl radical, while neither movement of the radical to another location, nor its rotation is involved.

The PGHS-1 radical (presumably on Tyr385) with the wide doublet (Fig. 3, V) is hydrogen bonded [146]. This follows from the value of the g_x-component of the radical's g-tensor measured by the HF EPR spectroscopy: It is shifted to the lower values when compared to tyrosyl radicals in other systems. As the evolution from the wide doublet to the narrow singlet progresses, g_x increases continuously, which is consistent with a *continuous* decrease of the hydrogen bond strength. This continuous change of the g_x component has allowed the authors to favour the single site hypothesis, rather than the view that the spectral change is associated with a radical transfer from one tyrosine to another [146]. However, the changes in the g-tensor alone detected by the HF EPR spectroscopy could not explain the observed doublet-to-singlet transformation measured by the X-band EPR spectroscopy; clearly some changes in the hyperfine interaction must be taking place as well. To account for this, the authors suggested a continuous change of the ring rotation angle [146], because the hyperfine splitting constants on two methylene protons in a tyrosyl radical depend on this angle according to the McConnell relation (6). However, it is not necessary to suggest the rotation in order to account for a change in the hyperfine interaction. A new empirical result that follows from TRSSA (Section 5.2.1) is that g_x is in a linear relationship with the spin density ρ_{C1} [71]:

$$g_x = -0.0359\rho_{C1} + 2.02160 \quad (10)$$

Therefore, a change in g_x caused by a change in the hydrogen bond strength would automatically mean a change in ρ_{C1}, and, linked with it via (7), the changes in the hyperfine splitting constants for the methylene protons. During the doublet-to-singlet evolution in PGHS-1, g_x is increasing from 2.0068 to 2.0078 [146]. According to (10), this corresponds to a decrease of the spin density on atom C1 from 0.412 to 0.384. In accordance with the McConnell relation (6), a directly proportional decrease should occur in the hyperfine splitting constants for the methylene protons, even without any rotation of the ring. This decrease in the values of $A^{\beta 1}_{iso}$ and $A^{\beta 2}_{iso}$, plus the change in g_x[6], plus the minor changes in all other hyperfine interactions[7] would result in a notable change of the detected X-band EPR lineshape. In fact, these changes are sufficient to account for the doublet-to-singlet evolution in PGHS-1 without suggesting a ring rotation: the use of TRSSA in the simulation of the PGHS-1 wide doublet and the PGHS-1 indomethacin-inhibited singlet yielded rather close values of the rotation angle θ of 37° (or 83°) and 39° (or 81°) respectively [71]. However, the g-factors and associated with them values of spin density on C1, were markedly different for the two radicals, thus being the main cause of the differences in the X-band spectra.

[6] The value of g_x, being variable in different tyrosyl radicals, is often thought not to be critical for the X-band spectra but, in fact, the change in g_x considered here can smear partly or completely, the pattern of the hyperfine structure of the spectrum, otherwise resolved in an X-band spectrum.

[7] The change of the spin density on one atom (C1) cannot take place without a change of the spin density on other atoms.

6.4.4. Catalase-peroxidase

Catalase-peroxidases represent a group of enzymes which exhibit typical peroxidase activity towards a broad group of substrates, but unusual for peroxidases they also exhibit a high turnover rate of H_2O_2 disproportionation, comparable with the monofunctional catalases [115]. Amino acid sequence analysis shows little homology to catalases, but a high homology to yeast C*c*P and eukaryotic ascorbate peroxidase [157,158]. Like other haem peroxidases, the resting (ferric) form of catalase-peroxidases from different organisms reacts with hydroperoxides to form Compound I [Fe^{4+}=O por$^{\bullet+}$] [114,115,159]. In *Mycobacterium tuberculosis* catalase-peroxidase (KatG), Compound I decays rapidly with the formation of a protein bound free radical. The pattern of the radical formation and evolution, as evidenced by a freeze-quench EPR study [160], is surprisingly similar to that in PGHS treated with peroxides. A doublet EPR signal appears within 6.4 ms after the addition of PAA (Fig. 3, AC) and increases over a few hundreds of milliseconds. A narrower singlet then develops (Fig. 3, AD) and finally replaces the doublet after 10 s, when the continuously growing total concentration of both kinds of radical reaches the maximum of 0.5 equivalent of initial haem concentration. Both EPR lines have been assigned to tyrosyl radicals [160]. Following a peptide mapping study, Tyr353 has been suggested as a site of free radical formation [161]. The replacement of this tyrosine with phenylalanine resulted in a lower yield of radicals, although still with doublet and singlet lineshapes [161].

