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Hypochlorite-induced oxidation of amino acids, peptides and proteins

Review Article

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Summary. Activated phagocytes generate the potent oxidant hypochlorite (HOCl) via the release of the enzyme myeloperoxidase and hydrogen peroxide. HOCl is known to react with a number of biological targets including proteins, DNA, lipids and cholesterol. Proteins are likely to be major targets for reaction with HOCl within a cell due to their abundance and high reactivity with HOCl. This review summarizes information on the rate of reaction of HOCl with proteins, the nature of the intermediates formed, the mechanisms involved in protein oxidation and the products of these reactions. The predicted targets for reaction with HOCl from kinetic modeling studies and the consequences of HOCl-induced protein oxidation are also discussed.

Keywords: Hypochlorite – Myeloperoxidase – Protein oxidation – Chloramines – Chloramides – Radicals – Oxidative stress – Fragmentation

Abbreviations used: BSA, bovine serum albumin; CMP, cytidine 5′-monophosphate; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance spectroscopy; GC-MS, gas chromatography-mass spectrometry; GSH, reduced glutathione; HOCl, the physiological mixture of hypochlorous acid and its anion hypochlorite; HSA, human serum albumin; LDL, low-density lipoprotein; *N*-Ac, *N*-acetyl group; NMR, nuclear magnetic resonance; RNHCl, alkyl chloramine; RR′NCl, dialkyl chloramine; TMP, thymidine 5′-monophosphate; UMP, uridine 5′-monophosphate

Formation of HOCl

Activation of phagocyte cells both *in vivo* and *in vitro* is known to result in the generation of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{-\bullet}$) via a respiratory burst, and the release of the heme enzyme myeloperoxidase (Weiss and LoBuglio, 1982). This enzyme catalyses the reaction of H_2O_2 with physiological concentrations of Cl^- ions to give the potent oxidant HOCl (Kettle and Winterbourn, 1997). HOCl plays a major role in killing bacteria and invading pathogens *in vivo* (Thomas, 1979; Weiss and LoBuglio, 1982). However, excessive or misplaced generation of HOCl can cause tissue damage (Jesaitis and Dratz, 1992; Winterbourn and Kettle, 2000), and this process is believed to be important in the progression of a number of diseases including atherosclerosis, chronic inflammation and some cancers (Weitzman and Gordon, 1990; Heinecke, 1999). HOCl, once formed, reacts readily with a wide variety of biological molecules including proteins, DNA (Prutz, 1996), lipids (Winterbourn et al., 1992), cholesterol (Carr et al., 1996), NADH (Prutz, 1996) and free thiols and disulfides (Prutz, 1996; Winterbourn and Brennan, 1997).

Summary of HOCl reactions with biomolecules excluding proteins

Reaction with DNA is known to result in the formation of short-lived chloramine (RNHCl, RR′NCl) species (Hayatsu et al., 1971; Hawkins and Davies, 2001b) which can lead to the dissociation of double stranded DNA due to the disruption of hydrogen-bonding (Prutz, 1998a). The kinetics of reaction of HOCl with individual nucleosides has been determined (Prutz, 1996; Prutz, 1998a), and it has been shown that the base moiety is the major target. Reaction with both exocyclic (RNH_2) and heterocyclic (RNHR′) amine functions to give chloramines are the major primary processes, with reaction at the latter more rapid than at the former (Prutz, 1996; Prutz, 1998a). Evidence has also been presented for chlorine transfer between these sites, particularly from the heterocyclic species to the more stable exocyclic sites (Prutz, 1996; Prutz, 1998a). These chloramines appear to undergo both one- and two-electron decay

routes, with the former resulting in the generation of nitrogen-centered radicals. This process is stimulated at elevated temperatures and in the presence of metal ions. The chemistry of the resulting radicals has been explored in some detail (Hawkins and Davies, 2001b; Hawkins and Davies, 2002). The heterocyclic chloramines formed with thymidine and guanosine are also readily repaired by thiols and other primary amines via chlorine transfer with regeneration of the parent compound (Prutz, 1998b). In addition to these reactive chloramines, a number of stable chlorinated products, including 5-chlorocytosine, 5-chlorouracil, 8-chloroadenine and 8-chloroguanosine have been characterized (Whiteman et al., 1997; Henderson et al., 1999; Masuda et al., 2001).

Chlorohydrins are formed on addition of HOCl to the double bonds of unsaturated fatty acids and cholesterol, and these can be readily detected by GC-MS (reviewed (Winterbourn and Kettle, 2000)). These products can have physiological effects including destabilization of membrane structures and cytotoxicity, but the rate of formation of these materials is relatively slow, and hence these materials are relatively minor products in most situations (Carr et al., 1996; Carr et al., 1997; Vissers et al., 2001; Pattison et al., 2003).

Some antioxidants react rapidly with HOCl and rate constants have been determined for reduced glutathione (GSH), ascorbate, some phenols and hydroquinones (Folkes et al., 1995; Prutz, 1996; Pattison et al., 2003). Reaction with GSH and related thiols and thio-ethers is fast and is discussed further below. Reaction with ascorbate occurs with k_2 ca. $6 \times 10^6\,M^{-1}\,s^{-1}$ (Folkes et al., 1995), whereas reaction with the phenols and hydroquinones that have been examined are somewhat slower (k_2 ca. $50\,M^{-1}\,s^{-1}$ Pattison et al., 2003).

In most biological systems, however, reaction with these alternative targets, with the exception of reaction with GSH and possibly ascorbate, is likely to be uncompetitive with reaction with amino acids, peptides and proteins as a result of their abundance and their high reactivity with HOCl (Pattison and Davies, 2001). The treatment of isolated proteins with HOCl is known to result mainly in the alteration of amino acid side-chains, with some protein fragmentation and cross-linking/aggregation also observed. These data are reviewed below.

Kinetics of reaction of HOCl with amino acids, peptides and proteins

The reactions of HOCl with proteins have been studied extensively. The majority of the studies have focused on the products formed, their structural effects, and their pathological consequences. Which materials are formed is determined to a major extent by the rates of reaction of HOCl with the individual components (e.g. side-chain groups, backbone) of proteins.

The rates of some of the reactions of HOCl with amino acids and biological substrates have been determined by competitive kinetic studies and stopped flow methods (Winterbourn, 1985; Armesto et al., 1993; Antelo et al., 1995a; Folkes et al., 1995; Prutz, 1996; Prutz, 1998a; Armesto et al., 2000; Pattison and Davies, 2001). In 1985, Winterbourn determined the relative reaction rates of selected amino acids with HOCl by competitive reaction with monochlorodimedon (Winterbourn, 1985). The order of reactivity was determined as Cys > Met > Cystine > His > Ser > Leu, but the specific sites of reaction (α-amino *vs.* side-chain) were not determined. More recently, the rates of reaction of HOCl with Met and Cys have been reported in basic aqueous solutions (pH > 10) (Armesto et al., 2000). These authors have also studied the mechanistic details of the reactions of various amines with HOCl at different pH values by stopped-flow techniques (Armesto et al., 1993; Antelo et al., 1995b; Armesto et al., 1998). They conclude that ^-OCl is more reactive than the protonated acid (HOCl), and reacts most readily with protonated amine groups ($R-NH_3^+$). Thus, maximal rates of reaction occur at pH values (usually pH ~ 8.5) that are the average of the pK_a values for HOCl and the amine. Recently, second-order rate constants have been determined for the reactions of HOCl (at physiological pH, 7.4, in aqueous solution) with all of the potential reactive sites within a protein (Pattison and Davies, 2001). The order of reactivity for the various side-chains was found to be: Met > Cys ≫ Cystine ~ His ~ α-amino > Trp > Lys ≫ Tyr ~ Arg > Gln ~ Asn (Table 1) (Pattison and Davies, 2001). The second-order rate constants for

Table 1. Second-order rate constants determined for the reactions of HOCl with amino acid side-chain groups, α-amino groups and backbone amides (Pattison and Davies, 2001)

