RESEARCH COMMUNICATION

Radical-induced chain oxidation of proteins and its inhibition by chain-breaking antioxidants

Jiří NEUŽIL,* Janusz M. GEBICKI† and Roland STOCKER*‡

*Biochemistry Group, Heart Research Institute, 145 Missenden Road, Camperdown Sydney, N.S.W. 2050, Australia, and †School of Biological Sciences, Macquarie University, Sydney, N.S.W. 2109, Australia

Exposure of proteins to oxygen-centred radicals results in dramatic changes in their structure, stability and function; properties that have been studied in many laboratories from a qualitative viewpoint. To allow a quantitative evaluation, we subjected aerated solutions of BSA to hydroxyl and superoxide anion radicals generated radiolytically under conditions where all radicals formed reacted with the protein (as judged by maximum damage to BSA). We observed that for each radical generated approx. 15 amino acids were consumed initially. Similar results were found with lysozyme and melittin. Such a massive consumption of BSA's amino acids was not observed when irradiations were carried out under anaerobic conditions. When bilirubin or Trolox (a water-soluble analogue of vitamin E) was added at a 2-fold molar excess over BSA, initial consumption of all measured amino acids, except tryptophan, decreased 4-fold. The total mass of amino acids initially protected (from consumption) exceeded the mass of antioxidants consumed by more than a factor of 20. Such protection of amino acids was not observed when the antioxidant-inactive acetyl Trolox was used. These results suggest that radical-mediated oxidation of proteins can proceed via a previously unrecognized chain reaction that may be inhibited by chain-breaking antioxidants.

INTRODUCTION

Free radicals have been proposed to cause oxidative damage to biological molecules involved in the development of many severe disorders of humans, including atherosclerosis, cancer, rheumatoid arthritis, post-ischaemic re-oxygenation injury, and parasitic and viral infections [1–4]. As a result, much work has been conducted aimed at understanding modification of lipids by free radicals and its inhibition by antioxidants *in vivo* and *in vitro*. In comparison, radical-induced protein modifications have received less attention, although it is recognized that radical 'damage' to proteins can adversely affect their activity, stability and function [5,6]. Recently, interest in (transient) protein radicals has arisen from their putative roles in the normal catalytic function of various enzymes [7–10].

To prevent radical-induced damage, biological systems contain antioxidant defences that include non-proteinaceous, small molecular compounds. Among them, α-tocopherol, the most active form of vitamin E, is best known as a protective agent for lipids [11] and proteins [12,13]. Bilirubin (BR) has been suggested to function as a physiological antioxidant [14–16] as it efficiently protects human albumin-bound fatty acids from peroxyl-radical-mediated oxidation *in vitro* and, when incorporated into liposomes, can act as a chain-breaking antioxidant which is as efficient as α-tocopherol [14]. An earlier report also indicated a beneficial role for BR in protecting albumin's amino acids from photo-oxidative damage [17]. The known radical-trapping activity of BR together with its abundance in the circulation and extravascular space in the form of an albumin complex, suggest that the pigment might protect the albumin from radical-mediated damage.

Here, we report on the oxidative modification of BSA, lysozyme and melittin induced by γ-irradiation under conditions that allowed quantification of the oxidation processes in the absence and presence of BR and the water-soluble vitamin E analogue, Trolox (TOH).

MATERIALS AND METHODS

BSA (fatty-acid free), melittin (from bee venom) and BR were purchased from Sigma, lysozyme (from hen egg white) from Boehringer, TOH (2-carboxy-2,5,7,8-tetramethyl-6-chromanol) from Aldrich, and Chelex-100 from Bio-Rad. Acetyl Trolox (TOAc) was synthesized from TOH by Dr. C. Suarna (Heart Research Institute, Sydney, Australia) by O-acylation using acetic anhydride. The authenticity and purity of TOAc were confirmed by its u.v. spectrum, n.m.r. spectroscopy and h.p.l.c. analysis. Other chemicals used were of the highest purity available. All buffers and solutions were treated thoroughly with Chelex-100 before irradiation to remove contaminating transition metals. The concentration of transition metals in the Chelex-treated solutions, as well as in the irradiated BSA samples, was below the catalytically significant level as judged by the ascorbate autoxidation method [18].

