Characterization of non-covalent oligomers of proteins treated with hypochlorous acid

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Hypochlorous acid (HOCl) is a potent oxidant produced by myeloperoxidase that causes aggregation of many proteins. Treatment of apohaemoglobin and apomyoglobin with HOCl produced a regular series of oligomer bands when the proteins were separated by SDS/PAGE under reducing conditions. Aggregation was detectable at a HOCl/protein molar ratio of 0.5:1 and was maximal at ratios of 10:1–20:1. Dimers formed within 1 min of adding HOCl, and further aggregation occurred over the next 30 min. No convincing evidence for covalent cross-linking was obtained by amino acid analysis, peptide analysis or electrospray ionization-MS of HOCl-modified apomyoglobin. The latter showed an increase in mass consistent with conversion of the two methionine residues into sulphoxides. A 5-fold excess of HOCl generated approximately three chloramines on the apomyoglobin. These underwent slow decay. Protein carbonyls were formed and were almost entirely located only on the polymer bands. Conversion of positively into negatively charged groups on the protein by succinylation caused preformed aggregates to dissociate. Treatment of apomyoglobin with taurine chloramine generated methionine sulphoxides but few protein carbonyls, and did not result in aggregation. We conclude that aggregation was due to strong, non-covalent interactions between protein chains. We propose that formation of protein carbonyls and possibly chloramines, along with methionine oxidation, alters protein folding to expose hydrophobic areas on neighbouring molecules that associate to form dimers and higher-molecular-mass aggregates. This process could lead to the formation of aggregated proteins at sites of myeloperoxidase activity and contribute to inflammatory tissue injury.

Key words: haemoglobin, myeloperoxidase, myoglobin, neutrophil, protein aggregation, protein oxidation.

INTRODUCTION

Large numbers of neutrophils infiltrate sites of infection and act as the first line of defence against invading micro-organisms. When these cells are activated, they undergo an oxidative burst that results in the production of a number of oxidants, including hypochlorous acid (HOCl) [1]. [At pH 7.4, hypochlorous acid is present as its anion (hypochlorite) and the protonated form in almost equal amounts (pK 7.5). We use the term HOCl to describe this mixture.] This potent oxidant is generated from hydrogen peroxide and chloride in a reaction catalysed by myeloperoxidase [2]. HOCl is a strong microbicidal agent, capable of killing bacteria, fungi and viruses [1]. It also reacts readily with proteins and may be responsible for damage to host tissue at sites of inflammation. HOCl modifies proteins in a number of ways, including conversion of cysteine residues into disulphides and higher oxidation products, conversion of methionine into its sulphoxide, tryptophan oxidation, tyrosine chlorination and conversion of amino groups into chloramines [3,4]. Protein carbonyls (aldehydes) can subsequently form from chloramines via the loss of HCl and hydrolysis of the imine [5].

A striking finding with a number of proteins is the formation of polymers or aggregates on treatment with HOCl. Proteins that have been shown to undergo aggregation include fibronectin [6], α_{s-1} casein [7], apolipoprotein B and Lp(a) from LDL (low-density lipoprotein) [8–10], apolipoprotein A-I from HDL (high-density lipoprotein) [11,12], ovalbumin [13], α_2-macroglobulin [14] and Mn-superoxide dismutase [15]. Other proteins, such as serum albumin [16], glucose-6-phosphate dehydrogenase [17] and Cu/Zn-superoxide dismutase [18], are much less prone to aggregation.

Aggregation of proteins is attracting increasing interest as a pathological mechanism, for example in neurodegenerative diseases [19–21]. Protein aggregation by HOCl could contribute to inflammatory tissue injury, including the early stages of atherosclerosis. Modification of apolipoprotein B by HOCl is associated with high uptake of LDL by macrophages and the generation of foam cells. With HDL, apolipoprotein A-I oxidation is linked to impaired receptor recognition and lipid transfer [8,9,22,23]. Immune complexes such as IgG–anti-IgG are cross-linked when they are incubated with activated neutrophils or the myeloperoxidase/hydrogen peroxide/chloride system, suggesting that such aggregation may be involved in autoimmune responses [24]. In addition, aggregation has been proposed to cause the lysis of red blood cells that occurs as a result of exposure to HOCl [25]. It may also lead to the disruption of normal tissue organization when fibronectin is oxidized at sites of inflammation [6].

The mechanism of HOCl-mediated protein aggregation, and the properties that make some proteins more susceptible than others, have not been established. Aggregation has been observed as high-molecular-mass bands on SDS/PAGE or by size exclusion chromatography, even under reducing conditions, and is generally assumed to represent intermolecular covalent cross-linking of the protein. Cross-linking via dityrosine formation has been suggested [6,7,15]. In the case of the S100A8 protein of neutrophils, a sulphinamide linkage between an oxidized cysteine residue of one molecule and a lysine of another has been demonstrated [26]. This mechanism could operate for other proteins [27], but not all the proteins that aggregate contain free thiols, so it cannot be a universal explanation. Lysine residues have been implicated in some studies, and a mechanism involving Schiff-base formation between an aldehyde generated as a result

Abbreviations used: DNP, 2,4-dinitrophenylhydrazine; ESI, electrospray ionization; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; TNB, 5-thio-2-nitrobenzoic acid.

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of chloramine breakdown and a lysine on another molecule has been proposed [7,8,13]. However, there is no direct evidence for this or indeed any covalent linkage.

In view of its potential significance for oxidative injury by neutrophils, we have sought to characterize the mechanism of HOCl-mediated aggregation and to identify the molecular changes involved. We have studied human haemoglobin and horse heart myoglobin, as well characterized model proteins that readily undergo aggregation, and used the haem-depleted apo forms to avoid complications due to the reaction of HOCl with the haem groups. By following aggregation by SDS/PAGE and MS, we found no evidence for covalent cross-linking, and conclude that aggregation is due to rapid, strong, non-covalent interactions between protein chains.

MATERIALS AND METHODS

Materials

HOCl was from Reckitt and Coleman, Auckland, New Zealand. Its concentration was determined either by using TNB (5-thio-2-nitrobenzoic acid) [28] or by measuring A_{292} at pH 12 (ε_{292} 350 $M^{-1} \cdot cm^{-1}$). Taurine chloramine was prepared by adding an equal volume of HOCl (20 mM; pH 7.4) to a solution of taurine (100 mM) while vortexing. The chloramine content was determined after 10 min using TNB. Horse heart (met)myoglobin was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cyanmetmyoglobin was prepared by adding 1 mM KCN to a 10 mg/ml solution and removing excess KCN by gel filtration. The haemoglobin solution was prepared by lysis of washed human red blood cells and removal of membranes using carbon tetrachloride [29], and was used without further purification.

