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Review

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The role of protein-derived free radicals as intermediaries of oxidative processes

Abstract: The fact that proteins are the main target of reactive species formed in the cells and extracellular fluids has led to the realization of a great deal of research devoted to revealing the molecular and biological consequences associated with the presence of intermediary protein radicals. This review article describes and comments upon the main chemical pathways involving primary proteic radicals.

Keywords: carbonyls; chain reactions; protein-derived free radicals; reactive species; repair mechanisms.

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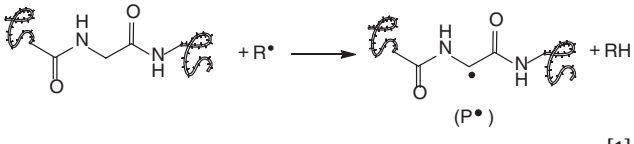
Introduction

Reactive species, such as free radicals and oxidants, have been proposed as an important source of oxidative damage to biological molecules. This deleterious effect has been directly associated with the development of human diseases, such as Alzheimer (1–3), autoimmune diseases (4, 5), cardiovascular diseases (6–8), Crohn's disease (9), kidney diseases (10, 11) and Parkinson's disease (12–14).

Among all biological molecules, proteins are the main target of oxygen- and nitrogen-derived reactive species owing to their abundance in cells and extracellular fluids. In fact, it has been estimated that these macromolecules scavenge between 50% and 75% of the primary reactive species produced *in vivo* (15). Proteins can react with different *in vivo*-generated reactive species such as alkoxyl, hydroxyl and peroxyl radicals, as well as with carbonate radical anion and nitrogen-centered reactive species, among others (16). It has been proposed that, after the reaction of these reactive species with proteins, a carbon-centered free radical ($P^{\bullet}$) can be generated either in the backbone or in the side chain of amino acid residues. $P^{\bullet}$ constitutes one of the first intermediaries formed in proteins when they are exposed to oxidative environments. There are different fates for $P^{\bullet}$ (17). The major pathway, and probably the most important in an aerobic medium, is its reaction with molecular oxygen to give a protein-derived peroxyl radical ($POO^{\bullet}$). This pathway prevails over most other reactions even at low oxygen tensions in metabolically active tissues. This does, however, take into account that the reaction between the carbon-centered radical and O_2 generally occurs at, or near, the diffusion-controlled limit (i.e., k ca. 10^9 l mol^{-1} s^{-1}) (18). A secondary fate for $P^{\bullet}$ is its dimerization, a process probably likely to be uncommon in the presence of oxygen, but its occurrence has been reported in some special cases (19–24). The third fate corresponds to the possibility that $P^{\bullet}$ can be repaired, although at a relatively slow rate compared to the high reactivity towards O_2 (*vide infra*).

The mechanism of $P^{\bullet}$ and $POO^{\bullet}$ formation has been extensively described by different research groups (17, 25, 26), and, when the attack involves the main backbone of the protein, it can be represented by reactions **1** and **2**, respectively:



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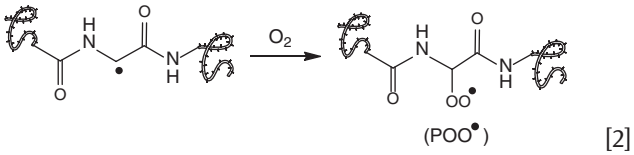


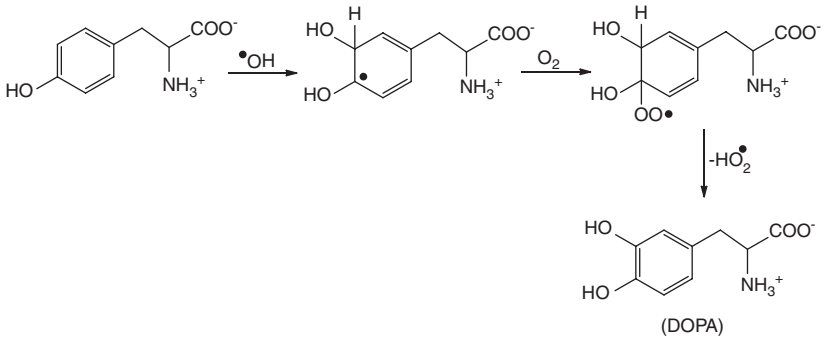
[2]

where $R^{\bullet}$ represents a free radical such as an alkoxyl, hydroxyl, peroxyl or carbonate free radical, among others.