Irradiations were carried out using a ^{60}Co source (dose rate 44.3 Gy/min) and all samples were gassed throughout irradiations with either oxygen [leading to formation of hydroxyl radical ($^{\bullet}$OH) and superoxide radical ($O_2^{-\bullet}$) at a constant rate of 12.4 μM/min each] or N_2 (resulting in formation of $^{\bullet}$OH only at a rate of 12.4 μM/min). For anaerobic irradiations, samples were pre-gassed thoroughly with N_2. Unless indicated otherwise, 5 mg/ml (75 μM) BSA solutions in 0.1 M phosphate buffer

Abbreviations used: BR, bilirubin; $HO_2^{\bullet}$, hydroperoxyl radical; ODS, octadecyl silica; $^{\bullet}$OH, hydroxyl radical; $O_2^{-\bullet}$, superoxide anion radical; ROS, reactive oxygen species; TOH, Trolox; TOAc, acetyl Trolox.

‡ To whom correspondence should be addressed.

(pH 7.4) were used. Melittin was used at a concentration of 5 mg/ml (0.7 mM) and lysozyme at 20 mg/ml (1 mM) unless indicated differently. Some samples were supplemented with either BR (added to the BSA solution as an aliquot of a freshly prepared stock solution in 50 mM NaOH), TOH or TOAc (both dissolved in water) at a final concentration of about 150 μM. At various time points, aliquots were withdrawn into Eppendorf tubes, placed in solid CO_2 and kept frozen until analysed.

Tryptophan levels were determined by three independent methods. In the first method, the protein in the thawed BSA solutions was precipitated with ethanol (1:4, v/v). After centrifugation, the supernatant was used for antioxidant analysis (see below), while the pellet was washed twice with ethanol, resuspended in 6 M guanidinium chloride, and its tryptophan content determined by fluorescence analysis ($\lambda_{ex.} = 280$ nm, $\lambda_{em.} = 345$ nm, 5 nm bandwidths). The other two methods for tryptophan determination involved acid hydrolysis of the BSA samples followed by either amino acid analysis (see below) or a specific h.p.l.c. assay [19]. In the latter, tryptophan was separated on an octadecyl silica (ODS) column (100 mm × 3.2 mm, 3 μm particle size; Applied Biosystems, Foster City, CA, U.S.A.) eluted with 100 mM chloroacetic acid (pH 2.4) containing 3% acetonitrile at 0.6 ml/min and the eluent monitored in a fluorimeter ($\lambda_{ex.} = 280$ nm, $\lambda_{em.} = 345$ nm).

For hydrolysis, aliquots of the BSA (or lysozyme or melittin) samples were lyophilized in tapered h.p.l.c. autosampler microvials (Alltech, Homebush, N.S.W., Australia). These vials (eight at a time) were then placed into a Millipore PicoTag vial containing 1 ml of 6 M HCl supplemented with 1% (v/v) phenol plus 10 μl of thioglycollic acid, the PicoTag vial was gassed thoroughly with N_2, evacuated and heated to 110 °C for 24 h. The hydrolysate was again lyophilized, dissolved in water before being used for the tryptophan h.p.l.c. assay (see above) or derivatized with *o*-phthalaldehyde reagent [20]. The time interval between the onset of derivatization and subjection to amino acid h.p.l.c. analysis was kept at 2 min for each sample. Amino acid derivatives were separated on an ODS column (150 mm × 4.6 mm, 3 μm particle size; Supelco, Bellefonte, PA, U.S.A.) eluted with a gradient of solvent A (methanol/tetrahydrofuran/20 mM sodium acetate, pH 5.4, adjusted with acetic acid, 20:2.5:77.5, by vol.) and solvent B (same solvents at 80:2.5:17.5, by vol.). The gradient was obtained as follows: 2 min at 100% A, then to 60% B in 23 min, then to 100% B in 5 min. The flow rate was 1 ml/min and the eluent was monitored in a fluorimeter set at $\lambda_{ex.} = 340$ nm and $\lambda_{em.} = 440$ nm. Homoarginine, added to the thawed samples before hydrolysis, was used as an internal standard. All samples were analysed in duplicate, with an overall precision of the assay of ±4% ($n = 3$).

