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# RADICAL-INDUCED DAMAGE TO PROTEINS: E.S.R. SPIN-TRAPPING STUDIES

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The reactions of hydroxyl radicals generated from $Fe^{II}/H_2O_2$ and $Cu^{II}/H_2O_2$ redox couples with a variety of proteins (BSA, histones, cytochrome c, lysozyme and protamine) have been investigated by e.s.r. spin trapping. The signals obtained, which are generally anisotropic in nature, characterize the formation of partially-immobilized spin-adducts resulting from attack of the HO· radicals on the protein and subsequent reaction of the protein-derived radicals with the spin trap. Similar spin adducts are observed on incubation of two haem-proteins (haemoglobin and myoglobin) with $H_2O_2$ in the absence of added metal ions implying a reaction at the haem centre followed by internal electron transfer reactions.

Two strategies have been employed to obtain information about the site(s) of radical damage in these proteins. The first involves the use of a variety of spin traps and in particular DMPO: with this particular trap the broad spectra from largely immobilized radicals show characteristic $a(\beta\text{-H})$ values which enable carbon-, oxygen- and sulphur-centred radicals to be distinguished. The second involves the use of enzymatic cleavage of first-formed adducts to release smaller nitroxides, with isotropic spectra, which allow the recognition of $\beta$-proton splittings and hence information about the *sites* of radical damage to be obtained. These results, which allows backbone and side-chain attack to be distinguished, are in agreement with *random* attack of the HO· radical on the protein and are in accord with studies carried out on model peptides. In contrast the use of less reactive attacking radicals [$N_3$·, ·$CH(CH_3)OH$] and oxidising agents ($Ce^{4+}$) provides evidence for *selective* attack on these proteins at particular residues.

KEY WORDS: Radicals, electron spin resonance, hydrogen peroxide, hydroxyl, spin trap, protein damage.

## INTRODUCTION

A variety of external factors (such as ionizing and UV radiation and exposure to certain chemicals) and endogenous processes (such as the respiratory burst of phagocytes and leakage from the mitochondrial electron-transport chain) can lead to the formation of free radicals in biological systems.[1] One result of these events is the generation under aerobic conditions of both hydrogen peroxide and a variety of hydroperoxides, such as those derived from lipids. In the presence of certain transition metals, $H_2O_2$ and lipid hydroperoxides are known to be degraded, to hydroxyl (HO·) and superoxide ($O_2^-$ ·) radicals from the former and both peroxyl ($RO_2$·) and alkoxyl (RO·) radicals from the latter.[2] The generation of such species is known to lead to extensive modification of cellular proteins, including fragmentation, cross-linking, decreased fluorescence, and changes in amino-acid composition, as well as secondary, tertiary and quaternary structure, and loss of function.[3] However, the radicals which might be involved in such reactions have not been observed directly and evidence is lacking concerning the detailed mechanisms of these processes.

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The technique of electron spin resonance (e.s.r) spin-trapping has been used extensively in a number of chemical[4,5] and biological systems[6–8] to characterize radical generation, but in general this methodology has been successfully employed to investigate only relatively small, mobile, species, and little attention has been given to the possibility of using the technique to trap the large, relatively immobile species which may be formed during radical-induced damage to proteins (and which give rise to highly anisotropic spectra with relatively broad lines[9–12]).

We set out to explore the possibility that, despite this potential drawback, spin-trapping could be employed as a useful tool in conjunction with other methods in studying the behaviour of proteins. In particular, we wished to ascertain whether the (broad) signals could, under appropriate circumstances, be analysed to give *quantitative* information on radical formation and reaction, and *structural* information, e.g. on the site(s) of initial attack and on the nature of radicals formed in subsequent reactions (for instance with oxygen).

Our approach has involved the use of the Fenton reaction [reaction (1)] and related redox couples to generate the hydroxyl radical, whose subsequent reaction(s) with proteins was to be explored. Initial experiments were designed to investigate the protein BSA, bovine serum albumin, together with a variety of spin traps (see below). Subsequent experiments involved the use of a variety of other proteins chosen so as to have a range of molecular weight and structure. We also aimed to explore the possibilities both that enzymatic cleavage of bulky, long-lived spin adducts could provide further information about sites of radical damage and that judicious choice of simpler model compounds could assist in structural analysis.

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + HO\cdot + HO^- \qquad (1)$$

## EXPERIMENTAL

### *Chemicals*

All proteins and enzymes were obtained from Sigma Chemical Co. and used as supplied. All other chemicals were commercial samples of high purity and used as supplied, with the exception of the spin trap DMPO which was purified before use as described previously.[13] All solutions were prepared using deionised water; stock solutions of MNP were obtained by stirring overnight at room temperature.

### *E.s.r. Spectroscopy*

Spectra of incubations carried out at room temperature in an aqueous-sample cell were recorded using a Bruker ESP 300 spectrometer equipped with 100 kHz modulation, a Bruker ER 035H gaussmeter for field calibration and a Bruker ESP 1600 data system. Hyperfine coupling constants were measured directly from the field scan; g-values were measured relative to a standard sample. All experiments using Fe(II)/EDTA were carried out by the addition of the iron complex, prepared in deionised water (thoroughly degassed using oxygen-free nitrogen), to a mixture containing all the other components, all solutions being typically at pH 7. EDTA was omitted from experiments at low pH. For most experiments the mixed solutions were not deoxygenated.

Photolytic experiments were carried out using a 100 W Varian mercury/xenon lamp incident, through a 50% grating, on an aqueous sample cell inserted into the cavity of the spectrometer. Low-temperature experiments were performed using a Bruker ER 411 variable-temperature unit using nitrogen as the cooling gas.

### *Simulations*

Spectra of some partially immobilized nitroxides were simulated using a programme kindly provided by Dr N.J.F. Dodd (Christie Hospital, Manchester) based on a model by Ehrenberg and coworkers[14] and run on a Vax main-frame computer.

### *Enzyme Digestion*

Enzyme degradation of proteins which had been reacted with HO· in the presence of the spin trap were carried out at 37°C under aerobic conditions for 10–30 min using protease (*ca.* 15 units/ml final concentration), chymotrypsin (86.3 units/ml final concentration) or trypsin (20,000 units/ml).

