Reaction of HOCl with amino acids and peptides: EPR evidence for rapid rearrangement and fragmentation reactions of nitrogen-centred radicals

Clare L. Hawkins and Michael J. Davies *

The Heart Research Institute, 145 Missenden Road, Camperdown, Sydney, NSW 2050, Australia

EPR spin trapping together with UV–VIS spectroscopy has been employed to examine the reaction of HOCl with amino acids and some small peptides. Evidence has been obtained for the formation and subsequent decomposition of short-lived chloramine derivatives from free amine groups present on both amino acid side-chains and at the N-terminus. Radical formation, detected by EPR spin trapping, occurs concurrently with chloramine decomposition. This process is enhanced by, but does not require, the presence of added Fe^{2+}. With some substrates nitrogen-centred (aminyl, $RNH^{\bullet}$ or $RNH_2^{\bullet+}$) radicals, formed from cleavage of the N–Cl bond of the chloramine, can be detected. These initial aminyl radicals undergo a variety of hydrogen atom abstraction, rearrangement and fragmentation reactions to give carbon-centred species. Evidence has been obtained for both inter- and intra-molecular (1,2- and 1,5-) hydrogen atom abstraction reactions, decarboxylation and β-scission processes. The last of these only occurs to a significant extent where the resulting radical is highly stabilised, for example, by aromatic substituents, and results in loss of the amino acid side-chain. Studies with *N*-acetyl derivatives and peptides are consistent with reaction of HOCl at amide (peptide) bonds to give transient chloramides which rapidly decompose to give (undetected) amidyl [$^{\bullet}N(R)C(O)R'$] radicals. These species undergo rapid 1,2-hydrogen atom shift reactions to give (stabilised) α-carbon radicals with most peptides. Evidence has also been obtained for the occurrence of hydrogen atom abstraction and decarboxylation reactions with these substrates.

Introduction

Oxidants such as $O_2^{\bullet-}$ and H_2O_2 are known to be generated during the respiratory burst of activated phagocyte cells both *in vitro* and *in vivo*.[1] These cells also release the heme enzyme myeloperoxidase which catalyses the reaction of H_2O_2 with physiological concentrations of Cl^- to give the powerful oxidant HOCl.[2] This species has a pK_a of 7.59, thus a mixture of HOCl and $^-$OCl is present at physiological pH; HOCl is used below to designate this mixture.

HOCl plays an important role in mammalian defences against invading micro-organisms as a result of its potent bactericidal activity.[3] Excessive or misplaced generation of HOCl is also known to cause damage to mammalian tissues, and this is believed to be important in some human disease such as atherosclerosis, chronic inflammatory disorders and some cancers.[1] Previous studies have shown that proteins are major targets for HOCl,[4,5] though this oxidant also reacts with a wide variety of other biological targets including DNA,[6] lipids,[7] cholesterol,[8] NADH,[9] and free thiols and sulfides.[9–11] Various amino acid side-chains have been shown to be particularly susceptible to attack and modification by HOCl.[12,13] It is known that tyrosine is readily chlorinated by HOCl to give 3-chlorotyrosine,[14] and this product has been employed as a marker of HOCl-mediated damage to proteins.[15] Cysteine and methionine react rapidly with HOCl to give oxyacids and cystine (from cysteine) and sulfoxides (from methionine).[10–13] The latter type of reaction has been shown to inactivate α_1-proteinase inhibitor.[16] Amino acids which lack the above functional groups also react with HOCl *via* the free amino group which results in the generation of unstable chloramine intermediates ($RNHCl/RNH_2^+Cl$); subsequent decomposition of these species gives NH_3, CO_2 and aldehydes.[17–22] The free side-chain amino group of lysine is also converted to an unstable chloramine.[23] Similar reactions also occur with peptides though reaction has been suggested to occur only at the free N-terminal amino nitrogen and reactive side-chain sites, and not at the amide nitrogen of peptide bonds.[20,21] This has however been disputed.[24]

Though the products of reaction of HOCl with amino acids and some peptides are reasonably well characterised, the mechanism(s) by which these products arise is less well understood. Radicals have not generally been implicated as intermediates, though it has been suggested that inactivation of α_1-proteinase inhibitor involves radicals on the basis of the protection afforded by the radical scavenger butylated hydroxytoluene.[16] In recent studies we have shown[25] that the reaction of HOCl with proteins results in the formation (amongst others) of both high- and low-molecular-weight, protein-derived, nitrogen-centred radicals derived from lysine side-chain amino groups. These species arise *via* the formation and subsequent decomposition of chloramines formed at this site. In the light of these observations we have now examined radical formation from chloramines and chloramides generated from reaction of HOCl with a number of free amino acids and small peptides, by use of electron paramagnetic resonance (EPR) spectroscopy with spin trapping, and UV–VIS spectroscopy.

Results and discussion

UV–VIS spectroscopic studies

Free amino acids. Previous studies have shown that HOCl reacts with most amino acids to generate unstable chloramines which have significant optical absorption bands in the 220–340 nm region, with λ_{max} typically between 250 and 260 nm.[19,22,26] This process, together with the loss of the parent absorption band from $^-$OCl at 292 nm,[26] has been employed to investigate the occurrence and rate of reaction of HOCl with a variety of amino acids and derivatives and the subsequent decomposition of the chloramines so generated.

Initial studies were carried out with glycine (0.1 M) and HOCl

Table 1 Characteristics and life-times of the UV absorptions of amino acid-derived chloramine species

Amino acid	λ_{max}/nm	$t_{\frac{1}{2}}$/min
Alanine	251	>60
Arginine	252	38.2
Aspartic acid	253	8.9
Asparagine	249	>60
Glutamic acid	254	24.5
Glutamine	248	47.6
Glycine	255	>60
Isoleucine	252	>60
Leucine	252	28.4
Lysine	250	53.0
N(α)-Acetyllysine	250	>60
N(ε)-Acetyllysine	250	>60
Serine	249	24.4
Threonine	252	53.1
Valine	251	55.0
Proline	260	2.2

(2.5 mM) at pH 7.4; this resulted in immediate loss of the $^{-}$OCl absorption band at 292 nm and the detection of a new absorption band with λ_{max} *ca.* 255 nm; this reaction occurred within the mixing time, and its kinetics could therefore not be monitored. Similar behaviour was observed with other amino acids (see Table 1). The new absorption bands, which are assigned to chloramine species, decreased in intensity over time with the half-life dependent on the substrate employed; the $t_{\frac{1}{2}}$ values for each chloramine are given in Table 1.

No chloramine absorptions were detected in experiments with histidine, tryptophan, tyrosine or phenylalanine due to the intense absorptions from the aromatic side chains of these amino acids, nor with cysteine and methionine, though in each case complete loss of the $^{-}$OCl absorption band was detected indicating that reaction had occurred. The lack of chloramine species with cysteine and methionine is believed to be due to preferential reaction with the sulfur-containing side chains.

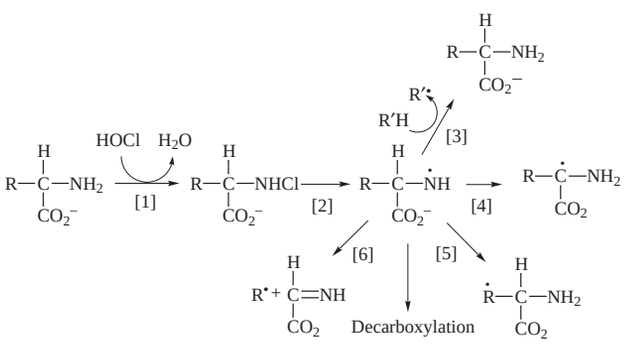
***N*-Acetylamino acid derivatives and peptides.** No absorptions due to chloramides were detected in the 220–700 nm wavelength region on reaction of HOCl with any *N*-acetylamino acid, though the initial absorption band of $^{-}$OCl at 292 nm was lost indicating that reaction does occur. Chloramine absorptions were observed with *N*(α)- and *N*(ε)-acetyllysine, and all the (unblocked) peptides as a result of reaction with the remaining free amino group in these materials. No evidence for a chloramide was obtained with *N*(α),*N*(ε)-diacetyllysine or peptides where all free amino groups are blocked. Indirect evidence for reaction at amide sites was obtained from examination of the yield of chloramines formed at the (N-terminal) amino group of free amino acids compared to homopeptides. Thus $(Gly)_2$ gave an *ca.* 10% lower yield of chloramines compared to Gly with fixed concentrations of HOCl. This is consistent with the free amino group being *ca.* 10-fold more reactive than amide site(s). Overall these experiments suggest that HOCl can react with amide groups to give very short-lived chloramide intermediates which decompose rapidly, but that this process is less favoured than reaction at free amino sites.

