Review

Generation and propagation of radical reactions on proteins

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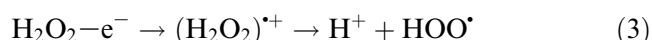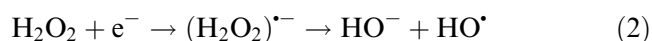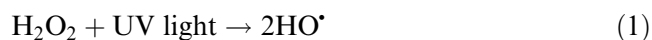
Abstract

The oxidation of proteins by free radicals is thought to play a major role in many oxidative processes within cells and is implicated in a number of human diseases as well as ageing. This review summarises information on the formation of radicals on peptides and proteins and how radical damage may be propagated and transferred within protein structures. The emphasis of this article is primarily on the deleterious actions of radicals generated on proteins, and their mechanisms of action, rather than on enzymatic systems where radicals are deliberately formed as transient intermediates. The final section of this review examines the control of protein oxidation and how such damage might be limited by antioxidants. © 2001 Elsevier Science B.V. All rights reserved.

1. Generation of radicals on proteins

Radicals can be generated in both chemical and biological systems by multiple pathways. In general, radicals are formed by either the direct cleavage of bonds (e.g. reaction 1) or by electron transfer reactions (e.g. reaction 2 or 3). In most biological systems the latter process predominates, though the former can be important when the system is exposed to energetic radiation (e.g. γ-radiation, UV light). Transfer of an electron to, or from, a molecule results in the formation of radical anions (reaction 2) or radical cations (reaction 3). In most cases these are relatively short-lived species which react rapidly with a range of targets to yield other radicals, though there are some exceptions (e.g. the superoxide radical, $O_2^{\bullet-}$, and nitro radical anions). The various processes and mechanisms which give rise to the various types of radicals (and other oxidants) formed in biological systems will not be covered further here, as they have been covered extensively in other articles and books (e.g. [1–3]).

$$H_2O_2 + UV \text{ light} \rightarrow 2HO^\bullet \quad (1)$$

$$H_2O_2 + e^- \rightarrow (H_2O_2)^{\bullet-} \rightarrow HO^- + HO^\bullet \quad (2)$$

$$H_2O_2 - e^- \rightarrow (H_2O_2)^{\bullet+} \rightarrow H^+ + HOO^\bullet \quad (3)$$

Radicals undergo a variety of reactions including hydrogen abstraction, electron transfer (oxidation or reduction of the substrate), addition, fragmentation and rearrangement, dimerisation, disproportionation and substitution (concerted addition and elimination)

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with amino acids, peptides and proteins. These reactions have been the subject of a number of reviews (e.g. [2,4–17]). This article will concentrate on the reactions of radicals, once formed, on proteins and peptides, rather than reactions of free amino acids and their radicals, and will provide a broad overview of the major types of processes that occur, rather than being an exhaustive catalogue of all possible reactions. Space restrictions also prohibit an exhaustive bibliography, so only key early papers and recent work have been referenced.

The major techniques which have been employed to detect radical intermediates on proteins are fast optical (or occasionally infrared) spectroscopy, conductivity measurements and electron paramagnetic (or spin) resonance (EPR or ESR) spectroscopy. The former techniques are powerful methods for studying reaction kinetics, but are not specific for radicals. In contrast, EPR spectroscopy is specific for radicals and hence structural assignments can be definitive; kinetic information is, however, more difficult, though not impossible, to obtain using this technique. The information obtained from these techniques is therefore complementary. Methods for the separation (HPLC, TLC, column chromatography, electrophoresis) and identification (mass spectrometry, NMR, sequencing techniques) of products are covered in a number of excellent volumes (e.g. the Methods in Enzymology series).

A wide range of different radicals can be formed on reaction of a protein with an attacking radical. This is due to the varied nature of the amino acid side chains which offer a multitude of possible sites of attack, in addition to attack on the backbone. The nature of the radicals formed on peptides and proteins depends on the nature and reactivity of the attacking radical. Thus electrophilic radicals (e.g. HO•, alkoxyl radicals) preferentially oxidise electron-rich sites, whereas nucleophilic species (such as phenyl and many other carbon-centred radicals) attack electron-deficient sites.

Whilst the positional selectivity and rates of radical attack on free amino acids are relatively well characterised (reviewed in [14,18]), the situation with peptides and proteins is less clear. There is a wide variation in the magnitude of the rate constants for attack by species such as HO• on free amino acids (from 1×10^7 dm^3 mol^{-1} s^{-1} for Gly to approx.

1×10^{10} dm^3 mol^{-1} s^{-1} for Trp, His and Cys [18]), and this can be readily accounted for in terms of preferential attack at sites remote from the deactivating (powerfully electron withdrawing) protonated amine group at the α-carbon of free amino acids, and the presence of radical stabilising groups on some side chains. Thus with free amino acids, side chain radicals usually predominate. Furthermore, the deactivating effect of the protonated amine group is exerted over long distances with attack on hydrocarbon side chains (e.g. Val, Leu, Ile) skewed towards the most remote sites [19–25]. Thus the ratio of attack at potential sites is different to that expected on the basis of the greater stability of tertiary > secondary > primary carbon-centred radicals arising from the increased number of electron releasing (stabilising) alkyl groups.

The selectivity of attack on side chains is also markedly affected by the presence of a functional group which can stabilise the resulting radicals. Thus hydrogen atom abstraction occurs preferentially at positions adjacent to electron delocalising (stabilising) groups such as hydroxy groups (in Ser and Thr), carboxyl and amide functions (in Asp, Glu, Asn, Gln), and the guanidine group in Arg [26–37]. In contrast, the protonated amine function on the Lys side chain has a similar effect to the protonated amine group on the α-carbon. This results in hydrogen abstraction at sites remote from both groups, and hence products arising mainly from the C-4 and C-5 positions on Lys [21,22,28,38]. Addition reactions are usually faster than hydrogen atom abstraction reactions, as there is no bond breaking involved in the transition state [1,2]. Hence, addition to the aromatic rings of Phe, Tyr, Trp and His, and the sulphur atoms of Met and cystine predominates over abstraction from the methylene (-CH$_2$-) groups. The adduct species formed with the aromatic rings are stabilised by delocalisation on to neighbouring double bonds [39,40]. The only major exception occurs with Cys where hydrogen abstraction from the thiol (-SH) group is particularly fast [2,41–43].

Though the presence of such electron-rich radical stabilising groups also appears to play a role in determining the selectivity of radical attack on peptides and proteins this is less marked. Thus, the conversion of the deactivating amine group on the α-carbon into an (electron delocalising) amide function through the

formation of a peptide bond increases both the extent and rate constant for attack of radicals, such as HO$^•$, at the α-carbon, thereby resulting in significant levels of backbone oxidation [25,44]. The range of values of the rate constants for HO$^•$ attack on amino acid derivatives (e.g. *N*-acetylated species) or simple two amino acid peptides (e.g. the Gly-X series) is much smaller (see Table 1) than that observed with the free amino acids [18]. This effect is even more marked with larger peptides, as judged by the small amount of data available, where the rate constants for attack are, in all cases, around 10^9–10^{10} dm^3 mol^{-1} s^{-1} (Table 1). The α-carbon radical formed as a result of hydrogen atom abstraction from the backbone is particularly stable as a result of electron delocalisation on to both the neighbouring amide group (on the N-terminal side) and the carbonyl function (on the C-terminal side) [45–47]. This has important consequences for radical transfer reactions (see below). Not all α-carbon radicals are of equal

stability, however, and there is evidence for preferential formation at Gly residues in peptides [9,48,49]. This has been postulated to arise because of steric interactions between the side chain and backbone groups which prevents the α-carbon radical achieving planarity (and hence effective electron delocalisation) for those residues with bulky side chains [50,51]. This results in the secondary α-carbon radical formed from Gly being more stable than the tertiary α-carbon radical formed from other amino acids in peptides. With small peptides significant levels of attack therefore occur on the backbone as well as at side chain sites, though exact quantification of the extent of backbone versus side chain oxidation in anything but the smallest substrates is lacking. The situation with larger structures where secondary and tertiary structures are present is more complex and less well understood as a result of the additional influence of other factors. Theoretical calculations have shown that the stability of α-carbon radicals varies with secondary structure as a result of the constraints that such structure plays on the geometry of the α-carbon radical [47,52,53]. These species are therefore less stable, and hence attack at the α-carbon would be expected to be less favourable, when present in sheet or helix conformations. Furthermore secondary and tertiary structures may play a significant role in blocking ready access of radicals present in bulk solution to backbone sites as a result of the outward protrusion of the side chains. This would suggest that side chain reactions may play a more important role in the chemistry of intact globular or sheet proteins than in the chemistry of disordered structures or small random coil peptides. The limited experimental evidence available to date is in accord with this suggestion, with reaction at side chains sites appearing to predominate in larger peptides [25,54,55].

