Free Rad. Res. Comms., Vol. 18, No. 6, pp. 353-367
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RADICAL-INDUCED DAMAGE TO BOVINE SERUM ALBUMIN: ROLE OF THE CYSTEINE RESIDUE

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(Received March 9th 1993)

The reactions of cerium(IV) and the hydroxyl radical [generated from iron(ii)/H_2O_2] with bovine serum albumin (BSA) have been investigated by EPR spin trapping. With the former reagent a protein-derived thiyl radical is selectively generated; this has been characterized via the anisotropic EPR spectra observed on reaction of this radical with the spin trap DMPO. Blocking of the thiol group results in the loss of this species and the detection of a peroxyl radical, believed to be formed by reaction of oxygen with initially-generated, but undetected, carbon-centred radicals from aromatic amino acids. Experiments with a second spin trap (DBNBS) confirm the formation of these carbon-centred species and suggest that damage can be transferred from the thiol group to carbon sites in the protein. A similar transfer pathway can be observed when hydroxyl radicals react with BSA.

Further experiments demonstrate that the reverse process can also occur: when hydroxyl radicals react with BSA, the thiol group appears to act as a radical sink and protects the protein from denaturation and fragmentation through the transfer of damage from a carbon site to the thiol group. Thiol-blocked BSA is shown to be more susceptible to damage than the native protein in both direct EPR experiments and enzyme digestion studies. Oxygen has a similar effect, with more rapid fragmentation detected in its presence than its absence.

KEY WORDS: Radicals, EPR, protein damage, thiyl radicals, hydroxyl radicals, hydrogen peroxide, fragmentation, denaturation

INTRODUCTION

Free radicals generated both as a result of external factors (such as exposure to radiation and certain chemicals) and endogenous processes (for example, the respiratory burst, mitochondrial electron transfer and metal-catalysed oxidation) are known to produce widespread damage to a number of biological targets.[1] Thus it is known that the generation of species such as hydroxyl, peroxyl, alkoxyl and superoxide radicals can lead to extensive modification of cellular proteins, including fragmentation, cross-linking and changes in amino-acid composition.[2-5] These alterations often result in changes in structure and conformation, and loss of function. Evidence has been presented for the rapid degradation of the majority of these damaged materials by intracellular enzymes, either directly if the damaged protein is intracellular, or after endocytosis if the altered material is extracellular.[3,5-8] Such processing, which is much more rapid than that of native protein, does not appear to be totally efficient, as a recent report[8] has demonstrated that a small fraction of altered BSA is catabolised much less rapidly than unaltered, native, BSA and hence

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accumulates within cells; this may be an important factor in certain diseases (e.g. atherosclerosis) and ageing.[8]

However, the radical species generated within proteins which lead to such alterations and changes in function are not well characterised and evidence is lacking concerning both the detailed mechanisms of these reactions and the role of particular amino acids. In particular the thiol group of cysteine residues may play an important role in free-radical damage to proteins, as it is known that in the absence of oxygen aggregation of proteins can occur via the formation of disulphide bonds,[9-12] and that the loss of activity of certain enzymes is due to oxidation of such residues to sulphoxides or sulphonic acids.[13-15] The role of some of the more reactive amino acids has been explored by Prutz and co-workers[16,17] who have demonstrated that under certain circumstances (particularly the absence of oxygen) an electron-transfer mechanism may operate which leads to the transfer of the radical species from the initial site of damage to amino acids elsewhere in the protein and in particular to tyrosine, tryptophan and cysteine residues, which results in the formation of phenoxyl, tryptophanyl and thiyl radicals, respectively.

In order to obtain further information as to the role of the thiol group in radical-induced damage to proteins, and BSA in particular, we have carried out an EPR spin-trapping study; this technique is known to give valuable information on the generation of thiyl radicals[18-20] and on the identity of protein-radical intermediates produced when proteins are exposed to radical-generating systems such as metal ion/peroxide couples.[21]

EXPERIMENTAL

Chemicals

Bovine serum albumin (fatty-acid free) was obtained from Sigma Chemical Co. and used as supplied, with the exception of experiments with the thiol-blocked protein; this was prepared as described previously.[22] All other chemicals were commercial samples of high purity and used as supplied, with the exception of the spin traps 5, 5-dimethyl-l-pyrroline N-oxide (DMPO), which was purified before use by treatment with activated charcoal, and 3,5-dibromo-4-nitrosobenzene-sulphonic acid (sodium salt; DBNBS) which was synthesised as described previously.[23]

EPR Spectroscopy

Spectra of incubations carried out at room temperature in an aqueus-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 determined directly from the field scan. Experiments using Fe(II)/EDTA were carried out by the addition of the preformed iron complex (prepared in deoxygenated, deionised, water) to the other reaction components; all other solutions were prepared in potassium phosphate buffer (50 mM, pH 7.4) using normoxic, deionised, water. In experiments in which the effect of oxygen was examined, the samples were degassed, before mixing, using repeated freeze-pump-thaw cycles on a vacuum line.

Simulations

Experimental spectra of partially immobilized spin-adducts were simulated using a programme, originally developed for slowly-tumbling nitroxides[24] and adapted (by Dr. Adrian Whitwood, Department of Chemistry, University of York) to include additional isotropic β-hydrogen splittings, run on an IBM-compatible 486 DX PC.

