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Observation of Protein-derived (BSA) Oxygen-centered Radicals by EPR Spin-trapping Techniques

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Electron paramagnetic resonance (EPR) spin-trapping experiments, employing the novel spin-trap DEPMPO, provide evidence for the formation of protein-peroxyl radicals from the reaction of bovine serum albumin (BSA) or lysozyme with HO˙ in the presence of O_2. Spin-trapping leads to the detection of anisotropic spectra of partially immobilized protein-peroxyl spin-adducts; positive identification is based on a novel spectrum simulation approach (through which broadened *anisotropic* spectra are simulated and compared with experiment) and by comparison of results with those obtained when $MeO_2^{\cdot}$ is trapped and the adduct frozen in a solid matrix.

Keywords: Spin-trapping; Anisotropy; Protein peroxyl; DEPMPO; Spectral simulations

INTRODUCTION

It has been widely claimed that free radicals are present in biological media, both through the operation of important biological functions and also by way of adventitious processes, such as leakage in the respiratory chain.[1–5]

Proteins are one of the most important targets for free radicals *in vivo* and it has been suggested that radical attack can lead to a various types of damage including aggregation, fragmentation, amino-acid modification and changes in proteolytic properties.[6–14] Specific diseases (e.g. atherosclerosis) may result from protein attack.[15–17] Recently, it has also been shown that hydroperoxides, which are believed to result from the radical reactions of peptides and proteins in the presence of oxygen (see Scheme 1),[18] cause diminution of cellular defence mechanisms (e.g. via loss of biological reductants such as ascorbic acid and glutathione).[19] It is also believed that such hydroperoxides are highly susceptible to one-electron reduction in the presence of low-valent transition-metal ions (e.g. Fe^{II}) to give further oxygen-centered radicals whose reactions may include the initiation of cellular damage, to DNA and other components.[20–23]

Spin-trapping techniques, in conjunction with electron paramagnetic resonance (EPR) spectroscopy, are now used increasingly in the detection and identification of reactive free radicals in biological systems.[24–26] Cyclic nitrones with a five-membered ring [e.g. DEPMPO (1) and DMPO (2), see scheme 2] are especially useful traps which provide information on the nature of the trapped radical (e.g. carbon-, sulfur-, or oxygen-centered), largely as a result of the well-established angular dependence of the β-proton splitting in the resulting adducts; for the novel phosphorylated spin-trap DEPMPO (1), additional structural information can be derived from the phosphorus splitting.[27–31] It has also been claimed that such cyclic nitrones can

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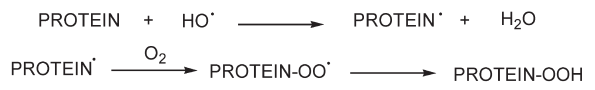
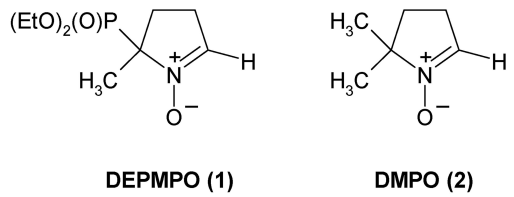


SCHEME 1

effectively trap peroxyl radicals, as might be anticipated, though as has been pointed out by Mason, the appropriate adducts obtained with DMPO in water, are in fact those of the corresponding alkoxyl radicals ($RO^{\cdot}$), these presumably having been formed from self-termination reactions of the appropriate peroxyl radicals.[32] For example, Dikalov and Mason[33] have provided evidence that signals from DMPO adducts previously assigned to polyunsaturated fatty acid peroxyl radicals ($PUFA-O_2^{\cdot}$) are in fact the corresponding PUFA-$O^{\cdot}$ alkoxyl adducts. Peroxyl adducts are clearly very elusive and as far as we are aware, no macromolecular (e.g. protein-derived) peroxyl radicals have ever been detected, not least because of the relatively weak bond to be formed with the spin-trap and the anticipated steric hindrance of the reaction. Nevertheless, spin-trapping of a species assigned to methylperoxyl radical with DEPMPO in water has been described previously[30] and the apparent ability of DEPMPO to trap peroxyl radicals (with $a_{H\beta}$ typically 0.9–1.15 mT) and give spectra distinct from the corresponding alkoxyl spin adducts (with $a_{H\beta}$ typically 0.8–0.9 mT) has been reported.(Karoui *et al.* unpublished work and Ref. [34]).