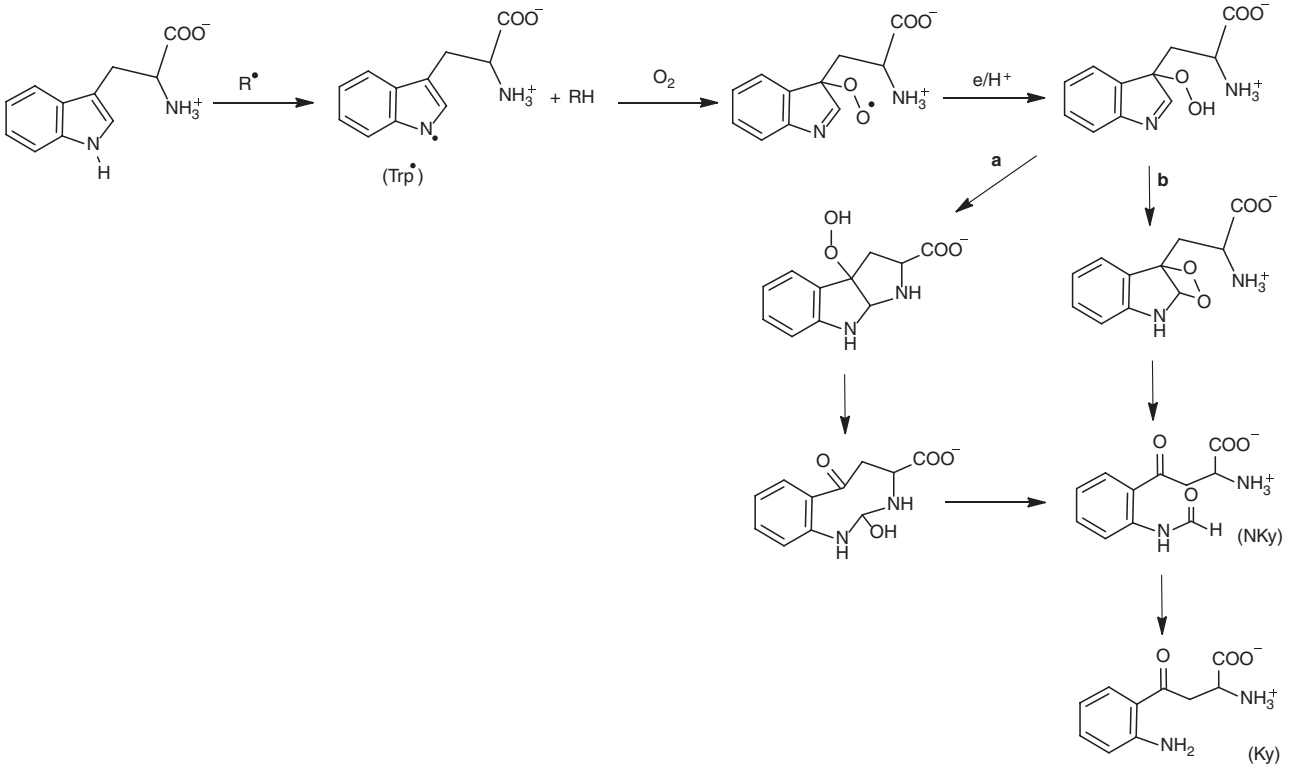
As is inferred from reaction **1**, the chemical environment of the α-carbon strongly modulates the ability of the hydrogen to be subtracted by a particular reactive species.

Since glycine residues contain only hydrogens in this position, the lack of steric or electronic effects explains its large capacity to form P• and POO• (17). Conversely, for most amino acid residues, the presence of side chains makes the possible abstraction of a hydrogen atom from the α-carbon by reactive species difficult. New oxidizable targets are also incorporated. In fact, the oxidation of amino acid side chains and the corresponding formation of P• and peroxyl radicals have been widely studied. Studies on γ radiolysis of valine, leucine and lysine aqueous solutions have demonstrated the formation of peroxyl radicals on their side chains (25). In the case of tyrosine, one of the pathways of its reaction with hydroxyl radical is that it forms a phenoxyl secondary free radical that reacts with oxygen, forming the corresponding peroxyl radical derivative. After the elimination of $HO_2^•$ and ketol intermediate formation, a 3,4-dihydroxyphenylalanine is generated as a final product (Scheme 1) (25, 27).

Tryptophan residues can also be oxidized by mechanisms involving peroxyl radical intermediates. Reactive species can abstract the hydrogen atom at position 1 of tryptophan, generating a N• free radical. After delocalization and interaction with oxygen, it forms a peroxyl radical, leading to *N*-formyl kunurenine (NKy) and



Scheme 1 Mechanism of POO• formation during tyrosine oxidation induced by hydroxyl radicals.



Scheme 2 Mechanism of tryptophan oxidation induced by reactive species.

kynurenine (Ky) as final products (Scheme 2) (25, 28–30). This mechanism has been frequently proposed, in spite of the fact that Candeias et al. (31) reported a low kinetic rate constant (lower than 10^6 M^{-1} s^{-1}) for the reaction between tryptophanyl radical and oxygen.

