Absolute rate constants for the formation of nitrogen-centred radicals from chloramines/amides and their reactions with antioxidants

David I. Pattison,[a] Michael J. Davies *[a] and Klaus-Dieter Asmus [b]

[a] *The Heart Research Institute, 145 Missenden Rd, Camperdown, NSW 2050, Australia*
[b] *Faculty of Chemistry, Adam-Mickiewicz-University, Grunwaldska 6, 60-780 Poznan, Poland*

Received (in Cambridge, UK) 12th March 2002, Accepted 29th May 2002
First published as an Advance Article on the web 20th June 2002

Pulse radiolysis techniques have been employed to investigate the one-electron reduction of a variety of chloramines and chloramides. These include models for the side-chain of Lys (6-aminohexanoic acid chloramine and α-*N*-acetyl-Lys chloramine), Gly chloramine, β-alanine chloramine and two models of protein backbone amides, the chloramides of cyclo-$(Gly)_2$ and cyclo-$(Ala)_2$. The molar absorption coefficients and stabilities of these chloramines/amides have been determined. The one-electron reduction of these chloramine/amide species by hydrated electrons occurs with second-order rate constants of the order of 10^9–10^{10} M^{-1} s^{-1}, and results in cleavage of the N–Cl bonds to yield nitrogen-centred radicals and chloride ions (as measured by high performance ion chromatography). The reactivities of the nitrogen-centred radicals have been investigated with the readily oxidisable quenchers, hydroquinone and Trolox. These quenchers were used as models of the *in vivo* antioxidants, ubiquinol-10 and α-tocopherol, and react with second-order rate constants between 2×10^7 and 1×10^8 M^{-1} s^{-1}. No evidence was obtained in these pulse radiolysis experiments for a rapid rearrangement of the oxidising nitrogen-centred radicals to reducing carbon-centred radicals, though such reactions have been indicated in previous EPR studies.

Introduction

The oxidation of proteins by the powerful oxidant hypochlorous acid (HOCl) has been studied extensively.[1–5] This interest is due to the detection of specific marker compounds for HOCl-induced protein damage (*e.g.* 3-chlorotyrosine) in many disease states, including atherosclerosis, arthritis and other inflammatory diseases.[4–7] HOCl is produced *in vivo* by activated phagocytes as part of the body immune defense system, but misplaced, or excessive production of HOCl leads to damage to proteins, DNA and other biological compounds.[4,5,8,9]

Some of the major targets for HOCl-mediated protein oxidation are the free amino groups of the Lys side-chains, and those of the N-terminus, to yield moderately stable chloramine species.[10–12] Chloramines are capable of oxidising further substrates (*e.g.* thiols, thioethers *etc.*) but are less reactive than HOCl.[13–15] It has been shown by EPR spectroscopic studies that nitrogen-centred radicals are formed during decomposition of chloramines *via* cleavage of the N–Cl bond.[1,2,12] The rates of formation of nitrogen-centred radicals from chloramines were enhanced by addition of one-electron reductants, such as reducing metal ions (*e.g.* Fe^{2+}, Cu^{+}) or superoxide radicals.[1,2,12,16] Once formed, nitrogen-centred radicals can undergo a variety of rearrangement reactions to give carbon-centred species, *via* intramolecular 1,2 or 1,5 H-shifts, β-scission or decarboxylation processes or intermolecular H atom abstraction reactions.[12,17–20]

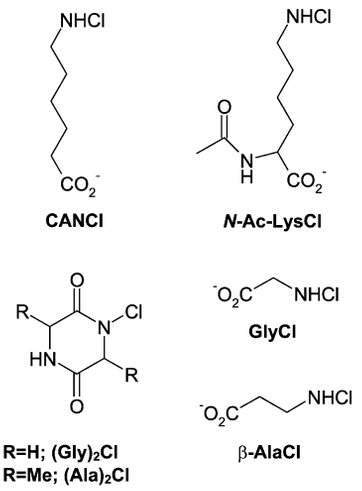
Thus, the identities of radicals that are formed *via* decomposition of chloramines are well characterised. However, the reactivities of nitrogen-centred radicals, and the rearranged carbon-centred radicals, with potential biological targets have not been studied in detail, particularly in aqueous solutions. A recent report[21] showed that the pulse radiolysis technique could be used to investigate the rate of reduction of *N*-chloro,*N*-phenylglycine by hydrated electrons (e^-_{aq}). This resulted in formation of the aminyl radical of *N*-phenylglycine, with a second-order rate constant, $k = (2.9 \pm 0.1) \times 10^9$ M^{-1} s^{-1}. The subsequent reactivity of this radical was not investigated.