SCHEME 2

We report the use of EPR spectroscopy in conjunction with spin-trapping to study the formation of such species in the reaction of bovine serum albumin (BSA) and lysozyme with $HO^{\cdot}$ and O_2. DEPMPO has been employed on account both of the extra information potentially available, and the recognition that its adducts of $O_2^{-\cdot}$ have a significantly longer lifetime in aqueous solution than the corresponding adducts of DMPO and related acyclic nitrones.[28–35] Further, the EPR spectrum of the $HO_2^{\cdot}$ adduct of DEPMPO shows the existence of *trans* (90%) and *cis* (10%) diastereoisomers (arising from *trans* or *cis* addition of the peroxyl radical relative to the bulky phosphorus group), the former of which shows line-broadening characteristic of conformational change also noted for peroxyl adducts in organic solvents.[36,37] These unusual conformational properties provide a highly distinctive EPR spectral pattern which is expected to be reflected in the spectra of peroxyl adducts.[27]

We have previously demonstrated that analysis via automated spectral simulation of anisotropic or immobilized spectra (with appropriate nitrogen, hydrogen and phosphorus splittings) can substantially assist the process of obtaining averaged hyperfine splittings with which to characterize the spectra of macromolecular radicals or spectra recorded in frozen matrices.[38–40] This method has been employed here in conjunction with an approach which employs frozen aqueous solutions, the latter providing further advantages including the increase in the signal-to-noise level (resulting from the increase in volume of the sample in the EPR cavity) and the increased stability anticipated for the peroxyl adducts at low temperature in a matrix.

EXPERIMENTAL

Chemicals

BSA and lysozyme were purchased from Sigma and used as supplied. DEPMPO was synthesized as described previously.[41] All solutions were prepared in 100 mM phosphate buffer at pH 7.0, using high-purity deionized water. For all experiments, Fe(II)-ethylenediamine tetraacetic acid (Fe^{2+}/EDTA) solutions were freshly made up in high-purity deionized water only, without degassing and used immediately after solid ($FeSO_4{\cdot}7H_2O$, EDTA) dissolution. All concentrations given are those after mixing.

EPR Experiments

The EPR experiments typically involved mixing of solutions of the protein (5–10 mM), DEPMPO (2.5 mM), hydrogen peroxide (2.0 mM) and Fe^{2+}/EDTA (2.0 mM) (added last). Experiments were typically carried out at 0°C in an ice bath. The mixture was then transferred rapidly to an EPR flat cell and the EPR spectrum recorded; for experiments carried out at low temperature, mixtures were introduced into an NMR tube and then frozen directly in the EPR cavity in a Dewar; samples were not degassed before use.

For formation of DEPMPO/CH_3–$OO^{\cdot}$ (experiment under O_2), oxygen saturation was obtained by bubbling O_2 (1 h) through the buffered solution prior to reaction. For reaction of BSA and lysozyme, the buffer solution was saturated with oxygen before protein dissolution (1 h), and then O_2 was blown on the surface of the protein solution (1 h) with stirring.

The methoxy adduct of DEPMPO was generated by nucleophilic addition of sodium methoxide solution (1 M) to DEPMPO (final concentration: 100 mM) in methanol with subsequent aerial oxidation. The mixture was then diluted 3 times with phosphate buffer. The solution was deoxygenated before EPR measurements by bubbling with N_2 gas.

EPR Spectroscopy

The EPR measurements were carried out on Bruker ESP 300 and EMX (X-band) spectrometers at room temperature unless otherwise stated. Reagent concentrations and spectrometer settings are given in the text and in the relevant figure legends.

Computational Approach

The EPR simulation program, employed previously,[40] simulates the effect of slow tumbling by using effective tensorial elements for Zeeman and hyperfine interactions. The principal axes of interactions (namely the g, A_N, A_H and A_P tensors) are assumed to be parallel. Residual motion in slowly tumbling radical-adducts is taken into account by inclusion of an orientation-dependence of the line-width tensors. All tensorial elements were adjusted by combining iteration and least-square procedures, with a search for the minimum square deviation between experimental and computed spectra.

For each spectrum simulation reported, we also started from different but realistic parameter sets and the iteration converged to the same (optimum) values. Iterations trapped in local minima (which were rejected) were recognized by the significantly larger least square error. The program is available upon request from Prof. A. Rockenbauer (E-mail: rocky@cric.chemres.hu).

RESULTS AND DISCUSSION

Model Experiments: Characterization of DEPMPO Adducts of Methylperoxyl and Methoxy Species

With the aim to study protein peroxyl radicals and to establish the efficacy of this spin-trapping and simulation approach, we first carried out experiments with $HO^{\cdot}$ and DMSO (a reaction which generates the methyl radical). In anoxic conditions, addition of Fe^{2+} to a mixture of DMSO (10% v/v), H_2O_2 and DEPMPO in buffer solution led to the formation of a 12-line spectrum characteristic of the DEPMPO/$CH_3^{\cdot}$ adduct, with $a_N = 1.50$, $a_P = 4.74$, $a_H = 2.21$ mT (Fig. 1a). However, on saturating the solution with oxygen a different radical is trapped with a much smaller overall width (and a_H ca. 1 mT, Fig. 1b). This species is assigned to the DEPMPO/$CH_3OO^{\cdot}$ spin adduct rather than the methoxy adduct for reasons given below; apparently the methyl radical reacts with O_2 at a diffusion-controlled rate to form $CH_3OO^{\cdot}$ which is trapped with DEPMPO to give a peroxyl adduct.

