Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation

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Stimulated monocytes and neutrophils generate hypochlorite (HOCl) via the release of the enzyme myeloperoxidase and hydrogen peroxide. HOCl damages proteins by reaction with amino acid side-chains or backbone cleavage. Little information is available about the mechanisms and intermediates involved in these reactions. EPR spin trapping has been employed to identify radicals on proteins, peptides and amino acids after treatment with HOCl. Reaction with HOCl gives both high- and low-molecular-mass nitrogen-centred, protein-derived radicals; the yield of the latter increases with both higher HOCl:protein ratios and enzymic digestion. These radicals, which arise from lysine side-chain amino groups, react with ascorbate, glutathione and Trolox. Reaction of HOCl-treated proteins with excess methionine eliminates radical formation, which is consistent with lysine-derived chloramines (via homolysis of N–Cl bonds) being the radical source. Incubation of HOCl-treated proteins, after removal of excess oxidant, gives rise to both nitrogen-centred radicals, over a period of hours, and time-dependent fragmentation of the protein. Treatment with excess methionine or antioxidants (Trolox, ascorbate, glutathione) protects against fragmentation; urate and bilirubin do not. Chloramine formation and nitrogen-centred radicals are therefore key species in HOCl-induced protein fragmentation.

INTRODUCTION

Activation of neutrophils, both *in vitro* and in certain diseases (e.g. inflammatory conditions, atherosclerosis), results in the production of highly reactive species, such as $O_2^{-\bullet}$ and H_2O_2, and the release of the enzyme myeloperoxidase [1]. Reaction of the latter with H_2O_2 in the presence of physiological concentrations of Cl^- results in the formation of the powerful oxidant HOCl [2]. This species has a pK_a of 7.59 [3], therefore a mixture of both the protonated form and $^-$OCl will be present at pH 7.4; HOCl is used below to designate this mixture. HOCl is believed to be the major oxidant produced by neutrophils under physiological conditions [2].

HOCl reacts with a wide variety of biological molecules and is known to cause tissue damage [4,5]. Reaction of HOCl with amino acids, peptides and proteins has been the subject of extensive study, and it has been demonstrated that such processes are important in both bacterial cell killing [6,7] and human disease [8–12]. Active myeloperoxidase has been identified in human atherosclerotic plaque tissue [11], as have HOCl-modified apolipoprotein B-100 [from low-density lipoproteins (LDLs)] and other proteins [13]. Treatment of proteins with HOCl results in direct oxidative damage to the protein (alteration of amino acid side-chains, protein fragmentation and dimerization) [9,10,12,14–17], and renders the protein more susceptible to degradation by proteolytic enzymes [8,18]. For example, exposure of fibronectin to HOCl results in changes to both the primary and tertiary structure of the protein and increases the susceptibility of the protein to degradation by elastase [16].

Certain amino acids are particularly susceptible to modification by HOCl. Tyr and Phe (free amino acids or in proteins) undergo ring chlorination, and these materials have been employed as markers of HOCl-induced damage [17,19]. Cysteine and methionine react rapidly to give oxyacids and cystine (from cysteine) and sulphoxides (from methionine) [5,20,21]. The latter type of reaction has been shown to inactivate α_1 proteinase inhibitor [22,23]. The free amino groups of lysine residues, which are present in many proteins at much higher levels than other reactive residues, and hence are major sites of attack, are converted into semi-stable chloramines. These species have been proposed to undergo hydrolysis to aldehydes which can form inter- or intra-molecular cross-links with free amine groups [10,15,24]. Hydrolysis of lysine side-chain chloramines on the apolipoprotein B-100 protein of LDL has been reported to induce LDL aggregation via intermolecular Schiff-base formation [10]. Reactions of chloramines may be of particular biological significance, as these materials would be expected to diffuse a considerable distance, and hence cause damage at remote sites, as a result of their longer lifetimes compared with the primary oxidants H_2O_2 and HOCl [25].

Apart from the formation of chloramines at lysine side-chains (and N-terminal amino groups), relatively little is known about the mechanism(s) of HOCl-mediated fragmentation and dimerization of proteins. Radicals have not generally been considered as important intermediates, although evidence has been presented for their involvement in HOCl-mediated inactivation of α_1 proteinase inhibitor, on the basis of the protective effect of the radical scavenger, butylated hydroxytoluene [23]. In this study, EPR spectroscopy with spin trapping has been employed to examine radical generation on reaction of HOCl with proteins and related materials. The effects of HOCl treatment on protein integrity have been examined by SDS/PAGE and protein carbonyl formation.

MATERIALS AND METHODS

The water used was filtered through a four-stage Milli Q system (Millipore-Waters, Lane Cove, New South Wales, Australia) equipped with a 0.2 μm-pore-size final filter. pH control was achieved by use of 50 mM phosphate buffer, pH 7.4. The amino acids, peptides, BSA, human serum albumin (HSA) (both fatty-

Abbreviations used: BCA, bicinchoninic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DNPH, 2,4-dinitrophenylhydrazine; HOCl, the physiological mixture of hypochlorous acid and its anion $^-$OCl; HSA, human serum albumin; LDL, low-density lipoprotein; TNB, 5-thio-2-nitrobenzoic acid.

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acid free) and other proteins were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), except Gly-Lys-Gly and Gly-Gly-Lys (Bachem, Switzerland), and the isotopically labelled lysine derivatives [Cambridge Isotope Laboratories (Andover, MA, U.S.A.) or C/D/N isotopes (Quebec, Canada)]. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO; Sigma Chemical Co.) was purified before use by treatment with activated charcoal. All other chemicals were of analytical reagent grade. HOCl solutions were prepared daily by dilution of a concentrated stock solution [approx. 1 M in 0.1 M NaOH (BDH, Poole, Dorset, U.K.)] into 50 mM phosphate buffer, pH 7.4; when concentrated solutions of HOCl were employed the pH was adjusted back to pH 7.4 if neccessary. HOCl concentrations were determined from the absorbance of the chloramine adduct generated with taurine (0.1 M) at 252 nm, based on a molar absorption coefficient of 429 $M^{-1} \cdot cm^{-1}$ [26]. Protein concentrations were determined using the bicinchoninic acid (BCA) assay as outlined previously [27]. Chelex treatment of solutions had no significant effect on the results obtained.

Enzyme degradation of proteins, which had been reacted with HOCl in the presence of the spin-trap DMPO, was carried out at 20 °C under aerobic conditions for 0–30 min using Pronase E (a mixture of proteases with different proteolytic activities, 2500 units $\cdot ml^{-1}$ final concentration; Boehringer Mannheim, Castle Hill, New South Wales, Australia).