Enzyme Digestion

Enzymatic degradation of proteins which had been allowed to react with radical-generating systems in the presence of DBNBS were carried out at 25°C under normoxic conditions using protease (*ca.* 15 units/ml final concentration).

RESULTS AND DISCUSSION

Spin-trapping studies

(a) DMPO

In order to investigate whether protein-derived thiyl radicals could be directly detected using EPR spin-trapping with the spin trap DMPO (which has been previously used to detect low-molecular-weight thiyl radicals[19]), initial experiments were carried out using Ce^{4+} to oxidise the single cysteine residue in BSA (at position 34). This particular reagent was chosen as it is a relatively selective oxidant and has been previously shown to oxidise thiyl groups in organic molecules and proteins;[18,25] by selectively generating this radical in relatively high yields it was hoped that its spin-adduct could be identified (and hence its presence in other reaction systems determined).

Reaction of a solution of BSA (0.5 mM) with a Ce^{4+}-NTA complex (25 and 50 mM respectively) in the presence of DMPO (25 mM) in phosphate buffer, pH 7.4, resulted in the detection of a relatively weak, broad, four-line spectrum. The intensity of this spectrum (and hence concentration of the spin adduct) was improved when the concentrations of both BSA (1.5 mM) and DMPO (50 mM) were increased (see Figure 1a). Analysis of this spectrum shows that it consists of absorptions from two species; one of these species has an isotropic spectrum (with relatively sharp lines which reflect the occurrence of rapid molecular motion) and the other is relatively anisotropic (i.e. with lines broadened as a result of slow molecular motion); this indicates that both low-molecular-weight and high-molecular-weight radicals have been trapped by DMPO. When this experiment was repeated using reagents which had been previously degassed by the freeze/thaw method, the isotropic components of the spectrum disappeared (Figure 1b). Omission of either the Ce^{4+}-NTA complex or the spin trap from either type of experiment resulted in the loss of all signals whereas omission of the protein resulted in observation of the well characterised oxidation product of the spin trap, DMPOx.[26]

The anisotropic EPR spectrum observed both in the presence and absence of oxygen, which has the dominant triplet pattern expected for a slowly-tumbling nitroxide, is believed to be a spin-adduct of a **sulphur**-centred radical because of the selectivity of Ce^{4+} for the thiol residue and because the size of the β-hydrogen splitting (1.3 mT) detected on all three main peaks is comparable to (although slightly lower than) the typical β-hydrogen splittings of known sulphur-centred spin-adducts

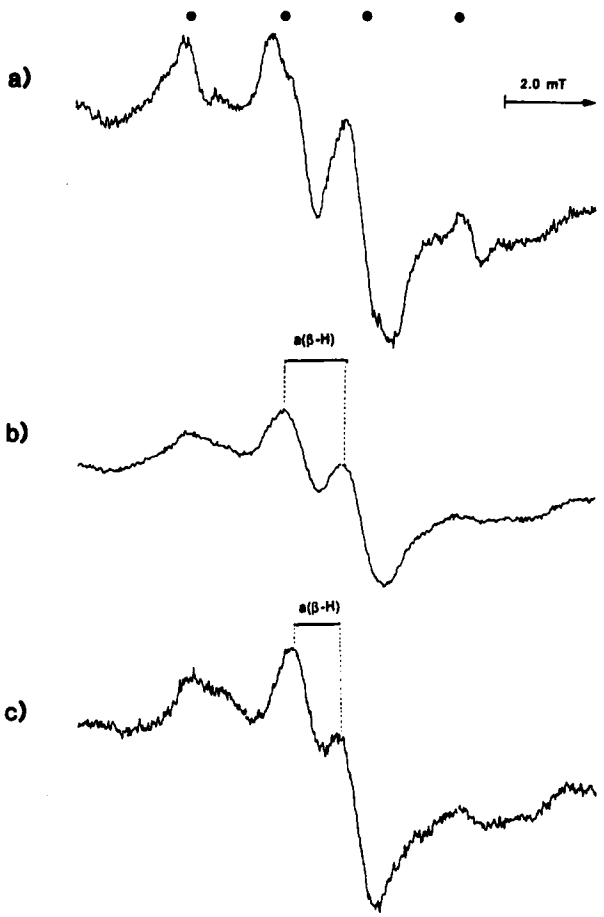


FIGURE 1 (a) EPR spectrum detected when native BSA (1.5 mM) reacts with Ce^{4+} (25 mM) and the spin trap DMPO (50 mM) at pH 7.4 in the presence of oxygen. An isotropic component (•) is marked (see text). (b) Anisotropic EPR spectrum of a protein spin-adduct obtained when the experiment is repeated in the absence of oxygen; the β-proton doublet is indicated. (c) Anisotropic EPR spectrum obtained when thiol-blocked protein (BSA-NEM) reacts with Ce^{4+} (25 mM) and DMPO (50 mM) at pH 7.4 in the presence of oxygen. The β-proton doublet is indicated.