The selectivity of attack at different sites becomes more marked with less reactive attacking radicals. This can be readily rationalised, as less reactive radicals usually have late transition states where there is significant radical character on the incipient radical site. Radical stabilising factors also play a more important role. Thus, selective damage has been observed with some peptides and proteins as a result of attack by ^{t}BuO$^•$, Br$^•$, *N*-bromosuccinimidyl and triplet ketones (reviewed in [14,56]). Too few data

Table 1
Rate constants for reaction of HO$^•$ with selected peptides and proteins (selected data from [2,18])

Substrate	Rate constant (dm^3 mol^{-1} s^{-1})
N-Ac-Gly	4×10^8
N-Ac-Ala	4.7×10^8
N-Ac-Ser-NH$_2$	7.6×10^8
N-Ac-Val	1.4×10^9
N-Ac-Leu	3.1×10^9
N-Ac-Met	6.7×10^9
Gly-Gly	2.4×10^8
Gly-Ala	3.5×10^8
Gly-Ser	7.0×10^8
Gly-Val	1.6×10^9
Gly-Leu	2.5×10^9
Gly-Ile	3.1×10^9
Gly-Pro	1.7×10^9
Gly-Phe	6.2×10^9
Gly-Tyr	6×10^9
Gly-His	1.0×10^{10}
Gly-Trp	1.2×10^{10}
N-Ac-Gly-Gly	7.8×10^8
cyclo(Gly-Gly)	1.2×10^9
(Gly)$_3$	7.3×10^8
(Gly)$_4$	4.5×10^8
cyclo(Ala-Ala)	1.8×10^9
N-Ac-(Ala)$_3$	3.0×10^9
Lysozyme	$\simeq 5 \times 10^{10}$
Human serum albumin	7.8×10^{10}

are available at present to determine whether there is a definite correlation between the rate constants for reaction and the percentage decrease in amino acid content in peptides and proteins under well-defined conditions.

One confounding factor in the determination of the sites of radical attack on large peptides and proteins arises from the fact that at least some of these reactions are chain processes, with a greater concentration of amino acids damaged than initial attacking radicals generated. Chain lengths of up to 15 have been reported (i.e. 15 amino acids consumed per $HO^•$ generated) which is modest by comparison with lipid peroxidation, but large enough to confound product analyses [5,57]. The nature of the chain carrying species in these reactions is poorly defined. However, it is known that O_2 consumption during these reactions is modest (approx. 2 moles per mole attacking radical) indicating that non-O_2-dependent reactions play a major role [57]. Some potential key reactions are outlined in Section 2.

Selective damage to particular residues present in peptides and proteins can arise from the binding of a metal ion, or other initiating species, at a particular site on a peptide or protein [12]. Evidence has been presented for the formation of radicals at specific sites on a number of proteins including catalase [58,59], BSA [60], β-amyloid precursor protein [61], iron regulatory protein-2 [62] and mitochondrial $F_1ATPase$ [63], as well as small peptides [28,64,65]. In each case discrete, well-defined, fragments were detected, and this has been ascribed to complexation/binding of the metal ion to particular sites, and subsequent generation of $HO^•$ or other highly reactive species which would be expected to react in their immediate vicinity thereby inducing site-selective damage. In the case of iron regulatory protein-2, such oxidative damage may play a functional role in the regulation of iron uptake [62]. With thyrotropin releasing hormone, copper complexation occurs at the His residue in the sequence ∼ Glu-His-Pro ∼, with $HO^•$ abstracting a hydrogen atom from the α-carbon site of Pro [64]. Subsequent reaction with O_2 gives strand breakage, with formation of an aldehyde and a new N-terminal amide (i.e. ∼ Glu-His-Pro-NH_2).

Selective modification of His residues has also been observed with glutamine synthetase [66,67],

and human growth hormone [68], Met modification during treatment of peptides with Fe^{3+}/O_2/ascorbate [69], His and Met in human relaxin treated with mixtures of either Fe^{3+} or Cu^{2+}/ascorbate/O_2 [70], and Trp loss in peptides exposed to $Fe^{3+}/O_2^{•-}$ [71]. A similar rationale has been suggested for the enhanced loss of certain amino acids exposed to metal ion-generated $HO^•$ compared to the same radical produced by radiolysis (reviewed in [12]). The amino acids lost most rapidly (His, Cys, Met, Lys, Arg, Trp) in each of these proteins are those most likely to be involved in metal ion binding. In some cases other oxidants have been invoked (e.g. metal ion-peroxy species, high oxidation state Ni^{3+} complexes, or H_2O_2) [65,69,71,72]. Site-specific oxidation may also occur in model systems where metal ions are employed to generate radicals in the absence of strong ligands to chelate the metal ion, with resultant binding of the metal ion to the protein. Metal ion-hydroperoxide complexes (M^{n+}-OOH) have been shown to be formed in some systems and these can be selective oxidants [73–75]. Such complexes can induce site-specific cleavage reactions on proteins (e.g. [73–75]), with the localisation of the metal ion at a particular site allowing the three-dimensional structure in the vicinity of the metal ion to be probed (e.g. [76,77]).

Site-specific protein oxidation can also be a characteristic of damage induced by both lipid peroxidation (e.g. [78–80]) and sugar autoxidation. In the former case damage is thought to be localised at the interface between the lipid and protein regions. The chemistry of the resulting protein radicals is likely to be identical to that described below. Two different categories of site-specific damage can be defined in sugar autoxidation: firstly, damage arising from where the sugar molecule attaches to the protein, and secondly damage which arises in the vicinity of covalently linked sugar molecules which undergo metal ion catalysed, or autoxidation, reactions. The nature of these reactions and the damage they generate remain to be fully elucidated, though it is clear that both types of process can result in protein damage [81–85].
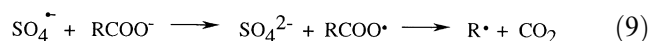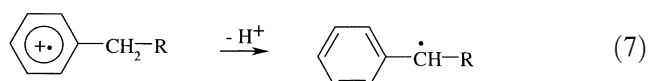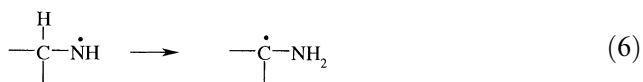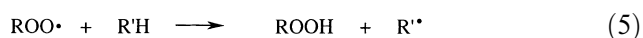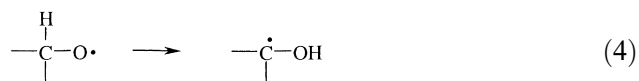
The remainder of this review article discusses how the propagation of protein damage resulting from attack by an initiating radical occurs and how this damage may be controlled.

2. Propagation of protein radical damage

2.1. Side chain radical chemistry
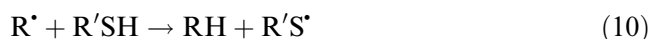
2.1.1. Carbon-centred radicals

Reaction of most radicals with large peptides and proteins results in the initial formation of carbon-centred radicals at either side chains or α-carbon sites. These species can be formed via hydrogen abstraction from C-H bonds (side chain or α-carbon), or from radical addition to an aromatic ring. Carbon-centred radicals can also be generated via secondary reactions of other species, such as further reactions of alkoxyl [2,86–88], peroxyl [89–93], or nitrogen-centred radicals [94,95] (reactions 4–6 respectively), as a result of deprotonation of aromatic side chain radical cations (reaction 7), electron attachment to either aromatic rings or carbonyl functions (reaction 8) and subsequent protonation, or decarboxylation of carboxylate anions (reaction 9; reviewed in [96]).

$$-\overset{H}{\underset{|}{C}}-O\cdot \longrightarrow -\overset{\cdot}{\underset{|}{C}}-OH \qquad (4)$$

$$ROO\cdot + R'H \longrightarrow ROOH + R''^{\bullet} \qquad (5)$$

$$-\overset{H}{\underset{|}{C}}-\overset{\bullet}{N}H \longrightarrow -\overset{\bullet}{\underset{|}{C}}-NH_2 \qquad (6)$$

$$\langle\!\langle\!\!+\!\cdot\rangle\!\rangle\!-CH_2\!-R \overset{-H^+}{\longrightarrow} \langle\!\langle\rangle\!\rangle\!-\overset{\bullet}{C}H\!-R \qquad (7)$$

$$e^-_{aq} + {}^-OOCCH_2NH_3^+ \longrightarrow \cdot C(O^-)(O^-)CH_2NH_3^+ \qquad (8)$$

$$SO_4^{\bullet-} + RCOO^- \longrightarrow SO_4^{2-} + RCOO^{\bullet} \longrightarrow R\cdot + CO_2 \qquad (9)$$

The major fate of carbon-centred radicals formed on proteins is dimerisation in the absence of O_2, and reaction to form peroxyl radicals in its presence [97]. Reaction with O_2 usually occurs at diffusion-controlled rates (10^9–10^{10} dm^3 mol^{-1} s^{-1}), with dimerisation a somewhat slower process [97]. The rate constants for dimerisation are more structure-dependent than reaction with O_2, and this can be an important factor in governing the behaviour of radicals on pro-

teins. As dimerisation involves two radicals, that are usually present only at low concentrations, reaction with O_2 usually predominates when it is present. Highly stabilised/delocalised radicals (e.g. Tyr phenoxyl radicals) are the only major exception to this, with dimerisation (k approx. 5×10^8 dm^3 mol^{-1} s^{-1}) through carbon atoms on the aromatic ring not markedly affected by O_2 [98–100].