## RESULTS

### *Spin-Trapping Experiments with BSA*

Initial studies were carried out using a solution containing BSA (0.5 mM), $Fe^{II}$-EDTA (2 mM), $H_2O_2$ (0.1 M) and the spin trap 2-methyl-2-nitrosopropane (MNP) at pH 7.4. The strong e.s.r. spectrum obtained (recorded a few minutes after mixing the solutions, see Figure 1) was found to be stable for a considerable period of time (a strong signal could still be detected after *ca.* 24 h); its formation required the presence of all the components of the mixture. An identical signal (though with some variation in intensity) was observed when the iron and peroxide concentrations were varied (in the ranges 1–8 mM and 80–240 mM respectively), and throughout the pH range 2–7.4.

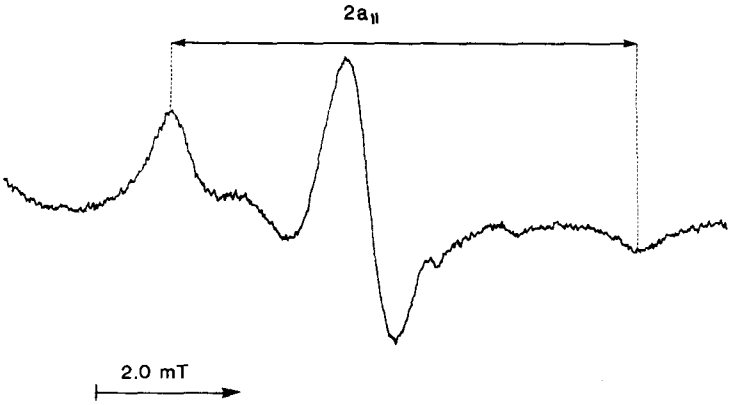



FIGURE 1 E.s.r. spectrum of a motionally-restricted nitroxide (with $a_{||}$ 3.2 mT) obtained when bovine serum albumin is exposed to ·OH (from $Fe^{II}$-EDTA and $H_2O_2$ at pH 7.4) in the presence of the spin trap 2-methyl-2-nitrosopropane (MNP).

When these experiments were repeated under deoxygenated conditions, essentially similar spectra were obtained. A similar signal was obtained via photolytic generation of ·OH from hydrogen peroxide (0.2 M) in the presence of the spin trap MNP (saturated) and BSA (0.5 mM). It is suggested that in each case the first-formed hydroxyl radical [reaction (1)] reacts with the protein to give a radical (designated P·) which subsequently adds to the trap [reactions (2) and (3)].

$$HO\cdot + P(BSA) \rightarrow H_2O + P\cdot \quad (2)$$

$$\underset{MNP(1)}{P\cdot + Me_3CNO} \rightarrow \underset{\quad\quad\quad O\cdot}{P\text{–}N\text{–}CMe_3} \quad (3)$$

The broad nature of the e.s.r. spectrum (Figure 1) is consistent with the formation of a slowly tumbling and partially immobilized spin-adduct, with some local freedom of motion of bonds around the nitroxide centre (and as illustrated, for example, by the proposed analysis of the patterns of the spectra obtained from solutions of spin-labelled polybenzylglutamate in DMF[15,16]).

The broad nature of the signal does not allow isotropic coupling constants to be unambiguously determined, but the overall splitting between the outermost features (designated as $2a_{\|}$, with $a_{\|}$ 3.2 mT in this case) is believed to be characteristic of the system and, in particular of the extent of immobilization (see later): the value of $a_{\|}$ observed for a particular radical may vary from a maximum value associated with a *completely* immobilized nitroxide ($T_{zz}$) to a much smaller value indicative of averaging in $a$ values by some local motion.

A detailed set of simulations has not been attempted at this stage and indeed it would be unlikely to provide a single reliable analysis of spectroscopic parameters and the rates (or nature) of motional processes. However, Figure 2 illustrates how the general features of the observed spectra can be reproduced with a programme which is believed[14] to be appropriate for spin-labelled proteins: some *tumbling* motion of the nitroxide (within a cone of semi-cone angle $\beta$ which governs $a_{\|}$) is coupled with more rapid but *restricted* rotational motion ($\Phi$). This simulation employed input data of $a_{\|}$

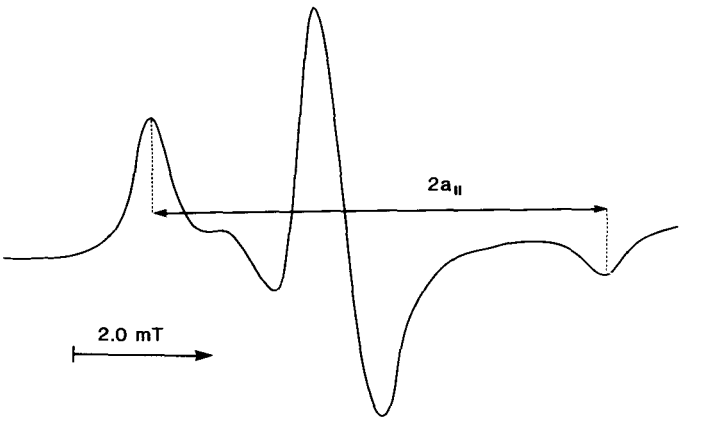



FIGURE 2 Example of a simulated e.s.r. spectrum of a motionally restricted BSA adduct of MNP (see Figure 1) showing characteristic effect of an input value of 3.2 mT for $a_{\|}$ (for other data, see text).

3.2 mT, $T_{xx}$ 0.63, $T_{yy}$ 0.58, $T_{zz}$ 3.36 mT and $g_{xx}$ 2.0088, $g_{yy}$ 2.0061, and $g_{zz}$ 2.0027[17] together with $\tau$ 2 × $10^{-8}$ s (local tumbling motion) and $\tau$ 5 × $10^{-9}$ s (internal restricted rotation, with $\Phi$ 60°). Further studies are being carried out to determine whether detailed fittings and more meaningful interpretations can be achieved.