EPR studies

Free amino acids. The presence of radical intermediates during the reaction of HOCl with amino acids, *N*-acetyl derivatives and some peptides has been examined using EPR in conjunction with the spin traps 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DMPO; 167 mM) or 3,5-dibromo-4-nitrosobenzene sulfonic acid (DBNBS; 2.5 mM). Spectra were recorded of reaction mixtures typically containing the substrate (50 mM), HOCl (6.25 mM) and a spin trap at pH 7.4, though some experiments were also carried out at lower pH values where indicated. Sequential spectra were recorded for up to 60 min after initiation of the reaction. The substrate was always present in large excess to ensure complete consumption of HOCl, to prevent formation of multiple chlorinated species (*e.g.* dichloramines, $RNCl_2$) which would complicate analysis, and to minimise direct reaction of HOCl with the spin trap; the latter is of particular importance with DMPO due to the rapid formation of chloroimines.[27] It was generally found that radical concentrations were significantly increased on addition of Fe^{2+} (0.25 mM) to the reaction mixture, though in many cases identical behaviour was observed with no additions. In some experiments weak signals from spin trap degradation products such as the $SO_3^{\bullet -}$ adduct to DBNBS [*a*(N) 1.27, *a*(2H) 0.07 mT], and DMPO–OH$^{\bullet}$ from DMPO [*a*(N) 1.49, *a*(H) 1.49 mT] were observed. The low concentration of the latter adduct, and its observation in only a few cases even with very high trap concentrations, strongly suggest that HO$^{\bullet}$ is not generated in these reactions (*e.g. via* pseudo-Fenton reactions with HOCl); this is in accord with some previous observations,[28,29] but not others.[30]

The radicals detected are summarised in Table 2. Assignment of the observed signals was made on the basis of the observed splitting patterns and comparison of the hyperfine coupling constants with previous data from other reaction systems. Assignments were confirmed by computer simulation and correlation coefficients between experimental data and simulation were usually greater than 0.95. In cases where unequivocal assignment was initially not possible, isotopically labelled (^{2}H or ^{13}C) substrates were employed to provide additional information.

With all the free amino acids examined the radicals observed can be accounted for *via* the initial generation of a chloramine species at a free amino group (Scheme 1, reaction 1), decom-



Scheme 1

position of this species to nitrogen-centred radicals (reaction 2), and subsequent rapid reaction of these species *via* a variety of structure-dependent pathways including: i) inter-molecular hydrogen atom abstraction (reaction 3), ii) intra-molecular hydrogen atom abstraction (reactions 4 and 5), iii) decarboxylation, and iv) β-scission with loss of the side-chain (reaction 6). Evidence for the occurrence of each of these processes is summarised below.

Formation of nitrogen-centred radicals.—In a small number of cases direct evidence for the formation of nitrogen-centred radicals has been obtained. Thus lysine (as previously[25]), ornithine, asparagine and tyrosine gave EPR signals with distinctive small 1:1:1 triplet splittings (in addition to the large 1:1:1 nitrogen coupling from the nitroxide nitrogen) when high concentrations of DMPO (typically 150–200 mM, though concentrations down to 10 mM could be used with lysine) were employed (Fig. 1). The high trap concentrations required to observe these signals in most cases implies that these nitrogen-centred species can undergo rapid alternative reactions. The source of the nitrogen-centred radical observed with lysine has been identified as the side-chain amino group by use of ^{15}N-labelling.[25] In the case of lysine (and *N*-acetyl-Lys, see Table 3) the dependence of the observed signals on the pH of the reaction mixture was investigated. No changes in the

Table 2 Parameters of the radicals observed on reaction of HOCl with amino acids in the presence of the spin traps DBNBS and DMPO

Substrate	Spin trap	Hyperfine coupling constants/mT[a] *a*(N)	*a*(H)	*a*(other)	Assignment
Alanine	DBNBS	1.34	0.89 (2H)		Side chain at C(3)
	DMPO	1.58	2.23 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct
Valine	DBNBS	1.39			C(2) or side chain at C(3)
	DMPO	1.58	2.44 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct
Nor-valine	DBNBS	1.42	1.19 (2H)		Side chain at C(5)[b]
		1.35	0.63 (1H)		Side chain at C(3) or C(4)
Leucine	DBNBS	1.42	1.18 (2H)		Side chain Me group[b]
		1.42	0.48 (1H)	0.18 (1N)	Decarboxylation at C(2)
		1.31	0.53 (1H)		Side chain at C(3)
	DMPO	1.63	2.29 (1H)		Carbon-centred
Isoleucine	DBNBS	1.42	1.18 (2H)		Side chain Me group[b]
		1.42			C(2) or side chain at C(3)
	DMPO	1.60	2.33 (1H)		Carbon-centred
Lysine, [4,4,5,5- $^2H_4$]-lysine, [6-^{13}C]lysine	DBNBS	1.36	0.92 (1H)		Side chain at C(3)
		1.31	0.35 (1H)		Decarboxylation at C(2)
	DMPO	1.49	1.82 (1H)	0.29 (1N)	Nitrogen-centred (side chain)[c,d]
		1.56	2.35 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct
[3-^{13}C]Lysine	DBNBS	1.36	0.92 (1H)	0.88 (^{13}C)	Side chain at C(3)
		1.31	0.35 (1H)	0.88 (^{13}C)	Decarboxylation at C(2)
[1-^{13}C]Lysine	DMPO	1.56	2.35 (1H)		Carbon-centred
		1.56	1.88 (1H)	1.21 (^{13}C)	$^{13}CO_2^{\bullet -}$ adduct
Lysine-OMe	DBNBS	1.35	0.83 (1H)		Side chain at C(3)
Ornithine	DBNBS	1.33	0.38 (1H)		Side chain at C(3) or decarboxylation at C(2)
	DMPO	1.46	1.80 (1H)	0.30 (1N)	Nitrogen-centred (side chain)[b,c]
		1.56	2.35 (1H)		Carbon-centred
		1.56	1.85 (1H)		$CO_2^{\bullet -}$ adduct
Histidine	DBNBS	1.42	0.72 (1H)		Side chain at C(3)
		1.33	0.50 (1H)		Decarboxylation at C(2)
	DMPO[e]	1.56	2.33 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct
1-Methylhistidine	DBNBS	1.42	0.72 (1H)		Side chain at C(3)
		1.33	0.50 (1H)		Decarboxylation at C(2)
		1.44	1.03 (2H)		Methyl group
	DMPO	1.56	2.33 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct
Arginine	DBNBS	1.38	0.92 (1H)		Side chain CH_2 group
		1.35	0.33 (1H)		Decarboxylation at C(2)
	DMPO	1.57	2.34 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct
Glutamine	DBNBS	1.33	0.43 (1H)		Side chain CH_2 group or decarboxylation at C(2)
	DMPO	1.57	2.29 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct
Asparagine	DMPO	1.54	1.79 (1H)	0.25 (1N)	Nitrogen-centred
		1.56	2.23 (1H)		Carbon-centred
Aspartic acid	DBNBS	1.31	0.68 (1H)		Side chain at C(3)
		1.31	0.89 (2H)		Decarboxylation at C(3)
[3-^{13}C]Aspartic acid	DBNBS	1.31	0.68 (1H)	0.64 (^{13}C)	Side chain at C(3)
		1.31	0.89 (2H)	0.64 (^{13}C)	Decarboxylation at C(3)
Glutamic acid, [2,4,4-$^2H_3$]glutamic acid	DBNBS	1.31	0.33 (1H)		Side chain at C(3)
		1.35	0.61 (1H)		Secondary species seen at long time points
Tyrosine	DBNBS	1.47	1.19 (2H)	0.07 (2H)[f]	β-scission at C(3)
		1.42	0.88 (1H)		Side chain at C(3)
	DMPO[e]	1.46	1.79 (1H)	0.29 (1N)	Nitrogen-centred[c]
		1.56	2.33 (1H)		Carbon-centred
		1.56	1.92 (1H)		Carbon-centred
[3,3-$^2H_2$]Tyrosine	DBNBS	1.47		0.18 (2D)	β-scission at C(3)
				0.07 (2H)[f]	
		1.42[g]			Side chain at C(3)
Phenylalanine	DBNBS	1.47	1.19 (2H)		β-scission at C(3)
		1.42	0.88 (1H)		Side chain at C(3)
	DMPO	1.56	2.38 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct
Proline	DBNBS	1.36	0.39 (1H)		Side chain at C(3)/C(5)
	DMPO	1.58	2.32 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct
[4,4-$^2H_2$]Proline	DBNBS	1.36	0.39 (1H)		Side chain at C(3)/C(5)
Hydroxyproline	DBNBS	1.35	0.37 (1H)		Side chain at C(3)/C(5)
Proline-OMe	DBNBS	1.36	0.39 (1H)		Side chain at C(3)/C(5)
	DMPO	1.52	2.23 (1H)		Carbon-centred
Threonine	DBNBS	1.35	0.92 (2H)		Side chain at C(4)
	DMPO	1.63	2.27 (1H)		Carbon-centred
		1.56	1.88 (1H)		$CO_2^{\bullet -}$ adduct