For BR analysis, supernatant resulting from the ethanol precipitation was subjected to h.p.l.c. [21] using an ODS column (75 mm × 4.6 mm, 5 μm particle size; Supelco). For TOH and TOAc, an aliquot of the same supernatant was resolved on an ODS column (250 mm × 4.6 mm, 5 μm particle size; Supelco) eluted with methanol/H_2O/50 mM $NaClO_4$/ethyl acetate (45:35:5:15, by vol.) at 1 ml/min and the eluent monitored at 283 nm.

Oxygen consumption during γ-irradiation was measured with an O_2 electrode (Rank Brothers, Cambridge, U.K.) located near the ^{60}Co source, at a site where the dose rate measured was lower (i.e. 8.15 Gy/min, corresponding to 2.03 μM of each $^{\bullet}OH$ and $O_2^{-\bullet}$ per min) than that in the normal experimental position. Protein concentrations were determined before irradiation using the bicinchoninic acid assay supplied by Sigma. Total nitrogen content was determined by the micro-Kjeldahl method.

RESULTS AND DISCUSSION

Hydroxyl ($^{\bullet}OH$), hydroperoxyl ($HO_2^{\bullet}$) and superoxide anion radicals ($O_2^{-\bullet}$) are physiologically important primary reactive oxygen species (ROS), with reactivities decreasing in the order $^{\bullet}OH \gg HO_2^{\bullet} > O_2^{-\bullet}$. A convenient and chemically clean system yielding $^{\bullet}OH$ and $O_2^{-\bullet}$ is ^{60}Co γ-irradiation of aerated water, where both radicals are formed in approx. equimolar amounts; under anaerobic conditions only $^{\bullet}OH$ is formed. As the yields of these primary radicals are known from the energy (radiation) dose rate and irradiation time, this system makes it possible to obtain a material balance under 'substrate-saturating' conditions, i.e. when all primary radicals react with the substrate rather than with each other [22]. The results in Figure 1(a) show that for a given radical dose the maximum loss of BSA's tryptophan residues was obtained at ≥ 4 mg of BSA/ml. For lysozyme, the maximum loss of tryptophan residues was at about 15 mg/ml and for melittin at > 5 mg/ml. These results suggest that 4 mg of BSA/ml corresponded to the 'substrate-saturating' condition, in agreement with previous work using formation of protein hydroperoxide as another parameter of radical-induced oxidative damage [23]. We used 5 mg of BSA/ml for all subsequent experiments, a much higher concentration than that used in some earlier semi-quantitative studies of radical-mediated protein damage [24,25]. Lysozyme and melittin were used at 20 and 5 mg/ml respectively.