The similarity of the free radical formation in catalase-peroxidase and PGHS treated with peroxides is unexpected and might be coincidental because there is no structural homology between the enzymes. Note that the doublets (spectra V, W vs. AC in Fig. 3) and the singlets (spectra X–Z vs. AD in Fig. 3) in the two enzymes are not identical as one would certainly expect from two unrelated proteins. However, while radical transfer and transformation in proteins is a common process, PGHS and KatG are both characterised by the time scale of the doublet-to-singlet spectral change, that is relatively rare—the process is complete after a few seconds (or even faster in the case of PGHS-2). The rate of this process is too slow as compared to the very fast intramolecular electron transfer, undetectable by conventional EPR techniques (in the picosecond and nanosecond range as in DNA photolyase [100]), and it is too fast as compared to some common changes in the EPR spectra observed over minutes and likely to be associated with the intermolecular radical (electron) transfer [35,39,65,66]. This rate of the EPR signal evolution might be consistent with the characteristic time range of protein conformational change during the reaction. The question of whether the doublet-to-singlet evolution in PGHS-1, PGHS-2 or KatG is associated with a tyrosyl radical transfer to a different tyrosine or with a conformational change of the same residue has not been unequivocally solved [149,160]. The evidence has been presented supporting the single site hypothesis for PGHS-1 [146] and the radical transfer to another Tyr for PGHS-2 [149]. The mutagenesis work addressing this issue for KatG is under way now [160,161]. If the signal evolution in KatG is associated with a conformational change of the same tyrosine residue, then it seems more likely that the nature of this change would be a hydrogen bond relaxation, rather than the phenoxyl ring rotation.

An analysis of all 42 tyrosine residues in 2 homologous subunits (A and B) of the newly available structure of *M. tuberculosis* catalase-peroxidase (PDB file 1SJ2 [162]; entry 1SJ2 in PRRAD) shows that there are nine tyrosine residues with the rotational angle θ of the phenol ring, averaged over subunits A and B, that could result in a doublet EPR spectrum[8], if the residue were in the neutral tyrosyl radical form (Table 6).

It has been demonstrated before that four of the nine tyrosines listed in Table 6, Tyr98, Tyr229, Tyr304 and Tyr426, cannot be responsible for the observed EPR signals, since their replacement with phenylalanine in the mutagenetic experiments did not eliminate either doublet or singlet [160]. Therefore, one of only five residues (shown in a bold font in Table 6) can be the site of the radical as far as the rotational conformation of the ring is considered: Tyr64, Tyr197, Tyr413, Tyr638 and Tyr711 (Fig. 10). These five tyrosines are conserved to a different degree in the four catalase-peroxidases for which the three dimensional structure is known (Table 7). Future EPR studies of catalase-

Table 6
Selected 9 of total 21 tyrosine residues in *M. tuberculosis* catalase-peroxidase (PDB file 1SJ2) with the ring rotation angle θ close to either 30° or 90° (the conformation that could give a doublet EPR spectrum, if the residue were a free radical)[a]

Tyrosine	Angle θ averaged over subunits A and B (°)	Angle θ is close to the target value of	$\lvert\theta-\theta_{target}\rvert$ (°)
Tyr98	30.1	30	0.1
Tyr229	27.1	30	2.9
Tyr64	−85.8	−90	4.2
Tyr304	82.7	90	7.3
Tyr413	82.0	90	8.0
Tyr638	80.4	90	9.6
Tyr426	−79.8	−90	10.2
Tyr197	79.5	90	10.5
Tyr711	42.5	30	12.5

[a] Angle θ is defined on the interval of −90°–+90° (Section 5.2.1). Therefore the conformations for which θ is greater than 90° are presented with equivalent angles that are accordingly less, by absolute value, than −90°.

