REVIEW ARTICLE

Biochemistry and pathology of radical-mediated protein oxidation

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Radical-mediated damage to proteins may be initiated by electron leakage, metal-ion-dependent reactions and autoxidation of lipids and sugars. The consequent protein oxidation is O_2-dependent, and involves several propagating radicals, notably alkoxyl radicals. Its products include several categories of reactive species, and a range of stable products whose chemistry is currently being elucidated. Among the reactive products, protein hydroperoxides can generate further radical fluxes on reaction with transition-metal ions; protein-bound reductants (notably dopa) can reduce transition-metal ions and thereby facilitate their reaction with hydroperoxides; and aldehydes may participate in Schiff-base formation and other reactions. Cells can detoxify some of the reactive species, e.g. by reducing protein hydroperoxides to unreactive hydroxides. Oxidized proteins are often functionally inactive and their unfolding is associated with enhanced susceptibility to proteinases. Thus cells can generally remove oxidized proteins by proteolysis. However, certain oxidized proteins are poorly handled by cells, and together with possible alterations in the rate of production of oxidized proteins, this may contribute to the observed accumulation and damaging actions of oxidized proteins during aging and in pathologies such as diabetes, atherosclerosis and neurodegenerative diseases. Protein oxidation may also sometimes play controlling roles in cellular remodelling and cell growth. Proteins are also key targets in defensive cytolysis and in inflammatory self-damage. The possibility of selective protection against protein oxidation (antioxidation) is raised.

INTRODUCTION

Radical-mediated protein oxidation has been studied throughout the century. In the first decade, Dakin (e.g. [1,2]) published detailed chemical studies of the oxidation of leucine and other amino acids by Fenton systems (transition-metal ions plus hydrogen peroxide), and protein aggregation and (apparently) fragmentation were detected by others. Soon after the discovery of glutathione, Hopkins appreciated that this reductant could be both an anti- and a pro-oxidant, the latter depending on the presence of transition-metal ions [3], and so could inactivate proteins. Several authors assessed the proteolytic susceptibility of oxidized proteins, and demonstrated biphasic effects, whereby limited oxidation leads to enhanced susceptibility, while more extensive oxidation may be associated with increasing resistance [4,5].

The body of the present review focuses on the literature since 1950, and is prefaced by a synopsis of radical sources, and of the chemistry of protein oxidation, to place the discussion of biochemistry and pathology in a comprehensible framework. A complementary review will detail the chemistry of protein oxidation (M. J. Davies, unpublished work).

SOURCES OF RADICALS IN EXPERIMENTAL AND BIOLOGICAL SYSTEMS

The primary free radical in most oxygenated biological systems is the superoxide radical ($O_2^{-\bullet}$), which is in equilibrium with its protonated form, the hydroperoxyl radical ($HO_2^{\bullet}$). The major sources of these radicals are modest leakages from the electron transport chains of mitochondria, chloroplasts and endoplasmic reticulum. Although $O_2^{-\bullet}$ is relatively unreactive in comparison with many other radicals, biological systems can convert it into other more reactive species, such as peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and hydroxyl ($HO^{\bullet}$) radicals. The last of these can originate from the Fenton reaction, in which the metal ion redox cycles, with reduction effected by $O_2^{-\bullet}$ and oxidation by its dismutation product, hydrogen peroxide (H_2O_2). Iron and copper are biologically important transition-metal ions, with their reduced forms capable of rapidly cleaving organic (including lipid) hydroperoxides, forming radicals that can initiate chain reactions, ultimately giving stable products such as lipid hydroxides.

Experimentally, many biologically important radicals can be generated in defined qualities and quantities by the γ-radiolysis of water. Linear fluxes of $ROO^{\bullet}$ can also be generated by decomposition of thermolabile azo compounds such as 2,2′-azobis-(2-amidinopropane) hydrochloride. More complex experimental systems can involve the metal-ion-catalysed autoxidation of a variety of molecules, such as sugars. In these cases, it is extremely difficult to quantify the radical fluxes.

We will also refer briefly to the actions of the two-electron (non-radical) oxidant hypochlorite. This is a major product of stimulated neutrophils, which produce superoxide radicals which dismute to H_2O_2 and then convert it into hypohalous acids by the action of myeloperoxidase in the presence of halides. Although the non-radical nature of this oxidant makes it chemically distinctive, its occurrence in biological systems makes it appropriate for brief review.

For more detailed discussions of some of these oxidative systems, see [6,7].

SYNOPSIS OF THE CHEMISTRY OF PROTEIN OXIDATION

Much of the chemistry of the products of protein oxidation (M. J. Davies, unpublished work) was elucidated by Dakin. He detected the formation from amino acids of carbonyls, such as

Abbreviations used: apoB, apolipoprotein B; 4-HNE, 4-hydroxynonenal; LDL, low-density liopoprotein; PBN, *N*-t-butyl-α-phenylnitrone.

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Table 1 New moieties generated by biological protein oxidation and their genesis

When $HO^{\bullet}$ attack is indicated, this is not intended to discriminate between the several possible $HO^{\bullet}$-generating mechanisms (metal-ion-dependent and -independent) indicated in the text or known. Reactive nitrogen species (RNIs) similarly refers to NO, peroxynitrite, the peroxynitrite–CO_2 adduct and the products of interactions of hypochlorite and hydrogen peroxide with inorganic nitrite. Reactions of ozone and other pollutants are not considered here.

Oxidative insult	Product	Selected refs.
Tyr + $HO^{\bullet}$ or RNIs	Dopa	[28]
Tyr + HOCl	3-Chlorotyrosine	[223]
Tyr + RNIs	3-Nitrotyrosine	[294]
Tyr + $HO^{\bullet}$, or one-electron oxidation of Tyr or HOCl, followed by radical–radical combination	Dityrosine	[134,295]
Phe + $HO^{\bullet}$; one-electron oxidation; RNIs?	*o*- and *m*-tyrosine	[295]
Phe + $HO^{\bullet}$ before or after dimerization	Dimers of hydroxylated aromatic amino acids	[192]
Trp + $HO^{\bullet}$, or one-electron oxidation	*N*-Formylkynurenine; kynurenine	[22,132]
Trp + $HO^{\bullet}$, or one-electron oxidation	5-Hydroxytryptophan; 7-hydroxytryptophan	[132,296,297]
His + $HO^{\bullet}$, or one-electron oxidation	2-Oxohistidine	[19]
Glu + $HO^{\bullet}$ in presence of O_2	Glutamic acid hydroperoxide	[27,31]
Leu + $HO^{\bullet}$ in presence of O_2	Leucine hydroperoxides and hydroxides; α-ketoisocaproic acid; isovaleric acid; isovaleraldehyde; isovaleraldehyde oxime; carbonyl compounds	[14,27,29,31,298]*
Val + $HO^{\bullet}$ in presence of O_2	Valine hydroperoxides and hydroxides; carbonyl compounds	[13,29]
Lys + $HO^{\bullet}$ in presence of O_2	Lysine hydroperoxides and hydroxides; carbonyl compounds	[27,31,299]†
Pro + $HO^{\bullet}$ in presence of O_2	Proline hydroperoxides and hydroxides; 5-hydroxy-2-aminovaleric acid; carbonyl compounds	[27,31,299,300]
Arg + $HO^{\bullet}$ in presence of O_2	5-Hydroxy-2-aminovaleric acid	[300]
Ile + $HO^{\bullet}$ in presence of O_2	Isoleucine hydroperoxides; isoleucine hydroxides?; carbonyl compounds?	[27,31]
Gly: hydrogen-atom abstraction from α-carbon followed by reaction with $CO_2^{\bullet-}$ radicals; could arise from a succession of oxidations of other amino acids	Aminomalonic acid	[301,302]
Met + $HO^{\bullet}$ or one-electron oxidation	Methionine sulphoxide	[94,303,304]
Cys + $HO^{\bullet}$, or other hydrogen-atom-abstracting species	Cystine; oxy acids	[305,306]
Non-aromatic side chains exposed to radicals?	Protein carbonyls	[190]
All amino acids exposed to photo-oxidation, oxidizing radicals or HOCl	RCHO species formed by decarboxylation and deamination	[307–309]

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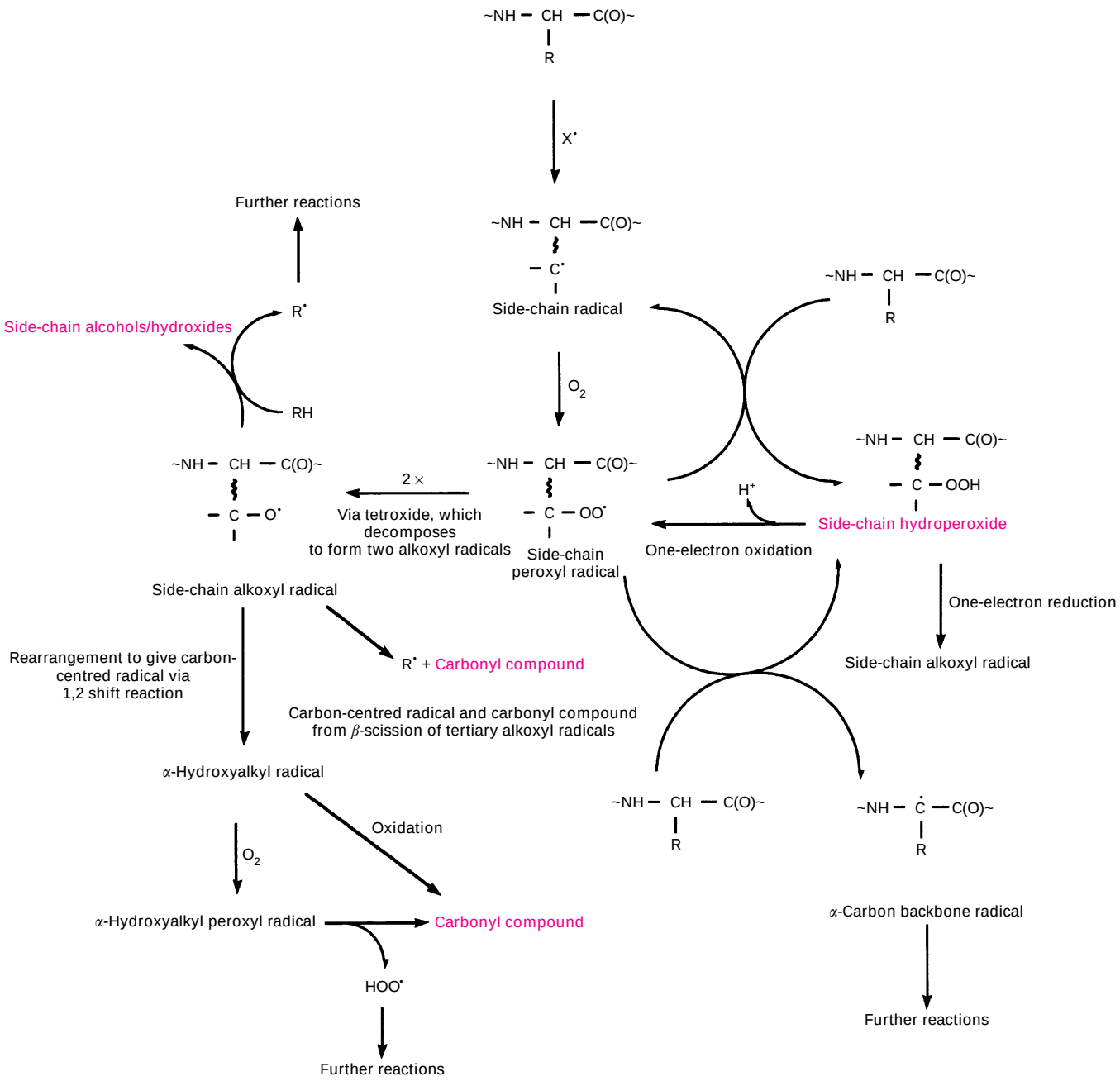
the oxo acids and aldehydes with the same or one less carbon atom than the parent amino acid; e.g. glycine giving rise to glyoxal and glyoxylic acid, formaldehyde and formic acid; alanine giving rise to acetaldehyde and acetic acid, etc. This general scheme has been confirmed for many amino acids, including aromatic amino acids, although other reactions may also occur. Notable quantitative studies were contributed by Garrison, who also proposed a 'peptide α-amidation' scheme for the oxidative breakage of polypeptide backbones ([8,9]; reviewed in [10]), which has been confirmed in some cases [11], and seems to be a common component of chain-fragmentation reactions.