The anti-dinitrophenyl–KLH, rabbit IgG fraction, biotin–XX conjugate (anti-DNP–biotin, where KLH is keyhole limpet haemocyanin and DNP is 2,4-dinitrophenylhydrazine) was purchased from Molecular Probes (Eugene, OR, U.S.A.) and the streptavidin–biotinylated horseradish peroxidase complex was from Amersham Life Sciences Ltd. All other chemicals were from BDH Laboratory Supplies (Poole, Dorset. U.K.) or Sigma. PBS, pH 7.4, contained 10 mM phosphate and 140 mM chloride.

Preparation of apohaemoglobin and apomyoglobin

The apoproteins were prepared by adding 1 vol. of either red blood cell lysate (0.1 g of haemoglobin/ml) or myoglobin (0.1 g/ml in water) to 9 vol. of ice-cold acidified acetone [1 % (v/v) HCl + 0.1 % (v/v) mercaptoethanol] while vortexing [29]. Solutions were centrifuged for 5 min at 500 ***g*** and supernatants discarded. Pellets were washed with ice-cold acetone, re-centrifuged, air dried, dissolved in water, then dialysed into PBS. Protein concentrations were determined using the Bio-Rad Protein Assay.

Treatment of proteins with HOCl

HOCl (typically 100 μl of 0.3–24 mM neutralized solution in PBS) was added to an equal volume of protein solution (10 mg/ml) while vortexing. This resulted in HOCl/protein molar ratios of between 0.5:1 and 40:1. Reactions were carried out at room temperature in PBS unless stated otherwise. They were stopped by the addition of SDS/PAGE loading buffer containing 5 % (v/v) β-mercaptoethanol to quench the HOCl. In cases where the proteins were not electrophoresed after HOCl treatment, methionine or thiodipropionic acid was added at fives times the final HOCl concentration to stop the reaction.

SDS/PAGE

A discontinuous mini-gel system, consisting of a 7–18 % (w/v) acrylamide gradient separating gel and a 3 % (w/v) stacker gel, was used to separate aggregated proteins [30]. Samples were diluted into loading buffer containing 5 % (v/v) β-mercaptoethanol and the gels were run at 200 V (constant) and 50 mA for approx. 50 min. Gels were stained overnight with colloidal Coomassie Blue.

Detection of protein amine groups and chloramines

Protein amine groups were detected with fluorescamine [31]. Chloramine content was determined by adding aliquots of protein solution to solutions of TNB while vortexing, and measuring the decrease in absorbance at 412 nm (ε_{412} 14 100 $M^{-1} \cdot cm^{-1}$) after 5 min in the dark [28]. Unreacted HOCl, as distinct from protein chloramines, was detected by reaction with monochlorodimedon as described previously [3].

Amino acid analyses

Apomyoglobin was treated with a 5:1 molar excess of HOCl for 15 min and the reaction was stopped by the addition of methionine. The protein was separated by size exclusion chromatography on a Sephacryl S-100 column equilibrated with PBS into two peaks, one running in the monomer position and the other aggregate fraction eluting in the exclusion volume. Both fractions were hydrolysed overnight in 6 M HCl. Amino acid analysis was performed after derivatization with *o*-phthalaldehyde by HPLC using an Adsorbosphere OPA (Alltech) column with fluorescent detection [excitation wavelength (λ_{ex}) 325 nm; emission wavelength (λ_{em}) 465 nm] [32].

Dityrosine was detected in acid hydrolysates by reverse-phase HPLC using a Brownlee SPHERI-5 ODS column with fluorescent detection (λ_{ex} 280 nm; λ_{em} 400 nm) and water containing acetonitrile (8 %, v/v) and trifluoroacetic acid (0.1 %, v/v) as solvent. A dityrosine standard was prepared by reaction of 1 mM tyrosine with 100 μM hydrogen peroxide in the presence of 10 μg/ml horseradish peroxidase [33].

The fluorescence due to tryptophan residues was measured at excitation and emission wavelengths of 275 and 355 nm respectively. The appropriate wavelengths were determined on the basis of spectral scans of L-tryptophan.

Succinylation of apomyoglobin

Solid succinic anhydride (50 mg) was added to 4 ml of treated or untreated apomyoglobin solution (4.5 mg/ml in 0.5 M borate buffer, pH 8.8) to give a 25-fold excess of anhydride over protein amine groups [34]. The anhydride was added slowly and the pH was monitored. When necessary, 5 M NaOH was added to maintain the pH at 8.8. Samples of the treated protein were diluted into equal volumes of loading buffer and loaded on to 12 % gels for SDS/PAGE.

Determination of protein carbonyls

Proteins were treated with HOCl or taurine chloramine and then reacted with DNP prior to electrophoresis as described by Levine et al. [35]. SDS/PAGE gels were run as described above and

then electroblotted for 1 h at 100 V using a Bio-Rad Transblot apparatus. Carbonyls were detected on the blots using anti-DNP–biotin [35] followed by streptavidin–biotinylated horseradish peroxidase complex with peroxidase staining. Total protein carbonyls were also quantified by ELISA after derivatization with DNP and reaction with anti-DNP–biotin [36]. The assay was calibrated with HOCl-modified serum albumin which was standardized colorimetrically. It has been shown previously that there is a linear relationship between concentrations measured by ELISA and colorimetrically when albumin is treated with increasing concentrations of HOCl [36].

ESI (electrospray ionization)-MS

Control and treated apomyoglobin samples were dissolved in a solution of 30 % (v/v) acetonitrile and 1 % (v/v) formic acid at a concentration of 0.15 mg/ml. Aliquots of 10–20 μl of each sample were injected directly on to a VG Platform quadrupole analyser operating in positive-ion mode at a flow rate of 10 μl/min. The source was maintained at 60 °C and the probe was charged at + 3000 V. Mass ranges of 700–1800 *m/z* were scanned every 2 s. A cone voltage ramp of 40–80 V was applied over the *m/z* ranges. The charge series generated from the α chain of human haemoglobin was used as a calibration standard [37]. Data were processed using Mass-Lynx software and transformed on to a true molecular mass scale using maximum entropy software supplied with the instrument.

