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Chemical Modification of Lysozyme, Glucose 6-Phosphate Dehydrogenase, and Bovine Eye Lens Proteins Induced by Peroxyl Radicals: Role of Oxidizable Amino Acid Residues

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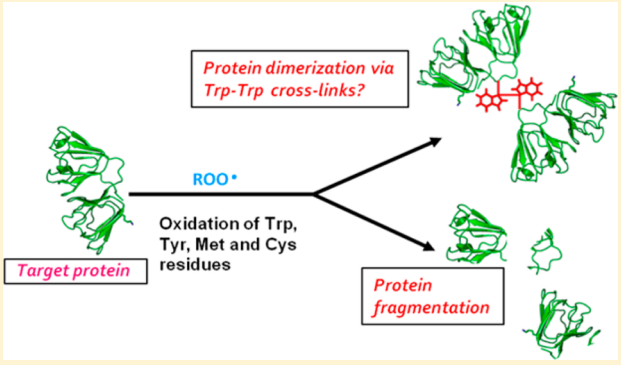
ABSTRACT: Chemical and structural alterations to lysozyme (LYSO), glucose 6-phosphate dehydrogenase (G6PD), and bovine eye lens proteins (BLP) promoted by peroxyl radicals generated by the thermal decomposition of 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) under aerobic conditions were investigated. SDS−PAGE analysis of the AAPH-treated proteins revealed the occurrence of protein aggregation, cross-linking, and fragmentation; BLP, which are naturally organized in globular assemblies, were the most affected proteins. Transmission electron microscopy (TEM) analysis of BLP shows the formation of complex protein aggregates after treatment with AAPH. These structural modifications were accompanied by the formation of protein carbonyl groups and protein hydroperoxides. The yield of carbonyls was lower than that for protein hydroperoxide generation and was unrelated to protein fragmentation. The oxidized proteins were also characterized by significant oxidation of Met, Trp, and Tyr (but not other) residues, and low levels of dityrosine. As the dityrosine yield is too low to account for the observed cross-linking, we propose that aggregation is associated with tryptophan oxidation and Trp-derived cross-links. It is also proposed that Trp oxidation products play a fundamental role in nonrandom fragmentation and carbonyl group formation particularly for LYSO and G6PD. These data point to a complex mechanism of peroxyl-radical mediated modification of proteins with monomeric (LYSO), dimeric (G6PD), and multimeric (BLP) structural organization, which not only results in oxidation of protein side chains but also gives rise to radical-mediated protein cross-links and fragmentation, with Trp species being critical intermediates.



1. INTRODUCTION

Proteins are one of the main targets of free radical-mediated oxidative processes in cells, with reaction of free radicals with proteins frequently leading to a progressive loss of protein function, conformational changes, post-translational modifications, aggregation, cross-linking, and fragmentation.[1−4]

One of the major types of free radicals formed in cells are peroxyl radicals, as these are ubiquitous species formed on reaction of carbon-centered radicals (formed by multiple mechanisms on proteins, lipids, DNA, carbohydrates, and low molecular mass materials) with molecular oxygen. The majority of these reactions occur at or close to the diffusion-controlled limit, making peroxyl radicals major intermediates in cellular oxidative events. One of the methods commonly used to investigate the chemistry of peroxyl species is via the use of (water- or lipid-soluble) thermo-labile azocompounds that generate peroxyl radicals at a known defined rate over long incubation times in the presence of molecular oxygen. The water-soluble compound AAPH (2,2′-azobis-(2-amidinopropane) hydrochloride) has been widely employed to generate aqueous peroxyl radicals. AAPH-derived peroxyl radicals have been shown to react with biological-relevant targets by abstracting a hydrogen atom or an electron, thereby generating

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a secondary free radical on the target molecule. If this substrate is a protein, downstream protein-derived radicals can participate in further oxygen-mediated chain reactions resulting in the formation of protein hydroperoxides and carbonyls, and other chemical alterations to amino acid residues. These modifications can subsequently give rise to conformational changes (e.g., unfolding), aggregation, cross-linking and/or fragmentation processes, with the protein structure and sequence/conformation playing an important role in the eventual outcome. A number of groups have quantified hydroperoxides formed on free amino acids and a wide range of proteins exposed to oxygen-derived radicals generated by γ-irradiation and other mechanisms.[5−7] These studies have also been extended to the oxidation of proteins within cells, with oxygen-centered radicals generated by either irradiation or thermolabile azo compounds.[8,9]

The aim of study reported here was to examine the reaction between proteins present in the ocular globe and defined amounts of peroxyl radicals generated by AAPH, with particular emphasis on the downstream consequences for these proteins in terms of side-chain modification/loss, fragmentation, aggregation, and cross-linking, and the specific role of different amino acid residues in these processes. The selected proteins were lyzosyme (LYSO), glucose-6-phosphate dehydrogenase (G6PD), and bovine lens proteins (BLP). LYSO is a monomeric globular enzyme and comprises a single polypeptide chain of 129 amino acids whose sequence and three-dimensional conformation are well characterized.[10] This protein is found in tears at high concentrations (between 2.4 and 10 mg/mL), and a common source is from chicken egg white. Its function is to protect the conjunctiva that covers the ocular globe from infection.[11] G6PD is a dimeric globular enzyme[12] present in the epithelial cells that cover the ocular lens and plays an important role in protection against oxidative stress due to its capacity to supply the NADPH required by the enzymes glutathione reductase, which is present at high concentrations in the eye's lens, and thioredoxin reductase, as well as multiple other cellular processes. A decrease in G6PD concentration has been reported in cataracts[13−15] and aged ocular lenses.[16] Bovine lens proteins (BLP) are primarily crystalline species that possess a highly ordered multimeric arrangement and are densely packed. This conformation provides and is critical to the transparency required for normal lens function.[17−19] Oxidation of these Crystallin proteins, with the formation of hydroxylated protein side chains (presumed to arise via hydroperoxides) and other products, has been associated with the formation of nuclear cataracts.[20]

2. EXPERIMENTAL PROCEDURES

2.1. Materials. Glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (G6PD); lysozyme from chicken egg white (LYSO); Trolox; xylenol orange (XO); 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH); ferrous ammonium sulfate; trichloroacetic acid (TCA); 2,4-dinitrophenylhydrazine (DNPH); 4 M methanesulfonic acid containing 0.2% w/v tryptamine; *o*-phthaldialdehyde; deoxycholic acid, sodium salt; methionine sulfoxide (MetSO); amino acid standards (500 μM); thioglycolic acid (mercaptoacetic acid); sodium perchlorate monohydrate, 98%; and sodium borohydride were purchased from Sigma. 2-Mercaptoethanol and sodium acetate trihydrate were purchased from Fluka. Anhydrous sodium hydroxide was purchased from MP Biomedicals Inc. Glacial acetic acid was purchased from Lab Scan Analytical Sciences. HCl (32% w/v) was purchased from Chem Supply. Orthophosphoric acid (approx 85% w/v, i.e., 14.6 M) was purchased from BDH. Methanol (HPLC grade) was purchased from Mallinckrodt and tetrahydrofuran and acetone (both HPLC grade) from Merck. The OxyBlot Protein Oxidation Detection Kit was purchased from Chemicon International, Temecula, CA.