*Variation of the Spin Trap*

Use of the spin trap 3,5-dibromo-4-nitrosobenzenesulphonic acid (DBNBS) (2) under similar reaction conditions led to the observation of a similar broad spectrum (with $a_{\|}$ 2.9 mT), whose formation presumably reflects the reactions (1), (2) and (4). With the spin trap α-phenyl-N-t-butylnitrone (PBN), (3), a similar broad signal ($a_{\|}$ 3.2 mT) was also observed [reaction (5)] together with a sharp, isotropic signal [with $a$(N) 1.58, $a$(β-H) 0.28 mT] characteristic of the hydroxyl-radical adduct of the trap[18] (reaction 6), whose observation confirms that the source of damage is indeed the hydroxyl radical formed in the Fenton reaction.[5] The intensity of the two signals varied with [PBN] and [BSA] in a manner which confirms that competitive attack occurs by ·OH on the two substrates [knowledge of the rate constant for trapping of HO· on PBN[19] (8.5 × $10^9$ $dm^3$ $mol^{-1}$ $s^{-1}$) allows the rate of reaction of ·OH with BSA to be estimated as in the range 5–10 × $10^{11}$ $dm^3$ $mol^{-1}$ $s^{-1}$, which is in reasonable agreement with pulse-radiolysis studies on human serum albumin.[20] With the spin trap 5,5-dimethyl-1-pyrroline-N-oxide [DMPO (4)], the spectra observed not only showed sharp isotropic features of the corresponding ·OH adduct [a(N) = a(H) = 1.49 mT[18]] but also a different type of broad spectrum (Figure 3) whose appearance is modified compared with those described earlier, by the incorporation of a splitting associated with the β-proton in the corresponding adduct; this signal is assigned to a carbon-centered adduct on the basis of the size of this splitting (*ca.* 2.38 mT).


DBNBS (2) (4)

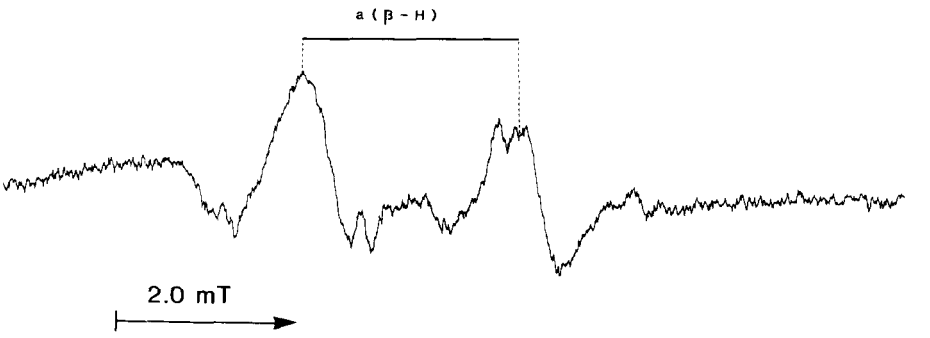
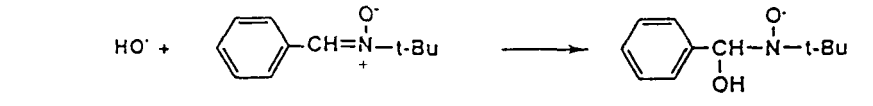


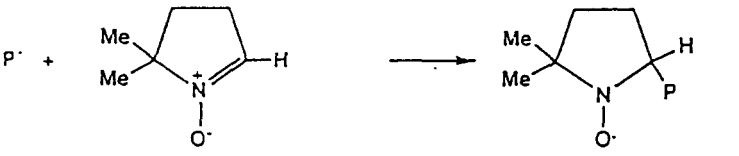

FIGURE 3 E.s.r. spectrum of a motionally-restricted adduct obtained from a carbon-centred radical (from ·OH and BSA) and the spin trap DMPO: the characteristic doublet splitting [with $a$(β-H) *ca.* 2.38 mT] is indicated (contrast Figure 1).


(5)

PBN (3)




(6)




(7)

DMPO (4)

Although all of the traps were employed in subsequent experiments, we generally used DMPO (to obtain information about adduct structure) and DBNBS, since in the latter case optimum signal intensity was normally obtained.

*Spin Trapping of Radicals from Different Proteins*

Use of the $Fe^{II}$-EDTA/$H_2O_2$/DBNBS system as described above gave similar broad e.s.r. signals from lysozyme, histone proteins 2A and 2S, cytochrome c and protamine. For the haem proteins methaemoglobin and metmyoglobin, similar signals derived from the proteins were also obtained in the *absence* of added iron: these findings are consistent with previous reports[21–24] of the generation and detection, by e.s.r. spectroscopy, of globin-derived radicals resulting from reaction of $H_2O_2$ at the haem centre. Table I shows the values of $a_{\|}$ obtained from the major components of these spectra (in some cases *mixtures* of radicals may be present), together with the molecular weights of the proteins.[25] It can be seen that the values of $a_{\|}$ are clearly not governed merely by the bulk (or molecular weight) of the protein though the larger proteins do indeed show a generally greater extent of immobilisation implied by the larger values of $a_{\|}$. Of particular note, however, is the spectrum from histone 2A (Figure 4) which has a significant contribution from an almost isotropic spectrum [comprising a mixture of nitroxide radicals, one a triplet $a$(N) 1.36 mT and the other a triplet of doublets, $a$(N) 1.39, $a$(H) 0.80 mT]: this appears to reflect the formation of two relatively small (or at least rapidly tumbling) nitroxides, either by fragmentation of larger species or by attack on external parts of the protein with motional freedom. These possibilities are discussed further below.