[8] A tyrosyl radical would give a doublet EPR spectrum if the hyperfine splitting constants for the two methylene protons are significantly different, i.e. when one of the two protons is close to the ring plane and the other proton forms an angle of 120° with the plane, that is when θ=30° and when θ=90°. Thus, we are looking for the tyrosines with angle θ being within an arbitrary chosen range of, say, 25° around the values of 30° and 90°. This is only the necessary, but not sufficient condition for a doublet EPR spectrum.

peroxidase from these organisms will certainly provide us with information about the location of the radical formed in each case. For example, if catalase-peroxidase from *Haloarcula Marismortui* in its reaction with PAA shows a similar pattern of radical formation as the *M. tuberculosis* enzyme, then Tyr197 (*M. tuberculosis* numbering) is not the radical site because the *H. Marismortui* enzyme does not have a tyrosine at that position.

The formation of protein-bound radicals in the reaction of PAA with another catalase-peroxidase, from *Synechocystis* PCC6803, has been studied by a combination of site directed mutagenesis, isotopic labelling, X-band and HF EPR spectroscopy [115]. Like in many other peroxidases and catalases, Compound I [Fe^{4+}=O por$^{\bullet+}$] has been detected. Its decay resulted in formation of more than one type of protein bound radical with the integral EPR spectrum shown in Fig. 3 (AE). Fully deuterated tyrosine and tryptophan residues, as well as partly deuterated tyrosine, were used to show that the EPR lineshape AE (Fig. 3) represents a superposition of the signals from the radicals on tyrosine and tryptophan residues. Site directed mutagenesis study has allowed the authors to suggest the site of the tryptophan radical as Trp106. Radical formation on Trp106 has been suggested to involve an intramolecular radical transfer over a string of residues and hydrogen bonded water molecules: Trp122, His123, Arg119, seven water molecules, the haem 6-propionate group and Trp106. The site of the tyrosyl radical has not been assigned [115]. When spectrum AE was recorded on a wider field scale, a component was revealed at $g \approx 2.03$ (compare 'dashed' and 'solid' versions of signal AE, AE_d and AE_s, Fig. 3). This component has not been assigned. However, its position fits very well to the $g_{||}$ of the peroxyl radicals (compare with the spectra of Mb, Fig. 3). Considering the fact that a tryptophanyl radical has been suggested to be formed in this system, it seems very probable that it would react with O_2, like in the myoglobin systems, to form a peroxyl species. If so, spectrum AE in Fig. 3 is probably a superposition of the Trp-OO• signal (of the radical formed on Trp106) and a Tyr• signal (of a tyrosyl radical of unknown location but with a conformation similar to that of the tyrosyl radicals in BLC, TIP7 and SW Mb, as can be concluded from the similarity of the linewidth of their EPR signals, Fig. 3).

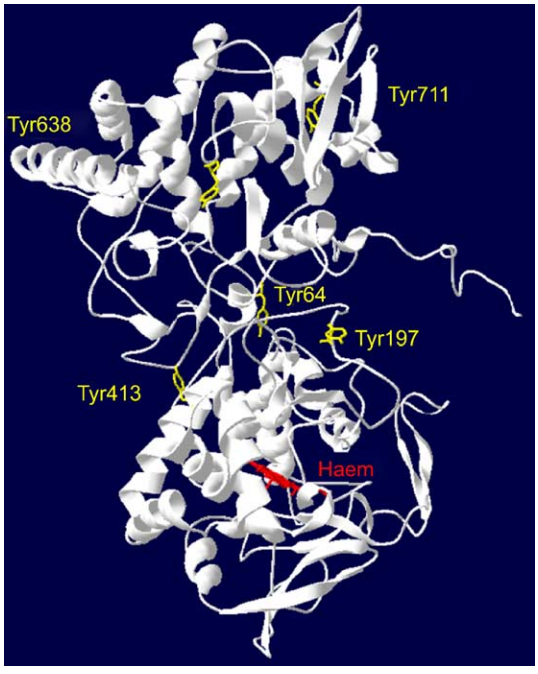


Fig. 10. *M. tuberculosis* catalase-peroxidase and the five tyrosines which could be responsible for a doublet EPR spectrum. The rotation angle of the phenol ring in these three tyrosines is close to either 30° or 90° (see Table 6). Subunit A of structure file 1SJ2 [162] is shown.