(1.4–1.8 mT).[19] There is a possibility, however, that the spin-adduct may derive from the reaction of Ce^{4+} with other amino-acids in the protein – for example, the aromatic amino-acids which are particularly sensitive to oxidation. To test this hypothesis, the reaction was repeated using a solution of BSA pretreated with N-ethylmaleimide (NEM, to block the thiol residue[22]). The resulting EPR spectrum (which is only observed in the presence of oxygen) is shown in Figure 1c; it is clearly different from that obtained with the native protein. This spectrum has a β-hydrogen splitting of 0.9 mT and is assigned to the spin-adduct of an **oxygen**-centred radical (possibly a peroxyl species) since the β-hydrogen splitting is comparable to those of typical **oxygen**-centred adducts (1.0–1.5 mT).[26]

The isotropic spectrum observed when the native protein reacts with Ce^{4+}-NTA

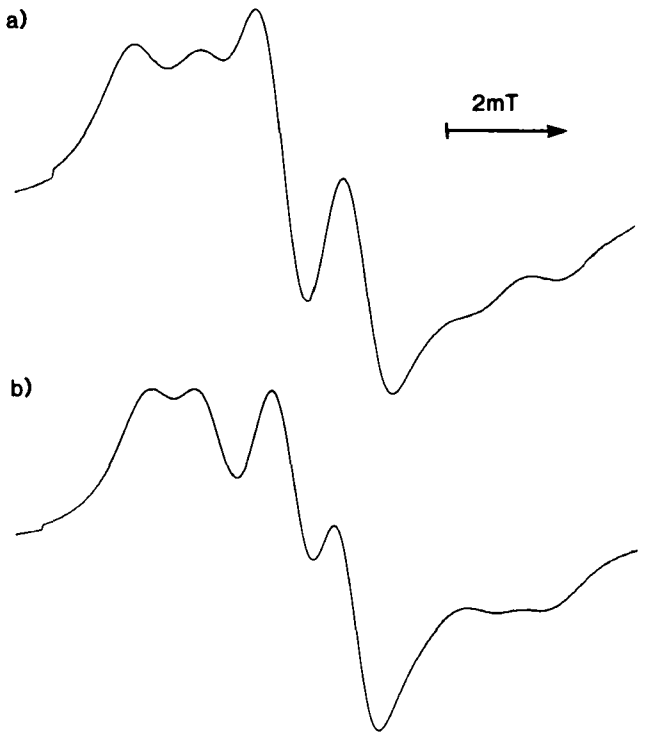
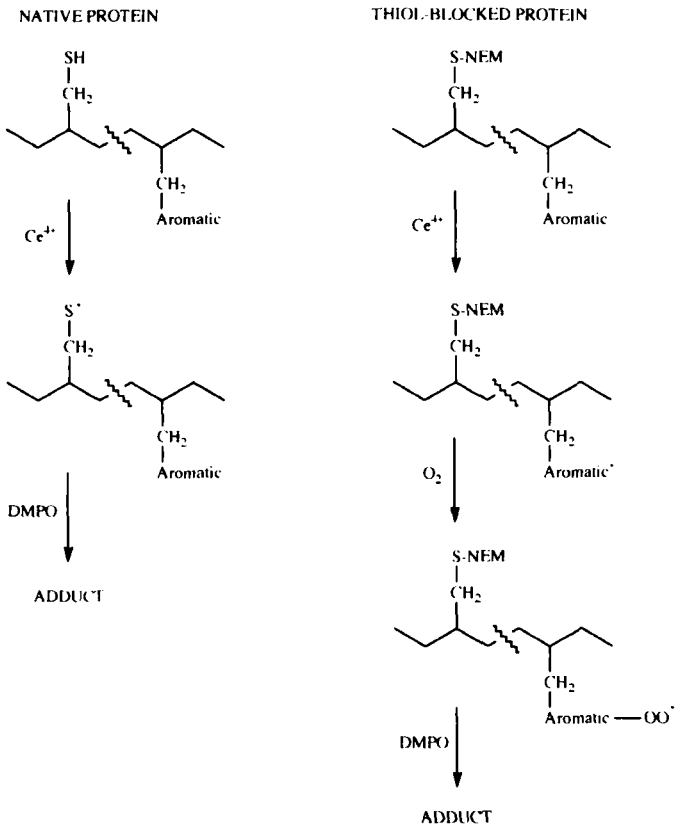


FIGURE 2 Simulated EPR spectra of a sulphur-centred spin-adduct (a) and an oxygen-centred spin adduct (b). The spectra were calculated using the program given in ref. 24, using the parameters described there (g_{xx} 2.0080, g_{yy} 2.00610, g_{zz} 2.00270; a_{xx} 0.58 mT, a_{yy} 0.58 mT, a_{zz} 3.08 mT, d_{xy}, d_{zz} 10^7 s^{-1}) together with an extra isotropic double splitting [(a) 1.3 mT, (b) 0.9 mT].

in the presence (but not absence) of oxygen is also believed to arise from the trapping of an oxygen-centred radical on the basis of its β-hydrogen coupling (1.3 mT). The exact identity of this species cannot be determined from the present data though it is believed, by comparison with previous data, that this may be either an alkoxyl or peroxyl radical, arising from either further reactions of the thiyl radical or oxidation of the aromatic amino acids.[27,28]

Further support for the assignment of the anisotropic spectra to sulphur- and oxygen-centred radical adducts is provided by simulations carried out with a programme suitable for modelling the EPR spectra of nitroxides undergoing slow or restricted motion.[24] Our approach, in which we have adapted the programme to incorporate the addition of an (isotropic) β-hydrogen doublet, has been to assume that there is no change in the size of the β-hydrogen splitting as the mobility of the spin-adduct decreases, an assumption which appears to be valid for the majority of partially mobile (as opposed to completely immobilised) adducts. Simulations of the observed spectra using the experimentally determined β-hydrogen splittings are shown in Figure 2.