During the oxidation of aliphatic amino acids by $HO^{\bullet}$, hydroxylated derivatives, notably of the side chains, are formed. These were partially characterized by Kopoldova and co-workers (e.g. [12]), and have been fully defined for valine [13] and leucine [14]. During the oxidation of aromatic residues, the formation of phenoxyl radicals from tyrosine, and their conversion into dityrosine and further products, can occur, especially if there are no reductants to repair the tyrosyl radicals (e.g. thiols, vitamin E) and if there are vicinal tyrosyl radicals [15,16]. Hydroxylation of phenylalanine, tyrosine and tryptophan is also a characteristic reaction of hydroxyl radicals [17,18], and similar reactions of histidine (giving 2-oxohistidine) are important [19]. Fenton chemistry can generate both the aliphatic and aromatic [20] products. Table 1 summarizes some products of protein oxidation; Schemes 1 and 2 illustrate some of the major reactions believed to be important in the oxidation of side chains and the backbone respectively.

A more recent advance has been the realization that, in the presence of O_2, there is a protein-radical chain reaction, with modest O_2 consumption and a chain length of seven or more [21,22], as indicated but not identified in earlier data [23,24]. The possible participation of a range of radicals in this chain process has been demonstrated by detailed EPR spin-trapping studies [25,26]. Alkoxyl radicals apparently have a greater importance in protein oxidation chains [25] than they do in lipid peroxidation, in which peroxyl radicals are the key chain-propagating species [6].

Two categories of reactive, but non-radical, intermediates in protein oxidation have been identified. Reductive moieties, notably dopa formed from tyrosine, can reduce transition-metal ions, thus enhancing reactions with hydroperoxides, and are also able to induce radical formation in reactions with O_2 [27,28]. The other category is the hydroperoxides formed particularly on aliphatic side chains, but probably also on main-chain α-carbons [14,26,27]. These can be decomposed by transition-metal ions to give further radicals, which may propagate reaction chains. The hydroperoxides may also be reductively detoxified to hydroxides, probably without radical formation [29]. Hydroperoxides on proteins were first detected in the 1940s [30], but were only chemically characterized more recently [27,31].

The precise stoichiometry of the protein oxidation chain is



Scheme 1 Major reactions of aliphatic side-chain radicals formed during protein oxidation in the presence of oxygen

Species detected as products of side-chain oxidation are depicted in colour.

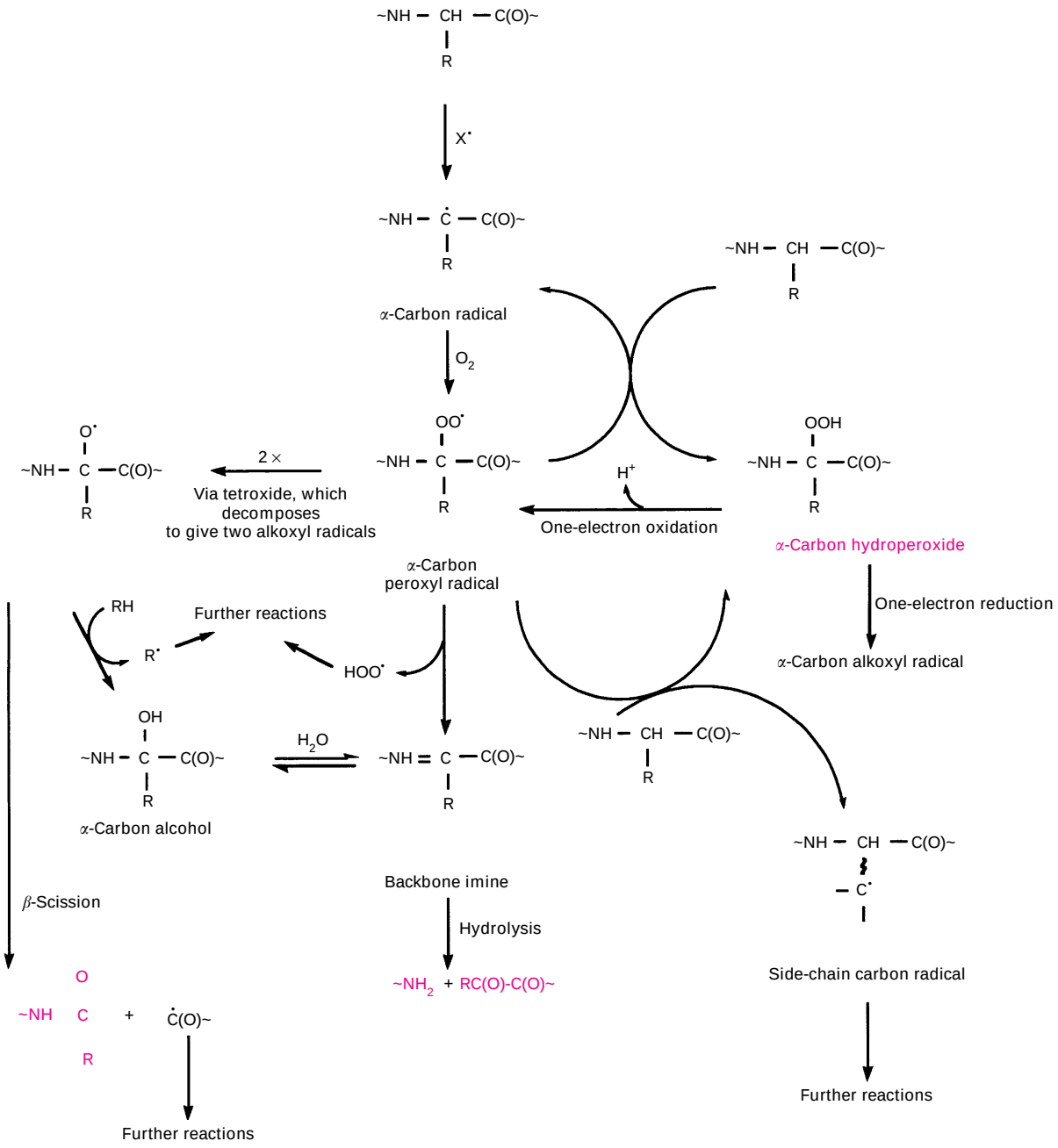
unknown. It is clear that there are several propagation reactions that do not require O_2, consistent with the low O_2 consumption observed, but the overall reaction is O_2-dependent, probably as a result of a requirement to form peroxyl (and possibly alkoxyl) radicals from carbon-centred species.

BIOCHEMISTRY OF PROTEIN OXIDATION

Reversible and irreversible inactivation of proteins by radicals

Damage by radiation-induced radicals

Early radiation studies on lysozyme [32], ribonuclease [33] and other enzymes were carried out mainly in the absence of O_2. They showed that $HO^•$ was the most effective inactivator, and characterized other more selective (but less efficiently inactivating) species such as $(SCN)_2^{-•}$, $Br_2^{-•}$, $Cl_2^{-•}$ and $I_2^{-•}$. For example, $(SCN)_2^{-•}$ was found to react with an important tryptophan residue in pepsin and so inactivate the enzyme, although damage could be reversed by the same radical [34]. Inactivation by the hydrated electron has also been reported (e.g. [35]), but its significance, and that of the above-mentioned selective radicals, for biological systems may be limited. In studies on D-amino acid oxidase, it was found that removal of the coenzyme FAD enhanced radical damage and inactivation, illustrating that



Scheme 2 Major reactions of backbone radicals formed during protein oxidation in presence of oxygen

Species detected as products of backbone oxidation are depicted in colour.

conformation [36] and ligands can affect the extent of inactivation (see [6] for a review of inactivation studies).

Damage by metal-ion-catalysed systems

Since 1981, Stadtman and colleagues have examined the inactivation of proteins in cell-free systems involving the metal-ion-catalysed autoxidation of ascorbate and/or hydrogen peroxide; in some cases the metal ions were derived from metalloproteins. These systems are now termed 'metal-catalysed oxidation systems', instead of the previous confusing term 'mixed-function oxidation systems'.

These studies developed from earlier work on the oxidative inactivation of glutamine phosphoribosylpyrophosphate amidotransferase in extracts from *Bacillus subtilis* [37,38]. This system is O_2-dependent and is perturbed by metal-ion chelators, but its chemistry has not been fully defined. A similar inactivation occurs in a mutant strain lacking important proteinases; oxidation of a (4Fe–4S) centre appears to be important.

Although metal-catalysed oxidation systems are mechanistically diverse, they are like Fenton systems and involve H_2O_2; thus they can be blocked by catalase. They inactivate a wide range of enzymes (e.g. [39–41]) and are suggested to be important for both proteolytic turnover [39] and the accumulation of

proteins during aging [42]. The inactivation of one of the most studied enzymes, glutamine synthetase, is influenced by its adenylation state [39], which also regulates the enzyme and some multi-enzyme cascades [43,44].