MALDI-TOF (matrix-assisted laser desorption/ionization time of flight) MS of tryptic digests

Apomyoglobin was treated with a 5-fold molar excess of HOCl for 15 min and electrophoresed as described above. The gel was negatively stained with $CuCl_2$ [38]. Dimer and monomer bands were excised and the gel fragments destained, suspended in 20 μl of 0.1 M ammonium bicarbonate, pH 8, containing trypsin (0.5 μg), crushed and incubated for 2–3 h at 37 °C [39]. Peptides were extracted by repeated sonication of the gel fragments in a 1:1 (v/v) solution of acetonitrile and 0.5 % (v/v) trifluoroacetic acid. Resulting peptide supernatants were lyophilized, suspended in 0.5 % trifluoroacetic acid and then mixed 1:1 (v/v) with a solution of 10 mg/ml α-cyano-4-hydroxycinnamic acid in acetonitrile and 0.5 % trifluoroacetic acid. Monoisotopic masses were obtained in positive-ion reflector mode with an accelerating voltage of 20 000 V, a grid voltage of 74 %, a delay time of 100 ns and no voltage on the guide wire. Masses were calibrated with a close external standard containing angiotensin I (*m/z* 1296.69) and corticotropin-(18–39) (*m/z* 2465.20). All spectra were analysed using PerSeptive GRAMS/32 software.

RESULTS

Characteristics of the aggregation reaction

Treatment of human apohaemoglobin and horse heart apomyoglobin with HOCl produced high-molecular-mass polymers that could be separated by SDS/PAGE under reducing conditions. As shown in Figure 1, apohaemoglobin gave bands that ran in positions corresponding to dimers, trimers, tetramers and higher-molecular-mass complexes. Dimers were formed at the lowest ratio used of 0.5 mol of HOCl per mol of apohaemoglobin. Trimers and tetramers became evident at ratios of 1:1 and 2:1, and larger aggregates were produced at higher ratios. With the largest amounts of HOCl, the distinct pattern of aggregation was

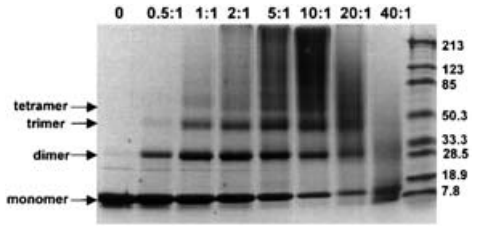

Figure 1 Formation of high-molecular-mass aggregates on treatment of apohaemoglobin with HOCl

Apohaemoglobin was treated with increasing amounts of HOCl at the molar ratios shown for 15 min at room temperature, and separated by SDS/PAGE under reducing conditions as described in the Materials and methods section. Molar ratios are expressed per haemoglobin monomer, and a ratio of 5:1 corresponds to 3.2 μmol of HOCl per 10 mg of protein. The positions of the protein monomer (15.5 kDa), dimer (31 kDa), trimer (46.5 kDa) and tetramer (62 kDa) and the masses of the marker proteins (kDa) are shown. The gel is representative of five experiments.

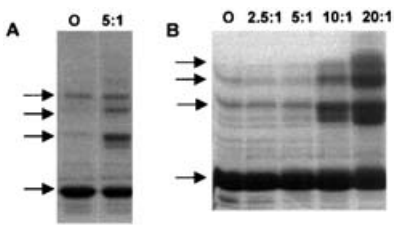

Figure 2 HOCl-mediated aggregation of (A) apomyoglobin and (B) cyanmetmyoglobin

The proteins were treated with HOCl for 15 min at the molar ratios shown and separated by SDS/PAGE. The positions corresponding to the protein monomer (16.9 kDa), dimer (33.9 kDa), trimer (50.8 kDa) and tetramer (67.8 kDa) were determined from the mobilities of marker proteins and are marked with arrows. The gels are representative of two or more experiments.

lost and the proteins ran as a smear. This could be due either to formation of aggregates that were too large to enter the separating gel, or to fragmentation [16,40]. Varying the concentrations of apohaemoglobin and HOCl but keeping the molar ratios the same did not affect the extent of aggregation (results not shown).

Polymer bands were also seen with apomyoglobin, at comparable molar ratios of HOCl to those used with apohaemoglobin (Figure 2A). To ensure that aggregation was not peculiar to the apoproteins, which have less organized structures and greater instability than the native forms, cyanmetmyoglobin was also examined. The binding of cyanide decreases the susceptibility of the haem group to oxidation and makes it unreactive with HOCl. Cyanmetmyoglobin was aggregated when treated with HOCl, although the higher ratio of oxidant to protein of 10:1 was required to generate dimers (Figure 2B).

Time course of the aggregation reaction

The time course was investigated with apohaemoglobin and a 5-fold molar excess of HOCl. HOCl concentration measurements showed that approx. 90 % was consumed in the first 30 s, with less than 5 % still present after 3 min. Formation of protein dimers occurred within 30 s, coinciding with the period when most of the HOCl reacted with the protein (Figure 3). Trimers and tetramers gradually became evident after 5 min or more of treatment, and higher aggregates accumulated over 45 min. The pattern of aggregation was unchanged after 72 h at room temperature (results not shown). The reaction could not be reversed by the addition of reducing agents such as mercaptoethanol or by boiling the treated

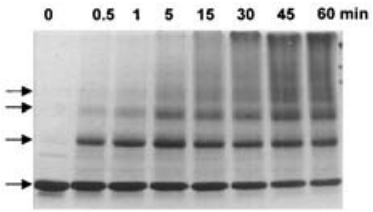

Figure 3 Time course of aggregation of apohaemoglobin

The protein was treated with a 5-fold molar excess of HOCl for the times shown and separated as in Figure 1. The arrows mark the positions corresponding to the monomer, dimer, trimer and tetramer, determined from marker proteins as in Figure 1. The gel is representative of five experiments.