Thiol-exchanged BSA was obtained by dissolving BSA (1.5 mM) in a solution of 3-mercaptopropionic acid (150 mM) with stirring for 15 min, before separation of the modified protein from excess thiol on a PD-10 Sephadex column (Pharmacia, Uppsala, Sweden) eluted with 50 mM phosphate buffer, pH 7.4. Reductive methylation of BSA, which has previously been shown to block reaction of lysine residues with HOCl [10], was carried out essentially as described previously [10,28]. Briefly, 50 μl of freshly prepared sodium borohydride (40 mg $\cdot ml^{-1}$) was added to 2 ml of BSA (10 mg $\cdot ml^{-1}$ in 0.2 M borate buffer, pH 9) at 4 °C before the addition of 14 aliquots of formaldehyde (18 %, v/v) at 5 min intervals. A further 10 μl of sodium borohydride was added after ten additions of formaldehyde. The BSA was separated from excess reagents using a PD-10 Sephadex column eluted with 50 mM phosphate buffer, pH 7.4. The proportion of modified amino groups present on the reductively methylated BSA was determined by fluorescamine fluorescence [29]. A solution containing 150 μl of BSA (2.5 mg $\cdot ml^{-1}$) in 1.5 ml of 50 mM phosphate buffer, pH 8, was vortex-mixed during the addition of 0.5 ml of fluorescamine (15 mg in 50 ml of acetone) and the fluorescence was measured ($\lambda_{excitation}$ 390 nm, $\lambda_{emission}$ 475 nm). The degree of blocking achieved was consistently between 80 and 85 %. This treatment did not affect the yield of free thiol groups present on the BSA as assayed using 5,5′-dithiobis-(2-nitrobenzoic acid) [30].

Proteins were separated by the method of Laemmli [31] using either 8 % (BSA samples) or 12 % (ribonuclease A samples) polyacrylamide gels. Protein samples were added to an equal volume of 60 mM Tris/HCl buffer, pH 6.8, containing glycerol (10 % v/v), 2-mercaptoethanol (5 % v/v), SDS (2 % w/v) and Bromophenol Blue (0.01 % w/v); these were then heated at 95 °C for 5 min before cooling and loading on to the gel. Bands were visualized using Coomassie Blue or silver staining. Gels were scanned using a Bio-Rad GelDoc 1000 system (Bio-Rad, Hercules, CA, U.S.A.), and the density of the parent protein band was determined over a linear range using the Bio-Rad molecular analysis software.

Carbonyl groups formed on BSA were quantified as described previously [32]. Briefly, an equal volume of 2,4-dinitrophenylhydrazine (DNPH; 10 mM in 2.5 M HCl) was added to a solution containing the oxidized protein (2.5 mg $\cdot ml^{-1}$); this was incubated in the dark for 15 min before the addition of trichloroacetic acid (20 % final concentration) and centrifugation to precipitate the protein. The supernatants were discarded and the protein pellet was washed three times with 1 ml portions of ethanol/ethyl acetate (1:1) to remove any free DNPH. The protein was re-suspended in 1 ml of guanidine HCl (6 M) at 37 °C for 15 min with vortex mixing. Carbonyl contents were determined from the maximum absorbance in the range 355–390 nm using a molar absorption coefficient of 22000 $M^{-1} \cdot cm^{-1}$ [32]. Protein concentrations after re-suspension in guanidine HCl were determined by the BCA assay and the carbonyl contents were corrected for any protein loss.

Chloramine formation was quantified by use of 5-thio-2-nitrobenzoic acid (TNB), as described previously [16]. TNB was prepared from 5,5′-dithiobis-(2-nitrobenzoic acid) (1 mM) by exposure to NaOH (0.1 M) for 2 min before dilution into 50 mM phosphate buffer, pH 7.4. BSA (40 μM) was incubated with HOCl (0.25–2.5 mM) for 15 min at room temperature before separation from excess HOCl on a PD-10 Sephadex column and addition to TNB at various time intervals, thus preventing reaction of unreacted HOCl with the TNB. The concentration of TNB remaining after reaction with BSA-derived chloramines was determined at 412 nm using a molar absorption coefficient of 13600 $M^{-1} \cdot cm^{-1}$ [33].

EPR spectra were recorded at room temperature using a Bruker EMX X-band spectrometer with 100 kHz modulation and either a standard rectangular (ER 4102ST) or cylindrical (ER 4103TM) cavity. Samples were contained in a standard, flattened, aqueous-sample cell and recording of spectra was initiated within 2 min of the start of the reaction, except when specified otherwise. Hyperfine couplings were measured directly from the field scan and confirmed, in the majority of cases, by spectral simulation using the program WINSIM [34]. The accuracy of such simulations was assessed by visual comparison with the experimental data, and by comparison of the calculated correlation coefficients between simulated and experimental data, which were > 0.95. Typical EPR spectrometer settings were: gain 2×10^6, modulation amplitude 0.1 mT, time constant 0.16 s, scan time 84 s, resolution 1024 points, centre field 347.5 mT, field scan 10 mT, power 25 mW, frequency 9.76 GHz, with four scans averaged.

RESULTS

Formation of radicals on reaction of HOCl with proteins

(i) Reaction with HSA and BSA

Reaction of either HSA or BSA (1.25 mM) with a 4-fold molar excess of HOCl in the presence of DMPO (200 mM) gave broad signals, due to slowly tumbling adducts, with some partially resolved isotropic splittings superimposed (Figure 1a). These signals were detected within 120 s after addition of HOCl (the dead time taken to fill the EPR cell and tune the spectrometer). The concentration (signal intensity) of these adducts decreased over a period of 60 min. No significant differences were observed on use of Chelex-treated solutions compared with untreated solutions, eliminating any possible contribution of trace metal ions to these reactions. In the absence of BSA or HSA, a signal from a single radical species, arising from direct reaction of HOCl with the spin trap, was detected as observed previously [35]. This species has significantly different hyperfine-coupling constants from those detected in the presence of the proteins.

The relatively stable protein-derived adducts observed with the complete reaction system were subsequently incubated with

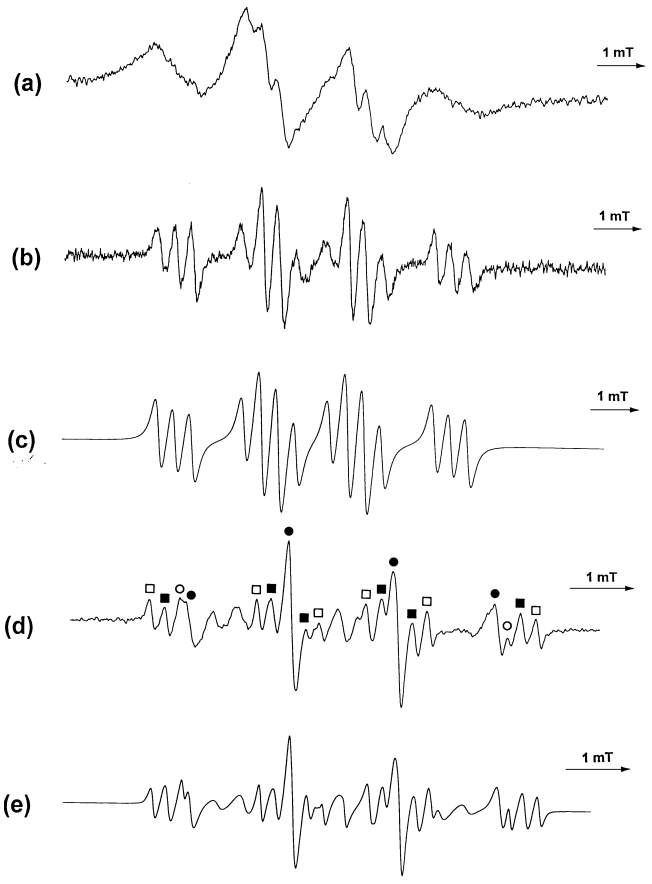


Figure 1 EPR spectra observed on reaction of HOCl with BSA in the presence of the spin-trap DMPO