Residue	$k_2/M^{-1}\,s^{-1}$	Residue	$k_2/M^{-1}\,s^{-1}$
Met	3.8×10^7	Lys	5.0×10^3
Cys	3.0×10^7	Tyr	44
Cystine	1.6×10^5	Arg	26
His	1.0×10^5	Backbone amides	$< 10^a$
α-amino	1.0×10^5	Asn	0.03
Trp	1.1×10^4	Gln	0.03

[a] The second-order rate constant for backbone amides varies over several orders of magnitude, depending on the environment. This value is a maximal estimate based on studies with cyclic dipeptides

HOCl with several models of the protein backbone were also determined, and the rates were highly dependent on the peptide bond environment (Pattison and Davies, 2001). Cyclic dipeptides reacted most rapidly with HOCl (k ca. $20\,M^{-1}\,s^{-1}$ per amide group), but incorporation of a charged side-chain reduced the reactivity approximately 10 fold. Similar studies with *N*-acetyl blocked amino acids, di- and tri- peptides showed a similar charge dependency on the rates of reaction, but the rates were generally much slower (k, 10^{-3}–$10^{-1}\,M^{-1}\,s^{-1}$) (Pattison and Davies, 2001). The reactivities of amide bonds in the tripeptide models were greater than for the blocked amino acids as the mid-chain amides are less deactivated than the terminal amides. Previous studies on the reaction of HOCl with *N*-methylacetamide ($k \sim 0.05\,M^{-1}\,s^{-1}$ at pH 6.3) (Antelo et al., 1995b) and *N*-Ac-Ala (k, $1.6 \times 10^3\,M^{-1}\,s^{-1}$ at pH 7.0) (Jensen et al., 1999) yielded similar rate constants. Overall these data indicate that the majority of reaction, particularly with low excesses of HOCl, occurs primarily with side-chain (and the N-terminal α-amino group) rather than backbone sites.

In addition to direct attack of HOCl on reactive protein components, protein oxidation can also occur via secondary processes. Several kinetic studies have been undertaken to determine the reactivities of secondary species (e.g. chloramines) with potential reactive sites of proteins (Table 2) (Prutz, 1996; Prutz, 1998a; Peskin and Winterbourn, 2001) and other biomolecules (e.g. DNA, NADH and antioxidants; Table 3) (Prutz, 1998a; Prutz, 1999; Prutz et al., 2000). The rates of reaction of taurine and glycine chloramines with several thiols have been investigated, and these are more than five orders of magnitude slower than thiol oxidation by HOCl (Table 2) (Peskin and Winterbourn, 2001). The reaction rates for taurine chloramine became more rapid as the pK_a of the thiols decreased (Peskin and Winterbourn, 2001). This phenomenon may have important implications in determining which protein thiol groups are oxidized by low concentrations of chloramines. The rates of oxidation of other secondary targets by chloramines formed on pyrimidine nucleotides have also been studied (Prutz, 1999). Those formed on the heterocyclic RR′NH positions of thymidine and uridine derivatives have been found to oxidize GSH, disulfide bonds, free amine groups (of taurine and Gly-Gly-Gly) and peptide amide bonds (cyclo-$(Gly)_2$) with rates only 10–100 times slower than HOCl itself (Prutz, 1998a; Prutz, 1999). Conversely, chloramines formed on the exocyclic RNH_2 positions of cytidine derivatives reacted only with GSH, at rates ca. 10^4 times slower than HOCl. Similar studies (Prutz, 1999) have shown that chlorine transfer from the chloramide of cyclo-$(Gly)_2$ to GSH, Met, and disulfide bonds also occurs, but the rates are between 10^3 and 10^6 times slower than with HOCl, depending on the substrate (Table 2).

Table 2. Summary of some of the apparent second-order rate constants determined for oxidation reactions mediated by chloramines/amides

Chloramine/amide	Target substrate	$k_2/M^{-1}\,s^{-1}$
Taurine chloramine[a]	Cys	205
	GSH	115
	Met	39
Gly chloramine[a]	Cys	350
	GSH	228
	Met	197
N-Ac-Lys chloramine[a]	Cys	479
	GSH	259
	Met	52
Cyclo-$(Gly)_2$ chloramide[b]	GSH	$>5 \times 10^4$
	Met	275

[a] From (Peskin and Winterbourn, 2001). [b] From (Prutz, 1999)

Table 3. Summary of some of the apparent second-order rate constants determined for oxidation reactions mediated by chloramines of pyrimidine nucleotides

Chloramine	Target substrate	$k_2/M^{-1}\,s^{-1}$
CMP (exocyclic)	GSH	2×10^3
	Linear $(Gly)_3$	No reaction
TMP (heterocyclic)	GSH	4.3×10^6
	Linear $(Gly)_3$	5.8×10^3
UMP (heterocyclic)	GSH	4.7×10^6
	Linear $(Gly)_3$	1.4×10^4

[a] From (Prutz, 1998a)

Kinetic modeling of HOCl-mediated protein oxidation

The kinetic data obtained for the reactions of HOCl with the reactive sites of proteins have been incorporated into a computational model for HOCl-mediated protein oxidation (Pattison and Davies, 2001). The model can be used as a guide to predicting the effect of HOCl oxidation on any protein for which the amino acid composition is known. The model incorporates the second-order rate constants determined for individual reactive sites of the protein, together with the amino acid composition of the protein of interest, to provide a prediction of the percentage of HOCl reaction with various protein components, and the extent of depletion of each reactive site, with varying excesses of HOCl.

The predictive value of the model is potentially limited by the assumption that all reactive sites are equally accessible to attack; this is unlikely to be the case in globular proteins where some reactive residues (e.g. Met and Trp) are typically buried in hydrophobic regions of the protein. Likewise, the kinetics of HOCl reactions with backbone amide model compounds are known to be affected by the presence of charged groups in their vicinity (Pattison and Davies, 2001), a phenomenon which cannot be readily incorporated into the computational model. Similarly, some residues, such as Cys and His, have variable pK_a values depending on their environment, and their rates of reaction with HOCl have been shown to vary with their pK_a values (Pattison and Davies, 2001; Peskin and Winterbourn, 2001). Again, these effects cannot be readily accounted for in the model.

Despite the limitations described above, the predicted reactivity of HOCl matches experimental data remarkably well (Pattison and Davies, 2001). Protein fragmentation of BSA has been observed experimentally with a 70-fold excess of HOCl (Hawkins and Davies, 1998b), and the model predicts that significant backbone attack (a presumed prerequisite for fragmentation) starts to occur at similar molar excesses (Pattison and Davies, 2001). Similarly, the order of depletion of residues in HSA and human plasma treated with increasing excesses of HOCl are adequately predicted by the computational model, as are the chloramine yields formed on histone proteins (Pattison and Davies, 2001; Hawkins et al., 2002).

In addition to reconciling experimentally observed data, the model has been used to predict the levels of HOCl that may be present *in vivo*. It was assumed, from modeling predictions, that an approximately 20-fold molar excess of HOCl was required over the lifetime of tissue proteins, to achieve the levels of 3-chlorotyrosine observed in atherosclerotic plaques (Hazen and Heinecke, 1997). This led to an estimate of the total HOCl concentration to which proteins are exposed over their biological lifetime of ca. 50 mM, assuming the protein concentration in plasma is $\sim$2.5 mM (Vander et al., 1980). This approach may overestimate HOCl exposure *in vivo*, as the model does not account for secondary reactions such as chlorine transfer from chloramines (Domigan et al., 1995), which might result in the formation of additional 3-chlorotyrosine (see below).

Overall however, this computational model provides a simple, but effective method of predicting which residues in a protein (or more complex systems, such as the nucleosome (Hawkins et al., 2002)) are likely to be oxidized by HOCl (Pattison and Davies, 2001). Further developments of the model to include secondary reactions of, for example, chloramines, will make this a more useful tool, and work towards this goal is in progress.

