Protein hydroperoxides can give rise to reactive free radicals

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Proteins damaged by free-radical-generating systems in the presence of oxygen yield relatively long-lived protein hydroperoxides. These hydroperoxides have been shown by e.p.r. spectroscopy to be readily degraded to reactive free radicals on reaction with iron(II) complexes. Comparison of the observed spectra with those obtained with free amino acid hydroperoxides had allowed identification of some of the protein-derived radical species (including a number of carbon-centred radicals, alkoxyl radicals and a species believed to be the CO_2 radical anion) and the elucidation of novel fragmentation and rearrangement processes involving amino acid side chains. In particular, degradation of hydroperoxide functions on the side chain of glutamic acid is shown to result in decarboxylation at the side-chain carboxy group via the formation of the CO_2 radical anion; the generation of an identical radical from hydroperoxide groups on proteins suggests that a similar process occurs with these molecules. In a number of cases these fragmentation and rearrangement reactions give rise to further reactive free radicals ($R^{\bullet}$, $O_2^{-\bullet}/HO_2^{\bullet}$, $CO_2^{-\bullet}$) which may act as chain-carrying species in protein oxidations. These studies suggest that protein hydroperoxides are capable of initiating further radical chain reactions both intra- and inter-molecularly, and provide information on some of the fundamental mechanisms of protein alteration and side-chain fragmentation.

INTRODUCTION

Free radicals can be generated either as by-products of normal cellular redox processes or via the interaction of cells and tissues with a variety of external agents and processes (e.g. thermal or photochemical reactions, irradiation with γ- or X-rays and neutrons, or the action of xenobiotics) [1,2]. Subsequent reactions of these species can produce cellular damage as a result of their interaction with nucleic acids, carbohydrates, lipids and proteins; as a consequence free radicals have been implicated in many pathological states [1–6]. Although considerable amounts of information are available on the action of radicals with, for example, DNA and lipids [1,3,5], relatively few studies have been carried out on their effects on proteins despite the fact that these are acknowledged to be important targets and may have considerable significance *in vivo* [6–9].

Until recently, radical-induced damage to proteins was considered to be mainly a chain-terminating process (i.e. radical generation on or within the protein did not result in the initiation or propagation of further radical-induced processes) and it was thought that the products of the damage produced on the protein (for example, protein scission, cross-linking, chemical modification of side chains) were relatively inert; these damaged materials are thought to be subsequently degraded by intra- and extra-cellular enzymes [8–11]. Although it appears to be true that most of the products are indeed relatively inert, it has been recently demonstrated [12,13] that two types of material, i.e. protein-bound reducing moieties (subsequently identified as mainly 3,4-dihydroxyphenylalanine [14]) and protein peroxides, are capable of initiating further chemical reactions. Thus the protein-bound reducing moieties have been shown to be able to reduce transition metals [12–14] resulting in redox cycling of these species (which are normally present solely in their oxidized states), and the protein peroxides have been demonstrated to consume important cellular reductants such as ascorbate and GSH (but not NADH or NADPH) via redox reactions [12]. Although this latter type of process results in increased oxidant stress as a result of a decrease in cellular antioxidant defences, repair of these molecules in enzymic reactions mediated by dehydroascorbate reductase and glutathione reductase [2] might be expected under normal conditions to migitate against prolonged or pronounced effects.

The possibility that these protein peroxides can be catalytically degraded (by, for example, iron or copper ions) to yield highly reactive and damaging free radicals, as observed with organic and lipid peroxides [15,16], has not been addressed in depth [12]; neither the identity of the species involved nor the consequences of these processes for the protein or the system as a whole are known. In the present study, e.p.r. spectroscopy in conjunction with spin trapping has been employed to examine such processes directly; previous studies have demonstrated the feasibility of this approach [17–19]. Evidence is provided for the generation of a wide variety of protein-derived reactive radical species, which may play an important role in the initiation and propagation of radical chain reactions *in vivo*, and the occurrence of novel side-chain fragmentation processes of glutamate-derived radicals which are suggested to be of importance in modifying protein structure and recognition.

EXPERIMENTAL

Materials

Solutions were made up in distilled water purified by passage through a four-stage Milli Q system equipped with a 0.2 μm-pore-size final filter. All glassware was cleaned by heating for 6–7 h in conc. HNO_3, washing in distilled water and drying. BSA (Cohn fraction V, fat-free), lysozyme, insulin and catalase were supplied by Boehringer-Mannheim. Superoxide dismutase (from bovine blood) and all amino acids and spin traps were from Sigma and used as supplied except for 5,5-dimethyl-1-pyrroline

Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; AAPH, 2,2′-azobis-(2-amidinopropane) hydrochloride; PBN, *N*-*t*-butyl-α-phenylnitrone; MNP, 2-methyl-2-nitrosopropane.