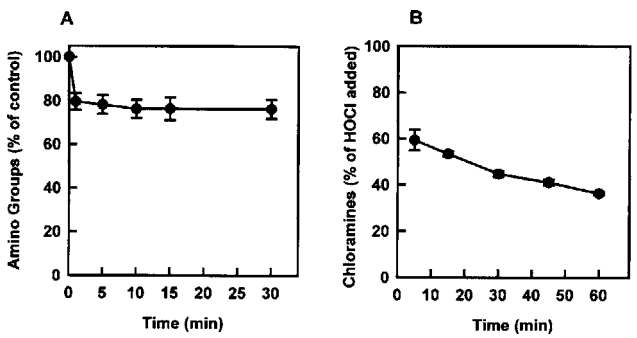

Figure 4 Loss of protein amine groups (A) and formation of chloramines (B) after treatment of apomyoglobin with HOCl

The protein was treated with a 5-fold molar excess of HOCl. Amine groups were measured with fluorescamine and chloramines with TNB, as described in the Materials and methods section. In (**A**) the data are the means ± S.D. of three experiments. In (**B**) the data are the means ± S.D. of eight values from four experiments. Where error bars are not shown, they fall within the symbol size.

proteins in SDS/PAGE loading buffer. A similar time course was seen with apomyoglobin (results not shown).

Involvement of chloramines and protein carbonyls in aggregation

The most susceptible amino acids to oxidation by HOCl include cysteine, methionine, tyrosine, tryptophan and lysine [3,41]. As all gels were run under reducing conditions, aggregation was not due to the generation of disulphide bonds. Furthermore, the lack of cysteine residues in horse myoglobin rules out the involvement of this residue in the process.

Apomyoglobin contains 19 lysine residues plus the N-terminal amine. The conversion of these amino groups into chloramines was followed over the period during which aggregation occurred. Within 1 min of treating apomyoglobin with a 5-fold excess of HOCl, 20 % of the total amino groups were lost (Figure 4A). There was little further decay. Chloramines at 5 min accounted for 60 % (3 of the 5 mol) of the HOCl initially added to the protein (Figure 4B). Approximately one-third of these chloramines decayed over 60 min.

The involvement of chloramines in aggregation was followed by adding methionine to apomyoglobin at 1 min, when all of the HOCl had reacted with the protein. Chloramines are reduced by methionine (and other thioethers such as thiodipropionate) back to the parent amines. At the 1 min time point, most of the dimerization had already occurred. Further incubation resulted in the accumulation of high-molecular-mass aggregates, which was impaired, although not prevented, when methionine (Figure 5) or

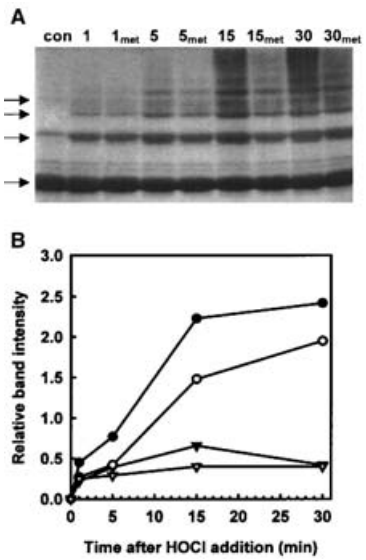

Figure 5 Inhibition of aggregation by methionine

(**A**) Duplicate samples of apomyoglobin were treated with HOCl at an oxidant/protein molar ratio of 5:1. After 1 min, methionine at a 5-fold excess over the HOCl concentration was added to one sample (lanes labelled met). Both samples (with and without methionine added) were incubated, and portions were removed at various times after the addition of HOCl (shown in min above the lanes) for electrophoresis. The arrows show the positions of monomer and polymer bands as in Figure 2; con, control protein. The gel is representative of three experiments. (**B**) Densitometry of dimer (▼, ▽) and polymer (●, ○) bands relative to the monomer in the respective samples. Data are from (**A**), without (▼, ●) and with (▽, ○) methionine.

Table 1 Protein carbonyl formation on apomyoglobin treated with HOCl or taurine chloramine

Oxidant	Oxidant/protein molar ratio	Protein carbonyls (nmol/mg) 15 min	60 min
None	–	0.07	0.07
HOCl	5:1	1.94	3.51
	10:1	2.56	3.44
Taurine chloramines	5:1	0.10	0.41
	10:1	0.15	0.84

thiodipropionate (results not shown) was present. The effect was most evident after a 15 min incubation (Figure 5). These results point to a role for chloramines at least in the latter phase of the aggregation process.

As chloramines are potential precursors of protein carbonyls [5,8], we determined whether carbonyls were formed during protein aggregation. When HOCl-oxidized apomyoglobin was treated with the carbonyl reagent DNP, analysis by ELISA showed substantial formation of carbonyls within 15 min and a further increase at 1 h (Table 1). The relationship between carbonyl formation and aggregation was examined by SDS/PAGE and Western blotting with an anti-DNP antibody (Figure 6A). With apohaemoglobin, carbonyls were present in all of the oligomeric bands, with the monomer showing only low-intensity staining at the higher oxidant exposure. With apomyoglobin, carbonyls were associated predominantly with the higher-molecular-mass aggregates, and were almost undetectable in the monomer. This contrasts with the protein gel (Figure 6B) in which the oxidized monomers were major bands. Data from densitometry scans show

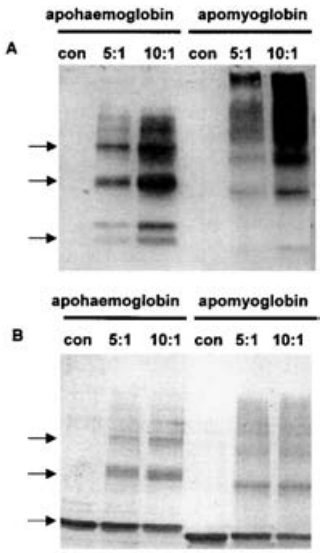

Figure 6 Detection of protein carbonyls in HOCl-treated proteins

Apohaemoglobin and apomyoglobin were treated with HOCl for 15 min at oxidant/protein molar ratios of 5:1 and 10:1. Samples were reacted with DNP, electrophoresed and either Western blotted and incubated with anti-DNP antibody (**A**) or stained for protein (**B**). HOCl/protein molar ratios for each lane are shown; con represents DNP-treated control protein. Arrows show the positions of monomer, dimer and trimer bands established from marker proteins as in Figure 1. The blot is representative of two experiments and the gel of three experiments.

Table 2 Comparison of protein gels and carbonyl blots for relative intensities of monomer and polymer bands after treatment of apohaemoglobin and apomyoglobin with HOCl

Percentages were determined by densitometry from the results shown in Figure 6. The carbonyl content and the proportions of dimer and polymer in the untreated proteins were set at zero. Band X represents the band just above the monomer, present only in the apohaemoglobin blot.