These results establish firstly that it is indeed possible to generate thiyl radicals on the protein (as previously suggested[18,20,21]) and that these radicals can be directly detected as stable, relatively long-lived, spin-adducts to the spin trap DMPO.



Secondly, it is shown that BSA, which has had its thiyl group blocked with NEM, reacts with Ce^{4+}-NTA to give rise to a spin-adduct which is believed to be a peroxyl radical; this may arise from addition of oxygen to carbon-centred radicals derived, ultimately, from oxidation of aromatic amino acids by this metal ion (Scheme 1).

The success of these experiments raises the question as to whether thiyl radicals can also be detected when the hydroxyl radical reacts with BSA: it is known that the thiol group is the most sensitive amino acid with regard to oxidation by ·OH (with k 3.4×10^{10} dm^3 $mol^{-1}s^{-1}$)[29] and therefore it might be expected that a thiyl radical would be generated. However, previous experiments carried out using Fe^{2+}-EDTA and H_2O_2 (2 mM and 100 mM respectively) as a source of hydroxyl radicals gave only signals which were assigned to **carbon-centred** radical adducts.[21] The failure to detect a thiyl-radical adduct suggests that the thiol is not a major site of initial ·OH attack on the protein (as might be predicted on the basis of a single cysteine residue out of a total of 588 amino acids, despite the differences in rate constants for ·OH attack), though it does not rule out a role for this group in the radicals which are detected.

To study this possibility the reaction between BSA (1.0 mM) and ·OH [generated from Fe^{2+}-EDTA (both 2 mM) and H_2O_2 (0.1 M)] and DMPO (6 mM) was re-examined using BSA which had been pretreated with NEM to block the cysteine residue. The EPR spectrum observed (Figure 3a) is dramatically different to that obtained with the native protein (Figure 3b). When the experiment (with the

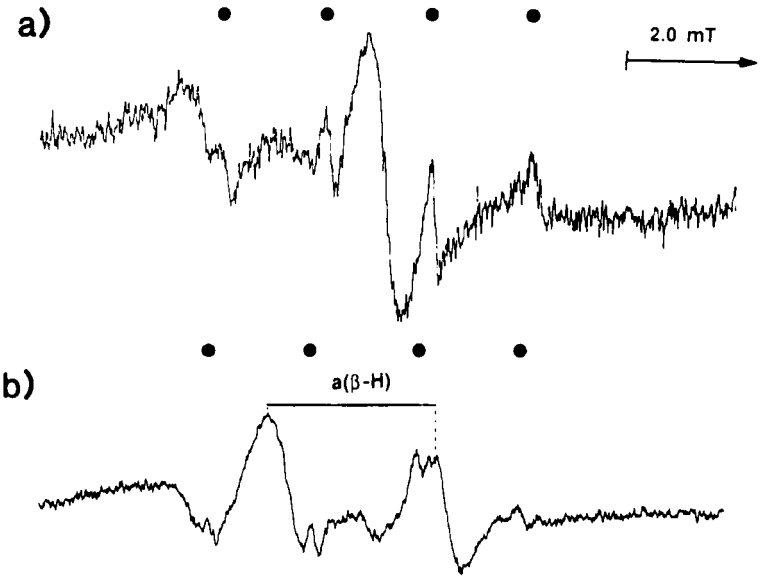
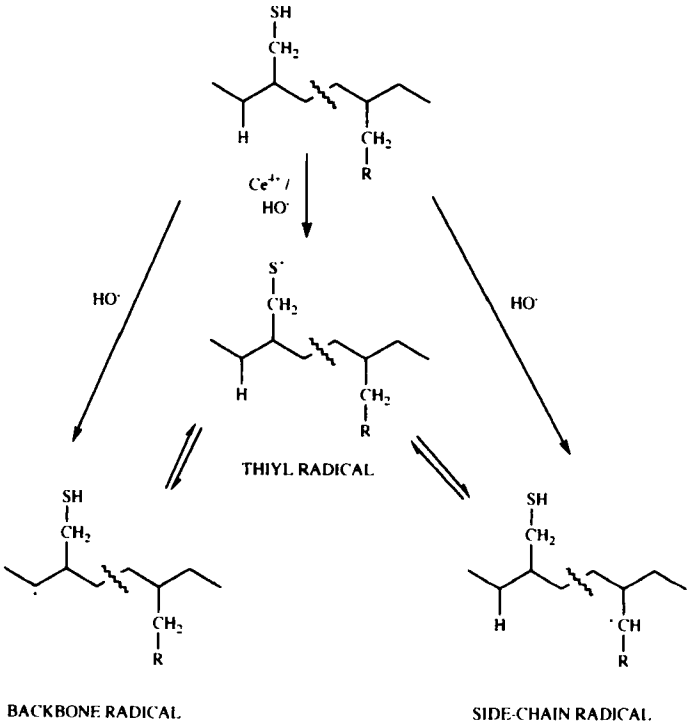