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N-oxide (DMPO) which was purified before use by treatment with activated charcoal. 2,2′-Azobis-(2-amidinopropane) hydrochloride (AAPH) was supplied by Polysciences, and [5-^{13}C] glutamate was obtained from CDN isotopes.

Preparation of hydroperoxides

Irradiation was carried out using a ^{60}Co facility as described previously [20], on oxygenated solutions (maintained by slow continuous bubbling with O_2) of 2 mg/ml protein or 1 mM amino acids made up in 10 mM Chelex-treated phosphate buffer, pH 7.4, at a dose rate of 36 Gy/min for 30 min. After irradiation, catalase (49 units/ml) was added to destroy radiation-generated H_2O_2; this treatment does not remove amino acid- or protein-derived hydroperoxides or interfere with any of the assays performed. Protein hydroperoxide samples were also prepared by treatment with AAPH or Fe^{2+}/H_2O_2 as described previously [20] with the treated protein being subsequently separated from low-molecular-mass materials by filtration on Sephadex G-25M columns. For experiments in which the radical-generating fraction(s) from the protein samples were investigated, samples were loaded and subsequently eluted from Pharmacia PD-10 Sephadex G-25M columns, using the method described by the manufacturers. All hydroperoxide samples were kept at −80 °C before use, which was within 3 weeks of preparation; this period of storage has been shown to have little effect on the peroxide levels [20], which were assayed using an iodometric method [21].

E.p.r. spectroscopy

E.p.r. spectra were obtained at room temperature (20 °C) using a Bruker ESP300 X-band spectrometer equipped with 100 kHz modulation and standard aqueous flat cells. Computer simulations of experimental spectra were carried out using a program written by Dr. M. F. Chiu [22] and adapted by Dr. A. C. Whitwood (both Department of Chemistry, University of York) to run on a VAX mainframe cluster. This program can simulate the isotropic spectra of up to ten radicals simultaneously using Lorentzian line shapes and takes into account the relative concentrations of each species and variations in linewidth, g values, multiplicity of splittings and, where necessary, second-order effects and exchange processes.

RESULTS

Incubation of γ-irradiated BSA (0.9 mg/ml protein; about 45 μM hydroperoxide final concentration) with Fe^{2+}–EDTA (100 μM; 1:1 complex), under either aerobic or anaerobic conditions, in the presence of the spin trap DMPO (0.166 M) resulted in the immediate detection of a complex mixture of spin adducts by e.p.r. spectroscopy (Figure 1a). The intensity of these signals, and hence spin-adduct concentrations, reached a maximum during the first 8 min of incubation and decreased slowly thereafter. Weak signals were observed on omission of the Fe^{2+} complex presumably because of thermal and/or photochemical degradation of the hydroperoxide at ambient temperatures under fluorescent laboratory lighting; this is in accord with previous studies on the stability of these hydroperoxides [12,20]. Omission of the γ-irradiated protein or inclusion of non-irradiated protein resulted in the detection of very low concentrations of spin adducts which are attributed to the autoxidation of the iron complex which results in formation of $O_2^{-\bullet}$ and subsequently HO$^\bullet$. In experiments where the γ-irradiated samples were not pretreated with catalase, the intensity of the signals from the $O_2^{-\bullet}$ and HO$^\bullet$ adducts increased but the presence and intensity of other signals were not affected, demonstrating that the observed radicals arise from reaction of the hydroperoxides on the protein with the added iron(II) chelate and not with the haem protein. In experiments carried out in the absence of the spin trap, no radical species were detected.

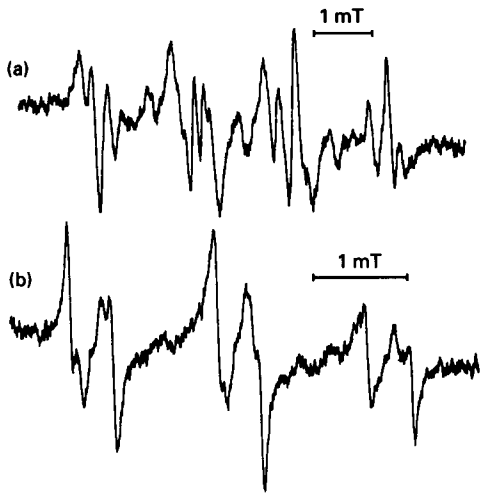


Figure 1 E.p.r. spectra observed on reaction of γ-irradiated BSA with Fe^{2+}–EDTA under normoxic conditions in the presence of spin traps

(**a**) With DMPO (0.166 M) as the trap; (**b**) with PBN (25 mM) as the trap. In each case the γ-irradiated protein, produced as described in the Experimental section, was incubated at 0.9 mg/ml with Fe^{2+}–EDTA (100 μM, 1:1 complex) in 50 mM phosphate buffer (pH 7.4) at 20 °C in a standard aqueous flat cell inserted into the cavity of the e.p.r. spectrometer (see the Experimental section). Spectrometer settings: gain 2×10^5, modulation amplitude, 0.05 mT, time constant 1.25 s, scan time 600 s, centre field 347.5 mT, field scan 8 mT (**a**) or 5 mT (**b**), power 30 mW, frequency 9.77 GHz.