2.2. Isolation of Water-Soluble Bovine Lens Proteins. Bovine lenses (approximately 18 months old) were obtained from the slaughter-house. Twenty bovine lenses were decapsulated and stirred in 50 mM Tris-HCl buffer, pH 7.4, containing 0.2 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and 0.05% NaN_3. The suspension was homogenized and centrifuged at 20000*g* for 30 min at 4 °C (Sorvall Superspeed RC2-B). The supernatant was then extensively dialyzed against deionized water at 4 °C. Potassium phosphate buffer was added in order to achieve a protein concentration of 50 mg mL^{-1}, and 0.5 M phosphate buffer, pH 7.4, was also added.

2.3. Protein Oxidation by Peroxyl Radicals. The oxidation of proteins was examined using peroxyl radicals generated by the thermal decomposition of AAPH (10 mM) at 37 °C in air-saturated aqueous solutions.[21] All aqueous solutions were prepared in 100 mM Chelex-treated phosphate buffer, pH 7.4.[22]

2.4. SDS−PAGE Analysis. Protein cross-linking and fragmentation were determined by SDS−PAGE analysis.[23] Samples of preoxidized protein solutions were boiled for 5 min in a 62.5 mM Tris buffer (pH 6.8) solution containing 2% sodium dodecylsulfate (SDS), 10% glycerol, 100 mM β-mercaptoethanol (as reducing agent), and traces of bromophenol blue (as a tracking dye). Acrylamide (3%) stacking gel, 12% and 8% acrylamide resolving gels, and a running buffer comprising 25 mM Tris, 400 mM Gly, and 0.1% SDS, pH 8.3, were used. Electrophoresis was performed at 100 V over 1−2 h. Gels were stained with 0.1% Coomassie Brilliant Blue and destained in methanol/acetic acid over 48 h. Gels were scanned, and the quantification of cross-linked and fragmented materials performed using ImageJ software.

2.5. Quantification of Carbonyl Residues. Carbonyl concentrations on preoxidized proteins were determined according to the method described by Levine et al.[24] Briefly, 330 μL aliquots of oxidized protein solutions (3 mg/mL) were incubated with 1.6 mL of 0.2% DNPH (prepared in 2 N HCl) for 30 min. Proteins were precipitated with ice-cold TCA (20% final concentration) and centrifuged (1970*g*) for 10 min. The resultant pellets were washed three times with a 1/1 (v/v) solution of ethanol/ethyl acetate and then redissolved in 6 M guanidine (10 min, 37 °C). The absorbance was measured at 370 nm, and the carbonyl concentration estimated using an extinction coefficient of 22000 M^{-1} cm^{-1}.

2.6. OxyBlot Detection of Protein Carbonyls Separated by SDS−PAGE. AAPH-incubated samples were treated with 2,4-dinitrophenylhydrazine prior to the resolution of the proteins by SDS−PAGE. The gels were transferred onto a nitrocellulose membrane and developed with polyclonal antibodies to the 2,4-dinitrophenylhydrazone moiety as described in the manufacturer's instructions (Chemicon International, Temecula, CA).

2.7. Quantification of Protein Hydroperoxide Formation. Total protein hydroperoxides were quantified by the FOX assay.[25,26] Briefly, 420 μL of protein solutions were incubated with 2 mL of the FOX reagent (0.25 mM ferrous ammonium sulfate and 125 μM xylenol orange (XO) in 25 mM sulfuric acid) for 30 min, after which the absorbance was determined at 560 nm. The concentration of protein hydroperoxides was determined by preincubating the solutions with 20 μL of catalase (CAT, 715 U/mL) for 15 min to remove H_2O_2, before analysis. Protein hydroperoxide concentrations were determined using a calibration curve generated using commercial H_2O_2, and the data are therefore expressed as H_2O_2 equivalents.

2.8. Quantification of Free Thiols. Free protein thiols were quantified using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) via the measurement of released 5-thio-2-nitrobenzoate anion at 412 nm.[27,28] Briefly, 40 μM protein was mixed with 96 μM DTNB. After 1 h of incubation at 21 °C, the absorbance was determined at 412 nm. The concentration of thiols was estimated from the absorbance using a calibration curve constructed using reduced glutathione.

2.9. Transmission Electronic Microscopy (TEM). BLP samples (10 μL protein solution at a concentration of 0.3 mg/mL) were

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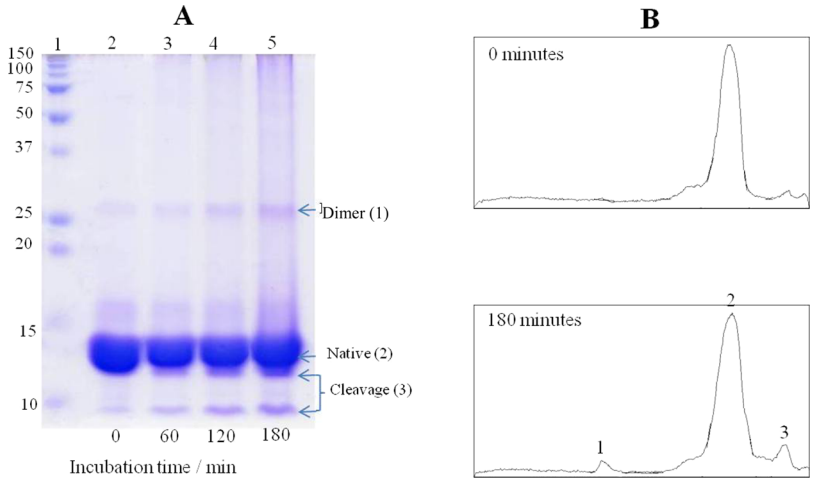

Figure 1. (A) SDS−PAGE analysis of LYSO (208 μM) incubated with peroxyl radicals generated from 10 mM AAPH in 100 mM phosphate buffer, pH 7.4, at 37 °C for 60, 120, or 180 min. (B) Representative densitometric quantification of the SDS−PAGE gel shown in A; peak 1 corresponds to the dimer, peak 2 corresponds to native protein, and the area labeled peak 3 corresponds to (one or more) fragments.