[a] ±0.02 mT. [b] Radical short-lived. [c] Only observed in the absence of Fe^{2+}. [d] Parameters for radical adduct detected at pH values between 7.4 and 6. At pH 4 an alternative species with parameters *a*(N) 1.40, *a*(H) 1.84, *a*(N) 0.31 mT is detected; for further details see text. [e] DMPO–OH$^{\bullet}$ also observed. [f] Splitting due to the hydrogens at positions 2 and 6 on the aromatic ring of DBNBS. [g] Further unresolved fine structure observed.

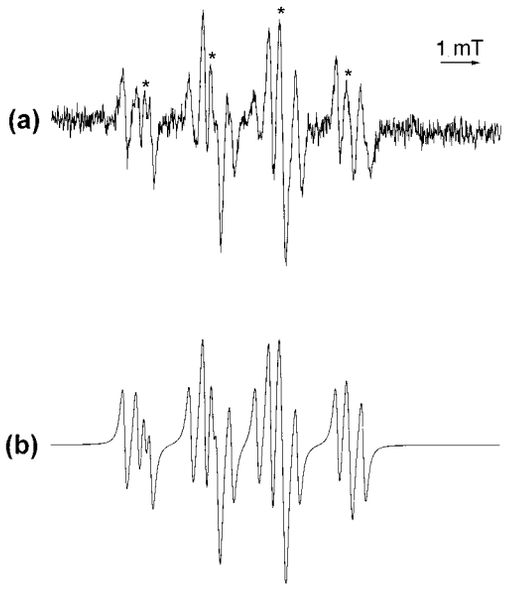


Fig. 1 (a) EPR spectrum observed on reaction of tyrosine (saturated solution) with HOCl (6.25 mM) in the presence of DMPO (167 mM) at pH 7.4. Signals assigned to a nitrogen-centred radical adduct formed from the tyrosine amino group, and DMPO–OH$^{\bullet}$ (lines marked [*]). (b) Computer simulation of the spectrum in (a) using the parameters in Table 2.

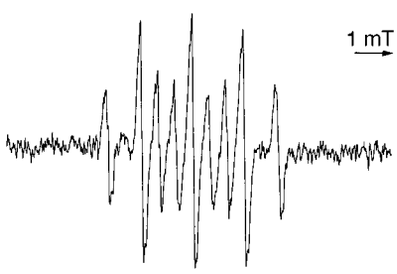


Fig. 2 EPR spectrum observed on reaction of alanine (50 mM) with HOCl (6.25 mM) and Fe^{2+} (0.25 mM) in the presence of DBNBS (2.5 mM) at pH 7.4. Signal assigned to the adduct of $^{\bullet}CH_2CH(NH_3^+)COO^-$.

spectra were detected at pH 6, but on lowering the reaction pH to *ca.* 5, a broadening of the signals from the nitrogen-centred radical adduct was observed; further decreases in the pH (to *ca.* 4) allowed a second signal to be discerned. This second signal has also been assigned to a nitrogen-centred radical adduct species, with somewhat different hyperfine coupling constants [*a*(N) 1.40, *a*(H) 1.84, *a*(N) 0.31 mT]. These results are interpreted in terms of the presence of the neutral aminyl radical adduct (*i.e.* trapped RNH$^{\bullet}$) at pH 7.4 and the aminyl radical-cation adduct (trapped $RNH_2^{\bullet +}$) at pH values < *ca.* 5.

Inter-molecular hydrogen abstraction.—EPR signals which can be assigned to carbon-centred, side-chain-derived, species were detected with most amino acids (see Table 2). For example alanine in the presence of DBNBS gave a signal [*a*(N) 1.34, *a*(2H) 0.89 mT] which increases in intensity over 60 min, and on the addition of Fe^{2+} (Fig. 2). These signals were not observed in the absence of the trap, the substrate, or HOCl. These signals are attributed to the trapped side-chain radical $^{\bullet}CH_2CH(NH_3^+)COO^-$; this species is also believed to be trapped with DMPO [*a*(N) 1.58, *a*(H) 2.23 mT]. This radical is postulated to arise *via* inter-molecular hydrogen atom abstraction from an undetected nitrogen-centred (aminyl) radical present on another alanine molecule; this process may involve either the neutral (RNH$^{\bullet}$) or protonated ($RNH_2^{\bullet +}$) form of this species (see below). Similar side-chain-derived radicals are observed with the majority of other amino acids (see Table 2). In some cases this reaction appears to be particularly favourable, for example with tyrosine, histidine and phenylalanine, where stabilised C(3)-derived (benzyl) radicals are formed.

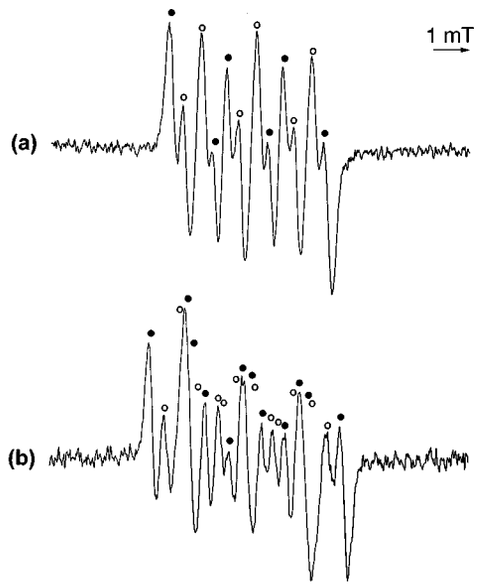


Fig. 3 EPR spectra observed on reaction of (a) lysine and (b) [2-^{13}C]lysine (both 50 mM) with HOCl (6.25 mM) and Fe^{2+} (0.25 mM) in the presence of DBNBS (2.5 mM) at pH 7.4. Signals marked [●] assigned to a carbon-centred radical adduct formed at C(3). Features marked [○] are attributed to a carbon-centred radical adduct generated at C(2) as a result of decarboxylation.

Intra-molecular hydrogen shifts.—The reaction of HOCl with lysine exhibits a complex pH and time dependence with two radical adducts detected with DBNBS [radical (1): *a*(N) 1.36, *a*(H) 0.92 mT; radical (2): *a*(N) 1.31, *a*(H) 0.35 mT] which increased in intensity over 60 min [Fig. 3(a)]. The ratio of these signals was pH dependent, with signal 2 becoming dominant at pH values below 6. The latter species is believed to be a C(2)-derived radical arising *via* decarboxylation (see below). The former species, which dominates at pH 7.4, is assigned to a carbon-centred radical. Experiments with [4,4,5,5-$^2H_4$]lysine and [6-^{13}C]lysine have provided evidence that this species is not centred at C(4), C(5) or C(6) as no changes were observed in the EPR spectra obtained from these substrates. Experiments with [2-^{13}C]lysine gave additional (doublet) ^{13}C couplings [0.88 mT; see Fig. 3(b)] for both radical adducts, confirming that these are C(2)- and C(3)-derived with the additional doublet splitting in the latter case being a β-^{13}C splitting (*cf.* α- and β-^{13}C couplings of 0.54 mT and 0.48 mT for di-*tert*-butyl aminoxyl[31]). These carbon-centred radicals are believed to be the source of the carbon-centred adduct(s) observed with DMPO.