Table 7
Five tyrosine residues of *M. Tuberculosis* catalase-peroxidase, suggested to have a doublet EPR spectrum, if in the tyrosyl radical form (see Table 6), and the homologous tyrosines in this enzyme from three other organisms

Organism	Residue					PDB file
M. Tuberculosis	Tyr64	Tyr197	Tyr413	Tyr638	Tyr711	1SJ2 [162]
B. Pseudomalle	Tyr68	Tyr201			Tyr719	1MWV [167]
Synechococcus PCC7942	Tyr51	Tyr184			Tyr691	1UB2[a]
H. Marismortui	Tyr52		Tyr404	Tyr628	Tyr702	1ITK [158]

[a] Wada, K., Tada, T. Crystal structure of catalase-peroxidase from *Synechococcus* PCC 7942. To be published.

7. Conclusions

I. Various methods have been successfully used by many researchers to identify the types of the free radicals formed in the reaction of haem proteins with hydroperoxides: Trp•, Trp-OO•, Tyr•, Cys•, Cys-OO•, His• or (His–Tyr)•. Our knowledge about the sites of these radicals in the proteins is also profound. But we still know very little about the mechanism of the radical formation, migration and transformation. In an attempt to generalize the current knowledge about the reaction mechanism, the following sequence of events can be suggested.

I.I. The first act of interaction of a haem protein in the oxidized state with a peroxide is formation of a complex between the haem and the peroxide, probably in a singly deprotonated form (Section 5.3.1). This very short lived complex has only been detected in the SW metMb/H_2O_2 system when the haem area of the protein was genetically modified [86].

I.II. Two electron reduction of the peroxide creates the oxoferryl haem iron state and a porphyrin π-cation radical. The oxoferryl state can be auto-reduced, either via direct intramolecular oxidation (Section 5.3.2) of a

protein residue or via the protonation mechanism (2) linked to oxidation of substrate molecule. The porphyrin π-cation radical plays a functional role in a number of enzymes. The life time of this radical in many enzymes is long enough for these species to be detected experimentally. Such radical is more difficult to detect in the oxygen binding haem proteins implying a significantly faster electron transfer rate from the globin to the haem.

I.III. The primary radical formed on a protein as a result of the porphyrin π-cation radical reduction is a cation radical of a residue with a low oxidation potential (Trp or Tyr). The cation radical, being a charged species, can oxidize another Trp or Tyr on the same protein with a very fast rate (tens of ps), thus creating a very fast transfer of the cation radical over a cascade of Trp and Tyr residues. If the terminal reducing agent for the final residue in the cascade is missing, the radical character can travel backwards with a lower rate (Section 5.3.2), thus creating a smeared (delocalised) cation radical. The deprotonation of the cation radical at a particular residue stops the very fast cation radical transfer at that residue and results in formation of a neutral radical, tryptophanyl or tyrosyl, which can be observed or spin trapped experimentally, with a much lower ability to be intramolecularly transferred any further (Section 5.3.2). Difference in the pK_1 values for the Trp and Tyr cation radicals, defined by the molecular environment of individual residues, governs the quantitative distribution of the neutral radicals of different kinds observed experimentally, this distribution thus being pH-dependent and would also depend on the temperature at which the reaction is conducted.

I.IV. If molecular oxygen is present in the medium, it acts as a natural spin trap for the tryptophanyl radicals but not for tyrosyl radicals (Section 5.1). Therefore in the majority of haem protein/peroxide systems the two main free radical species observed experimentally are tyrosyl radical and tryptophan peroxyl radical. Both Trp-OO• and Tyr• can oxidize other molecules (Section 3), i.e. either exogenously added substrate or the protein itself. The latter case is treated as intermolecular radical transfer (Section 5.3.3). The radicals shown to be formed as a result of the intermolecular transfer are found close to the surface of the proteins (Cys110• in human Mb, Tyr151• in SW Mb, Tyr146• in HH Mb and possibly in SW Mb). The solvent exposed radicals can interact with each other to form protein oligomers with the formation of Cys110–Cys110 cross-links in human Mb and Tyr151–Tyr151 cross-links in SW Mb. The thiyl radical on cysteine, like the tryptophanyl radical, can interact with molecular oxygen to form a peroxyl radical (Section 5.1).