To investigate quantitatively the extent of damage sustained by BSA exposed to ROS, we irradiated BSA solutions for various periods of time in the presence and absence of O_2, and measured the loss of individual amino acids after hydrolysis of the protein. The results in Table 1 show that under aerobic conditions all of the measured constituent amino acids were susceptible to destruction and that the extent of their loss was high for all amino acids and increased with increasing length of irradiation. This agrees with results published previously for BSA [25] and insulin [26]. What is new, however, is the demonstration of effectiveness of the ROS in the destruction of BSA's amino acids. Many studies have shown that, at neutral pH values and in the presence of O_2, the $^{\bullet}OH$ radicals cause most of the chemical changes to proteins, the $O_2^{-\bullet}$ radicals being largely unreactive towards amino acids [25,27]. Calculation of the number of amino acids lost from BSA (Table 1) shows that, in the early phases of irradiation, about 15 amino acids were lost for every radical, corresponding to about 30 amino acids for every $^{\bullet}OH$ reacting with the protein. This indicates that a destructive chain reaction followed the initial interaction between $^{\bullet}OH$ and BSA. The massive loss of amino acids was not due to an artefact in work-up and/or analysis, as in the case of tryptophan the results of the amino acid analysis corresponded closely with those from two other methods using different work-up and analysis procedures (Figure 1b). Furthermore, the total nitrogen content determined in non-irradiated and irradiated stored samples was identical (results not shown), ruling out the possibility of post-irradiation loss of protein. The removal of O_2 from the irradiation mixture protected the amino acids of BSA from detectable loss (Table 1) within the first 40 min of irradiation, in agreement with another report [28]. It follows from our results that the presence of O_2 is required for the chain reaction to proceed. However, measurement of O_2 consumption showed that O_2 is not a chain carrier in the process of amino acid oxidation, as the calculated G value (O_2 consumption) [29] was 1.99, i.e. for every mol of $^{\bullet}OH$ generated 0.7 mol of O_2 was consumed in reactions involving BSA.

To verify further the occurrence of a chain reaction during irradiation of aerated BSA solutions, we included small amounts

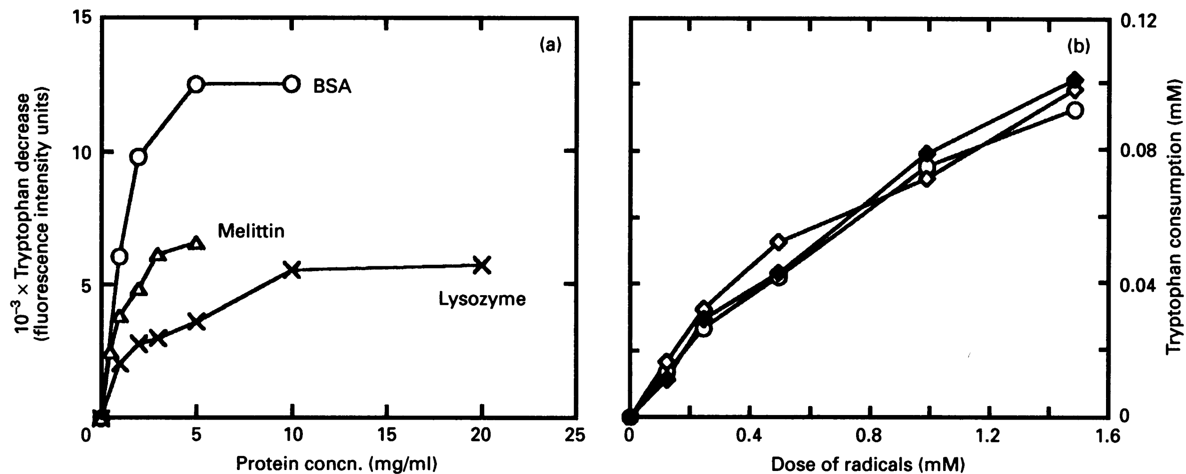


Figure 1 Saturation curves of BSA, lysozyme and melittin, and degradation of BSA's tryptophan residues during radiolysis under O_2-saturated conditions

(**a**) The graph shows the loss of tryptophan moieties during γ-radiolysis as a function of protein concentration. Tryptophan loss is expressed as the difference in tryptophan-specific fluorescence of non-irradiated (control) polypeptide and that exposed to approx. 0.5 mM $^{\bullet}OH + O_2^{-\bullet}$ (BSA, ○; lysozyme, ×; melittin, △). Samples were gassed continuously with O_2 for formation of $^{\bullet}OH + O_2^{-\bullet}$ (12.4 μmol/min each). After 20 min of radiolysis, samples were assayed for tryptophan using the spectrofluorimetric method described in the Materials and methods section. (**b**) Aerated BSA solutions were irradiated for various periods of times and the remaining tryptophan content determined by either tryptophan-specific fluorescence of non-hydrolysed BSA (○), amino acid h.p.l.c. (◇), or tryptophan-specific h.p.l.c. (◆) (see the Materials and methods section).