(**a**) Complete system: HOCl (5 mM), BSA (1.25 mM), DMPO (200 mM). (**b**) As (**a**) except after incubation with Pronase E (0.2 mg/ml, 15 min). (**c**) Computer simulation of spectrum (**b**) using the hyperfine-coupling constants given in the text. (**d**) As (**a**) except with a 75:1 molar ratio of HOCl:BSA. Signals in (**a**) are assigned to large, protein-derived radical adducts. Signals in (**b**) are assigned to protein-derived nitrogen-centred radical adducts. Signals in (**d**) are attributed to two carbon-centred radicals (features marked □ and ■) together with a further unidentified radical adduct (○). These adducts are believed to arise from the trapping of radicals generated during fragmentation of the protein. Features marked (●) are assigned to DMPO-OH. Other (unmarked) absorption lines in the centre of spectrum are due to a breakdown product of the spin trap. (**e**) Computer simulation of spectrum (**d**) using the hyperfine-coupling constants given in the text.

the proteolytic enzyme Pronase E to release small fragments from the spin-trapped protein; these should give isotropic spectra as a result of their increased rates of tumbling in solution [36]. Incubation with this enzyme mixture (0.2 mg·ml^{-1}, 20 °C, 15 min) resulted in the detection of spectra with sharp (isotropic) lines, consistent with the release of low-molecular-mass spin adducts from the initial macromolecule adducts (Figure 1b). The resolution of these hyperfine couplings has allowed two different radical adduct species to be identified. One of these is the well-characterized DMPO-OH adduct [37]. The second has both β-hydrogen and β-nitrogen couplings [a(N) 1.50, a(H) 1.79, a(N) 0.29 mT]. This distinctive signal is assigned to a novel protein-derived nitrogen-centred spin adduct on the basis of the small 1:1:1 triplet from the second nitrogen atom (arising from the nuclear spin of this nucleus, $I = 1$), the relatively low value of the β-hydrogen coupling, which is indicative of the trapping of a heteroatom-centred radical, and the similarity of these parameters to those from other nitrogen-centred radicals [37,38]. Confirmation of the presence of these two adducts and their hyperfine-coupling constants was obtained by computer simulation of the experimental spectra obtained after treatment with Pronase E (Figure 1c).

In experiments where the protein concentration was kept constant at 400 μM and the HOCl concentration was varied between 1.6 mM and 40 mM (i.e. HOCl:protein molar ratios between 4:1 and 100:1) identical signals were detected, although higher HOCl concentrations gave better-resolved (more isotropic) spectra. This is ascribed to an increased extent of protein damage, with protein fragmentation resulting in a greater yield of low-molecular-mass material. Somewhat similar behaviour was observed when the HOCl concentration was kept constant (5 mM) and the protein concentration decreased (i.e. the ratio of HOCl to protein increased). Sharper, more-resolved signals were again observed, but at high HOCl:protein ratios ($> 50:1$) further radical adduct species were also detected. These additional features, which were most intense at ratios of approx 75:1, have been assigned to two carbon-centred adducts [a(N) 1.55, a(H) 2.03 mT and a(N) 1.57, a(H) 2.45 mT] and a further adduct from which only the outer lines could be discerned [a(N) approx. 1.54, a(H) 1.58 mT] (Figure 1d). These assignments and coupling constants were confirmed by computer simulation (Figure 1e). These carbon-centred radicals are believed to arise from fragmentation of the protein induced by HOCl.

(ii) Protein blocking experiments

Thiol groups are known to react rapidly with HOCl [4,5], so the effect of blocking the free thiol group (Cys-34) on BSA and HSA was examined using a thiol-exchange method employing 3-mercaptopropionic acid ($HSCH_2CH_2CO_2H$). EPR studies on reaction mixtures containing thiol-exchanged BSA (0.5 mM), HOCl (5 mM) and DMPO (200 mM) gave spectra identical with, but more intense than, those observed with native BSA. No signals were observed with 3-mercaptopropionic acid treated with HOCl in the presence of DMPO but in the absence of protein. These results suggest that the radicals observed with HOCl-treated BSA do not arise from reaction of HOCl at the free thiol group. No EPR signals from thiyl radical adducts to DMPO were detected, although it is known that such species can be detected using this trap [39]. The increased radical adduct concentration on blocking the thiol group implies that some reaction does occur at the thiol group (as observed in previous studies with cysteine and GSH with a stoichiometry of 2–4 HOCl molecules consumed per thiol group [5,6]) and that this results in a lower concentration of HOCl available to react at the sites which give rise to the species observed with native BSA.

Lysine residues are major targets for HOCl, with this reaction giving semi-stable chloramine (N–Cl) species via nucleophilic reaction [40]. The formation of chloramines and their potential role in the generation of the observed radicals was investigated by: (1) chemical blocking of the lysine amino groups (by reductive methylation); and (2) removal of the chloramine groups by reaction with excess methionine (50 mM) [4] before addition of the spin trap. In both cases only very weak signals were observed. These observations suggest that the terminal side-chain amino groups of lysine residues react with HOCl to give chloramines which subsequently generate the novel nitrogen-centred radicals detected with DMPO.

(iii) Time course of chloramine formation and decay

Confirmation of the formation and subsequent decomposition of chloramines on BSA upon treatment with HOCl was obtained