Some metal-catalysed oxidation systems can cause selective damage in cell-free systems, such as to the histidine residues in glutamate synthetase (reviewed in [45]), but this is accompanied by other alterations; in this case, both protein fragmentation [46] and changes in hydrophobicity. Limited oxidation increases hydrophilicity, while further oxidation increases hydrophobicity [47]. Early studies suggested that modification of one (of 16) histidines both inactivated and increased proteolytic susceptibility [48]; later studies showed that enhanced proteolytic susceptibility requires the modification of at least two histidines per subunit [49]. By the stage at which this takes place, other modifications also occur, e.g. at Arg-344 [50], yet only 0.7 mol of carbonyl/mol of protein is present [49], so most altered histidine residues do not contain carbonyls. Incorporation of tritium (from tritiated borohydride) into the enzyme at various stages of oxidation revealed eight (unidentified) radioactive peaks using amino acid HPLC after protein hydrolysis. Some of these may represent carbonyls. As the chromatographic pattern was relatively constant at different times, it was argued that the range of products is also constant. However, during the period when the first histidine was oxidized (< 60 min of oxidation), less than six of these species were formed. By 120 min little further histidine oxidation had occurred, yet a further two or three amino acid derivatives were formed. Unfortunately, the earliest time point reported was 20 min, making it difficult to conclude that the critical event for inactivation is the histidine oxidation, since other modifications had also occurred. Consumption of other individual amino acids was low when compared with that of histidine, and could not be detected; the aggregate loss of all amino acids was not quantified, and may have been significant.

In vivo studies of glutamine synthetase inactivation have been carried out in other species, such as the fungus *Neurospora crassa*, where the inactivated enzyme has been detected *in vivo* [51]. Furthermore, when living *Klebsiella pneumoniae* [52] is switched from anaerobic to aerobic conditions (or exposed to H_2O_2), several enzyme activities, including that of glycerol dehydrogenase, are lost, apparently by oxidative inactivation. Purification of this enzyme when 90% inactivated (from cells exposed to H_2O_2) and comparison with the native form has shown no detectable differences in either subunit molecular size or amino acid composition (including -SH groups). The lack of such gross changes suggests that limited modifications are sufficient for inactivation, although the inactivated enzyme was more hydrophobic, and its intact oligomer migrated in gel filtration as if slightly larger than the native form. This emphasizes a major problem in studying oxidative inactivation *in vivo*: even analysis of the inactivated enzyme from these cells gave no evidence that inactivation was due to protein oxidation, although it was clearly initiated by an oxidative affront.

Inactivation of non-enzymic proteins by metal-catalysed oxidation has also been studied extensively. Thus α_1-proteinase inhibitor is inactivated on oxidation of a susceptible methionine residue, and H_2O_2 inactivates a neutrophil cytosolic serine-proteinase inhibitor (serpin), possibly via a similar process [53]. In some cases, these reactions of H_2O_2 and methionine may be nucleophilic (molecular) rather than radical-mediated. The proteinase subtilisin is dependent on a methionine residue both for its activity and for its susceptibility to H_2O_2 *in vitro*; replacement of this residue by site-directed mutagenesis decreases inactivation of the enzyme by H_2O_2 [54]. In contrast, Fenton systems produce a range of amino acid derivatives that inactivate α_1-proteinase inhibitor (e.g. [55]); they also inactivate proteinase inhibitors and neutrophil elastase, which lack the susceptible methionine, with comparable efficiency [56]. Thus methionine-containing inhibitors are not automatically more vulnerable, and susceptibility depends on the radical or oxidizing conditions. Which systems are important *in vivo* are not known.

Damage by singlet oxygen and photochemical reactions

Singlet oxygen ($^1O_2$) and radical species can participate in photosensitizer-induced inactivation of enzymes, as exemplified by studies on purified catalase, or catalase within cells [57]. Photosensitized inactivation of protein kinase C due to calphostin [58] has been shown to be reversible and dependent on O_2. Within cells this process becomes irreversible, and oxidation appears to occur mainly on the membrane portion of the complex.

Damage by endogenous radicals

It is known that a number of enzymes contain an intrinsic radical species while in their active form (reviewed in [59]). These radicals can be present on tyrosine, modified tyrosine, tryptophan, modified tryptophan, glycine or thiols. For example, during catalysis ribonucleotide reductase contains a tyrosyl radical, and probably also a thiyl radical, in order to abstract a hydrogen atom from the ribose ring of the substrate [60].

The reaction of these protein radicals with O_2 (or possibly other species) can cause enzyme inactivation by backbone cleavage rather than side-chain alterations. Reduction of the intrinsic tyrosyl radical on the R2 subunit of the enzyme can be accompanied by cleavage of the R1 subunit into 26 and 61 kDa fragments, respectively from the N- and C-termini of R2. The precise site and mechanism of this reaction [61] remain to be elucidated. Similarly, inactivation of anaerobically grown *Escherichia coli* pyruvate formate lyase, which contains an intrinsic glycyl radical, can occur in the presence of O_2 or hypophosphite and appears to involve glycyl radical reactions. This process is initially reversible, since damage is both localized and limited in extent [62]. Reversal may involve loss of O_2 from ROO$^\bullet$, a relatively facile process when the resultant carbon-centred species is stabilized (as it will be here, since backbone α-carbon radicals are capto-dative species stabilized by both the carbonyl and amide functions). This glycyl α-carbon radical occurs in the sequence -Ser-Gly-Tyr- which is cleaved with formation of an N-terminal oxalyl residue from the glycine [11], consistent with the α-amidation fragmentation mechanism [10].

Many oxidative enzymes, such as lipoxygenases and the cytochrome *P*-450 family, can also generate radical species during their interaction with substrates, such that 'self-inactivation' occurs. Thus lipid peroxyl radicals are released during lipoxygenase action, and the product hydroperoxides of linoleic acid can inactivate it when iron is available (from the enzyme or elsewhere). Inactivation is enhanced by O_2, and such reactions give rise to the 'hydroperoxidase' activity of this enzyme [63].

Protein damage by radicals or carbonyls arising from other biological processes

Radicals produced during lipid autoxidation can inactivate α_1-proteinase inhibitor by oxidation of Met-358 [64]. Similarly, the formation of haemichrome is concomitant with lipid oxidation during Fe(II)-induced oxidation of liver slices [65]. Later studies with liver homogenates also demonstrated a slow inactivation of glutathione peroxidase [66].

End-products of lipid oxidation, such as malondialdehyde [67] and 4-hydroxynonenal (4-HNE) [68], are also inactivating agents, possibly via Schiff-base formation. Schiff bases are short-lived species formed by the reaction of carbonyl groups with amines, and can be formed, for example, during exposure of proteins to: (i) lipid-derived aldehydes [69], (ii) autoxidizing sugars (some of which form vicinal dicarbonyl compounds [70]), and (iii) amino acid-derived aldehydes, as mentioned earlier. Thus binding of the apolipoprotein B (apoB) protein of low-density lipoprotein (LDL) to its cellular receptor is perturbed by reaction with 4-HNE at modest levels [71], and grossly altered when aggregating, supra-pathological, levels are used [72]. With some lipid carbonyls, Schiff-base formation may be of limited importance compared with Michael addition reactions [73,74]. In insulin (which lacks cysteine), histidine residues are selectively modified by Michael addition of 4-HNE [75]. With dialdehydes, Schiff-base formation and Michael addition appear to be simultaneous, thus cross-linking lysine residues [76]. 4-HNE also inactivates glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase; in the latter case both intra- and intermolecular cross-links appear to be formed [77].

Mechanisms of reversal of oxidative lesions

Thiol groups

Some of the (limited) modifications discussed above are transiently reversible, and such reversal may cause re-activation. Conversion of -SH groups into disulphides and other oxidized species (e.g. oxyacids) is one of the earliest observable events during the radical-mediated oxidation of proteins, with the ratio of disulphide to oxygenated materials depending on the environment. For example, H_2O_2 causes preferential thiol (and subsequently methionine) consumption in lens crystallins *in vitro*, whereas most other amino acids show little change; this may be a non-radical (molecular) reaction [78]. Higher sulphides and persulphides may also be generated on proteins [79], resulting in conformational changes [78].

Inactivation of enzymes by limited -SH oxidation has been studied, mainly with oxidized thiols (e.g. GSSG) as oxidants, so as to restrict the range of reactions. Thus treatment of aldose reductase with GSSG results in mixed disulphide formation, conformational changes and inactivation of the enzyme, which can be reversed by GSH [80]. Reversible S-thiolation has also been detected on proteins in cells exposed to radicals [81]. Furthermore, the respiratory burst of human monocytes results in rapid and reversible S-thiolation of a number of cytosolic proteins [82]. Thus biological thiols, such as GSH and cysteine, can influence oxidant-induced protein inactivation either by direct reaction with the radicals or indirectly, by forming reversible bonds with (normally free) thiols on the proteins. The latter should be reversible (e.g. [80,82]); it is also an essential process in *de novo* protein folding and in the maintenance of conformation.

Methionine

The oxidation of methionine residues, discussed above for proteinase inhibitors [83,84], can be reversible. t-Butyl hydroperoxide behaves like H_2O_2, but only oxidizes exposed methionines [85], presumably because its increased steric bulk limits access. Treatment of isolated human erythrocyte glyceraldehyde-3-phosphate dehydrogenase and glycophorin with ozone also leads to the loss of both methionines. In the latter, no other amino acids are oxidized, and the reactions are probably nucleophilic [86]. In the former, however, cysteine and aromatic residues are also oxidized [87], suggesting a radical-mediated process. A number of neutrophil neutral proteinase inhibitors, isolated *E. coli* glutamine synthetase and BSA when exposed to ozone also show methionine oxidation and lesser oxidation of histidine and aromatic residues [83,88]. The formyl-Met-Leu-Phe-triggered respiratory burst of neutrophils also results in oxidation of the methionine in the formyl-Met-Leu-Phe [89].

Two pathways may exist for the reversal of methionine oxidation. Firstly, the initial radical species derived from methionine (i.e. the radical cation or the corresponding hydroxylated material) can be reduced by suitable donors such as the water-soluble tocopherol analogue Trolox C [90]. Secondly, methionine sulphoxide can be reduced by methionine sulphoxide reductase, which is present in many mammalian cells [91,92]. The latter may be the major re-activating process with α_1-proteinase inhibitor [64]. Further oxidation products of methionine sulphoxide (e.g. the sulphone [93]) cannot be reversed by the reductase. Methionine oxidation and repair may be a biological control mechanism [94], analogous to reversible thiolation.