Carbon-centred protein radicals can also abstract hydrogen atoms from suitable donors. This type of process may be of major importance in proteins when the concentration of O_2 is low, and dimerisation is prevented by steric factors. Such reactions would be expected to occur most readily with targets containing weak X-H bonds such as thiols, and it has been shown that this type of reaction (reaction 10) can act as a repair reaction for the carbon-centred radical [42,101] (see also Section 2.3.2 for further discussion).
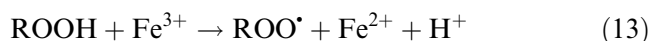
$$R^{\bullet} + R'SH \rightarrow RH + R'S^{\bullet} \qquad (10)$$

In the absence of O_2, some substituted carbon-centred species undergo slow unimolecular elimination reactions. Thus α-hydroxyalkyl radicals with β-amino groups (e.g. those formed from Ser and Thr) can release NH_3 [102]. This process may occur with some side chain-derived radicals, e.g. those formed at C-5 of 5-hydroxylysine (reaction 11).

$$R - {}^{\bullet}CH(OH) - CH_2NH_3^+ \rightarrow$$
$$R - C(=O) - CH_2^{\bullet} + NH_4^+ \qquad (11)$$
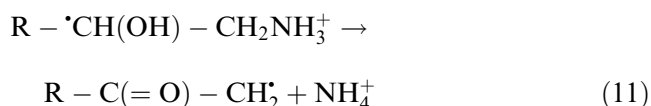
2.1.2. Peroxyl radicals

As discussed above, most carbon-centred radicals react rapidly with O_2 to give peroxyl species (reaction 12 [97]). Peroxyl radicals can also be generated, in the absence of O_2, from metal ion-catalysed decomposition of hydroperoxides (reaction 13 [103–105]).

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \qquad (12)$$

$$ROOH + Fe^{3+} \rightarrow ROO^{\bullet} + Fe^{2+} + H^+ \qquad (13)$$

Peroxyl radicals undergo a number of reactions that result in the formation of carbonyl groups (aldehydes or ketones), alcohols and hydroperoxides. Peroxyl radicals undergo ready dimerisation reac-

tions (reaction 14, R = H or alkyl) with other peroxyl radicals or related species such as $O_2^{\cdot-}/HOO^{\cdot}$; reactions with the latter species are more likely with proteins for steric reasons. Subsequent decomposition of the tetroxide species formed via reaction 14 or direct termination reactions yields carbonyl groups and alcohol (reactions 15–19). These radicals can also carry out hydrogen or electron abstraction reactions (reactions 20 and 21 [89–93]) to give hydroperoxides [106]. Reactions 20 and 21 are likely to be important processes for protein peroxyl radicals when the radical flux is low, and probably account for the high yield of peroxides detected on some oxidised proteins (see below).

$$2\ ROO^{\cdot} \rightarrow ROO - OOR \tag{14}$$

$$ROO - OOR \rightarrow RO - OR + O_2 \tag{15}$$

$$ROO - OOR \rightarrow 2\ RO^{\cdot} + O_2 \tag{16}$$

$$2\ RR'CH - OO^{\cdot} \rightarrow 2\ R'CH = O + 2\ R^{\cdot} + O_2 \tag{17}$$

$$2\ RR'CH - OO^{\cdot} \rightarrow RR'CH - OH + RR'C = O + O_2 \tag{18}$$

$$2\ RR'CH - OO^{\cdot} \rightarrow 2\ RR'C = O + H_2O_2 \tag{19}$$

$$ROO^{\cdot} + R' - H \rightarrow ROOH + R'^{\cdot} \tag{20}$$

$$ROO^{\cdot} + e^- \rightarrow ROO^- + H^+ \rightarrow ROOH \tag{21}$$

Peroxyl radicals with α-substituted heteroatoms can undergo rapid unimolecular elimination of $HOO^{\cdot}/O_2^{\cdot-}$ (reaction 22) [97,107–109]. This process occurs with side chains containing either α-hydroxyl or α-amino groups [97,106,110]. Thus, carbon-centred radicals formed at C-6 on lysine side chains react rapidly with O_2 to give peroxyl radicals which readily eliminate NH_4^+ and $HOO^{\cdot}$ to yield α-amino-adipate-δ-semialdehyde (reaction 23; reviewed in [17]). Similar reactions occur with α-amide functions, which are of importance in backbone damage (see later) [111–114]. This type of reaction can also occur with peroxyl radicals formed on aromatic rings (reaction 24) [115]. These elimination reactions may be key reactions in protein chain oxidation, as they result in damage to one amino acid, and the release of further radicals that can propagate damage.

$$\tag{22}$$

$$\tag{23}$$

$$\tag{24}$$

Thermal or metal ion-catalysed decomposition of hydroperoxides formed by reactions 20 and 21 can also generate further radicals (e.g. reaction 13 [104,105]), and hence, participate in chain reactions [116,117]. Aliphatic side chains (e.g. Val, Leu, Ile and Pro) give particularly high yields of hydroperoxides [117–119], though these can also be formed on aromatic rings and other side chains on reaction with oxidants such as singlet oxygen ($^1O_2$) [120,121].

The yields of each of these products have yet to be fully elucidated and there is a dearth of kinetic data for the reactions of side chain peroxyl radicals on peptides and proteins. Factors which are likely to play a major role in influencing the product concentrations include: (i) the rate of reaction of the initial carbon-centred radical with O_2 (usually $k\ 10^9$–10^{10} $dm^3\ mol^{-1}\ s^{-1}$), (ii) the rate of reaction of the peroxyl radical with hydrogen atom donors, (iii) the radical flux and the rates of the various radical-radical termination reactions, (iv) steric and electronic effects which alter the ratio of bi- to unimolecular reactions, and (v) the stability of any tetroxides (see reaction 14) and hydroperoxides formed.

2.1.3. Alkoxyl radicals

Alkoxyl radicals can be generated from peroxyl radicals via a tetroxide (reactions 14 and 16), or one-electron reduction (reaction 25) of alkyl hydroperoxides (generated via reactions 20 and 21) or dialkylperoxides (RO-OR; from reaction 15). Alkoxyl radicals undergo rapid addition and hydrogen abstraction reactions, as well as facile unimolecular fragmentation and rearrangement reactions [2,86–

88]. Most primary and secondary alkoxyl radicals undergo rapid (formally 1,2-) hydrogen shift reactions (reaction 26; k approx. 10^6–10^7 s^{-1}) which result in the formation of α-hydroxyalkyl radicals [86,87,122]. These reactions compete with intra- (usually 1,5-hydrogen shifts; reaction 27) and intermolecular hydrogen abstraction processes to give alcohols [87,122]. β-Fragmentation reactions (e.g. reaction 28) occur with tertiary alkoxyl radicals, where 1,2-hydrogen shift reactions are impossible, and with primary or secondary alkoxyl radicals where a particularly stable carbon-centred radical and aldehyde/ketone are formed. Relief of steric strain also plays a role in determining the extent of these reactions, which can be rapid in aqueous solution ($k > 10^6$ s^{-1}) [88,123–126]. These processes can result in the transfer of damage from a side chain to the backbone (see below). Other fragmentation reactions can also occur, with decomposition of a C-4 hydroperoxide group on the Glu side chain resulting in the loss of the adjacent side chain carboxyl group as $CO_2^{\bullet-}$ via a β-scission reaction (reaction 29 [104]).

$$ROOH + e^- \rightarrow RO^{\bullet} + HO^- \tag{25}$$

$$RR'CH - O^{\bullet} \rightarrow {}^{\bullet}CRR'OH \tag{26}$$

$$-CH_2CH_2CH_2CH_2 - O^{\bullet} \rightarrow -{}^{\bullet}CHCH_2CH_2CH_2OH \tag{27}$$

$$R''R'RC - O^{\bullet} \rightarrow R''R'C = O + R^{\bullet} \tag{28}$$

 (29)
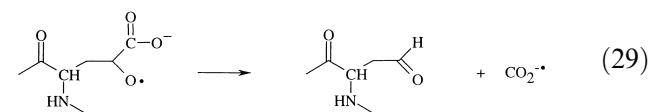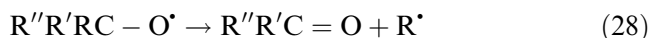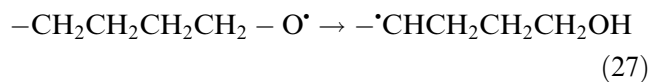
2.1.4. Thiyl radicals

Thiyl radicals (RS$^{\bullet}$) are readily generated by either hydrogen abstraction from a free thiol group (reaction 10) or by cleavage of disulphide linkages. The latter reaction can occur photolytically (reaction 30) and by addition of an electron (reduction) followed by rapid fragmentation of the radical anion (reaction 31; reviewed in [127]).