Replacement of the spin trap DMPO with a second nitrone compound, *N*-*t*-butyl-α-phenylnitrone (PBN; 25 mM) also resulted in the detection of complex mixture of spin-adduct species with a similar time dependence (Figure 1b). The generation and detection of spin adducts with each of these spin traps is not unique to γ-irradiated BSA; qualitatively similar behaviour was observed with γ-irradiated lysozyme and insulin (Figure 2) and BSA that had been peroxidized with either a thermolabile azo initiator (AAPH) or a Fenton (Fe^{2+}/H_2O_2) system, although the relative and absolute concentrations of the spin-adduct species detected were somewhat different in each case.

With all three proteins, the spin adducts detected with both spin traps give rise to spectra that are either completely isotropic or only partially anisotropic, suggesting that the species that give rise to these absorptions have considerable freedom of motion (i.e. are tumbling rapidly in solution). These observations are consistent with the detection of either low-molecular-mass spin adducts (i.e. fragments arising from degradation of the protein) or protein-derived species with considerable local freedom of motion (cf. a previous study which suggested that local motion rather than overall molecular tumbling is the more important arbiter of spin-adduct spectral anisotropy [17]). The former could be envisaged as arising during the initial irradiation period or during the subsequent degradation of the protein-bound hydroperoxides by the added Fe^{2+}–EDTA. The first of these two explanations is inconsistent with both previous electrophoretic and h.p.l.c. size-exclusion chromatography studies [23]. This was confirmed by the results of experiments where γ-irradiated BSA samples were fractionated on Sephadex G-25M columns before treatment with added Fe^{2+}–EDTA; in these studies the radical-

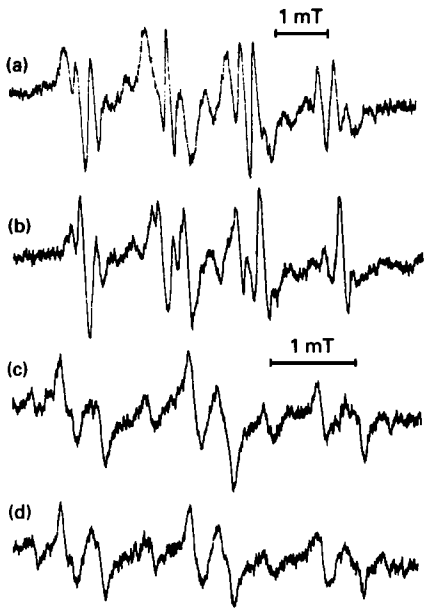


Figure 2 E.p.r. spectra observed on reaction of γ-irradiated lysozyme and insulin with Fe^{2+}–EDTA under normoxic conditions in the presence of spin traps

(**a**) Lysozyme with DMPO as spin trap; (**b**) insulin with DMPO as spin trap; (**c**) lysozyme with PBN as spin trap; (**d**) insulin with PBN as spin trap. Incubation and other experimental conditions were as Figure 1.

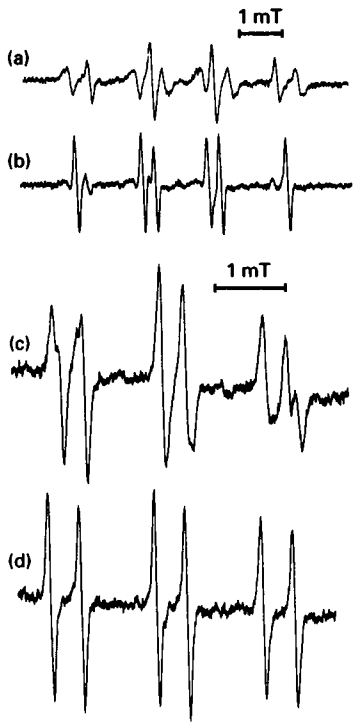


Figure 3 E.p.r. spectra observed on reaction of γ-irradiated valine and glutamate with Fe^{2+}–EDTA under normoxic conditions in the presence of spin traps

(**a**) Valine with DMPO as spin trap; (**b**) glutamate with DMPO as spin trap, (**c**) valine with PBN as spin trap, (**d**) glutamate with PBN as spin trap. Incubation and other experimental conditions were as in Figure 1 except that γ-irradiated amino acids were present at 0.57 mM and spectrometer settings for (**a**) and (**b**) were: gain 5×10^5, time constant 0.32 s, scan time 300 s.

adduct-producing material was co-eluted with intact protein. The observation of identical species in these experiments confirms that the catalase (or haem produced from degraded catalase) added after irradiation, which is removed by this treatment, is not involved in the generation of the radicals detected.