Table 1 Changes in amino acid contents of BSA on γ-radiation

Irradiations were carried out and amino acids determined as described in the Materials and methods section. The numbers given in parentheses after the individual amino acids represent the published number of residues present in intact BSA. Numbers under individual radiation times denote the number of individual amino acids remaining. The results represent the mean values of three separate experiments with the S.D. values shown in Figure 2(**b**). The abbreviations Asx and Glx stand for aspartic acid plus asparagine, and glutamic acid plus glutamine respectively.

		Number of amino acids remaining after irradiation										
		Anaerobic			Aerobic, −BR				Aerobic, +BR			
Amino acid	Irradiation time...	0 min	20 min	40 min	0 min	20 min	40 min	60 min	0 min	20 min	40 min	60 min
Ala (47)		46.1	45.4	49	45.2	30.7	27.3	25.8	43	40.4	37	35.3
Arg (22)		20.5	22.5	21	20.8	14	12.1	11.2	20.1	18.9	16.5	14.7
Asx (52)		48	54.1	49.6	51.2	37.4	35.1	32.3	52.5	54	49.9	48.8
Glx (79)		75	77.6	78.2	78.5	67.5	56.9	48.7	76.4	72.6	68.3	65
Gly (14)		14.5	15	15.9	16.1	11.6	9.8	8.7	15.7	15.8	13.9	12.1
His (17)		16	15.8	17	15.3	17	13.5	9.2	14.9	14.9	14.1	12.9
Ile (14)		13.2	12.8	14.1	13.8	10	8.7	8	14.5	13.6	13	12.3
Leu (61)		58.4	57.1	57.6	57.9	39.4	35.1	33.6	60.3	54.9	52.1	48.6
Lys (60)		58.1	57.1	57.6	56.8	42.6	39.2	37.8	57.9	54.8	53.1	48.6
Met (4)		3.5	3.5	3.9	3.7	3	3.1	2.8	4.2	4.2	4	3.8
Phe (26)		24.7	25	24	26.2	18.9	16.2	15	25.3	24	21.6	19.7
Ser (28)		27.2	26.9	27.1	29.3	20.2	16	17	30.4	29.2	27	25
Thr (34)		29	31	34	31	23.6	19.4	17.4	29.5	29.2	26.6	25.4
Trp (2)		2	1.9	1.7	1.8	1.3	0.9	0.7	1.9	1.3	1.1	0.7
Tyr (20)		19.3	19.5	20.6	20.6	13.6	12.9	11.5	20.3	19.3	17.2	15.6
Val (36)		33.6	34	34.5	32.8	24.3	21.6	19.7	34.1	32	30.6	29

of the known chain-breaking antioxidants BR and TOH and compared their effect with that of TOAc, which lacks antioxidant activity. Under aerobic conditions (where $^{\bullet}OH$ and $O_2^{-\bullet}$ radicals were generated at a rate of 12.4 μM/min each) TOAc was consumed at the rate of 0.64 μM/min, suggesting that the TOAc added at a 2-fold molar excess over BSA scavenged only about 2.5% of the radicals formed. Such low efficacy of radical scavenging by TOAc is expected considering the lack of selectivity of the extremely reactive $^{\bullet}OH$ [29], the low reactivity of $O_2^{-\bullet}$ with TOAc, and the vast excess (by mass) of amino acid substrate over TOAc used in our experiments (since each molecule of BSA contains 582 amino acid residues). In sharp contrast with TOAc, the initial rates of consumption of the antioxidants TOH and BR, added separately at the same concentration as TOAc, were much higher, i.e. 11 and 9 μM/min respectively (Figure 2a). Antioxidants were not consumed when added to BSA that had been irradiated previously (results not shown), thereby eliminating the possibility that antioxidant consumption resulted from reaction with stable oxidizing moieties known to be present in irradiated BSA [30]. Also, $O_2^{-\bullet}$ produced during aerobic