Schiff bases

Schiff-base adducts formed on reaction of an amine with a carbonyl are unstable [95], and so may be reversible inactivating lesions. Schiff-base formation is, however, often followed rapidly by Amadori rearrangements (e.g. [96]), and hence the window for possible reversal is small. The thioether linkage formed by Michael reactions of protein -SH groups with 4-HNE is unlikely to be reversed biologically. During hypochlorite oxidation of LDL, in which protein is the preferential target, lysine-derived aldehydes seem to form protein cross-links that are initially reversible [97].

Reversible and irreversible protein unfolding

Very limited protein unfolding occurs in most of the examples of (cell-free) protein inactivation given above. Similarly, *in vivo* alterations to glycerol dehydrogenase during exposure of *Klebsiella* to H_2O_2 [52] involve minor size and hydrophobicity changes. This is in agreement with the small free-energy changes (0.25–0.5 kJ/mol) measured during the *in vitro* inactivation of glutamine synthetase [98].

In vitro studies also show that alteration of protein size occurs to only a limited extent during oxidation. Thus BSA exposed to $HO^{\bullet}$ (from radiolysis) in the presence of O_2 yields only small amounts of molecules of changed size, as detected by non-denaturing HPLC gel filtration, even though dose-dependent generation of smaller fragments can be seen by SDS/PAGE [99,100]. In the absence of O_2, $HO^{\bullet}$ causes substantial cross-linking. Similar observations have been made during prolonged *in vitro* autoxidation of lens crystallins [101]; later studies identified aggregates by light scattering and size-exclusion chromatography [102]. Thus oxidatively generated fragments probably remain associated, at least initially, in a conformation similar to that of the native protein.

As mentioned above, inactivation often involves limited conformational change. On the other hand, limited *in vitro* oxidation of glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase by $^1O_2$ or $HO^{\bullet}$ causes changes to the intrinsic fluorescence [103]. This raises the question as to whether limited conformational changes (or even large-scale unfolding) can be reversed, perhaps independently of the repair of the chemical lesions responsible for them. Very limited unfolding can be reversed (e.g. [103]), but there have been no systematic studies with radical-damaged proteins.

Protein disulphide isomerase [104] is concerned with re-pairing of -SH groups, while chaperonins are important in controlling correct folding after protein synthesis [105]. In theory, the various conformations accessible to a given polypeptide are exchangeable, and these proteins can facilitate the exchange, but the energy barriers to such interconversions may be too great for them to occur at a detectable rate under physiological conditions. The likelihood of the correct refolding of a substantially damaged protein is probably low, even if it is recognized by chaperonins.

The situation during oxidative insult may, however, be different. It is possible that chaperonins among the heat-shock protein families that are induced by oxidative stress may protect against the irreversible denaturation of a partially unfolded molecule. Thus macrophage colony-stimulating factor, which enhances $O_2^{-\bullet}$ output, induces the synthesis of heat-shock proteins of 60, 70 and 90 kDa, and confers enhanced resistance to H_2O_2; the chaperonins may contribute to this effect [106] and protect against autoxidative damage during the respiratory burst. A parallel induction of chaperonins arises on exposure of the intracellular facultative bacterium *Francisella tularensis LVS* to H_2O_2; this is presumably a defence against oxidative stress produced by the host macrophages [107]. Many human cells also respond to oxidative stress by the induction of heat-shock proteins (e.g. [108]). Complexes of partially unfolded proteins with chaperones may trigger further responses in these systems.

The lens of the eye is especially vulnerable to oxidative damage, as a result of both high exposure levels to UV light and the fact that the crystallin proteins are exceptionally long-lived. Thus it is not surprising that α-crystallin is a chaperone that can restrict the aggregation of other proteins, and hence opacity and cataract formation [102]. Oxidation of this crystallin *in vitro* diminishes its chaperone capacity, and α-crystallin isolated from senile human lenses shows such decreased chaperone activity and enhanced oxidation.

Enhanced susceptibility to degradation by proteinases is often employed as a criterion of unfolding. This criterion is usually, but not always, fulfilled with oxidized proteins. Thus alcohol dehydrogenase is relatively more resistant to elastase and proteinase K than several other proteins after limited oxidation by $^1O_2$ or $HO^\bullet$ [103]. It is known that extensively oxidized proteins may be denatured and aggregated to such an extent that proteolytic access is restricted rather than enhanced; the distinction between limited and extensive is, of course, arbitrary and wholly dependent on the protein under study. The other major component of irreversible protein oxidation is fragmentation, and when this is extensive it seems inevitably to lead to loss of conformation.

Protein fragmentation and polymerization

Measurement

The measurement of oxidative protein fragmentation, which does not always involve the formation of new N-termini, is not straightforward. In most cases only qualitative measurements have been made. Quantification by SDS/PAGE under reducing conditions (e.g. [109]) is difficult, as the fragments are often small and difficult to retain (during electrophoresis and/or staining), and individual fragments may stain differentially depending on their composition and degree of oxidation [110].

There have been attempts to correlate fragmentation with terminal amino group generation. When amino groups were measured in proteins exposed to $HO^\bullet$ after unfolding induced by guanidine hydrochloride, modest increments in total amino groups were observed after low doses, with losses at higher doses [111]. Similarly, exposure of lysozyme to Cu(II)/H_2O_2 resulted in an initial loss of ϵ-amino groups of lysine, and a subsequent increase in soluble amino groups; if the lysine groups were blocked prior to oxidation, only modest increases were observed [112]. Thus, until precise measurements of the competing pathways are obtained, the release of trichloroacetic acid-soluble amino groups is an unreliable index of fragmentation. Accurate quantification of released materials requires knowledge of the exact amounts of protein present. Determination of Kjeldahl nitrogen or total amino acids after hydrolysis provides such data, although this can be difficult to achieve with oxidized fragments.

A more reliable method of measuring fragmentation is to use proteins radioactively labelled in either main- or side-chain sites. These can be produced by biosynthesis, reductive methylation [110] or iodination. With the last of these, routinely used in cellular metabolism studies, the free iodide generated can be problematical and needs to be removed by treatment with silver nitrate.

Features of radical-mediated protein fragmentation and polymerization

A series of important studies [109,113–115] has shown that, even though $HO^\bullet$ attack on BSA causes extensive O_2-dependent fragmentation, this process can be selective. Cross-linking (both reductant-sensitive and -resistant links) is also observed, and predominates in the absence of O_2. Fragment formation can be detected during anoxic irradiation, at least when lactate dehydrogenase is the target [116]. These observations have been confirmed and extended [24,99,100,110,117]. $HO^\bullet$ and $O_2^{-\bullet}$ act synergistically [99], possibly as a result of additional decay pathways for the α-carbon peroxyl radicals by reaction with $O_2^{-\bullet}$/$HOO^\bullet$ The reasons for selective cleavage are not entirely clear; they may include differences in accessibility of sites to the attacking species, the stability of the protein radicals formed, and the occurrence of radical transfer reactions.

The sizes of the fragments generated from BSA, haemoglobin and myoglobin by radiolytic $HO^\bullet$ are compatible with fragmentation at proline residues [109,115]. Indeed proline (and histidine) is an important site of $HO^\bullet$ attack on BSA, although the predicted product(s) from cleavage at this site could not be detected, while new products arising from proline were formed [118]. This putative pathway has been observed in simple peptide systems [119], but is inconsistent with the reported stability of the α-carbon radical formed from proline (see [120]).

We have re-assessed the fragment size data obtained by Schuessler and co-workers from BSA, haemoglobin and myoglobin [109,114,115], and conclude that they can be explained more readily by cleavage at glycine (M. J. Davies, unpublished work), whose α-carbon radical is especially stable [121]. Glycine residues also appear to be important in the fragmentation of calf skin collagen induced by a xanthine oxidase system [122]. Cleavage is probably induced by $HO^\bullet$ in this system, as the primary $O_2^{-\bullet}$ produced is inactive in chain breakage. Of the N-terminal amino acids generated by fragmentation, 90% were glycine, even though the parent molecule contains only approx. 30% glycine. Selective fragmentation has also been observed with the apoB protein of LDL during either radiolysis or metal-ion-catalysed damage [123]; it is difficult to to assess the relevance of cleavage at proline or glycine in this case, due to difficulties in measuring fragment sizes. The importance of glycine residues during radical attack on proteins in aqueous physiological systems (cf. studies in organic solvents [124,125]) will only be resolved by sequencing of the fragments.

Comparison of the data from metal-ion/peroxide with those from radiolytic systems shows that the fragmentation patterns are distinct; this has been confirmed by direct comparison

[126–128]. The selective nature of metal-ion-catalysed oxidation may be a consequence of the localization of the metal ions at particular sites on the protein [129–131].

For some proteins (e.g. haemoglobin), unlike BSA, the extent of $HO^{\bullet}$-induced fragmentation is similar with or without O_2. This may arise as a result of the presence of the iron–protoporphyrin IX prosthetic group, which may liberate sufficient O_2 from H_2O_2 generated during radiolysis to permit fragmentation and restrict cross-linking. A similar rationale may explain the absence of cross-linked material from BSA treated with Cu(II)/H_2O_2 [126]. Both aggregation and fragmentation have been observed in a similar system with lysozyme [112], but the aggregation is coincident in time with the loss of lysine amino groups, and may be non-covalent.

Using proteins that lack one of Tyr, Trp or His, Guptasarma et al. [132] demonstrated the importance of His in covalent protein cross-linking in the presence of O_2. Lys was also demonstrated to be involved; these cross-links may involve Schiff-base derivatives. In the Tyr-containing proteins bovine pancreatic trypsin inhibitor, RNase A and crystallins, dityrosine cross-links were not observed. Interestingly, these workers also noted that treatment of melittin (which lacks Tyr) with $HO^{\bullet}$ induced changes in protein fluorescence characteristic of the formation of dityrosine, demonstrating the danger of identification of dityrosine on this basis. Definitive identification can be obtained by the use of HPLC separation combined with mass spectral identification (e.g. [133]) and co-elution. This has been done in some (e.g. myeloperoxidase-catalysed oxidation of proteins and exogenous Tyr [134]), but not all [135], studies.

Aggregation of fibrinogen as a result of treatment with Cu(II)/ascorbate is probably a special case, since chain cleavage may lead to aggregation [136], as it does during proteolytic conversion into fibrin. However, oxidized fibrinogen is not cleaved normally by thrombin [137], in agreement with early irradiation studies [138]. Fibrinogen appears to be the plasma protein that is most sensitive to *in vitro* metal-ion-catalysed carbonyl formation [139].

Protein oxidation in solution, in membranes and in lipoproteins

The immediate environment of polypeptides influences the nature and extent of their reactions with radicals. The key factors are the protein concentration (and if there is more than one, the nature and relative concentrations), and the nature, extent and location of the radical flux. The yield of oxidation products for a given radical flux increases with protein concentration until the system is saturated (i.e. all incident radicals react with the protein rather than with other components). Under saturating conditions and with non-selective oxidants, different proteins would be expected to interact with the incident radicals in a manner dependent on their relative volume occupancy. Whether this results in proportionate damage depends on whether inter-protein radical transfer occurs and whether the oxidation chains on each protein are of comparable length.