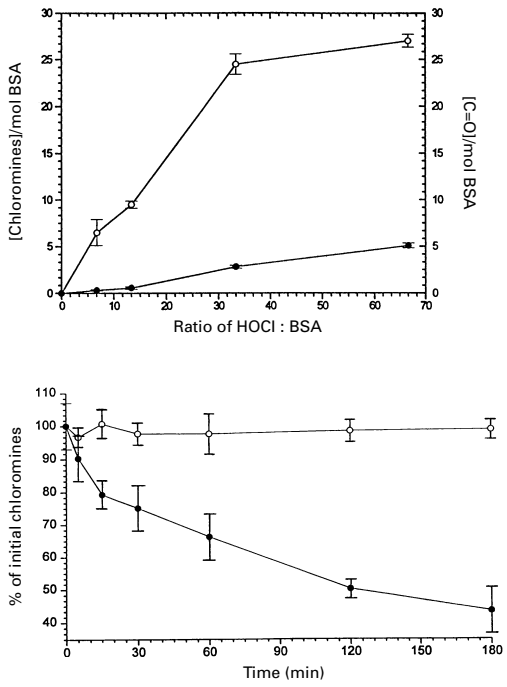


Figure 2 Formation and decay of chloramines and generation of carbonyl compounds

Chloramine formation and decay were measured using the TNB assay and carbonyl formation was assessed as DNPH-derivatives, as outlined in the Materials and methods section. Top: chloramine formation (○) and carbonyl generation (●) on BSA (40 μM) after treatment with HOCl (0.25–2.5 mM). Data are means (±S.D.) of triplicate determinations. Bottom: decay of chloramines formed on BSA (40 μM) after treatment with HOCl (2 mM) and subsequent separation of the protein from excess oxidant on a Sephadex PD-10 column. Samples were subsequently incubated at 4 °C (○) or 37 °C (●) and chloramine levels were assayed at the times indicated. Data are mean values (±S.D.) of six determinations.

using the TNB assay (see the Materials and methods section), with the treated protein being separated from any excess HOCl (which also oxidizes TNB) before the assay. Chloramines were detected at all HOCl:BSA ratios tested (Figure 2, top), with this process being particularly efficient at lower molar ratios (< 40:1, HOCl:BSA). The plateau in chloramine yield at higher ratios is ascribed to reaction of HOCl with other (non-lysine) sites which do not give chloramines. The decay of these species, once generated, was also investigated at two different temperatures: 4 °C and 37 °C (Figure 2b). Decay was rapid under the latter conditions, but not the former.

(iv) Time course of radical formation

The time course of radical formation from BSA-derived chloramines was investigated by adding DMPO at various time points after BSA had been treated with HOCl (HOCl:BSA molar ratio of 50:1, samples kept at room temperature under laboratory light). Protein-derived, nitrogen-centred radical adduct formation could still be detected by EPR up to 6 h after mixing the protein and HOCl, although the signal intensity became progressively weaker over this time period. A more rapid loss in the intensity of the spin adduct signals was observed when the HOCl-treated BSA was kept at 37 °C, as expected from the results shown in Figure 2 (bottom).

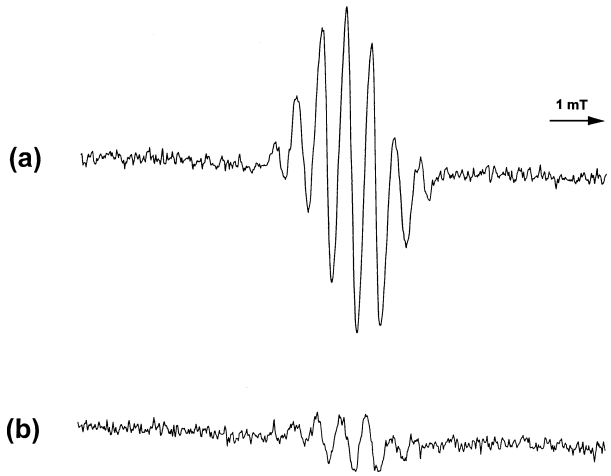


Figure 3 EPR spectra observed on addition of Trolox to reaction mixtures containing BSA and HOCl

(**a**) Trolox (0.4 mM) was added to reaction mixtures containing BSA (0.3 mM) and HOCl (7 mM). (**b**) As (**a**), except in the presence of 25 mM methionine. Signals are assigned to the Trolox phenoxyl radical.

(v) Formation of protein carbonyls

Previous studies have shown that chloramines can hydrolyse to give aldehydes and ammonia [10,20,24]. The extent of this (non-radical) process, which presumably competes with the above radical reactions, was assessed by quantifying carbonyl groups on HOCl-treated BSA using DNPH (see the Materials and methods section). Reaction of BSA (40 μM) with increasing amounts of HOCl (0.25–2.5 mM) resulted in increasing yields of carbonyl groups (Figure 2a). However, even when high molar ratios of HOCl:protein were employed (> 50:1), and with long incubation periods (60 mins) before carbonyl quantification, only low yields of carbonyls were obtained (approx. 5 mol/mol of protein). Under all circumstances the concentration of chloramines produced (and subsequently lost) on the protein was found be significantly higher than the concentration of carbonyl groups generated. This suggests that (non-radical) hydrolysis of protein-derived chloramines to produce aldehydes is not the major reaction pathway under the conditions employed in this study.

(vi) Effect of antioxidants

Addition of the radical scavenger Trolox (0.4 mM) to reaction mixtures containing BSA (0.3 mM) and HOCl (7 mM), in the absence of the spin-trap DMPO, resulted in the observation of an intense seven-line EPR signal, which is attributed to the Trolox-derived phenoxyl radical (Figure 3a) [41]. This signal was only observed in the presence of all of the components of the reaction mixture and was not observed when HOCl was incubated (for up to 60 min) with Trolox alone. Direct oxidation of Trolox by HOCl to the phenoxyl radical does not therefore occur to any major extent under these conditions. The intensity of the signal obtained with the complete system was significantly reduced when excess methionine (25 mM) was added before the addition of Trolox (Figure 3b). The formation of this phenoxyl radical is ascribed to oxidation of Trolox by radicals derived from the protein chloramines.

Analogous behaviour was observed when ascorbate (25 mM) was added to BSA (2.5 mM) which had been previously treated with excess HOCl (25 mM, i.e. HOCl:protein 10:1) and excess oxidant removed on a Sephadex column. In these experiments strong signals from the ascorbyl radical were detected; control experiments with untreated BSA gave only weak signals. In experiments where GSH was incubated with the HOCl-treated BSA, DMPO (200 mM) was also added. With low concentrations of GSH (0.5–10 mM), a dose-dependent loss of the BSA-derived radical adducts was observed. With higher concentrations of GSH, a further signal assigned to the glutathione thiyl radical was detected {with parameters a(N) 1.54, a(H) 1.62 mT as described previously [37]}. Control samples with untreated BSA gave only weak signals. In both cases the detection of antioxidant-derived signals is ascribed to oxidation of the antioxidant by radicals formed on HOCl-treated BSA as a result of decomposition of chloramine species. In contrast, when urate (saturated solution) was added, no significant loss of the BSA-derived radicals was observed.

Experiments were also carried out where the HOCl-pretreated BSA was incubated for various periods of time before addition of Trolox. Quantitatively similar Trolox phenoxyl radical signals were observed when Trolox was added either immediately after separation of the protein from excess oxidant, or 3 h after initiation of the reaction with the samples kept at 20 °C. In contrast, a significant reduction in the Trolox phenoxyl radical concentration was observed with samples kept at 37 °C for 3 h.