Signals from only one adduct could be discerned, presumably from the *trans* adduct. We believe that, under our conditions, the minor (*cis*) adduct may be present but is masked by the signals from the dominant adduct. The optimum spectrum simulation (Fig. 1c) was achieved by considering an exchange between two conformers of this adduct, as noted previously for $HO_2^{\cdot}$.[30–37] An adequate simulation of the spectrum was obtained without inclusion of a contribution from a second adduct. As DEPMPO/$CH_3OO^{\cdot}$ and DEPMPO/$HOO^{\cdot}$ adducts exhibit closely similar splitting patterns, the experiment was repeated in the presence of superoxide dismutase, SOD, which is expected to remove $HO_2^{\cdot}$ and $O_2^{\cdot-}$; the spectrum was unaffected, from which we conclude that it is not therefore from the DEPMPO/$HOO^{\cdot}$ adduct.

In order to provide further evidence that spectra attributed to peroxyl adducts [$MeOO^{\cdot}$ and protein-derived peroxyl (from BSA and lysozyme)], are not due to adducts of the appropriate alkoxyl radicals, we generated the methoxy adduct of DEPMPO via nucleophilic attack of MeO^- on the nitrone and subsequent oxidation.[42] The isotropic spectrum obtained (Fig. 1d) is a mixture of two diastereoisomeric adducts [simulated with isotropic parameters, (*trans*):$a_N = 1.34$, $a_P = 4.74$, $a_H = 0.92$ mT; (*cis*) : $a_N = 1.36$, $a_P = 4.02$, $a_H = 0.83$ mT], i.e. a distinctly different pattern from those observed for methyl peroxyl adduct. Similar results have been obtained in experiments in which alkoxy adducts of DEPMPO were produced from alcohols (ROH, R = C_nH_{2n+1}, $n = 1$ to 10) and lead tetra-acetate or photolysis of diethylperoxide (EtOOEt) in the presence of DEPMPO.[37]

Reactions of BSA and Lysozyme with $HO^{\cdot}$ Under Normoxic Conditions

Addition of Fe^{2+}/EDTA to an aqueous solution of BSA, H_2O_2 and DEPMPO in normoxic conditions, at 0°C led to the immediate detection of a strong and anisotropic EPR signal shown in Fig. 2a and as observed previously[40] at 25°C in both anoxic and normoxic conditions. The optimized spectrum simulation shown in Fig. 2b was obtained using the parameters listed in Table I; the agreement between experimental and calculated spectra is excellent. The large *average* β-hydrogen splitting obtained (2.13 mT) and the *average* β-phosphorus value (4.82 mT) are typical of the spin-trapping of a *carbon*-centered radical and can be compared to isotropic values obtained for (small) carbon-centered radicals.[27] Further evidence to support this