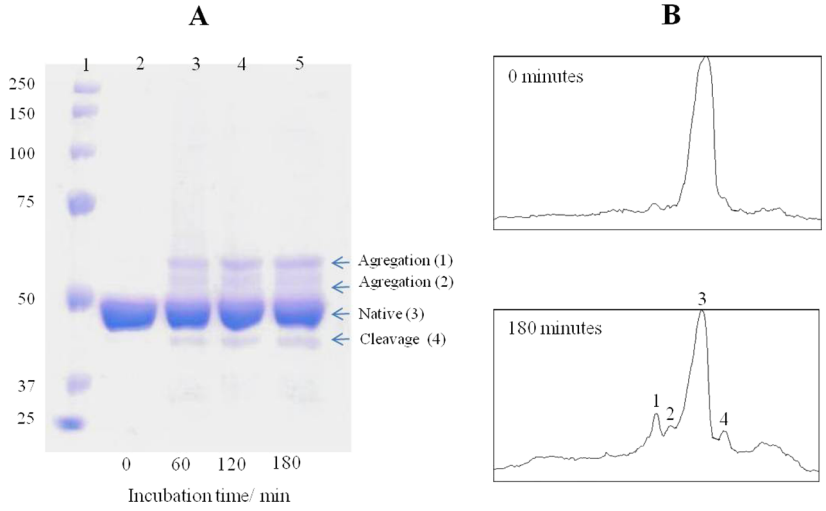

Figure 2. (A) SDS−PAGE analysis of G6PD (11.6 μM) incubated with peroxyl radicals generated from 10 mM AAPH in 100 mM phosphate buffer solution, pH 7.4, at 37 °C for 60, 120, or 180 min. (B) Representative densitometric quantification of the SDS−PAGE gel shown in A; peak 1 corresponds to aggregate-1, peak 2 corresponds to aggregate-2, peak 3 corresponds to the native protein, and peak 4 corresponds to one or more fragments.

adsorbed for 2 min onto copper grids. Excess protein solution was blotted off, and the protein sample was washed twice with nanopure water. The samples were negatively stained for 2 min using 10 μL of 1% (v/v) uranyl acetate. Electron micrographs were recorded at a nominal magnification of 50000.

2.10. Amino Acid Analysis. Amino acid analysis was performed on protein samples digested with methanesulfonic acid (MSA) by HPLC with precolumn *o*-phthaldialdehyde (OPA) derivatization.[29,30] Control and oxidized protein samples (<0.5 mg) were precipitated by the addition of 50% w/v TCA and incubation on ice for 5 min, followed by centrifugation at 5 °C for 2 min at 13000*g*. The pellets obtained were then washed twice with ice-cold acetone and recentrifuged for 2 min at 13000*g*. The pellets were then dried and resuspended in 150 μL of MSA and introduced into a PicoTag hydrolysis vessel. The vessels were then evacuated using a vacuum pump and regassed with N_2 at least three times, after which the samples were incubated at 110 °C overnight. The samples were then neutralized using 150 μL of freshly prepared 4 M NaOH, filtered, and diluted with nanopure water. Then, 40 μL of diluted samples was transferred into HPLC vials placed and kept at 5 °C prior to derivatization with 20 μL of activated OPA and injection. Incomplete OPA reagent was activated immediately before use by the addition of 5 μL of 2-mercaptoethanol to 1 mL of OPA reagent. Standard curves of parent amino acids and oxidation products were prepared by the addition of 10 μL of amino acid standards and 5 μL of MetSO (1 mM stock) to 980 μL of nanopure water with subsequent dilution to give, 1, 2, 3, and 5 μM standards.

Samples were eluted from the HPLC column using a gradient elution system as described previously[30] with Buffer A consisting of 400 mL of methanol, 50 mL of tetrahydrofuran, 1450 mL of nanopure water, and 100 mL of 1 M sodium acetate at pH 5.0, and Buffer B consisting of 1600 mL of methanol, 50 mL of tetrahydrofuran, 250 mL of nanopure water, and 100 mL of 1 M sodium acetate at pH 5.0. The HPLC equipment (Shimadzu SCL-10Avp)) was fitted with a Beckman Coulter Ultrasphere ODS 4.6 mm × 25 cm, 5 μm pore size column (No. 235329) and a Beckman Coulter Ultrasphere ODS 4.6 mm × 4.5 cm (No. 243533) guard column. The flow rate was 1 mL min^{-1}, with the column oven (Shimadzu CTO-10Avp) set at 30 °C. The fluorescence detector (Shimadzu RF-10AXL) was set with λ_{EX} 340 nm and λ_{EM} 440 nm.

2.11. Analysis of Tyr-Derived Oxidation Products by HPLC..[20,30] Protein samples (0.5 mg) were treated with 10 μL of freshly prepared 1 mg/mL sodium borohydride for 5 min at 21 °C, precipitated, dried, and transferred to PicoTag hydrolysis vessels as