	Density distribution (%)							
	Apohaemoglobin				Apomyoglobin			
HOCl/protein ratio...	5:1		10:1		5:1		10:1	
Band	Protein	Blot	Protein	Blot	Protein	Blot	Protein	Blot
Monomer	39	3	28	6	23	0	20	3
Band X	–	7	–	10	–	–	–	–
Dimer	26	26	32	20	12	6	15	6
Polymers	35	64	40	63	65	94	65	91

that there were proportionately more carbonyls on the higher polymer bands (Table 2).

Amino acid modification

If HOCl causes covalent linkages between the polypeptide chains, new products derived from cross-linked amino acids would be expected on amino acid or peptide analysis. To investigate this, apomyoglobin was treated with HOCl and, after stopping the reaction at 15 min, was fractionated into two peaks using size exclusion chromatography. Aggregated protein was eluted at the exclusion volume, and oxidized monomer had the same retention time as the untreated protein. There was no evidence of discrete polymers, and SDS/PAGE of the aggregated protein showed predominantly high-molecular-mass material and only a minor band in the dimer position (results not shown). Amino acid

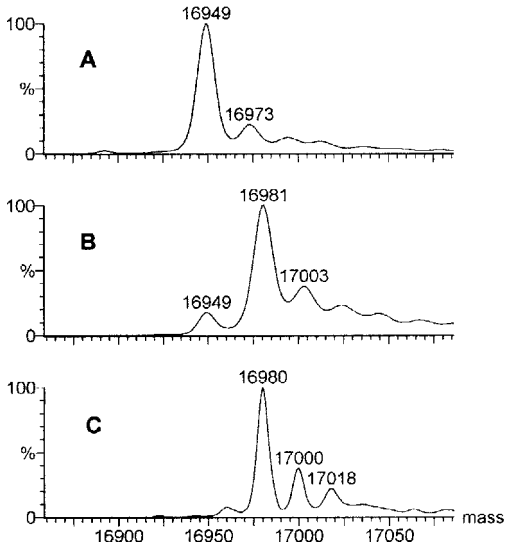

Figure 7 ESI-MS of HOCl- and taurine chloramine-treated apomyoglobin

Untreated apomyoglobin (**A**) and apomyoglobin treated for 15 min with a 5-fold molar excess of HOCl (**B**) or a 10-fold molar excess of taurine chloramine (**C**) were analysed by ESI-MS. Reactions were stopped by the addition of thiodipropionic acid. The peaks with molecular masses approx. 22 and 38 Da higher than those of the major peaks are likely to be sodium and potassium adducts.

analysis identified only one new product that was not present in the monomer or untreated control. It co-eluted with authentic α-aminoadipic acid, a known oxidation product of lysine derived from breakdown of lysine chloramine [7,42].

The dityrosine and 3-chlorotyrosine content of HOCl-treated apomyoglobin accounted for less than 0.1% and 0.4% respectively of the two tyrosine residues in the protein. Treatment with HOCl led to gradual decrease in protein fluorescence attributable to the two tryptophan residues, with a 15% loss over the first 5 min.

Tryptic digests of control apomyoglobin and oxidized monomer and dimer, isolated from SDS/12%-PAGE slab gels, were compared using MALDI-TOF MS. There was no clear evidence of novel peptides in the dimer that could have arisen from covalently cross-linked protein. The yield of the N-terminal tryptic peptide (residues 1–16) was decreased in the dimer compared with the control and monomer. The residues in this peptide most susceptible to HOCl are the N-terminal amino group and two tryptophan residues. The peptide corresponding to residues 119–133 was absent in the dimer and a new peak at $M+16$ was present. This peptide contains methionine at position 131, and the increased mass could be explained by its oxidation by HOCl to give the sulphoxide. This peptide was also partially oxidized in the oxidized monomer. The peptide containing methionine-55 was not evident in the digest, and no other unusual peptides were detected in the dimer.

ESI-MS of HOCl-treated apomyoglobin

On ESI-MS, control apomyoglobin had the predicted mass of 16949 Da (Figure 7A), while the mass of the HOCl-treated protein was 16981 Da (Figure 7B). This increase of 32 Da is equivalent to the addition of two oxygen atoms, and could be accounted for by oxidation of the two methionine residues to methionine sulphoxides. The mass increase is unlikely to be due to chloramine (replacement of H with Cl would give an increase of 34 Da),

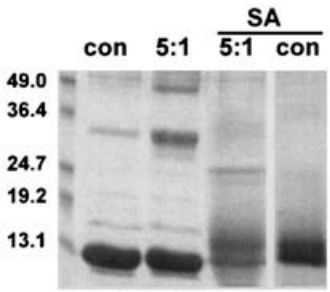

Figure 8 Reversal of aggregation by succinylation of HOCl-treated apomyoglobin

Apomyoglobin was treated with HOCl at an oxidant/protein molar ratio of 5:1 for 15 min. Solid succinic anhydride was added to aliquots of control (con) and treated protein (lanes labelled SA), as described in the Materials and methods section, and the proteins were separated by SDS/PAGE. The masses of marker proteins (kDa) are shown. The gel is representative of three experiments.

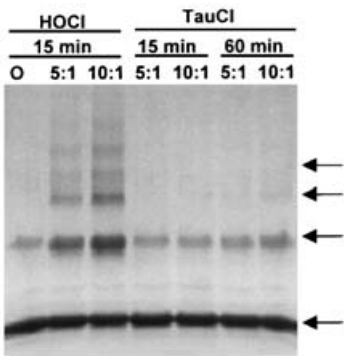

Figure 9 Lack of apomyoglobin aggregation with taurine chloramines

Apomyoglobin was treated with HOCl or taurine chloramine at oxidant/protein molar ratios of 5:1 and 10:1 for the times stated and then separated by SDS/PAGE. Arrows correspond to positions of monomer and polymer bands as in Figure 2. The gel is representative of two experiments.

as chloramines had been reduced prior to analysis. Peaks near $M + 23$ were seen in both samples and are likely to be sodium adducts. There was no convincing evidence for the presence of a covalently linked dimer in the HOCl-treated apomyoglobin. A small peak with a mass of 33962 Da was seen. However, its signal was only 5 % of that of the monomer peak, which is much less than the proportion of dimer seen by SDS/PAGE. Also, it had a mass exactly twice that of the 16981 Da peak, and is most probably a gas-phase $2M + 1$ species that did not dissociate during analysis.