(vii) Reaction with other proteins

The formation of lysine-derived nitrogen-centred radicals on reaction of HOCl with other proteins was also investigated in order to examine whether this is a general phenomenon. Reaction of histone H1 with HOCl (HOCl:protein molar ratio of 5:1) gave a sharp isotropic EPR signal together with a broader anisotropic signal. The hyperfine-coupling constants of the former are very similar to those assigned to the nitrogen-centred radical detected with BSA and HSA [a(N) 1.46, a(H) 1.79, a(N) 0.29 mT] and are therefore assigned to a similar, or identical, species. The major signal detected with this protein was much more isotropic (i.e. the radical is tumbling more rapidly) than those observed with HSA and BSA. This may reflect the smaller overall size of this protein and/or attack on the lysine-rich mobile N- and C-termini of this protein. The broad, anisotropic signal may reflect similar types of reaction on the globular core of the protein. No further species were observed on increasing or decreasing the HOCl:protein ratio.

Similar behaviour to that observed with HSA and BSA was detected with other proteins (myoglobin, ribonuclease A, and histones H2B and H3), although the intensity of the nitrogen-centred radical signal varied. In many cases the initial signals were somewhat broad in nature and were more clearly resolved after addition of Pronase E. With other proteins (histone H4, melittin) only weak or no (lysozyme, protamine) nitrogen-centred radical adduct signals were observed. In some cases (e.g. histone H4) other signals assigned to carbon-centred radical adducts [a(N) 1.54, a(H) 2.02 mT] were observed. The variation in signal intensity between proteins appears to depend, at least in part, on the lysine content of the protein; those with the highest number of lysine residues give the most intense spectra from the nitrogen-centred radical adduct. Thus lysine residues appear to be a major site of reaction of HOCl with many proteins, with this process yielding chloramine species, which subsequently decompose to give nitrogen-centred radicals.

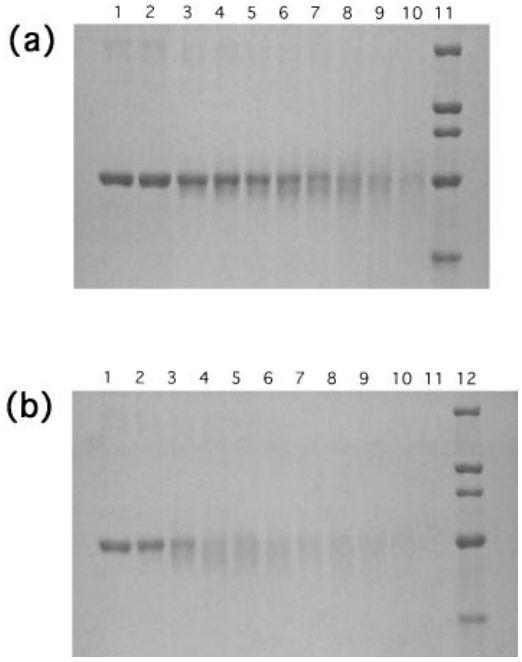


Figure 4 SDS/PAGE gels of (a) BSA and (b) reductively methylated BSA treated with HOCl

Increasing amounts of HOCl give rise to an increasing extent of fragmentation of BSA as shown by the loss in the parent band; this occurs at lower HOCl:BSA ratios with the reductively methylated BSA than with native BSA. (**a**) Lane 1, BSA (15 μM) alone; lanes 2–10, BSA (15 μM) treated with a 10-, 50-, 60-, 70-, 80-, 90-, 100-, 125- and 150-fold molar excess of HOCl respectively; lane 11, molecular-mass markers. (**b**) Lane 1, reductively methylated BSA (15 μM) alone; lanes 2–11, reductively methylated BSA (15 μM) treated with a 10-, 25-, 50-, 60-, 70-, 80-, 90-, 100-, 125- and 150-fold molar excess of HOCl respectively; lane 12, molecular-mass markers. Gels were developed with Coomassie Blue.

Effect of HOCl on protein integrity

(i) SDS/PAGE studies of fragmentation

The effect of HOCl treatment on protein integrity was assessed by SDS/PAGE. BSA was treated with various concentrations of HOCl and subsequently incubated (20 °C, laboratory light) for 15 min. Excess methionine (50 mM) was then added to remove excess HOCl or unreacted chloramine species before electrophoresis. Reductively methylated BSA samples were also examined, as were samples generated on reaction of BSA with a fixed concentration of HOCl and subsequently treated with excess methionine, either immediately or at time intervals up to 6 h.

Significant loss of the parent protein band (as indicated by either Coomassie Blue or silver staining) was only observed with HOCl:protein ratios $> 70:1$ when short incubation periods were employed before addition of the methionine (Figure 4a). Fragmentation does not appear to be completely random, as there are indications of discrete protein fragments at lower molecular mass, although there is also evidence of smearing of the bands, particularly at very high HOCl:protein ratios. Addition of excess methionine immediately after treatment of the protein with HOCl, rather than after 15 min, gave essentially identical results, indicating that fragmentation is rapid at high HOCl:protein ratios. A progressive increase in fragmentation of the protein (as assessed by loss of the parent protein band) was observed with low HOCl:protein ratios as the incubation time of the protein with HOCl increased. This suggests that the fragmentation of the

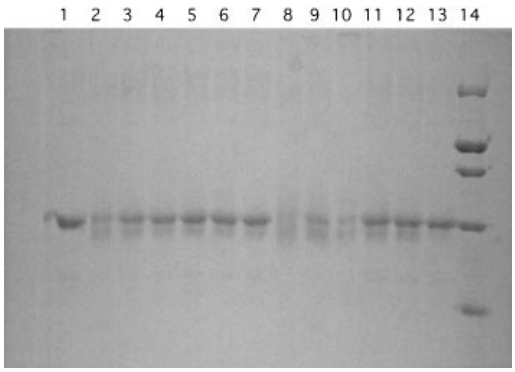


Figure 5 SDS/PAGE of BSA exposed to two different concentrations of HOCl in the presence of various amounts of Trolox

Increasing amounts of Trolox result in a reduction in the fragmentation of the BSA, as shown by the increase in the staining of the parent band at both concentrations of HOCl employed. Lane 1, BSA (15 μM) alone; lanes 2–7, BSA (15 μM) treated with a 75-fold molar excess of HOCl in the presence of 0, 1.5, 3.75, 6, 7.5 and 9 mM Trolox respectively; lanes 8–13, BSA (15 μM) treated with a 100-fold molar excess of HOCl in the presence of 0, 1.5, 3.75, 6, 7.5 and 9 mM Trolox respectively; lane 14, molecular-mass markers. Samples were incubated at 20 °C for 15 min before addition of 50 mM methionine to remove excess chloramines and halt reaction. Samples (10 μg/well) were subsequently loaded on to gel and run as described in the Materials and methods section. The gel was developed with Coomassie Blue.