Another way to generate peroxyl radicals is related to the initial attack toward cysteine residues. They are present in most human proteins, and their intracellular content is comparable to that of the most abundant intracellular low-molecular-mass thiol, glutathione (32, 33). Primary thiyl radicals ($RS^{\bullet}$) are formed in the reaction of thiol compounds with free radicals. Secondary reactions of $RS^{\bullet}$ result in half-lives of the order of microseconds, hindering its analysis in biological systems. However, in recent years and by using electron paramagnetic resonance spin trapping and other experimental protocols, it has been possible to assess its presence (34). $RS^{\bullet}$ can undergo several reactions; some of them are represented below (35, 36):

$$R\text{-}S^{\bullet} + R\text{-}S^{\bullet}\ (Y^{\bullet}) \rightarrow R\text{-}S\text{-}S\text{-}R\ (R\text{-}S\text{-}Y) \quad [3]$$

$$R\text{-}S^{\bullet} + O_2 \rightleftharpoons R\text{-}SOO^{\bullet} \quad [4]$$

$$R\text{-}S^{\bullet} + NO \rightarrow R\text{-}SNO \quad [5]$$

$$R\text{-}S^{\bullet} + GS^{-} \rightarrow [R\text{-}S\text{-}S\text{-}G]^{\bullet -} \quad [6]$$

$$R\text{-}S^{\bullet} + PH \rightleftharpoons RSH + P^{\bullet} \quad [7]$$

Reaction **3** corresponds to a radical-radical reaction, and reaction **4** shows the reaction with molecular oxygen to give a thiyl peroxyl radical whose secondary reactions can lead to sulfinic, sulfenic and sulfonic acids. Reaction **5** represents a *S*-nitrosation process, and reaction **6** corresponds to a *S*-glutathiolation, where GS^{-} denotes the thiolate form of glutathione.

The abstraction of a hydrogen atom is indicated in reaction **7**. The hydrogen abstraction by thiyl radicals from the side group or C-H bonds in model peptides (37) and proteins (38, 39) has been a matter of increasing interest in recent years owing to the biological significance of these kinds of reactions. The intermolecular reactions of thiyl radicals with amino acids within model peptide structures, *N*-acetylamino acid amides and diketopiperazines, proceed with rate constants of the order of 10^3–10^5 M^{-1} s^{-1} (40) and have been reported as a reversible process in some systems (41, 42). The hydrogen abstraction by thiyl radicals from C-H bonds in proteins takes place intramolecularly, with a control of selectivity that depends on the protein secondary structure (38). The α-C-H bonds of amino acids located inside of α-helices and β-sheets have higher homolytic C-H bond dissociation energies compared to the α-C-H bonds of amino acids in extended conformations (38). The set of reactions **3**–**7** supports the fact that $POO^{\bullet}$ can also be formed through secondary processes following the initial interaction of free radicals with SH bonds in the proteins.

In spite of the lower reactivity of peroxyl radicals than that of other reactive species such as hydroxyl, carbonate or alcoxyl radicals (43), once $POO^{\bullet}$ is generated (either in the backbone or in the side chains of amino acid residues) it is able to induce processes such as chain radical reactions, dimerization, triggering scission or hydrogen abstraction. Therefore, $POO^{\bullet}$ can be considered as a reactive species able to increase the initial damage inflicted by other reactive species. Attention is increasingly given to these processes since they lead to irreversible protein modifications and can be implicated in the pathological conditions mentioned above.

Role of peroxyl radicals in chain reactions

A simple mechanism for protein modification mediated by peroxyl radicals produced from the thermolysis of an azocompound [such as AAPH, 2,2′-azo-bis(2-amidinopropane) dihydrochloride] under aerobic conditions is depicted in the following set of reactions **8** to **13**:

$$R\text{-}N{=}N\text{-}R \rightarrow 2R^{\bullet} + N_2 \quad [8]$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad [9]$$

$$PH + ROO^{\bullet} \rightarrow P^{\bullet} + ROOH \quad [10]$$

$$P^{\bullet} + O_2 \rightarrow POO^{\bullet} \quad [11]$$

$$2ROO^{\bullet} \rightarrow \text{Non-radical products} \quad [12]$$

$$2POO^{\bullet} \rightarrow \text{Non-radical products} \quad [13]$$

where $R^{\bullet}$ and $ROO^{\bullet}$ are the azocompound-derived alkyl and peroxyl radicals, respectively.

These reactions lead to a global process whose rate law depends on the solute (protein) concentration. In particular, this oversimplified mechanism predicts that the rate law of protein modification should depend on the protein concentration.

In this context, it has been reported that, in systems involving azocompounds and catalase, superoxide dismutase, or acid phosphatase, at low protein concentration, proteins follow a first-order kinetic and the kinetic order of the initiator (azoderivative) is equal to 0.5. In contrast, at high protein concentrations, proteins follow a zero-order kinetic and the initiator kinetic order (azoderivative)

is 1 (44, 45). Furthermore, in reactions **8–13**, which rely on conditions of quantitative capture of the primary radicals ($ROO^{\bullet}$) by proteins, up to one amino acid can be modified by each radical introduced into the system. In spite of the fact that these speculations are proven in several systems such as the oxidation of horseradish peroxidase, sticholysin II hemolytic toxin and yeast alcohol dehydrogenase (46–48), some apparent contradictions have been found in other systems:

- stoichiometric values (*n*, defined as the moles of proteins oxidized for each peroxyl radical introduced into the system) larger than 1 (44, 45, 49–51)
- kinetic order in proteins at high protein concentrations considerably higher than 0 (50).