FIGURE 3 (a) EPR spectrum obtained from the reaction of thiol-blocked BSA (1.0 mM) with ·OH (generated from the reaction between 2 mM Fe^{2+}/EDTA and 0.1 M H_2O_2) and the spin trap DMPO (6 mM) at pH 7.4 in the presence of oxygen. The spectrum of the hydroxyl-radical adduct is also indicated (●). (b) The spectrum of a spin-adduct of a carbon-centred radical obtained which the reaction is repeated with native BSA. The spectrum of the hydroxyl-radical adduct is also indicated (●).

thiol-blocked protein) was repeated using degassed solutions the intensity of the observed signals decreased considerably. Each of these spectra consist of two components; an isotropic signal (●) due to the hydroxyl-radical adduct to the trap and an anisotropic signal. With the thiol-blocked protein this latter component, which appears not to have a resolved β-hydrogen splitting, may correspond either to a spin-adduct of a radical formed via a mechanism which involves a peroxyl-radical intermediate (as it is not observed in the absence of O_2) or may even correspond to a peroxyl radical adduct itself (with a very low β-hydrogen splitting). Evidence which supports the latter hypothesis is provided by the known low β-hydrogen splittings for peroxyl radicals (see ref. 26 and earlier) and the results of experiments in which known peroxyl-radical adducts were gradually restricted in motion by freezing, whereby a gradual broadening and hence loss of the β-H splitting was observed. The large β-proton splitting in the anisotropic spectrum in Figure 3b is characteristic of a carbon-centred radical-adduct.[21]

These results show firstly that reaction of native BSA with ·OH and DMPO gives a carbon-centred spin-adduct, which is not detected when the protein is pretreated with NEM. This suggests, therefore, that at some stage in the mechanism by which when the native protein reacts with ·OH, damage is transferred from sulphydryl (presumably thiyl) radical intermediates to carbon-centred radicals (see Scheme 2). It is believed, however, that the thiol is not the primary site of attack by ·OH, since it is known that direct oxidation of cysteine (with Ce^{4+}) gives a **sulphur**- and not a carbon-centred spin-adduct. It is also expected that the relatively unselective ·OH will react randomly at the surface of the protein. It is therefore suggested that the results are best interpreted as providing support for the mechanism which has been



SCHEME 2

suggested by Prutz,[16,17] in which the thiol acts as a "sink" in an electron-transfer process which takes place in the protein following initial random attack by ·OH on the protein.

Secondly, we note that reaction of thiol-blocked BSA with ·OH and DMPO does not give high concentrations of carbon-centred spin-adducts despite the fact that carbon-centred radicals are undoubtedly formed as a result of random attack of ·OH on the protein surface. This probably arises as a result of two factors; firstly the rapid reaction of the carbon-centred radicals with oxygen to give peroxyl radicals (believed to be the species observed in these experiments) and a slow rate of trapping of these large carbon-centred radicals by DMPO.

(b) DBNBS

In order further to investigate transfer of radical damage additional experiments have been carried out using the spin trap DBNBS. It has been previously shown[21] that reaction of ·OH with BSA in the presence of this trap gives rise to an anisotropic spectrum which (on the basis of enzyme degradation and model compound studies) is assigned to a mixture of spin-adducts of secondary and tertiary carbon-centred radicals as a result of random attack of ·OH on both the backbone (α-carbons) and side-chains of the protein. To investigate whether the generation of these spin-adducts is dependent on the presence of a free thiol group (as might be expected on

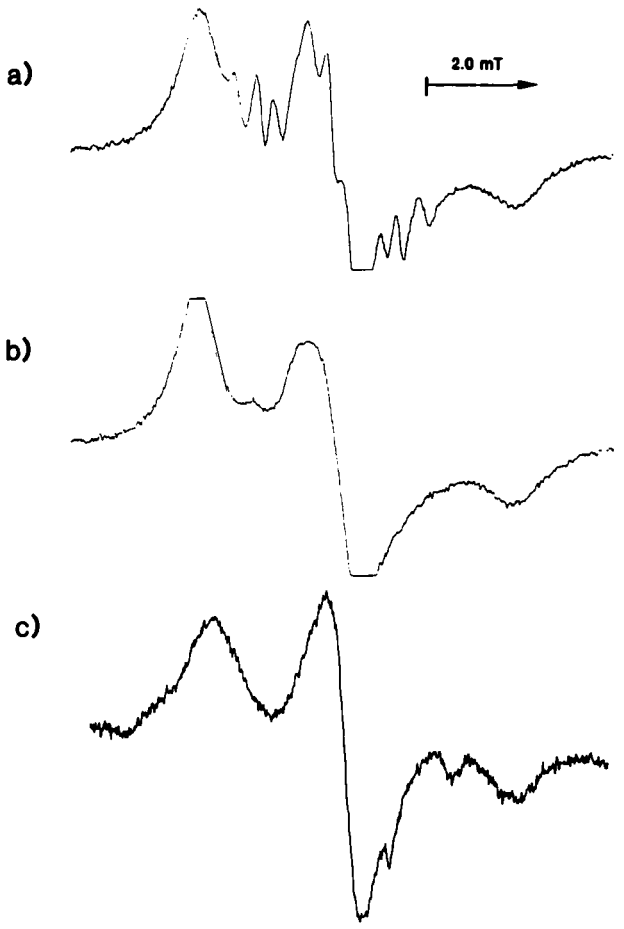


FIGURE 4 (a) EPR spectrum of protein-derived spin adducts obtained when thiol-blocked BSA (1 mM) reacts with Fe^{2+}/EDTA (2 mM), H_2O_2 (0.1 M) and the spin-trap DBNBS (10 mM) at pH 7.4 in the presence of oxygen. (b) EPR spectrum from HO·, native BSA, and DBNBS [conditions as in (a)]. (c) As 4(b) but experiment carried out in absence of oxygen.