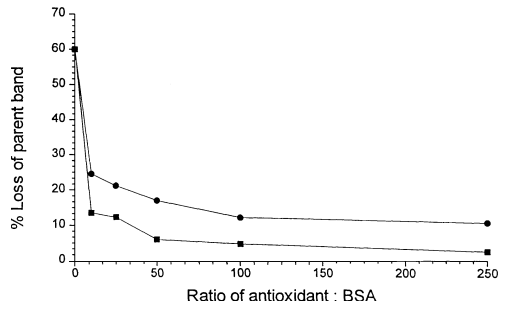


Figure 6 Protective effect of the antioxidants GSH and ascorbate against the HOCl-mediated fragmentation of BSA

HOCl-treated BSA (HOCl : BSA molar ratio 50 : 1, with the treated protein separated from excess HOCl on a Sephadex PD-10 column) was incubated at 37 °C in the presence of either GSH (■) or ascorbate (●) at the stated antioxidant : BSA ratios. Reaction was stopped after 180 min by addition of excess methionine (50 mM) to remove unreacted chloramines, then protein samples (10 μg/well) were loaded on to gels and subjected to electrophoresis. The extent of fragmentation was determined by measuring the loss of the parent band after staining with Coomassie Blue (see the Materials and methods section). Data are from a single experiment.

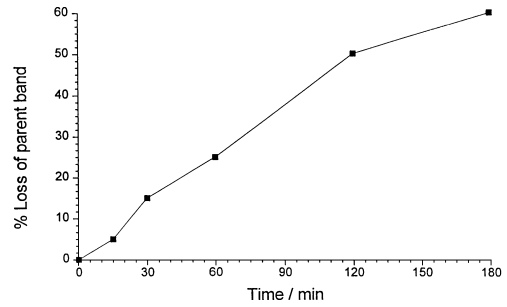


Figure 7 Effect of increasing incubation time on the extent of BSA fragmentation induced by BSA-derived chloramines

BSA treated with HOCl (HOCl : protein molar ratio 50 : 1) was separated from any excess HOCl on a Sephadex PD-10 column and incubated at 37 °C. After the indicated times, excess methionine (50 mM) was added to remove unreacted chloramines and stop the reaction. Protein samples were loaded (10 μg/well) on to gels, subjected to electrophoresis and developed with Coomassie Blue. Loss of the parent protein band was subsequently quantified as described in the Materials and methods section. Data are from a single experiment.

protein backbone can arise from rapid direct reaction (probably at backbone amide groups) at high HOCl : protein ratios and via a slower process involving lysine-derived chloramines at low HOCl : protein ratios. The HOCl : protein molar ratio ($> 70 : 1$) needed to generate efficient initial fragmentation correlates well with the number of side-chain sites in this protein that are believed to react rapidly with HOCl ($1 \times$ Cys, $4 \times$ Met, $2 \times$ Trp, $59 \times$ Lys; 66 in total).

When reductively methylated BSA was employed (80–85 % of the lysine residues blocked; see the Materials and methods section), significant fragmentation was observed at much lower HOCl : protein ratios (approx. 25 : 1) (Figure 4b). This suggests that reaction at lysine (and possibly other reactive) side-chains occurs preferentially over attack at the backbone and that this limits rapid backbone fragmentation; it is likely, however, to result in side-chain alterations which would not be detectable on these gels. When these lysine side-chain sites are blocked by methylation, a greater extent of reaction occurs at other sites (including backbone amide groups), which subsequently give rise to rapid backbone cleavage. This hypothesis was tested with ribonuclease A which has only ten lysine residues and four other HOCl-reactive side-chains (4 Met) compared with the 59 Lys (and 66 total HOCl-reactive side-chains) in BSA. With this protein, significant fragmentation of the protein backbone was observed at HOCl : protein molar ratios of $\geqslant 15 : 1$.

(ii) Effect of antioxidants

Radical-scavenging antioxidants might be expected to exert a protective effect against protein fragmentation by scavenging HOCl-derived radicals (see above). Addition of Trolox to BSA before HOCl (HOCl : BSA molar ratio 75 : 1) afforded significant protection (as assessed by loss of the parent band) at a molar ratio of Trolox : protein $\geqslant 250 : 1$ (Figure 5). Addition of Trolox after HOCl also provided protection, although this was less efficient (results not shown). Similar protection was observed with GSH and ascorbic acid (Figure 6); in these experiments the HOCl-treated BSA was separated from excess HOCl by Sephadex chromatography before addition of GSH or ascorbate in order to prevent any direct reaction of HOCl with these agents. No protection was observed with either urate (at BSA : urate molar ratios $\leqslant 1 : 500$) or bilirubin (BSA : bilirubin molar ratio 1 : 1 or 1 : 2). These data support the hypothesis that HOCl-mediated protein fragmentation is radical-mediated and inhibitable by some, but not all, putative antioxidants.

(iii) Role of chloramine-derived radicals in fragmentation

The role of chloramine-derived radicals in fragmentation was investigated in studies where HOCl-treated BSA (HOCl : BSA molar ratio 50 : 1, i.e. under conditions where most attack would be expected to occur with reactive side-chains) was separated from excess HOCl on a Sephadex column and then incubated for various periods at 37 °C. Excess methionine was then added to remove unreacted chloramines and stop further reaction. Protein fragmentation was observed under these conditions (Figure 7) and increased with increasing incubation time over the period when chloramine decay was detected (cf. Figure 2b). Excess

Table 1 Parameters of radical adducts observed on reaction of HOCl with lysine and related materials in the presence of DMPO

*$\pm$0.01 mT; †a broad signal is also observed with this material.

Substrate	Hyperfine-coupling constant (mT)*			Assignment
	a(N)	a(H)	a(other)	
Lysine	1.49	1.82	0.29 (1 N)	N-centred radical (ϵ-NH_2)
$^{15}N(\epsilon)$-Lysine	1.49	1.85	0.38 (1 ^{15}N)	N-centred radical (ϵ-$^{15}NH_2$)
$N(\alpha)$-Acetyl-lysine	1.49	1.85	0.29 (1 N)	N-centred radical (ϵ-NH_2)
$N(\epsilon)$-Acetyl-lysine	1.60	2.54		C-centred radical
	1.56	2.19		C-centred radical
Gly-Lys	1.49	1.86	0.29 (1 N)	N-centred radical (ϵ-NH_2)
Gly-Gly-Lys	1.48	1.85	0.29	N-centred radical (ϵ-NH_2)
Gly-Lys-Gly	1.50	1.83	0.30	N-centred radical (ϵ-NH_2)
Polylysine†	1.50	1.89	0.29	N-centred radical (ϵ-NH_2)
Ornithine	1.49	1.82	0.30 (1 N)	N-centred radical (ϵ-NH_2)
Taurine	1.46	1.79	0.33 (1 N)	N-centred radical

methionine afforded protection, suggesting that chloramine-derived radicals can induce fragmentation. This fragmentation is less efficient than that observed with higher ratios of HOCl. The preferential reaction of HOCl at lysine side-chains, when these are available for reaction, therefore appears to both slow and decrease, but not halt, fragmentation induced by HOCl.