The *selective* formation of the C(3) hydrogen atom abstraction radical from lysine at pH 7.4 would be unexpected if inter-molecular hydrogen atom abstraction is the major reaction, as the initial nitrogen-centred species would be expected to show similar selectivity to (electrophilic) oxygen-centred radicals such as HO$^{\bullet}$, which preferentially reacts at C(4) and C(5) (*i.e.* at sites remote from the de-stabilising protonated amino groups).[32] This selectivity is therefore proposed to be due to an intra-molecular 1,5-hydrogen atom shift reaction of the side-chain aminyl radical (*via* a 6-membered ring transition state) which results in the selective formation of the C(3) radical. This is in accord with previous studies which have shown that nitrogen-centred radical-cations can mediate selective intra-molecular hydrogen atom abstraction reactions *via* 6-membered ring transition states (the Hofmann–Loeffler reaction[33,34]). In experiments with the shorter side-chain analogue ornithine, selective hydrogen atom abstraction by the initial nitrogen-centred radical (detected with DMPO) was also detected with only a single carbon-centred radical detected with DBNBS. The parameters of this adduct [*a*(N) 1.33, *a*(H) 0.38 mT] are different to the two adducts detected on reaction with authentic HO$^{\bullet}$

Table 3 Parameters of the radicals observed on reaction of HOCl with *N*-acetyl amino acids and peptides in the presence of the spin traps DBNBS and DMPO

		Hyperfine coupling constants/mT[a]			
Substrate	Spin trap	*a*(N)	*a*(H)	*a*(other)	Assignment
N-Acetylglycine	DBNBS	1.39	0.63 (1H)	0.21 (N)	H-abstraction at C(2)
		1.38	0.82 (2H)	0.23 (1N)	Decarboxylation at C(2)
				0.07 (2H)[b]	
Gly-Gly	DBNBS	1.36	0.83 (2H)	0.25 (1N)	Decarboxylation at C-terminal C(2)
	DMPO	1.54	2.00 (1H)		Carbon-centred
N-Ac-Gly-Gly	DBNBS	1.39	0.79 (2H)	0.25 (1N)	Decarboxylation at C-terminal C(2)
Gly_n (n = 3–5)	DBNBS	1.39	0.83 (2H)	0.25 (1N)	Decarboxylation at C-terminal C(2)
Gly-Gly-Gly	DMPO	1.54	2.00 (1H)		Carbon-centred
N-Acetylalanine, Gly-Ala	DBNBS	1.39			H-abstraction at C(2)[c]
		1.39	0.69 (1H)	0.25 (1N)	Decarboxylation at C-terminal C(2)[d]
Ala-Gly	DBNBS	1.39	0.93 (2H)	0.22 (1N)	Decarboxylation at C-terminal C(2)
N-Acetylvaline	DBNBS	1.39			H-abstraction at C(2)
N-Acetylleucine	DBNBS	1.42			H-abstraction at C(2)
N(α)-Acetyllysine	DBNBS	1.33			H-abstraction at C(2)
		1.38	0.73 (1H)		Side chain [not C(3)]
	DMPO	1.49	1.80 (1H)	0.28 (1N)	Nitrogen-centred on side chain[e]
		1.56	2.35 (1H)		Carbon-centred
N(ε)-Acetyllysine	DBNBS	1.35	0.67 (1H)		Side chain
	DMPO	1.60	2.54 (1H)		Carbon-centred
		1.56	2.19 (1H)		Carbon-centred
N(α),*N*(ε)-Diacetyllysine	DBNBS	1.33			H-abstraction at C(2)
		1.38	0.75 (1H)		Side chain [not C(3)]
Gly-Lys, Gly-Gly-Lys	DBNBS	1.33			H-abstraction at C-terminal C(2)
		1.38	0.73 (1H)		Lys side chain [not C(3)]
	DMPO	1.49	1.82 (1H)	0.29 (1N)	Nitrogen-centred on Lys side chain
		1.56	2.35 (1H)		Carbon-centred
Lys-Gly	DBNBS	1.36	0.92 (1H)		Lys side chain at C(3)
N-Acetylhistidine	DBNBS	1.33			H-abstraction at C(2)
Gly-His	DBNBS	1.33			H-abstraction at C-terminal C(2)
		1.36	0.72 (1H)		His side chain at C(3)
	DMPO	1.52	1.98 (1H)		Carbon-centred
N-Acetylaspartic acid	DBNBS	1.33			H-abstraction at C(2)
N-Acetylglutamic acid	DBNBS	1.32			H-abstraction at C(2)
N-Acetyltyrosine	DBNBS	1.33			H-abstraction at C(2)
Gly-Tyr	DBNBS	1.36			H-abstraction at C-terminal C(2)
Tyr-Gly	DBNBS	1.33			H-abstraction at N-terminal C(2)
Tyr-Tyr	DBNBS	1.49	1.19 (2H)	0.07 (2H)[b]	β-scission
		1.33			H-abstraction at C(2)
N-Acetylphenylalanine	DBNBS	1.33			H-abstraction at C(2)
		1.44	0.72 (1H)		Side chain C(3)
		1.36	0.61 (1H)	0.25 (1N)	Decarboxylation at C(2)[d]
N-Acetylphenylalanine-OMe	DBNBS	1.44	0.72 (1H)		Side chain C(3)
		1.33			H-abstraction at C(2)
N-Acetylproline, *N*-Acetylhydroxyproline	DBNBS	0.97			Acyl type radical [$^{\bullet}$CO(R)]
Gly-Pro	DBNBS	1.33			H-abstraction at C-terminal C(2)
		1.39	1.19 (1H)		Pro side chain
Gly-Hyp	DBNBS	1.33			H-abstraction at C-terminal C(2)
Pro-Gly	DBNBS	1.39	0.37 (1H)		Pro side chain [not C(4)]

[a] ±0.02 mT. [b] Splitting due to the hydrogens at positions 2 and 6 on the aromatic ring of DBNBS. [c] Only observed at pH ≥ 7. [d] Only observed at pH ≤ 6. [e] Parameters for radical adduct detected at pH values >5. At pH 4 an alternative species with parameters *a*(N) 1.42, *a*(H) 1.84, *a*(N) 0.32 mT is observed. This second signal is assigned to the protonated aminyl radical.

[generated using an Fe^{2+}–EDTA/H_2O_2 redox couple; *a*(N) 1.36, *a*(H) 0.69 mT and *a*(N) 1.36, *a*(H) 0.50 mT]. The exact identity of the former adduct has not been established; it is tentatively assigned to the C(3) radical formed by a 1,4-hydrogen atom shift reaction, but an alternative assignment to a C(2) radical formed by decarboxylation (see below) cannot be excluded. The formation of a tertiary radical at C(2) *via* a 1,5-shift is probably not favoured in this case due to the de-stabilising influence of the protonated amino group on radicals formed at this site in the free amino acid.[35,36]

Further evidence for intra-molecular 1,5-hydrogen atom shift reactions of aminyl radicals arises from the results obtained with leucine and isoleucine. These amino acids, in contrast to valine where a tertiary radical [presumed to be the C(3) species formed *via* inter-molecular abstraction] is detected, give high yields of adducts from radicals with the partial structure $^{\bullet}CH_2R$ [*cf.* Figs. 4(a),(b)]. These are thought to arise from hydrogen abstraction at the side-chain methyl groups. Similarly nor-valine (2-aminopentanoic acid) gives rise, at short reaction times, to spectra consistent with the trapping of a primary radical [presumed to be *via* hydrogen atom abstraction at C(5)] rather than a secondary radical. At longer reaction times a second signal from a (more stable) secondary radical dominates. The preferential attack at these primary sites compared to tertiary or secondary positions, is ascribed to the occurrence of a favourable intra-molecular 1,5-hydrogen-atom abstraction reaction by the aminyl radical with leucine, isoleucine and nor-valine.