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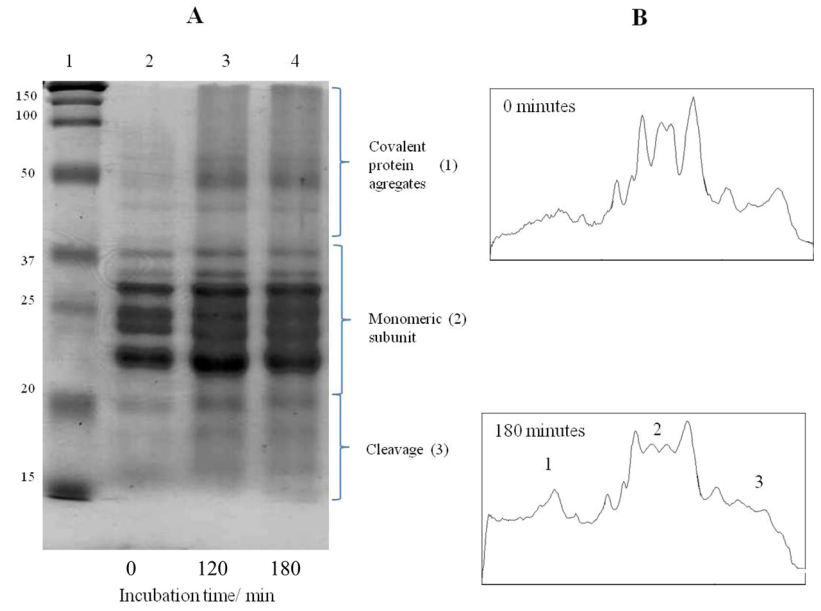
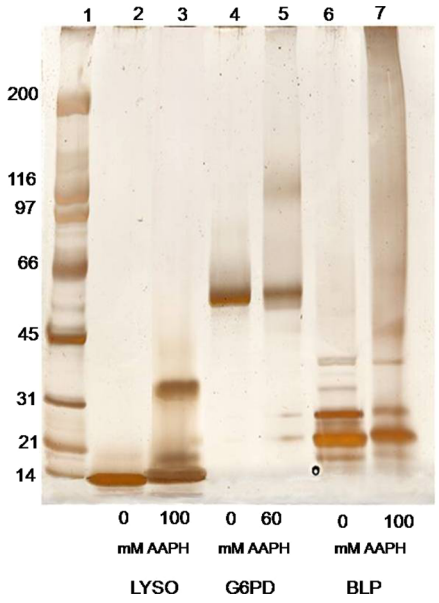


Figure 3. (A) SDS−PAGE analysis of solutions of BLP (120 μM) incubated with peroxyl radicals generated from 10 mM AAPH in 100 mM phosphate buffer, pH 7.4, at 37 °C for 120 or 180 min. (B) Representative densitometric quantification of the SDS−PAGE gel shown in A; peak 1 is assigned to covalent protein aggregates, peak 2 is due to the native protein, and peak 3 corresponds to one or more protein fragments.



	Fraction of native protein involved in crosslinking processes
LYSO	0.33
G6PD	0.31
BLP	0.77

Figure 4. SDS−PAGE analysis of a solution of BLP, LYSO, and G6PD (120, 208, and 11.6 μM, respectively) incubated with peroxyl radicals generated AAPH in 100 mM phosphate buffer solution, pH 7.4, at 37 °C for 180 min (Lanes 3, 5 and 7). The values indicated at the right of the Figure are the extents of cross-linking (in μmol) per μmol parent protein for each species.

described above. One milliliter of 6 M HCl and 50 μL of thioglycolic acid were then added to the bottom of the PicoTag vessel and the vessels evacuated and incubated overnight (17 h) as described above. The samples were then dried using a centrifugal vacuum concentrator (Christ RVC 2-33, Johns Morris, Australia, fitted with a Savant RT 490 refrigerated condensation trap, Savant vacuum gauge and LH LeyboldTrivac D8A pump). The pellets were resuspended in 200 μL of nanopure water and centrifuged at 16000*g* for 2 min at 5 °C prior to placement (20 μL of sample) in the autoinjector at 5 °C. Samples were eluted from the HPLC column as described previously,[30] using a gradient mobile phase with Buffer A consisting of 10 mM sodium perchlorate/10 mM H_3PO_4 and Buffer B 80% v/v methanol/water. The fluorescence detector was set at λ_{EX} 280 nm and λ_{EM} 410 nm for di-Tyr analysis.

3. RESULTS

3.1. Cross-Linking and Fragmentation Measurements by SDS−PAGE. The data shown in Figures 1−3, obtained for LYSO, G6PD, and BLP, respectively, provide evidence for the simultaneous occurrence of protein fragmentation and irreversible cross-linking on exposure to defined amounts of aqueous peroxyl radicals. For LYSO incubated with AAPH for 60, 120, and 180 min, the oxidized samples show clear evidence of fragmentation and cross-linking (Figure 1A). Densitometric analysis of these chromatograms (Figure 1B) indicates that in addition to the peak from the native protein (2), two weaker bands ascribed to dimers (1) and fragments (3) are present. The presence of a clear band at ∼10 kDa is consistent with

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nonrandom cleavage of LYSO by peroxyl radicals, resulting in the formation of discrete species of well-defined mass. The densitometric quantification of the fragments arising from oxidation of this protein may be underestimated by the production of low molecular mass products (<5000 Da) that would not be retained on the gel and also, depending on their composition and extent of oxidation, by differential staining of these species.[6]

Figure 2A shows the SDS−PAGE of native G6PD and G6PD after exposure to AAPH-derived radicals. Both the gel image and the derived densitometric chromatogram indicate the presence of fragments and aggregates produced by peroxyl radical-mediated protein oxidation. Interestingly, the apparent molecular masses of the aggregates are smaller than those expected of a dimer, and the fragments are concentrated over a small range of masses. This suggests that aggregation involves one native protein and a fragment obtained by selective (nonrandom) cleavage of native G6PD. Dimers were only observed with much larger doses of peroxyl radicals (data not shown).

Figure 3 shows data obtained for BLP proteins. It can be seen, as reported previously,[31,32] that these proteins comprise a series of polypeptides with molecular masses between 18 and 32 kD. A series of bands of higher molecular mass were generated on peroxyl radical-mediated oxidation of these proteins. As a consequence, densitometric analysis of the gel images was more complex. The gels obtained from BLP treated with AAPH show the presence of multiple different aggregated forms, as well as protein fragments (Figure 3A). The (apparently random) cross-linking is, in the case of BLP, particularly evident at high AAPH-derived radical doses (Figure 4), a behavior not observed with LYSO and G6PD. These data are summarized in Tables 1−3. Comparison of the results

Table 1. Decrease of Native Protein Bands (Expressed as Percentage) after 120 and 180 min of Incubation with 10 mM AAPH[a]

incubation time	120 min	180 min
LYSO	10.3	9.9
G6PD	12.6	14.2
BLP	15.1	23.2

[a]Percentages were evaluated relative to the native band at zero time as 100%.