$$RS - SR + light \rightarrow 2\,RS^{\bullet} \tag{30}$$

$$RS - SR + e^- \rightarrow RS^{\bullet} + RS^- \tag{31}$$

Thiyl radicals react rapidly, but reversibly, with O_2 to form peroxyl radicals RSOO$^{\bullet}$ (reaction 32); these can isomerise to sulphonyl radicals RS($=$O)O$^{\bullet}$ and give rise to oxyacids and sulphinyl (RSO$^{\bullet}$) radicals [128]. At physiological pH values, reaction with excess thiol anion (RS$^-$) to give a disulphide radical anion (reaction 33) competes with reaction with O_2 [2,41–43]. The disulphide radical anion also reacts readily with O_2 via electron transfer to give the disulphide and $O_2^{\bullet-}$ (reaction 34) [2,41–43]. Thiyl radicals readily dimerise, and thereby give rise to (inter- or intramolecular) protein cross-links, though the occurrence of such reactions may be limited by steric and electronic factors [2,41–43].

$$RS^{\bullet} + O_2 \rightarrow RSOO^{\bullet} \tag{32}$$

$$RS^{\bullet} + RS^- \rightarrow (RSSR)^{\bullet-} \tag{33}$$

$$(RSSR)^{\bullet-} + O_2 \rightarrow RSSR + O_2^{\bullet-} \tag{34}$$
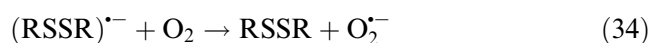
2.1.5. Nitrogen-centred radicals

Reaction of hypochlorous acid (HOCl) with proteins can result in the formation of unstable chloramines (RNHCl) or chloramides (R-C(O)-NCl-R′) via reaction with amines and amides [94,95,129–132]. Decomposition of these species can give nitrogen-centred radicals via cleavage of the N-Cl bond (reaction 35) [94,95]. These nitrogen-centred radicals undergo various rearrangement reactions to give carbon-centred radicals via mechanisms analogous to those outlined above for alkoxyl radicals [94,95]. It has also been demonstrated that reaction of HO$^{\bullet}$ with free Gly can give rise to nitrogen-centred radicals as a result of one-electron oxidation of the free amine group to give an aminium radical cation (RNH$_2^{+\bullet}$) or the neutral aminyl radical (RNH$^{\bullet}$) [133]. Whether these reactions occur with other free amino acids and with backbone amide groups has yet to be determined.

$$RNH_2 + HOCl \rightarrow H_2O + RNHCl \rightarrow RNH^{\bullet} + Cl^- \tag{35}$$
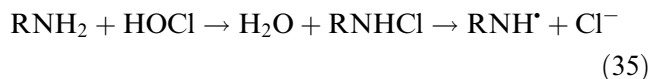
Intramolecular abstraction appears to be a particularly favoured process with side chain aminyl radicals (e.g. those formed from the ε-amino group of Lys side chains), and this generates carbon-centred

radicals at either C-3 or the α-carbon [94,95]. The chemistry of such radicals on proteins, and their significance, is still largely unknown.

2.1.6. Ring-derived radicals

Reaction with aromatic side chains is generally by addition to the ring, though the initial adducts (e.g. **1**, reaction 36) often undergo rapid further reactions (e.g. reactions 36–38). Hydrogen abstraction from the ring and side chain methylene (-CH$_2$-) groups are minor processes. Reaction of HO$^\bullet$ with Tyr residues results in the formation of adduct radicals (substituted cyclohexadienyl species) which can react further to give 3,4-dihydroxyphenylalanine (DOPA; reactions 36 and 37) [39,40,134], or rapidly eliminate water, in both acid- and base-catalysed reactions, to give phenoxyl radicals (reaction 38). DOPA is formed as a result of disproportionation of two initial ring-derived radicals in the absence of O$_2$ (reaction 36 [135,136]), whereas in its presence, peroxyl radical formation is followed by rapid elimination of HOO$^\bullet$ (reaction 37 [137,138]). The latter pathway gives higher yields of DOPA, as one DOPA is generated per initial ring radical, whereas in the absence of O$_2$, two initial ring-derived radicals are required to form one DOPA. DOPA formation has been used as a marker of tyrosine and protein oxidation [17], though this species can react further and give rise (for example) to DNA damage [139].

(36)

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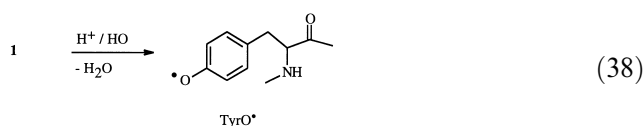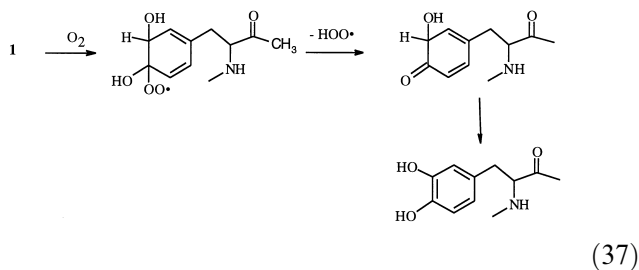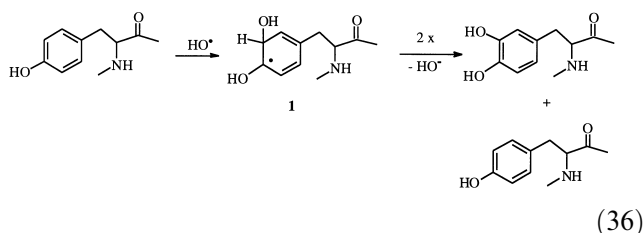
Phenoxyl species are also generated efficiently and rapidly by selective oxidants, such as N$_3^\bullet$, via one-electron oxidation of the phenolic ring, to form a radical cation, and subsequent rapid loss of the phenolic proton. They are also generated on a large number of haem, and other proteins, via enzymatic reactions; recent studies include [140–144]. This topic has been reviewed [145,146]. Phenoxyl radicals can dimerise to yield hydroxylated biphenyls (di- or bityrosine, reaction 39, k approx. 5×10^8 dm^3 mol^{-1} s^{-1} [135,136,147]) resulting in protein cross-linking. Cross-links between the *ortho* site and the oxygen atom have also been characterised (reaction 39) [98]. Reaction of phenoxyl radicals with O$_2$ is slow, $k < 1 \times 10^3$ dm^3 mol^{-1} s^{-1}, and hence the yield of dityrosine is not significantly affected by the O$_2$ concentration [100,148]. Phenoxyl radicals have been implicated in the oxidation of a number of biological targets, including other amino acids, peptides, proteins, lipoproteins and antioxidants [149–155].


(39)
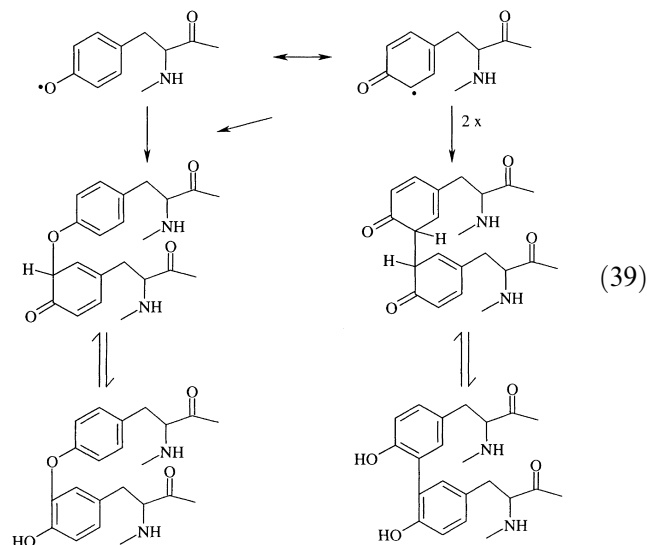
Similar reactions are observed with Trp, with initial addition occurring to either the benzene ring (approx. 40%; reaction 40) or the pyrrole moiety (approx. 60%; reaction 41) [156–158]. In the absence of O$_2$, the benzene ring-derived radicals give either low yields of 4-, 5-, 6- and 7-hydroxytryptophans or lose water to give the neutral indolyl radical, **3** (25–30%, reaction 42) [156,157,159,160]. Indolyl radicals react slowly with O$_2$ [161,162], but react rapidly with O$_2^{\bullet-}$ (k 4.5×10^9 dm^3 mol^{-1} s^{-1}) to give a hydroperoxide (reaction 43 [158,162,163]). The remaining benzene ring-derived radicals react with O$_2$ to form peroxyl

radicals, some 30% of which eliminate $HOO^{\bullet}/O_2^{\bullet-}$ to give hydroxylated products. The peroxyl radicals formed on reaction of the initial C-3 pyrrole ring-derived radical with O_2 undergo a ring opening reaction to give *N*-formylkynurenine (reaction 44 [157,158,164]). This peroxyl species has been implicated in the oxidation of other biological targets [154,155,165]. The formation of hydroxylated products and *N*-formylkynurenine, and the loss of fluorescence from the parent amino acid, have been employed as markers of Trp oxidation (reviewed in [17]).