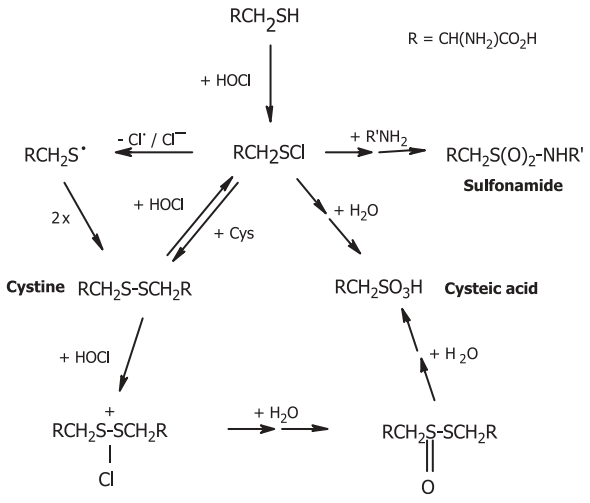
It should also however be borne in mind that the above arguments may not be applicable in cases where HOCl is not produced in free solution and has ready access to all reactive sites. Evidence has been presented for the association/binding of myeloperoxidase, the major source of HOCl *in vivo*, with various biological molecules including extra-cellular matrix (Britigan et al., 1996; Daphna et al., 1998), and low-density lipoproteins (Yang et al., 1999; Carr et al., 2000). This arises from the highly basic nature of this protein and hence the resultant affinity with negatively charged macromolecules such as some highly negatively charged glycosaminoglycans (Daphna et al., 1998). This may result in ''site-specific'' HOCl formation and subsequent damage to a different set of targets to those that would be predicted on kinetic grounds for HOCl itself; some evidence has been presented for this type of selective damage (Yang et al., 1999).

Products of HOCl reactions with amino acid side-chains

The oxidation of proteins by HOCl can lead to side-chain modifications that do not result in fragmentation or aggregation. Many of these reactions occur via two-electron oxidation processes with suitable electron-rich (nucleophilic) centers such as thiols, sulfides, amines and to a lesser extent amides and aromatic rings. Some of these modifications are well characterized (e.g. sulfur-containing residues, Tyr), while the identities of other products are ambiguous (e.g. Trp) or have not been studied in any detail (e.g. His, Arg). The nature of these oxidized side-chain products will be discussed in turn below, in the approximate order of their reactivity with HOCl (Pattison and Davies, 2001).

Sulfur-containing side-chains; Cys, Met and Cystine

The reactions of HOCl with Cys proceed via chlorination of the thiol group to yield unstable sulfenyl chloride intermediates that undergo further reactions with nucleophiles (Pereira et al., 1973; Drozdz et al., 1988; Fu et al., 2002). In aqueous solutions the sulfenyl chloride can react with water, to ultimately form cysteic acid (RSO_3H; Scheme 1) (Pereira et al., 1973; Drozdz et al., 1988). The mechanism of hydrolysis is complex and appears to involve a series of reactions with sulfenic (RSOH) and sulfinic (RSO_2H)

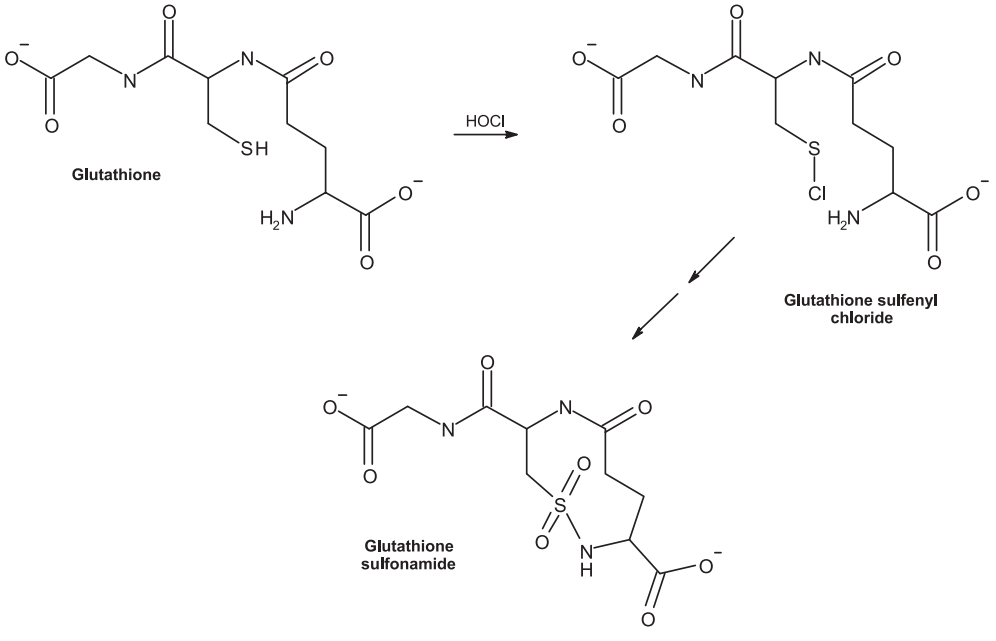


Scheme 1. Summary of the mechanisms and products of oxidation of Cys and Cystine residues by HOCl

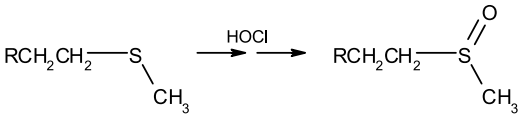
acids as intermediates (Savige and Maclaren, 1966; Capozzi and Modena, 1974).

In the presence of other Cys side-chains, the sulfenyl chloride intermediates can react with the thiol group of a second Cys molecule to generate the disulfide, cystine (Scheme 1) (Pereira et al., 1973; Drozdz et al., 1988). Cystine can also be oxidized by HOCl, possibly via a sulfenyl chloride intermediate $RS^{+}(Cl)SR$, to ultimately give rise to more cysteic acid via hydrolysis to RS(=O)SR (Scheme 1) (Savige and Maclaren, 1966; Pereira et al., 1973; Capozzi and Modena, 1974; Drozdz et al., 1988). Oxidation of model thiol (5-thio-2-nitrobenzoic acid; TNB) and disulfide (5,5′-dithiobis(2-nitrobenzoic acid); DTNB) compounds by HOCl yielded analogous products (Silverstein and Hager, 1974).

It has recently been shown that sulfenyl chloride intermediates can react with nitrogen centers, via two-electron processes to yield further products (Winterbourn and Brennan, 1997; Pullar et al., 2001; Raftery et al., 2001; Fu et al., 2002). The guanidine groups of Arg side-chains, α-amino groups, and side-chain amines of Lys are all capable of reacting with sulfenyl chlorides to form sulfenamide (RSN-) crosslinks. Further reactions of sulfenamides with oxygen yield sulfinamides (RS(O)N–) and sulfonamides ($RS(O)_2N$–) (Fu et al., 2002). These products have been identified in *in vitro* studies of small model peptides (Fu et al., 2002) and intact proteins (Raftery et al., 2001) by electrospray MS techniques. Winterbourn et al., have shown that glutathione sulfonamide is generated following treatment of GSH with HOCl (Winterbourn and Brennan, 1997; Pullar et al., 2001). In this case an intramolecular condensation is believed to occur between a sulfonyl chloride intermediate (RSO_2Cl) of the Cys side-chain, and the amino group of the glutamyl side-chain (Scheme 2). It has been proposed that glutathione sulfonamide may be a specific marker of HOCl oxidation



Scheme 2. Mechanism of formation of glutathione sulfonamide following treatment of GSH with HOCl



Scheme 3. Mechanism of Met oxidation mediated by HOCl to yield methionine sulfoxide