Reversal of aggregation by succinylation

With no convincing evidence for covalent cross-linking, we investigated whether the polymer bands on SDS/PAGE represented the products of non-covalent interactions between protein chains that could be dissociated by treatment with succinic anhydride. Succinylation converts positively charged amines into negatively charged carboxylates, leading to increased electrostatic repulsion and expansion of the protein [34]. In the absence of succinylation, a typical pattern of aggregation was observed when apomyoglobin was treated with HOCl. However, when the protein was succinylated after HOCl treatment, the aggregates dissociated and the pattern of protein staining was similar to that seen when control protein was succinylated (Figure 8).

Comparison of HOCl and taurine chloramine treatment of apomyoglobin

To investigate whether methionine sulphoxide and protein carbonyl formation are critical for aggregation, apomyoglobin was treated with taurine chloramine. Treatment of proteins with chloramines has been shown to cause methionine oxidation but very little carbonyl formation [43]. We first established that this was the case with apomyoglobin. As shown by ESI-MS (Figure 7C), taurine chloramine caused the same mass increase as did HOCl, consistent with oxidation of the two methionine residues to sulphoxides. However, protein carbonyl formation at 15 min was only about 3 % of that seen with HOCl (Table 1). In contrast with HOCl, taurine chloramine caused very little aggregation of the apomyoglobin (Figure 9). After 15 min, there was no more dimer than in the control. An increase in intensity of the dimer band was just evident at 1 h with the 10:1 HOCl/protein ratio, coincident with an increase in carbonyl content (Table 1).

DISCUSSION

We have shown that HOCl caused aggregation of apohaemoglobin and apomyoglobin to give a regular series of oligomer bands on SDS/PAGE under reducing conditions. Aggregation required low amounts of HOCl, with dimers detectable at 0.5 mol of HOCl per mol of protein; maximum aggregation was seen at molar ratios of 10:1–20:1. Dimers were formed within 1 min, when all of the HOCl had been consumed, and further aggregation occurred over the next 30 min. Methionine slowed aggregation, suggesting the involvement of chloramines, at least in the latter stage of the process. However, it was not reversed by methionine or thiol reductants.

Of the amino acid residues that are susceptible to HOCl, the involvement of cysteine in aggregation can be ruled out, as it is not present in horse myoglobin. At a 5:1 molar ratio of HOCl to protein, evidence from MS indicated that both methionines were converted into sulphoxides. There was also rapid loss of approx. 20 % of the 20 amino groups in the protein and recovery of approx. 3 of the 5 mol of HOCl as chloramines. Therefore methionine sulphoxides and chloramines can account for the HOCl consumed. Losses of tryptophan and tyrosine were minor. Gradual decay of the chloramines during the course of the reaction was the likely source of the protein carbonyls that accumulated. From other studies [44–46] we would predict that the main source of carbonyls was the less stable chloramine formed on the N-terminus.

Based on the formation of distinct dimers and polymers of apohaemoglobin and apomyoglobin that withstood dissociation with SDS, we expected that aggregation by HOCl would involve covalent cross-linking. However, we found no evidence for this mechanism. There was little if any dityrosine formation, and the absence of cysteine involvement also excludes sulphinamide or sulphonamide bonds between oxidized thiols and ε-amine groups of lysine residues on different chains, as observed with model peptides and the S100A8 protein of neutrophils [26,27]. Covalent linkage via Schiff bases, as has been proposed [7,8], is also not supported by our data. These linkages should dissociate when treated with DNP [47], rather than the aggregate bands being retained, as we observed. We also saw no new products on amino acid or peptide analysis that could have arisen from a covalent link between two amino acids.

Other findings also point to aggregation being non-covalent. The strongest evidence in favour of this conclusion is the

dissociation of aggregated apomyoglobin when its negative charge was increased by succinylation. The resultant increase in electrostatic repulsion between chains was sufficient to allow the aggregates to break apart on SDS/PAGE. ESI-MS analysis, showing that almost all of the protein was monomeric and only a small peak at exactly twice the monomer mass, also supports a non-covalent linkage. SDS-resistant non-covalent aggregation has been observed for other proteins [48,49].

We propose that the mechanism of aggregation involves unfolding of the protein and an increase in surface hydrophobicity. This mechanism has been proposed for other oxidized proteins, and can be achieved through oxidation of methionine residues to sulphoxides [50–52]. Because the sulphoxides are more hydrophilic, they tend to move from internal positions to a more aqueous environment, exposing neighbouring hydrophobic residues [51]. However, generation of methionine sulphoxide alone is not sufficient to explain the aggregation of apomyoglobin. Despite conversion of both methionine residues into sulphoxides on treatment with HOCl, not all of the protein was aggregated. Protein carbonyls appear to play a critical role. Western blotting with an anti-DNP antibody produced the striking finding that protein carbonyls were present predominantly in the higher polymer bands. Furthermore, treatment with taurine chloramine, which caused methionine oxidation, produced few protein carbonyls and caused no aggregation. Carbonyl groups should increase hydrophobicity by replacing positively charged amines, and it may be a combination of protein carbonyl and methionine sulphoxide formation that leads to aggregation. However, it is likely that chloramines also contribute, particularly in the rapid phase of dimer formation. Little conversion into carbonyls would be expected during that time, and, in the case of apomyoglobin, the dimer band showed relatively little carbonyl staining. As chloramines are not charged at neutral pH, they should favour hydrophobic interactions. Also, chlorine exchange is slow, so few chloramine groups should form on the protein during exposure to taurine chloramine. A role for chloramines and/or carbonyls in aggregation is also implied from studies of LDL in which the blocking of amine groups caused inhibition [9].

We used the apo forms of myoglobin and haemoglobin to avoid HOCl reacting with the haem groups, but also observed aggregation of cyanmetmyoglobin. The higher concentrations of HOCl needed with cyanmethaemoglobin could be due to greater modification being required to disrupt the more ordered structure conferred by the haem groups. Many proteins have been shown to aggregate when treated with HOCl [6–15]. Covalent cross-linking has often been assumed, but the same mechanism as described here could apply. The resistance of some proteins to aggregation [16–18] could reflect a lesser degree of unfolding and exposure of hydrophobic regions.

Protein misfolding, as a result of either mutation or post-translational modification, is now well recognized as a pathological mechanism [20,21]. Aggregated proteins are formed during aging [53], in diabetes [21] and in neurodegenerative diseases, including Creutzfeldt–Jacob disease, Huntington's disease, Alzheimer's disease and Parkinson's disease [19,21,54]. Mechanisms exist to remove damaged, partially oxidized proteins. The exposure of hydrophobic residues as a result of oxidation-induced protein unfolding triggers degradation by the 20 S–26 S proteasome complex [55]. This is enhanced by the presence of a few oxidized amino acids [56], but highly aggregated proteins are poor substrates for proteolysis. They can impede clearance and result in the storage of other oxidized proteins [53].