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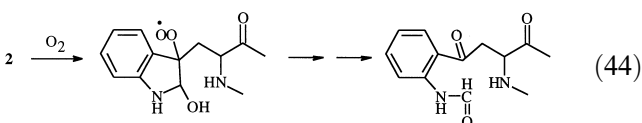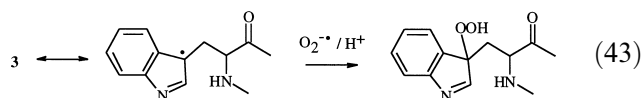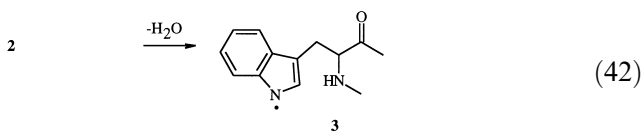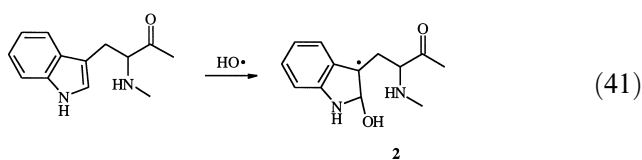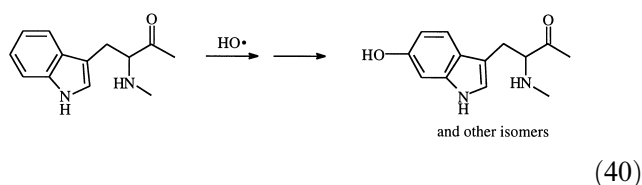
Reactions of radicals such as $HO^{\bullet}$ with His are complex, with initial addition occurring at C-2, C-4 and C-5 of the imidazole ring (reaction 45 [166–169]). These radicals can react with O_2 to give peroxyl radicals or undergo base-catalysed loss of water to give a stabilised diazacyclopentadienyl radical [166,168]. The final products of these reactions have not been completely characterised, but include 2-oxo-histidine, asparagine, aspartic acid, hydroxylated derivatives and hydroperoxides [170,171].

(45)
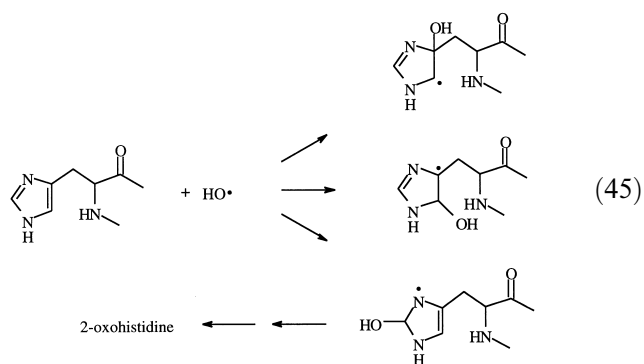
Electron transfer reactions resulting in the formation of radical anions and radical cations are also common with aromatic side chains. Thus, reaction of the solvated electron with Phe generates a transient radical anion which rapidly protonates to give a cyclohexadienyl radical (reaction 46 [172]). Ring radical cations are generated with all the aromatic amino acids on reaction with powerful oxidants, such as $SO_4^{\bullet-}$ (reaction 47 [101]), and on direct photo-ionisation [173]. The charge on these species is rapidly lost by a number of processes including hydration (thereby yielding hydroxylated products) and loss of a proton from an adjacent C-H (Phe), N-H (with His or Trp) or O-H bond (with Tyr) [96,174].
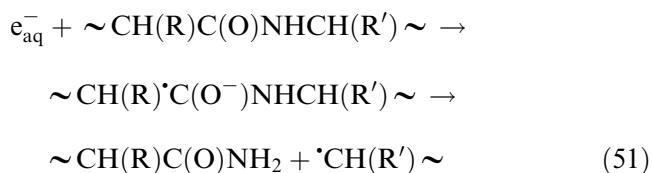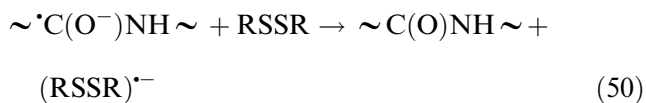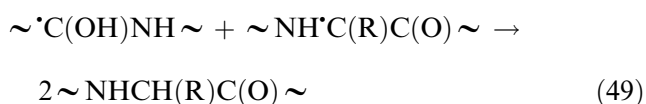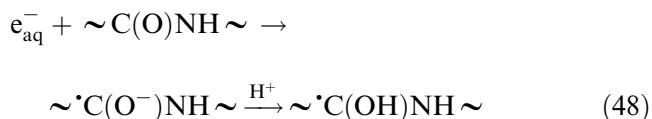

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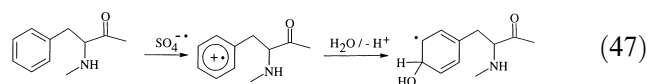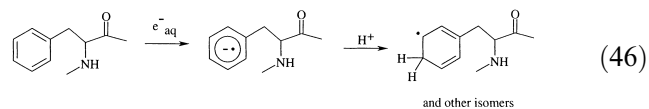
2.2. Backbone radical chemistry

2.2.1. Carbon-centred radicals

Hydrogen abstraction from the α-carbon position accounts for more than 90% of the radicals formed with a series of alanine-derived peptides on reaction with $HO^{\bullet}$. This is due to the greater stability of the α-carbon radical, over the primary alkyl radical formed on hydrogen atom abstraction from the methyl side chain [25,56,175]. However, the yield of such backbone-derived radicals decreases markedly when there are side chains present which can form stabilised radicals, or when steric factors play a role

(see above). α-Carbon radicals decay mainly by dimerisation in the absence of O_2 [176]. The products of these reactions are well characterised with small peptides, where cross-links between all radical sites have been detected [44,176], but not with proteins. In the former case significant yields of cross-links involving side chain-derived radicals have been identified; in the presence of O_2, peroxyl and alkoxyl species are also generated [99,177,178].

α-Carbon-centred radicals are also generated on addition of the solvated electron to backbone carbonyl groups (reaction 48 [179–181]). The resulting midchain α-hydroxy α-amido radicals, formed on protonation of the initial adduct, decay primarily via reaction with other radicals in the absence of O_2. Thus, reaction with an α-carbon radical results in the repair of both species (reaction 49 [182]). The initial adduct species also undergo electron transfer reactions with acceptors such as disulphide (reaction 50) or His residues [183]. Main chain cleavage via reaction 51 is believed to be a minor process [184,185], though EPR studies have detected radicals from this reaction [181,186].

$$e_{aq}^- + \sim C(O)NH \sim \rightarrow$$

$$\sim {}^\bullet C(O^-)NH \sim \xrightarrow{H^+} \sim {}^\bullet C(OH)NH \sim \qquad (48)$$

$$\sim {}^\bullet C(OH)NH \sim + \sim NH{}^\bullet C(R)C(O) \sim \rightarrow$$

$$2 \sim NHCH(R)C(O) \sim \qquad (49)$$

$$\sim {}^\bullet C(O^-)NH \sim + RSSR \rightarrow \sim C(O)NH \sim +$$

$$(RSSR)^{\bullet -} \qquad (50)$$

$$e_{aq}^- + \sim CH(R)C(O)NHCH(R') \sim \rightarrow$$

$$\sim CH(R){}^\bullet C(O^-)NHCH(R') \sim \rightarrow$$

$$\sim CH(R)C(O)NH_2 + {}^\bullet CH(R') \sim \qquad (51)$$

2.2.2. Peroxyl radicals

α-Carbon peroxyl radicals undergo a complex series of reactions (Scheme 1) which result in backbone cleavage (reviewed in [6]). These species have been assumed to rapidly eliminate $HOO^\bullet$ to give acyl imines that subsequently react with water to form

the corresponding amides and carbonyl compounds (Scheme 1). However, studies on cyclo(Gly$_2$) and cyclo(Ala$_2$) have shown that these peroxyl radicals undergo only slow loss of $HOO^\bullet$. At high pH, ionisation of the -NH- group (pK_a 10.8 and 11.2 for cyclo(Gly$_2$) and cyclo(Ala$_2$) respectively) results in the rapid (base-catalysed) elimination of $O_2^{-\bullet}$. This process gives a single product (reaction 52), whereas at lower pH values, where slow loss of $HOO^\bullet$ is observed, bimolecular decay predominates and multiple species are formed (reaction 53). The rate of water addition to such imines is structure-dependent, with reaction at the -C=N- bond in cyclo(Gly)$_2$ approx. two orders of magnitude faster than that with cyclo-(Ala)$_2$ [113]. It is unclear what influence the cyclic nature of these substrates has on the kinetics of these reactions, or the stability of imines, as analogous studies have not been carried out with linear peptides. Hydrogen atom abstraction by backbone α-carbon peroxyl radicals yields to α-carbon hydroperoxides [105,114], whereas cross-termination reactions with $O_2^{-\bullet}$ and $HOO^\bullet$ yields alkoxyl radicals via reactions analogous to reactions 14–16 [114]. The hydroperoxides can undergo both thermal and catalysed (metal ion, UV light) decomposition to give further alkoxyl radicals (see below) [104,105].