No definitive evidence has been obtained for the occurrence of rapid 1,2-hydrogen atom abstraction reactions (*cf.* data for alkoxyl radicals in aqueous solution[37,38]), though it is possible that such reactions are occurring and that the species generated by this process rapidly undergo further reaction (*e.g.* decarboxylation, see below). Such 1,2 shifts might however not be expected

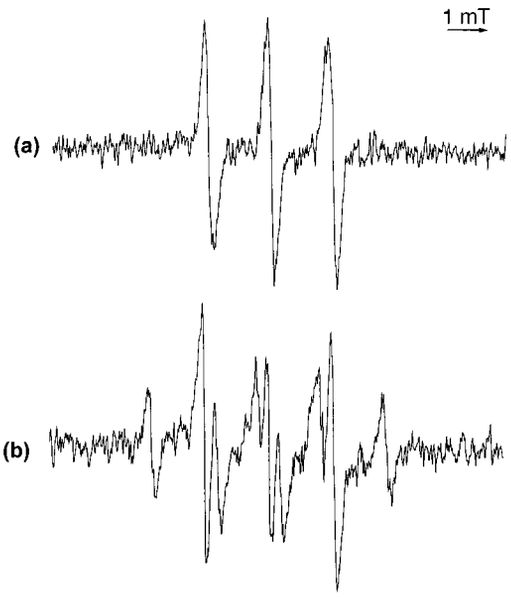


Fig. 4 EPR spectra observed during the early stages of reaction of (a) valine (50 mM) and (b) leucine (50 mM) with HOCl (6.25 mM) and Fe^{2+} (0.25 mM) in the presence of DBNBS (2.5 mM) at pH 7.4. Signal in (a) assigned to a carbon-centred radical produced on hydrogen abstraction at either C(2) or C(3). Signal in (b) assigned to a carbon-centred radical adduct formed by hydrogen abstraction from a side chain methyl group.

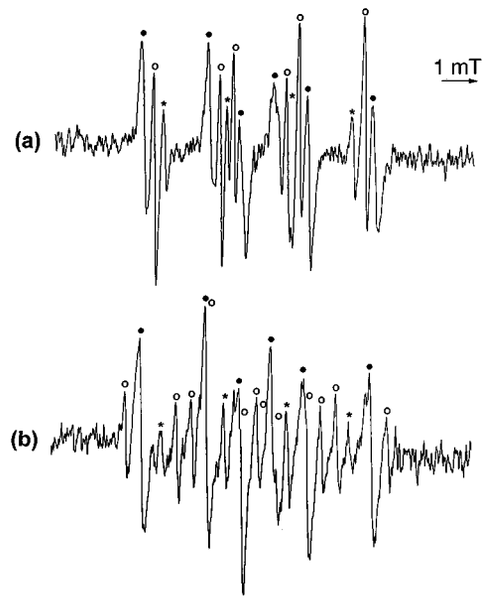
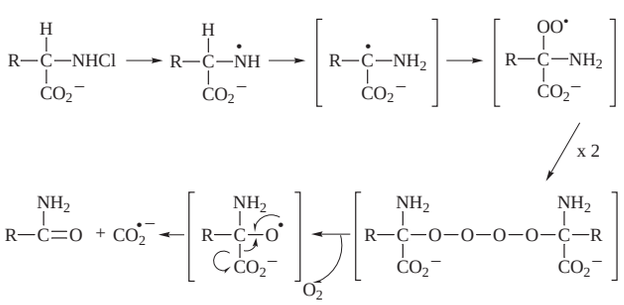


Fig. 5 EPR spectra observed on reaction of (a) lysine and (b) [1-^{13}C]-lysine (both 50 mM) with HOCl (6.25 mM) and Fe^{2+} (0.25 mM) in the presence of DMPO (167 mM) at pH 7.4. Signals marked [●] assigned to a lysine-derived side-chain carbon-centred radical adduct. Features marked [○] assigned to the $CO_2^{\bullet -}$ adduct in (a) and the $^{13}CO_2^{\bullet -}$ adduct in (b). Signals marked [*] are due to DMPO–OH$^{\bullet}$.

to be favoured with the free amino acids due to the formation of an α-carbon [C(2)] radical [or C(6) in the case of the lysine side-chain] with a neighbouring protonated amino group. The situation with amide groups (*e.g.* with *N*-acetyl derivatives and peptides) where this deactivating influence is removed is different (see below).

Decarboxylation.—Evidence has been obtained for the occurrence of two different processes which give rise to decarboxylation of free amino acids; in one case $CO_2^{\bullet -}$ is formed, in the other CO_2 is lost.

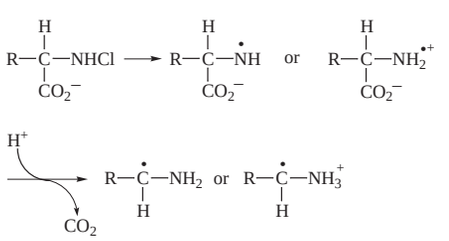
The first mode of decarboxylation involves the formation of $CO_2^{\bullet -}$ from the carboxyl function. This process has been observed with a number of the amino acids using DMPO as the spin trap, and the identity of this species has been confirmed for lysine using [1-^{13}C]lysine [Figs. 5(a),(b)]. The formation of this species is oxygen dependent, suggesting that this is a secondary radical. One possible mechanism of formation of this species involves the initial generation of an α-carbon [C(2)]-centred radical and subsequent reaction with oxygen, formation of an alkoxyl radical at this site (*via* dimerisation of the peroxyl radical and decomposition of the corresponding tetroxide), and subsequent β-scission (Scheme 2). The latter reactions in



Scheme 2

this scheme have been previously demonstrated to occur when α-carbon alkoxyl radicals are generated from the corresponding hydroperoxide.[39] The initial α-carbon radical required for such a process may arise *via* a rapid 1,2-hydrogen atom shift of the initial aminyl radical; the corresponding process with alkoxyl radicals in aqueous solution is fast (*k ca.* 10^7 s^{-1}).[37,38]

The second decarboxylation process occurs primarily at lower pH (≤6) and results in the formation of α-aminoalkyl radicals which can be readily identified in some cases by the presence of distinctive additional nitrogen couplings from their adducts with DBNBS (*cf.* data for leucine in Table 2). In other cases (*e.g.* lysine, histidine, arginine) the additional nitrogen coupling from the amino group is not observed; this is in accord with previous studies on such radicals using this trap and has been ascribed to steric factors affecting the radical conformation.[35] The identity of the radicals has been confirmed in some cases by ^{13}C-labelling. This process does not occur with the corresponding methyl esters (*e.g.* lysine methyl ester). This decarboxylation may arise *via* direct reaction of HOCl with the carboxyl group (*cf.* previous reports[40]) or *via* initial formation of a neutral or protonated aminyl radical. The latter are known to induce decarboxylation of α-carboxyl functions (Scheme 3).[41] The pH dependence of this reaction may therefore be due



Scheme 3

either to a requirement for a protonated aminyl radical (see data above on the pH-induced changes with lysine, and a previous report that the pK_a of protonated dialkylaminyl radicals is *ca.* 6.5–7.3[42]), or for high concentrations of HOCl (rather than $^-$OCl) for the direct decarboxylation process.