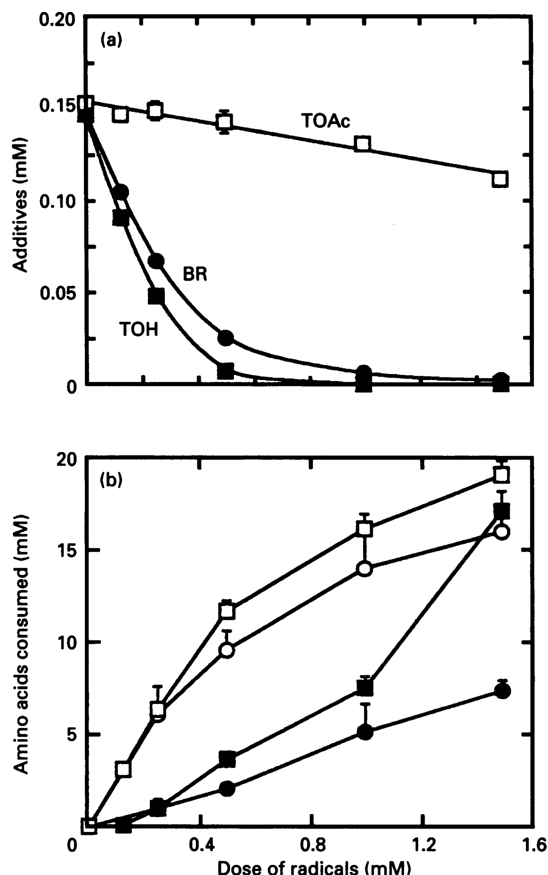


Figure 2 Protective effect of antioxidants on irradiation-induced consumption of BSA's amino acids

Aerobic BSA solutions (5 mg/ml) were supplemented with 150 μM of either BR (●), TOH (■) or TOAc (□). The control (BSA only) solutions were supplemented with the appropriate volume of 50 mM NaOH. Samples were exposed to various doses of $^{\bullet}OH$ and $O_2^{-\bullet}$ before the content of remaining antioxidants (**a**) and amino acids (**b**) were determined by h.p.l.c. as described in the Materials and methods section. The consumption of amino acids of control (non-supplemented) BSA is also shown (○). The extent of consumption of individual amino acids was calculated from their respective peak heights in the h.p.l.c. analyses and by taking into account their relative contribution to the overall amino acid mass of BSA. The radical dose shown refers to $^{\bullet}OH$ plus $O_2^{-\bullet}$, generated at 12.4 μmol/min each. The results shown represent mean values ± S.D. of three independent experiments.

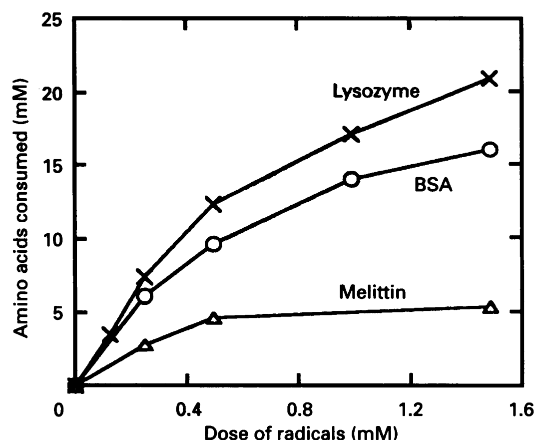


Figure 3 Consumption of amino acids during irradiation of BSA, lysozyme and melittin

The total loss of amino acids of BSA (○), lysozyme (×) and melittin (△) during irradiation under O_2-saturated conditions is expressed as described in the legend to Figure 2 and plotted as a function of radical dose that refers to $^{\bullet}OH$ plus $O_2^{-\bullet}$.