Broadly similar results to those described above were obtained with the spin traps PBN or DMPO. When PBN was employed, the sharp-lined signals from the hydroxyl adduct were also detected (as described for BSA); the rate constants of attack of ·OH on these proteins were estimated (as above) to be in the range $10^{11}$–$10^{12}$ $dm^3\ mol^{-1}\ s^{-1}$. Figure 5a shows an interesting example of a DMPO-trapped radical from protamine. The use of this cyclic spin trap allows a typical carbon-centred radical to be characterised (via the $a$(H) value of 2.38 mT[18]): the relatively sharp-lined spectrum indicates that the nitroxide has considerable freedom of motion. When this sample was then

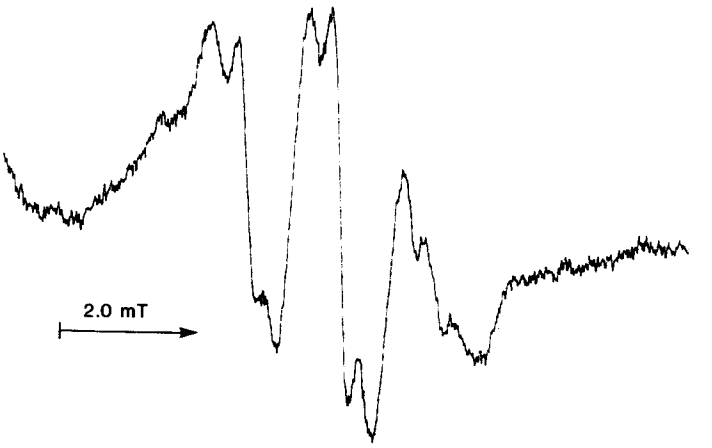



FIGURE 4 E.s.r. spectra of relatively mobile radical adducts of DBNBS following reaction of histone 2A with ·OH at pH 7.4.

TABLE I
E.s.r. parameters ($a_{\|}$ in mT) for a variety of protein-derived radical-adducts of DBNBS[a]

| Protein | Relative molar mass | Source of radical damage (·OH)[b] | $a_{\|}$/mT[a] |
|---|---|---|---|
| BSA | 66,000 | $Fe^{II}$-EDTA, $H_2O_2$ | 2.92 |
| Methaemoglobin | 64,500 | $Fe^{II}$-EDTA, $H_2O_2$<br>$H_2O_2$[c] | 2.89<br>2.94 |
| Myoglobin | 18,800 | $Fe^{II}$-EDTA, $H_2O_2$<br>$H_2O_2$[c] | 2.80<br>2.61 |
| Lysozyme | 14,600 | $Fe^{II}$-EDTA, $H_2O_2$<br>$CuSO_4$, $H_2O_2$ | 2.91<br>2.84 |
| Histone 2A | *ca.* 15,000 | $Fe^{II}$-EDTA, $H_2O_2$ | 1.36 $a$(N)[d]<br>1.36 $a$(N)[d]<br>0.80 $a$(H)[d] |
| Histone 2S | *ca.* 15,000 | $Fe^{II}$-EDTA, $H_2O_2$<br>$CuSO_4$, $H_2O_2$ | 3.06[e]<br>3.06 |
| Cytochrome c | 12,400 | $Fe^{II}$-EDTA, $H_2O_2$ | 2.91 |
| Protamine | 4,400 | $Fe^{II}$-EDTA, $H_2O_2$ | [d] |

[a] Details of major component (±0.05 mT). Generated by reaction of the protein in aqueous solution at pH 7.4 with ·OH ($Fe^{II}/H_2O_2$) and 3,5-dibromo-4-nitrosobenzenesulphonic acid (DBNBS).
[b] For conditions see text.
[c] No exogenous metal ion required.
[d] Sharp isotropic spectra obtained (see text).
[e] Some mobile components are detected.

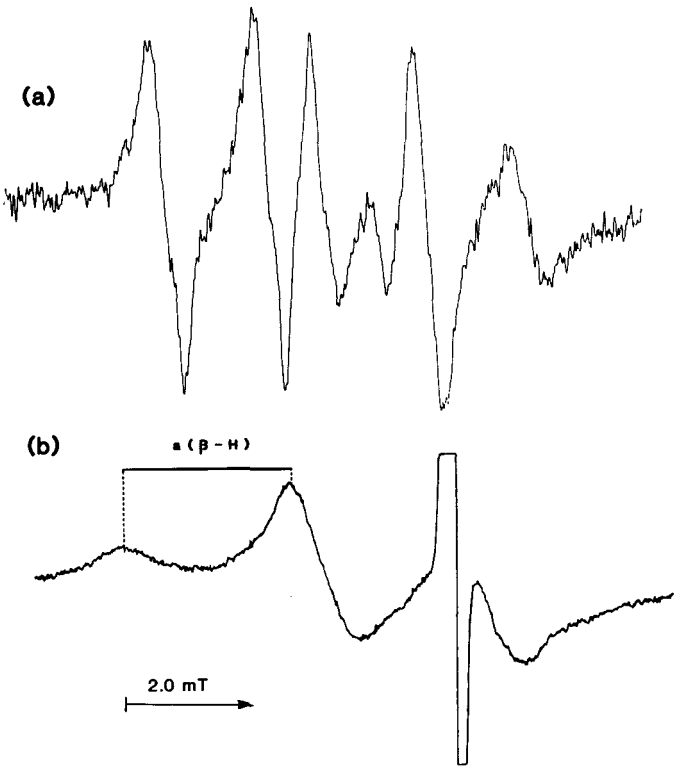



FIGURE 5 (a) Isotropic e.s.r. spectrum of a carbon-centred radical-adduct of DMPO, showing considerable freedom of motion, obtained from protamine, $Fe^{II}$-EDTA and $H_2O_2$ in the presence of the trap. (b) Anisotropic e.s.r. spectrum (fully immobilized) from the protamine-derived radical trapped with DMPO, recorded when the sample from (a) was frozen to 231 K.

frozen (at 231 K) the broadened spectrum obtained (Figure 5b) retains the clearly indicated proton splitting of *ca.* 2.4 mT, as would be expected. This also closely resembles the spectrum obtained from BSA-DMPO (Figure 3), providing confirmation of the analysis.