Remote decarboxylation at a side-chain carboxyl group has been detected with aspartic acid, with the identity of the carbon-centred radical formed confirmed using [3-^{13}C]aspartic acid. This behaviour was not observed with glutamic acid, suggesting that (at least in this case) decarboxylation is not due to direct reaction of HOCl with the carboxylic acid group but is

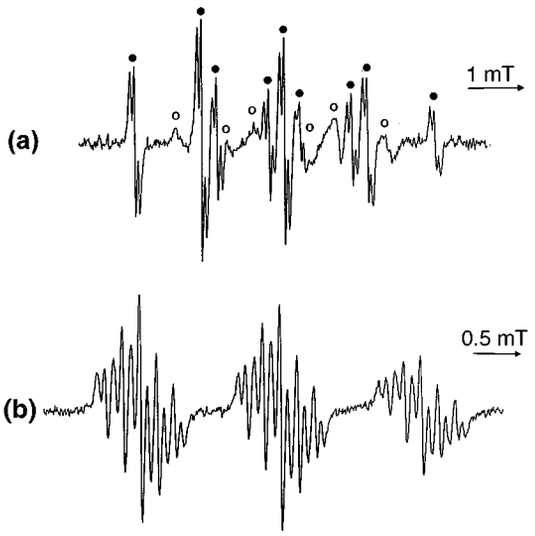
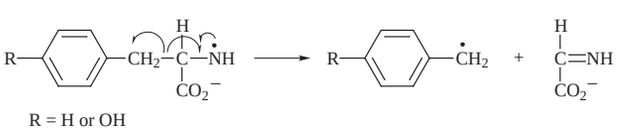


Fig. 6 EPR spectra observed during the early stages of reaction of (a) tyrosine and (b) [3,3-$^2H_2$]tyrosine (both saturated solutions) with HOCl (6.25 mM) and Fe^{2+} (0.25 mM) in the presence of DBNBS (2.5 mM) at pH 7.4. Features in (a) assigned to two different carbon-centred radical adducts formed at C(3) as a result of β-scission [●] and hydrogen abstraction [○] respectively. Signals in (b) assigned to the adduct of the carbon-centred radical at C(3) formed as a result of β-scission of the deuterated substrate.

instead a result of a particularly favourable intra-molecular electron transfer to the initial aminyl radical formed from a chloramine species at the free amino group. This hypothesis is supported by the data obtained with *N*-acetylaspartic acid (see below) where no decarboxylation is observed.

β-Scission.—Reaction of HOCl with tyrosine or phenylalanine in the presence of DBNBS resulted in the detection of short-lived signals [*a*(N) 1.47, *a*(2H) 1.19, *a*(2H) 0.07 mT] which decayed rapidly over *ca.* 10 min [Fig. 6(a)]. A further triplet of doublets signal [*a*(N) 1.42, *a*(H) 0.88 mT] was observed at longer reaction times. The former signal suggests the presence of a radical with partial structure $^{\bullet}CH_2R$ with the further small 1 : 2 : 1 triplet attributed to coupling to the C(2) and C(6) hydrogens on the aromatic ring of DBNBS. This type of radical could arise from cleavage of the amino acid side-chain on either side of C(3) with cleavage of the C(2)–C(3) bond more likely on thermodynamic groups (see Scheme 4). This mode of cleavage



Scheme 4

has been confirmed, in the case of tyrosine, using [3,3-$^2H_2$]tyrosine which results in collapse of the large 1 : 2 : 1 triplet (from the -CH_2- hydrogens) to a small quintet (1 : 2 : 3 : 2 : 1) (from -C[^{2}H]$_2$-) [Fig. 6(b)]. The second signal [triplet of doublets, Fig. 6(a)] has likewise been confirmed as arising from hydrogen atom abstraction at C(3) to give the stabilised (substituted) benzyl radical. The latter is probably formed *via* an inter-molecular hydrogen atom abstraction process, though deprotonation at this site after initial oxidation of the aromatic ring to the radical-cation cannot be ruled out.

The cleavage of the C(2)–C(3) bond observed with these two substrates is believed to arise *via* β-scission of the initial nitrogen-centred radical (which has been trapped with tyrosine, see above) in a manner analogous to tertiary alkoxyl radicals.[43] This process would be expected to be particularly favourable with these substrates, compared to other modes of reaction, as it results in the generation of stabilised benzyl radicals. β-Scission reactions of nitrogen-centred species have been proposed in a previous product study.[44]

***N*-Acetyl amino acids and peptides.** Reaction of HOCl with *N*-acetyl derivatives and small peptides at pH 7.4 in the presence of DBNBS resulted, in the majority of cases, in the detection of radicals resulting from loss of a hydrogen atom from the α-carbon [C(2)] (see Table 3). Different behaviour was observed at lower pH values (see below). Nitrogen splittings from the N-terminal amino group of such C(2) radicals were not observed (*cf.* previous data[35]). In some cases no signals were observed when DMPO was employed as the spin trap; in those cases where carbon-centred species were observed these are thought to be due to the species also detected with DBNBS.

The predominant formation of α-carbon [C(2)] radicals with *N*-acetylamino acids is believed to occur *via* the formation of short-lived chloramide species which decompose rapidly to give (undetected) amidyl radicals which subsequently undergo 1,2-hydrogen atom shift reactions and/or inter-molecular hydrogen atom abstraction to give carbon-centred radicals at side-chain sites. The former process appears to predominate in most cases, presumably as a result of the stability of α-carbon radicals (*via* the capto-dative effect) which do not have a neighbouring protonated amine function.[36] Previous studies have shown that there is little delocalisation of the unpaired electron onto the carbonyl group of amidyl radicals,[45] and that these species react primarily through the nitrogen atom rather than oxygen.[46,47]

In the case of the *N*-acetylamino acids there is only one possible α-carbon [C(2)] site. However with the di- (and higher) peptides this species could theoretically be formed at either the N-terminal α-carbon [C(2)] or at mid-chain/C-terminal positions depending on whether initial reaction with HOCl occurs at the N-terminal amino group or at amide groups down the chain. Given the higher rate of reaction at the free amino group compared to amide positions, the former is likely to predominate. This hypothesis is supported by the data from Tyr-Gly, where the α-carbon [C(2)] radical formed at the two possible positions would give different spectra—only a radical formed at the Tyr position, presumably as a result of a 1,2-shift of an initial nitrogen-centred species formed at the N-terminal amino group, was observed. With increasing peptide chain lengths, and higher HOCl concentrations, reaction at mid-chain sites will presumably become more important (*cf.* the detection of backbone fragmentation with proteins[25]).

Significant yields of side-chain derived radicals were only observed with lysine-containing peptides, where radicals appear to be generated as a result of reaction at both the side-chain amino group (as observed with the free amino acid) and also at the amide or free amino groups on the backbone, and with derivatives and peptides with aromatic side-chains where hydrogen atom abstraction at C(3) occurs readily presumably due to the stability of these species. The detection of a radical with partial structure $^{\bullet}CH_2R$ from the dipeptide Tyr-Tyr suggests that β-scission processes can also occur with aminyl radicals formed at N-terminal amine groups (*cf.* data with the free amino acid above). The corresponding amidyl radical does not appear to behave in a similar manner {*cf.* the α-carbon [C(2)] radicals detected with *N*-Ac-Tyr and Gly-Tyr}.

In some cases further species were detected at longer reaction times; these species were more prominent and dominant at lower pH (≤6). These additional species are ascribed to radicals formed by decarboxylation at the C-terminus. Thus $(Gly)_n$ peptides gave signals from radical adducts with partial structure $^{\bullet}CH_2NR$ (Fig. 7). The mechanism of this decarboxylation process is not fully understood, but may be similar to that observed with the free amino acids at pH values ≤6. In these cases amidyl radicals may be involved due to the remote nature of the free amine function from the free carboxyl group in some of the longer peptide chains, or the decarboxylation may involve a direct reaction between the carboxyl function and HOCl.

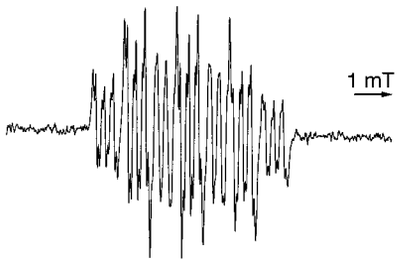


Fig. 7 EPR spectrum observed after 60 min of reaction of Gly-Gly (50 mM) with HOCl (6.25 mM) and Fe^{2+} (0.25 mM) in the presence of DBNBS (2.5 mM) at pH 7.4. Signal assigned to the adduct of a carbon-centred radical formed at C(2) at the C-terminus *via* decarboxylation of the peptide.