Intra-peptide and -protein transfer of radicals has been demonstrated directly (reviewed in [6,140]). Indirect evidence also supports the occurrence of such reactions; thus aromatic residues are normally damaged more extensively than aliphatic residues (although all are usually damaged to a significant degree), despite the fact that many of these residues are internal to a protein structure and hence not readily accessible to radicals in solution. Inter-chain interactions are a prerequisite for cross-linking reactions.

With metal-ion-catalysed oxidations, the location of the active transition-metal ion becomes important, and residues such as His, Cys, Lys and Met, which bind metal ions, may localize reactions to their vicinity. The differential capacities of proteins to bind metal ions and render them either redox-inactive (e.g. transferrin, lactoferrin) or active also influences the distribution of damage among protein populations. Few studies have directly addressed the quantitative differences in distribution of damage in heterogeneous systems.

Protein oxidation by lipid-derived species

It is well established that lipid radicals may damage proteins (e.g. [141]; reviewed in [142]). Such reactions show differences from those observed with radiolytic systems, as exemplified by the protein fragmentation patterns observed with model aqueous/multiphasic systems [127]. In systems that contain lipid hydroperoxides and soluble proteins, metal-ion-catalysed reactions appear to be central. Thus amino acid consumption varies somewhat between proteins, as might be expected if site specificity plays a role [143]. Furthermore, protein inactivation by peroxidizing lipid is often associated with the binding of lipid components to the protein. Thus, in membranes, competition and interactions between protein and lipid oxidation are expected.

Early studies emphasized the fluorescent cross-links that can form between lipid oxidation products and proteins, and their possible contribution to ceroid, lipofuscin and other 'age pigments' found in cells. The demonstration of such fluorophores *in vitro* has not generally been followed by the precise chemical definition of the materials *in vivo* (reviewed in [143]).

The interactions between lipids and proteins during protein oxidation in membranes have been studied using the mitochondrial outer membrane enzyme monoamine oxidase, selectively labelled (covalently) with a radioactive binding inhibitor. Monoamine oxidase can be delipidated subsequently to labelling. When present, lipid consumes some of a radiolytic radical flux, such that the extent of monoamine oxidase fragmentation is decreased. The fragmentation yield in these circumstances is also lower than that with BSA. However, after initial radiolytic oxidation, membrane-bound monoamine oxidase is much more susceptible than soluble BSA to further damage by metal ions. This is apparently due to radicals derived from the lipid hydroperoxides formed in the initial oxidation [101,144–146]. Metal-ion-catalysed damage to unirradiated membranes seems to affect lipid and protein in parallel, and is restricted by α-tocopherol [145]. Membranes can also influence radical damage to proteins by physical separation of the target from the radical source [147] or by sequestration of transition-metal ions away from hydroperoxides [148].

Lipoproteins are specialized lipid–protein environments, and their relevance to atherosclerosis (see below) has lead to significant work on their oxidation. Lipid oxidation products, notably aldehydes, can modify the lysine residues of apoB, and thereby decrease binding to the classical LDL receptor, while increasing its endocytosis by other routes, such as the scavenger receptor. LDL oxidation by metal-catalysed oxidation causes generalized loss of amino acids, although aspartate and glutamate are increased [149], consistently with the damage to histidine and proline residues. Fragmentation also takes place, as demonstrated by selective radiolytic techniques [123]. During metal-ion and radiolytic attack, lipid and protein oxidation are often concurrent, and occur even while much vitamin E remains in the particles. In contrast, hypochlorite selectively attacks the protein, consuming mainly lysine, tryptophan, cysteine and methionine residues, and giving rise to chloramines [97,150]. Exogenous tyrosine can be cross-linked to apoB tyrosines by hypochlorite

[150], although it is not clear whether this occurs with physiological levels of tyrosine.

Protein oxidation in isolated organelles and other complex systems

Studies on phage inactivation by radicals, and its sensitization by O_2 and localization by transition-metal ions, are indicative of both protein and DNA damage (e.g. [151]), although protein damage and DNA–protein cross-linking were directly studied only to a limited extent [152]. Damage to mitochondrial electron-transport proteins by hypochlorite [153] and other agents has been studied, and noted to be enhanced by vitamin E deficiency [154]. Mitochondria exposed to exogenous radicals lose control of ion balance, notably of calcium transport; protein oxidation as well as proteolysis may be important in such changes [145,155,156]. Leakage of electrons from the chains, leading to radical fluxes and self-inactivation, may also be important, especially as mitochondria seem to be a major radical-generating site and contain more oxidized DNA than nuclei [157,158]. Again, this may be associated with alterations in enzymic proteolysis as well as protein oxidation [159,160]. These studies indicate close parallels between lipid and protein oxidation in mitochondria.

In chloroplasts, electron-transport proteins are continuously exposed to radicals derived from electron leakage and photochemical reactions. This is important, for example, in the inactivation of photosystem II, in which a 32 kDa herbicide-binding protein is oxidatively modified prior to proteolytic degradation [161,162].

Microsomal oxidation reactions depend largely on the cytochrome *P*-450 family, and these enzymes seem to undergo suicidal inactivation, independently of lipid oxidation. In this case the substrate probably forms radicals in, or near, the enzyme active site, leading to haem damage and inactivation [163,164]. Ascorbate can protect microsomes against protein damage involving cytochrome *P*-450, which seems to be a conventional metal-catalysed oxidation system, using the iron of the cytochrome [165,166].

In these membranous systems, therefore, there may be both lipid-dependent and -independent pathways of protein oxidation, just as there may be protein-independent lipid oxidation. In contrast, in extracellular connective tissue, protein oxidation appears to be an isolated process, removed from lipid, although occurring in the vicinity of high concentrations of proteoglycans. During radiolytic or Fenton-derived radical attack on cartilage discs, polypeptides are preferentially attacked by HO$^{\bullet}$ [167,168], although these radicals can also depolymerize the non-proteinaceous components [169].

Enzymic removal of oxidized proteins

Like most partially denatured proteins, modestly oxidized proteins are usually more sensitive to proteolytic attack by most proteinases [39,99,117], whereas heavily oxidized proteins generally show decreased susceptibility [101,117]. The latter may depend on local chemical and structural changes rendering parts of the molecules poorly digestible [170], in agreement with the fact that certain aldehyde derivatives of proteins can be less susceptible to proteolysis than the parent protein; for example 4-HNE- (but not malondialdehyde-) modified apoB in LDL [171]. Thus one might expect that oxidized proteins in general are efficiently removed from cells and organisms, assuming that they are accessible to appropriate proteinases.

This argument has lead to several proposals that features of oxidized proteins constitute 'signals' or 'markers' for rapid proteolytic removal in living cells. Alteration in protein -SH/S–S status was one of the first signals proposed [172,173]. Developing earlier work [37,38] Stadtman and colleagues [39] integrated metal-catalysed oxidation with initiation of proteolysis. They suggested that limited modifications (e.g. to one histidine residue in glutamine synthetase) might suffice to signal degradation. However, even in simple lysate systems this proposed relationship between susceptibility to degradation and single modifications to the target is difficult to verify, since many alterations occur concomitantly (see above; [49]). These difficulties are multiplied when modifications within living cells are considered. Even in the most carefully studied systems [52,174–177], one can only rigorously conclude that a wide range of oxidative modifications are concomitant with elevated proteolytic removal. Similar observations have been made with isolated mitochondria and choloroplasts, so that the idea probably has generality.

Within cells there are many potential proteolytic sites (mitochondria, lysosomes, proteasomes) for degradation of oxidized proteins, some of which originate locally, others of which are transported there. Selective transport of certain cytosolic and organelle proteins to lysosomes for degradation appears to be important [178–180]; in contrast, selective reverse translocation of newly synthesized unfolded proteins from the endoplasmic reticulum to the proteasome can occur [181].

The endocytic supply of oxidized protein substrates is one of few approaches in which one can define the quantities of substrates, their catabolic rate and their locations within the cell. ApoB of oxidized LDL follows a normal transport pathway within macrophages, but is poorly degraded and accumulates (in lysosomes) to an elevated level compared with normal LDL apoB [171,182]. The precise importance of apoB itself is not clear, since oxidized lipids are also supplied. However, 4-HNE-derivatized apoB in otherwise unaltered LDL, as well as oxidized (lipid-free) BSA, are also poorly degraded, suggesting that protein modifications are important [171,183].

For intracellularly generated oxidized proteins, the degradation sites may be multiple, and require identification. Evidence for a crucial role for the proteasome in the degradation of such proteins is unconvincing [184]. The support from studies using antisense RNA inhibition of proteasome function [185] appears to be undermined by gross re-utilization of radioactive amino acids, such that the measured 'degradation' rates are implausible and more probably reflect protein synthesis rates.

Further work is needed to compare the absolute rates of catabolism and the half-lives of oxidized and native proteins. This requires knowledge of both pool sizes and catabolic rates. Microinjection studies have shown that the proportional degradation of oxidized proteins can be more rapid than that of the parent [186]. However, difficulties remain in extrapolating data from these necessarily artificial conditions (microinjection, inappropriate pool sizes) to normal conditions. One promising approach may be to use purified oxidized amino acids as substrates for protein synthesis, thereby generating controlled amounts of 'oxidized' proteins without oxidative affront to the cells. A detailed review of the proteolysis of oxidized proteins is available [187].