One example of a nitrogen-centred radical that has been studied in aqueous solutions is the succinimidyl radical. The reduction of *N*-chlorosuccinimide by e^-_{aq} in H_2O occurs rapidly ($k = 1.6 \times 10^{10}$ M^{-1} s^{-1}) to yield chloride ions, and the nitrogen-centred succinimidyl radical.[22] The succinimidyl radical is capable of oxidising tris(2,2′-bipyridyl)-Ru(II) with a second-order rate constant, $k = 1.3 \times 10^9$ M^{-1} s^{-1},[22] and abstracts hydrogen atoms from acetonitrile and methanol with second-order rate constants of $k = 1.2 \times 10^5$ M^{-1} s^{-1} and $k = 1 \times 10^7$ M^{-1} s^{-1}, respectively.[23] In addition to intermolecular oxidation reactions, the succinimidyl radical undergoes a first-order ring-opening reaction to yield $^{\bullet}CH_2CH_2C(O)NCO$ ($k = 7.8 \times 10^4$ s^{-1}).[22] Similar studies with *N*-chloroglutarimide have shown that it reacts rapidly with e^-_{aq} ($k = 1.9 \times 10^{10}$ M^{-1} s^{-1}) to form the glutarimidyl radical, which undergoes further oxidation and H-abstraction reactions approximately ten times faster than the succinimidyl radical.[23] No evidence for ring-opening reactions was obtained for the glutarimidyl radical.[23]

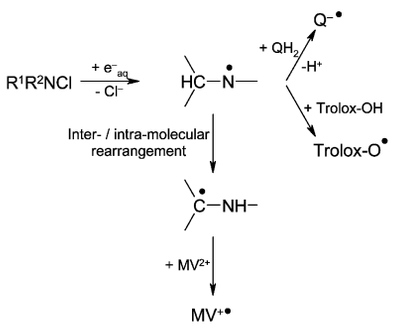
Despite the scarcity of data for the reactivity of aminyl and amidyl radicals in aqueous solutions, several studies have been undertaken in organic solvents.[24–27] However, it is well established that the reactivities of analogous alkoxyl radicals vary dramatically between organic and aqueous/alcoholic solutions,[26,27] and it might be expected that a similar solvent dependency exists for aminyl and amidyl radicals. In particular, it has been demonstrated that alkoxyl radicals do not undergo 1,2-shifts in organic solvents, but these reactions are facilitated in aqueous solutions due to the role of H_2O in the reaction.[28] It might be expected that analogous aminyl radicals would behave in a similar manner.

In this paper, the rates of reduction of a series of chloramines and chloramides (Schemes 1 and 2) by e^-_{aq} have been investigated by pulse radiolysis techniques. Among the chloramines

DOI: 10.1039/b202526d



Scheme 1 Structures of the chloramines/amides used in these studies: 6-aminohexanoic acid chloramine, CANCl; α-*N*-acetyl-Lys chloramine, *N*-Ac-LysCl; Gly chloramine, GlyCl; β-Ala chloramine, β-AlaCl; cyclo-$(Gly)_2$ chloramide, $(Gly)_2Cl$; cyclo-$(Ala)_2$ chloramide, $(Ala)_2Cl$.



Scheme 2 Proposed reaction scheme for the reduction of chloramines/amides by e^-_{aq} and the subsequent radical reactions with oxidisable (QH_2 and Trolox) and reducible (MV^{2+}) quenchers.

selected for study were α-*N*-acetyllysine chloramine (*N*-Ac-LysCl) and 6-aminohexanoic acid chloramine (CANCl), which have been used to model the reactivity of Lys side-chain chloramines generated during HOCl-mediated protein oxidation. Two other chloramines, namely glycine chloramine (GlyCl) and β-alanine chloramine (β-AlaCl), have also been investigated as models for α-amino chloramines, and to determine the effects of neighbouring groups on the reactivity of the resulting nitrogen-centred radicals. The cyclic chloramides, $(Gly)_2Cl$ and $(Ala)_2Cl$, are suitable models for protein backbone chloramides, and are readily generated from reaction of cyclo-$(Gly)_2$ (glycine anhydride) or cyclo-$(Ala)_2$ (alanine anhydride) with HOCl.[29] The subsequent reactivity of the oxidising nitrogen-centred radicals (Scheme 2) has been investigated with hydroquinone (QH_2; a simple model for the antioxidant, ubiquinol-10) and with Trolox (a water-soluble analogue of Vitamin E). Further rearrangement reactions (Scheme 2) of the nitrogen-centred radicals to give reducing carbon-centred radicals have been investigated with methyl viologen (MV^{2+}).