These anomalies have been attributed (50–52) to the occurrence of chain reactions driven by peroxyl radicals:

$$PH + POO^{\bullet} \rightarrow P^{\bullet} + POOH. \quad [14]$$

$P^{\bullet}$ reacts with oxygen in agreement with reaction **11**, extending the chain reactions until the occurrence of a termination process according to reaction **13** or to alternate reaction **15**:

$$P^{\bullet} + P^{\bullet} \rightarrow P\text{-}P. \quad [15]$$

Chain reactions have been evidenced in some studies such as that carried out by Lissi and Clavero (50), employing lysozyme as substrate, where nearly three tryptophan groups were modified per radical introduced into the system. Also, Arenas et al. (19) reported that other amino acid residues, such as tyrosine and methionine, were also modified by peroxyl radicals, up to an average of 4.5 amino acids oxidized by each AAPH-derived peroxyl radical produced (19). These chain reactions operate independently of the free radical produced in the initiation step. For example, it has been noted that, following the reaction between bovine serum albumin and hydroxyl radicals in aerobic conditions, nearly 30 amino acids are modified per radical introduced into the system (51). Similar chain reactions take place employing other proteins (lysozyme, n=7) and polypeptides (melittin, n=15) (51).

These radical-mediated chain reactions can involve side chain-to-side chain, backbone-to-backbone, side chain-to-backbone and/or, finally, backbone-to-side chain processes (52), and can be promoted by the vicinity of the free unpaired electron and the target amino acid residue. The process can take place by electron and/or hydrogen atom transfer, and tunnelling effects could contribute to a high reaction rate (53).

Another peculiar aspect of these types of data is the large (average) number of amino acid residues that are modified by each inactivated protein (48, 54). These low inactivation efficiencies have also been observed in protein oxidation processes initiated by radiation or Fenton-like mechanisms (51, 55). For example, lysozyme lost half of its activity only after the modification of 42 amino acids per protein when γ radiation was employed as a radical source. Interestingly, when a similar degree of inactivation was promoted by Fenton's reaction, a smaller number (13.5) of damaged amino acids were observed (55). The most likely explanation for these extensive damages was the occurrence of intra-protein chains (51, 52). The difference between both results can be explained in terms of the differences in the rate of input of radicals. Fenton's reaction provides a faster rate of radical production and, hence, shorter free-radical chains. In fact, if this type of process is taking place, the oxidized ensemble should comprise intact proteins (those that have not been damaged) and proteins with a large number of modifications that (probably) have completely lost their activity as an enzyme, toxin or carrier. In other words, damaged amino acids are not randomly distributed over the whole oxidized ensemble but are concentrated in those proteins that have suffered intra-protein oxidation chains. This oxidized ensemble will comprise a few heavily damaged (inactive) protein molecules and 'intact' (active) proteins. This implies that the chain reaction must be mainly intramolecular and hence nearly independent of the protein concentration. This appears as contradictory to Lissi and Clavero's (50) results obtained in the AAPH-lysozyme system, where a chain process appears as operating only at high protein concentrations. However, if because of the low reaction rate of termination steps involving two $POO^{\bullet}$ radicals, the chains are terminated by reactions involving the primary radicals, such as

$$ROO^{\bullet} + POO^{\bullet} \rightarrow \text{Non-radical products}, \quad [16]$$

the decrease in $ROO^{\bullet}$ steady-state concentration associated with an increase in protein concentration would favor the number and length of the intramolecular chains at high protein concentrations.

Role of peroxyl radicals in protein aggregation and fragmentation

Several reactions confirm the relevant role that $POO^{\bullet}$ plays in the chain of reactions where proteins can

undergo when they are exposed to radicals (52). Among these reactions, $POO^{\bullet}$ can undergo dimerization (reaction **17**) or give rise to hydroperoxides (reactions **18** and **19**).

$$POO^{\bullet} + POO^{\bullet} \rightarrow POO\text{-}OOP \quad [17]$$

$$POO^{\bullet} + P'\text{-}H \rightarrow POOH + P'^{\bullet} \quad [18]$$

$$POO^{\bullet} + e^{-} \rightarrow POO^{-}/+H^{+} \rightarrow POOH \quad [19]$$

In addition, the tetroxide derivative generated in reaction **17** can undergo a decomposition process producing alkoxyl radicals ($PO^{\bullet}$) in agreement with reaction **20:**