Table 2. Percentage[a] of Cleavage and Cross-Linking Bands after 120 and 180 min of Protein Incubation with AAPH (10 mM)

		LYSO	G6PD	BPL
120 min	cleavage	5.9 ± 2.0	3.9 ± 0.7	6.7 ± 3.0
	cross-linking	4.4 ± 1.4	8.7 ± 0.5	8.4 ± 2.8
180 min	cleavage	6.2 ± 1.5	4.5 ± 0.8	8.9 ± 4.0
	cross-linking	3.7 ± 0.6	9.6 ± 0.6	14.2 ± 7.3

[a]The percentages of fragmentation and protein cross-linking were obtained from the values shown in Table 1, using the intensity values of the bands corresponding to these fractions (Figures 1−3). These values were employed without any correction, assuming that all the bands in the lanes do not stain differentially depending on their composition and oxidation degree, which is a matter of controversy.[64]

obtained with the three proteins show (i) that the fragmentation (cleavage) to cross-link ratios range from 1.67 (LYSO at 180 min) to 0.51 (G6PD at 120 min); (ii) that the fragmentation to cross-linking ratio increases with incubation time for LYSO and decreases for G6PD and BLP; (iii) that LYSO is the most stable of the tested proteins and BLP the most susceptible to damage; and (iv) that at low radical doses, selective (nonrandom) cleavage of BLP occurs, while at higher doses almost random cleavage is observed.

Table 3. Fragmentation/Cross-Linking Band Ratio after 120 and 180 min of Protein Incubation with AAPH (10 mM)

incubation time	LYSO	G6PD	BPL
120 min	1.4	0.5	0.8
180 min	1.7	0.5	0.6

3.2. TEM Studies. To gain further insight into the protein modifications induced by AAPH-generated peroxyl radicals, TEM experiments were carried out to examine changes in morphology and protein size of BLP. In the case of LYSO and G6PD, representative images could not be obtained on the effect of peroxyl radicals on these proteins. TEM analysis of native BLP showed the presence of small particles of nearly spherical shape (Figure 5A), whereas AAPH-treatment resulted in the generation of irregular particles with high degrees of aggregation (Figure 5B).

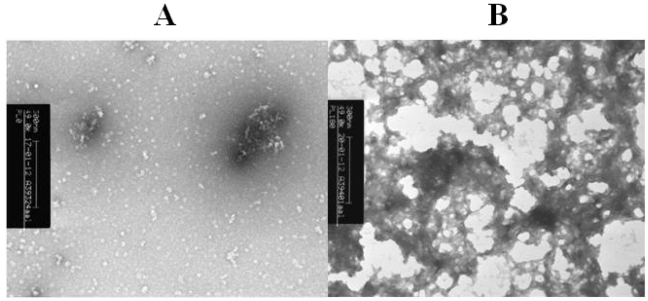

Figure 5. Image analysis of BLP (120 μM) negatively stained with 1% uranyl acetate. (A) native BLP. (B) BLP incubated with 100 mM AAPH for 180 min in 100 mM phosphate buffer, pH 7.4, at 37 °C.

3.3. Oxidation of Specific Amino Acids. Evaluation of the loss of parent amino acids from controls and oxidized proteins were carried out employing higher AAPH concentrations to minimize errors associated with the determination of small decreases on large background levels. Under these conditions, the most modified amino acids were Cys, Met, Trp, Tyr, and Lys (with the latter only decreased significantly in the case of G6PD). The extent of loss of individual types of each amino acid on all three proteins and their percentage decreases are summarized in Table 4. These data indicate that (a) the number of modified groups per native protein (LYSO 4.4; G6PD 18.3; BLP 4.4, Table 4) is higher than the fraction of native protein involved in the cross-linking process (LYSO 0.33; G6PD 0.31; BLP 0.77, Figure 4); (b) that Tyr residues are not the only source of cross-linking; and (c) that the relative consumption of amino acids depends on the protein. Thus, in LYSO the extent of modification decreases in the order Trp > Met ≫ Tyr; for G6PD, the order is Met > Trp > Lys ≫ Tyr; and for BLP, the order is Cys ≈ Met > Trp ≫ Tyr. In the case of LYSO, this trend is in agreement with the relative reactivity of the constituent amino acids with peroxyl radicals.[33] Furthermore, the lack of free Cys residues in LYSO and

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Table 4. Extent of Amino Acid Modification Determined by HPLC with Fluorescence Detection in Proteins Treated with 100 mM AAPH for 180 min at 37 °C in 100 mM Phosphate Buffer at pH 7.4[a]

	Cys	Met[b]	Trp	Tyr	Lys	TOTAL
LYSO		0.5 (50%)	3.0 (50%)	0.3 (10%)	0.6 (10%)	4.4
G6PD		7.4 (90%)	2.5 (36%)	0.8 (4%)	7.6 (23%)	18.3
BLP	0.9 (90%)	0.9 (90%)	2.4 (63%)	0.2 (6%)	no modification	4.4

[a]Results are expressed as the number of amino acids consumed per native protein and as percentage of their initial amount (values in parentheses). [b]Methionine is already partially oxidized to methionine sulfoxide (50% in LYSO, 10% in G6PD, and 73% in BLP).

Table 5. Quantification of Carbonyls and Protein Hydroperoxides Present in Protein Samples after Incubation of the Indicated Protein with AAPH in 100 mM Phosphate Buffer, pH 7.4, for 180 min at 37 °C

		LYSO		G6PD		BLP	
		10 mM AAPH	100 mM AAPH	10 mM AAPH	60 mM AAPH	10 mM AAPH	100 mM AAPH
carbonyls	carbonyls per generated radical	0.08 ± 0.01	0.03 ± 0.001	0.002 ± 0.02	0.006 ± 0.003	0.016 ± 0.01	0.013 ± 0.001
	carbonyls/protein	0.06 ± 0.01	0.23 ± 0.01			0.020 ± 0.01	0.15 ± 0.01
protein hydroperoxides	hydroperoxides per generated radical	0.24 ± 0.01	0.08 ± 0.01	0.09 ± 0.07	0.06 ± 0.01	0.008 ± 0.001	0.05 ± 0.01
	hydroperoxides/protein	0.17 ± 0.01	0.58 ± 0.04			0.01 ± 0.001	0.56 ± 0.08

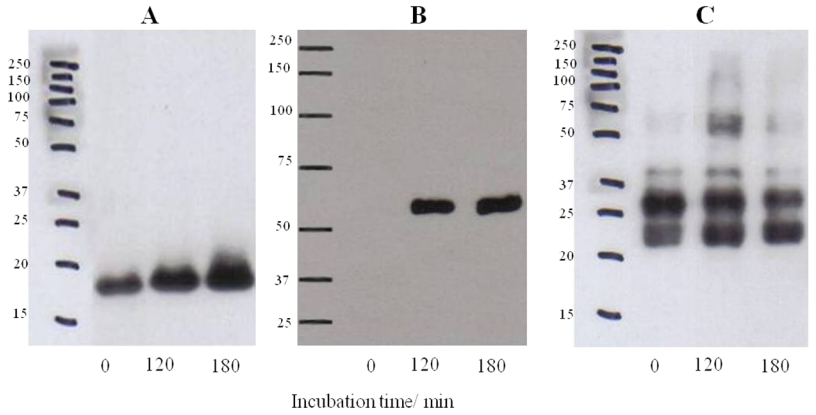

Figure 6. Detection of protein carbonyls by Western blotting (using the Oxyblot system) on (A) LYSO, (B) G6PD, and (C) BLP that had been incubated with 10 mM AAPH for 120 or 180 min, in 100 mM phosphate buffer, pH 7.4, at 37 °C.