the basis of the above results) the reaction of ·OH with thiol-blocked BSA was studied. Reaction of this treated protein (1 mM) with ·OH [from Fe^{2+}-EDTA (both 2 mM) and H_2O_2 (0.1 M)] and DBNBS (10 mM) gave the spectrum shown in Figure 4a; this is similar to that obtained with the native protein (Figure 4b) though the former is both less intense (i.e the radical concentration is lower) and also contains isotropic components which are not observed in the latter case. When these experiments were repeated in the absence of O_2 (achieved by freeze/thawing) little change was observed in the spectrum from the thiol-blocked protein whereas quite marked changes [believed to be due to an increase in mobility of the adducts(s)] were observed with the native protein (Figure 4c).

The effect of blocking the thiol decreases the intensity of the anisotropic signal and results in the detection of isotropic spin-adducts; the latter may or may not be present in the EPR spectrum obtained from the native protein (as they may simply be

obscured by the stronger signal of the anisotropic spin-adduct). Since the intensity of the anisotropic signal decreases when the thiol is blocked it is believed that this signal corresponds to a carbon-centred radical which requires for its formation, at least to some extent, the presence of free thiol in the protein, but it can also be formed by direct attack by ·OH. The presence of isotropic spin-adducts in the spectrum suggest that the effect of blocking the thiol may be to induce denaturation or fragmentation of the protein. An alternative explanation that the blocking of the thiol group simply causes transference of the site of ·OH attack to other sites/regions of the protein, to give more mobile components; this is felt to be unlikely in view of the earlier results and the indiscriminate nature of ·OH attack. The close resemblance of the isotropic spectrum (which appears to comprise a mixture of a triplet and a triplet of doublets) to the EPR spectrum obtained when BSA spin-adducts are digested with proteolytic enzymes[21] supports the hypotheses that fragmentation of the protein may have occurred, and that the presence of the thiol group protects the protein from fragmentation and/or denaturation by acting as a radical sink.

Further evidence that the effect of blocking the thiol group is to increase damage to the protein was obtained from studying the effects of urea (8 M) on the spin-adduct spectra. It would be expected that the addition of this denaturing agent would have dramatic effects on spin-adduct mobility [and hence the size of the overall splitting between the outermost features of the spectra ($a_{\parallel}$)] if the protein had retained its original globular conformation, but relatively small effects if it had already been denatured. In accord with this, little change was observed with the thiol-blocked protein (suggesting that the protein was already denatured), but dramatic effects were seen with the native protein (Figure 5). The reaction of Ce^{4+} with BSA in the present of DBNBS was also studied, since if proteins generate mainly thiyl radicals (as suggested by the above results) which then transfer damage, then carbon-centred radicals would be detected as spin-adducts to this trap: their identification should be straightforward, especially as thiyl radical adducts to nitroso compounds are, in general, unstable and hence not detectable without using flow techniques.[30] Reaction of BSA (1 mM) with Ce^{4+}-NTA (17 and 34 mM respectively) in the presence of DBNBS (10 mM) gave the intense EPR spectrum shown in Figure 6a, which corresponds to that of a highly immobile spin-adduct (with $a_{\parallel}$ *ca.* 3.22 mT); this is assigned to a tertiary carbon-centred radical adduct, since proteolytic digestion of this species gives an isotropic triplet spectrum with no β-hydrogen splittings (Figure 6b). [This anisotropic spectrum itself is an unusual shape, which could suggest that there are a mixture of species present; none of these are believed to be a thiyl-radical adduct which would have a significantly offset spectrum due to the higher g-value of this type of spin-adduct.[30]]

This result provides further evidence that carbon-centred radicals are being formed, at least in part, via oxidation of the thiol group by Ce^{4+} and subsequent transfer of damage (see Schemes 1 and 2). To confirm this, these experiments were repeated with thiol-blocked BSA. In this case the spectrum is considerably weaker in intensity and the spin adduct(s) detected are somewhat more mobile in nature (with lower $a_{\parallel}$: see Figure 6c); this spectrum is lost completely if oxygen is removed from the reagent solutions before the reaction is carried out. These results confirm that the carbon-centred radicals observed when the native protein reacts with Ce^{4+} do indeed derive, at least in part, from initial generation of a thiyl radical and subsequent transfer of damage presumably via hydrogen abstraction from neighbouring amino acids by the RS· radical (cf. Scheme 2 and recent work on such reactions[31-33]). The species obtained with the thiol-blocked protein is believed to derive from reaction of Ce^{4+}

