Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1655 (2004) 372–380
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Review

Tryptophan or tyrosine? On the nature of the amino acid radical formed following hydrogen peroxide treatment of cytochrome *c* oxidase

Dimitri A. Svistunenko*, Mike T. Wilson, Chris E. Cooper

Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, UK

Received 13 March 2003; accepted 24 June 2003

Abstract

It has been reported that different amino acid radicals are formed following the addition of hydrogen peroxide to cytochrome *c* oxidase (C*c*O) from bovine heart or from *Paracoccus denitrificans*. A broad unresolved signal in the electron paramagnetic resonance (EPR) spectra of bovine C*c*O has been assigned to a tryptophan radical, probably Trp126 [Rigby et al. *Biochemistry* 2000, 39, 5921–5928]. In the *P. denitrificans* enzyme, a similarly broad signal but with a well-resolved hyperfine structure was shown to originate from a tyrosyl radical and was tentatively assigned to the active site Tyr280 [MacMillan et al. *Biochemistry* 1999, 38, 9179–9184]. We confirm that the EPR signal from *P. denitrificans* C*c*O can be simulated using spectral parameters typical for known Tyr radicals in other systems. However, the rotational conformation of the phenolic ring of Tyr280 is inconsistent with our simulation. Instead, the simulation parameters we used correspond to the rotational conformation of ring that matches very accurately the conformation found in Tyr167, a residue that is close enough (~ 10 Å) to the binuclear centre to readily donate an electron. The broad unresolved EPR signal in the bovine oxidase has been thought previously to be inconsistent with a tyrosyl radical. However, we have simulated a hypothetical EPR spectrum arising from a Tyr129 radical (the equivalent of Tyr167 in *P. denitrificans* C*c*O) and showed that it is similar to the observed broad signal. The possibility exists, therefore, that the homological tyrosine amino acid (Tyr167/Tyr129) is responsible for the EPR spectrum in both the *Paraccoccus* and the bovine enzyme. This correspondence between the two enzymes at least allows the possibility that this radical may have functional importance.

Keywords: Tyrosine; Tryptophan; Free radical; Electron transfer; Cytochrome *c* oxidase; Hydrogen peroxide; EPR spectroscopy

1. Introduction

Jerry Babcock made many important contributions to bioenergetics through his insightful application of spectroscopic methods. Some of the most groundbreaking involved determining the role of amino acids as stable free radical sites, capable of catalysing electron transfer or redox chemistry [1]. His work was particularly influential in analysing the nature of the stable ‘‘dark free radical’’ observed in plants and showing that this originated from a tyrosine residue residing between the water splitting site and the ‘‘special pair’’ of chlorophyll molecules [2]. In more recent years, those of us in the cytochrome *c* oxidase (C*c*O) field benefited from Jerry’s rekindled interest in this enzyme [3]. In this latter work, one of his last, he demonstrated the same clarity of thought and experimental innovation that always characterised his research.

There is a variety of redox states of cytochrome oxidase where the spectroscopically observed signals appear unable to account for all the electrons that have been donated to the binuclear haem-copper active site. Jerry provided experimental evidence that the ‘‘lost electron’’ in this mechanism might be donated from a tyrosine residue (Tyr244 in bovine, Tyr280 in *Paracoccus denitrificans*), known to be covalently bound to a histidine residue in the binuclear haem-copper active site [3]. The same electron-donating site should also be available following hydrogen peroxide addition to the oxidised enzyme. In this paper we address this issue, and argue that an electron can be donated by a tyrosine residue 10 Å from the binuclear centre. It is a great shame, and our

Abbreviations: EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; MCD, magnetic circular dichroism; C*c*O, cytochrome *c* oxidase; PDB, Protein Data Bank

* Corresponding author. Tel.: +44-1206-873-149; fax: +44-1206-872-592.

E-mail address: svist@essex.ac.uk (D.A. Svistunenko).

0005-2728/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2003.06.006

scientific loss, that we would not be able to hear Jerry's opinions of this idea.

2. Electron paramagnetic resonance (EPR) spectra of bovine and *P. denitrificans* C*c*O mixed with H_2O_2

Hydrogen peroxide is a two-electron oxidant. Following its addition to ferric haem proteins the iron is generally oxidised to a ferryl ($Fe^{4+}=O^{2-}$) state and an electron is abstracted from the protein [4]. The latter can arise from donation by the porphyrin ring itself (e.g. catalase, horse radish peroxidase) or from other redox centres either tryptophan (e.g. yeast cytochrome *c* peroxidase), tyrosine (e.g. prostaglandin H synthase) or a second (ferrous) haem centre (e.g. cytochrome *c* peroxidase from *Pseudomonas aeroginosa*). These enzymes, peroxidases and catalases, are all designed to utilise hydrogen peroxide as a substrate. However, hydrogen peroxide addition is frequently used as a probe of the redox states, only transiently accessible during steady state turnover in many oxidases (e.g. cytochrome P450, cytochrome oxidase). In this case, the data must be treated with some caution as the nature of the species produced may not be on the normal reaction pathway in the absence of peroxide. In particular, any free radicals detected are frequently produced in amounts that are not stoichiometric with the metal sites—either these radicals are highly reactive and are quenched or (possibly) they are on side pathways to the primary reaction. This is exemplified by the situation of myoglobin and haemoglobin, proteins that have evolved as neither oxidases nor peroxidases, but in which the peroxide/ferryl/free radical pathway is linked to the aetiology of a range of disease processes [5].

A number of studies utilising either peroxide or oxygen (with the mixed valence species) as an oxidant for cytochrome oxidases have illuminated the nature of the highly oxidising redox states available to the binuclear centre. Two species, termed **P** and **F**, possessing distinct optical spectra, can be produced in varying amounts depending on the experimental conditions [6–8]. **P** and **F** have been detected in the "normal" reaction cycle of the enzyme [9] and both are now generally agreed to contain iron in the ferryl redox state. Given this assignment, it follows that a further electron must be donated by the enzyme in order to break the oxygen–oxygen bond. One suggestion is that the copper in the binuclear centre is oxidised from Cu^{II} to Cu^{III}. Alternatively, as suggested by Jerry Babcock, the electron may instead be donated from a tyrosine near the binuclear centre [5], generating a radical detectable by iodide trapping [3]. In both these cases, it is unlikely that the resulting spin system would be easily detected by conventional EPR spectroscopy (as the unpaired electron would be so close to the $S=1$ ferryl iron). However, it is possible to detect unpaired electrons by EPR following peroxide addition to C*c*O [7,8,10]. In 1995, Fabian and Palmer [7] reported the formation of two individual free radicals in bovine C*c*O on addition of hydrogen peroxide. The two radicals had a common *g*-factor, close to 2.00, but different peak-to-trough widths, 45 and 11 G (Fig. 1A), and different pH and microwave power saturation characteristics. The absence