G6PD and the low number of Cys residues in BLP points to a modest (at best) role for this amino acid in cross-linking.

Considering the relatively low rate of peroxyl radical formation (8 μM min^{-1})[21] and that the initial protein concentrations were 208 and 120 μM, for LYSO and BLP respectively, which corresponds to concentrations of AAPH-sensitive amino acid residues of 3.3 and 1.1 mM for each protein (Table 4), it appears that over the reaction times examined an excess of reactive target would be present. The latter would imply a zero order kinetic limit in protein and first order kinetics for AAPH. Taking into account these protein concentrations and the data presented in Table 4, it is possible to estimate the total concentration of amino acids consumed by peroxyl radicals. After 3 h of incubation with 100 mM AAPH, a total concentration of amino acids consumed of 0.9 and 0.5 mM can be estimated for LYSO and BLP, respectively. This implies that after 3 h, when 1.4 mM of peroxyl radicals were generated, the total concentration of amino acids consumed is of approximately the same magnitude as the concentration of the peroxyl radicals initially formed.[21]

3.4. Quantification of Protein Carbonyls and Hydroperoxides. The yield of protein carbonyls and hydroperoxides generated on incubation of the proteins with AAPH was assessed. Table 5 summarizes the data obtained normalized per mol of protein and also per radical introduced into the system. After 3 h of incubation with 10 mM AAPH, the yield of carbonyls and protein hydroperoxides formed differed between the proteins examined, with the lowest levels of protein hydroperoxides detected on BLP and the lowest levels of carbonyls on G6PD. Additional experiments were carried out using higher AAPH concentrations. Incubation of the proteins for 3 h with 60 or 100 mM AAPH gave rise to higher protein carbonyl and protein hydroperoxide levels, suggesting that the rate of formation of these species occurs at a faster rate than their further reaction. However, even with these higher AAPH levels, the overall concentrations of carbonyls and protein hydroperoxides formed remained low. The concentration of hydroperoxides detected was unaffected by the addition of catalase (data not shown), indicating that H_2O_2 is not a major component of the detected peroxides.

While these data could be interpreted as indicating that carbonyls and protein hydroperoxides are not major intermediates, it should be noted that both of these species can react further, and hence, the levels detected at the end of the incubation time are unlikely to represent the total amounts formed.[22,34] In the case of carbonyls, these can undergo reactions with nucleophiles (e.g Lys, Arg, Cys, and His residues) to give Schiff bases and further products.[35]

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3.5. Identification of Carbonyl Groups on SDS−PAGE Bands. In order to examine potential sites of formation of carbonyls on the proteins examined, Western blotting of carbonyl groups present on the proteins separated by SDS−PAGE was carried out using the OxyBlot system. With AAPH-treated LYSO and G6PD, the majority of the carbonyl groups were localized in the band corresponding to the protein monomer (Figure 6). For BLP, similar carbonyl levels were detected in the low-molecular-mass fractions of both the AAPH-treated and control samples. Carbonyl groups were also detected in the AAPH-treated samples at positions on the membrane corresponding to cross-linked materials.

3.6. Quantification of Dityrosine. The role of dityrosine, a known cross-linking agent, in the observed protein aggregation was examined by quantifying the levels of this cross-linked material. The levels of this product, expressed as the number of molecules per protein, were 3.7×10^{-4} for LYSO and 9.9×10^{-4} for BLP. The dityrosine levels formed on oxidized G6PD were below the limits of detection.

4. DISCUSSION

The damage induced by the incubation of biological molecules, including proteins, with AAPH in the presence of oxygen is widely acknowledged to be due to the formation of peroxyl radicals.[4] Organic peroxyl radicals are typically less reactive than hydroxyl radicals (HO·); however, this lower reactivity and hence longer lifetime may result in a greater selectivity of damage to target molecules.[2,6,36] This assumption is supported by the data obtained in the current study where only a small number of different amino acid residues (Cys, Met, Tyr, and Trp (and Lys in the case of G6PD)), were observed to be modified. This contrasts with previous results obtained for LYSO and HO·, where most amino acids were modified.[37] Furthermore, the types and percentages of each amino acid side chain modified in LYSO was observed to follow the known reactivity of the *free* amino acids toward peroxyl radicals, i.e., Trp > Met > Tyr.[1] However, this relative reactivity was not the same for each of the three protein systems considered. This would imply that, at least in some circumstances, the reactivity of a given amino acid is modulated by its environment and/or secondary/tertiary structure.[38] The consumption of Lys observed with G6PD was also unexpected, as this residue is not particularly reactive toward peroxyl radicals;[39] this may suggest that Lys consumption occurs via secondary reactions (*vide infra*).