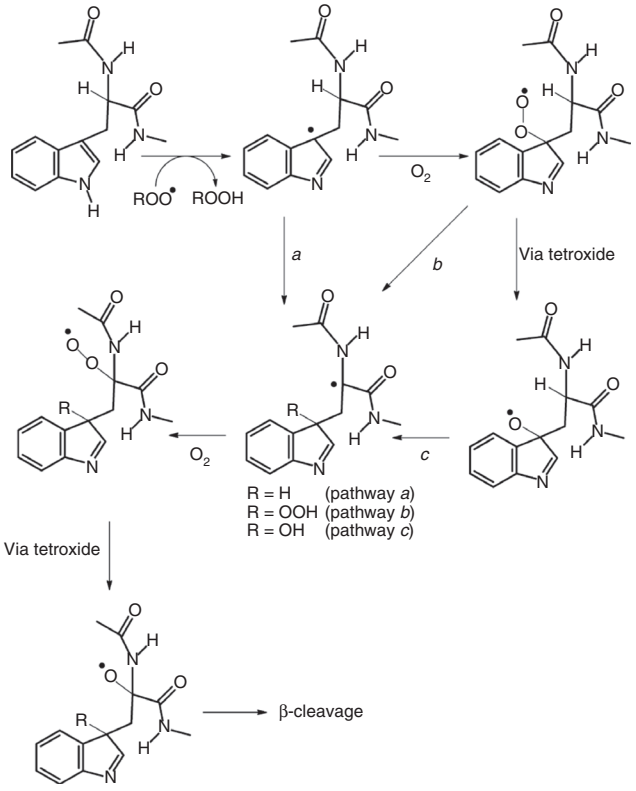
$$POO\text{-}OOP \rightarrow 2PO^{\bullet} + O_2. \quad [20]$$

Both protein hydroperoxides (*vide infra*) and $PO^{\bullet}$ are able to participate in other reactions, extending the initial damage inflicted by reactive species. In particular, $PO^{\bullet}$ is more reactive than $POO^{\bullet}$ (43) and can undergo rapid hydrogen abstraction reactions, as well as facile unimolecular fragmentation and rearrangements (52), leading to the formation of carbonyl groups. In fact, $PO^{\bullet}$ formation in the main polypeptide chain, followed by β scission, has been considered as an important source of carbonyl groups in oxidized proteins (56). However, results have recently shown that carbonyls are present in non-fragmented or aggregated proteins, which contradicts the former possibility (19). Fragmentation and oligomerization are typical processes present in free-radical reactions of proteins triggered by oxygen- and nitrogen-derived reactive species. These processes are mostly irreversible (with the exception of association through the formation of S-S bonds). In spite of the relatively low quantum yields, both processes, and particularly oligomerization, are very important since they change the solubility of the proteins, leading to insoluble aggregates with deleterious effects, such as those present in cataracts (57). Dimerization can follow reactions in the side groups and/or in the polypeptide chains. The last process is particularly important for very reactive free radicals, such as hydroxyl radicals. In contrast, it can be expected that the less reactive radicals, such as $POO^{\bullet}$, selectively attack more reactive targets whose secondary reactions lead to aggregation. A typical example is di-tyrosine whose formation starts with a hydrogen abstraction from the phenol group. This dimerization is typical of free-radical reactions and has been frequently used as a marker of the extent of protein oxidation and also as a urinary biomarker of oxidative stress conditions (17, 58, 59).

In the context of protein fragmentation, glycine residues have been shown to be the most relevant amino acid residues in this process (17). The mechanism is associated with the formation of a carbon-centered free radical, with the consecutive $POO^{\bullet}$ formation, and with $PO^{\bullet}$ through reactions **17** and **20**. Recently, tryptophan-derived peroxyl radicals have also been proposed as an alternative pathway of protein fragmentation initiated by oxidation in the reactive side group of this amino acid (Scheme 3) (19).

It is interesting to note that the side chain-to-backbone damage transfer (as presented in Scheme 3) is similar to that observed when a carbon-centered free radical is formed by the action of hydroxyl radicals on aliphatic side chains (53, 56). Additionally, the same hydrogen abstraction from the tryptophan-indole ring (Scheme 3) has been put forward as the initial step in covalent dimerization of human superoxide dismutase promoted by carbonate radicals (22).

It has been demonstrated that protein fragmentation and aggregation are extremely dependent on the target protein. For example, as is depicted in Table 1, the damage to lysozyme and human serum albumin (HSA) inflicted by AAPH-derived peroxyl radicals in similar experimental conditions led to different yields of such processes (60).



Scheme 3 Proposed mechanism for protein fragmentation through tryptophan-derived peroxyl radicals (19).

Table 1 Quantum yields (Φ) of lysozyme and HSA modifications elicited by AAPH-derived peroxyl radicals.

Target protein	$\Phi_{oligomer}\times10^3$	$\Phi_{fragmentation}\times10^3$	$\Phi_{fragmentation}/\Phi_{carbonyl}$
Lyso	140±40	300±10	2.3±0.4
HSA	21±8	10±4	0.06±0.01

Values represent the rate of protein modification/rate of peroxyl radical production (60).