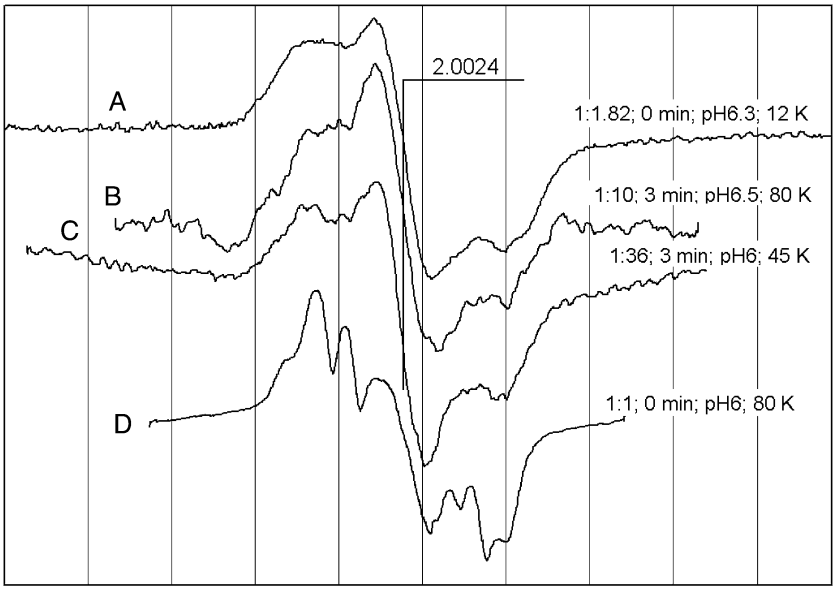

Fig. 1. The low temperature EPR spectra of the free radicals formed in bovine (A [7], B [10], C [11]) and *P. denitrificans* (D [12]) C*c*O after H_2O_2 treatment. The following parameters are indicated next to each spectrum: molar ratio oxidase:H_2O_2; time of reaction before a sample was frozen, min; pH of the reaction; temperature of spectrum registration. Oxidase concentrations were 110 μM (A), 100 μM (B), 14 μM (C) and 200 μM (D). The spectra presented in original papers were scanned, digitized and plotted on a common magnetic field axis (horizontal). A shift along the field axis was allowed for every spectrum in accordance with the different microwave frequencies used in the different X-band spectrometers. The gridlines are drawn at a 20-G interval. The *g*-factor of the narrow central feature in the bovine C*c*O spectra (2.0024 [10]) is indicated.

of a resolved hyperfine structure in the EPR spectra of these two species made it difficult to assign them to particular structures. Similar unresolved EPR spectra of bovine C*c*O mixed with H_2O_2 were reported later (Fig. 1B and C) [8,10,11].

The narrow signal was shown to have an electron nuclear double resonance (ENDOR) spectrum very similar to that of the horse radish peroxidase/H_2O_2 system. It was therefore assigned to a porphyrin cation radical [10]. Fabian and Palmer [7] originally rejected the possibility that either of the species observed in the bovine C*c*O/H_2O_2 system can be a porphyrin π-cation radical, as porphyrin π-cation radicals have specific optical and magnetic circular dichroism (MCD) spectra characteristics [13] which were not observed in the oxidase system. However, the fact that the concentration of the radicals causing the EPR signals in C*c*O seems to be much lower than the ferryl iron species and that it has not been possible to assign the EPR signals to a unique optical state of the enzyme, Rigby et al. [10] suggested that the characteristic optical/MCD signatures for a porphyrin π-cation radical might be too small to be readily detectable.

The ENDOR spectrum of the broad EPR free radical feature in the oxidase/H_2O_2 system was compared to that of cytochrome *c* peroxidase, known to originate from a tryptophan radical [14]. Although the comparison did not yield conclusive evidence for the formation of a Trp radical in bovine C*c*O, data obtained from H_2O/D_2O substitution provided additional support. A tryptophan amino acid, most probably Trp126 was therefore suggested as the originator of the "broad feature" [10]. The broad EPR signal is thought to be associated with the **F**-related state termed **F**·, which is usually formed at low pH and is isoelectronic with the $\mathbf{P_M}$ state, formed with CO and oxygen at high pH, but has a visible absorption band similar to that of **F** [11].

In contrast to bovine C*c*O, the broad radical spectrum of *P. denitrificans* oxidase with H_2O_2 (Fig. 1D) was assigned to a tyrosyl radical because the spectrum changed dramatically when (2,3,5,6-^{2}H)tyrosine was supplemented to the *P. denitrificans* cell culture [12]. A triplet spectrum was observed instead of the spectrum shown in Fig. 1D, and this was interpreted in terms of the unaffected hyperfine interaction with the methylene protons of a Tyr radical. MacMillan and coauthors suggested that Tyr280, the closest tyrosine to the binuclear centre, was the most likely site for the radical. This residue has an unusual covalent bond between its C3 (or C5) atom and the ε-nitrogen of His276, a ligand to Cu_B in the binuclear centre [15]. The importance of Tyr280 for Cu_B ligation and for the catalytic activity of the oxidase has been demonstrated [16].

3. Why do similar proteins give different radicals on H_2O_2 exposure?

Could it be that structurally similar enzymes under similar conditions of exposure to H_2O_2 exhibit formation of a radical on different amino acid residues—in the case of bovine oxidase a Trp but in *P. denitrificans* oxidase a Tyr? There is no reason why this should not be possible, but if this is the case then the role of such radicals may not be central to enzyme mechanism—the view we share with Rigby et al. [10]. We would like to address here an alternative possibility, namely that the radicals are on equivalent amino acids in the protein structure. We hope to show that the broad EPR signal in the bovine oxidase (Fig. 1A–C), in fact, originates from the same Tyr species as the broad signal in *P. denitrificans* oxidase (Fig. 1D), despite the resolution of the hyperfine coupling in the two systems being different. Whilst not disagreeing with the assignment in *P. denitrificans* to a Tyr radical [12], we will show that the suggested Tyr280 cannot account for the observed spectral features.

4. Tyrosyl radicals in proteins

It should be noted that Tyr (Fig. 2) is the 'usual suspect' when assigning a free radical spectrum of a protein to a specific amino acid residue. Tyrosyl radicals have been reported in ribonucleotide reductase from different sources

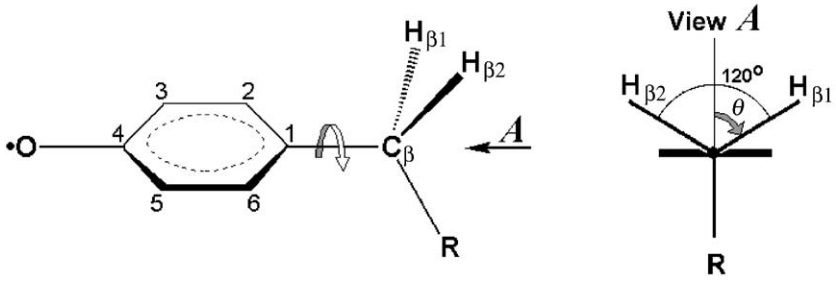

Fig. 2. Tyrosine and its radical form (shown here) can attain various conformations due to free rotation of the ring around the C_β–C1 bond. View *A* is defined here as when a tyrosine is looked at along C_β–C1 bond from C_β to C1 (and not otherwise), the ring plane being horizontal and C_β–R bond being below the ring plane. The rotation angle θ is defined for view *A* as shown (linked to $H_{\beta 1}$), positive angles corresponding to the clockwise directions.