Attempts to determine the approximate molecular mass of the radical adduct species by fractionation of the preformed protein-derived nitroxide spin adducts using short Sephadex G-25M size-exclusion columns were only partially successful because of the decay of the adduct species; although the only nitroxide-containing fraction was eluted with the intact protein, the concentration of these adducts (as judged by their e.p.r. signal intensities) was considerably reduced which could be interpreted as either decay of intact protein-derived species or the formation of lower-molecular-mass species and the decay of these species (to undetectable levels) during the separation process.

The rapid motion of the adducts observed with either spin trap (which have rotational correlation times, τ_c, $> 10^{-10}$ s, as judged by the degree of anisotropy of the experimental spectra) were found to be unaffected by the addition of 5 M guanidinium chloride, suggesting that the species detected are slightly anisotropic (i.e. partially constrained) as a result of the physical size of the attached group(s) rather than a consequence of the steric constraints imposed by either (non-covalent) binding of a low-molecular-mass species to the protein or the secondary/tertiary structure of the protein. In contrast, addition of SDS (1.25%, w/v) produced broadening of the observed signals which is attributed to either the formation of micelles or the binding of the detergent to the protein-derived adduct.

In contrast with the highly anisotropic spectra observed with other protein–radical adducts [17,18,24], for which the hyperfine coupling constants are difficult to determine, the spectra obtained in this study yield valuable information as to the type of radical species trapped. Thus the spectra obtained with both γ-irradiated BSA and BSA treated with AAPH or Fe^{2+}/H_2O_2, in the presence of DMPO, can be assigned, by comparison with data from the literature [25], to at least one carbon-centred (alkyl) radical adduct (with coupling constants approx. a_N 1.58, a_H 2.28 mT), a species believed to be the $CO_2^{-\bullet}$ adduct (with a_N 1.557, a_H 1.860 mT), an adduct with a_N 1.49, a_H 1.50 mT which is assigned to either the hydroxyl radical adduct or an alkoxyl radical adduct, and a species that does not have any β-hydrogen splittings; this last radical is believed to be a degradation product of the spin trap. The relative concentrations of these adducts (as determined by computer simulation) for γ-irradiated BSA and the corresponding ratios for the AAPH-treated BSA are dramatically different, with considerably less of the species believed to be the $CO_2^{-\bullet}$ adduct in the latter case. These changes are attributed to differences in the reactivity and selectively of the initial attacking radicals ($HO^\bullet$ and $ROO^\bullet$ respectively) which result in significantly different yields and sites of hydroperoxide formation on the protein. Similar spectral analyses are proposed for the spectra observed with γ-irradiated lysozyme and insulin although again the relative concentrations of the adducts are different; this is believed to the due to differences in the protein structures and composition and suggests that individual proteins may give unique 'fingerprints'. Addition of large amounts of additional catalase to these samples and preincubation of the samples before the addition of Fe^{2+}–EDTA diminished the signal intensity, but did not completely remove the signals assigned to the hydroxyl/alkoxyl radical adduct, suggesting that at least part

Table 1 Hyperfine coupling constants of radical adducts generated on decomposition of γ-irradiated proteins and amino acids

The protein and amino acid samples were prepared and treated as described in the Experimental section. The carbon-centred species detected with DMPO, PBN and MNP are believed to be due to trapping of the same species in each case.