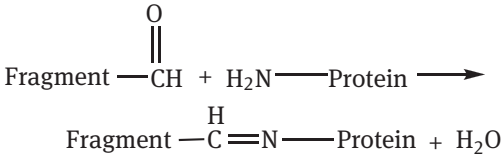
The data presented in Table 1 would suggest that

- carbonyl groups in the protein are a poor predictor of protein damage;
- the yield (Φ) of carbonyl group formation in HSA is considerably smaller than that during fragmentation. This implies that this process is not regulated by β scission of protein-derived alkoxyl radicals, leading to carbonyl formation and main backbone cleavage.

As mentioned previously, the relevance of fragmentation and aggregation processes strongly depends on the protein target. Particularly, reactions between two different proteins to crosslinking by reactions triggered by free radicals arise as a plausible process in biological environments. Oxidation of protein mixtures gives the opportunity to assess such kinds of reactions. Data obtained using a mixture containing lysozyme and HSA, two proteins characterized by different charges at physiological pH (7.4), could lead to the production of non-covalent aggregates (prior to oxidation), as shown in Table 2.

These data would indicate that physical association prior to oxidation favors the covalent binding of the proteins, when the process is mediated either by AAPH-derived peroxyl radicals or by riboflavin photolysis.

An interesting aspect of protein fragmentation and oligomerization promoted by peroxyl radicals is the non-random cleavage and dimerization, indicating that the initial attack and/or the cleavage takes place at selected positions along the main chain and/or in specific lateral groups (19). These reactions could be associated with the selectivity of peroxyl radicals on amino acid residues and also by the selectivity of secondary reactions between protein-protein (P-P), protein-fragments (P-F) and/or fragments-fragments (F-F). Data obtained employing AAPH as the free-radical source are consistent with the dimerization of lysozyme by the P-P type, whereas in the case of glucose-6-phosphate dehydrogenase (G6PD), particularly at low radical doses, it seems to be of the P-F type. In addition, an efficient reaction such as



[21]

would explain the presence of aggregates with molecular weights lower than that of P-P dimer and the lack of carbonyls in the fragments (19).

Table 2 Quantum yields (Φ) of dimer formation in the oxidation of lysozyme (Lyso) and HSA mixtures (60).

ROS source	$\Phi_{Lyso\text{-}Lyso}\times10^3$	$\Phi_{HSA\text{-}HSA}\times10^3$	$\Phi_{Lyso\text{-}HSA}\times10^3$
AAPH pyrolysis[a]	2.0±0.3	<0.2	40±6
Riboflavin[b]	0.9±0.1	0.04±0.005	2.3±0.6

[a]Values represent the rate of protein modification/rate of peroxyl radical production. [b]Photosensitized processes induced by riboflavin irradiation.

Protein hydroperoxides as intermediaries in the development of biological damage initiated by ROS

In 1992, it was reported (61) that protein hydroperoxides (POOH) can consume key cellular reductants. The potential biological significance of such reactions is that it may cause depletion of ascorbate and glutathione, and thus constitute a source of oxidative stress (61). Simultaneously to this oxidizing capacity (61), a second type of reactive species, protein-bound reducing moieties (PBRedM), was detected, which were recognized by their capacity to reduce cytochrome *c*. PBRedM species are even preserved in proteins from which hydroperoxides have been removed by the reduction by ascorbate or glutathione. This long-lived reducing capacity was attributed to a tyrosine hydroxylation pathway that produces catechols, such as 3,4-dihydroxyphenylalanine-like molecules, which are known to be able to reduce transition metals (62, 63).

The hydroperoxides generated when amino acids, peptides and proteins are exposed to radicals in the presence of O_2 are stable in the absence of exogenous catalysts (e.g., redox-active transition metal ions). However, they decompose rapidly in the presence of these agents to give a variety of free radicals including alkoxyl, peroxyl and carbon-centered free-radical species. The formation of these free radicals allows the propagation of oxidative damage to other biomolecules (64) including lipids, proteins (65, 66), antioxidants (67, 68) and DNA (69–73).

Peroxidized proteins react with DNA molecules giving rise to strong intermolecular crosslinks whose extension

depends on the number of POOH present in the oxidized proteins (74). The mechanism proposed for this crosslink reaction would require the initial formation of alkoxyl radicals in a reaction mediated by a DNA-bound metal (M) (74):

$$\text{DNA-M}^{n+} + \text{POOH} \rightarrow \text{DNA-M}^{(n+1)+} + \text{PO}^{\bullet} + \text{HO}^{-} \quad [22]$$

followed by

$$\text{PO}^{\bullet} + \text{DNA} \rightarrow \text{PO-DNA}^{\bullet} \quad [23]$$

to give the covalent crosslink. It is stated that any protein peroxidized by ROS in a cell can potentially form such crosslinks if it comes into close proximity to DNA by diffusion or during functional association. The DNA-protein crosslinks have received increasing attention in the last decade in regard to their induction (72, 73), repair (71) and biological consequences (69, 71).