(52)

(53)
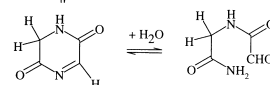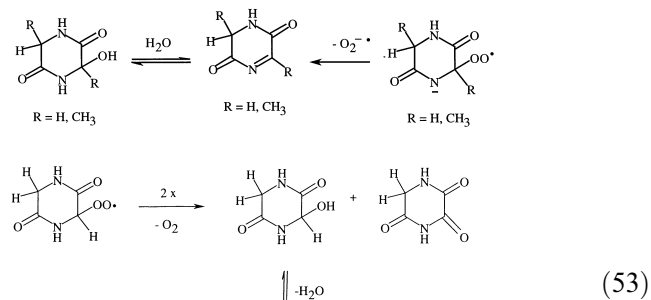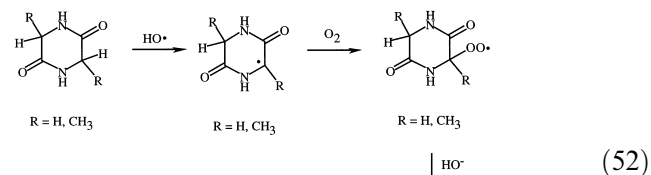
2.2.3. Alkoxyl radicals

Backbone-derived alkoxyl radicals, formed via

cross-termination reactions of peroxyl radicals or decomposition of backbone hydroperoxides, undergo rapid β-scission (fragmentation) reactions. This results in the formation of carbonyl groups and acyl radicals of partial structure $^{\bullet}C(O)NHR$ (see Scheme 1 and reaction 54) [105]. With C-terminal α-carbon alkoxyl radicals, $CO_2^{-\bullet}$ (or $^{\bullet}C(O)NH_2$ in the case of C-terminal amides) is released [105]. Analysis of the products obtained from protein cleavage reactions should indicate the percentage of backbone cleavage occurring via the alternative pathways shown in Scheme 1. Preliminary studies with peptides suggest that the alkoxyl radical pathway can be important (Mortimer et al., unpublished). Fragments with new N-termini, as opposed to products with blocked N-termini that arise via the imine pathway, have been detected with oxidised proteins; these materials may be alkoxyl radical β-scission products [73,187–189]. However, these studies have been carried out with metal ion-dependent systems, where metal ion binding and other reactive species may play a role. Fragments consistent with the alkoxyl radical pathway have also been detected during backbone cleavage of the R1 subunit of ribonucleotide reductase, where a backbone, Gly-derived, α-carbon radical is involved [190].
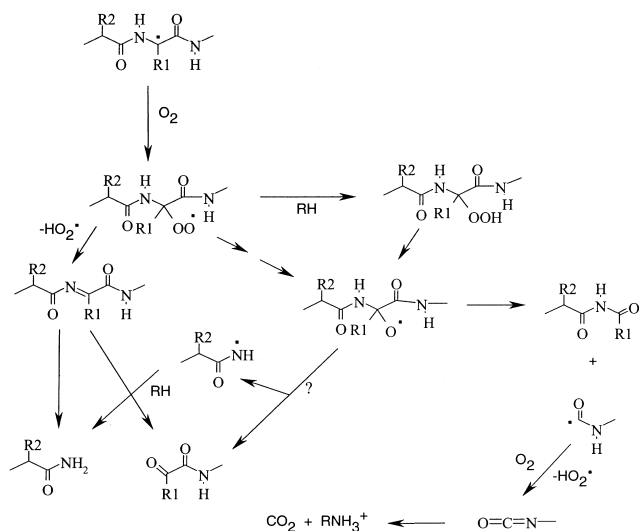
Scheme 1. Potential mechanisms of backbone cleavage in proteins arising from initial hydrogen atom abstraction from an α-carbon site.

$$\sim C(O) - NH - C(O^{\bullet})(R) - C(O) - NHR \sim \rightarrow$$

$$\sim C(O) - NH - C(R) = O + {^{\bullet}}C(O) - NHR \sim$$

(54)
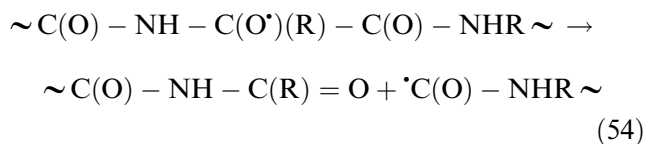
2.2.4. Nitrogen-centred radicals

Reaction of HOCl with proteins can result in backbone cleavage [94,191,192]. This process may involve nitrogen-centred radicals formed on decomposition of intermediate chloramide species [94,192], or non-radical dehydrochlorination and hydrolysis of the imines [131,193]. The inhibition of protein fragmentation by chloramine/chloramide scavengers (e.g. methionine) and radical scavengers (Trolox C) suggests a role for radicals in these reactions [94,192], though it is unclear whether these arise from direct formation of backbone radicals or via damage transfer from reactive side chain sites (e.g. Lys amine groups).

2.3. Transfer between sites

2.3.1. Side chain to side chain

A number of side chain to side chain transfer reactions have been characterised, particularly with aromatic side chain-derived radicals. These studies have yielded important information about the mechanisms and pathways of transfer of oxidising equivalents and electrons through proteins (reviewed in [184,194–196]). The reduction potentials of peptide radicals suggest that the ultimate sink for oxidising equivalents is likely to be Tyr residues (or Trp in the absence of these side chains) [194]. Thus, peptide radicals are able to oxidise Tyr residues via the formation of the ring radical cation, and subsequent deprotonation to give the phenoxyl radical (reaction 38). These reactions are equilibria, so Tyr phenoxyl radicals can be repaired by high concentrations of thiols such as Cys, yielding thiyl radicals [194,197]. This process is enhanced by excess thiol anion, as the thiyl radicals generated are removed via the formation of the disulphide radical anion.

Reaction of Trp with $N_3^{\bullet}$ results in the generation of the neutral indolyl radical. If such species are generated on peptides or proteins that also contain Tyr residues, rapid oxidation of the latter residues to give phenoxyl radicals is observed via electron transfer

[184,195]. This type of transfer process has been investigated in a 62 amino acid peptide (erabutoxin B) that contains single Trp (Trp-25) and Tyr (Tyr-29) residues. Slow transfer is observed in this case, even though these residues are only 1.3 nm apart; this is attributed to the rigid nature of this peptide that contains four disulphide bonds [198]. Rapid Trp→Tyr transfer is, however, observed on reduction (and blocking to prevent re-oxidation) of the disulphide bonds [198]. This study suggests that rapid electron transfer requires either direct contact of the reactive residues, or contact via suitable intermediate species, and that the peptide backbone does not provide a transfer pathway. Studies with peptides containing Trp and Tyr residues held at defined distances apart by rigid spacers (e.g. Pro residues) have shown that the rate of transfer depends on interresidue distance [194,198,199]. However, in many proteins the transfer pathway is unclear due to the presence of multiple Tyr and Trp residues. Transfer reactions have also been demonstrated between other residues; examples are given in Scheme 2 [194]. Such reactions may explain the rapid loss of Trp, Tyr and Phe residues in many proteins, despite these being buried in the hydrophobic core and inaccessible to species present in bulk solution [194].