[17–19], in ovine prostaglandin H synthase [20], in *Mycobacterium tuberculosis* catalase-peroxidase treated with peroxyacetic acid [21], in *Dactylium dendroides* galactose oxidase [22,23], in *Phanerochaete chrysosporium* glyoxal oxidase [24], in bovine catalase treated with H_2O_2 [25], in human haemoglobin, soybean leghaemoglobin, horse and sperm whale myoglobins treated with H_2O_2 [26–29], and in photosystem II [30,31]. Indeed, Jerry Babcock was among the first to suggest a possible involvement of a Tyr radical in the catalytic cycle of C*c*O and to recognise the similarity in the mechanisms of O–O bond formation in PSII and O–O bond cleavage in C*c*O [32,33]. However, in spite of the fact that a Tyr radical appears so frequently in protein systems, the possibility that it could be responsible for the broad EPR signal in bovine oxidase was dismissed from the very beginning, since the overall line width of the signal was much larger than that of the known tyrosyl radicals in proteins at that time [7]. We believe that this interpretation needs to be revised.

We report in this paper a simulation of the EPR spectrum of the radical formed on addition of H_2O_2 to *P. denitrificans* C*c*O, performed with the parameters typical for a Tyr radical (Fig. 3 and Table 1). The three components of the *g*-tensor are taken to be equal to those of the Tyr radical in *Salmonella typhimurium* ribonucleotide reductase [19]. The hyperfine splitting constants *A* for C3 and C5 hydrogen atoms were measured accurately by ENDOR for the tyrosyl Y_D radical in photosystem II [34]. The data on the C2 and C6 hydrogen atoms splitting constants, also for a tyrosyl Y_D, were taken from Ref. [31]. Euler angle ϕ is the angle between the *x*-axes of the *g*-tensor and of corresponding hyperfine splitting tensor. The individual line width (ΔH) components were considered variables in our simulation but were restrained by the condition that these values should be within a narrow interval of 3–5 G and should obey an axial anisotropy, i.e $\Delta H_y = \Delta H_z$. The only parameters which were allowed to vary in a significant interval, though again within an axial symmetry [34], were the hyperfine splitting constants of the methylene protons β1 and β2.

Table 1
The simulation parameters for the Tyr radical EPR spectrum B in Fig. 3[a]

	x	*y*	*z*	ϕ (°)	Reference
g	2.00897	2.00437	2.00217		[19]
$A^{\beta 1}$	53.78	46.17	46.17	0	[b]
$A^{\beta 2}$	39.66	34.05	34.05	0	[b]
A^{C3}	−25.6	−8.0	−19.1	22	[34]
A^{C5}	−27.5	−8.0	−20.5	−22	[34]
A^{C2}	4.75	7.54	1.12	10	[31]
A^{C6}	4.75	7.54	1.12	−10	[31]
ΔH	5.0	3.6	3.6		[b]

[a] *A*-values are given in MHz, ΔH (line width) in G.
[b] These parameters were varied to attain the best fit to the experimental spectrum.

5. Relating EPR spectra to structure: rotational conformation of phenoxyl ring in tyrosyl radical and phenolic ring in tyrosine

Knowing the parameters that provide a good correspondence between the simulated and experimental spectra (Fig. 3), we can find the angle of the phenoxyl ring rotation in the radical and then try to determine which Tyr residue in the crystal structure has a similar conformation.

The isotropic value of the hyperfine splitting constant for a β-proton is given by the empirical McConnell relation [35,36]:

$$A^{\beta}_{\mathrm{iso}} = \rho(B' + B'' \cos^2\theta) \quad (1)$$

where ρ is the spin density on C1 atom; θ is the phenoxyl ring rotation angle as defined on Fig. 2; B' and B'' are constants, B' being commonly neglected in practical applications and B'' being equal to 58 G (162 MHz) [37].

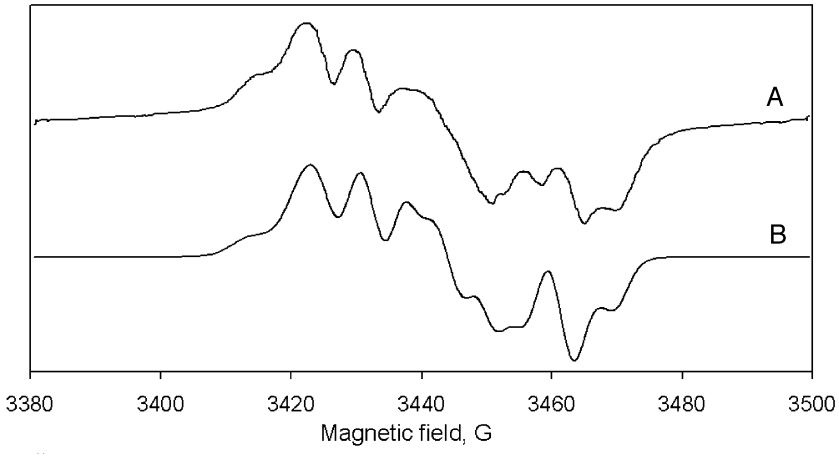

Fig. 3. (A) The EPR spectrum of the free radicals formed in *P. denitrificans* C*c*O on H_2O_2 treatment [12] (the same as spectrum D from Fig. 1); (B) the simulation of a Tyr radical spectrum using the parameters indicated in Table 1. The program simpow6 was used to simulate the spectrum (Mark Nilges, Illinois EPR Research Center, ftp://rbc6000.scs.uiuc.edu/pub/PowderPattern/simpow6).

Considering the angle between two methylene protons being equal to 120° (Fig. 2), we can write the system of equations:

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

The hyperfine splitting constants for the methylene protons (Table 1), averaged over directions x, y and z, can be considered as the isotropic values. This brings us to the system with two unknowns, θ and ρB″:

$$\left.\begin{aligned} 48.71 &= \rho B'' \cos^2\theta \\ 35.92 &= \rho B'' \cos^2(\theta - 120^\circ) \end{aligned}\right\} \quad (3)$$

This system has two pairs of solutions on the interval $-90 < \theta^\circ < +90$, with a particular value of ρB″ for each pair, the two possible values of θ within each pair being symmetrical around the values of -30° and $+60^\circ$, the two rotation angles at which the hyperfine splitting constants are equal for two methylene protons (Fig. 4).