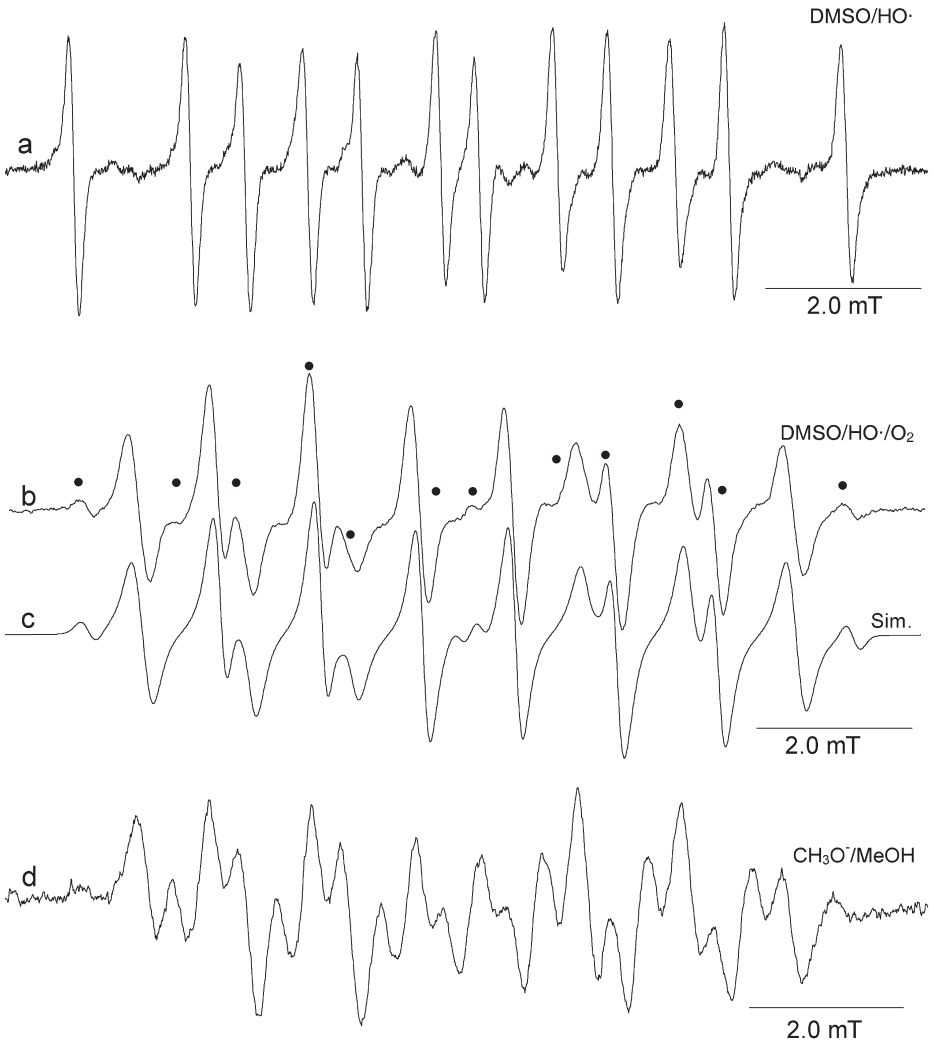


FIGURE 1 (a) EPR spectrum of DEPMPO/$CH_3^{\cdot}$ radical adduct formed in reaction of DMSO with Fe^{2+}/H_2O_2 in presence of DEPMPO, (b) EPR spectrum of DEPMPO-/CH_3–$OO^{\cdot}$ radical adduct formed in reaction of DMSO with Fe^{2+}/H_2O_2 in presence of DEPMPO and O_2 at 0°C, the weak signals indicated (•) are attributed to the methyl-radical adduct (3.0%), (c) simulated spectrum (for parameters see Table I). (d) EPR spectrum of methoxy adducts of DEPMPO formed in reaction of MeONa with DEPMPO in MeOH followed by aerial oxidation, dilution 3 times with phosphate buffer and N_2 degassing. Concentrations: (a) DEPMPO 10.0 mM, DMSO 10% (v/v), Fe^{2+} 2.0 mM, H_2O_2 2.0 mM, (b) DEPMPO 10.0 mM, DMSO 10% (v/v), Fe^{2+} 2.0 mM, H_2O_2 2.0 mM and O_2 saturation at 0°C. (d) DEPMPO (final concentration after dilution with phosphate buffer (pB)) 33.3 mM, MeOH 33% (v/v). EPR spectrometer settings: (a) and (b) modulation amplitude 0.2 mT, time constant 81.92 ms, sweep time 41.94 s, gain 5.0×10^4, microwave power 10 mW, (d) modulation amplitude 0.2 mT, time constant 163.84 ms, sweep time 83.89 s, gain 1.0×10^5, microwave power 20 mW.

conclusion was obtained from the results of an experiment in which a denaturing agent (urea, at a final concentration of 9 M) was added to the reaction mixture (after trapping). The resultant narrower and sharper spectrum of a less immobilized spin-adduct (characterized by decrease in the difference between the calculated $A_{\|}$ and $A_{\perp}$ values for nitrogen) and the typical β-hyperfine splittings, confirm that a carbon-centered radical is obtained (see Fig. 2c and d) as the protein's tertiary structure is denatured to the expected random coil with more freedom of motion.[40]

Reaction of $HO^{\cdot}$ (from Fe^{2+}/EDTA and H_2O_2) with lysozyme in the presence of DEPMPO in normoxic conditions at 0°C led to the detection of the anisotropic spectrum shown in Fig. 2e, with simulation shown in Fig. 2f. A small amount of the DEPMPO/$HO^{\cdot}$ spin-adduct was also detected (estimated by spectrum simulation to be 6%). This spectrum is attributed to a carbon-centered radical adduct, again on the basis of the β-proton coupling constant; the spectrum is markedly less anisotropic than that of the corresponding BSA adduct (with much more freedom of motion, as judged by the decrease in the difference between the $A_{\|}$ and $A_{\perp}$ values for nitrogen, and as expected from more rapid tumbling of smaller molecules.

Although the anisotropic spectra in Fig. 2a and e can be effectively simulated assuming only one species in each case, we cannot rule out the possibility