Disulphide bonds (cystine residues) can act as a major sink for electrons arising from electron transfer by reducing species. Thus initial addition of solvated electrons to both the backbone carbonyl groups of peptide bonds and at some side chain sites (e.g. aromatic residues) can result in the ultimate reduction of cystine groups [179,180,200,201]. Such reactions are sufficiently rapid to compete with reaction with O_2 [184]. The yield of initial electrons that end up at disulphide sites depends on the protein; with lysozyme it is approx. 65% whereas with RNase A it is approx. 20% [202]. The latter observation is of particular interest as the disulphide groups in this protein are internalised, and inaccessible to species in bulk solution [202]. Transfer occurs via hydrogen bonding networks, with the backbone acting as an efficient conduit, unlike the oxidative pathway [196,202]. Information on the rates and pathways of transfer cannot be readily obtained in many of these systems due to the random nature of the initial electron addition. Studies with modified metallopro-
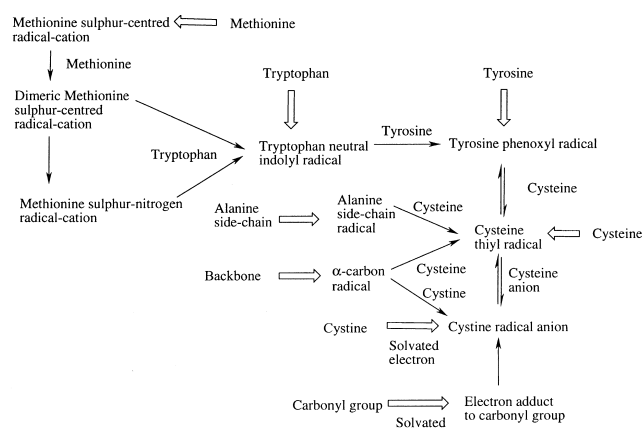
Scheme 2. Transfer of oxidising and reducing equivalents within proteins. Initial oxidation/reduction reactions are indicated by broad arrows. Subsequent transfer reactions are indicated by narrow arrows. Adapted from [194].
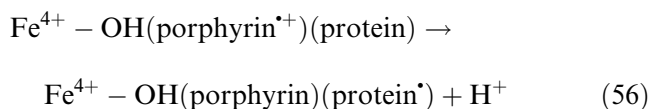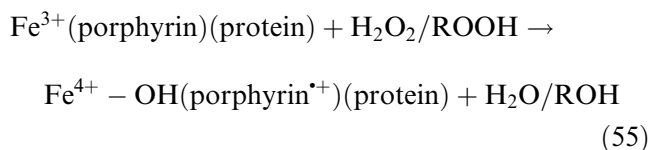
teins have, however, provided information about the mechanisms and control of electron transfer within proteins [203,204]. Thiyl radicals generated from the cystine radical anions via the reverse of reaction 3 can oxidise Tyr residues, thereby providing a point of convergence of the oxidative and reductive pathways.

Less powerful reductants, such as $CO_2^{\cdot-}$, do not undergo rapid electron transfer with main chain carbonyl groups. However, this species can reduce the buried disulphide bonds of RNase A, suggesting that there are electron transfer pathways that do not involve the initial reduction of backbone carbonyl functions. These reactions are slower than those of the solvated electron and are ill defined [202].

Reduction of the iron atom in ferric cytochrome c occurs readily when this protein is exposed to $HO^{\cdot}$ [205]. This has been postulated to occur via initial reaction at the protein surface, and formation of reducing moieties; approx. 50% of the initial $HO^{\cdot}$ has been reported to give metal ion reduction making this a remarkably efficient process. How this occurs is poorly understood, though it may involve short-lived species such as trisulphides (RSSSR) and catechols [206,207].

Damage transfer has also been observed in haem proteins where the initial radical generation occurs at the haem moiety. Reaction of H_2O_2 (and other two electron oxidants) with the Fe^{3+} form of a number of haem proteins (e.g. haemoglobin, myoglobin, leghaemoglobin) gives rise to protein (globin) radicals via

the generation of a Fe^{4+}-oxo (ferryl) porphyrin radical cation (reaction 55; reviewed in [14,208]). The latter undergoes electron transfer with the surrounding protein, resulting in the formation of one or more protein radicals (reaction 56).

$$Fe^{3+}(porphyrin)(protein) + H_2O_2/ROOH \rightarrow$$

$$Fe^{4+} - OH(porphyrin^{\bullet+})(protein) + H_2O/ROH$$
$$(55)$$

$$Fe^{4+} - OH(porphyrin^{\bullet+})(protein) \rightarrow$$

$$Fe^{4+} - OH(porphyrin)(protein^{\bullet}) + H^+ \quad (56)$$

There appear to be multiple sites for these radicals, with Tyr-, Trp-, His-, Cys- and carbon-centred sites having being reported with various forms of these proteins (reviewed in [14]). The Tyr-, Trp-, His- and Cys-derived radicals probably arise via oxidation of the side chain to a radical cation, and subsequent rapid deprotonation to give a neutral radical as discussed earlier. It is not clear whether these species are sequential sites on a single oxidation pathway, or whether these radicals arise via alternative pathways, and which, if any, are in equilibrium. Site-directed mutagenesis studies have shown that replacement of key Tyr residues in myoglobin alters the radical(s) formed, suggesting that Tyr residues are either the site(s) of these species or conduits to their formation [209,210]. However, replacement of *all* the Tyr residues still results in radical formation, suggesting that the removal, or blocking of a key pathway, merely alters the site of oxidation to another residue. Evidence has been presented for Trp-14 being a major site for the Mb-derived radical, even though this residue is more remote from the haem ring than Trp-7. This has been rationalised in terms of the relative orientation of these residues compared to the porphyrin ring [209,211,212]. Significant radical populations have also been shown to be present at Tyr-103 (as a phenoxyl radical) in equine and many other types of Mb [149,155,210], and Cys-110 (as a thiyl radical) in the human protein [213].

These globin-derived radicals can generate intermolecular cross-links, oxidise -SH, Tyr and Trp groups on other proteins, and form haem-protein cross-links (e.g. [149,154,155,213–215]). The C-3 per-

oxyl radical on Trp-14, or the Tyr-103 phenoxyl species can also oxidise species present in bulk solution, including thiols, ascorbic acid, phenols, vitamin E analogues and lipids [216–222]. Modified proteins resulting from these reactions are toxic to cells [223].

2.3.2. Side chain to backbone and vice versa

There is evidence for radical transfer from side chain sites to the α-carbon, but few known examples of transfer from α-carbon sites; this is as expected in the light of the known stability of α-carbon radicals. Thus only transfer to the most readily oxidised side chains (aromatic, Cys and cystine) is observed. The repair of carbon-centred radicals, including α-carbon species, by thiols has been studied extensively and shown to occur in some proteins (reviewed in [101]). Reaction of α-carbon radicals with cystine occurs by homolytic substitution to give cross-linked thioethers [224].

Backbone to side chain transfers can occur readily when the radical is centred on other sites apart from the α-carbon. Thus (nitrogen-centred) amidyl radicals generated by photolysis of *N*-haloamino acid derivatives (e.g. those formed on reaction of HOCl with backbone amide groups) readily abstract hydrogen atoms from side chain sites, with intramolecular 1,5-hydrogen abstraction being particularly rapid (Clark and Easton, unpublished data). Abstraction of side chain hydrogen atoms has also been shown to occur with excited state carbonyl functions on the backbone, particularly when geometrical constraints prevent intramolecular reactions to give α-carbon-centred radicals; these reactions can also occur with excited carbonyl functions on side chains [225]. While 1,5- and 1,6-hydrogen atom transfer reactions are not unusual, the efficiency of intramolecular hydrogen abstraction decreases as the transition state ring size increases. Larger transition states have been invoked to explain some long-range photochemically induced transfer reactions of oligopeptide-linked anthraquinones. These reactions involve 1,19- and 1,21-hydrogen atom transfer, and are highly regioselective for coupling of the α-carbon of a Gly residue to a specific carbonyl group on the anthraquinone [226]. The efficiency of these reactions may be attributable, at least in part, to the rigid structure of the anthraquinone, but it seems likely

that such reactions are also facilitated by the stability of the product α-carbon radicals. Such reactions may occur to only a limited extent with most side chain-derived, carbon-centred, species due to the rapid reaction of such radicals with O_2, though there is a lack of definitive information on this point. This type of process has, however, been detected with peptides in the absence of O_2.

Hydrogen abstraction at the γ-carbon position on side chains can yield dehydropeptides via peroxyl radical formation [6]. The dehydropeptides undergo base hydrolysis to give a new amide function and a keto acid. Thus initial side chain damage can result in backbone cleavage via the intermediacy of a peroxyl species. The significance of these reactions on proteins remains to be established. The three-dimensional structure of a peptide can also affect the chemistry of side chain-derived peroxyl radicals. Thus the products (amides, α-ketoglutaric acid, pyruvic acid) obtained from poly-Glu on reaction with HO• are relatively constant over the pH range 6–8, and similar to those detected for N-Ac-Glu over the pH range 3–8 [4,29]. However, with poly-Glu at pH values < 6, the amide and pyruvic acid yields decrease abruptly, whereas that of α-ketoglutaric acid remains constant. This pH dependence has been explained in terms of a changes in polypeptide conformation, with poly-Glu adopting a random coil conformation at pH values > 6, and a helical structure at lower pH values. The former structure allows various sections of the macromolecule to interact freely (both intra- and intermolecularly), thereby giving product yields similar to those from model compounds, whereas the rigid helical conformation is thought to hinder intramolecular self-dimerisation reaction of side chain peroxyl radicals, thereby favouring other decay pathways [6].