When allowing $B'' = 58$ G [37], the two solutions with ρB″ = 60.4 G result in an unrealistic value of $\rho = 1.04$ (not only must ρ be less than 1, it should be considerably less than 1 because of the delocalisation of the spin density over the ring system). On the contrary, from the pair of solutions with ρB″ = 20.4 G, it follows that $\rho = 0.35$, which is in a good correspondence with the experimentally obtained data for the Tyr radical in *E. coli* ribonucleotide reductase (0.38) [38] and for the photosynthetic Tyr radical Y_D (0.37) [30,39]. The value of ρ we obtained is also very close to the calculated ρ for a related model compound (0.39) [40], if we adopt the value of B″ = 52.4 G calculated and used in that work.

The two possible rotation angles -22.5° and -37.5° are indistinguishable as far as the EPR method is concerned. Since each of the angles is only 7.5° different from $\theta = -30^\circ$, whichever of the two is the case in reality, the conformation of the tyrosine hosting such radical will be very unusual, with the R–C1 bond positioned practically in the ring plane. A similar conformation has only been reported once: for the Tyr103 radical formed in horse myoglobin following peroxide treatment [28]. Our next step will be to find out how many Tyr residues in *P. denitrificans* C*c*O are in a conformation close to either of these two angles.

6. Which Tyr residue in *P. denitrificans* C*c*O hosts the radical?

There are 46 Tyr residues in subunits A, B and C of *P. denitrificans* C*c*O; the fourth subunit D does not have any Tyr residues (Protein Data Bank http://www.rcsb.org/pdb/ file 1QLE). We have analyzed the rotational conformations of the phenolic ring in all tyrosine residues and expressed the result in terms of θ defined on the interval $(-90^\circ; +90^\circ)$ (Table 2). Clearly, ATyr280 cannot be responsible for the observed radical since its θ angle is very different from any of the two possible angles. In addition, Tyr280 generally seems an unlikely candidate from the point of view of our simulation which was performed for an intact (not bound) Tyr whereas Tyr280 has a covalent bond between C3 (or C5) and His276. A radical on Tyr280 would lack a hyperfine interaction with one of the C3/C5 protons ($I = 1/2$) and should have, instead, an interaction with the histidine's N_{δ}

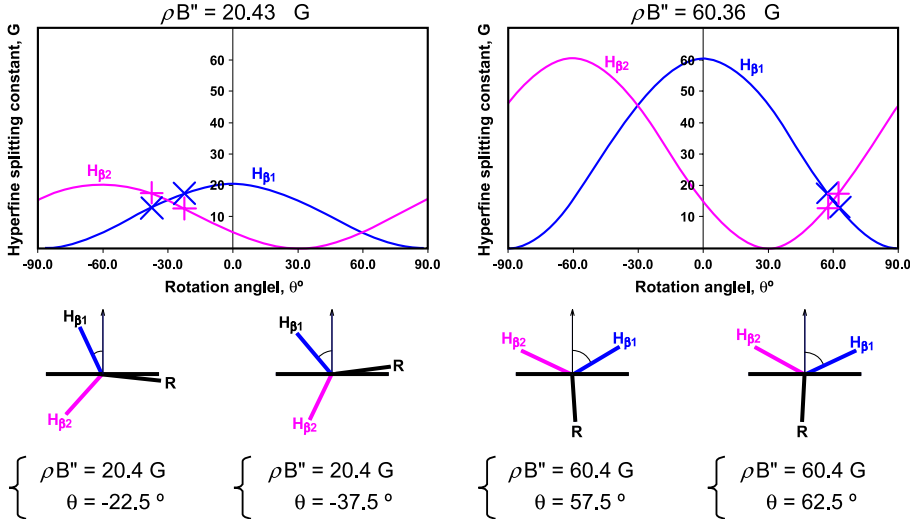

Fig. 4. The four solutions of system {3}, i.e. the four possible combinations of values θ (degree) and ρB" (G) that give the isotropic splitting constants for the methylene protons $H_{\beta 1}$ and $H_{\beta 2}$ consistent with empirically determined anisotropic values given in Table 1. Note that $((-22.5^\circ)+(-37.5^\circ))/2 = -30^\circ$ and $(57.5^\circ + 62.5^\circ)/2 = 60^\circ$.

Table 2
Phenolic ring rotation angle θ in all Tyr residues of *P. denitrificans* C*c*O (PDB file 1QLE) arranged in three different ways[a]