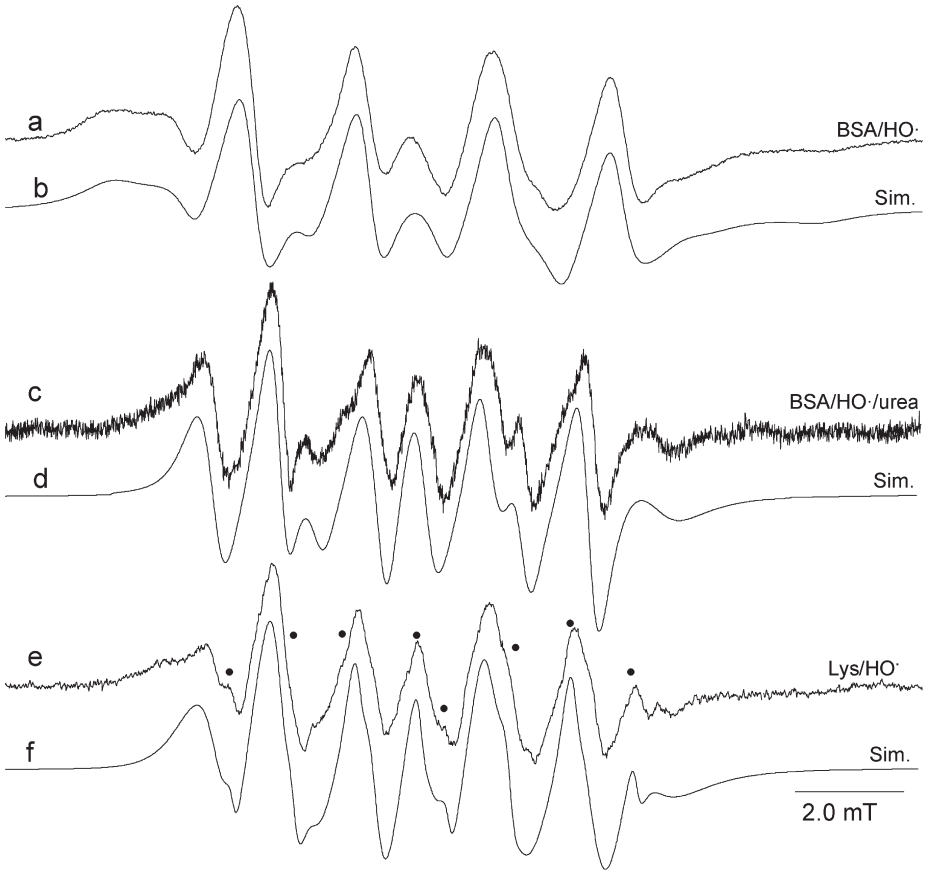


FIGURE 2 (a) EPR spectrum of carbon-centered BSA-derived radical adduct formed in the reaction of BSA with $Fe^{2+}/EDTA/H_2O_2$ in presence of DEPMPO, (b) simulated spectrum, (c) EPR spectrum of carbon-centered BSA-derived radical adduct formed in reaction of BSA with $Fe^{2+}/EDTA/H_2O_2$ in presence of DEPMPO after denaturation with urea (9 M), (d) simulated spectrum, (e) EPR spectrum of carbon-centered lysozyme-derived radical adduct formed in reaction of lysozyme with $Fe^{2+}/EDTA/H_2O_2$ in presence of DEPMPO, the weak signals indicated (•) are attributed to the hydroxyl-radical adduct (6%), (f) simulated spectrum, (for parameters see Table I). Concentrations: (a) (c) (e) DEPMPO 2.5 mM, BSA 5.0 mM, (e) Lysozyme 10.0 mM, Fe^{2+}/EDTA 2.0 mM, H_2O_2 2.0 mM at 0°C, (c) urea (9 M). EPR spectrometer settings: modulation amplitude 0.25 mT, time constant 20.48 ms, sweep time 167.7 s, (a), (c) microwave power 20 mW.

TABLE I Simulated parameters of EPR spectra of spin-adducts proteins obtained in the presence of DEPMPO at pH 7.0

			Hyperfine coupling constant (mT)									
	Temp (°C)	% of adduct	$A_{N\|\|}$	$A_{N\perp}$	$\langle a_N \rangle$	$A_{H\|\|}$	$A_{H\perp}$	$\langle a_H \rangle$	$A_{P\|\|}$	$A_{P\perp}$	$\langle a_P \rangle$	Adduct
BSA/HO·	25		2.86	1.10	1.69	2.12	2.13	2.13	5.24	4.61	4.82	BSA-C·
BSA/HO·/urea	25		1.65	1.39	1.47	1.90	2.21	2.00	5.07	4.47	4.87	BSA-C·
BSA/HO·/O_2	25		2.76	0.65	1.35	0.90	1.45	1.27	5.87	4.72	5.11	ROO·
	−50		3.03	0.6	1.41	0.92	1.13	1.06	5.87	4.9	5.22	ROO·
Lysozyme/HO·	25		1.70	1.39	1.49	1.99	2.09	2.02	5.21	4.53	4.75	Lysozyme-C·
Lysozyme/HO·/O_2	25		1.50	1.25	1.33	1.00	1.30	1.20	4.77	5.20	4.91	ROO·
DMSO/HO·	25*				1.51			2.21			4.74	Me·
DMSO/HO·/O_2	25*†	50			1.34			0.90			4.80	MeOO·
		50			1.31			1.15			4.98	
	−20		2.31	0.83	1.32	1.0	1.27	1.18	5.87	4.99	5.22	MeOO·
	−50		2.80	0.61	1.34	0.89	1.26	1.14	5.67	4.91	5.16	MeOO·
	−100		2.91	0.41	1.24	0.98	1.11	1.07	5.83	4.87	5.19	MeOO·
MeONa/MeOH	25*‡	37.5			1.36			0.83			4.02	MeO–
		62.5			1.34			0.92			4.76	
	−100		2.80	0.69	1.39	0.59	0.60	0.60	4.81	3.76	4.11	MeO–

Anisotropic spectrum unless indicated otherwise. *Isotropic spectrum. †Optimum simulation shows exchange between two conformers. ‡*cis* and *trans* adducts, see text.