Source	Spin trap	Assignment of trapped radical	Hyperfine splittings (mT) a_N	a_H
BSA	DMPO	Carbon-centred*	1.580	2.280
Insulin	DMPO	$CO_2^{-\bullet}$	1.557	1.860
Lysozyme	DMPO	Alkoxyl	1.490	1.500
	PBN	$CO_2^{-\bullet}$	1.570	0.460
	PBN	Carbon-centred*	1.580	0.270
Glutamate	DMPO	$CO_2^{-\bullet}$	1.557	1.860
	DMPO	Carbon-centred	1.566	2.250
	DMPO	Alkoxyl	1.490	1.500
	PBN	$CO_2^{-\bullet}$	1.580	0.460
	MNP	Alkoxyl	2.672	–
Isoleucine	DMPO	Carbon-centred*	1.576	2.264
	DMPO	Alkoxyl	1.490	1.500
	DMPO	$RO_2^{\bullet}/O_2^{-\bullet}$	1.430	1.170, 0.130
	PBN	Carbon-centred	1.640	0.330
	PBN	Carbon-centred	1.520	0.347
	MNP†	Tertiary carbon-centred‡	1.470	–
Leucine	DMPO	Carbon-centred	1.587	2.254
	DMPO	Carbon-centred	1.588	2.350
	DMPO	Alkoxyl	1.490	1.500
	DMPO	$RO_2^{\bullet}/O_2^{-\bullet}$	1.430	1.170, 0.130
	PBN	Carbon-centred	1.520	0.340
	PBN	Carbon-centred	1.600	0.330
	MNP†	Secondary carbon-centred§	1.540	0.182
	MNP†	Alkoxyl	2.670	–
Lysine	DMPO	Carbon-centred*	1.555	2.243
	DMPO	Alkoxyl	1.490	1.500
	PBN	Carbon-centred	1.560	0.530
	PBN	Carbon-centred	1.640	0.390
	MNP†	Alkoxyl	2.580	–
	MNP†	Secondary carbon-centred‖	1.635	0.381
Proline	DMPO	Carbon-centred*	1.540	2.265
	DMPO	Alkoxyl	1.490	1.500
	DMPO	$RO_2^{\bullet}/O_2^{-\bullet}$	1.430	1.170, 0.130
	PBN	Carbon-centred	1.520	0.307
	PBN	Carbon-centred	1.570	0.460
	PBN	Carbon-centred	1.530	0.613
	MNP†	Alkoxyl	2.563	–
	MNP†	Tertiary carbon-centred	1.590	–
Valine	DMPO	Carbon-centred	1.582	2.222
	DMPO	Carbon-centred	1.580	2.450
	DMPO	Alkoxyl	1.490	1.500
	PBN	Carbon-centred	1.507	0.333
	PBN	Carbon-centred	1.600	0.347
	MNP†	Secondary carbon-centred‖	1.587	0.635
	MNP†	Alkoxyl	2.563	–
	MNP†	Tertiary carbon-centred¶	1.680	–
	MNP†	Tertiary carbon-centred	1.440	–

* More than one species present; hyperfine splittings are approximate values due to broad overlapping lines.
† Signals from t-BuNHO$^{\bullet}$ also present; believed to be formed via reduction of the spin trap by α-hydroxyalkyl radicals, and subsequent protonation.
‡ Believed to be due to the adduct of $^{\bullet}C(OH)(CH_3)CH(CH_3)CH(NH_3^+)COO^-$ formed via reactions (1) and (5) given in the Discussion section.
§ Believed to be due to the adduct of $^{\bullet}CH(OH)CH(CH_3)CH_2CH(NH_3^+)COO^-$ formed via reactions (1) and (6) given in the Discussion section.
‖ Believed to be due to an adduct with the partial structure $^{\bullet}CHR^1R^2$ formed via a hydrogen-shift reaction of an initial alkoxyl radical.
¶ Believed to be due to an adduct with the partial structure $^{\bullet}C(OH)R^1R^2$ formed via reactions (1) and (5) given in the Discussion section.

of this signal is due to the latter species. The partial inhibition of this signal by catalase present in the reaction mixture also suggests that some H_2O_2 is formed during, or as a result of, the reactions taking place on addition of Fe^{2+}–EDTA.

The species observed with the second nitrone spin trap PBN are likewise assigned to mixtures of carbon-centred radicals with hyperfine coupling constants a_N 1.57, a_H 0.46 mT and a_N 1.58, a_H 0.27 mT. In this case signals from hydroxyl/alkoxyl radical adducts were not observed, presumably as a result of their low concentration and/or rapid decay.

Further information on the identity of these radical adducts was obtained from similar studies carried out with the amino acids glutamate, lysine, proline, valine, leucine, and isoleucine, which are known to produce significant levels of amino acid

hydroperoxides on γ-irradiation [20], and three different spin traps, DMPO, PBN and the nitroso spin trap 2-methyl-2-nitrosopropane (MNP). The last of these traps was employed in an attempt to obtain further information on the exact identity of the species present, as this trap gives adduct species in which the added radical is directly bonded to the nitroxide function rather than one carbon removed; the spectra of such adducts often therefore exhibit additional (diagnostic) hyperfine coupling [25]. All of these γ-irradiated amino acids gave detectable spin adducts on addition of Fe^{2+}–EDTA (see, for example, Figure 3), under either aerobic or anaerobic conditions, in the presence of DMPO, PBN or MNP. In contrast, experiments with the amino acid alanine, which does not give a high yield of hydroperoxide(s) on γ-irradiation [20], did not give any detectable radical adducts. With the first of these traps, carbon-centred and hydroxyl/alkoxyl radical adducts were detected in all cases with parameters similar to, or identical with, those determined from the spectra of the irradiated proteins; with PBN, carbon-centred species were observed which again match some of those detected in the protein experiments. Use of the nitroso trap MNP yielded considerable further information on the exact identity of some of these carbon-centred species as a result of the additional hyperfine couplings present in the spectra of MNP radical adducts. A summary of the radical adducts detected and the proposed assignments (made by comparison of the experimentally determined values with data from the literature [25]) are presented in Table 1.