Part 1		Part 2			Part 3		
	θ, °		θ, °	|θ-(-22.5)|		θ, °	|θ-(-37.5)|
ATyr35	38.75	CTyr269	-19.04	3.46	ATyr167	-39.50	2.00
ATyr52	-78.38	BTyr40	-8.24	14.27	ATyr429	-45.54	8.04
ATyr64	16.18	ATyr167	-39.50	17.00	CTyr193	-48.73	11.23
ATyr93	66.48	ATyr328	-2.45	20.05	CTyr210	-49.20	11.70
ATyr114	44.71	ATyr429	-45.54	23.04	CTyr269	-19.04	18.46
ATyr135	62.52	CTyr193	-48.73	26.23	ATyr138	-59.29	21.79
ATyr138	-59.29	CTyr210	-49.20	26.70	ATyr475	-62.45	24.95
ATyr167	-39.50	ATyr402	6.63	29.13	BTyr40	-8.24	29.27
ATyr177	-83.27	ATyr494	6.90	29.41	ATyr328	-2.45	35.05
ATyr267	60.95	CTyr114	8.89	31.39	BTyr154	-74.21	36.71
ATyr280	61.23	ATyr138	-59.29	36.79	ATyr52	-78.38	40.88
ATyr305	77.92	ATyr64	16.18	38.68	ATyr402	6.63	44.13
ATyr328	-2.45	ATyr475	-62.45	39.95	ATyr494	6.90	44.41
ATyr339	63.50	BTyr154	-74.21	51.71	ATyr177	-83.27	45.77
ATyr402	6.63	CTyr88	32.83	55.33	CTyr114	8.89	46.39
ATyr406	83.41	ATyr52	-78.38	55.88	ATyr522	-84.61	47.11
ATyr407	50.62	BTyr149	35.25	57.75	CTyr191	-88.15	50.65
ATyr114	85.65	BTyr226	36.73	59.23	ATyr64	16.18	53.68
ATyr429	-45.54	ATyr177	-83.27	60.77	CTyr88	32.83	70.33
ATy430	87.77	ATyr35	38.57	61.07	BTyr149	35.25	72.75
ATyr440	39.35	ATyr440	39.35	61.85	BTyr226	36.73	74.23
ATyr475	-62.45	ATyr522	-84.61	62.11	ATyr35	38.57	76.07
ATyr478	69.73	CTyr8	41.98	64.48	ATyr440	39.35	76.85
ATyr484	56.40	CTyr191	-88.15	65.65	CTyr8	41.98	79.48
ATyr494	6.90	ATyr114	44.71	67.21	ATyr114	44.71	82.21
ATyr509	50.11	CTyr253	48.91	71.41	CTyr253	48.91	86.41
ATyr522	-84.61	ATyr509	50.11	72.61	ATyr509	50.11	87.61
BTyr40	-8.24	ATyr407	50.62	73.12	ATyr407	50.62	88.12
BTyr122	50.67	BTyr122	50.67	73.17	BTyr122	50.67	88.17
BTyr125	85.14	ATyr484	56.40	78.90	ATyr484	56.40	93.90
BTyr127	81.87	BTyr212	58.44	80.94	BTyr212	58.44	95.94
BTyr149	35.25	CTyr205	59.37	81.87	CTyr205	59.37	96.87
BTyr154	-74.21	ATyr267	60.95	83.45	ATyr267	60.95	98.45
BTyr212	58.44	ATyr280	61.23	83.73	ATyr280	61.23	98.73
BTyr226	36.73	ATyr135	62.52	85.02	ATyr135	62.52	100.02
BTyr239	77.66	ATyr339	63.50	86.00	ATyr339	63.50	101.00
CTyr8	41.98	ATyr93	66.48	88.98	ATyr93	66.48	103.98
CTyr60	81.86	ATyr478	69.73	92.23	ATyr478	69.73	107.23
CTyr88	32.83	BTyr239	77.66	100.16	BTyr239	77.66	115.16
CTyr114	8.89	ATyr305	77.92	100.42	ATyr305	77.92	115.42
CTyr191	-88.15	CTyr60	81.86	104.36	CTyr60	81.86	119.36
CTyr193	-48.73	BTyr127	81.87	104.37	BTyr127	81.87	119.37
CTyr205	59.37	ATyr406	83.41	105.91	ATyr406	83.41	120.91
CTyr210	-49.20	BTyr125	85.14	107.64	BTyr125	85.14	122.64
CTyr253	48.91	ATyr114	85.65	108.15	ATyr114	85.65	123.15
CTyr269	-19.04	ATy430	87.77	110.27	ATy430	87.77	125.27

[a] Part 1 is sorted ascending by Tyr number. Part 2 is sorted ascending by difference $|\theta^\circ - (-22.5^\circ)|$. Part 3 is sorted ascending by difference $|\theta^\circ - (-37.5^\circ)|$. The residues with θ less than 20° different with either of the two θ-values provided by the simulation of the experimental EPR spectrum are underlined. The residue originally suggested to be responsible for the radical (ATyr280) [12] is also marked.

($I=1$). This would have a dramatic effect on the EPR spectrum.

The accuracy of the angle θ determination from a protein's crystal structure is probably not greater than 15°, and in most cases is mush less (a few degrees), as can be judged from the comparison of the angles found for a particular Tyr residue when the structures reported by different authors are analysed. The accuracy of θ for the Tyr radical found from the simulation is ~ 2°. We suggest therefore that we look at differences of up to 20° between angle θ found from the crystal structure and θ determined by EPR. There are three Tyr residues that satisfy this criterion, if θ is considered to be − 22.5° (top three entries in Part 2, Table 2) and five residues if the angle is − 37.5°, two of which are the same as in the − 22.5° case (top five entries in Part 3). Thus, we have to analyze six Tyr residues as possible candidates for hosting the radical: ATyr167, ATyr429, BTyr40, CTyr193, CTyr210 and CTyr269.

As the free radicals observed in C*c*O on addition of H_2O_2 were not formed in samples pretreated with cyanide or formate or exposed to CO, the radical formation must be associated with the binuclear haem a_3-Cu_B centre [10]. Once a primary radical is formed in the vicinity of the binuclear centre, it could migrate rapidly over long distances to another location. Therefore, the free radical observed is not necessarily located on the closest Tyr to the binuclear centre. However, it is a reasonable conjecture that the probability that a free radical is 'trapped' by a particular Tyr residue decreases as the distance from the haem increases. Thus, it is unlikely that any of Tyr residues in the Tyr cluster in chain C, CTyr193, CTyr210 and CTyr269 (PDB file 1QLE for *P. denitrificans* C*c*O) is responsible for the radical observed as the cluster is about 40 Å from the binuclear centre. The distance from haem a_3 to ATyr429 is ~ 27 Å and that to BTyr40 is ~ 24 Å. Although free radicals can migrate over such distances in proteins, the last of the six candidate Tyr residues, ATyr167, is significantly closer to the binuclear centre and should be considered as the most likely candidate. The phenolic oxygen of ATyr167 is 12.9 Å from a_3 and 10.1 Å from Cu_B. Tyr167 is thus not only in the conformation closest to that suggested by the EPR spectral analysis ($|\Delta\theta| = 2^\circ$, Table 2), it is also the closest of the possible Tyr residues to the binuclear centre. We conclude therefore that this Tyr residue is the most likely site of the experimentally observed radical in *P. denitrificans* C*c*O.

7. Can the same Tyr residue in *P. denitrificans* and bovine C*c*O host the radical?

Fig. 5 shows the Trp and Tyr residues that are found in both *P. denitrificans* and bovine oxidases in the vicinity of the binuclear centre. Tyrosine (129/167) is highly conserved in the seed sequence alignment in the Pfarm A database (PF00115). In the four structures of COX 1 available, this tyrosine (ATyr167 in *P. denitrificans*, ATyr129 in bovine, ATyr175 in *R. Sphaeroides* and ATyr136 in Ba3 C*c*O from *T. Thermophilus*) occupies the same position. The Tyr-His motif is probably the site of primary radical formation. Recently, a chemical analogue of this motif was shown to have a decreased value of pK_a, as compared to the value of intact tyrosine, which is