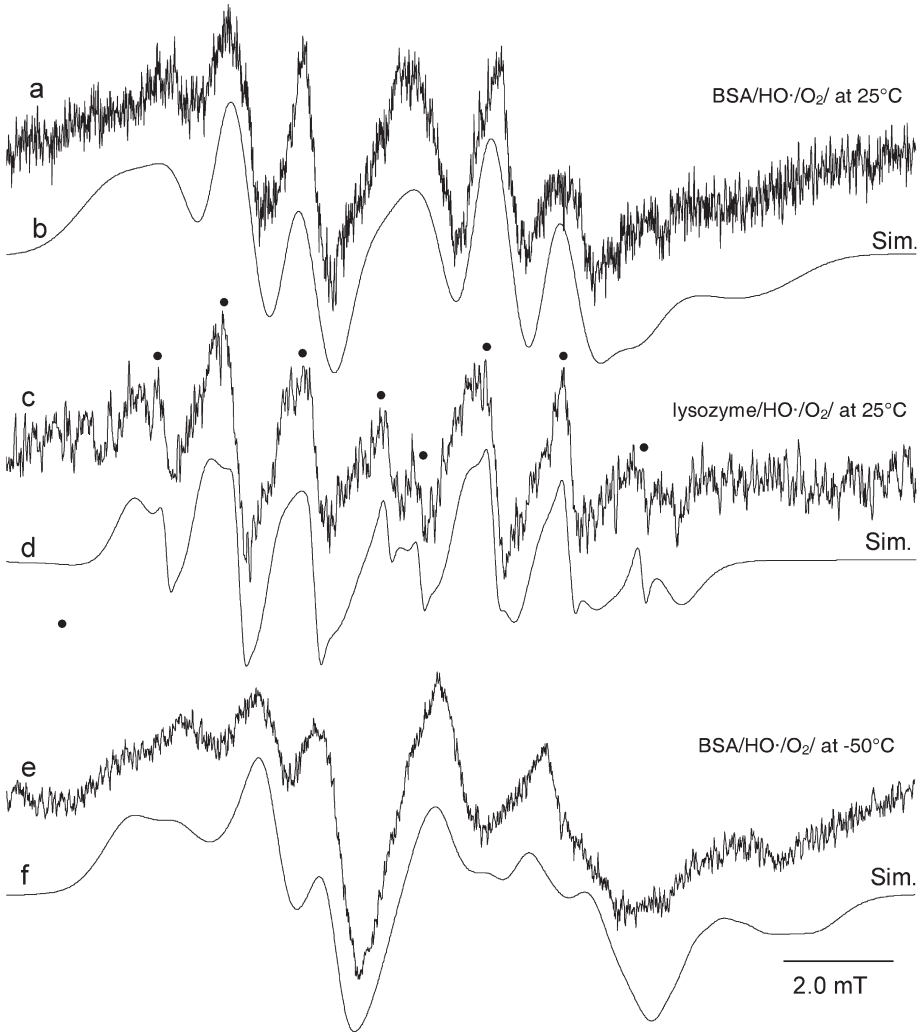


FIGURE 3 (a) EPR spectrum of a peroxyl-centered BSA-derived radical adduct formed in reaction of BSA with $Fe^{2+}/EDTA/H_2O_2$ in the presence of DEPMPO and O_2 at 0°C, (b) simulated spectrum, (c) EPR spectrum of a peroxyl-centered lysozyme-derived radical adduct formed in the reaction of lysozyme with $Fe^{2+}/EDTA/H_2O_2$ in presence of DEPMPO and O_2 at 0°C, the weak signals indicated (•) are attributed to the hydroxyl-radical adduct (4%), (d) simulated spectrum, (e) as (a) but in solid matrix at −50°C, (f) simulated spectrum (for parameters see Table I). Concentrations: DEPMPO 2.5 mM, BSA 5.0 mM, (c) Lysozyme 10.0 mM, Fe^{2+}/EDTA 2.0 mM, H_2O_2 2.0 mM. EPR spectrometer settings: modulation amplitude 0.25 mT, time constant 163.84 ms, sweep time 671.1 s, (c and e) sweep time 167.77 s, gain 2.0×10^5, microwave power 50 mW, (c) microwave power 20 mW.

that a variety of carbon-centered radicals (with closely similar parameters) are obtained. Direct flow experiments involving BSA and lysozyme and $HO^{\cdot}$ (from Ti^{3+}/H_2O_2) led to the observation of anisotropic spectra in which the broad EPR signals are characteristic of the formation of radicals with partial structures $^{\cdot}CH(CO_2^-)CH_2-$ or $^{\cdot}CH(CONH_2)CH_2-$ (Clément *et al.* unpublished work). This is in accord with Davies' findings.[22,23] We suggest that, in the present study, radicals of this type have been trapped, though details of different individual adducts cannot be resolved from the broad spectra obtained.

Reaction of BSA and Lysozyme with $HO^{\cdot}$ in the Presence of O_2

Addition of Fe^{2+}/EDTA to an O_2-saturated buffered solution of BSA or lysozyme, H_2O_2 and DEPMPO led to the immediate detection of completely different anisotropic spectra (Fig. 3a and c).