irradiation did not appear to contribute significantly to the observed consumption of TOH and BR, as the latter proceeded at similar rates independent of the rate of $O_2^{-\bullet}$ production, as judged by irradiations carried out in solutions gassed with either O_2 or N_2O/O_2 (9:1, v/v) (results not shown) [29]. In contrast, when irradiations were carried out in the absence of O_2, TOH and TOAc were consumed at similar low rates (results not shown). As the rate constants for the reaction of $^{\bullet}OH$ radicals with TOAc, TOH and BR are expected to be similar, these results indicate that most of the irradiation-induced consumption of the two antioxidants was not due to direct scavenging of $^{\bullet}OH$. These results are consistent, however, with the occurrence of a chain reaction during γ-irradiation of BSA whereby chain-breaking antioxidants primarily scavenge secondary (radical) oxidant(s) formed as transient product(s) after the initial reaction of $^{\bullet}OH$ with the protein.

The presence of chain reactions on BSA is supported further by the inhibitory effects of the chain-breaking antioxidants TOH and BR (but not TOAc) on irradiation-induced loss of amino acids (Table 1, Figure 2b). Both antioxidants, when added to the solution at a 2-fold molar excess over BSA, afforded substantial protection to all detected amino acids, except tryptophan, thereby reducing the chain length (calculated per $^{\bullet}OH + O_2^{-\bullet}$) from about 15 to some 3–4. In both cases, the total amount of amino acids protected from radical-induced loss exceeded the amount of antioxidant present initially and that accounted for only about 0.5% of the total target mass for $^{\bullet}OH$ in the solution. This suggests that the antioxidants acted, at least in part, by scavenging the chain-carrying (propagating) radical(s). In contrast, the presence of TOAc had no significant effect on the amounts of amino acids consumed (Figure 2b).

To find out whether the above chain reaction is a general feature of protein oxidation, we also irradiated melittin (an oligopeptide of $M_r = 2847$) and lysozyme ($M_r = 14300$) under O_2. The results obtained (Figure 3) indeed indicated that seven and more than 15 amino acids were consumed initially during irradiation of melittin and lysozyme respectively for every radical generated.

We are unaware of any previous report demonstrating a chain reaction during radical-mediated oxidation of proteins, although a detailed amino acid analysis of γ-irradiated BSA has been carried out by Davies and co-workers [25]. While unrecognized by these authors, we calculated a chain length of about seven residues (per $^{\bullet}OH$ generated) from the data in their Table 1 for irradiations carried out under O_2-saturated conditions as used in this study [25]. A smaller chain-length value than ours may be the result of the low (5 μM) BSA concentration used in their studies, whereas our experiments were conducted under 'saturating' albumin concentrations (approx. 75 μM). Lissi and Clavero [31] suggested that short reaction chains could explain some of their observations made during the aqueous peroxyl-radical-mediated inactivation of lysozyme.

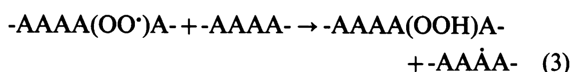
The mechanism of protein chain oxidation and the nature of the 'chain-carrying' radical(s) are not known at present. We propose the following tentative scheme: in most cases the initiating reaction will be that of $^{\bullet}OH$ with the protein amino acids, followed, under aerobic conditions, by addition of O_2 to

the protein alkyl radical to form the protein amino acid peroxyl radical:

$$\text{-AAAAA-} + {}^{\bullet}\text{OH} \rightarrow \text{-AAA}\dot{\text{A}}\text{A-} + \text{H}_2\text{O} \quad (1)$$

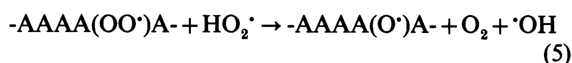
$$\text{-AAA}\dot{\text{A}}\text{A-} + \text{O}_2 \rightarrow \text{-AAAA(OO}^{\bullet}\text{)A-} \quad (2)$$