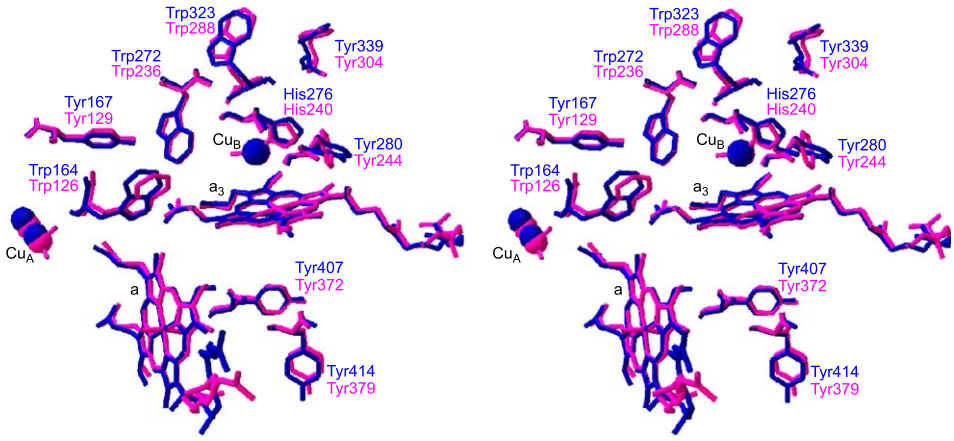

Fig. 5. A stereo view of the Tyr and Trp residues found in both *P. denitrificans* C*c*O (blue) and bovine C*c*O (pink) within 15 Å of haem a_3. PDB files 1QLE and 1OCC were used to generate the picture (note that these files do not provide visualisation of the covalent bonds between His276 and Tyr280 in *P. denitrificans* and between His240 and Tyr244 in bovine C*c*O).

thought to be important for facilitating proton delivery to the binuclear centre [41]. In the case of *P. denitrificans* oxidase, the Tyr-His radical could readily be transferred to Tyr167, the most likely residue responsible for the EPR detectable radical. It is quite possible that this transfer includes an intermediate radical state on one of the Trp residues of the triad Trp164, Trp272 and Trp323. It is also possible that, in the case of bovine oxidase, the radical does not finally go to Tyr129, but is localised instead on one of these tryptophans, e.g. on Trp126 as was suggested in [10]. Indeed, a role of tryptophan residues in a radical transfer mechanism in bovine C*c*O treated with organic peroxides, as well as similar roles for tyrosine and cysteine residues, has been suggested recently [42]. However, considering the great degree of similarity between the two oxidases, one should also consider the possibility that the process of radical transfer might also be similar in the two enzymes. Could it be Tyr129, not Trp126, that harbours the EPR-detectable radical in bovine C*c*O?

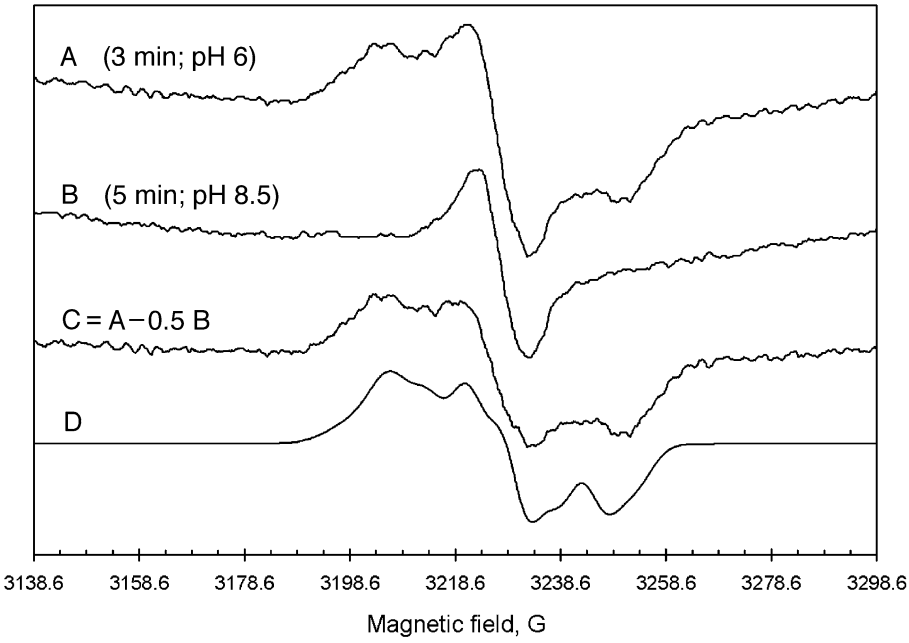

Fig. 6. A simulation of the broad EPR signal in 14 μM bovine C*c*O formed following the addition of 0.5 mM H_2O_2. Two samples were frozen at 3 min after initiation of the reaction at pH 6 (A) and at 5 min of reaction at pH 8.5 (B) [11]. Signal A is identical to signal C in Fig. 1. Signals A and B are digitized images of the spectra presented in the original paper [11]. C represents a pure line shape of the broad signal after the narrow singlet B was subtracted with a factor of 0.50. Signal D is a Tyr radical simulated spectrum (the parameters are given in Table 3) for a phenoxyl ring conformation characteristic of Tyr129, the homologous residue to Tyr167 in *P. denitrificans* C*c*O. Simpow6 was used to simulate the spectrum (see credits in Fig. 3 caption).

Both Tyr129 in bovine oxidase and Tyr167 in *P. denitrificans* are characterised by a similarly unusual conformation of the phenolic ring – sthe R–C1 bond in both residues is located practically in the ring's plane (Fig. 5) with θ angle being close to $-30°$ ($-29.5°$ and $-39.5°$, respectively, PDB files 1OCC and 1QLE). This means that if Tyr129 were in a free radical state, its EPR spectrum should be unusually wide (~ 40 G), like the observed spectrum in *P. denitrificans* C*c*O (Fig. 1D), or the horse Mb spectrum attributable to the Tyr103 radical [28] for which $\theta = -31.68°$ (PDB file 1WLA). We have used the bovine C*c*O spectra published in Ref. [11] (Fig. 6A and B) to obtain a pure line shape of the broad signal (Fig. 6C). We then simulated this broad signal (Fig. 6D) using basically the parameters we used to simulate the *P. denitrificans* C*c*O spectrum (Fig. 3 and Table 1). However, this time we did not vary the hyperfine splitting constants for the methylene protons, but calculated them assuming that the spin density on C1 atom of Tyr129 radical (bovine C*c*O) is the same as that for Tyr167 radical in *P. denitrificans* C*c*O ($\rho B'' = 20.4$ G). The other parameter we need in order strictly to define the isotropic hyperfine splitting constants for the methylene protons is the rotation angle θ, which we found to be $-29.5°$ in the 1OCC PDB file. Thus, the only parameters we are left with to vary in order to attain a correspondence to the experimental spectrum are the line width components. These we had to increase to 8, 6 and 6 G (see Table 3).

The simulated spectrum corresponds well to the pure line shape of the broad signal that we have obtained by a spectral subtraction procedure (Fig. 6C and D). We conclude therefore that the wide EPR signal reported for bovine C*c*O [7,8,10,11] *can* originate from Tyr129. The only significant difference required from the *Paracoccus* tyrosyl spectrum (apart from a slightly different θ) is an increase in the individual line widths from an average value of $(5+3.6+3.6)/3 = 4.1$ G to $(8+6+6)/3 = 6.7$ G. This is only a 2.6 increment, but it results in a loss of the observed hyperfine structure. How justified is this increase? This is difficult to determine in the absence of a complete quantum mechanical description of the tyrosyl radicals in question. We do note, however, that there are precedents in other proteins, where tyrosyl radicals are characterised by an unresolved singlet EPR line at low temperature, although their spectra are well resolved in the liquid phase, e.g. human Hb and sperm whale Mb treated with H_2O_2 [26].