The overall width of these spectra is clearly compatible with immobilized macromolecular structures with little residual motion. Good fits (Fig. 3b and d) are obtained for simulations with average splittings of $\langle a_N \rangle = 1.35$, $\langle a_P \rangle = 5.11$, $\langle a_H \rangle = 1.27$ mT for BSA and $\langle a_N \rangle = 1.33$, $\langle a_P \rangle = 4.98$, $\langle a_H \rangle = 1.20$ mT for lysozyme (see Table I for further details). The much smaller (average) β-proton splittings, typical of oxygen-centered radicals, are clearly apparent. We estimate that the extra line width of these anisotropic spectra did not require consideration of conformational exchange for adequate simulation unlike small isotropic peroxyl spin adducts. We would expect the addition of bulky peroxyl radicals to be more selective than $HOO^{\cdot}$ and that the presence of the bulky peroxyl moiety may

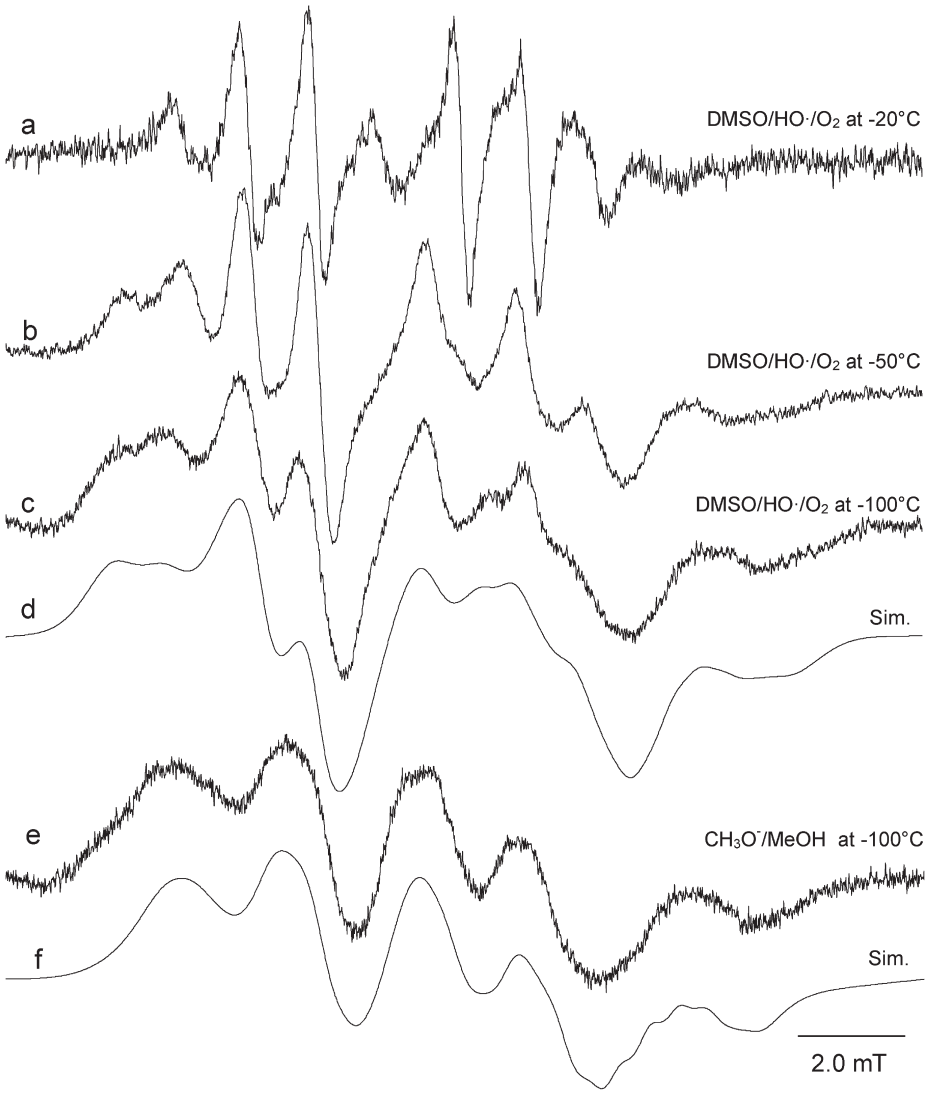


FIGURE 4 (a) EPR spectrum of DEPMPO/$CH_3OO^{\cdot}$ radical adduct formed in reaction of DMSO with Fe^{2+}/H_2O_2 in presence of DEPMPO and O_2 at 0°C, in solid matrix at −20°C, (b) at −50°C (c) at −100°C, (d) simulated spectrum, (e) EPR spectrum of the methoxy adduct of DEPMPO at −100°C formed in reaction of MeONa with DEPMPO in MeOH at room temperature followed by aerial oxidation, dilution with buffer (3 times) and N_2 degassing, (e) simulated spectrum (for parameters see Table I). Concentrations: (a), (b) and (c) DEPMPO 10.0 mM, DMSO 10% (v/v), Fe^{2+} 2.0 mM, H_2O_2 2.0 mM and O_2 saturation at 0°C, (e) DEPMPO (final concentration after dilution with phosphate buffer) 33.3 mM, MeOH 33% (v/v). EPR spectrometer settings: (a), (b) and (c) modulation amplitude 0.2 mT, time constant 81.92 ms, sweep time 167.77 s, gain 1.0×10^5, microwave power 20 mW, (e) modulation amplitude 0.2 mT, time constant 327.68 ms, sweep time 1342.18 s, gain 2.0×10^5, microwave power 20 mW.