Alkoxyl radicals formed at C-3 (the β-carbon) on peptides and proteins undergo β-scission reactions to give α-carbon species ([227]; Headlam et al., submitted). Alkoxyl radicals formed on Ala side chains readily lose formaldehyde to generate the corresponding Gly α-carbon species (reaction 57). This process predominates over other reactions of such alkoxyl radicals (e.g. 1,2-hydrogen shifts) due to the stability of the α-carbon radical, and appears to occur with a range of C-3 side chain alkoxyl radicals

(Headlam et al., submitted).

$$(57)$$
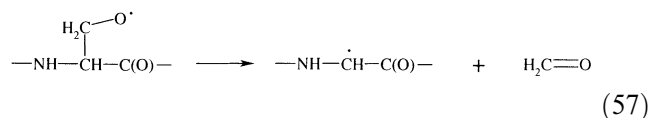
Other side chain-derived alkoxyl radicals may also transfer damage to the α-carbon site. Thus alkoxyl radicals formed at remote sites on large side chains (e.g. Lys, Arg, Leu and Ile) might be expected to abstract a hydrogen atom from α-carbon sites via an intramolecular 1,5-hydrogen atom shift (e.g. reaction 58). Direct evidence for such reactions is lacking, though such processes are known to occur rapidly with model compounds (k approx. 8×10^6 s^{-1}), even when the product radical is poorly stabilised [87]. 1,4-Hydrogen shifts are less favourable, and hence slower, than 1,5 (and 1,6) reactions [87,122].

$$(58)$$
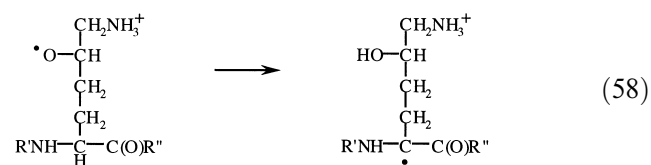
The thiyl radical formed from the Cys residue in GSH can abstract a hydrogen atom from a suitably placed backbone α-carbon site (reaction 58) [228–230]. This process occurs readily, unlike most hydrogen abstraction reactions by thiyl radicals (the reverse reaction – repair of a carbon-centred radical by a thiol, is usually favoured), due to the stability of the α-carbon radical. Thus reaction 59 occurs with GSH at pH values where the γ-glutamyl amine function is deprotonated, even though this involves a nine-membered transition state [229]. Analogous thiyl to α-carbon transfer occurs with the thiyl radical from homocysteine when the amino group is deprotonated [231]. This process does not occur readily with Cys as it would involve a strained transition state [231].

$$(59)$$
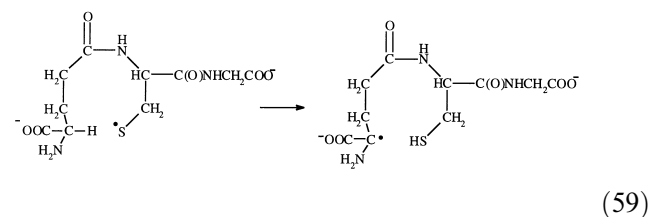
Initial oxidation at the sulphur atom on the Met side chain can also result in damage transfer to neighbouring amino acids in peptides, such as N-terminal Thr or Ser residues [232–234]. This process requires a free N-terminal amine and hence is probably of limited relevance in proteins.

All of these processes provide routes to backbone cleavage via initial side chain damage, and may be of particular importance in proteins where secondary structures (e.g. α-helix or β-pleated sheet) may limit direct access of the attacking radical to α-carbon sites.

2.3.3. Backbone to backbone

The yield of primary amides released from oligo-peptides after radiolysis is higher than would be expected from simple stoichiometric reactions [6]. This may involve backbone alkoxyl radicals, formed from the corresponding peroxyl radicals or hydroperoxides, in intra- or intermolecular hydrogen abstraction reactions. The increased primary amide yield with large peptides compared to *N*-Ac amino acids may be due to a change from an inefficient intermolecular process, to efficient intramolecular reaction involving a favourable six-membered transition state. This is borne out by product analyses; on going from *N*-Ac-Gly to di-Gly the yields change from $G(NH_3) \simeq G(carbonyl) \simeq G(HO^\bullet) \simeq 3$, to $G(NH_3) \simeq 4.8$, $G(HCOOH) \simeq 1.7$ and $G(CHOCOOH)$ 1.9 respectively [175]. Furthermore, with Gly-Ala, both

glyoxylic and pyruvic acids are formed with $G(carbonyl) \simeq 2$ and $G(NH_3)$ 4.8 [175]. The same type of process probably occurs with homo-polypeptides, as poly-Ala gives yields of $G(NH_3) \simeq 4$, $G(RCO-COOH) \simeq 1.2$, $G(RCOOH) \simeq 3.0$ and $G(CO_2) \simeq 2.4$ [112]. This can be readily accounted for by the β-scission reaction of backbone alkoxyl radicals outlined above, and release of further radicals [105].

3. Control

Though the prevention of protein oxidation is of immense industrial and biological importance, there is relatively little mechanistic or kinetic data available on the reaction of protein-derived radicals with antioxidants. This is probably due, at least in part, to the difficulties in generating known yields of specific radicals on proteins in the absence of other competing species. Some data have been obtained on the repair of amino acid radicals at neutral pH by Trolox C (a water-soluble vitamin E analogue) (see Table 2) [235]. The observation that the Trp radical cation reacts more rapidly than the neutral indolyl radical suggests that these processes occur by electron transfer [235], i.e. the reverse of the transfer processes discussed above involving oxidising and reducing species. Repair of Trp-derived radicals on lysozyme by Trolox C has also been examined, and shown to be a fast process (k 2–5×10^7 dm^3 mol^{-1}

Table 2
Rate constants for reaction of amino acid and protein-derived radicals with antioxidants (data from [235,236])

Amino acid or protein-derived radical	Antioxidant	Rate constant (dm^3 mol^{-1} s^{-1})
Trp neutral indolyl radical	Trolox C	5×10^7
Trp neutral indolyl radical in lysozyme		2–5×10^7
Trp indolyl radical cation		2×10^9
Tyr phenoxyl radical		4×10^8
Met-derived radical		7×10^8
His-derived radical		8×10^8
Trp neutral indolyl radical	Vitamin E in SDS micelles	1×10^8
Trp neutral indolyl radical in lysozyme		$< 1 \times 10^7$
Tyr phenoxyl radical in lysozyme in the presence of SDS		2.6×10^4
Trp neutral indolyl radical	Uric acid	1.5×10^7
Trp neutral indolyl radical in lysozyme		1.2×10^7
Tyr phenoxyl radical in lysozyme in the presence of SDS		5.4×10^6
Trp neutral indolyl radical	Ascorbic acid	9×10^7
Trp neutral indolyl radical in lysozyme		8.3×10^7
Tyr phenoxyl radical in lysozyme in the presence of SDS		1.1×10^7

s^{-1} [235]). The observation that repair of such a buried residue occurs with a rate constant similar to that for the free amino acid supports the hypothesis that repair reactions are efficient and can occur over large distances; these reactions may involve the various types of radical transfer processes discussed above. In contrast, vitamin E present in sodium dodecyl sulphate (SDS) micelles, though it can repair free Trp-derived radicals, cannot rapidly repair the same species when present in lysozyme [236]. Trolox C has been shown to repair Tyr phenoxyl radicals in a number of proteins [217–221], and α-tocopherol has been demonstrated to repair Tyr phenoxyl radicals in lysozyme [236]. Other phenolic compounds (e.g. *n*-propyl gallate and sesamol) and vitamin C act in a similar manner [236,237]. Trolox C, vitamins C and E, and thiols have also been shown to react with protein and peptide peroxyl and alkoxyl radicals [154,212,216]. These latter reactions do not, however, result in the repair of the initial radical lesion, as the peptide remains as an oxidised species (either alcohols or hydroperoxides), though these reactions would be expected to inhibit chain reactions within a protein by exporting the radical in to bulk solution.

Cys, *N*-Ac-Cys, and GSH can react with Tyr phenoxyl radicals on proteins, though this reaction is reversible, and 'repair' will only be effective if the thiyl radical is removed (e.g. via reaction with O_2 or thiol anions) before it reacts further [238]. Uric acid can also repair both Trp indolyl radicals and Tyr phenoxyl radicals (in the presence of SDS) in lysozyme [236]. The modest difference between the rate constants for repair of the free Trp indolyl radical and that of the same radical in lysozyme again suggests that these are electron transfer reactions. Bilirubin (and other molecules) which bind strongly to HSA or BSA appear to protect such proteins; however, it is unclear whether this is due to repair of radicals formed on the protein, or whether these materials are merely providing an alternative target for the initial attacking species [239,240].

4. Concluding remarks

There is a growing body of evidence for the involvement, and importance, of protein oxidation in a wide spectrum of physiological pathways and human diseases, as well as industrial and biological processes (reviewed in [2,3,8,10,11,14,15,17]). Though considerable progress has been made since the beginning of this century in our understanding of the fundamental mechanisms of oxidation of amino acids, peptides, and proteins, the overall picture is still far from complete, and there remain considerable areas of uncertainty. These include: knowledge of how the 3-D structure of proteins affects the fundamental chemical reactions that occur during protein oxidation; the role of chain reactions in the propagation of damage and the nature of the chain carrying species; whether and how the products formed on proteins differ from those detected with amino acids and small peptides and the absolute yields of these materials; and how such reactions can be prevented or ameliorated. Recent advances in analytical techniques, the increasing availability of 3-D structures, and our ability to generate specific altered protein structures by site-directed mutagenesis, should allow many of these questions to be answered in the near future.

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