Results and discussion

Determination of molar absorption coefficients and chloramine/amide stability

The chloramine/amide solutions were prepared by adding a small molar excess (1.1–1.2) of the desired amine or amide to HOCl to ensure complete consumption of the HOCl without the formation of dichlorinated products.[10] The molar absorption coefficients of the chloramines were determined by plotting the direct UV absorbance at 252 nm against the chloramine concentration (measured by the oxidation of

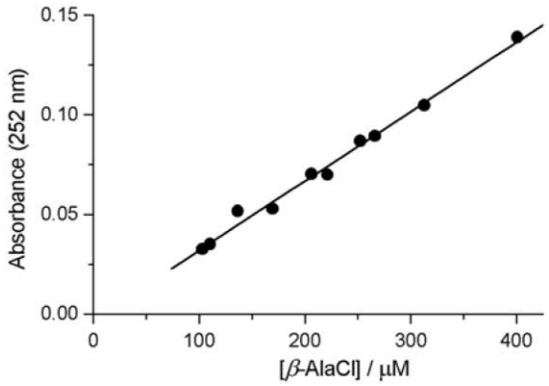


Fig. 1 Determination of the molar absorption coefficient for β-AlaCl at 252 nm. β-AlaCl concentrations were determined by the TNB assay, and the absorbance at 252 nm was determined relative to a β-Ala baseline. The molar absorption coefficients are given in Table 1

5-mercapto-2-nitrobenzoic acid (5-thio-2-nitrobenzoic acid) to the corresponding disulfide; the TNB assay) as shown for β-AlaCl (Fig. 1). The calculated values are very similar to molar absorption coefficients that have been determined for other chloramines (ε = 340–370 M^{-1} cm^{-1}) using different experimental methods.[30,31] The molar absorption coefficients for the two cyclic chloramide compounds could not be determined due to interfering absorbances from the parent compounds themselves, and other reaction products.

The reactions of HOCl with 6-aminohexanoic acid and Gly, to yield CANCl and GlyCl respectively, gave almost 100% yields of the chloramines. Stability studies carried out with the TNB assay showed that neither chloramine decayed by more than 5% at 22 °C over a period of 3 h (the maximum period for carrying out the pulse radiolysis experiments described below). Negligible loss was observed at 4 °C. The reactions of HOCl with $(Gly)_2$ and $(Ala)_2$ to form $(Gly)_2Cl$ (66% conversion) and $(Ala)_2Cl$ (45% conversion) were less efficient under the conditions used to prepare the stock solutions for pulse radiolysis. However, the chloramides formed were reasonably stable with < 20% decay of either chloramide at 4 °C after 4 h, and *ca.* 50% loss of both chloramides at 22 °C after 4 h.

The stability of CANCl has been investigated in the presence of both QH_2 and Trolox to ensure that thermal reactions do not interfere with the pulse radiolysis quenching experiments over the timescales employed. With a 2.5-fold Trolox excess over CANCl (320 μM CANCl, 800 μM Trolox), the concentration of CANCl (measured by the TNB assay) dropped to 95% of the initial value over a period of 3 h at 22 °C (Fig. 2a). These conditions are comparable to the concentrations and time period used for the pulse radiolysis experiments described below, and show that thermal reactions between Trolox and CANCl do not affect the pulse radiolysis data. A similar approach was employed to investigate the reaction between CANCl and QH_2, but preliminary experiments using aerated solutions showed that any $Q^{-\bullet}$ interfered with the TNB assay. Thus, the reaction was monitored directly by UV/vis spectroscopy. Under the conditions used for the pulse radiolysis experiments (where solutions are purged with N_2) there was less than 10% change in absorbance of the QH_2 peak at 285 nm over 1 h (280 μM CANCl, 350 μM QH_2; Fig. 2b), showing that significant reaction between the chloramines and QH_2 does not occur. In contrast, in aerated solutions, rapid absorbance changes were detected (280 μM CANCl, 350 μM QH_2), and over 30 min the QH_2 peak at 285 nm was diminished to *ca.* 50% of the initial intensity.