prevent conformational change. At this stage, we note that the $a_{\beta\text{-H}}$ and a_P values for these DEPMPO adducts are in the range expected for peroxyl adducts with this trap rather than alkoxyl adducts. The values of $a_{\beta\text{-H}}$ and a_P for alkoxyl adducts would be expected to be somewhat lower as described above and as observed for tyrosyl adducts derived from proteins[40] and small alkoxyl[34] adducts in which $a_{\beta\text{-H}}$ is in the range 0.5–0.9 mT and a_P from 4.5 to 4.8. The possible spin-trapping of protein alkoxyl radicals seems to be unlikely though not impossible. It has been shown that their formation by reaction of first-formed protein hydroperoxides with Fe^{2+} leads to β-scission in water very rapidly and EPR detection (by mean of spin-trapping) of carbon centered protein or protein fragments radicals.[22–45]

At 0°C under normoxic conditions, or under O_2 saturation, an increase in the BSA concentration (for example, from 2.25 to 5.0 mM) led to an improvement in the signal noise ratio of the spectrum. When the BSA concentration was kept at 5.0 mM, increasing the trap concentration (from 2.5 to 5, 10 and 50 mM) led to detection of the DEPMPO/$HO^{\cdot}$ spin-adduct. Furthermore the resulting anisotropic spectra (for [DEPMPO] = 5.0, 10.0 mM) were interpreted as a mixture of carbon- and peroxyl-centered spin adduct signal (data not shown). With [DEPMPO] = 50.0 mM only the hydroxyl adduct was detected.

When the sample was frozen at −50°C, the spectrum in Fig. 3e was obtained; decrease of temperature has little effect on the spectrum shape and parameters, as expected, due to the anticipated relatively low mobility of the spin adduct. Below −50°C the shape became even broader so that it could not be effectively simulated. The anisotropic spectra (see Fig. 3a, c and e) are discussed below in the context of the experiments involving methylperoxyl and methoxy adducts.

Study of the Methylperoxyl and Methoxy Adducts of DEPMPO in a Solid Matrix

Figure 4a, b and c show the spectra of spin-adducts assigned to DEPMPO/$CH_3OO^{\cdot}$ at −20, −50 and −100°C, respectively. Figure 4d shows the excellent fit obtained for simulation of the anisotropic spectrum at the lowest temperature. Again, effective simulations did not require consideration of chemical exchange between two conformers as observed for the $MeOO^{\cdot}$ adduct at room temperature. The close similarity between the low-temperature, immobilized spectrum (Fig. 4c) and that of DEPMPO/BSA-$OO^{\cdot}$ at −50°C (Fig. 3e) is remarkable and adds further weight to our conclusions.

On the other hand, simulation of the spectrum obtained for the methoxy adduct of DEPMPO at low temperatures (Fig. 4e and f) has $\langle a_H \rangle = 0.6$ and $\langle a_P \rangle = 4.11$ mT (see Table I), values which are characteristic of alkoxyl adducts and noticeably different from the peroxyl adducts. Good agreement between simulated and experimental spectra was obtained for a single species.

CONCLUSION

These spin-trapping results on the peroxidation of proteins strongly suggest that the appropriate peroxyl radical can be characterized by means of spin-trapping and can be distinguished from the appropriate alkoxyl spin adduct by use of DEPMPO. Particular support for our assignment derives from the comparison of EPR spectra of DEPMPO/CH_3-$OO^{\cdot}$ and DEPMPO/BSA-$OO^{\cdot}$ adducts in solid matrices at low temperature and from EPR simulation of anisotropic spectra of protein-derived species.

We note at this point one potential difference between the isotropic solution spectra parameters of typical DEPMPO adducts of small radical species and those recorded here for large molecules and/or at low-temperature. For the small, mobile adducts the spectra (and especially $a_{\beta\text{-H}}$) observed are believed to be averaged over a number of conformations which differ in the shape of the five-membered ring (pseudo-rotation between e.g. envelope and half-chair conformations).[36,37] With protein spin-adducts the anisotropy is, of course, due to slow tumbling of the spin-adduct itself, but we believe, additionally, that recorded spectra may largely reflect a preferred conformation within the five-membered ring, on account of the presence of the bulky addend. At low temperatures in particular, with small as well as with very bulky addends (for which this should be most marked) it is likely that the spectra detected reflect a predominant (or exclusive) conformation with five-membered cycle pseudo-rotation more or less frozen depending on the temperature and the addend size. This implies that average frozen and solution spectral parameters would not be expected to be identical. The changes in the nitroxide-nitrogen splitting indicated by the simulations may reflect the uncertainty in the analysis using this approach, though changes in solvation and ring geometry may be involved, as well as anisotropy of the averaging motion.

However, we believe that these considerations, whilst worthy of further study, do not affect our *overall* conclusions and the assignments, since the ranges of splittings characteristic of different adducts (alkyl, alkoxyl, peroxyl) are well separated.

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