Summation of the losses of individual amino acids per protein (Table 4) allows an estimate to be made of the total concentration of amino acids consumed for LYSO and BLP (0.9 and 0.5 mM, respectively). Interestingly, these values are of the same order of magnitude as the number of peroxyl radicals introduced into the system (~1.4 mM after 3 h of incubation with 100 mM AAPH using a peroxyl radical formation rate of 8 μM min^{-1}).[21] This can be ascribed to the protein high concentration and the low rate of radical generation, conditions that would favor the occurrence of processes first order in radicals (i.e., radical-protein as opposed to radical−radical reactions).[40] The lower efficiency observed for G6PD probably results from the lower concentration of protein employed in these experiments that would preclude quantitative trapping of the AAPH-derived peroxyl radicals.[40]

The observed protein cross-linking and fragmentation are postulated to involve the following initial reaction sequence:[1,41]

$$\text{AAPH} \rightarrow 2\text{R}\cdot + \text{N}_2 \quad (1)$$

$$\text{R}\cdot + \text{O}_2 \rightarrow \text{R-OO}\cdot \quad (2)$$

$$\text{R-OO}\cdot + \text{PH} \rightarrow \text{R-OOH} + \text{P}\cdot \quad (3)$$

$$\text{P}\cdot + \text{O}_2 \rightarrow \text{P-OO}\cdot \quad (4)$$

$$\text{P}\cdot + \text{P}\cdot \rightarrow \text{P−P} \quad (5)$$

$$\text{P−P} + \text{R-OO}\cdot \rightarrow \text{P−P}\cdot + \text{R-OOH} \quad (6)$$

$$\text{P−P}\cdot + \text{P}\cdot \rightarrow \text{multimeric aggregation} \quad (7)$$

$$\text{P}\cdot \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \text{fragmentation} \quad (8)$$

The reactions leading to dimerization or multimeric aggregation maybe explained, at least in part, by the modification of Trp and/or Tyr residues. These amino acids are known to be highly susceptible to oxidation by a wide range of one-electron oxidants.[1,2] In particular, Gracanin and co-workers showed, using LC/MS, that the oxidation of Trp residues in LYSO by AAPH-derived peroxyl radicals leads to multiple oxidation products including *N*-formyl-kynurenine and lower levels of alcohols, diols, and kynurenine.[42]

The radicals likely to be formed from Trp and Tyr side chains are delocalized species (indolyl and phenoxyl radicals, respectively) that have significant spin density on the carbon atoms of the aromatic rings. In the case of the Tyr phenoxyl radical, considerable electron density is present on the carbons ortho to the phenol group (positions 3 and 5). For Trp, significant electron density is present at C3 of the pyrrole ring as well as carbons in the benzene ring. Two downstream reaction pathways have been described for these free radicals, one of them mediated by oxygen giving rise to peroxyl radicals (for the Trp indolyl radical, with reaction occurring predominantly via C3) and the other generating intermolecular cross-links through radical−radical coupling, either through the carbon atoms (e.g., to give 3,3′-dityrosine from Tyr phenoxyl radicals) or through carbon−oxygen links (e.g., isodityrosine from Tyr phenoxyl radicals). The reaction of the Trp indolyl and Tyr phenoxyl radicals with oxygen is relatively slow (k ~5×10^6 and 1×10^3 dm^3 mol^{-1} s^{-1}, respectively) when compared to rate constants for radical−radical dimerization reactions (k ~5×10^8 dm^3 mol^{-1} s^{-1}), indicating that dimerization is likely to be a significant process, particularly when the radical flux is high and the oxygen concentration relatively low.[2,38,43−47] The low amounts of parent Tyr lost from the proteins exposed to AAPH-derived radicals, together with the low concentrations of dityrosine generated, argue against Tyr-Tyr radical coupling (either carbon−carbon or carbon−oxygen) being the principal pathway responsible for the protein cross-linking detected. In the case of BLP, which contains relatively low levels of free Cys side chains, formation of interprotein disulfide bonds may occur and induce cross-linking. However, these types of cross-links are unlikely to be the source of the observed aggregates (cf. Figures 3 and 4), because these SDS−PAGE was performed under reducing conditions that would remove such linkages. As peroxyl radicals react rapidly with free thiols, the oxidation of Cys residues would be expected to inhibit the formation of protein hydroperoxides in BLP, and this may account, at least in part, for the low levels of hydroperoxides detected on this protein (cf. data in Table 5) when the incubation was performed with 10 mM AAPH. Furthermore, it is known that hydroperoxides can undergo secondary molecular oxidation reactions with Cys residues (resulting in the formation of an

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alcohol and a sulfenic acid), a process that may further reduce the levels of protein hydroperoxides detected on BLP.[25]

Taking into account the extent of modification of the parent amino acids detected in the three proteins studied, only the extent of oxidation of Trp residues is consistent with the fraction of cross-linked proteins shown in Figure 4. Ditryptophan cross-links and indole dimers have been detected *in vitro* as a result of electrochemical oxidation[48−52] or acid-promoted dimerization of Trp containing peptides followed by oxidation.[53] Riboflavin-photosensitized modification of free Trp in aqueous solution also induces the generation of ditryptophan and oligomers of this amino acid via radical-mediated reactions.[54] Furthermore, it is interesting to note that Trp-free proteins, when oxidized by peroxyl radicals, generate considerably less chemiluminescence than Trp containing proteins, and this chemiluminescence has been related to protein hydroperoxide formation.[55]

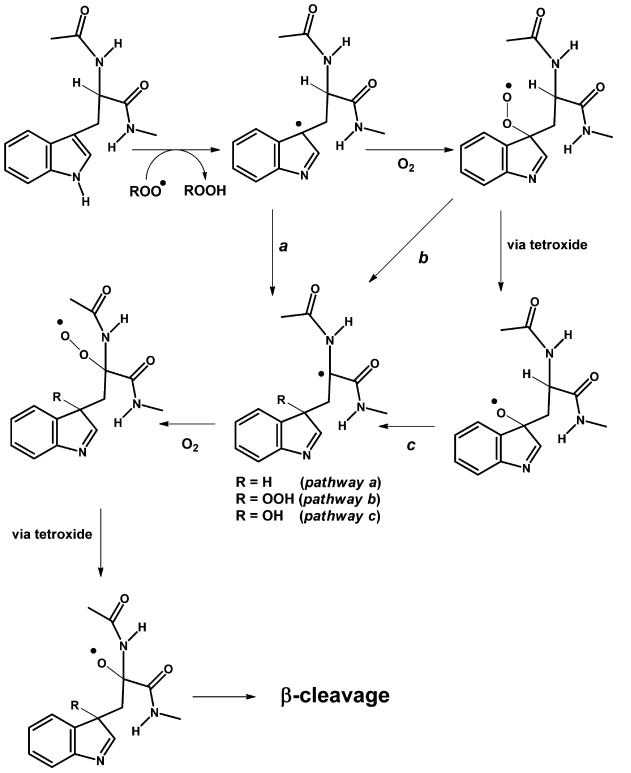
The observed cross-linking reactions appear to involve protein−protein (P−P), protein−fragment (P−F), and fragment−fragment (F−F) interactions (where P and F represent the native protein and fragments generated from reaction with peroxyl radicals, respectively). Fragmentation also appears to take place either at particular sites (G6PD and LYSO) or almost at random (BLP), producing a wide distribution of fragment masses. The occurrence of these two different processes depends on the protein under study; this again suggests a critical role for protein secondary and tertiary structures in the predominant reactions that take place.