Repair of protein radicals

There is general agreement that proteins are the major target for reactive species, and increased levels in protein oxidation products have been measured in aging and some diseases (75–80). As mentioned previously, the precursors of these products are P$^{\bullet}$ and POO$^{\bullet}$, two free radicals that could be reduced by biologically relevant derivatives such as glutathione and ascorbate. Studies employing THE pulse radiolysis technique have demonstrated the formation of tryptophan and tyrosine carbon-centered radicals during irradiation with nanosecond pulses (and fast transient spectra analysis) of dilute tryptophan and/or tyrosine-containing peptides and protein solutions (81, 82). In dilute solutions, such irradiation generates several decomposition products according to

$$e^{-} + H_2O \rightarrow HO^{\bullet}, H^{\bullet}, e^{-}_{aq}, H^{+}, H_2O_2. \quad [24]$$

In spite of this, for the absorption of a particular energy, the amount of these products can be seen by the wide range of species able to initiate the oxidation processes, which implies a highly complex system, especially in the presence of such complex targets as proteins. For this reason, experiments are usually carried out in the presence of N_2O (to produce HO$^{\bullet}$) and N_3 (to produce $N_3^{\bullet}$), according to

$$e^{-}_{aq} + N_2O + H_2O \rightarrow HO^{\bullet} + N_2 + OH^{-} \quad [25]$$

$$HO^{\bullet} + N_3^{-} \rightarrow N_3^{\bullet} + OH^{-}. \quad [26]$$

In contrast to HO$^{\bullet}$, azide radical ($N_3^{\bullet}$) is more selective, inflicting oxidative damage only to aromatic amino acids such as tryptophan and tyrosine (82). Tryptophan and tyrosine carbon-centered radicals are characterized by transient spectra with maxima at 405 and 510 nm, respectively. Following the formation and decay of the absorption intensity at these wavelengths, in the absence and presence of antioxidants, the kinetic rate constants have been determined for the repair of P$^{\bullet}$ in peptides and proteins mediated by glutathione (near 10^3–10^5 M^{-1} s^{-1}), ascorbate and urate (near 10^7–10^9 M^{-1} s^{-1} for both compounds) (81–83). Glutathione (43), a well-known low-molecular-weight antioxidant, repairs lysozyme tryptophan radical and *N*-acetyltryptophan amide radicals with kinetic rate constants ($7.8\pm0.4\times10^4$ and $1.2\pm0.2\times10^5$ M^{-1} s^{-1}, respectively) considerably higher than that of its reaction towards *N*-acetyl-tyrosine amide radicals ($3\pm2\times10^3$ M^{-1} s^{-1}) (82). The kinetic rate constants of the reactions between ascorbate and tyrosyl (TyrO$^{\bullet}$) or tryptophanyl (Trp$^{\bullet}$) radicals in the free amino acids, as well as those included in model peptides or proteins, are between two- and three orders of magnitude higher (ca. 10^7–10^8 M^{-1} s^{-1}) than that found in the presence of glutathione (67). Interestingly, both ascorbate and glutathione inhibit the transfer of electrons from tyrosine to Trp$^{\bullet}$ in proteins *in vitro*, but glutathione does not prevent the formation of POO$^{\bullet}$ or hydroperoxides. In this context, although the reaction between O_2 and the radicals located in most amino acids, to form peroxyl radicals, is fast [$k=(3\pm1)\times10^9$ M^{-1} s^{-1}] (84), it is necessary to take into consideration that the O_2 concentration in most mammalian tissues is commonly below 50 μmol/l, whereas that of ascorbate is at least 10 times higher. In this sense, the repair of protein-centered radicals by ascorbate, which are characterized by higher reaction rates than those of glutathione, should be competitive with the reaction with molecular oxygen (81).

The repair of Trp$^{\bullet}$ and TyrO$^{\bullet}$ in proteins by urate has been studied by pulse radiolysis in solutions saturated with N_2O (83). The electrode potential at pH 7 [$E^{\circ\prime}$ (urate radical/urate)≈+0.59 V, relative to the normal hydrogen electrode] (85) is lower than those of amino acid radicals and makes the reaction of urate with these radicals thermodynamically favorable. Domazou et al. (83) observed in chymotrypsin that urate efficiently repairs Trp$^{\bullet}$ with a kinetic rate constant of 2.7×10^8 M^{-1} s^{-1}, ca. 14 times higher than that derived for *N*-acetyltryptophan amide, 1.9×10^7 M^{-1} s^{-1}. In pepsin, urate repairs TyrO$^{\bullet}$ with a rate constant of 3×10^8 M^{-1} s^{-1}, ca. 12 times smaller than that reported for free tyrosine. The efficiency of urate at repairing protein-bound TyrO$^{\bullet}$ or Trp$^{\bullet}$ varies strongly with the protein (83, 86, 87), with no relation to the protein size. In conclusion, the fact that the amino acid radical is

bound to the protein does not constitute a barrier for its reaction with urate (83).

According to the values of the kinetic rate constants for the reactions between carbon-centered free radicals of the proteins and ascorbate (81) and urate (83), it would be expected that urate repairs protein radicals faster than ascorbate (83): (a) in tissues or compartments that contain urate at higher concentrations than ascorbate, such as plasma, heart and saliva (88–92); and (b) under conditions where ascorbate is unstable, e.g., at the acidic pH of the stomach.