Reaction of HOCl with peptides and amino acids

(i) Lysine and derivatives

Reaction of free lysine (80 mM) with HOCl (8 mM) in the presence of DMPO (100 mM) gave an intense signal with coupling constants very similar to those detected with proteins (see Table 1 and Figure 8a); this signal is assigned to a nitrogen-centred radical adduct. All components of the reaction mixture were required for observation of this signal. No further species were detected when the reactant concentrations were varied over a large range; less intense signals were observed at lower HOCl concentrations. The presence or absence of oxygen had no effect on these signals. Addition of excess methionine (100 mM) after treatment with HOCl, but before addition of DMPO, resulted in the loss of the EPR signals; this is consistent with chloramines being the precursors of the nitrogen-centred radical. Confirmation that this species is a nitrogen-centred radical adduct on the side-chain amino group was obtained using [^{15}N]lysine. Replacement of the ^{14}N nucleus with ^{15}N at the side-chain amino group would be expected to change the splitting from this nucleus from a relatively small 1:1:1 triplet splitting into a larger 1:1 doublet as a result of the different nuclear spin ($I = 0.5$) of the ^{15}N nucleus. This proved to be the case with a triplet of doublets of doublets signal [a(N) 1.49, a(H) 1.85, a(^{15}N) 0.4 mT] observed on reaction with HOCl in the presence of DMPO (Figure 8c). These assignments were confirmed by computer simulation of the experimental spectra (Figures 8b and 8d).

Nitrogen-centred radical adduct signals were also observed with N(α)-Ac-Lys, Gly-Lys, Gly-Lys-Gly and Gly-Gly-Lys on treatment with HOCl (see Table 1). N(ϵ)-Ac-Lys, where the side-chain amino group is blocked, gave only weak signals from carbon-centred radicals. No signals were detected with N(α)-, N(ϵ)-di-Ac-Lys. Experiments with polylysine resulted in the detection of both weak isotropic, nitrogen-centred radical adducts, and broader signals believed to be due to the same species present on the (slowly tumbling) polymer.

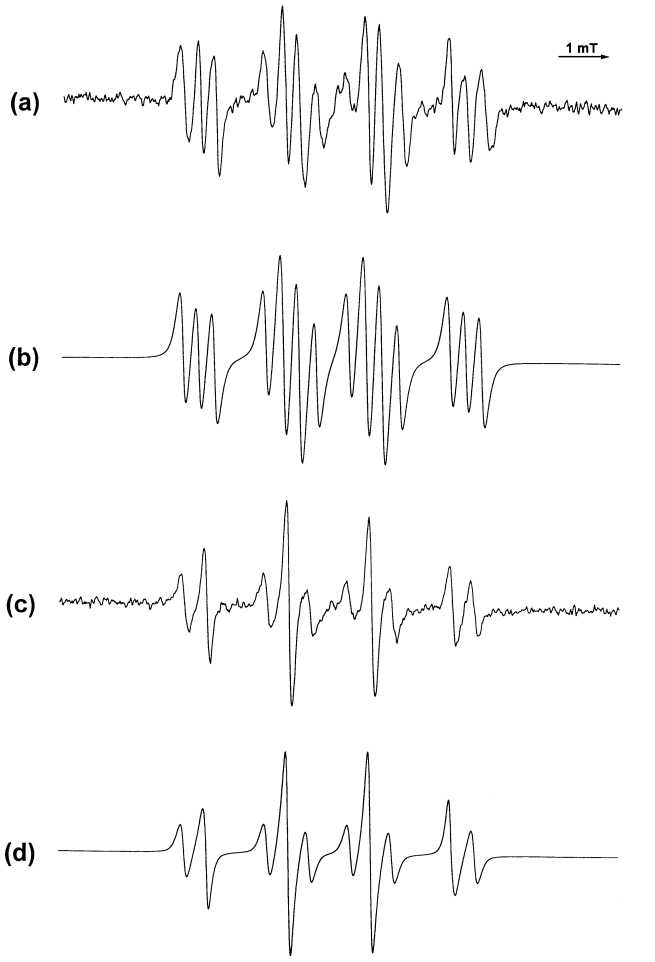


Figure 8 EPR spectra observed on reaction of lysine and $^{15}N(\varepsilon)$-lysine with HOCl in the presence of DMPO

(**a**) Lysine (80 mM) was treated with HOCl (8 mM) in the presence of DMPO (100 mM). The signal is assigned to a mixture of the nitrogen-centred radical adduct formed on the side-chain amino group and DMPO-OH. (**b**) Computer simulation of (**a**) using the parameters in Table 1. (**c**) As (**a**), except with $^{15}N(\epsilon)$-lysine (80 mM) in place of lysine. The signal is assigned to a mixture of the [^{15}N]nitrogen-centred radical adduct formed on the side-chain amino group and DMPO-OH. (**d**) Computer simulation of (**c**) using the parameters in Table 1.

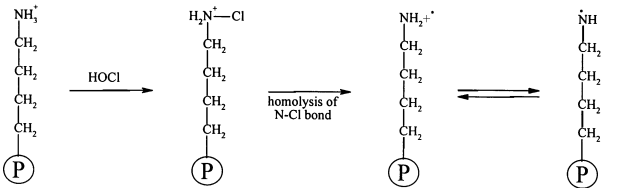
(ii) Other amino acids and derivatives

Replacement of lysine with the shorter side-chain analogue ornithine gave a nitrogen-centred radical adduct on treatment with HOCl (see Table 1). These signals were less intense than with lysine and could only be detected with 200 mM DMPO. Taurine behaved similarly (see Table 1). No nitrogen-centred radical adducts were detected with any other amino acid examined (His, Trp, Asn, Gln, Arg, Glu, Pro, Phe, Gly, Ala, Met, Cys), with the exception of Tyr. With most substrates, carbon-centred radicals were detected; the identity and mechanisms of formation of these species will be discussed in a future publication. With free Tyr, the formation of the nitrogen-centred radical adduct [a(N) 1.46, a(H) 1.79, a(N) 0.29 mT] was found to require the presence of the free N(α) amino group. No nitrogen-centred radical adducts were detected with N-Ac-Tyr or Gly-Tyr, ruling out the Tyr side-chain as the source of the nitrogen-centred radical adducts observed with the above proteins.

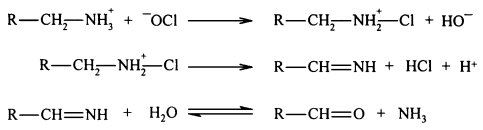
DISCUSSION

Reaction of HOCl with a number of proteins (HSA, BSA, myoglobin, ribonuclease A and some histones) has been shown to give rise to radicals which have been detected as spin adducts with DMPO. This observation is in contrast with previous studies which have reported that HOCl reacts by two-electron reactions without the involvement of radicals [4,10,16,24]. These radicals are observed over a wide range of HOCl concentrations and are protein-derived, as evidenced by the absence of these signals when protein was omitted from the reaction mixture, the partially anisotropic EPR signals and the increase in motion of these adducts on treatment with Pronase E. This digestion procedure has allowed the radicals to be identified as novel nitrogen-centred species on the basis of the additional (1:1:1) nitrogen coupling observed in the EPR spectra. Blocking the lysine residues on BSA by reductive methylation results in the loss of these signals, demonstrating that these radicals are formed from the terminal free amino group of lysine residues (see Scheme 1). Higher radical concentrations are observed with proteins with higher lysine contents when identical HOCl:protein molar ratios are employed. No signals from any other side-chain-derived radicals have been detected; this probably arises, at least in part, from the high mole-% of lysine side-chains in these proteins.