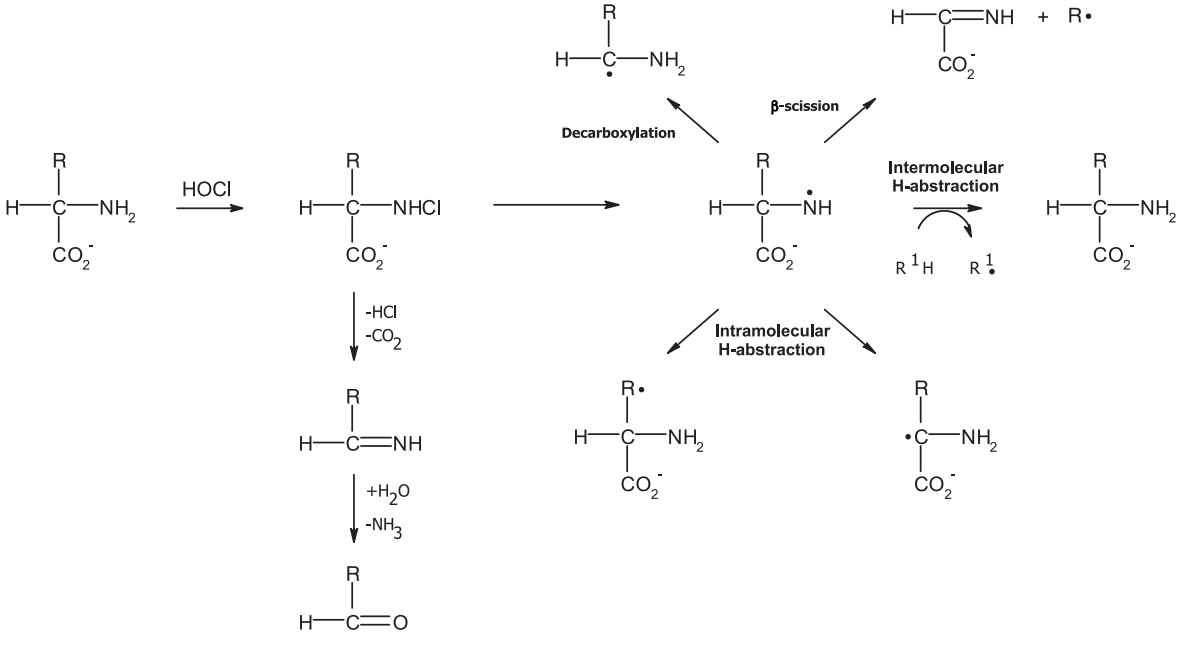
in biological systems, but further studies are required to determine the specificity of this product (Winterbourn and Brennan, 1997; Pullar et al., 2001).

Reaction of HOCl with thiols has also been shown to result in the generation of thiyl radicals (Davies and Hawkins, 2000). Evidence has been presented for the involvement of sulfenyl chlorides (RSCl) in these reactions, with the thiyl radicals arising from thermal (or metal ion catalyzed) decomposition of the S–Cl bond (Scheme 1). This process may compete with the other processes outlined above particularly when the thiol group is isolated on, or buried within, a protein and is unable to undergo ready reaction with a second thiol molecule or hydrolysis. The importance of this type of reaction remains to be established.

The oxidation of the Met side-chain by HOCl is less complex, with methionine sulfoxide (Scheme 3) the only product observed even with a 2.5 fold molar excess of HOCl over *N*-acetyl-Met (Drozdz et al., 1988; Naskalski, 1994). Methionine sulfoxide is a stable product of Met oxidation, and does not appear to undergo further reaction other than slow enzymatic reduction (e.g. by methionine sulfoxide reductase) to regenerate the starting material (Brot et al., 1981; Vogt, 1995; Moskovitz et al., 2001). It has been suggested that surface-exposed Met residues may act as a sacrificial antioxidant against oxidant (including HOCl-mediated) damage to proteins (Levine et al., 1999; Levine et al., 2000). However the slow rate of repair of this lesion, and the observation (see below) that such damage may result in (irreversible) unfolding of the protein suggests that this may not be an efficient defense mechanism.

Amines; α-amino groups and Lys

The reactions of amines, such as the Lys side-chain or terminal α-amino groups, with HOCl leads to the formation of unstable mono- and (with high excesses of HOCl) di-chloramines ($RNCl_2$ species) (Winterbourn, 1985; Thomas et al., 1986b; Folkes et al., 1995; Armesto et al., 1998; Hawkins and Davies, 1998b). These products are mild oxidizing agents, and can transfer chlorine to other substrates, regenerating the parent amine in the process (Weiss et al., 1983; Thomas et al., 1986b). In the absence of further oxidizable substrates, chloramines situated at an α-amino site decompose to yield aldehydes (e.g. Zgliczynski et al., 1968; Zgliczynski et al., 1971; Armesto et al., 1993; Anderson et al., 1997; Hazen et al., 1998a; Fu et al., 2000). This is believed to occur, for the free amino acids, via decarboxylation of chloramines to form unstable imines that undergo hydrolysis to aldehydes with the loss of ammonia (Scheme 4).



Scheme 4. Rearrangement reactions of amino acid nitrogen-centered radicals

The resulting ammonium ions may undergo further reaction with excess HOCl to generate monochloramine (NH_2Cl). These reactions have been shown to occur with the majority of free amino acids, to generate a family of aldehydes (Van Tamelen et al., 1968; Zgliczynski et al., 1968; Zgliczynski et al., 1971; Hazen et al., 1996a; Hazen et al., 1998a; Hazen et al., 1998b). These aldehydes, which include species such as glycolaldehyde, are able to undergo further reactions with proteins, particularly Schiff base formation with free amino groups (e.g. Lys side-chains) (Anderson et al., 1997; Hazen et al., 1997b). This is a potential pathway to the advanced glycation end (AGE) products observed in diabetic patients (Reddy et al., 1995). Aldehyde formation from chloramines formed at the N-terminus of small peptides has also been examined (e.g. (Stelmaszynska and Zgliczynski, 1978)), and carbonyls have been detected on a number of proteins after treatment with HOCl (e.g. on fibronectin (Vissers and Winterbourn, 1991), LDL (Hazell et al., 1994; Yang et al., 1997) and BSA (Hawkins and Davies, 1998b; Chapman et al., 2000)).

Lys-derived chloramines have also been proposed to undergo hydrolysis to aldehydes (reactions 1, 2) that can form inter- or intra-molecular cross-links with free amine groups (Clark et al., 1986; Hazell et al., 1994; see also section on protein dimerization below). However, this mechanism has been disputed, on the basis that carbonyl formation has only been detected with chloramines from free amino acids containing both a carboxylic acid and a free amino group on the α-carbon (Hazen et al., 1998a). No evidence was obtained for aldehydes generated from chloramines formed on the ε-amino group of Lys (Hazen et al., 1998b).

$$R\text{–}CH_2\text{–}NHCl \rightarrow R\text{–}CH{=}NH + HCl \quad (1)$$

$$R\text{–}CH{=}NH + H_2O \leftrightarrow R\text{–}CH{=}O + NH_3 \quad (2)$$

In addition to the generation of aldehydes as stable products via two electron processes, radical intermediates formed via one-electron pathways have been detected during thermal decomposition of both free Lys-, Lys-peptide- and protein-derived chloramines (Hawkins and Davies, 1998b). Decomposition of the chloramines in both thermal (reaction 3) and metal-ion catalyzed (reaction 4) processes, has been shown to give rise to reactive, nitrogen-centered, radicals (Hawkins and Davies, 1998b; Hawkins and Davies, 1998c). These radicals have been characterized using EPR spectroscopy with spin trapping, and the identity of the species confirmed, in the case of free Lys by use of ^{15}N labeling. The protein-derived, nitrogen-centered, radicals are also believed to arise from the decomposition of Lys-derived chloramines as removal of these intermediates (with excess Met) inhibits radical formation, as did blocking of the Lys side-chains by reductive methylation (Hawkins and Davies, 1998b).

$$RNHCl \rightarrow RNH^{\bullet} + Cl^{\bullet} \quad (3)$$

$$RNHCl + M^{n+} \rightarrow RNH^{\bullet} + Cl^{-} + M^{(n+1)+} \quad (4)$$

Studies with Lys and other amino acids have revealed that the initial nitrogen-centered radicals can undergo a number of rapid further rearrangement, and hydrogen atom abstraction, reactions (Scheme 4). The former include β-scission processes that result in loss of the side-chain or carboxyl groups, whereas the latter can be both inter- or intra-molecular (1,2-, 1,5- and 1,6-shifts) in nature. In each case further carbon-centered radicals are formed (Hawkins and Davies, 1998c). The resultant carbon-centered radicals are likely to react at diffusion-controlled rates with O_2 to give peroxyl radicals ($ROO^{\bullet}$). Subsequent reactions of these peroxyl radicals (which will include hydrogen atom abstraction reactions, disproportionation and dimerization processes, reviewed in (von Sonntag, 1987; Davies and Dean, 1997; Hawkins and Davies, 2001a)) may account for the detection of carbonyl products on decomposition of Lys chloramines. Whatever the mechanism of formation, the overall yield of carbonyls only appears to account for a relatively low percentage of the initial oxidizing equivalents (e.g. 5–10% for BSA over a wide range of HOCl concentrations (Hawkins and Davies, 1998b; Chapman et al., 2000)).