In only a single case, that of γ-irradiated glutamate, was a signal observed that was identical with that detected, and assigned to the $CO_2^{-\bullet}$ adduct to DMPO, with the radical-damaged proteins. Confirmation that this species is indeed the $CO_2^{-\bullet}$ radical arising from the side-chain carboxy group of this amino acid was obtained by the use of [5-^{13}C]glutamate; the presence of the ^{13}C isotope (which has nuclear spin, $I = 0.5$) results in an additional splitting both in the PBN adduct spectrum (of 1.175 mT, cf. a literature value of 1.17 mT [26]) and the DMPO adduct spectrum (of 1.22 mT).

DISCUSSION

Previous studies have demonstrated the ready formation of protein hydroperoxides on reaction of $HO^\bullet$ and other reactive radicals with a number of target proteins [12,20,27]. The yield of these materials is significant, with up to 0.4 peroxide group formed on BSA per $HO^\bullet$ generated during the γ-irradiation [12]. This observation together with the demonstration that 10–50% of the antioxidant capacity of human plasma is due to radical-scavenging by proteins [28] suggests that reaction between proteins and free radicals and hence protein hydroperoxide formation may be of major significance *in vivo* [10].

The protein hydroperoxides generated as a result of radical scavenging have been shown to be readily reduced by physiological reductants, such as ascorbate and glutathione (which may result in cellular depletion *in vivo* [12]), chemical agents such as $NaBH_4$, triphenylphosphine and dithionite [12], and the enzymic action of glutathione peroxidase (but not catalase) and rat liver extracts [12]. The degradation of these materials by metal ions has also been demonstrated [12], although the identity and mechanisms of formation of the radicals presumably produced as a result of these reactions have not been investigated.

This study provides evidence that a number of proteins that have been peroxidized using γ-irradiation, a Fenton system or peroxyl radicals, and subsequently exposed to redox-active metal ion complexes in either the presence or absence of oxygen produce reactive free radicals from protein hydroperoxides. The facile detection of these species as radical adducts to spin traps suggests that the free radicals produced from these protein hydroperoxides are intrinsically reactive and hence would be capable of initiating further damage if generated *in vivo*. The isotropic nature of the spin-adduct spectra together with the fact that they are generated by and react with water-soluble reagents suggests that these radicals are either present on the outside surface of the protein on exposed residues with considerable freedom of motion or are smaller low-molecular-mass fragments produced as a result of fragmentation of large protein radicals. The observation that the spectra of the protein-derived radicals are similar in nature to those produced from isolated amino acids is believed to reflect the occurrence of the initial peroxide function primarily on the side chain rather than the peptide backbone of the parent protein, although further work with model compounds, and in particular well-defined peptides, is needed to confirm this. This distribution would be in accord with the observation that fragmentation of the peptide backbone (as judged by SDS/PAGE) only occurs to a limited extent on the degradation of these proteins [23,29], although alteration of the protein side chains may well be much more significant (see below).