If the degradation of oxidized proteins *in vivo* is efficient, one would expect a small but finite pool of oxidized amino acids to be present in normal proteins. Table 1 indicates this to be the case, with relative levels of oxidized amino acids in proteins resembling their extents of generation during HO$^{\bullet}$ attack on proteins. Generally, hydroxylated aromatic residues are present at higher levels than aliphatic residues. Dityrosine is present at intermediate levels, and nitrotyrosine at low levels. This suggests that protein oxidation *in vivo* is a heterogeneous process giving

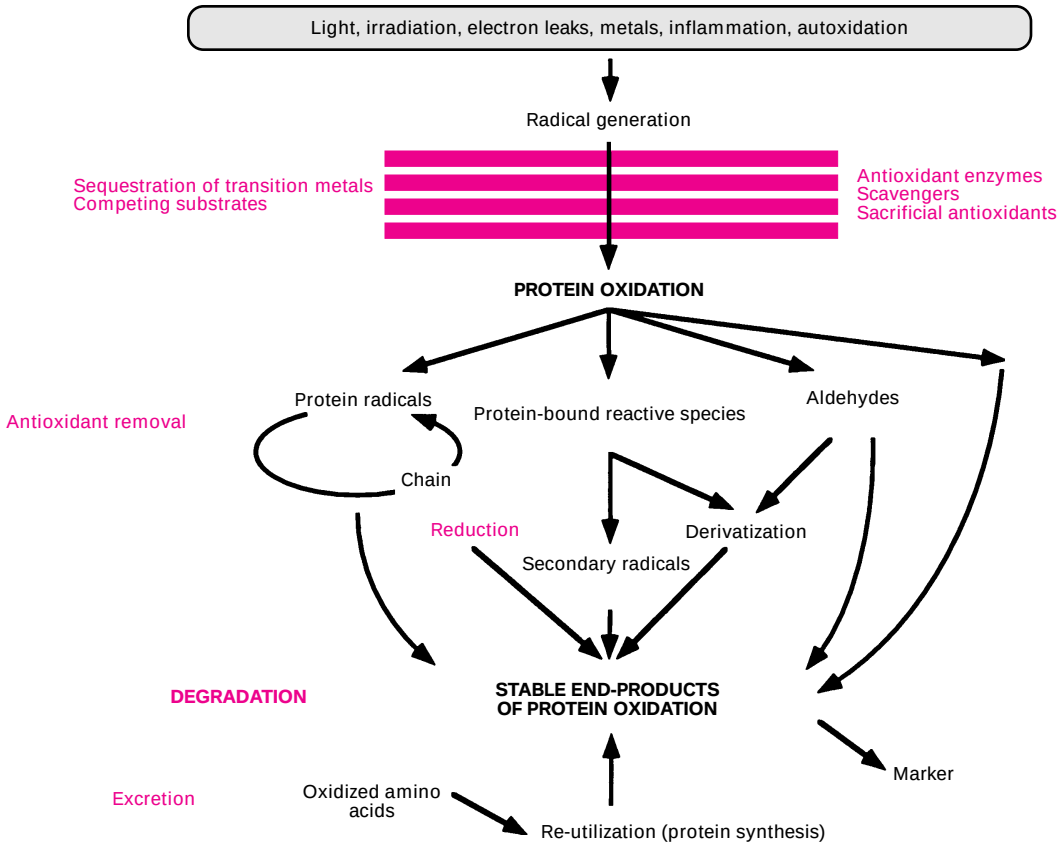


Figure 1 Postulated mechanisms of protein oxidation *in vivo*

Processes depicted in colour are those likely to result in the amelioration of protein damage *in vivo*.

rise to multiple products (as would be expected with HO$^{\bullet}$) rather than a highly selective one, and that all oxidized proteins are removed in parallel, so that there is no selective removal, for example, of those protein molecules containing hydroxyvaline in comparison with those containing dopa.

In aging and certain pathologies (discussed below), oxidized proteins accumulate, as has been demonstrated for inactive enzymes in nematodes [188]. This must indicate either physical separation of the oxidized molecules from proteolytic systems (as may apply in the lens), or that the rates of production and removal strike a new balance, while remaining mutually accessible. Claims of altered activities of individual degradative systems (e.g. the alkaline proteinases; [189]), even when confirmed, cannot be immediately interpreted as causal, since the routes of catabolism of oxidized proteins are not established, and may well be pleiotropic.

Fig. 1 summarizes the mechanisms by which oxidized proteins may undergo further reactions *in vivo*.

PHYSIOLOGY AND PATHOLOGY OF PROTEIN OXIDATION

It will ultimately be important to gain detailed information as to the pathways of protein oxidation *in vivo*; this is difficult at present. Protein carbonyl measurements [190] detect an incompletely characterized range of products, and are not straightforward with tissue samples [189,191]. The commonly observed tissue levels of around 1 nmol/mg of protein suggest that 5% of proteins contain a carbonyl function, and roughly one carbonyl group per 3000 parent amino acids. This is a remarkably high value given the specific amino acid modifications shown in Table 1. The simplest explanation is that the carbonyl assay overestimates protein oxidation in complex biological materials; this assay may be best restricted to purified proteins.

The hydroxy derivatives of leucine and valine can arise from HO$^{\bullet}$ attack. In contrast, 3-nitrotyrosine formation must involve reactive nitrogen intermediates, but nitric oxide, peroxynitrite, nitrite and reactions between hypochlorite and nitrogen-containing compounds are just some possible sources of such intermediates. Several of these species can also give rise to both hydroxylated aromatic residues and tyrosyl (phenoxyl) radicals, and hence dityrosine. Dityrosine can also be formed by the myeloperoxidase/chloride/H_2O_2 system, with either protein-bound or free tyrosine, as judged by model experiments. Were this to contribute *in vivo*, then dityrosine formation might be paralleled by 3-chlorotyrosine formation. Available data are complicated by the possible further oxidation of dityrosine [192]. It is also necessary to bear in mind the possibility of the artefactual generation of oxidized proteins during processing (e.g. [193]); this is a particular danger with post-mortem samples.

There are some physiological situations, such as the byssi of marine organisms from mussels to tunicates, in which oxidized proteins are clearly functionally important as adhesives. These unusual proteins contain very large portions of their tyrosine residues as dopa, as welll as further hydroxylated species. The specificity of the (apparently non-enzymic) oxidations is lower than that responsible for the formation of dopa or topa in active sites of certain redox enzymes. In accordance with this decreased specificity of oxidation, a variety of other unusual modified

amino acids are also found in these marine proteins, and some function as efficient metal chelators (see [194–196]).

Oxidized proteins in the control of cell growth and differentiation

Radicals are probably necessary mediators of cell growth control [197] and heat-shock protein synthesis [198]. The question is whether protein oxidation is an important factor in such mechanisms. Several transcription factors are redox-controlled, notably AP-1 and nuclear factor-κB. AP-1 may be important in regulation of cell growth [199], and the binding of AP-1 to DNA in 3B6 lymphocyte nuclear extracts is decreased when the cells are growth-inhibited by selenite or selenodiglutathione, which also oxidize thioredoxin. The functions of AP-1 and nuclear factor-κB are distinct, and may vary from system to system [200].

Protein oxidation products could be effectors of cell growth, but present evidence suggests simply a bystander role. Thus the level of protein carbonyls in senescent IMR-90 human fibroblast cells does not vary significantly with population doublings, even while growth control changes drastically [201]. Similarly, only fibroblasts taken from humans over the age of 60, or from Werner's and Progeria cases, show carbonyl levels higher than those in normal controls [202].

There is evidence that protein oxidation plays a significant role in cell differentiation. Several bacterial systems possess O_2-dependent mechanisms for remodelling their enzyme profile. Early studies by Turnbough and Switzer [37,38] examined the idea of metal-ion-catalysed inactivation of enzymes as a trigger for proteolytic removal. Two fascinating papers [52,203] concern protein catabolism in differentiating *Klebsiella aerogenes* and *K. pneumoniae* respectively. The argument is put forward [203] that inactivation of aspartokinases III (lysine-sensitive) and I precede and lead to their enzymic proteolysis when *K. aerogenes* is incubated in a nitrogen-lacking medium. However, a detailed analysis of these data and earlier immunological studies [39] showed only that the two processes occur very close together. Although lysate experiments indicate that both metal-ion-catalysed inactivation and proteolysis can contribute to enzyme inactivation, evidence that oxidative inactivation is responsible *in vivo* is lacking.

These same issues were subsequently carefully addressed [52] with reference mainly to glycerol dehydrogenase, which is inactivated and degraded when *K. pneumoniae* is moved from anaerobic to aerobic conditions. The enzyme was purified from cells before and after exposure to H_2O_2, which also inactivates it in intact cells. The purified inactivated enzyme showed enhanced susceptibility to proteolysis by subtilisin, but there was little clear evidence that it was oxidized. Chloramphenicol, an inhibitor of protein synthesis (and usually of protein degradation), prevented the inactivation and degradation of the glycerol dehydrogenase in cells exposed to O_2, but not in those exposed to H_2O_2, suggesting that some newly synthesized protein is necessary for inactivation and/or degradation triggered by O_2. Whether this is a protein component due to metal-catalysed oxidation, or a proteinase, is not clear. Thus the question of protein oxidation as a controlling step in the initiation of differentiative protein turnover remains to be settled.

Conidiation in *Neurospora crassa* is another differentiation system in which the role of protein oxidation has been studied. Conidiation comprises successively the germination of the conidium (an asexual spore) to generate filamentous hyphae by apical growth; later, aerial hyphae develop, and ultimately conidia form at their tips. There are thus three transitions, and each involves a 'hyperoxidant' state, as judged by chemiluminescence, alteration of GSH/GSSG and NAD(P)H/NAD(P)$^+$ ratios and excretion of GSSG [175,177,204–206]. Protein oxidation (measured by carbonyl groups) is kinetically and spatially correlated with these events. Reversible elevation of protein carbonyl levels (from high basal values of around 10 nmol/mg of protein) by up to 2.5-fold in less than 1 h was observed in association with each transition [174]. Reversal also took less than 1 h. The cell-free oxidative inactivation of glutamate dehydrogenase and glutamine synthetase from the fungus [51,207] created more acidic polypeptides, and importantly such acidic species were also observed *in vivo* and found to display enhanced susceptibility to proteinases. Loss of glutamine synthetase activity occurred shortly before each differentiation step, coincident with the increase in protein carbonyls [176]. Total protein turnover was also quantified, revealing highly selective degradation in the regions of elevated carbonyls. It seems that protein oxidation can therefore be a central mediator of the degradative/synthetic remodelling of protein profiles.

A possible involvement of protein oxidation in programmed cell death (apoptosis; see [208]) remains to be established. Some oxidized components, notably LDL, can initiate apoptosis [209]. The protein p53, which seems to be involved in the induction of apoptosis (e.g. [210]), may be regulated by alterations in protein thiols. Its action can be triggered by radiation exposure [211], and glutathione and other antioxidants can protect against this.

Protein oxidation in host defence and tissue catabolism

Physiological inflammatory reactions involve the recruitment of leucocytes to sites of wounding or infection, and connective tissue catabolism and repair. Both topics are discussed together here, although it should be noted that, in chronic inflammation, connective tissue breakdown can become pathological. Leucocyte recruitment usually occurs in response to chemotactic factors, a large number of which also stimulate radical production by the leucocyte respiratory burst. These radicals play roles in cytotoxicity against infecting organisms, and perhaps also in host connective tissue catabolism.

Ionic pump proteins involved in cellular homoeostasis are often primary targets in the lysis of infecting organisms by radicals produced by activated leucocytes. Subsequently, neutrophil-generated HOCl can induce efficient target bacterial cell protein fragmentation [212]. Thomas and colleagues [213–215] found that free and protein thiol groups in the bacteria were very sensitive to hypochlorite, and that N-Cl compounds were long-lasting aggressive products, functioning even after the supply of H_2O_2 (and hence hypochlorite) had ceased. They also observed protein fragments, and up to 50% of HOCl provided could be recovered as N-Cl-peptides.