Conclusions

This study demonstrates that the reaction of HOCl with free amino acids gives rise to chloramines at free amino groups which can be detected by their characteristic UV absorption bands. The formation of these species is rapid; their decomposition is slower and structure dependent. The decomposition of these intermediates occurs over a similar time scale as radical formation as detected by EPR spin trapping. The radical concentrations in the latter experiments are increased by the presence of Fe^{2+}, which is known to catalyse the decomposition of chloramines to radicals.[48,49] Chloramides were not detected with *N*-acetylamino acids or peptides with no free amino group (*cf.* the detection of analogous species in organic solvents[50]). Rapid reaction with these compounds does however occur as evidenced by the loss of the ^{-}OCl absorption band; these species presumably decay rapidly under the conditions employed in the present study and this is in accord with studies on simple amides in aqueous solution.[29]

With some substrates the initial nitrogen-centred (aminyl) radicals have been trapped. pH studies with free lysine and *N*-Ac-Lys suggest that, at least in these cases, the radical adducts are the neutral $RNH^{\bullet}$-derived species at pH 7.4, rather than the protonated ($RNH_2^{\bullet+}$-derived) adducts; the latter have been detected at lower pH values (5 and below). The pK_a values for these species are therefore somewhere between these values; a previous study has reported pK_a values for protonated dialkylaminyl radicals of *ca.* 6.5–7.3;[42] the (apparently) lower values obtained in this study may reflect the less substituted (mono-alkyl) nature of these radicals. It is therefore unclear which is the major reactive species in most of the reactions under study, though both the pH profiles observed with lysine and *N*-Ac-Lys, and the occurrence of additional reaction pathways at pH values < *ca.* 6 suggest that the neutral aminyl radical may be the major reactive species present at pH 7.4.

The high concentrations of DMPO required (0.1–0.2 M for ornithine) for the detection of these nitrogen-centred radicals suggest that alternative reactions of these radicals are fast. If it is assumed that these nitrogen-centred radicals react with DMPO at rates similar to those for alkoxyl and thiyl radicals (*i.e.* 10^7–10^8 dm^3 mol^{-1} s^{-1} [51]) then the rate constants for alternative (unimolecular) reactions must be $\geqslant 10^7$ s^{-1} in the case of ornithine. The nitrogen-centred species from lysine can however still be detected, though at much lower concentrations, with 10 mM DMPO, which suggests that the rate of rearrangement/fragmentation of this species is of the order of 10^6 s^{-1}. These values are of the same order of magnitude as the corresponding alkoxyl radical reactions.[37,38,43] In many cases only carbon-centred radicals arising from the rapid subsequent reaction of these radicals are observed. Evidence has been obtained for the formation of inter- and intra-molecular (1,5-, 1,2- and possibly 1,4-) hydrogen atom abstractions by these species. Previous studies with other amidyl radicals in organic solvents have also obtained evidence for intra-molecular 1,5- and 1,4-shift reactions, with the latter being much less favoured.[48,50,52] Little previous evidence has been obtained for the occurrence of 1,2-shift reactions with amidyl radicals; this process, like the corresponding alkoxyl radical reactions, may only occur to a significant extent in aqueous or alcoholic solvents due to the requirement for a water (or alcohol) molecule in the transition state.[53] The quantitative importance of these different reaction pathways is impossible to ascertain from the current data, due to the paucity of data on the kinetics of trapping of these types of radicals.

Decarboxylation appears to occur readily when there is a neighbouring carboxyl substituent. This process occurs *via* an oxygen-dependent pathway at pH 7.4, which is postulated to involve a 1,2-shift to give an α-carbon [C(2)] radical, and an alkoxyl radical which undergoes a β-scission process releasing $CO_2^{\bullet-}$ (*cf.* reactions of hydroperoxide-derived radicals[39]). An alternative decarboxylation reaction predominates at lower pH values and gives rise to α-aminoalkyl radicals; the mechanism of this latter process is less clear, though some evidence has been obtained to suggest that it does not occur *via* a direct reaction of HOCl with the carboxyl function (though such reactions are known to occur in other situations, *e.g.* with carbohydrates[54]), and may involve a protonated aminyl species. The products that would be expected from such a radical-mediated process are in accord with those that are observed experimentally.[17,22,55] Direct β-scission of the initial aminyl radical has been observed with some amino acids; this process only competes effectively with other processes when the radical formed *via* cleavage of the C(2)–C(3) bond generates a particularly stable radical—in this case benzyl (or substituted benzyl) species. Previous studies have suggested that β-scission only occurs under particularly favourable circumstances.[44,50]

Reaction of HOCl with *N*-acetylamino acids or small peptides gives radicals predominantly at α-carbon sites *via* reaction at *N*-terminal free amine groups or amide (peptide) bonds. It is proposed that these carbon-centred radicals are produced as a result of the rearrangement of initial nitrogen-centred radicals formed on cleavage of the N–Cl bond of the chloramine/chloramide species by a 1,2 shift reaction. Reaction at the free amine groups appears to be preferred, and is in accord with previous studies which have shown that *N*-terminal, or lysine side-chain, amino groups are favoured over amide groups at least when low concentrations of HOCl are employed.[20,25] Reaction at amide sites when either all free amino groups are blocked, or when high concentrations of HOCl are employed, with subsequent formation of α-carbon radicals may be of considerable biological significance as α-carbon radicals are known, in the presence of oxygen, to give rise to backbone fragmentation *via* the formation of peroxyl and/or alkoxyl radicals.[56] This data provides a rationale for the observation that significant levels of protein fragmentation are only detected above particular, protein-dependent, threshold concentrations of HOCl which correspond (approximately) to the number of HOCl-reactive side-chains in the protein, and that decreasing the number of such sites (*e.g.* by chemical blocking) results in an increased extent of protein backbone fragmentation.[25]

Experimental

Solutions were made up in high-purity, de-ionised water; metal-ion solutions were degassed before, and during, experiments using oxygen-free nitrogen. pH control was achieved by use of 50 mM phosphate buffer for experiments at pH 7.4; sulfuric acid was employed to achieve lower pH values in other experiments. All amino acids and derivatives were from Sigma or Bachem and used as supplied. Isotopically-labelled derivatives were obtained from Cambridge Isotope Laboratories or C/D/N isotopes. DMPO was purified before use by treatment with activated charcoal. DBNBS was synthesised as described previously.[57] Solutions of NaOCl were prepared daily by dilution of

a concentrated stock solution (BDH; approx. 1 M in 0.1 M NaOH) into 50 mM pH 7.4 phosphate buffer. The concentration of HOCl/$^{-}$OCl was determined by measuring the absorbance of the chloramine adduct generated on addition of HOCl to a solution containing taurine (0.1 M) at 252 nm, based on an extinction coefficient of 429 $dm^3\ mol^{-1}\ cm^{-1}$.[26]

EPR spectra were recorded at room temperature using Bruker EMX [with either a standard rectangular (ER 4102ST) or cylindrical (ER 4103TM) cavity] or Jeol RE-1X X-band spectrometers with 100 KHz modulation. Hyperfine couplings were measured directly from the field scan after calibration. Samples were contained in a standard, flattened, aqueous sample cell, and the recording of spectra was usually initiated within 120 s of the start of the reaction. Sequential spectra were recorded for up to 60 min after initiation of the reaction. Concentrations of reagents (after mixing) were as stated in the text and figure legends. The hyperfine couplings were confirmed, in the majority of cases, by spectral simulation using the program WINSIM.[58] The accuracy of each simulation was assessed by direct comparison with experimental data, with the correlation coefficient usually > 0.95. Typical EPR spectrometer settings were as follows; Bruker EMX spectrometer: gain 1×10^6, modulation amplitude 0.05 mT, time constant 81.92 ms, conversion time 40.96 ms, scan time 41.94 s, centre field 348 mT, field scan 10 mT, power 25 mW, frequency 9.76 GHz, resolution 1024 points, average of 8 scans; JEOL RE-1X spectrometer: gain 2×10^3, modulation amplitude 0.1 mT, time constant 0.3 s, scan time 480 s, centre field 334.5 mT, field scan 10 mT, power 10 mW, frequency 9.38 GHz.

The UV–VIS spectra were recorded on either Hitachi U-3210 or Perkin-Elmer Lambda 40 spectrophotometers.

Acknowledgements

The authors are grateful to the Arthritis and Rheumatism Council (UK) and the Australian Research Council for financial support, and Professor B. C. Gilbert, Professor R. T. Dean and Dr A. C. Whitwood for valuable discussions.