In the case of the repair of P$^{\bullet}$ and POO$^{\bullet}$ mediated by phenols, early works developed by Jovanovic and Simic (93) studied the ability of phenols to repair Trp$^{\bullet}$ (as well as other indole analogues) generated by a reaction with $Br_2^{\bullet-}$ during pulse radiolysis experiments in aqueous media at different pH values. Since the equilibrium

$$TrpH^{\bullet+} \rightleftharpoons Trp^{\bullet} + H^{+} \quad [27]$$

is a displacement to the right (pK_a of Trp$^{\bullet+}$=4.3) at physiological pH, the neutral form of Trp$^{\bullet}$ should be the species present in biological environments. Interestingly, no reaction of Trp$^{\bullet}$ with oxygen was evidenced; therefore POO$^{\bullet}$ was not generated in these experimental conditions. Because of its resonance, Trp$^{\bullet}$ slowly reacts with hydrogen donors such as phenols and thiols (93). In contrast, Trp$^{\bullet}$ readily reacts, by electron transfer, with good electron donors as ascorbate (93).

The ability of catechin, quercetin and rutin for repairing neutral Trp$^{\bullet}$ and TyrO$^{\bullet}$ in buffer, micelles and in the presence of human serum albumin has been reported. In agreement with ascorbate, glutathione and urate, the repair of Trp$^{\bullet}$ mediated by these flavonoids is faster than for TyrO$^{\bullet}$ (81–83, 86). In addition, from experiments carried out in SDS, CTAB and Triton X-100 micelles, the role that the environment plays in modulating the rate of the repair reaction was evidenced (86). In the presence of HSA, the ability of the flavonoids to repair Trp$^{\bullet}$ and TyrO$^{\bullet}$ was considerably decreased, a result probably associated with the limited accessibility of HSA-bound flavonoids to free Trp$^{\bullet}$ and TyrO$^{\bullet}$ radicals (86). In similar studies, but generated by pulse radiolysis, free radicals in ApoB present in LDL particles (94), or more recently in apoferritin (95), quercetin was shown to be highly efficient in repairing protein radicals (Trp$^{\bullet}$ and TyrO$^{\bullet}$).

The ability of phenols to repair P$^{\bullet}$ depends on their redox potential; phenols with low reduction potential values could be considered as better 'repairers' of protein radicals. However, the relevance of the repair of P$^{\bullet}$ radicals by natural polyphenols (flavonoids) could be minimized by the low concentrations that they reach in plasma after an intake of a polyphenolic-rich meal (mostly fruit, vegetables and some beverages) (96). In spite of the fact that the biological chemistry of different oxidants is becoming well understood, the actual relevance of the polyphenol-free radical reactions studied in *in vitro* conditions is often unclear (97). Taking into account that, physiologically, it is unlikely that polyphenol and carotenoid concentrations approach those of ascorbate, glutathione and urate, particularly in tissues or compartments, it is also unlikely that polyphenols have a biological role involving direct reaction with reactive species or protein-derived free radicals. Nonetheless, it has been suggested that the strong binding of flavonoids to proteins, in particular HSA, could increase the local concentration of these compounds, possibly favoring a protein free-radical repair process (86). In contrast, it has been postulated that the *in vivo* role of ascorbate, glutathione and urate is closely related to their ability to repair macromolecules. These processes are mostly driven by P$^{\bullet}$, since POO$^{\bullet}$ radicals do not play a relevant role in repair reactions. In fact, the reaction between POO$^{\bullet}$ and antioxidants (including phenols, ascorbate, glutathione and urate) should not be considered as a repair reaction, since the generated products (hydroperoxides) also have the capacity to trigger protein damage. In addition, it should also be considered that repair reactions on P$^{\bullet}$ are not always a means of restoring the biological function of proteins (67).

Concluding remarks and future perspectives

- Proteins are the main target of oxygen-, sulfur- or carbon-centered radicals.
- Secondary reactions of protein-centered radicals can lead to different modifications that include the formation of carbonyl and hydroperoxide derivatives, fragmentation and covalent aggregation.
- These protein modifications are not necessarily located at the site of the initial free radical attack; they can involve the occurrence of chain radical reactions and extend the damage to other biological components.

In tissues with low protein turnover (e.g., the eye lens and also organs during the senescence), the effect of chemical modifications induced by free radicals is accumulative and has significant functional and conformational consequences. As it is presented in this review, protein-derived free radicals, in particular carbon-centered and peroxyl

radicals, play a pivotal role in such processes. These free radicals can be formed by multiple pathways, implying that proteins under oxidative conditions follow a complex behavior. This subject has shown increasing interest from research studies and will stimulate new research works on this field.

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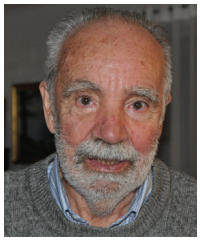

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