*Variation in the Attacking Radical*

In order to determine whether this spin-trapping approach can distinguish between damage inflicted by different attacking species, experiments were carried out using the lysozyme/DBNBS system with a variety of different attacking reagents. As Figure 6 illustrates, differences can indeed be recognized between spectra obtained via attack of $N_3\cdot$ (from $N_3^-$ and $\cdot OH$[26]), $\cdot OH$ itself, and $\cdot CHMeOH$ (from $\cdot OH$ and excess of ethanol). The differences, and, in particular, the clear changes in $a_{\parallel}$ suggests that these radicals attack at different sites on the protein as might be expected in terms of their vastly different reactivity and selectivity.[26,27] Two other types of behaviour were also observed: thus the proteins BSA and histone 2S showed some variation with the attacking radical (but to a much smaller extent), whereas for histone 2A and

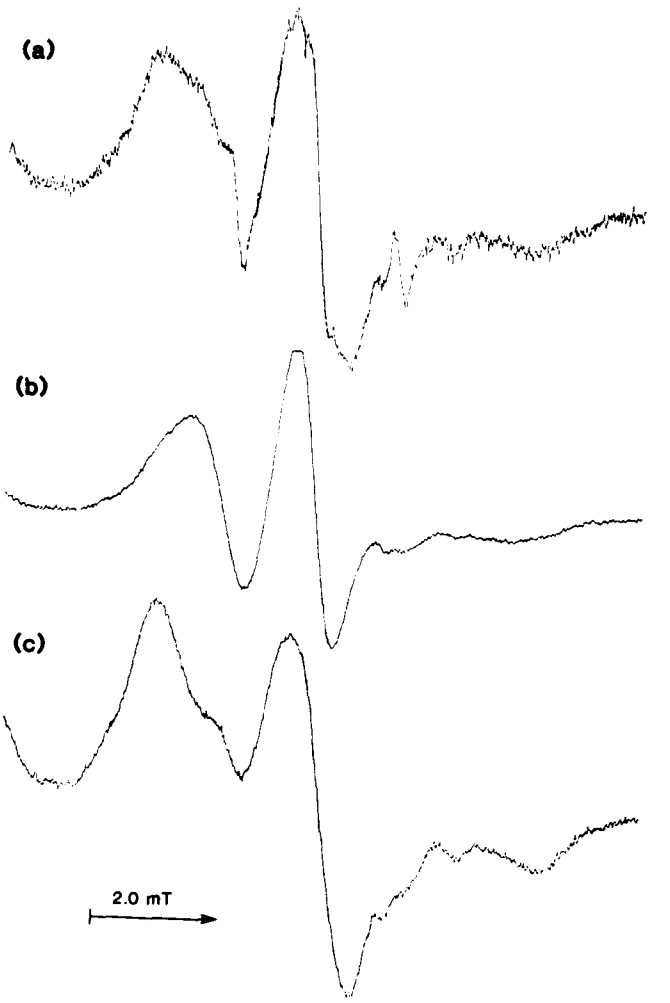



FIGURE 6 E.s.r. spectra of radical-adducts to DBNBS, obtained following reaction of lysozyme with (a) OH· (b) $N_3$· (c) ·CHMeOH.

protamine only ·OH was found to give rise to significant damage (at least as judged by the detection of signals from trapped radicals). The latter observation is consistent with the lack (in protamine) and low content (in histone 2A) of aromatic and sulphur-containing residues in these proteins, given that ·$N_3$, and possibly ·CHMeOH, prefer to react at these centres (rather than C–H bonds).

Reaction of $Ce^{4+}$ with a protein would be expected to occur[28] either at the sulphydryl groups (to give thiyl radicals) or at tyrosine residues (to give phenoxyl radicals, which may well not be trapped). As shown in Figure 7, the spectrum obtained from BSA and $Ce^{4+}$ in the presence of DMPO is distinctly different from that obtained from ·OH (Figure 3). The extra splitting observed (compared with those spectra lacking a $\beta$-proton splitting) is indicated: its value of *ca.* 1.6 mT strongly

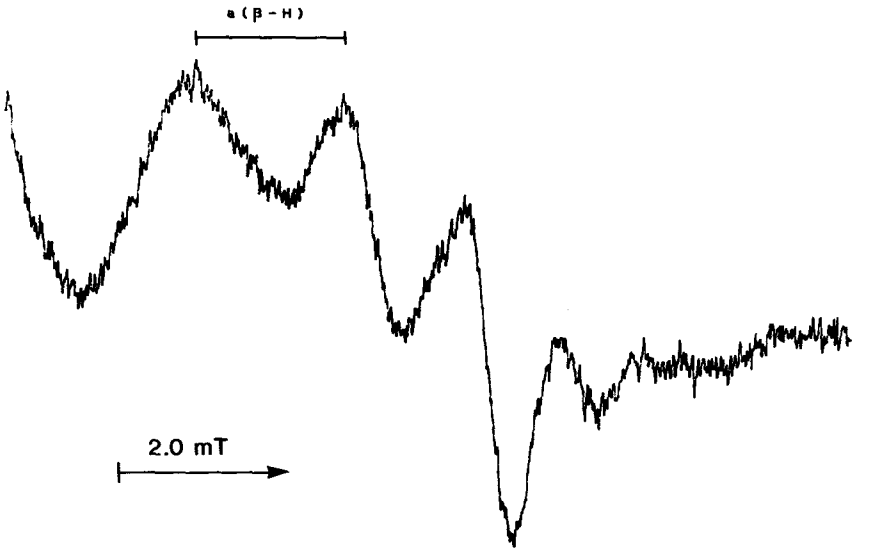



FIGURE 7 E.s.r. spectrum of a motionally-restricted nitroxide spin-adduct obtained on reaction of BSA with $Ce^{4+}$ in the presence of DMPO. The $\beta$-proton splitting (*ca.* 1.6 mT) is indicated.

suggests that in this reaction a thiyl radical (RS·) has been trapped.[18] This presumably arises from the *single* cysteine residue at position 34 in this protein, demonstrating that *selective* damage to proteins is possible.

Experiments with the $Cu^{II}/H_2O_2$ redox couple gave spectra which were qualitatively similar, in the majority of cases, to those obtained from $Fe^{II}/H_2O_2$, though the signals were often less intense. The two exceptions were BSA, where no signals were observed (possibly as a result of binding of the metal ion to the protein), and lysozyme. These results suggest, at least for the majority of systems, that the hydroxyl radical (or species with very similar *selectivity*) generated by the $Cu^{II}/H_2O_2$ couple, is again the attacking species; the differences observed with lysozyme may reflect either the formation of an alternative attacking species, a degree of site-selectivity (possibly due to binding of the metal ion), or an increased level of fragmentation or structural alteration to the protein. Further studies to investigate this phenomenon are in progress.