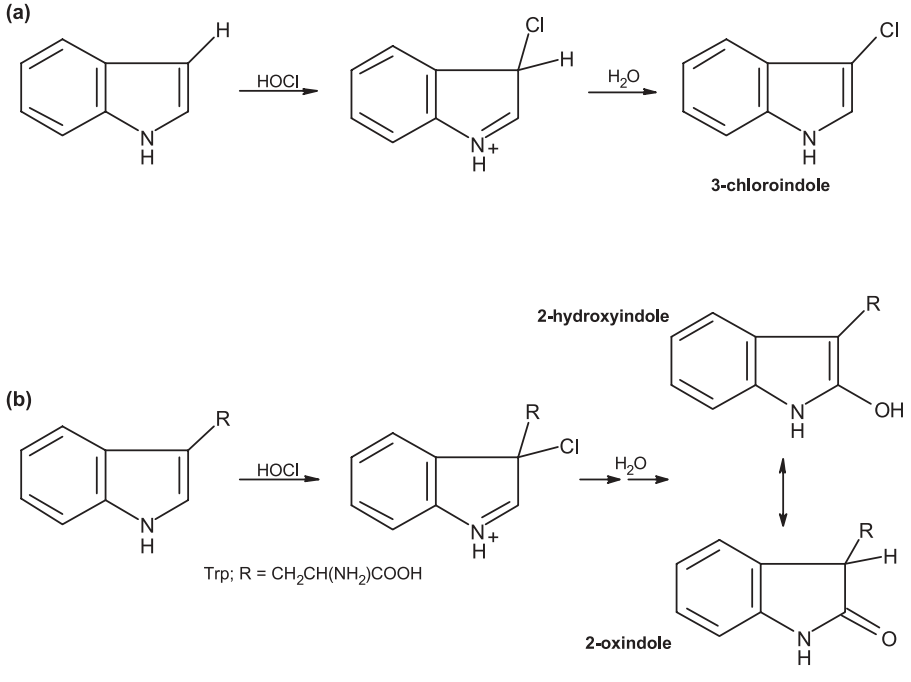
Heterocycle containing side-chains; His and Trp

The side-chain of His reacts rapidly with HOCl to form a short-lived chloramine as can the α-amino group in the free amino acid. Kinetic data suggests that reaction occurs almost equally at both sites at pH 7.4 (Pattison and Davies, 2001). However, the reactivity of the imidazole ring is highly sensitive to pH, thus changes in pH or in an environment where the pK_a of the side-chain is altered, the rate of reaction at the side-chain will be reduced. This will lead to preferential reaction at the α-amino group (Pattison and Davies, 2001). The ring-derived chloramine has been shown to rapidly transfer chlorine to other amine groups to generate more stable chloramines (e.g. on Lys residues (Pattison et al., unpublished results)) with regeneration of the parent compound. Chlorine transfer from His-derived chloramines has also been shown to occur to the exocyclic amines of the DNA bases, cytosine, guanosine and adenosine (Hawkins et al., unpublished data).

Though other products are also likely to be formed on the decomposition of these chloramines, these have yet to be characterized (Winterbourn, 1985; Pattison and Davies, 2001). It is possible that these processes give rise to carbonyl products (e.g. 2-oxo-Histidine), via either one- or two-electron processes, and these materials may play a role in protein aggregation (see below).

Oxidation of the Trp side-chain by HOCl has been investigated, but the final products of these reactions remain ambiguous. Drozdz et al., have suggested that the absorbance changes observed in the UV/visible region during the reaction of HOCl with *N*-Ac-Gly-Trp are due to the formation of 2-oxindole, and the multiply oxidized product, 5,7-dihydroxy-2-indolone (Drozdz et al., 1988). However, no additional techniques were utilized to confirm the identities of the products, and no mechanisms for the transformation were suggested. More recent studies have also suggested that these products are formed (Naskalski, 1994), but again no further characterization was undertaken (reviewed in Joule et al., 1995). Recent studies of the reactions of HOCl with melatonin and other indole derivatives have suggested that the products obtained depend on the substituents present on the indole ring (Dellegar et al., 1999). The products of these reactions have been separated and characterized by GC-MS, and in some cases by ^{1}H NMR spectroscopy. Reactions of HOCl with indoles that lack substituents at the 3-position are proposed to occur via an electrophilic aromatic substitution mechanism (Scheme 5) in which the 3-position is chlorinated (Dellegar et al., 1999). In contrast, HOCl reaction with indole rings substituted with an alkyl group at the 3-position (as is the case for Trp), such as melatonin and 3-methylindole, have been suggested to yield 2-hydroxyindoles (Scheme 5) (Dellegar et al., 1999). The 2-hydroxylated products have also been observed as products of unsubstituted indoles, which may occur through hydrolysis of the initial 3-chloroindole products. Thus, it is likely on the basis of these studies (Dellegar et al., 1999), that the major products of HOCl-mediated Trp oxidation are 2-hydroxyindole (which exists primarily as the tautomeric 2-oxindole (Joule et al., 1995)) derivatives of Trp, as proposed in the earlier studies (Drozdz et al., 1988; Naskalski, 1994). However, the HOCl-mediated oxidation products of Trp itself have not yet been characterized conclusively.

It has also been proposed that oxidation of Trp by HOCl can lead to formation of kynurenine and *N*-formylkynurenine, as characterized by UV/visible spectroscopy (Aspee and Lissi, 2002). The suggested mechanism involves reaction of HOCl at the α-amino site to form a monochloramine, followed by thermal (or metal-ion catalyzed) decomposition to yield radicals. These radicals can react inter-molecularly with other Trp side-chains to generate kynurenine. Similarly, when Lys chloramines



Scheme 5. Proposed mechanism for the reaction of HOCl with indole derivatives; (**a**) for indoles lacking a substituent at the 3-position, (**b**) for indoles with an alkyl substitutent at the 3-position (as is the case for Trp)

were added to free Trp, absorbance changes consistent with the formation of kynurenine and *N*-formylkynurenine were observed (Aspee and Lissi, 2002). Addition of the radical scavenger Trolox to the reaction mixture reduced the extent of kynurenine formation, consistent with the occurrence of a radical-mediated mechanism. The formation of kynurenine as a result of radical-mediated oxidation (e.g. by $HO^{\bullet}$) of Trp is well documented (Halliwell and Gutteridge, 1999).

Thus, there is evidence that Trp side-chains may be modified by at least two HOCl-mediated mechanisms. The first occurs via direct HOCl-mediated oxidation of the side-chain yielding 2-hydroxyindole derivatives of Trp. The second mechanism occurs via a secondary process, whereby decay of chloramines results in radical generation and kynurenine formation. A third potential mechanism, for which there is little direct experimental evidence to date, involves an intra-molecular version of the second mechanism with direct chlorination of the indolic nitrogen giving a secondary chloramine (Joule et al., 1995) which might subsequently decompose to give radicals and hence kynurenine.