Quenching of hydrated electrons by chloramines/amides

The reduction of various chloramines/amides (Scheme 1) by e^-_{aq} has been studied in 10% *tert*-butyl alcohol degassed with

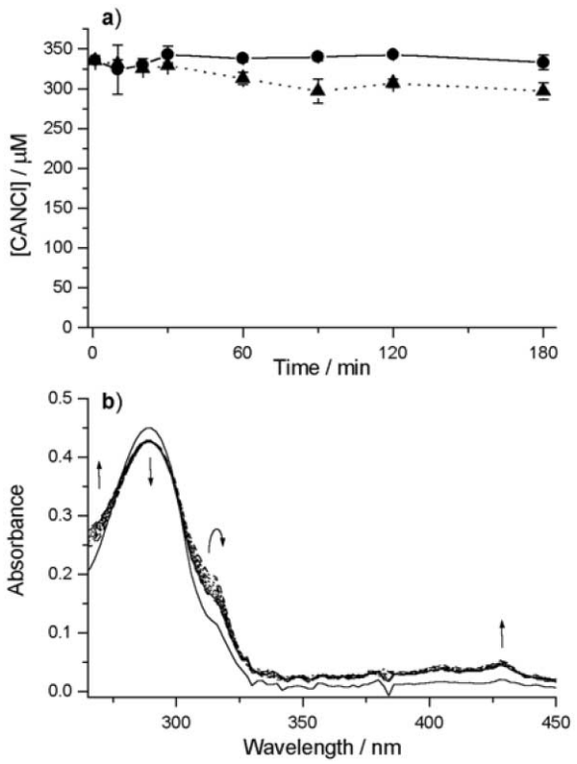


Fig. 2 Investigation of the thermal stability of CANCl in the presence of radical quenchers: a) CANCl stability in the absence of Trolox (●), and in the presence of 800 μM Trolox (▲) with concentrations determined by the TNB assay; b) UV/vis spectra measured every 6 min for the anaerobic (under N_2) reaction of 280 μM CANCl with 340 μM QH_2.

N_2. The rate of decay of e^-_{aq} was monitored at 720 nm, and increased linearly with the concentration of chloramine/amide present (Fig. 3). The second-order rate constants derived from

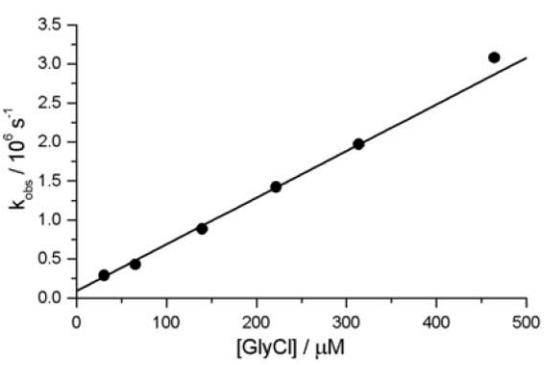


Fig. 3 Plot of the pseudo-first order rate of decay of e^-_{aq} (monitored at 720 nm in 10% *tert*-butyl alcohol degassed with N_2) in the presence of increasing concentrations of GlyCl.

these experiments are shown in Table 1.† Second-order rate constants for the reactions of e^-_{aq} with *N*-chloro,*N*-phenylglycine [21] and *N*-chloroglutarimide [23] have been determined previously as $k = (2.9 \pm 0.1) \times 10^9$ M^{-1} s^{-1}, and $k = 1.9 \times 10^{10}$ M^{-1} s^{-1}, respectively. These values are in close agreement with the rates determined here (Table 1).