Table 3
The simulation parameters for the Tyr radical EPR spectrum D in Fig. 6[a]

	x	*y*	*z*	ϕ (°)	Reference
g	2.00897	2.00437	2.00217		[19]
$A^{\beta 1}$	47.81	41.05	41.05	0	[b]
$A^{\beta 2}$	46.91	40.28	40.28	0	[b]
A^{C3}	−25.6	−8.0	−19.1	22	[34]
A^{C5}	−27.5	−8.0	−20.5	−22	[34]
A^{C2}	4.75	7.54	1.12	10	[31]
A^{C6}	4.75	7.54	1.12	−10	[31]
ΔH	8.0	6.0	6.0		[c]

[a] *A*-values are given in MHz, line width ΔH-values in G.

[b] The isotropic values for $A^{\beta 1}$ and $A^{\beta 2}$ were calculated within the following suggestions: (i) $\rho B''$' for the Tyr radical in bovine C*c*O is the same as for Tyr167 radical in *P. denitrificans* C*c*O, 20.4 G (see Fig. 4) and (ii) $\theta = -29.5°$, which is the value found from the crystal structure (PDB file 1OCC) for Tyr129 (homologous to Tyr167 in *P. denitrificans* C*c*O). Then the indicated *x*, *y* and *z* values for $A^{\beta 1}$ and $A^{\beta 2}$ were found from the isotropic values and with the suggestion that the degree of anisotropy $A^{\beta}_x / A^{\beta}_y$ is the same in Tyr129 in bovine C*c*O and in Tyr167 in *P. denitrificans* C*c*O.

[c] These parameters were varied to attain the best fit to the experimental spectrum.

8. Conclusion

We stress that the simulations reported here do not form an unequivocal proof of the identity of the amino acid radicals resulting in the broad EPR signal. Additional data are necessary to discriminate between the rival hypotheses. For example, we note that spin trapping data using 2-methyl-2-nitrosopropane (MNP) are consistent with the formation of a Tyr radical at some stage following H_2O_2 treatment of bovine C*c*O [43]. It is likely that appropriate mutagenesis and labelling studies will be required for a definitive assignment. Nevertheless, we have shown that it is at least possible to rationalise the EPR data with a model whereby a common amino acid is responsible for donating an electron to the active site binuclear centre following peroxide addition. This at least keeps the possibility "alive" that tyrosyl radicals, and in particular Tyr129(167) radical, may play a role in the mechanism of C*c*O.

Acknowledgements

This work was supported by the Wellcome Trust.

References

[1] G.T. Babcock, M. Espe, C. Hoganson, N. Lydakis-Simantiris, J. McCracken, W. Shi, S. Styring, C. Tommos, K. Warncke, Tyrosyl radicals in enzyme catalysis: some properties and a focus on photosynthetic water oxidation, Acta Chem. Scand. 51 (1997) 533–540.
[2] G.T. Babcock, Proteins, radicals, isotopes, and mutants in photosynthetic oxygen evolution, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 10893–10895.
[3] D.A. Proshlyakov, M.A. Pressler, C. DeMaso, J.F. Leykam, D.L. DeWitt, G.T. Babcock, Oxygen activation and reduction in respiration: involvement of redox-active tyrosine 244, Science 290 (2000) 1588–1591.
[4] C.E. Cooper, Ferryl iron and protein free radicals, in: C.A. Rice-Evans, R.H. Burdon (Eds.), Free radical damage and its control, Elsevier, Amsterdam, 1994, pp. 65–109.
[5] M.R.A. Blomberg, P.E.M. Siegbahn, G.T. Babcock, M. Wikström,