In the degradation of proteins of the extracellular matrix, there often seems to be an interaction between proteinase and radical systems of the leucocytes, operative near to the cell surface [216,217]. For example, exposing glomerular basement membranes to myeloperoxidase/H_2O_2/chloride can enhance their subsequent degradation. Model systems suggest that this is largely proteolytic, as antioxidants, scavengers and inhibitors of the respiratory burst have little effect. However, released oxidants inactivate secreted elastase, lysozyme and β-glucuronidase, and hence overall unchanged degradation was achieved with smaller amounts of enzyme, which could reflect co-operation with oxidants [217,218]. Others observed that PMA-triggered macrophages degrade collagen suspensions by a brief radical burst followed by proteolysis; the latter was restricted by proteinase inhibitors, the former by ascorbate or superoxide dismutase [219].

There are also examples of claimed oxidative activation of

proteolytic enzymes, such as urokinase by chloramines and hypochlorite [220]. There is good evidence for limited protein oxidation during inflammatory reactions; for example, broncheoalveolar fluids have elevated methionine sulphoxide/methionine ratios under conditions in which leucocyte activation is apparent [221]. Furthermore, oxidized proteins (and DNA) form inside the leucocytes during their triggering, as judged by protein carbonyls [222] and tyrosine modification [223], although it is not clear whether this affects protein function directly.

Oxidized proteins and toxicity to the host organism

Proteins are probably the most critical target of toxic damage, since their inactivation can have rapid and supra-stoichiometric effects, by virtue of their catalytic function. The inactivation of a small portion of an ion transporter (in the plasma membrane or mitochondria) is likely to have much more drastic consequences than a similarly extensive alteration of sterol molecules.

Most cytolysis ultimately depends on osmotic swelling and cell rupture, in contrast with the shrinkage mechanism of programmed cell death. Lipid damage may be separable from critical events, as lipophilic antioxidants often restrict lipid oxidation without decreasing cell lysis. Protein damage, including alterations of ion pumps, has been observed in a few cases. Changes in membrane potential may be observed very rapidly and, after a lag of 1–2 h, cell death ensues [224].

The oxidation of ionic transporters has been closely studied in relation to cardiac arrhythmias and ischaemia/reperfusion. An ischaemic period is followed by rapid re-oxygenation [225] and radical fluxes, as well as by potentially damaging leucocyte infiltration. Elegant systems for studying oxidative affronts to the heart and to cardiac cells have been developed, some using photosensitizers to localize oxidation, and/or to permit $^1O_2$ production [226]. Thus, after perfusion of hearts with the photosensitizer Rose Bengal, subsequent illumination leads, within seconds, to alterations in membrane transport and arrhythmias [227]. These events can be inhibited by antioxidants, and membrane protein -SH oxidation occurs, although the precise lesion has not been defined. The data are consistent with primary damage to the Na^+/H^+ exchanger [228]. The importance of calcium transporters, notably the Na^+/Ca^{2+} exchanger which can be inactivated through its -SH group [229], is also apparent. Alterations in cellular calcium may influence intracellular proteolysis and many other physiological systems.

Reperfusion of an ischaemic tissue can be accompanied by the release of redox-active transition-metal ions [230] and a burst of radicals [231] whose formation requires O_2. In working rat hearts, reperfusion is accompanied by a large rise in protein carbonyls within 5 min, followed by a return within 15 min almost to basal levels [232]. Similar observations have been made in relation to brain ischaemia and reperfusion; some antioxidants [233] and the spin trap *N*-t-butyl-α-phenylnitrone (PBN) [234] may decrease this. Several other antioxidant regimes can restrict overall damage. However, the precise interaction of these restricting regimes with protein oxidation is unknown, and there are disparities (see [235]) that require clarification. Site-specific modifications of selected proteins have been observed [236], and could explain these disparities.

PROTEIN OXIDATION IN AGING AND IN SELECTED PATHOLOGIES

Aging

Inactive forms of several proteins accumulate in aging cells, as shown for the nematode *Turbatrix aceti* [188]. 'Age pigments' that contain protein, such as lipofuscin, also accumulate in some aged tissues (see [237]). The key question is whether protein oxidation is primary or secondary in aging.

Protein carbonyl levels increase in several gerbil tissues with aging [234]. In contrast, increases in human fibroblasts aged *in vitro* or newly taken from older people are, respectively, nil [201] or slight [202]. Glutamine synthetase activity declines and inactive enzyme accumulates with aging [238]. However, there is little evidence that the inactive molecules are oxidized. The observation [239] that administration of the spin-trap PBN to rats prevents the age-related accumulation of protein carbonyls, loss of glutamine synthetase and neutral proteinase activities, and behavioural decrements is encouraging, but requires confirmation (see [189,240]). It seems unlikely that the levels of PBN achieved in this study would have any significant antioxidant effect.

Long-lived proteins of specialized tissues such as the eye permit the clearest indication that specific protein oxidation products, such as dopa, dityrosine, *o*- and *m*-tyrosine, and valine and leucine hydroxides, do accumulate (S. Fu, R. Dean, M. Southan and R. Truscott, unpublished work) in human cataractous lenses in association with loss of cysteine and methionine (e.g. [241]). In human non-cataractous lenses there seems to be little accumulation with age of *o*-tyrosine, but up to 33% increases in dityrosine, as well as vast increases in 'di-tyrosine' fluorescence [242].

In other tissues it is not clear how rates of production and removal of oxidized protein relate to the elevated levels measured; proteins in extracts of gerbil tissues show increased sensitivity to radiolytic oxidation with aging [243], but this is not the case for rat tissue extracts undergoing endogenous or metal-ion-catalysed oxidation [244]. Since the mechanisms of degradation of oxidized proteins are not established, it is perhaps premature to relate changes in the activity of individual proteolytic systems to the accumulation of oxidized proteins in aging (cf. [245]).

Drosophila has long been used in aging studies, and shows lipofuscin accumulation. Elegant studies by Sohal and coworkers [246] showed an age-related increase in oxidant production in mitochondria of the flight muscle, together with increases in protein carbonyls and indices of DNA damage. Thus a vicious circle, in which mitochondrial oxidative damage enhances further oxidant production and damage, may operate. Transgenic fly studies showed that overexpression of either catalase or superoxide dismutase singly had no effect on life span, whereas the simultaneous overexpression of both enzymes prolonged it [247,248]. This permitted an increased aggregate physical activity, and was associated with lower levels of protein carbonyls at various ages. The flies consumed enhanced amounts of O_2 in the last two-thirds of their lives, and 30% more overall. The overexpression of antioxidant enzymes is expected to help the flies resist the increased oxidative flux, but the changed O_2 consumption with age suggests that there are other factors involved. Whether the decreased pool sizes of oxidized proteins reflect altered generation or removal has not been directly tested. The levels of lipid oxidation products do not rise continuously with age [249], suggesting that either oxidative events or product removal must not be linear with time. It remains to be established whether protein oxidation is critical in the progression of aging, but these studies encourage further investigation (see [250]).

Diabetes

Diabetes is one of the most common inherited diseases, caused by the impaired production of insulin by pancreatic islet β-cells and/or by diminished tissue responses to insulin (insulin resistance). The consquence of this is that circulating blood glucose levels are chronically elevated, and this appears to be mainly

Table 2 Selected data on concentrations of oxidized amino acids in tissue proteins from humans under normal and pathological circumstances

'Unpublished work' refers to work of the present authors; results are means from three advanced human atherosclerotic plaques and three human LDL samples obtained fresh. In these studies, levels of the measured species in fresh human plasma expressed per parent amino acid were in most cases very slightly lower. The aorta samples used in the studies of Heinecke et al. [310] were obtained *post mortem*. ND, detailed data not found.

Product	Physiological levels	Pathological levels	Source
Dopa	85 pmol/mg of LDL protein (6/10000 tyrosines)	410 pmol/mg of protein (14/10000 tyrosines) in advanced human atherosclerotic plaques	Unpublished work
o- and *m*-tyrosine	62 and 35 pmol/mg of LDL protein (5 and 3/10000 phenylalanines) respectively	105 and 175 pmol/mg of protein (3.5 and 6/10000 phenylalanines) respectively in plaques	Unpublished work
		No increase in atherosclerotic aorta samples compared with normal	[310]
	5/10000 phenylalanines in human lenses of any age	Unchanged with age	[242]
N-Formylkynurenine; kynurenine	ND	ND	
Dityrosine	0.2 pmol/mg of LDL protein (0.02/10000 tyrosines)	150 pmol/mg of protein (5/10000 tyrosines) in plaques	Unpublished work
		10-fold elevated in aortic lesions in comparison with normal aortic samples; 0.03/10000 tyrosines in lens proteins from old people	[310]
	0.01/10000 tyrosines in human lens proteins from young people		[242]
Dimers of hydroxylated aromatic amino acids	ND	ND	
2-Oxohistidine	ND	ND	
Hydro(pero)xyleucine	β-Hydroxyleucine 2: 5 pmol/mg of LDL protein (0.1/10000 leucines)	β-Hydroxyleucine 2: 20 pmol/mg of protein (0.2/10000 leucines)	Unpublished work
Hydro(pero)xyvalines	β-Hydroxyvaline 1: 5 pmol/mg of LDL protein (0.1/10000 valines)	β-Hydroxyvaline 1: 10 pmol/mg of protein (0.1/10000 valines) in plaques	Unpublished work
3-Chlorotyrosine	Normal aorta: 0.8/10000 tyrosines	Atherosclerotic aorta: 4.2/10000 tyrosines	[310]
3-Nitrotyrosine	$<$ 10 pmol/mg of LDL protein	$<$ 10 pmol/mg of protein	Unpublished work*
	($<$ 1/10000 tyrosines)	($<$ 0.3/10000 tyrosines) in plaques	
		100-fold elevated in aortic lesion LDL compared with normal plasma LDL	[310]
p-Hydroxyphenylacetaldehyde	ND	ND	
Aminomalonic acid	0.04–0.3/1000 total amino acids in two *E. coli* strains	0.2/1000 glycines in post-mortem human plaque	[311]
5-Hydroxy-2-aminovaleric acid	0.15 nmol/mg of protein in 100000 ***g*** supernatants of young mouse livers	Unchanged in old mouse livers; elevated by hyperoxic exposure	[312]
Protein carbonyls	$\sim$ 1 nmol/mg of protein in many physiological tissue samples	$\leqslant$ 8 nmol/mg of protein in diseased brain samples	[190,191]

* Below UV-HPLC detection limit.

responsible for the major problems of diabetes, i.e. blindness, gangrene and kidney failure.