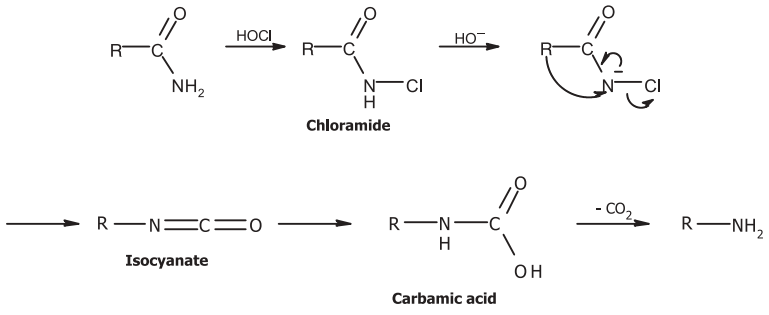
Aromatic side-chains; Tyr

Chlorination of Tyr is one of the most well characterized side-chain modifications induced by HOCl as a result of the use of the products of these reactions as specific markers for HOCl-mediated damage (Domigan et al., 1995; Kettle, 1996; Hazen et al., 1997a; Hazen and Heinecke, 1997; Winterbourn and Kettle, 2000). With the free amino acid, two major reactions can occur – reaction at the α-amino group and resultant formation of *p*-hydroxyphenylacetaldehyde (see above) (Hazen et al., 1996a), and reaction with the aromatic ring to give 3-chlorotyrosine. Both materials can react further to give respectively 3-chloro-4-hydroxyphenylacetaldehyde and 3,5-dichlorotyrosine (Fu et al., 2000). It has been proposed that the formation of 3-chlorotyrosine does not occur via direct reaction of the phenol ring with HOCl (Domigan et al., 1995), with this material arising from intra- (and possibly inter-) molecular chlorine transfer to the ring from other preferentially-generated chloramines (Domigan et al., 1995). Thus the chloramine formed on the N-terminal amino group of Gly-Gly-Tyr-Arg resulted in chlorination of the Tyr side-chain, but with free Tyr no ring chlorination was detected (Domigan et al., 1995). It was proposed that the chlorine transfer occurred via homolysis of the N–Cl bond with subsequent chlorine atom attack on the Tyr side-chain, but the possibility of a concerted electrophilic mechanism was not discounted (Domigan et al., 1995). However, in circumstances where no free amine site is available for chloramine formation (e.g. *N*-Ac-Tyr) it has been shown that direct chlorination of the phenolic side-chain by HOCl can occur, and this is supported by studies carried out on model compounds (Fu et al., 2000; Pattison and Davies, 2001). These data suggest that there are at least two mechanisms for ring chlorination; (i) direct HOCl attack, and (ii) chlorine transfer from initially generated chloramines. Hazen and co-workers have also provided evidence for a third mechanism involving the formation of chlorine gas (Cl_2) at low pH values, with these workers reporting that ring chlorination of Tyr is only observed at acidic pH values (Hazen et al., 1996b). More recent studies have cast doubt on this assertion (Fu et al., 2000).

Other nitrogen containing side-chains; Arg, Gln and Asn

The products arising from reaction of the side-chain of Arg with HOCl have not yet been studied in detail, though there is evidence for the formation of a chloramine at the guanidine group (Zhang et al., 2001, Pattison and Davies, unpublished results). In studies with the free amino acid reaction occurs preferentially with the α-amino group, with excess HOCl believed to react with the guanidine function to give a chloramine. These chlorinated species have been shown to inhibit endothelial cell nitric oxide synthase activity and hence $NO^{\bullet}$ formation (Zhang et al., 2001); this may be an important pathway to endothelial cell dysfunction. Whether the inhibition of nitric oxide synthase activity arises from one (or other) of the chloramines, or products arising from them, is unclear as yet. Further studies are in progress to determine the products of these reactions.

The products arising from HOCl-mediated oxidation of Gln and Asn side-chains in aqueous solution have not, to our knowledge, been reported in the literature. This is probably due, at least in part, to their slow rate of reaction with HOCl, rendering them relatively unimportant targets in proteins (Pattison and Davies, 2001). In contrast considerable data is available on the oxidation of amide functions in organic solvents (e.g. Neale et al., 1966; Neale, 1971; Johnson and Greene, 1975a; Johnson and Greene, 1975b). Evidence has been provided for the formation of chloramides as initial intermediates (e.g. Mauger and Soper, 1946; Beckwith and Goodrich, 1965; Johnson and Greene, 1975; Antelo et al., 1995b; Hawkins and Davies, 1998a). EPR spin trapping studies and product analysis have shown that these species can undergo decomposition via one-electron processes, particularly in



Scheme 6. Mechanism of the Hofmann rearrangement, which may have implications in the modification of Gln and Asn side-chains

the presence of metal ions or UV light, to give nitrogen-centered radicals, and these have been shown to undergo a number of rearrangement reactions to give carbon-centered species with reformation of the amide group (e.g. the Hofmann-Loffler reaction (Neale, 1971; Johnson and Greene, 1975a; Johnson and Greene, 1975b; Hawkins and Davies, 1998a)). The Hofmann rearrangement that is used in organic synthesis to convert primary amides into amines containing one less CH_2 group, also occurs via a haloamide intermediate (March, 1992). This two-electron process may also occur with proteins. The mechanism of this reaction is shown in Scheme 6, and proceeds via isocyanate and carbamic acid intermediates that are rapidly hydrolysed in aqueous solution. Thus, it is possible that the products of Gln and Asn side-chain oxidation are amine groups. These may then react further with HOCl to yield chloramines and associated decomposition products. The balance between these potential one- and two-electron processes has yet to be determined.

Reaction with backbone amides and protein fragmentation

Reaction of HOCl with the backbone amide groups of proteins and model peptide compounds results in the formation of chloramides (Thomas, 1979; Prutz, 1999). These chloramides are capable of oxidizing further biological substrates via chlorine transfer (Prutz, 1999), or can undergo further reactions. In non-aqueous environments, amidyl radicals have been implicated in a variety of rearrangement reactions (Neale, 1971; Johnson and Greene, 1975a; Johnson and Greene, 1975b). In the presence of water, backbone chloramides can undergo hydrolysis resulting in direct cleavage of the peptide bonds (Thomas, 1979), although the products of these reactions (in water) are poorly characterized. Metal-ion and UV-light catalyzed decomposition of backbone chloramides to give radicals has been extensively studied in organic solvents (Neale, 1971; Johnson and Greene, 1975a; Johnson and Greene, 1975b), but less so in aqueous solution. The amidyl radicals that result from such cleavage can undergo a number of rearrangement reactions, in aqueous solution, including 1,2-hydrogen atom shift reactions (which results in the formation of α-carbon radicals and hence backbone fragmentation; cf. data which suggests that the formation of these species are key intermediates in most mechanisms of backbone fragmentation (Davies and Dean, 1997; Dean et al., 1997; Hawkins and Davies, 2001a)) and remote hydrogen atom abstraction processes (1,5- and 1,6-shifts, e.g. (Neale, 1971; Johnson and Greene, 1975a; Johnson and Greene, 1975b)). The latter processes predominate in organic solvents and can result in remote functionalization and cyclization. The remote hydrogen atom abstraction processes result in transfer of damage from the backbone to the side-chains, and is therefore likely to protect against backbone cleavage. The propensity of different amidyl radicals to undergo these various pathways is believed to depend on the nature of the side-chains present on either side of the amidyl function in the protein, as evidence has been presented, in organic solvents, for the occurrence of hydrogen atom abstraction from side-chain substituents on both sides of the amidyl function (Mackiewicz and Furstoss, 1978). No evidence has been obtained for the involvement of the oxygen-centered radical tautomer ($R–C(O^{\bullet})=N–R'$) of the amidyl radical ($R–C(=O)–N^{\bullet}–R'$) in these processes.