*Structural Analysis of Spectra from Protein-Derived Adducts*

(*i*) *Enzymatic digestion.* In order to obtain further information from the broad spectra from spin-adducts we have explored the use of enzymatic digestion, whereby we aimed to generate smaller fragments from the damaged proteins (with the spin traps still attached) which will tumble rapidly and hence give sharper e.s.r. signals. These in turn should allow the resolution of small proton splittings, which depend upon the detailed local environment of the nitroxide moiety, and hence the identification of the initial radical. Protease and chymotrypsin digestion was indeed found to result in the liberation of one or more freely rotating (or partially restricted) radical adducts; the main exceptions were those examples, especially with DMPO, where the resulting adducts were too short-lived to give detectable signals at the end of the

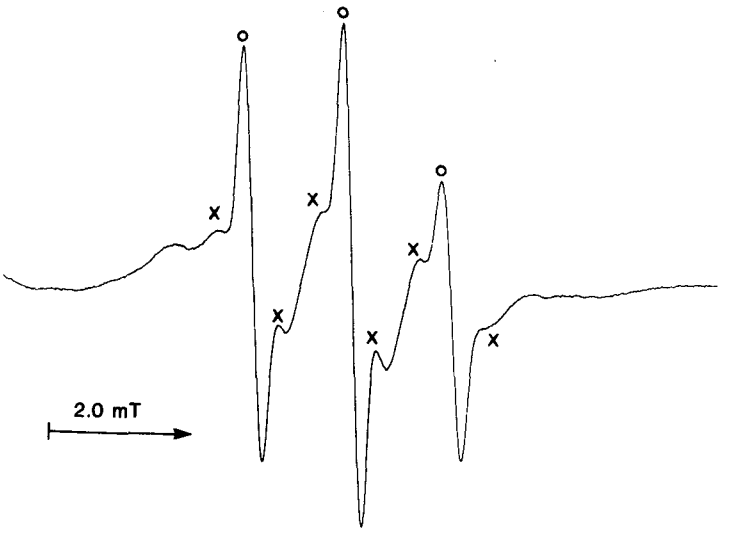



FIGURE 8 E.s.r. spectra of tertiary (O) and secondary ( × ) carbon-radical spin-adducts, recorded *ca.* 30 min after treatment of the BSA-DBNBS spin adduct (cf. Figure 1) with a non-specific protease enzyme.

digestion period. Figure 8 shows the sharper-lined spectra of two small nitroxides obtained 0.5 h after hydroxyl-damaged BSA, spin-trapped with DBNBS, was treated with protease. The spectrum (which is very similar to that observed from histone 2A without such treatment) contains traces of the broad signal expected from incompletely-released spin adducts together with signals from a relatively sharp triplet of *singlets* (i.e. with no further hyperfine splitting and hence possibly associated with a trapped *tertiary* carbon radical, with no α-protons) and a triplet of *doublets* [$a$(N) 1.42, $a(\beta$-H) 0.8 mT] which may hence be ascribed to a trapped *secondary* carbon-centred radical. Experiments to verify these assertions are described below.

Related experiments carried out with the DBNBS adducts of lysozyme, cytochrome c, methaemoglobin and metmyoglobin, gave only the sharp triplet spectrum referred to above, whereas protamine and histone 2A behaved like BSA in giving mixtures of triplets and doublets with $a$(H) *ca.* 0.8 mT. Use of trypsin generally led to the rapid disappearance of all e.s.r. signals.

(*ii*) *Experiments with model compounds.* In order to gain more information about the nature of the smaller nitroxides released by enzymatic cleavage, a series of experiments have been carried out with some model compounds (amino acids, di- and poly-peptides) using the spin traps DBNBS (so that $\beta$ C–H splittings should be detectable from protons attached to the first-formed radical's centre) and DMPO. The results obtained are given in Table II and some typical spectra are shown in Figure 9.

Most of the aliphatic amino acids studied showed a clear signal in the presence of DBNBS, characterized by a doublet of triplets. This is believed to be due to the formation and trapping of a radical in each case formed by abstraction of a secondary hydrogen atom in the side-chain (to give $\cdot CHR^1R^2$), though decarboxylation may prove a route to radicals $R\dot{C}HNH_2$ for methionine and phenylalanine.[28,29] The triplet

TABLE II

E.s.r. parameters for typical adducts obtained from DBNBS following spin trapping of radicals derived from ·OH and amino acids[a]

| Amino acid | Hyperfine pattern[b] | $a$(N)/mT[c] | $a(\beta$-H)/mT[c] | Addend radical |
|---|---|---|---|---|
| L-glutamic acid | doublet | 1.35 | 0.56 (1H) | secondary (side-chain) |
| L-arginine | doublet | 1.35 | 0.71 (1H) | |
| L-lysine | doublet | 1.36 | 0.75 (1H) | |
| D,L-leucine | singlet | 1.43 | – | tertiary |
| | doublet | 1.44 | 0.97 (1H) | secondary (side-chain) |
| | triplet ($CH_2$) | 1.40 | 1.18 (2H) | primary (side-chain) |
| D,L-alanine | triplet ($CH_2$) | 1.33 | 1.33 (2H) | primary (side-chain) |
| L-phenylalanine | singlet | 1.41 | – | α-Ċ |
| | doublet | 1.45 | 0.71 (1H) | secondary (side-chain) |
| L-tryptophan | singlet | 1.32 | – | α-Ċ |
| D,L-methionine | singlet | 1.38 | – | α-Ċ |
| | doublet | 1.35 | 0.63 (1H) | secondary (side chain or decarboxylated; see text) |

[a] For conditions, results with DMPO, and other amino acids see text.
[b] Splitting observed in addition to nitrogen splitting.
[c] ±0.01 mT.

of triplets detected from alanine and leucine presumably similarly reflect the formation of methylene-centred radicals via *side-chain* attack.