Comparison of the observed protein spin adducts with those detected with free amino acids and well-defined organic hydroperoxides [30–32] is consistent with previous suggestions that the peroxide groups on the protein are indeed hydroperoxides [12,20,27] rather than dialkylperoxides, peracids or related materials; the latter compounds have been previously shown to yield mainly alkyl or aryloxyl ($RCO_2^\bullet$) and carbon-centred radicals when treated with iron(II) chelates [31,32]. Detailed analysis of the adducts suggests that all of the observed species can be accounted for via the initial formation from the protein hydroperoxide of a protein alkoxyl radical on an amino acid side chain via a reductive process (reaction 1; see below). This intermediate can then (i) react with the spin trap (as observed with DMPO; alkoxyl radical adducts to PBN and MNP are relatively unstable [25]), (ii) undergo rearrangement or fragmentation reactions (c.f. the known reactions of other alkoxyl radicals [30–32]) to give carbon-centred species, or (iii) abstract a hydrogen atom (reaction 2) from a suitable donor to give an alcohol (a known product of the decomposition of these hydroperoxides; S. Fu and R. T. Dean, unpublished work). This last process, which may be a major pathway in the absence of a spin trap, could occur either intramolecularly, which would result in damage transfer within the initial protein, or intermolecularly, resulting in the initiation of damage on another protein molecule (or other biological targets in more complex systems). The rate and mechanism of fragmentation are believed to depend on the nature of the alkoxyl radical [30–32]. Tertiary alkoxyl radicals ($R^1R^2R^3CO^\bullet$) would be expected to undergo rapid β-scission reactions (reaction 3) to yield alkyl radicals [which would then be expected to react rapidly with molecular oxygen (reaction 4)], whereas secondary ($R^1R^2CHO^\bullet$) or primary ($R^1CH_2O^\bullet$) species would be predicted to give either α-hydroxyalkyl radicals as a result of 1,2-hydrogen-shift reactions (reactions 5 and 6) or carbon-centred radicals from 1,4 or 1,5-hydrogen shifts; these (unimolecular) processes are known to proceed rapidly ($k > 10^6\ s^{-1}$; [33,34]), and would give rise to the carbon-centred species observed with each spin trap. Results obtained with the peroxidized free amino acids are in agreement with these suggestions. The formation of α-hydroxyalkyl radicals via such reactions is supported by the detection of the radical *t*-BuNHO$^\bullet$ in experiments where MNP was used as the spin trap. This species is believed to arise via reduction of the spin trap and subsequent protonation of the radical anion; its observation is

therefore indicative of the presence of reducing radicals such as α-hydroxyalkyl species [35]. The presence of such radicals also provides a route to the H_2O_2 believed to be generated during the hydroperoxide-degradation process, as it is known [1] that such species react with O_2 at diffusion-limited rates to give peroxyl radicals which subsequently rapidly lose $HO_2^{\bullet}$; subsequent dismutation of the ionized form of this species ($O_2^{-\bullet}$; pK_a 4.7) would yield H_2O_2 (reactions 7–9) [1]. It should be noted that several of these processes (reactions 2, 3, 4 and 8) result in the generation of reactive radicals which can initiate further damage either to the same protein or another protein; these reactions may account for the occurrence of chain reactions during protein oxidation [36].

Reaction of higher-oxidation-state metal ion complexes [iron(III)] produced via reaction (1) with further protein hydroperoxide molecules (to yield protein peroxyl radicals directly) is expected (by comparison with model hydroperoxides) to be a slow process compared with reductive degradation to give the alkoxyl radical [37]. However, the regeneration of iron(II) via either this reaction or reduction of iron(III) by $O_2^{-\bullet}$ (as outlined above) and/or $CO_2^{-\bullet}$ (generated as described below) may result in catalytic rather than stoichiometric degradation of protein hydroperoxides by iron complexes.

$$\text{Protein–OOH} + M^{n+} \rightarrow \text{Protein–O}^{\bullet} + HO^- + M^{(n+1)+} \quad (1)$$

$$\text{Protein–O}^{\bullet} + \text{protein} \rightarrow \text{Protein–OH} + \text{protein}^{\bullet} \quad (2)$$

$$R^1R^2R^3CO^{\bullet} \rightarrow R^1R^2C{=}O + R^{3\bullet} \quad (3)$$

$$R^{3\bullet} + O_2 \rightarrow R^3OO^{\bullet} \quad (4)$$

$$R^1R^2CHO^{\bullet} \rightarrow {}^{\bullet}CR^1R^2(OH) \quad (5)$$

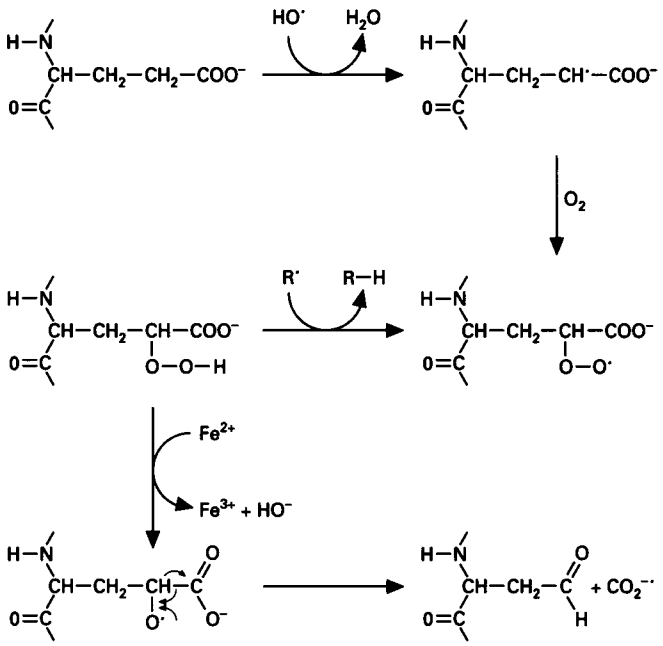
$$R^1CH_2O^{\bullet} \rightarrow {}^{\bullet}CHR^1(OH) \quad (6)$$