Protein modification by HOCl has received most consideration in relation to atherosclerosis, where both LDL oxidation and myeloperoxidase are implicated [57]. Aggregation of apoprotein B could be responsible for the uptake of LDL by the scavenger receptor [8,9], and apoprotein A-I aggregation is associated with aberrant recognition and lipid processing by HDL [12,22]. HOCl-induced membrane lysis could also be a result of non-covalent protein aggregation [25]. However, the mechanism could have much wider implications in inflammatory conditions where HOCl is produced by the action of myeloperoxidase. Our findings that non-covalent aggregation is rapid and requires relatively few residues on the protein to be modified suggests that it could be one of the more favourable reactions of HOCl. Outcomes could be altered function and the accumulation of damaged protein at sites of inflammation.

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REFERENCES

1 Klebanoff, S. J. (1999) Oxygen Metabolites from Phagocytes. In Inflammation: Basic Principles and Clinical Correlates (Gallin, J. I. and Snyderman, R., eds.), pp. 721–768, Lippincott Williams & Wilkins, Philadelphia
2 Kettle, A. J. and Winterbourn, C. C. (1997) Myeloperoxidase: A key regulator of neutrophil oxidant production. Redox Rep. **3**, 3–15
3 Winterbourn, C. C. (1985) Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. Biochim. Biophys. Acta **840**, 204–210
4 Pattison, D. I. and Davies, M. J. (2001) Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds. Chem. Res. Toxicol. **14**, 1453–1464
5 Hazen, S. L., d'Avignon, A., Anderson, M. M., Hsu, F. F. and Heinecke, J. W. (1998) Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to oxidize alpha-amino acids to a family of reactive aldehydes. Mechanistic studies identifying labile intermediates along the reaction pathway. J. Biol. Chem. **273**, 4997–5005
6 Vissers, M. C. M. and Winterbourn, C. C. (1991) Oxidative damage to fibronectin. I. The effects of the neutrophil myeloperoxidase system and HOCl. Arch. Biochem. Biophys. **285**, 53–59
7 Matoba, T., Shiona, T. and Kito, M. (1985) Cross-linking of α_{s-1} casein by sodium hypochlorite. J. Food Sci. **50**, 1738–1744
8 Hazell, L. J., van den Berg, J. J. and Stocker, R. (1994) Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation. Biochem. J. **302**, 297–304
9 Hazell, L. J. and Stocker, R. (1993) Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. Biochem. J. **290**, 165–172
10 O'Connell, A. M., Gieseg, S. P. and Stanley, K. K. (1994) Hypochlorite oxidation causes cross-linking of Lp(a). Biochim. Biophys. Acta **1225**, 180–186
11 Bergt, C., Marsche, G., Panzenboeck, U., Heinecke, J. W., Malle, E. and Sattler, W. (2001) Human neutrophils employ the myeloperoxidase/hydrogen peroxide/chloride system to oxidatively damage apolipoprotein A-I. Eur. J. Biochem. **268**, 3523–3531
12 Bergt, C., Oettl, K., Keller, W., Andreae, F., Leis, H. J., Malle, E. and Sattler, W. (2000) Reagent or myeloperoxidase-generated hypochlorite affects discrete regions in lipid-free and lipid-associated human apolipoprotein A-I. Biochem. J. **346**, 345–354
13 Olszowski, S., Olszowska, E., Stelmaszynska, T., Krawczyk, A., Marcinkiewicz, J. and Baczek, N. (1996) Oxidative modification of ovalbumin. Acta Biochim. Pol. **43**, 661–672
14 Wu, S. M. and Pizzo, S. V. (1999) Mechanism of hypochlorite-mediated inactivation of proteinase inhibition by alpha 2-macroglobulin. Biochemistry **38**, 13983–13990
15 MacMillan-Crow, L. A., Crow, J. P. and Thompson, J. A. (1998) Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. Biochemistry **37**, 1613–1622
16 Hawkins, C. L. and Davies, M. J. (1998) Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation. Biochem. J. **332**, 617–625
17 Ullrich, O., Reinheckel, T., Sitte, N. and Grune, T. (1999) Degradation of hypochlorite-damaged glucose-6-phosphate dehydrogenase by the 20S proteasome. Free Radical Biol. Med. **27**, 487–492