Modeling cytochrome oxidase: a quantum chemical study of the O–O bond cleavage mechanism, J. Am. Chem. Soc. 122 (2000) 12848–12858.
[6] J.M. Wrigglesworth, Formation and reduction of a 'peroxy' intermediate of cytochrome *c* oxidase by hydrogen peroxide, Biochem. J. 217 (1984) 715–719.
[7] M. Fabian, G. Palmer, The interaction of cytochrome oxidase with hydrogen peroxide: the relationship of compounds P and F, Biochemistry 34 (1995) 13802–13810.
[8] S. Junemann, P. Heathcote, P.R. Rich, The reactions of hydrogen peroxide with bovine cytochrome *c* oxidase, Biochim. Biophys. Acta 1456 (2000) 56–66.
[9] G.T. Babcock, M. Wikström, Oxygen activation and the conservation of energy in cell respiration, Nature 356 (1992) 301–309.
[10] S.E. Rigby, S. Junemann, P.R. Rich, P. Heathcote, Reaction of bovine cytochrome *c* oxidase with hydrogen peroxide produces a tryptophan cation radical and a porphyrin cation radical, Biochemistry 39 (2000) 5921–5928.
[11] P.R. Rich, S.E. Rigby, P. Heathcote, Radicals associated with the catalytic intermediates of bovine cytochrome *c* oxidase, Biochim. Biophys. Acta 1554 (2002) 137–146.
[12] F. MacMillan, A. Kannt, J. Behr, T. Prisner, H. Michel, Direct evidence for a tyrosine radical in the reaction of cytochrome *c* oxidase with hydrogen peroxide, Biochemistry 38 (1999) 9179–9184.
[13] M.J. Rodriguez Maranon, D. Mercier, R.B. van Huystee, M.J. Stillman, Analysis of the optical absorption and magnetic-circular-dichroism spectra of peanut peroxidase: electronic structure of a peroxidase with biochemical properties similar to those of horseradish peroxidase, Biochem. J. 301 (1994) 335–341.
[14] B.M. Hoffman, J.E. Roberts, C.H. Kang, E. Margoliash, Electron paramagnetic and electron nuclear double resonance of the hydrogen peroxide compound of cytochrome *c* peroxidase, J. Biol. Chem. 256 (1981) 6556–6564.
[15] C. Ostermeier, A. Harrenga, U. Ermler, H. Michel, Structure at 2.7 A resolution of the *Paracoccus denitrificans* two-subunit cytochrome *c* oxidase complexed with an antibody FV fragment, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10547–10553.
[16] T.K. Das, C. Pecoraro, F.L. Tomson, R.B. Gennis, D.L. Rousseau, The post-translational modification in cytochrome *c* oxidase is required to establish a functional environment of the catalytic site, Biochemistry 37 (1998) 14471–14476.
[17] P. Reichard, A. Ehrenberg, Ribonucleotide reductase—a radical enzyme, Science 221 (1983) 514–519.
[18] M. Sahlin, A. Graslund, A. Ehrenberg, B.M. Sjoberg, Structure of the tyrosyl radical in bacteriophage T4-induced ribonucleotide reductase, J. Biol. Chem. 257 (1982) 366–369.
[19] P. Allard, A.L. Barra, K.K. Andersson, P.P. Schmidt, M. Atta, A. Gräslund, Characterization of a new tyrosyl free radical in *Salmonella typhimurium* ribonucleotide reductase with EPR at 9.45 and 245 GHz, J. Am. Chem. Soc. 118 (1996) 895–896.
[20] P. Dorlet, S.A. Seibold, G.T. Babcock, G.J. Gerfen, W.L. Smith, A.L. Tsai, S. Un, High-field EPR study of tyrosyl radicals in prostaglandin H_2 synthase-1, Biochemistry 41 (2002) 6107–6114.
[21] S. Chouchane, S. Girotto, S. Yu, R.S. Magliozzo, Identification and characterization of tyrosyl radical formation in *Mycobacterium tuberculosis* catalase-peroxidase (KatG), J. Biol. Chem. 277 (2002) 42633–42638.
[22] G.T. Babcock, M.K. El-Deeb, P.O. Sandusky, M.M. Whittaker, J.W. Whittaker, Electron-paramagnetic resonance and electron nuclear double-resonance spectroscopies of the radical site in galactose-oxidase and of thioether-substituted phenol model compounds, J. Am. Chem. Soc. 114 (1992) 3727–3734.
[23] M.M. Whittaker, W.R. Duncan, J.W. Whittaker, Synthesis, structure, and properties of a model for galactose oxidase, Inorg. Chem. 35 (1996) 382–386.
[24] M.M. Whittaker, P.J. Kersten, N. Nakamura, J. Sandersloehr, E.S. Schweizer, J.W. Whittaker, Glyoxal Oxidase From *Phanerochaete-Chrysosporium* Is a New Radical- Copper Oxidase, J. Biol. Chem. 271 (1996) 681–687.
[25] A. Ivancich, H.M. Jouve, J. Gaillard, EPR evidence for a tyrosyl radical intermediate in bovine liver catalase, J. Am. Chem. Soc. 118 (1996) 12852–12853.
[26] D.A. Svistunenko, J. Dunne, M. Fryer, P. Nicholls, B.J. Reeder, M.T. Wilson, M.G. Bigotti, F. Cutruzzola, C.E. Cooper, Comparative study of tyrosine radicals in hemoglobin and myoglobins treated with hydrogen peroxide, Biophys. J. 83 (2002) 2845–2855.
[27] M.J. Davies, A. Puppo, Direct detection of a globin-derived radical in leghemoglobin treated with peroxides, Biochem. J. 281 (1992) 197–201.
[28] M.R. Gunther, B.E. Sturgeon, R.P. Mason, A long-lived tyrosyl radical from the reaction between horse metmyoglobin and hydrogen peroxide, Free Radic. Biol. Med. 28 (2000) 709–719.
[29] M.R. Gunther, R.A. Tschirret-Guth, H.E. Witkowska, Y.C. Fann, D.P. Barr, P.R. Ortiz de Montellano, R.P. Mason, Site-specific spin trapping of tyrosine radicals in the oxidation of metmyoglobin by hydrogen peroxide, Biochem. J. 330 (1998) 1293–1299.
[30] C. Tommos, X.-S. Tang, K. Warncke, C.W. Hoganson, S. Styring, J. McCracken, B.A. Diner, G.T. Babcock, Spin-density distribution, conformation, and hydrogen bonding of the redox-active tyrosine Yz in Photosystem II from multiple-electron magnetic-resonance spectroscopies: implications for photosynthetic oxygen evolution, J. Am. Chem. Soc. 117 (1995) 10325–10335.
[31] C.W. Hoganson, G.T. Babcock, Protein-tyrosyl radical interactions in photosystem II studied by electron spin resonance and electron nuclear double resonance spectroscopy: comparison with ribonucleotide reductase and in vitro tyrosine, Biochemistry 31 (1992) 11874–11880.
[32] C.W. Hoganson, G.T. Babcock, A metalloradical mechanism for the generation of oxygen from water in photosynthesis, Science 277 (1997) 1953–1956.
[33] C.W. Hoganson, M.A. Pressler, D.A. Proshlyakov, G.T. Babcock, From water to oxygen and back again: mechanistic similarities in the enzymatic redox conversions between water and dioxygen, Biochim. Biophys. Acta 1365 (1998) 170–174.
[34] S.E. Rigby, J.H. Nugent, P.J. O'Malley, The dark stable tyrosine radical of photosystem 2 studied in three species using ENDOR and EPR spectroscopies, Biochemistry 33 (1994) 1734–1742.
[35] H.M. McConnell, Indirect hyperfine interactions in the paramagnetic resonance spectra of aromatic free radicals, J. Chem. Phys. 24 (1956) 764–766.
[36] H.M. McConnell, D.B. Cheshunt, Theory of isotropic hyperfine interaction in p-electron radicals, J. Chem. Phys. 28 (1958) 107–117.
[37] R.W. Fessenden, R.H. Schuler, Electron spin resonance studies of transient alkyl radicals, J. Chem. Phys. 39 (1963) 2147–2195.
[38] C.W. Hoganson, M. Sahlin, B.-M. Sjöberg, G.T. Babcock, Electron magnetic resonance of the tyrosyl radical in ribonucleotide reductase from *Escherichia coli*, J. Am. Chem. Soc. 118 (1996) 4672–4679.
[39] K. Warncke, G.T. Babcock, J. McCracken, Structure of the Y_D tyrosine radical in photosystem II as revealed by ^2H electron spin echo envelope modulation (ESEEM) spectroscopic analysis of hydrogen hyperfine interactions, J. Am. Chem. Soc. 116 (1994) 7332–7340.
[40] F. Himo, A. Graslund, L.A. Eriksson, Density functional calculations on model tyrosyl radicals, Biophys. J. 72 (1997) 1556–1567.
[41] J.A. Cappuccio, I. Ayala, G.I. Elliott, I. Szundi, J. Lewis, J.P. Konopelski, B.A. Barry, O. Einarsdottir, Modeling the active site of cytochrome oxidase: synthesis and characterization of a cross-linked histidine-phenol, J. Am. Chem. Soc. 124 (2002) 1750–1760.
[42] Y.R. Chen, R.P. Mason, Mechanism in the reaction of cytochrome *c* oxidase with organic hydroperoxides: an ESR spin-trapping investigation, Biochem. J. 365 (2002) 461–469.
[43] Y.R. Chen, M.R. Gunther, R.P. Mason, An electron spin resonance spin-trapping investigation of the free radicals formed by the reaction of mitochondrial cytochrome *c* oxidase with H_2O_2, J. Biol. Chem. 274 (1999) 3308–3314.