II. The initial stage of interaction of the haem proteins with peroxides is not completely understood. The important questions to answer are which of the processes involved in the radical formation and transformation are intra- and which are intermolecular. The recently used approach of studying the mixtures of proteins with pre-defined different properties, which include native protein, apo-protein and site specific mutants (Sections 5.2.2.5 and 5.3.3), in a combination with the freeze-quench EPR technique that allows the detection of radicals in the millisecond range of reaction, seems promising in answering these questions in future studies.

III. Compound I can be detected over variable time after the initiation of the reaction with peroxides. Its intramolecular reduction by an amino acid residue results in formation of a protein bound cation radical that can be transferred very rapidly to the site of direct experimental detection, where the radical is deprotonated and transformed into a neutral radical (Section 5.3.2). Tryptophan and tyrosine residues have been identified as such sites. Tryptophan radicals in C*c*P and lignin peroxidase have been implicated in the catalytic function of the enzyme (Sections 6.1 and 6.2). The radical observed in bovine C*c*O and previously assigned to a tryptophan radical might need a re-assignment (Section 6.3). Tyrosine radicals, often found in enzymes, are also thought to be involved in the catalytic mechanism, e.g. Tyr385• in PGHS (Section 6.4.3), however, in some cases they might not play an important functional role as has been suggested for the tyrosyl radical in BLC (Section 6.4.1).

IV. Tyrosyl radical EPR spectra are very sensitive to the radical conformation defined by its environment. A simulation of the spectra can provide us with information about the rotation angle of the phenoxyl ring and the spin density on atom C1 of the tyrosine (Section 5.2.1). This information can be used in the identification of the tyrosine residue on which the radical has been formed (Sections 5.2.2.1, 5.2.2.2, 5.2.2.3, 6.3, 6.4.3 and 6.4.4). It is suggested that the site of the tyrosyl radical observed in two known isoforms of PGHS, when the enzyme is bound to a cyclooxygenase inhibitor before interacting with peroxides, is probably the same for both isoforms – Tyr504. This suggestion implies the same electron pathway in PGHS-1 and PGHS-2 complexed with the inhibitors, when reaction with peroxides is initiated (Section 6.4.3).

V. A new interpretation is given to the known effect of EPR signal evolution from the wide doublet to the narrow singlet in PGHS-1 during the reaction with peroxides. While strongly advocating the single site view and not supporting the idea of radical transfer to another site (Section 6.4.3), we suggest that the spectral changes during the reaction might be mainly caused by a relaxation of the hydrogen bond strength

in the radical, and not by the ring rotation (Section 6.4.3). The same mechanism might be responsible for the doublet-to-singlet EPR signal evolution in *M. tuberculosis* catalase-peroxidase (Section 6.4.4). The suggested interpretation thus explains in a simple way the similar effects that take place in homologically different enzymes.

VI. The method of site directed mutagenesis has proved to be useful in finding the location of the radicals observed in proteins. This method, however, can only confidently point to the residue which is not the radical's site (when the radical is still detected in the mutant with replaced residue). If, however, a mutation of a residue results in a loss of the detectable radical, it does not necessarily mean that the radical was on the mutated residue because a possibility always exists that the residue is on the pathway of electron transfer that leads to the formation of the detected radical elsewhere. Other methods of identification of the radical site in proteins are thus required. Identification of the tyrosyl radical residue in a protein by the rotational conformation of the phenoxyl ring becomes an important method now, after its accuracy has increased with the introduction of TRSSA (Section 5.2.1). Further development of this method can be seen in quantum mechanical calculations of the *g*-factors of the tyrosyl radicals as functions of their electrostatic environment, specific for tyrosines in different locations. A comparison of the calculated *g*-factors and those obtained from the experiments might significantly increase the accuracy of the tyrosyl radical site identification primarily based on a comparison of just rotation angles.

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