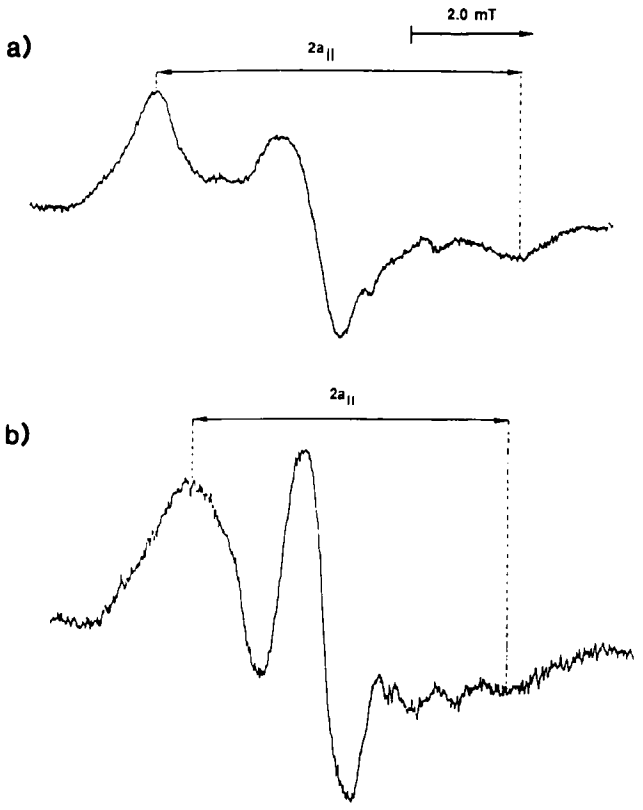


FIGURE 5 (a) EPR spectrum of spin adduct formed from BSA (0.5 mM) and DBNBS (2.5 mM) following reaction with the hydroxyl radical, generated from $FeSO_4$/EDTA (2 mM) and H_2O_2 (0.1 M) at pH 7. (b) EPR spectrum obtained by the addition of urea (final concentration 8 M) to the solution whose spectrum is shown in (a).

with aromatic amino acids (which are known to be susceptible to oxidation). However, the radicals are not observed in the absence of O_2, which suggests that they are side-chain or backbone carbon-centred radicals formed via peroxyl radical intermediates produced by addition of O_2 to the initially generated radicals. The peroxyl radicals themselves would not be expected to be observed with this trap as the adducts of such radicals to DBNBS are highly unstable; as noted earlier, they were observed with DMPO as the trap.

Enzymatic Digestion of Protein Spin-adducts

To obtain additional information about the role of the thiol in free-radical damage, the effects of blocking the thiol on the **rate** of enzyme digestion of spin-adducts were subsequently explored. From the results described previously, it is known that digestion with proteolytic enzymes of the spin-adducts obtained by reaction of native BSA with ·OH and DBNBS gives freely rotating spin-adducts which can be identified as secondary and tertiary carbon-centred radicals. When similar experiments were

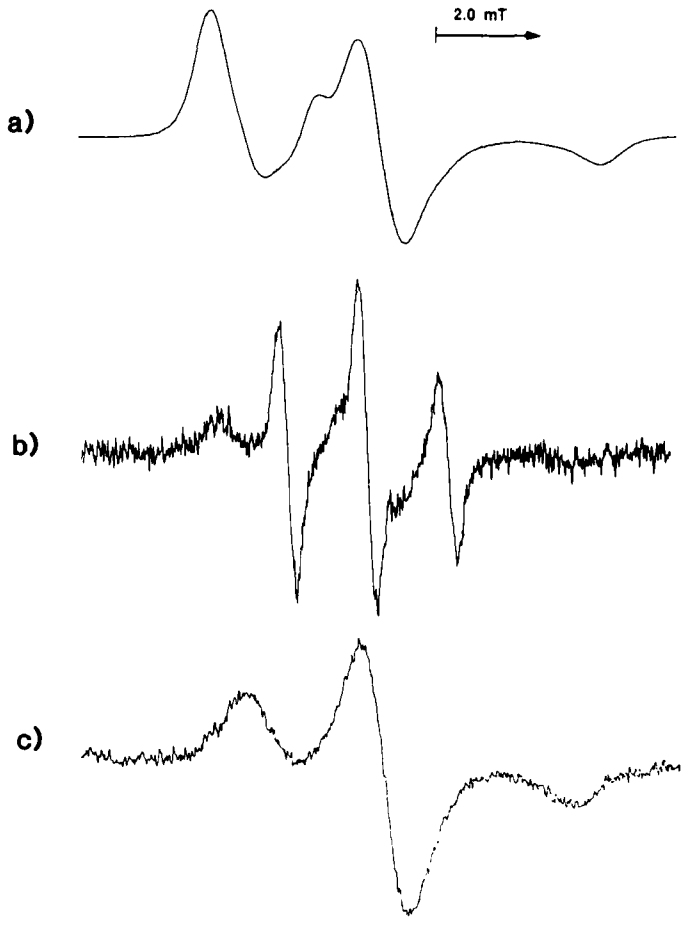


FIGURE 6 (a) Anisotropic EPR spectrum of a protein spin-adduct obtained when native BSA (1 mM) reacts with Ce^{4+} (17 mM) and DBNBS (10 mM) at pH 7.4 in the presence of oxygen. (b) Isotropic ESR spectrum obtained when the spin adduct shown in (a) is digested with non-specific protease enzyme (15 units/ml) at room temperature. (c) Spectrum obtained from thiol-blocked BSA (1 mM), Ce^{4+} (17 mM) and DBNBS (10 mM) at pH 7.4 in the presence of oxygen.

carried out with BSA (pretreated with NEM) it was found that similar spectra comprising secondary and tertiary carbon-centred radicals were obtained; however, it was observed that the rate of enzyme cleavage was much faster than that for native BSA (the rate of release of the tertiary carbon-centred radical with time is shown in Figure 7). The triplet signal was chosen for these studies as this adduct is more stable than the other (triplet of doublets) signal and hence is less likely to be affected by decay of the radical signal during the digestion process.