18 Auchere, F. and Capeillere-Blandin, C. (2002) Oxidation of Cu, Zn-superoxide dismutase by the myeloperoxidase/hydrogen peroxide/chloride system: functional and structural effects. Free Radical Res. **36**, 1185–1198
19 Koo, E. H., Lansbury, P. T. J. and Kelly, J. W. (1999) Amyloid diseases: abnormal protein aggregation in neurodegeneration. Proc. Natl. Acad. Sci. U.S.A. **96**, 9989–9990
20 Lomas, D. A. and Carrell, R. W. (2002) Serpinopathies and the conformational dementias. Nat. Rev. Genet. **3**, 759–768
21 Horwich, A. (2002) Protein aggregation in disease: a role for folding intermediates forming specific multimeric interactions. J. Clin. Invest. **110**, 1221–1232
22 Pussinen, P. J., Metso, J., Keva, R., Hirschmugl, B., Sattler, W., Jauhiainen, M. and Malle, E. (2003) Plasma phospholipid transfer protein-mediated reactions are impaired by hypochlorite-modification of high density lipoprotein. Int. J. Biochem. Cell Biol. **35**, 192–202
23 Marsche, G., Hammer, A., Oskolkova, O., Kozarsky, K. F., Sattler, W. and Malle, E. (2002) Hypochlorite-modified high density lipoprotein, a high affinity ligand to scavenger receptor class B, type I, impairs high density lipoprotein-dependent selective lipid uptake and reverse cholesterol transport. J. Biol. Chem. **277**, 32172–32179
24 Jasin, H. E. (1988) Oxidative cross-linking of immune complexes by polymorphonuclear leukocytes. J. Clin. Invest. **81**, 6–15
25 Vissers, M. C. M., Carr, A. C. and Chapman, A. L. P. (1998) A comparison of human red cell lysis by hypochlorous and hypobromous acid: Insights into the mechanism of lysis. Biochem. J. **330**, 131–138
26 Raftery, M. J., Yang, Z., Valenzuela, S. M. and Geczy, C. L. (2001) Novel intra- and inter-molecular sulfinamide bonds in S100A8 produced by hypochlorite oxidation. J. Biol. Chem. **276**, 33393–33401
27 Fu, X., Mueller, D. M. and Heinecke, J. W. (2002) Generation of intramolecular and intermolecular sulfenamides, sulfinamides, and sulfonamides by hypochlorous acid: a potential pathway for oxidative cross-linking of low-density lipoprotein by myeloperoxidase. Biochemistry **41**, 1293–1301
28 Kettle, A. J. and Winterbourn, C. C. (1994) Assays for the chlorination activity of myeloperoxidase. Methods Enzymol. **233**, 502–512
29 Rossi-Fanelli, A., Antonini, G. and Caputo, A. (1958) Studies on the structure of haemoglobin. I. Physicochemical properties of human globin. Biochim. Biophys. Acta **30**, 608–615
30 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
31 Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W. and Weigele, M. (1972) Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. Science **178**, 871–872
32 Jarrett, H. W., Cooksy, K. D., Ellis, B. and Anderson, J. M. (1986) The separation of *o*-phthalaldehyde derivatives of amino acids by reversed-phase chromatography on octylsilica columns. Anal. Biochem. **153**, 189–198
33 Pichorner, H., Metodiewa, D. and Winterbourn, C. C. (1995) Generation of superoxide and tyrosine peroxide as a result of tyrosyl radical scavenging by glutathione. Arch. Biochem. Biophys. **323**, 429–437
34 Klotz, I. M. (1967) Succinylation. Methods Enzymol. **11**, 576–580
35 Levine, R. L., Williams, J. A., Stadtman, E. R. and Shacter, E. (1994) Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. **233**, 346–357
36 Buss, I. H., Chan, T. P., Sluis, K. B., Domigan, N. M. and Winterbourn, C. C. (1997) Protein carbonyl measurement by a sensitive ELISA method. Free Radical Biol. Med. **23**, 361–366
37 Brennan, S. O. (1997) Electrospray ionization analysis of human fibrinogen. Thromb. Haemostasis **78**, 1055–1058
38 Lee, C., Levin, A. and Branton, D. (1987) Copper staining: a five-minute protein stain for sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. **166**, 308–312
39 Qin, J., Fenyo, D., Zhao, Y., Hall, W. W., Chao, D. M., Wilson, C. J., Young, R. A. and Chait, B. T. (1997) A strategy for rapid, high-confidence protein identification. Anal. Chem. **69**, 3995–4001
40 Hawkins, C. L. and Davies, M. J. (1998) Reaction of HOCl with amino acids and peptides: EPR evidence for rapid rearrangement and fragmentation reactions of nitrogen-centred radicals. J. Chem. Soc. Perkin Trans. **2**, 1937–1945
41 Peskin, A. V. and Winterbourn, C. C. (2001) Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate. Free Radical Biol. Med. **30**, 572–579
42 Diedrich, D. L. and Schnaitman, C. A. (1978) Lysyl-derived aldehydes in outer membrane proteins of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. **75**, 3708–3712
43 Dalle-Donne, I., Rossi, R., Giustarini, D., Gagliano, N., Di Simplicio, P., Colombo, R. and Milzani, A. (2002) Methionine oxidation as a major cause of the functional impairment of oxidized actin. Free Radical Biol. Med. **32**, 927–937
44 Handelman, G. J., Nightingale, Z. D., Dolnikowski, G. G. and Blumberg, J. B. (1998) Formation of carbonyls during attack on insulin by submolar amounts of hypochlorite. Anal. Biochem. **258**, 339–348
45 Yang, C. Y., Gu, Z. W., Yang, H. X., Yang, M., Wiseman, W. S., Rogers, L. K., Welty, S. E., Katta, V., Rohde, M. F. and Smith, C. V. (1997) Oxidation of bovine beta-casein by hypochlorite. Free Radical Biol. Med. **22**, 1235–1240
46 Yang, C., Wang, J., Krutchinsky, A. N., Chait, B. T., Morrisett, J. D. and Smith, C. V. (2001) Selective oxidation in vitro by myeloperoxidase of the N-terminal amine in apolipoprotein B-100. J. Lipid Res. **42**, 1891–1896
47 Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A.-G., Ahn, B.-W., Shalteil, S. and Stadtman, E. R. (1990) Determination of carbonyl content of oxidatively modified proteins. Methods Enzymol. **186**, 464–478
48 Maida, V., Bennardini, F., Bonomi, F., Ganadu, M. L., Iametti, S. and Mura, G. M. (2000) Dissociation of human alphaB-crystallin aggregates by thiocyanate is structurally and functionally reversible. J. Protein Chem. **19**, 311–318
49 Sagne, C., Isambert, M. F., Henry, J. P. and Gasnier, B. (1996) SDS-resistant aggregation of membrane proteins: application to the purification of the vesicular monoamine transporter. Biochem. J. **316**, 825–831
50 Davies, K. J. A. and Delsignore, M. E. (1987) Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure. J. Biol. Chem. **262**, 9908–9913
51 Chao, C. C., Ma, Y. S. and Stadtman, E. R. (1997) Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. Proc. Natl. Acad. Sci. U.S.A. **94**, 2969–2974
52 Friguet, B., Stadtman, E. R. and Szweda, L. I. (1994) Modification of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Formation of cross-linked protein that inhibits the multicatalytic protease. J. Biol. Chem. **269**, 21639–21643
53 Stadtman, E. R. and Berlett, B. S. (1997) Reactive oxygen-mediated protein oxidation in aging and disease. Chem. Res. Toxicol. **10**, 485–494
54 DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P. and Aronin, N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science **277**, 1990–1993
55 Giulivi, C., Pacifici, R. E. and Davies, K. J. (1994) Exposure of hydrophobic moieties promotes the selective degradation of hydrogen peroxide-modified hemoglobin by the multicatalytic proteinase complex, proteasome. Arch. Biochem. Biophys. **311**, 329–341
56 Grune, T., Reinheckel, T. and Davies, K. J. (1997) Degradation of oxidized proteins in mammalian cells. FASEB J. **11**, 526–534
57 Podrez, E. A., Abu-Soud, H. M. and Hazen, S. L. (2000) Myeloperoxidase-generated oxidants and atherosclerosis. Free Radical Biol. Med. **28**, 1717–1725

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