The protein peroxyl radical can convert into a hydroperoxide and a new protein radical (reaction 3), it may react with another protein peroxyl radical to yield singlet oxygen and reactive protein alkoxyl radicals (reaction 4), or it may combine with a hydroperoxyl radical (formed from the primary $O_2^{-\bullet}$) to form two reactive radicals and O_2 (reaction 5) [29,31]:



$$\text{-AAAA(OO}^{\bullet}\text{)A-} + \text{-AAAA-} \rightarrow \text{-AAAA(OOH)A-} + \text{-AA}\dot{\text{A}}\text{A-} \quad (3)$$

$$2\ \text{-AAAA(OO}^{\bullet}\text{)A-} \rightarrow 2\ \text{-AAAA(O}^{\bullet}\text{)A-} + {}^{1}\text{O}_2 \quad (4)$$



$$\text{-AAAA(OO}^{\bullet}\text{)A-} + \text{HO}_2^{\bullet} \rightarrow \text{-AAAA(O}^{\bullet}\text{)A-} + \text{O}_2 + {}^{\bullet}\text{OH} \quad (5)$$

Our results do not allow us to assign a primary role to any of these or other processes. However, the dependence of the chain reaction on O_2 and the low G (O_2 consumption) value suggest that, and could be largely explained if O_2-producing reactions (e.g. reactions 4 and 5) do take place readily. Independent of the precise mechanism, the important property of some of the secondary radicals formed is that they can act as 'carriers' in the chain reaction.

A number of reports in the literature can be seen as supportive evidence for some of the above-mentioned reactions. For example, protein hydroperoxides have been suggested to be formed in irradiated BSA [30]. Also, a protein tyrosinyl (peroxyl) radical, which in proteins is probably formed as a relatively stable transient radical intermediate [32], has been implied to be involved in the oxidation of organic substrates by myoglobin and H_2O_2 [32]. Interestingly, these authors observed that the protein radical initially formed readily transferred intramolecularly from one amino acid to another [33,34]. Our results with melittin, a tyrosine-free oligopeptide, would suggest, however, that the presence of tyrosine is not a prerequisite for a chain reaction. Thus protein (peroxyl, alkoxyl) radicals may transfer damage along amino acid residue of a polypeptide chain. While the chain-propagating reactions may be slow, the considerable chain lengths observed in our studies indicate that the propagating steps largely prevail over termination reactions. The latter presumably involve radical–radical reactions, e.g. those of a protein or chain-carrying radical with the primary radical(s) to give non-radical products. Considering the BSA concentration (75 μM) and radical flux (12,.4 μM/min for each ${}^{\bullet}OH$ and $O_2^{-\bullet}$) used in our experiments, and assuming that ${}^{\bullet}OH$ reacts with the same probability with either an unoxidized protein molecule, a protein carrying a radical, or a small molecular radical fragment, a termination reaction would occur only every 3 min. Given this length of time, it seems possible that a protein or transient radical could 'move along' the polypeptide chain and react even with the less reactive amino acid residues, thereby causing substantial damage. This proposed scenario is reminiscent of the situation with macromolecular peroxyl radicals in polymers [29] or the α-tocopheroxyl radical within lipoproteins [35], both of which have long lifetimes and consequently undergo chain-transfer reactions.

The chain process becomes interrupted when a chain-breaking antioxidant is present that effectively competes with the protein substrate for chain-carrying (protein) radicals. The water-soluble TOH would be expected to have ready access to these radicals. In the case of BR, the highly flexible nature of albumin, together with the high peroxyl-radical-scavenging activity of the pigment, appear to allow for efficient termination to take place throughout the protein molecule. The inability of the antioxidants to protect tryptophan may in part be due to addition (rather than H abstraction) of the initial ${}^{\bullet}OH$ to this amino acid, a reaction not expected to be intercepted by the antioxidants. Other antioxidants present in either the aqueous (e.g. vitamin C) or hydrophobic environment (e.g. α-tocopherol) may also be able to scavenge chain-carrying radical(s) thereby inhibiting protein-chain oxidation. The fact that we observed a chain in the course of ${}^{\bullet}OH/O_2^{-\bullet}$-mediated oxidation of melittin and lysozyme, too, indicates that this phenomenon is a general feature of proteins and peptides. It therefore seems probable that chain reactions represent a widespread, previously unrecognized feature of free-radical-mediated oxidation of proteins.

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