This mode of attack is believed to reflect the preference of ·OH attack at unactivated C–H bonds rather than the deactivated C–H bonds adjacent to the protonated amino groups. That attack can also occur at such sites (i.e. at α-C) is suggested by the detection of triplets of *singlets* in some cases (from leucine, phenylalanine, tryptophan and methionine), there now being no hydrogen on the (trapped) radical centre. Some amino acids failed to give detectable signals (including the relatively deactivated molecules glycine, asparagine and aspartic acid, and the amino acids tyrosine and cysteine, for which the rapid attack of ·OH would give oxygen- and sulphur-centred radicals, respectively, which would not be expected to give detectable spin adducts with this trap); some of the amino acids which gave evidence for side-chain derived radicals with DBNBS also gave a triplet of doublets with DMPO [$a$(N) ~ 1.56–1.6 mT, $a$(H) 2.30–2.38 mT], characteristic of carbon-centred radical adducts (presumably also side-chain derived).

Experiments with glycylglycine (Gly–Gly), Gly–Gly–Gly, Gly–Gly–Gly–Gly and Ala–Gly all gave rise to spectra (Table III and Figure 9) in which the major nitrogen splitting is further split into a hyperfine pattern characteristic of interaction with a proton and a second nitrogen – indicating that the trapped radical has the structure –NHĊHCO–, as expected for attack on the α-carbon (backbone). From the general similarity of the spectra – and the result for Ala–Gly – it seems likely that abstraction has occured preferentially at the $-CH_2-$ next to the *carboxylate* terminus [a position activated to attack by the presence of the adjacent nitrogen (+ M) and with delocalization onto both N and $CO_2^-$]. We believe that there are conformational implications in the variation in $a(\beta$-N) and $a$(H) for this series: $a$(H) in particular appears to be sensitive to the bulk (or length) of the attached chain.

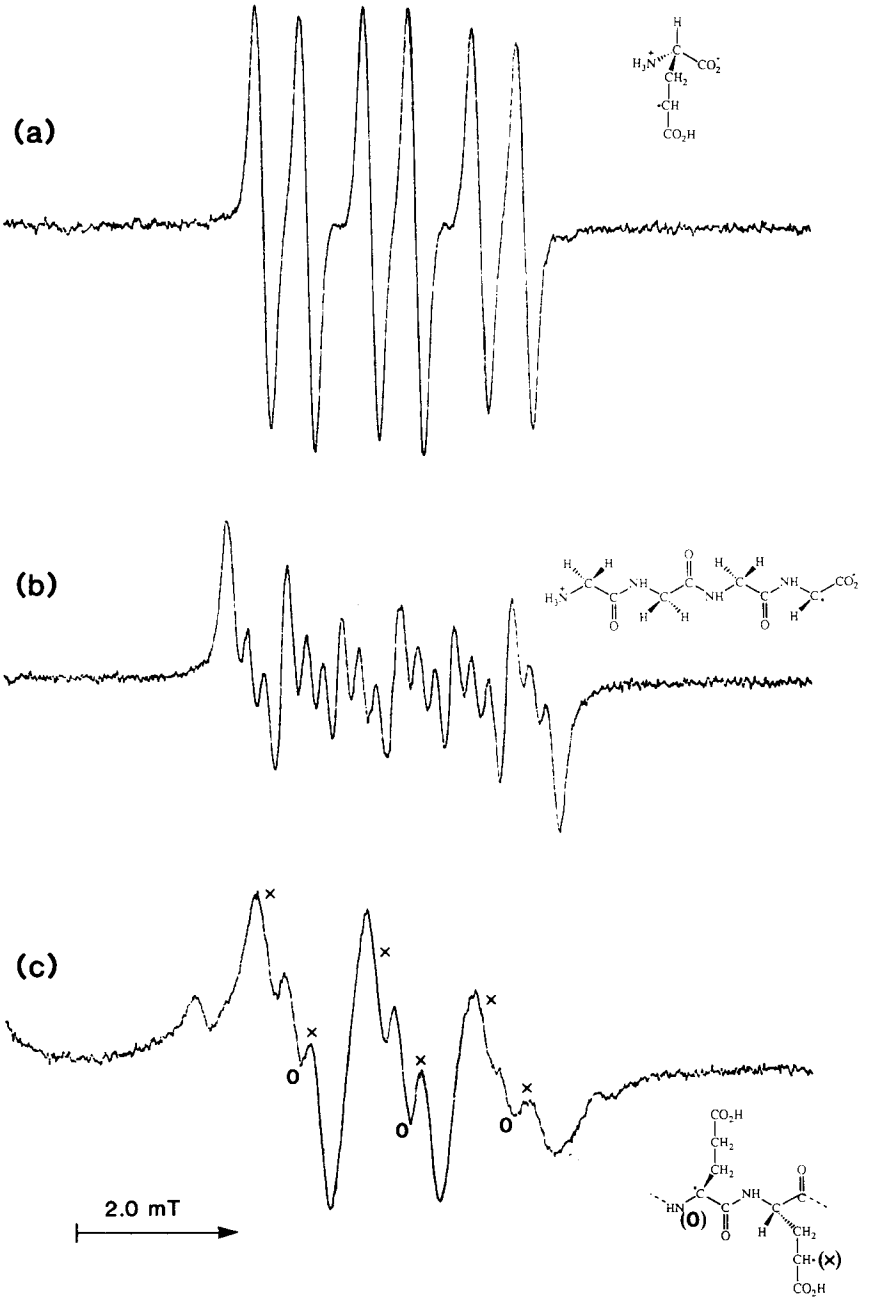



FIGURE 9 Examples of relatively isotropic spectra of nitroxide spin adducts obtained from the reaction of model compounds with ·OH (from $Fe^{II}$-EDTA and $H_2O_2$ at pH 7.4) in the presence of the spin trap DBNBS. Inserts indicate structures of radicals believed to be trapped in each system. (a) L-glutamic acid; (b) Gly–Gly–Gly–Gly; (c) Poly-L-glutamic acid.