These results establish that the release of tertiary carbon-centred radicals is indeed faster with the thiol-blocked protein than the native form. This implies that the peptide backbone of thiol-blocked protein is more accessible to enzymatic cleavage and this is believed to reflect an increase in radical-induced fragmentation or denaturation of the protein prior to spin trapping.

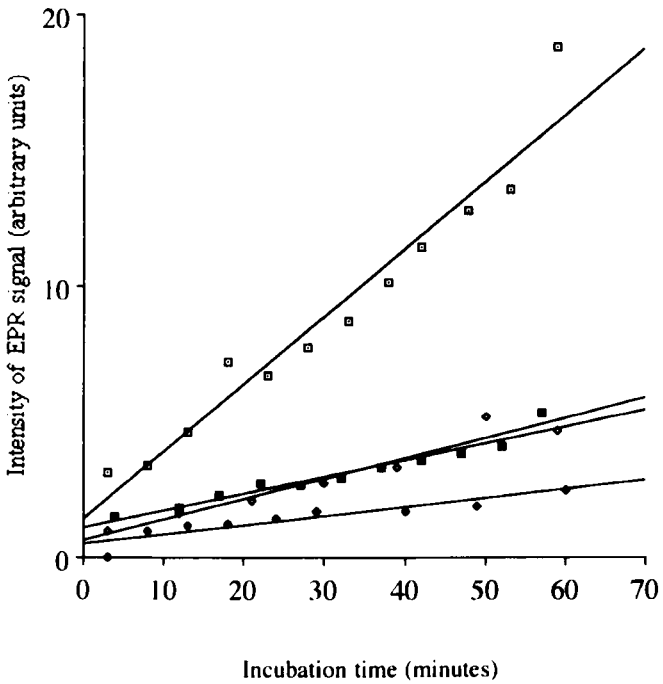


FIGURE 7 Variation with time in the intensity in the sharp isotropic triplet EPR signal [see for example Figure 6 (b)] following enzymic digestion of spin-trapped native (◇, ◆) or thiol-blocked (□, ■) BSA in both the presence (open symbols) and absence (filled symbols) of oxygen. Spin adducts were generated by reaction of the protein (1 mM) with Fe^{2+}/EDTA (2 mM), H_2O_2 (0.1 M) and DBNBS (10 mM) at pH 7.4 then subsequently digested with a non-specific protease enzyme (15 units/ml).

The results of the experiments described earlier support previous suggestions that oxygen plays a significant role in determining the extent of protein cross-linking and fragmentation after radical attack.[3,4] In order to confirm these observations the digestion experiments were repeated in the absence of oxygen. It was found that the rate of digestion, as measured by the increase in intensity of the isotropic triplet spectrum (which is a direct measure of the concentration of the low-molecular-weight tertiary-carbon-centred radical adducts), was considerably slower in the absence of oxygen than in its presence (see Figure 7). This confirms that the presence of oxygen increases the extent of denaturation and fragmentation.

CONCLUSIONS

Our spin-trapping results establish that the single cysteine residue in BSA is oxidized by cerium(IV) to give a thiyl radical, recognized via the anisotropic EPR spectra observed from adducts to DMPO. When the thiol group is blocked, experiments in the presence of oxygen lead to the detection of peroxyl adducts, probably formed via an oxidation pathway involving (undetected) carbon-centred radicals from the aromatic amino acids. Results with the spin-trap DBNBS (which is much less effective at trapping the first-formed thiyl radicals) provide crucial evidence for the

generation of these carbon-centred radicals: since this pathway is largely blocked while the thiol group is protected, we conclude that damage can be transferred from thiol to carbon sites in the protein, presumably via hydrogen transfer.

Reaction of the hydroxyl radical with BSA in the presence of both DMPO and DBNBS also allows carbon-centred radicals to be detected: reduction in signal intensity with thiol-blocked protein provides further evidence for sulphur-to-carbon transfer of oxidative damage.

However, several observations establish that, as might be expected, sulphur centres can also act to **protect** the protein from radical damage (via transfer of damage from carbon to sulphur). Thus the isotropic spectra seen from thiol-blocked protein with ·OH (in contrast to the anisotropic spectra detected from the native protein) confirm that radical attack can bring about denaturation or fragmentation. Further, enzymic degradation of the first-formed radical adducts is considerably faster for the thiol-blocked protein than the native (suggesting the damage to the peptide backbone has either occurred to a greater extent or is now more accessible), an increase which is also observed under oxygenated conditions (evidently as a result of peroxyl radical formation and the formation of irreparable lesions).

Acknowledgements

The authors are grateful to the Association for International Cancer Research for financial support and to Dr. Adrian Whitwood for assistance with the spectral simulations.

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Accepted by Professor B. Halliwell