EPR spectroscopy has shown that chloramine reduction by metal ions (Cu^+, Fe^{2+}, Ti^{3+}) or superoxide radicals ($O_2^{-\bullet}$) generates nitrogen-centred radicals of the general type >CH–N$^\bullet$–, which are able to undergo further rearrangement reactions to yield carbon-centred radicals, >C$^\bullet$–NH–,[12,17] although the yields of these rearrangement reactions are not known.[2,12,16] The stable products of CANCl (210 μM) reduction by e^-_{aq} (generated by steady-state γ-radiolysis) were investigated by ion chromatography. The yield of chloride ions released relative to the concentration of hydrated electrons generated within the solution was approximately 200% (Fig. 4). This shows that

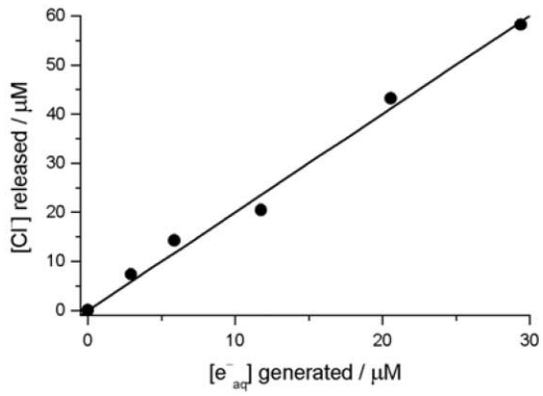
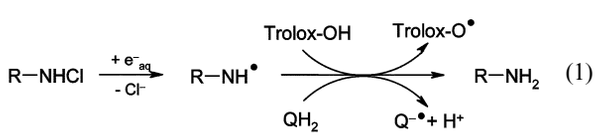


Fig. 4 Plot showing the increased release of chloride ions from a solution of CANCl (210 μM in 10% *tert*-butyl alcohol under N_2) following exposure to increasing concentrations of hydrated electrons (generated by steady state radiolysis with a ^{60}Co source with a dose rate of 37.5 Gy min^{-1}).

electron attachment to the N–Cl bond to give Cl^- and, by inference, nitrogen-centred radicals is the major process under these conditions, but there are also secondary reactions leading to further release of Cl^-. The mechanisms of these secondary reactions are the subject of further studies. The reactivity of the nitrogen-centred radicals generated by electron attachment to the N–Cl bonds was investigated further by pulse radiolysis experiments.

Quenching of oxidising radicals

The nitrogen-centred radicals generated by reduction of chloramines are expected to be strongly oxidising, by analogy with the nitrogen-centred radicals generated from deoxynucleosides and the succinimidyl and glutarimidyl species.[22,32,33] The reactivities of these oxidising radicals with two readily oxidisable quenchers, hydroquinone (QH_2) and Trolox, were investigated [eqn. (1)]. Pseudo-first order reactions of the nitrogen-centred



(1)

radicals with the quenchers were achieved by adding increasing quencher concentrations to chloramine/amide solutions (in 10% *tert*-butyl alcohol degassed with N_2). A typical trace (Fig. 5a) for quenching with QH_2 (monitored at 427 nm) showed a small instantaneous absorbance due to the absorbance of e^-_{aq}. This absorbance decayed as e^-_{aq} reacted with the chloramine/amide to generate nitrogen-centred radicals (inset, Fig. 5a). Aminyl/amidyl radicals are known to absorb weakly (typically $\varepsilon < 1500$ M^{-1} cm^{-1}) in the visible region of the spectrum,[21,22] but no absorbances due to nitrogen-centred radicals were observed in this study, probably due to the relatively poor signal-to-noise ratio. Immediately following the decay of e^-_{aq} a slow growth due to oxidation of the quencher was observed (inset, Fig. 5a). The rate of growth became faster as the added quencher concentration increased, and the second-order rate constants were obtained from linear plots of k_{obs} *versus* the quencher concentration (Fig. 5b, Table 1). At longer times a

† The errors in the second-order rate constants are formally expressed as 95% confidence limits. However, due to partial spontaneous decay of the chloramines/amides these errors only indicate the reproducibility of the kinetic determinations, and do not include systematic errors due to loss of chloramines/amides.