The converse process to the above – damage transfer from a side-chain site to the α-carbon position may also occur. Thus nitrogen-centered radicals formed on the side-chain amino group of Lys residues have been reported to be able to carry out inter- or intra-molecular hydrogen atom abstraction reactions, with targets including the hydrogen atom of the α-carbon site in protected amino acids or peptides (Hawkins and Davies, 1998b); abstraction from this site is particularly favorable as a result of the potential stabilization of the incipient radical center by the neighboring amide and carbonyl functions (Easton, 1997). The resulting α-carbon radicals are known precursors of

backbone cleavage (reviewed (Davies and Dean, 1997; Hawkins and Davies, 2001a)). The involvement of such damage transfer reactions in protein fragmentation reactions has yet to be confirmed. The hypothesis that radical intermediates play a role in HOCl-mediated protein damage is supported by the observation that protein fragmentation (as assessed by SDS-PAGE) is decreased in the presence of the antioxidants Trolox, ascorbate and GSH (Hawkins and Davies, 1998b).

Whatever the mechanism there is little doubt that high concentrations of HOCl can induce protein fragmentation. Early studies carried out with ovalbumin provided the initial evidence for HOCl-mediated protein degradation (Baker, 1947). In this study, protein fragmentation was observed on addition of approximately 3 mol of HOCl per amino acid residue (Baker, 1947). More recent studies have confirmed that fragmentation only occurs to a major extent with high molar excesses of HOCl. Low-molecular-weight protein fragments (as assessed by SDS-PAGE) have been observed in experiments with fibronectin treated with a 40–90-fold molar excess of HOCl (Vissers and Winterbourn, 1991), though at even higher excesses of HOCl these fragments were not observed possibly as a result of further degradation reactions (Vissers and Winterbourn, 1991). In contrast, studies carried out with collagen reported little or no fragmentation with less than 200-fold molar excesses of HOCl (Davies et al., 1993). Maximal HOCl-mediated fragmentation of the immunoglobulins IgG and IgM was observed on treatment of these proteins with 375-fold, and 800-fold, HOCl molar excesses respectively (Drozdz et al., 1995).

It has been proposed that the extent of protein fragmentation observed with a particular protein is dependent on the molar ratio of HOCl to reactive amino acid side-chains. Thus, ribonuclease A (with 14 HOCl-reactive side-chains) has been observed to undergo fragmentation more readily than BSA (66 HOCl-reactive side-chains) at fixed HOCl:protein molar ratios (Hawkins and Davies, 1998b). Furthermore, lower molar excesses of HOCl are required to induce fragmentation of BSA when the Lys side-chains have been chemically modified by reductive methylation, than for untreated BSA (Hawkins and Davies, 1998b). These observations are consistent with the suggestion that most protein fragmentation is mediated by backbone chloramides (R–C(O)–NCl–CHR^1–C(O)–R^2) formed via the direct reaction of the excess HOCl with the backbone (Thomas, 1979) and that this only occurs to a significant extent when all the more reactive side-chains have been consumed (see kinetics section above) (Hawkins and Davies, 1998b).

Fragmentation has also been observed on treatment of BSA with low molar excesses of HOCl (<50) after extended incubation periods (Hawkins and Davies, 1998b). Under these conditions, reaction of HOCl is expected to occur primarily with the amino acid side-chains. Under such conditions Lys-derived chloramines and/or the radicals derived from them may be responsible for protein fragmentation. This hypothesis is supported by the observation that removal of chloramines with excess Met, or incubation of the HOCl-treated protein in the presence of the radical scavenger Trolox, inhibits this process (Hawkins and Davies, 1998b).

Protein dimerization and aggregation

Treatment of proteins with HOCl can also result in the formation of protein cross-links and aggregates. Treatment of fibronectin with increasing concentrations of HOCl (40–160-fold molar excess) has been shown to result in the progressive loss of the parent protein and an increase in aggregated material (Vissers and Winterbourn, 1991). These aggregates were still detected under reducing conditions eliminating disulfide bond formation as a source of these cross-links (Vissers and Winterbourn, 1991), but cross-linking was inhibited by Met, implicating chloramines (Vissers and Winterbourn, 1991). Protein aggregate formation was paralleled by an increase in dityrosine formation arising from tyrosyl radicals (Vissers and Winterbourn, 1991). However, the yield of dityrosine was greater in experiments where HOCl was generated enzymatically using myeloperoxidase than with reagent HOCl, suggesting that the formation of tyrosyl radicals may be mediated by the peroxidase enzyme and not HOCl (Vissers and Winterbourn, 1991).

Similar results have been reported for LDL (O'Connell et al., 1994). Thus, high molecular weight apoprotein complexes that were resistant to reduction were observed with increasing molar excesses (25–250-fold) of HOCl (O'Connell et al., 1994). A corresponding increase in dityrosine formation was detected, however the concentration of dityrosine detected could not account for all the cross-links, even if it was assumed that all the dityrosine was formed intermolecularly (O'Connell et al., 1994). Furthermore, protein cross-linking by tyrosyl radicals is known to be a relatively inefficient reaction, generating only low yields of dityrosine in model proteins and LDL (Heinecke et al., 1993).

An alternative hypothesis to account for HOCl-mediated proteins cross-linking involves the formation and subsequent reactions of carbonyl groups arising from

chloramine decay, particularly those formed on Lys residues (Hazell et al., 1994). No protein aggregates were detected in experiments where LDL that had been chemically modified (by reductive methylation) to block Lys residues was treated with HOCl (Hazell et al., 1994). The observed cross-links were proposed to arise from hydrolysis of the Lys chloramines to aldehydes (see above) and subsequent reaction of these carbonyls with free amine groups, via Schiff base formation, either inter- or intramolecularly (reactions 5 and 6) (Clark et al., 1986; Hazell et al., 1994). A similar mechanism has been proposed to account for HOCl-mediated aggregation of BSA (Naskalski, 1994). Such a mechanism requires the formation of carbonyls from Lys side-chain chloramines, and this has been disputed (Hazen et al., 1998b) (see also above), so other mechanisms may also play a significant role.

$$\text{R–CH=O} + \text{R}^1\text{–CH}_2\text{–NH}_2 \leftrightarrow \text{R–CH=N–CH}_2\text{–R}^1 + \text{H}_2\text{O} \quad (5)$$

$$\text{R–CH=N–CH}_2\text{–R}^1 \rightarrow \text{R–CH}_2\text{–NH–CH}_2\text{–R}^1 \quad (6)$$

Recent studies with model peptides indicate that HOCl can form intra- and inter-molecular sulfenamide (RSNHR), sulfinamide (RS(O)NHR) and sulfonamide ($RS(O)_2NHR$) S–N cross-links between thiol groups and Lys or Arg residues (Fu et al., 2002). The suggested mechanism involves reaction of a thiol-derived sulfenyl chloride (RSCl) with an amino (Lys) or guanidine group (Arg) to form a sulfenamide (S–N) cross-link (Fu et al., 2002). This group may then react with oxygen to yield the sulfinamide and sulfonamide products (Fu et al., 2002). It has been proposed that this type of process may contribute to the cross-linking observed in HOCl-treated LDL (Fu et al., 2002). Novel sulfinamide bonds have also been reported in studies with HOCl-treated (cytoplasmic) S100A8 protein (Raftery et al., 2001). Disulfide cross-links may also be formed (and repaired by disulfide reduction systems) via the reaction of either sulfenyl chlorides with excess thiol, or via thiyl radical formation (see above).

His-derived chloramines, and the products derived from these species, may also contribute to protein cross-links (Chapman et al., unpublished data). Treatment of apomyoglobin with increasing concentrations of HOCl (5–40-fold molar excess) has been shown to give dimers, trimers and higher aggregates. This process was inhibited by addition of Met, implicating a role for chloramines as intermediates. The concentration of these protein aggregates was significantly reduced on chemical modification of either the His (using diethylpyrocarbonate) or Lys (by reductive methylation) residues, suggesting that both His- and Lys-derived chloramines may be important in mediating cross-link formation in this case (Chapman et al., unpublished data). Potential mechanisms which might give rise to such cross-links, include reaction of (intact) His residues with Lys-derived carbonyls, or reaction of an intact Lys amino group with a carbonyl formed on a His ring (e.g. a species such as 2-oxohistidine).