The data presented in Figure 1 are consistent with the major cross-link process with LYSO being a P−P type, while in the case of G6PD, the dominant process appears, at least at low radical doses, to involve linking of the parent protein to a fragment (i.e., P−F type); this generates aggregates whose molecular mass is considerably smaller than that of P−P cross-links (Figure 2). The different behavior of these two proteins may arise from the monomer (LYSO) versus dimeric nature of G6PD, with the P−F species observed with the latter possibly arising from reactions that result in the loss of part of one of the subunits and cross-linking of the remaining section to the other monomer unit.

The apparent nonrandom fragmentation of the main polypeptide chain observed with some of the proteins studied (see Figures 1−3) may arise from selective β-cleavage of the polypeptide chain, with this implying that the initial hydrogen-atom abstraction from the backbone of the polypeptide chain is also selective. A widely accepted pathway to backbone fragmentation involves initial abstraction of a hydrogen atom from α-carbon sites (i.e., the amino acid side-chain substituent site), with this giving rise to a carbon-centered radical that subsequently reacts with O_2 to yield an α-carbon peroxyl radical. Subsequent peroxyl radical dimerization yields a transient tetroxide (ROO-OOR) that rapidly decomposes to give two alkoxyl radicals that can fragment to cleave the backbone.[6] Alternatively the α-carbon peroxyl radical can undergo loss of $HO_2^{\bullet}$ to generate an imine that undergoes hydrolysis to cleave the backbone; both pathways result in the formation of carbonyl groups. Gly residues have been reported to be important in the fragmentation of some proteins[56,57] as the α-carbon radical formed from this amino acid is especially stable.

Nonetheless, the amino acid analysis performed in this work did not show any significant loss of Gly residues in the proteins on incubation with AAPH. Furthermore there are many more Gly residues present in the sequence of these proteins than the number of specific fragments detected. These data suggest either that a very selected number of Gly residues are involved (and hence the loss of these is below the levels that could be detected in the amino acid analysis experiments) or that other residues/pathways are involved. One potential alternative to the Gly pathway is via the oxidation of Trp residues (consistent with the observed residue loss) with the formation of a carbon-centered, peroxyl or alkoxyl radical at C-3 on the indolyl ring (pathways *a*, *b*, or *c* in Scheme 1, respectively), with these

Scheme 1. Proposed Mechanism Role of Trp on Protein Fragmentation



species potentially giving rise to hydrogen-atom abstraction from α-carbon sites on the polypeptide chain (either at the Trp residue α-carbon position or at other sites in close spatial proximity) and subsequent reactions as described above. This mechanism would result in backbone fragmentation and would introduce carbonyl groups on the formed fragments, in addition to those arising from oxidation of the Trp indole ring (e.g., the formation of kynurenine and *N*-formylkynurenine). This could account for the presence of carbonyls on polypeptides whose mass is not markedly different from those of the parent macromolecule.

A proposed sequence of reactions is given in Scheme 1. In this scheme *pathway a* may be favored due to the slowness of the reaction of Trp-derived radicals with molecular oxygen.[38] Likewise, *pathway b* could be promoted by the proximity of the free radical to the α-hydrogen and the involvement of a six-membered ring intermediate. *Pathway c* involves the participation of alkoxyl radicals that are more reactive than peroxyl radicals. This cleavage mechanism implies that each fragmentation would produce one carbonyl group, but this does not tally with the data obtained (e.g., for G6PD, cf. Figure 4 and Tables 2 and 5). With G6PD, the number of carbonyls detected is

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considerably smaller than the number of fragments, and the data presented in Figure 6 indicates that the carbonyls detected on LYSO and G6PD are almost exclusively detected on polypeptides proteins whose mass is close to that of the native protein. Two explanations can be advanced to account for these results: that backbone cleavage yields species other than detectable carbonyls (some of the β-cleavage reactions generate amides, which would not be detectable,[2] and/or that the materials bearing carbonyl groups react further with free NH_2 groups (at the N-terminus and/or present in Lys) to give Schiff bases (or downstream materials).

$$\text{P-NH}_2 + \text{F} = \text{CO} \rightarrow \text{P-F} \quad (9)$$

Both of these proposals appear reasonable, and the latter mechanism would explain both the consumption of Lys observed with G6PD and the presence of carbonyls on species with molecular masses between those of the parent protein (P) and dimers (P−P). This type of reaction would also explain the low yield of carbonyls observed after oxidation of this enzyme. Consistent with this hypothesis, it has been reported that some of the Lys residues on G6PD exhibit an anomalously high reactivity toward carbonyl groups, which is likely to reflect a shift in the pK_a of the amine function of some of the Lys residues (resulting in a greater yield of the more powerful nucleophilic RNH_2 form) due to the local environment of some of these residues in the enzyme.[58]

Overall, these studies using defined doses of well-characterized peroxyl radicals have provided important information on the mechanisms of aggregation and fragmentation of three well-characterized proteins. The data obtained indicate that oxidation of Trp and Tyr residues may be of particular importance with this relatively selective but widely encountered type of reactive oxidant in biological systems. The protein radicals generated as a consequence of the effect of the peroxyl radical on these molecules, represent a simple model to understand the behavior of BLP exposed to UVA/visible light in the presence of ascorbate and glucose decomposition products, which have been postulated to contribute to aging and cataractogenesis in the eye lens.[59,60] These decomposition products have a mixed Type I−Type II photosensitizing activity giving rise to both protein radical intermediates and singlet oxygen. The AAPH system used here allows the study of the radical-mediated processes, in the absence of singlet oxygen, which is known to produce protein cross-linking via the formation of hydroperoxides, and downstream His oxidation products, which react with nucleophilic sites on Lys, Cys, Trp, and Tyr.[61−63]

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Notes

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ABBREVIATIONS

AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; BLP, bovine eye lens proteins; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); F−F, fragment-fragment cross-linking; G6PD, glucose 6-phosphate dehydrogenase; HSA, human serum albumin; LYSO, lysozyme; MSA, methanesulfonic acid; OPA, *o*-phthaldaldehyde; P−P, protein−protein cross-linking; P−F protein-fragment cross-linking; TCA, trichloroacetic acid; TEM, transmission electron microscopy; XO, xylenol orange

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