Table 1 Second-order rate constants determined for the reduction of chloramines/amides by hydrated electrons, and for the reactions of the resulting oxidising nitrogen-centred radicals with the radical scavengers, QH_2 and Trolox[a]

Substrate	ε/M^{-1} cm^{-1}	$k_2(e^-_{aq}$ + substrate)/M^{-1} s^{-1}	$k_2(QH_2)$/M^{-1} s^{-1}	k_2(Trolox)/M^{-1} s^{-1}
CANCl	342 ± 16	(9.3 ± 0.6) × 10^9	(1.1 ± 0.1) × 10^8	(7.0 ± 0.3) × 10^7
N-Ac-LysCl	323 ± 45	(6.3 ± 0.7) × 10^9	(8.2 ± 0.7) × 10^7	Nd
GlyCl	351 ± 33	(6.5 ± 0.5) × 10^9	(7.7 ± 0.7) × 10^7	(4.6 ± 0.4) × 10^7
β-AlaCl	348 ± 27	(6.1 ± 0.7) × 10^9	(4.8 ± 0.7) × 10^7	(4.5 ± 0.3) × 10^7
$(Gly)_2Cl$	Nd	(1.5 ± 0.1) × 10^{10}	(5.4 ± 0.9) × 10^7	(2.1 ± 0.3) × 10^7
$(Ala)_2Cl$	Nd	(1.4 ± 0.1) × 10^{10}	(2.9 ± 1.2) × 10^7	Nd

[a] All errors are formally expressed as 95% confidence limits. However, due to partial spontaneous decay of the chloramines/amides these errors only indicate the reproducibility of the kinetic determinations, and do not include systematic errors due to loss of chloramines/amides. Nd, not determined.

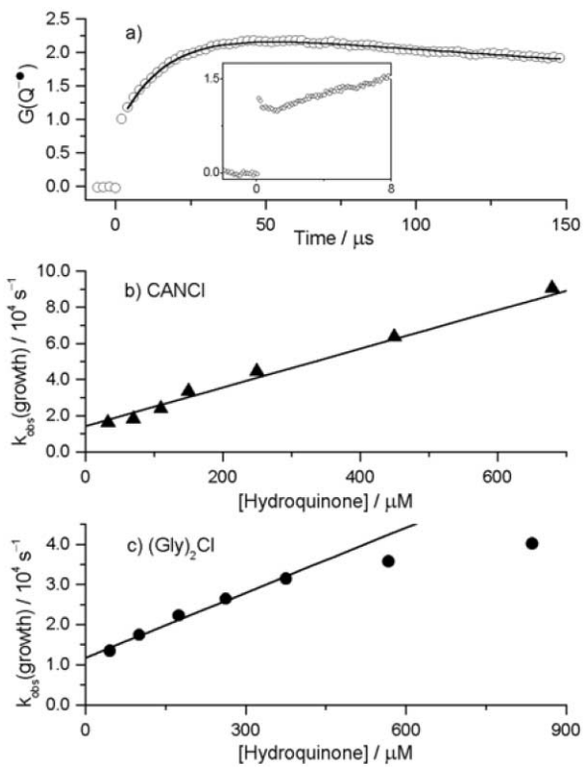


Fig. 5 Graphs showing the data obtained following pulse radiolysis of chloramines/amides in the presence of the readily oxidised quencher, QH_2: a) a kinetic trace (λ, 427 nm; [CANCl], 310 μM; [QH_2], 450 μM; 10% *tert*-butyl alcohol) showing a rapid absorption increase due to e^-_{aq} absorption, followed by growth and decay of $Q^{-\bullet}$. Raw data are shown as open circles, with the calculated fit shown by a solid line. Inset shows the absorbance changes occurring due to e^-_{aq} decay and $Q^{-\bullet}$ formation in the first 8 μs following the pulse. Absorbance is given in terms of *G*, which represents the approximate micromolar concentration per 10 J absorbed energy. b) Plot showing the pseudo-first order rate of formation of $Q^{-\bullet}$ following pulse radiolysis of 10% *tert*-butyl alcohol solutions (under N_2) containing 310 μM CANCl in the presence of increasing [QH_2]. c) Plot showing the pseudo-first order rate of formation of $Q^{-\bullet}$ following pulse radiolysis of 10% *tert*-butyl alcohol solutions (under N_2) containing 300 μM $(Gly)_2Cl$ in the presence of increasing [QH_2]. Linear fit provides a maximal estimate of the second-order rate constant for radical quenching by QH_2.

decay in absorbance was observed (Fig. 5a) due to radical–radical termination reactions between the quencher radicals and other radicals present, and these decays were allowed for in the determination of individual k_{obs} values (as described previously[18]). The oxidation of QH_2 and Trolox by the nitrogen-centred radicals is assumed to occur *via* electron transfer and subsequent deprotonation as described previously.[34,35] However, hydrogen abstraction is an equally acceptable mechanistic alternative and cannot be discounted on the basis of these results. One possibility to shed further light on this question would be an unambiguous identification of the initial protonation state of the semiquinone. Unfortunately, the resolution achievable under the experimental conditions did not allow any definite conclusions to be drawn in this direction. In any case, leaving this mechanistic question open has no bearing on the interpretation of all other data presented in this study.