The observation of DMPO-OH in many of these systems is thought to arise from processes other than direct trapping of HO$^{\bullet}$. Although previous studies have suggested that homolytic or metal-ion-catalysed processes (pseudo-Fenton reactions) can give rise to the formation of HO$^{\bullet}$ from HOCl [42], other studies have presented evidence inconsistent with this proposal (e.g. [43,44]). In the studies reported here, the identical behaviour of Chelex-treated compared with untreated solutions, the nature of the substrate-derived species and the observed selectivity of attack are inconsistent with the formation of free HO$^{\bullet}$. Previous studies with a wide variety of proteins have shown that HO$^{\bullet}$ reacts to give carbon-centred and thiyl radicals (from cysteine residues) [39,45], but not nitrogen-centred radicals. No evidence was obtained in the present study for the formation of carbon-centred radicals, except at very high HOCl concentrations. Under the latter conditions the carbon-centred radicals detected have been ascribed to species arising from protein fragmentation induced by protein-derived chloramines and chloramides. Similarly, the radicals detected with the peptides and free amino acids are very different from those observed in previous studies with HO$^{\bullet}$, where, for example, reaction with lysine side-chains occurs by hydrogen-atom abstraction primarily from C-4 and C-5 [46]. The DMPO-OH is therefore likely to arise from one of the many processes which can indirectly give rise to this species. One possibility is direct oxidation of DMPO to the radical-cation with subsequent hydration [47]. A second alternative is via formation and subsequent hydrolysis of the Cl$^{\bullet}$ adduct to the



Scheme 1 Formation of chloramines from reaction of HOCl with lysine side-chains on proteins and their decomposition to give nitrogen-centred radicals



Scheme 2 Formation of protein carbonyls from chloramines via (non-radical) hydrolysis (from [16])

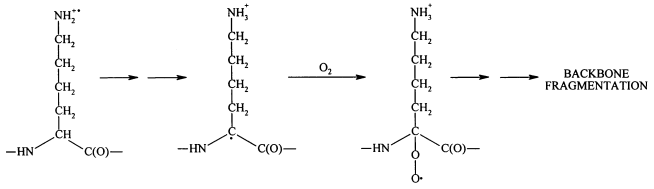
spin trap, with Cl$^{\bullet}$ arising from homolysis (thermal or photochemical) of N–Cl bonds (Scheme 1). A previous study [48] has shown that DMPO-Cl undergoes very rapid hydrolysis (via S_N1 or S_N2 processes) in aqueous solution (but not in organic solvents [49]) to give DMPO-OH.

The protein-derived nitrogen-centred radicals are believed to arise from the decomposition of lysine-derived chloramines, as removal of these semi-stable species (with excess methionine) inhibits radical formation. Analagous behaviour is observed with lysine-containing peptides, polylysine and the free amino acid. The formation of chloramines is rapid, as assessed by the TNB assay. Radical signals are also observed within minutes of initiation of reaction, suggesting that some chloramines must decay rapidly, though chloramines can still be detected after several hours at room temperature. This rapid formation and slower decay has been observed previously with model compounds by monitoring of the chloramine UV absorbance band at approx. 250 nm [24,25,44]. Thus radical formation from HOCl-treated BSA has been detected up to 6 h after treatment with HOCl. The rate of chloramine decomposition, and hence radical formation, is, however, enhanced at higher temperatures, supporting the suggestion that these radicals arise via thermal homolysis of the N–Cl bond.

Treatment of proteins with HOCl also induces both carbonyl formation (presumed to be primarily at side-chain sites) and protein fragmentation. The former process, which may arise from the hydrolysis of chloramines via a non-radical pathway (e.g. Scheme 2), accounts for $\leqslant 10\,\%$ of the added HOCl over a wide range of HOCl concentrations. Such processes may however be biologically significant, as they can result in inter- or intra-molecular cross-links via Schiff-base formation [10].

Protein fragmentation appears to occur via two separate processes: direct reaction with HOCl when the concentration of HOCl exceeds the concentration of reactive side-chain sites, and via subsequent reactions of chloramine-derived radicals. The extent of protein fragmentation observed with a particular protein appears to depend on the molar ratio of HOCl to reactive side-chains. Thus a much lower excess of HOCl is required to induce significant protein fragmentation for BSA on which the lysine side-chain sites have been blocked by reductive methylation, than for untreated BSA. Similarly, ribonuclease A (with 14 HOCl-reactive side-chain residues) fragments more readily than BSA (66 HOCl-reactive side-chains) at fixed HOCl:protein molar ratios. This fragmentation is ascribed to direct reaction of excess HOCl with the backbone in a process which can be ameliorated by the addition of excess methionine. The latter observation suggests that backbone chloramides [i.e. *R*-C(O)-NCl-CH*R*′-C(O)-*R*″] may be involved.

The detection of significant levels of backbone fragmentation of BSA after extended incubation periods with molar ratios of HOCl:protein $< 50:1$, where reaction would be expected to occur primarily with side-chain sites, is interpreted in terms of a role for lysine side-chain radicals in the generation of backbone damage. This hypothesis is supported by the observation that removal of chloramine species with excess methionine inhibits



Scheme 3 Generation of α-carbon backbone radicals by nitrogen-centred radicals present on lysine side-chains, and subsequent backbone fragmentation

this process. This reaction may involve inter- or intra-molecular transfer of damage from the side-chain amino nitrogen to the backbone (Scheme 3). This reaction probably involves hydrogen-atom abstraction from an α-carbon site (from the same lysine or possibly nearby residues). α-Carbon radicals are known precursors of backbone cleavage [39,50,51]. This transfer to particular α-carbon sites may explain the modest selectivity of fragmentation.

The protection against backbone fragmentation afforded by Trolox, ascorbate and GSH (as assessed by SDS/PAGE), and the detection of radicals from these materials by EPR spectroscopy, is in accord with protein fragmentation being mediated by radicals. Trolox, ascorbate and GSH, but not urate or bilirubin, are therefore protein antioxidants. These compounds are, however, relatively inefficient antioxidants, as high concentrations are required to give significant protection against fragmentation. This may be due to steric and electronic interactions which prevent the ready approach of these materials to the side-chain or backbone radicals involved in fragmentation [50,51]. This inefficiency is reinforced by the observation that protein-derived radicals can be detected in plasma treated with low concentrations of HOCl, even when endogenous antioxidants are present (C. L. Hawkins and M. J. Davies, unpublished work).

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