TABLE III

E.s.r. spectra of adducts derived from DBNBS following spin-trapping of radicals derived from ·OH and some di-, tri-, and poly-peptides[a]

| Peptide | Spectrum[b] | $a$(N)[c] | $a$(H)[c] | Addend radical |
|---|---|---|---|---|
| Gly–Gly | doublet, triplet | 1.40, 0.14 | 0.85 | |
| Gly–Gly–Gly | doublet, triplet | 1.41, 0.22 | 0.74 | |
| Gly–Gly–Gly–Gly | doublet, triplet | 1.40, 0.21 | 0.71 | |
| Gly–Gly–Gly–Gly–Gly | doublet, triplet | 1.41, 0.22 | 0.70 | |
| Gly–Ala | singlet | 1.34 | | α-Ċ[d] |
| Gly–Gly–Ala | singlet | 1.34 | | |
| Ala–Gly | doublet, triplet | 1.35, 0.13 | 0.97 | |
| Ala–Ala–Ala | singlet | 1.32 | | |
| Ala–Ala–Ala–Ala | singlet | 1.32 | | |
| Gly–Pro–Ala | singlet | 1.32 | | α-Ċ[d] |
| | doublet | 1.37 | 0.76 | Ċ–H (side-chain) |
| Gly–Phe–Ala | singlet | 1.43 | | α-Ċ[d] |
| | doublet | 1.45 | 0.80 | Ċ–H (side-chain) |
| Gly–Gly–Tyr–Arg | singlet | 1.33 | | α-Ċ[d] |
| | doublet | 1.37 | 0.76 | Ċ–H (side-chain) |
| Polyglutamic acid | singlet | 1.30 | | α-Ċ[d] |
| | doublet | 1.35 | 0.69 | Ċ–H (side-chain) |
| Polyaspartic acid | singlet | 1.35 | | α-Ċ[d] |
| Polyasparagine | singlet | 1.37 | | α-Ċ[d] |

[a] For conditions see text.
[b] In addition to main nitrogen triplet splitting.
[c] In mT: ±0.01 mT.
[d] Attack on the back-bone αC-carbons to give radicals of the type –NHĊRCO– or –NHĊRCOO⁻.

In accord with this interpretation the singlets formed from peptides with alanine at the carboxyl terminus (Gly–Ala, Ala–Ala–Ala, etc) are attributed to the radical obtained by hydrogen obstraction from the (activated) αC–H of this terminal amino acid i.e. $-NH\dot{C}(Me)CO_2^-$. The observation that there is no detectable $\beta$-nitrogen splitting in these adducts (possibly because they are more hindered than those of glycine derivatives), lends support to the suggestion (see above and below) that the *singlets* observed here and earlier characterize trapping of back-bone-derived radicals.

On the other hand, the signals from Gly–Pro–Ala, Gly–Phe–Ala and Gly–Gly–Tyr–Arg (Table III) suggest that, as might be expected, attack in these peptides can also occur respectively at proline (at unactivated methylene groups), at phenylalanine [to give the benzyl (side-chain) radical] and on the arginine side-chain. Thus, with certain amino acids with non-deactivated C–H groups, side-chain attack can compete effectively with attack on the back-bone C–H bonds.

Signals were less distinct, on the whole, when DMPO was employed as the trap. In several cases there was clear evidence for the production of a carbon-centred radical [with $a$(N) typically 1.57, $a$(H) 2.25 mT], but in some cases these spectra may represent mixtures of different species; separation into contributions from back-bone vs side-chain radicals does not appear possible at this stage.

Reaction of polyglutamic acid (mol. wt. 60–70,000) under similar circumstances led to the detection of signals which clearly comprise a mixture of two nitroxides — one with no extra hyperfine splitting, the other with a doublet splitting (Figure 9). Other polypeptides behaved similarly. The former radical in each case is, we believe,

associated with the trapping of radicals formed from attack on the back-bone (to give radicals of the type –NHĊRCO–), the latter with attack on the side-chain (as in the model compounds). The failure to detect a splitting from the second nitrogen (and hydrogen) in the former (unlike signals observed from attack at the α-carbon of the carboxyl terminal amino acid in Gly–Gly and Gly–Gly–Gly) is believed to reflect the conformation achieved by the much bulkier polypeptide chain.

The close similarity between some of the spectra observed after enzymatic cleavage and some of those obtained for model compounds is of particular interest: the mixtures of signals observed appears to indicate that both back-bone and side-chain radicals are formed in the initial damage to the proteins (to give nitroxides which are subsequently released as smaller fragments via enzymic cleavage).

The relatively sharp-lined spectra obtained *before* enzymatic cleavage for some proteins (e.g. histone 2A and protamine) is also of interest. This could presumably indicate either that the nitroxides detected are formed via *radical-induced* fragmentation, or that the spectra reflect the occurrence of radical damage in a portion of the molecule which has relative freedom of motion (e.g. random coil) in the native structure (or even as a result of radical-induced structural alterations). For histone 2A the latter explanation is preferred, as it is known that all of the core histone proteins have randomly coiled, unstructured N-termini and, in addition for histone 2A and 3, short C-termini tails comprising approximately 20% of the total amino acids.[30,31] It is therefore possible that the mixture of relatively mobile and partially immobilised species observed with histone 2A in these studies is due to attack on the mobile tails and globular core respectively.

## CONCLUSIONS

These studies have demonstrated that e.s.r. spin trapping, using a variety of spin traps, is a powerful technique which can be successfully employed to detect and investigate radical-induced damage to a wide variety of proteins. Spin adducts have been observed from a variety of different proteins, which have different sizes, structures and compositions, on exposure to a number of different attacking species, suggesting that this technique is widely applicable.

The spectra of the spin adducts tend to be broad on account of slow molecular tumbling but it has been shown that considerable information can, in principle, be obtained from such spectra by use of simulations, different spin traps (particularly DBNBS and DMPO), enzymatic digestion and comparison with data from small peptides and homopolymers. Application of these techniques has shown that exposure of proteins to $Fe^{II}/H_2O_2$ redox couples results in what appears to be random attack by hydroxyl radicals on the surface of the protein resulting in the formation of carbon-centered radicals on both the back-bone of the protein and the amino-acid side-chains; more selective damage to the protein occurs when species such as $N_3\cdot$, $CH_3\dot{C}HOH$ and $Ce^{4+}$ are employed. For some proteins (e.g. histone 2A), initial damage to the globular structure and a mobile tail can be clearly distinguished.

The production of these radicals is believed to be the initial stage in a process which ultimately leads to fragmentation, dimerisation, loss of enzymic activity etc. Further studies on the mechanism of these processes and the subsequent reactions of these radicals (for example, with oxygen) which lead to biological perturbations are in progress.

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