Glycation reactions between glucose and proteins and other biological molecules, notably the Maillard (browning) reaction, have long been considered a potential major part of these consequences. 'Autoxidative glycosylation', involving autoxidation of glucose via dicarbonyl intermediates and of protein-bound sugars, was proposed to be important [251,252]. Here we only consider the specific relevance of protein oxidation.

Metal-ion-catalysed autoxidation of sugars and products of protein glycation can generate radicals that initiate and propagate protein damage [128,251–253]. Cross-linking of collagen *in vitro* by exposure to glucose is virtually blocked under N_2 and in the presence of chelators, confirming the importance of oxidative events [254]. We have shown that proteins exposed to autoxidizing glucose contain protein-bound oxidizing and reducing species, later characterized as hydroperoxides and dopa [27,28,255]. We have also demonstrated substantial formation of a range of aliphatic amino acid hydroxides, dopa, dityrosine and hydroxylation products of phenylalanine during exposure of proteins to glucose (S. Fu, M.-X. Fu, J. W. Baynes, S. R. Thorpe and R. T. Dean, unpublished work).

Glyoxal and arabinose are major autoxidation products of glucose; the former can form *N*-ϵ-(carboxymethyl)lysine [256] and the latter can participate in the generation of the fluorescent pentosidine cross-links in proteins [257]. Ascorbate, and dicarbonyl sugars such as methylglyoxal and 3-deoxyglucosone, which are observed *in vivo*, may participate in autoxidative reactions contributing to browning. There are also other sources of glyoxal and related aldehydes such as those from amino acids (e.g. [1]). The protein-bound sugar Amadori intermediates are generally more readily autoxidized than free sugars, so the relative importance of autoxidation before and after protein binding may vary with the relative concentrations of the com-

ponents. *N*-ϵ-(Carboxymethyl)lysine and pentosidine accumulate *in vivo* with aging, and their levels can be elevated in diabetes and restricted in animal models by certain antioxidant regimes (see [70]). Current evidence points towards a significant role for sugar autoxidation, be it of free or protein-bound sugars, in the complications of diabetes.

Atherosclerosis

Diabetes predisposes to the age-related vascular disease atherosclerosis, and this may be partly because of oxidative features. Atherosclerosis involves vessel wall deposition of cells and lipids, forming the hallmark 'foam cells', and leads to cellular proliferation, wall thickening and calcium deposition. It is well established that macromolecule oxidation accompanies atherosclerosis; for example, around 30 % of the cholesterol linoleate accumulated in atherosclerotic vessel walls (it is lacking from normal walls) is oxidized [258]. Much of the accumulated lipid seems to come from LDL, and the modification of its protein apoB by aldehydes and lipid oxidation has been argued to be central in lipid deposition in atherosclerosis [259]. It is necessary to distinguish between direct protein oxidation and derivatizations secondary to lipid oxidation.

Here we consider only the evidence concerning direct apoB oxidation. ApoB in LDL can be readily oxidized and fragmented by radiolysis [123] or by metal-ion-catalysed oxidation [149]. *In vivo* evidence concerning oxidized apoB is limited, given ambiguities concerning 3-nitrotyrosine disposition between apoB and other proteins in plaque [260] and the low levels of this moiety and other oxidized amino acids in proteins (see Table 2), and given the focus on derivatized rather than directly oxidized proteins (e.g. [261]). Studies on hypochlorite-oxidized protein, in which antisera specificities have been carefully validated, suggest not only that hypochlorite-oxidized proteins are present in plaques, but also that apoB peptides are among them [262]. HOCl appears to attack the protein selectively [97,263].

If oxidized proteins are present in plaques, then we need to assess their relative rates of production and removal, and not simply to rely on pool sizes. Oxidative mechanisms may include metal-ion-dependent cellular processes, or putatively metal-independent ones, such as the action of HOCl. Yet extracellular fluids contain antioxidant and chelator capacity [264], and advanced plaques themselves are not deficient in aqueous or lipophilic antioxidants such as ascorbate and tocopherol [258]. Tocopherol-mediated peroxidation may be involved (see [265,266]). The inter- and/or intra-cellular site(s) of oxidation are not clear, and may require specialized conditions. A myriad of potentially pathogenic activities have been attributed to LDL and to some of its components at various poorly defined stages of oxidation (e.g. [267]). The possible roles of the oxidized protein components need to be considered.

Neurodegenerative diseases

Toxic roles for oxidized proteins, in preference to oxidized lipids, have been proposed in recent literature on Alzheimer's disease [268], even though the latter also accumulate [269]. In particular, the accumulation of aggregated amyloid β-protein in diseased brains as neurofibrillary tangles can occur through oxidation [270], and mitochondrial dysfunction has been envisaged as a radical source [271].

The content of protein carbonyls in Alzheimer's brain samples is greater than in age-matched controls [272], and this provides the clearest indication of greater accumulation of oxidized proteins in this disease. Brain regions show specific changes in this regard, and carbonyl levels correlate well with tangles [273]. Glycation and autoxidative glycosylation products also accumulate in Alzheimer's brains [274]. Importantly, the accumulation of oxidation products in particular regions of the brain seems to be related to specific cognitive defects [275]. In line with this, administration of the spin-trap PBN to gerbils retards both protein oxidation and such neurological defects (e.g. [239,276]).

How might these oxidized proteins act? Amyloid β-protein is cytotoxic, and induces oxidative damage in some brain cells [277]. This may involve a receptor response mechanism, although it has been claimed that the protein itself can generate radicals [278], even though freshly dissolved samples were inactive [279]. Variations between samples (e.g. [278]) and upon 'aging' of the materials make one wary of these data, as do the detailed characteristics of the EPR spectra. Regardless of this problem, radical metabolism [280] and protein oxidation could lead either to cross-linking and aggregation of proteins into tangles or to cytotoxic signals that alter ion transporters and neurotransmission [281], and in these ways may contribute specifically to the progression of Alzheimer's disease.

OUTLOOK: ON THE CONTROL OF PROTEIN OXIDATION

Mechanisms for the control of protein oxidation have been rather little studied. No substantial attempt has yet been made to elucidate the features of protein antioxidation that might be distinct from those of lipid antioxidation, e.g. as a result of the relatively different importance of peroxyl and alkoxyl radicals in the two processes. Factors such as control of radical generation, disposition of targets in relation to radical fluxes and breaking chain reactions seem likely to be important for both cases. Many of these basic mechanisms are discussed in earlier reviews [7,265].

Restricting the availability of transition-metal ions for oxidative interconversions is one key factor in antioxidation. In this respect proteins are often ambivalent, since they often present suitable binding sites, in the form of combinations of histidine, cysteine and other residues, but by binding metal ions they may either restrict the redox activity of the metal [126,282] or, if not, localize damage upon themselves [283].

A candidate specialized protein for the protection of protein thiol groups has been identified [284,285]. It restricts metal-ion-dependent protein oxidation involving thiols, but not systems in which thiol is replaced by ascorbate. The protein may be an analogue of superoxide dismutase, which dismutes $RSSR^{-\bullet}$ radicals, although it may also control metal-ion reduction (but not simply sequester metals). This protein may collaborate with the reversible thiolation mechanism discussed above.

Enzymic antioxidants, such as superoxide dismutase and catalase, and glutathione and phospholipid peroxidase, can remove peroxides and intermediate radicals. Sacrificial antioxidants, such as ascorbate, urate, vitamin E and quinols, can limit peroxidation chains by removing intermediate radicals, such as peroxyl radicals, and themselves become radical species, which can eventually be detoxified. For example, Trolox C can restrict radical damage to albumin [147]. There are indications of specialized protective mechanisms for proteins, notably bilirubin present on albumin, that may decrease protein damage by repairing protein radicals or by scavenging externally presented radicals [22]. When proteins are present in membranes or lipoproteins, agents (such as vitamin E plus vitamin C) that restrict lipid oxidation may consequently restrict protein oxidation, but it is unclear whether any also directly interact with protein radicals (cf. discussion of ubiquinol [286]).

The reactive intermediates (besides protein radicals) in protein oxidation, such as the protein hydroperoxides, may also be

selectively detoxified, and repair of some subsequent intermediates has been discussed above. For example, protein hydroperoxides are reduced efficiently by cells to stable (non-radical-generating) hydroxides, and there seems to be a cell surface mechanism for this; one can presume that there are intracellular counterparts also [29]. Metal-ion-mediated reduction can, however, also be accompanied by further radical fluxes [25,287], so at present it is not clear how protection (direct reduction) and radical generation are balanced. Lipoprotein lipid hydroperoxides are similarly reduced both in lipoproteins [288] and by cells [289].

Thioredoxin plus thioredoxin reductase can reduce protein disulphides to thiols, including those formed by the thiol-specific antioxidant enzyme upon reduction of alkyl hydroperoxides [290]. This activity is thought to be responsible for the involvement of the enzyme in the regulation of redox-sensitive transcription factors. In addition, thioredoxin reductase and thioredoxin have alkyl hydroperoxide reductase activity and are efficient electron donors to plasma glutathione peroxidase [291]. It is not known whether this reductase acts on protein hydroperoxides, although glutathione peroxidase and human plasma components can do so [29]. As discussed above, thiol-specific antioxidant enzyme also protects proteins against thiyl-radical-induced inactivation [292], although it is not known whether thiyl radicals on proteins are substrates for it.

In looking to future research and preventative prospects, several important aspects need to be considered regarding non-proteinaceous antioxidants for proteins. First, with the exception of cysteines, protein amino acids are less reactive than lipids containing bisallylic hydrogens, so that reactive radical oxidants ($HO^{\bullet}$, $RO^{\bullet}$) are of greater relevance to protein than to lipid (per)oxidation. Since the differences in rate constants of a radical with its targets decrease with increasing reactivity of the radical, it follows that the conventional scavenging antioxidants (α-tocopherol, ascorbate, urate, etc.) are less likely to offer specific protection to proteins than they do to lipids.

Secondly, the chain reaction of lipid peroxidation is probably the result of the predominant presence of a single chain-carrying species; in lipoproteins, α-tocopheroxyl radical is the likely chain carrier as long as vitamin E is present [293], whereas, after tocopherol depletion, lipid peroxyl radicals become the chain-carrying species. Thus an antioxidant for lipids can focus on the elimination of a single reactant, independent of the nature of the peroxidation-initiating radical [293]. In contrast, there are several chain-carrying species invloved during radical-induced protein (per)oxidation, such that individual antioxidants may perhaps be expected only to attenuate the process. On the other hand, the apparently more substantial role of alkoxyl radicals in protein than in lipid peroxidation may offer at least a selective target for inhibition of protein rather than lipid oxidation.

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