Overall it appears that cross-linking and aggregation occur more readily with HOCl-treated proteins than backbone cleavage. This is as would be predicted on the basis of the kinetic data discussed above. The aggregation arises primarily as a result of side-chain modification that is a rapid and facile process, whereas fragmentation only occurs when direct reaction with the backbone is a significant pathway. Thus, fragmentation only occurs to a major extent after reaction of HOCl with all the reactive side-chains.

Enzyme inhibition and loss of structural function

The reaction of HOCl with proteins can result in the inhibition of enzymatic activity as a consequence of modification of reactive amino acid side-chains at or near the enzyme active site, or via protein fragmentation or aggregation; the former is probably the most significant in many cases. Thus, it has been shown that HOCl can readily inactivate α_1-proteinase inhibitor due to the oxidation of a critical Met residue at or near the protease-binding site (Clark et al., 1981; Matheson and Travis, 1985). This inactivation is prevented by ascorbate (Halliwell et al., 1987), protein-conjugated bilirubin and biliverdin (Stocker and Peterhans, 1989), and butylated hydroxytoluene (Matheson and Travis, 1985). The protective action of the last of these suggests that radical intermediates may be important (Matheson and Travis, 1985).

Enzymes with Cys residues in the active site are also, as expected from the kinetic data discussed above, highly susceptible to HOCl-induced inactivation (Albrich et al., 1981). Papain, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and alcohol dehydrogenase (ADH) have highly reactive active-site Cys residues, and are all rapidly inactivated by equimolar amounts of HOCl (Albrich et al., 1981). Inactivation of GAPDH and creatine kinase (another thiol-dependent enzyme) has also been observed in cells treated with sub-lethal concentrations of HOCl (Schraufstatter et al., 1990; Pullar et al., 1999). Enzymes containing less reactive Cys residues including yeast aldolase and lactate dehydrogenase (LDH) required the addition of larger molar excesses of HOCl (>2.5-fold) to give inactivation (Albrich et al., 1981). Glutathione peroxidase

is also rapidly inactivated by HOCl (Aruoma and Halliwell, 1987).

The activity of the antioxidant enzymes catalase and superoxide dismutase (SOD) was reduced on treatment with HOCl (Aruoma and Halliwell, 1987). In this case, inactivation was only observed on addition of a high concentration of HOCl (180-fold and 750-fold molar excess for catalase and SOD respectively) (Aruoma and Halliwell, 1987). Reaction of HOCl with catalase also resulted in heme degradation. The loss of enzyme activity observed with these two enzymes may be associated with oxidative damage and fragmentation of the protein, rather than damage to a specific amino acid residue.

Recent studies with α_2-macroglobulin (α_2M), a high-affinity, broad-spectrum proteinase inhibitor, have shown that HOCl can abolish the ability of this enzyme to inhibit proteinases (Wu and Pizzo, 1999). The HOCl-induced inactivation of α_2M was attributed to protein unfolding, resulting in an increased susceptibility to proteolytic cleavage (Wu and Pizzo, 1999). This type of behavior has also been reported with other HOCl-treated proteins. Thus, it has been demonstrated that fibronectin has an increased susceptibility to degradation by elastase after treatment with increasing concentrations of HOCl (Vissers and Winterbourn, 1991). Similarly, both HOCl and pre-formed Ala- and Leu-derived chloramines have been shown to increase the susceptibility of collagen to degradation by collagenase (Davies et al., 1993). Recent work (Lindner et al., unpublished data) has shown that unfolding and loss of structural integrity occurs very readily with a range of proteins exposed to low molar excesses of HOCl (e.g. equimolar to 5-fold). Thus both lysozyme and trypsin inhibitor are completely converted to new structural forms with different elution times and profiles (on separation by size-exclusion HPLC) on treatment with 5-fold excesses of HOCl. Incubation of α-lactalbumin with low concentrations of HOCl has been shown to result in changes in the intrinsic fluorescence of Trp residues and loss of secondary and tertiary structure (as evidenced by circular dichroism measurements and 8-anilino-1-napthalene sulfonic acid (ANS) binding). When identical samples are analyzed by SDS-PAGE no changes are detected, consistent with these changes in structure being independent of fragmentation and aggregation.

Reaction of HOCl-damaged proteins with other targets

In addition to the direct chlorine transfer processes outlined above, there is additional evidence to suggest that HOCl-damaged proteins can induce changes to other biological targets. There is also a very considerable body of evidence for the induction of further damage by free chloramines derived from both amino acids (e.g. Lys-chloramine) and other compounds such as monochloramine (NH_2Cl), and taurine chloramine; this latter area is not discussed here, and has been reviewed elsewhere (Thomas et al., 1986a).

Recent studies have demonstrated that the protein-derived radicals formed on decomposition of chloramines on HOCl-treated proteins can mediate damage to other substrates (Hazell et al., 1999; Hawkins et al., 2002). It has been shown that the oxidation of lipids observed on treatment of LDL with HOCl is a secondary reaction, with the majority of the oxidant consumed by reaction with the single protein molecule (apolipoprotein B-100) present in these particles (Hazell et al., 1999). This is in accord with the kinetic data discussed above. The secondary oxidation of lipids has been shown to involve radicals by the use EPR spin trapping. Furthermore, the extent of lipid peroxidation was reduced on treatment of HOCl-treated LDL with either excess Met, which removes chloramines, and the (radical scavenging) spin trap DMPO. This suggests a role of both protein chloramines, and the radicals derived from them, in HOCl-induced LDL lipid peroxidation (Hazell et al., 1999).

It has also been shown that peptide and protein chloramines (as well as those from isolated Lys, His), can react with plasmid DNA to cause strand breaks (Hawkins et al., 2002). A role for radicals in chloramine-mediated DNA strand cleavage is supported by the protection afforded by the radical scavenger Trolox and spin trap DMPO (Hawkins et al., 2002). Chloramine-derived radicals have also been shown to react, by addition, to the C5–C6 double bond of pyrimidine nucleosides to yield carbon-centered, nucleoside-derived, radicals and specific, covalent, protein-nucleoside cross-links. Similar cross-links have been observed on reaction of Lys and His chloramines with DNA. This study suggests that in a cellular system, HOCl-induced damage to DNA may be mediated by reactions of pre-formed protein chloramines rather than, or in addition to, direct reaction with HOCl (Hawkins et al., 2002).

In summary, the available kinetic evidence is consistent with proteins being major targets for HOCl within complex biological systems. The majority of reaction occurs with the amino acid side-chains, with the order of reactivity being Met $>$ Cys $\gg$ Cystine $\sim$ His $\sim$ α-amino $>$ Trp $>$ Lys $\gg$ Tyr $\sim$ Arg $>$ Gln $\sim$ Asn. Reaction with backbone amide groups is a slow process. The rate constants determined for these reactions, together with protein

sequence data, now allow the sites of reaction to be predicted with a reasonable degree of certainty. The products of some of these reactions are well characterized (e.g. Met), but in other cases these are poorly understood and require further elucidation. In some cases the initial products are known to undergo further reactions (e.g. the sulfenyl chlorides generated from Cys residues and the chloramines from the α-amino group, Lys, and His side-chains), with these processes capable of inducing both oxidation of other target molecules (e.g. DNA, lipids, other proteins), as well as aggregation and to a much lesser extent fragmentation. The prevalence of aggregation over fragmentation, particularly with low HOCl excesses, appears to arise from the preferential modification of side-chains over the backbone, as a result of the considerably more rapid reaction at the former sites. Chemical alteration of the side-chains even at low levels (e.g. 1–5 fold excesses) can induce enzyme inactivation, with species having Met or Cys residues in their active site being particularly susceptible, and protein unfolding.

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