$${}^{\bullet}CR^1R^2(OH)/{}^{\bullet}CHR^1(OH) + O_2 \rightarrow {}^{\bullet}OOCR^1R^2(OH)/{}^{\bullet}OOCHR^1(OH) \quad (7)$$

$${}^{\bullet}OOCR^1R^2(OH)/{}^{\bullet}OOCHR^1(OH) \rightarrow HO_2^{\bullet} + R^1R^2C{=}O/R^1HC{=}O \quad (8)$$

$$2HO_2^{\bullet} \leftrightarrows 2H^+ + 2O_2^{-\bullet} \rightarrow H_2O_2 + O_2 \quad (9)$$

The occurrence of reactions (1)–(9) can account for all the species observed in the spin-trapping experiments for the $CO_2^{-\bullet}$ adduct, which is detected with both peroxidized glutamate and all the free-radical-damaged proteins. This species is believed to arise via a novel β-fragmentation process of an alkoxyl radical generated from a hydroperoxide function at C-4 (the penultimate carbon atom of the glutamate side chain); this position is a major site of $HO^{\bullet}$ attack on the parent amino acid and hence presumably the major site of the hydroperoxide function in the γ-irradiated sample [38]. The reactions believed to give rise to the $CO_2^{-\bullet}$ radical are outlined in Scheme 1 and are based on the known preference of alkoxyl radicals to fragment to yield the most stable pairing of radical (in this case $CO_2^{-\bullet}$) and molecule, which normally results in β-fragmentation occurring in the order $R^{\bullet} > Ph^{\bullet} > H^{\bullet}$ [39]. The occurrence of such a fragmentation process from an initial alkoxyl radical would also appear to be a plausible explanation for the known loss of CO_2 from isolated α-hydroperoxycarboxylic acids [40]. The detection of this radical with both free glutamate and each of the proteins tested suggests that the glutamate residues present in all three proteins tested are a major site of the hydroperoxide functions and is in accord with the hypothesis that the majority of the observed radicals are species on the exposed outer surface of the protein where the glutamate residues are found in each case.



Scheme 1

In none of the systems studied were any radicals detected that could be definitively assigned to radicals arising from decomposition of a hydroperoxide group formed at the α-carbon (i.e. on the peptide backbone), although this does not prove that such species are not formed, particularly at low concentration. The generation of only low concentrations of these intermediates is, however, consistent with the relatively minor contributions of peptide bond scission compared with the level of overall protein damage [23,29], as Garrison [41] has suggested that the formation of an alkoxyl radical at the α-carbon may result in backbone cleavage via reaction (10).

$$\begin{array}{l} \text{O}^{\bullet} \\ | \\ -\text{NH–C(R)–C(O)–NH} + O_2 \\ \\ \rightarrow -\text{NH–}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{(R)} + \text{O=C=N–} + HO_2^{\bullet} \end{array} \quad (10)$$

Garrison [41] has also demonstrated that side-chain damage can subsequently lead to backbone cleavage. However, the side-chain fragmentation processes we have observed do not appear to contribute significantly to the known slight backbone cleavage [23,29]. Nevertheless, these side-chain fragmentation processes may have important consequences for the structure, function and recognition of the protein. Each of the pathways elucidated above would result in the formation of both carbonyl (aldehyde or ketone) functional groups on the surface of the protein and further reactive free radicals ($R^{\bullet}$, $ROO^{\bullet}$, $HO_2^{\bullet}$ or $CO_2^{-\bullet}$) which may initiate further damage to either the parent protein or other biological targets and play a role as chain carriers in protein oxidation [36]. The formation of carbonyl species has been known for a considerable period of time [7] and is often used as a convenient marker of protein oxidation [2,7]. Such species may be involved in the observed cross-linking of damaged proteins (via the formation of Schiff's bases with amine functions [42]), recognition by enzymic degradative systems and alterations to the three-dimensional structure of a protein. The occurrence of this novel fragmentation process with glutamate side chains may be of particular importance in proteins with a high glutamate content, such as BSA (where it accounts for 10% of the total amino acid content and a much higher percentage of the surface

amino acids [43]), as it would result in the replacement of a charged highly hydrophilic residue on the surface of the protein with a relatively hydrophobic uncharged side chain. The significance of each of these side-chain fragmentation processes to the overall extent of damage is expected to depend on the protein studied (i.e. they are protein-specific), as the contribution from each of these processes depends on the exact amino acid composition of a protein, the position of the residues in the three-dimensional structure and the reaction conditions.

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