References

1 A. J. Jesaitis and E. A. Dratz, *The molecular basis of oxidative damage by leukocytes*, CRC Press, Boca Raton, 1992.
2 A. J. Kettle and C. C. Winterbourn, *Redox Report*, 1997, **3**, 3.
3 S. J. Weiss, M. B. Lampert and S. T. Test, *Science*, 1983, **222**, 625.
4 J. Arnhold, S. Hammerschmidt, M. Wagner, S. Mueller, K. Arnold and E. Grimm, *Biomed. Biochim. Acta*, 1990, **49**, 991.
5 L. J. Hazell, J. J. M. van den Berg and R. Stocker, *Biochem. J.*, 1994, **302**, 297.
6 W. A. Prutz, *Arch. Biochem. Biophys.*, 1998, **349**, 183.
7 C. C. Winterbourn, J. J. M. van den Berg, E. Roitman and F. A. Kuypers, *Arch. Biochem. Biophys.*, 1992, **296**, 547.
8 A. C. Carr, J. J. M. van den Berg and C. C. Winterbourn, *Arch. Biochem. Biophys.*, 1996, **332**, 63.
9 W. A. Prutz, *Arch. Biochem. Biophys.*, 1996, **332**, 110.
10 C. C. Winterbourn, *Biochim. Biophys. Acta*, 1985, **840**, 204.
11 C. C. Winterbourn and S. O. Brennan, *Biochem. J.*, 1997, **326**, 87.
12 C. Yang, Z. Gu, H. Yang, M. Yang, A. M. Gotto and C. V. Smith, *Free Rad. Biol. Med.*, 1997, **23**, 82.
13 C. Yang, Z. Gu, H. Yang, M. Yang, W. S. Wiseman, L. K. Rogers, S. E. Welty, V. Katta, M. F. Rohde and C. V. Smith, *Free Rad. Biol. Med.*, 1997, **22**, 1235.
14 N. M. Domigan, T. S. Charlton, M. W. Duncan, C. C. Winterbourn and A. J. Kettle, *J. Biol. Chem.*, 1995, **270**, 16 542.
15 S. L. Hazen and J. W. Heinecke, *J. Clin. Invest.*, 1997, **99**, 2075.
16 N. R. Matheson and J. Travis, *Biochemistry*, 1985, **24**, 1941.
17 E. E. Van Tamelen, V. B. Haarstad and R. L. Orvis, *Tetrahedron*, 1968, **24**, 687.
18 J. M. Zgliczynski, T. Stelmaszynska, W. Ostrowski, J. Naskalski and J. Sznajd, *Eur. J. Biochem.*, 1968, **4**, 540.
19 J. M. Zgliczynski, T. Stelmaszynska, J. Domanski and W. Ostrowski, *Biochim. Biophys. Acta*, 1971, **235**, 419.
20 W. E. Pereira, Y. Hoyano, R. E. Summons, V. A. Bacon and A. M. Duffield, *Biochim. Biophys. Acta*, 1973, **313**, 170.
21 T. Stelmaszynska and J. M. Zgliczynski, *Eur. J. Biochem.*, 1978, **92**, 301.
22 V. C. Hand, M. P. Snyder and D. W. Margerum, *J. Am. Chem. Soc.*, 1983, **105**, 4022.
23 R. A. Clark, S. Szot, M. A. Williams and H. M. Kagan, *Biochem. Biophys. Res. Commun.*, 1986, **135**, 451.
24 R. J. Selvaraj, B. B. Paul, R. R. Strauss, A. A. Jacobs and A. J. Sbarra, *Infect. Immun.*, 1974, **9**, 255.
25 C. L. Hawkins and M. J. Davies, *Biochem. J.*, 1998, **332**, 617.
26 E. L. Thomas, M. B. Grisham and M. M. Jefferson, *Methods Enzymol.*, 1986, **132**, 569.
27 B. M. R. Bandara, O. Hinojosa and C. Bernofsky, *J. Org. Chem.*, 1994, **59**, 1642.
28 L. K. Folkes, L. P. Candeias and P. Wardman, *Arch. Biochem. Biophys.*, 1995, **323**, 120.
29 C. L. Hawkins and M. J. Davies, *Free Rad. Biol. Med.*, 1998, **24**, 1396.
30 E. S. Yakutova, Y. S. Dremina, S. A. Yevgina, A. N. Osipov, V. S. Sharov, O. M. Panasenko and Y. A. Vladimirov, *Biophysics*, 1994, **39**, 241.
31 A. R. Forrester, in *Magnetic Properties of Free Radicals*, Landolt-Bornstein New Series, Springer-Verlag, Berlin, 1977, group 11, vol. 9, part c 1.
32 C. L. Hawkins and M. J. Davies, *Biochim. Biophys. Acta*, 1997, **1360**, 84.
33 S. F. Nelsen, in *Free Radicals*, ed. J. K. Kochi, Wiley-Interscience, 1973, vol. 2, p. 527.
34 C. J. Michejda, D. H. Campbell, D. H. Sieh and S. R. Koepke, in *Organic Free Radicals*, ed. W. A. Pryor, ACS Symposium Series vol. 69, American Chemical Society, Washington, 1978, p. 292.
35 M. J. Davies, B. C. Gilbert and R. M. Haywood, *Free Rad. Res. Commun.*, 1991, **15**, 111.
36 C. J. Easton, *Adv. Detailed Reaction Mechanisms*, 1991, **1**, 83.
37 V. M. Berdnikov, N. M. Bazhin, V. K. Federov and O. V. Polyakov, *Kinet. Kat.* (*Engl. Transl.*), 1972, **13**, 986.
38 B. C. Gilbert, R. G. G. Holmes, H. A. H. Laue and R. O. C. Norman, *J. Chem. Soc., Perkin Trans. 2*, 1976, 1047.
39 M. J. Davies, *Arch. Biochem. Biophys.*, 1996, **336**, 163.
40 S. K. Chakrabartty, in *Oxidation in Organic Chemistry*, *Part C*, ed. W. S. Trahanovsky, Academic Press, New York, 1978, p. 343.
41 K.-O. Hiller, B. Masloch, M. Gobl and K.-D. Asmus, *J. Am. Chem. Soc.*, 1981, **103**, 2734.
42 R. W. Fessenden and P. Neta, *J. Phys. Chem.*, 1972, **76**, 2857.
43 B. C. Gilbert, P. D. R. Marshall, R. O. C. Norman, N. Pineda and P. S. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1981, 1392.
44 Y. L. Chow and J. N. S. Tam, *J. Chem. Soc., C*, 1970, 1138.
45 W. C. Danen and R. W. Gellert, *J. Am. Chem. Soc.*, 1972, **94**, 6853.
46 Y. L. Chow and T. C. Joseph, *J. Chem. Soc., Chem. Commun.*, 1969, 490.
47 T. C. Joseph, J. N. S. Tam, M. Kitadani and Y. L. Chow, *Can. J. Chem.*, 1976, **54**, 3517.
48 R. S. Neale, *Synthesis*, 1971, 1.
49 G. Sosnovsky and D. J. Rawlinson, *Adv. Free Rad. Chem.*, 1972, **4**, 203.
50 R. A. Johnson and F. D. Greene, *J. Org. Chem.*, 1975, **40**, 2186.
51 M. J. Davies and G. S. Timmins, in *Biomedical Applications of Spectroscopy*, eds. R. J. H. Clark and R. E. Hester, John Wiley, Chichester, 1996, p. 217.
52 R. A. Johnson and F. D. Greene, *J. Org. Chem.*, 1975, **40**, 2192.
53 P. E. Elford and B. P. Roberts, *J. Chem. Soc., Perkin Trans. 2*, 1996, 2247.
54 R. L. Whistler and K. Yagi, *J. Org. Chem.*, 1961, **26**, 1050.
55 H. L. Slates, D. Taub, C. H. Kuo and N. L. Wendler, *J. Org. Chem.*, 1963, **29**, 1424.
56 M. J. Davies and R. T. Dean, *Radical-Mediated Protein Oxidation: from chemistry to medicine*, Oxford University Press, Oxford, 1997, pp. 1–443.
57 H. Kaur, K. H. W. Leung and M. J. Perkins, *J. Chem. Soc., Chem. Commun.*, 1981, 142.
58 D. R. Duling, *J. Magn. Reson.*, 1994, **104B**, 105.

Paper 8/02949K
Received 20*th April* 1998
Accepted 5*th June* 1998