The second-order rate constants determined for the reactions of nitrogen-centred radicals are similar for both QH_2 and Trolox (Table 1). The nitrogen-centred radicals obtained from the models of the Lys side-chain chloramine exhibit the fastest kinetics with both species. The rates for the GlyCl and β-AlaCl derived nitrogen-centred radicals are slightly slower. This effect is probably due to the electron delocalising properties of the nearby carboxylic acid substituent. The rate determined for the GlyCl-derived nitrogen-centred radical with QH_2 is in close agreement with data reported for the aminyl radical generated by $HO^\bullet$ reaction with Gly in basic solutions, $k = 7.4 \times 10^7$ M^{-1} s^{-1}.[18] The nitrogen-centred radicals derived from the cyclic chloramides reacted more slowly, probably due to increased radical stability induced *via* electron delocalisation into the neighbouring amide bond, and possibly steric interactions. It should be noted that the data obtained for $(Gly)_2Cl$ with QH_2 deviated from linearity at higher [QH_2], thus the given second-order rate constant is a maximal estimate, determined by fitting only the linear portion of the curve (Fig. 5c). The reason for this deviation from linearity is not known and will not be speculated upon at this point.

The yields‡ of QH_2 and Trolox oxidation were also determined from the kinetic traces. The experimental conditions were selected to give e^-_{aq} as the major primary reducing species (G = 2.7), with a small contribution from $H^\bullet$ (G = 0.6). The oxidising contribution of $HO^\bullet$ was removed by addition of 10% *tert*-butyl alcohol, which converted $HO^\bullet$ to the mildly reducing $^\bullet CH_2C(CH_3)_2OH$ radicals (G = 2.7). Typically, the maximum yields of QH_2 oxidation initiated by chloramine reduction were G = 2.7–3.2 (Fig. 6). These results are consistent with a practically quantitative conversion of e^-_{aq} to $Q^{-\bullet}$, suggesting that the primary product of chloramine reduction by e^-_{aq} is the oxidising nitrogen-centred radical. The yield of $Q^{-\bullet}$ is slightly higher ($\Delta G \sim 0.5$) than can be accounted for by e^-_{aq} alone, and is probably due to $H^\bullet$-mediated reduction of the chloramines. An identical experiment was carried out with an N_2O (instead of N_2) degassed solution of CANCl to rapidly quench e^-_{aq}, and leave $H^\bullet$ as the major reactive species. In this case, the maximum yield of QH_2 oxidation was $G \sim 0.7$, consistent with reduction of CANCl by $H^\bullet$ to yield species (presumably nitrogen-centred radicals) that are capable of oxidising QH_2.

The maximum QH_2 oxidation yields, G = 1.7–2.0, obtained by reduction of the cyclic chloramides were lower than with chloramines (Fig. 6), and show that there is incomplete conversion of e^-_{aq} to $Q^{-\bullet}$. The reaction of e^-_{aq} with parent $(Gly)_2$ was investigated at 720 nm, and yielded a second-order rate

‡ Yields are quoted as the number of species formed per 100 eV of absorbed energy. G = 1 corresponds to 0.1036 μM J^{-1} in SI units.

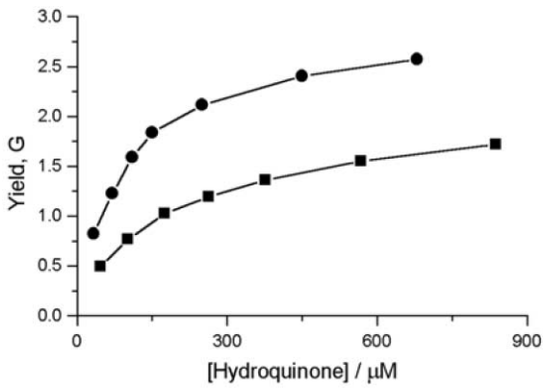
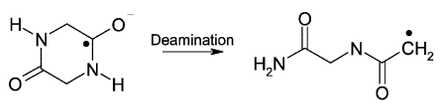


Fig. 6 Plot showing the yields of $Q^{-\bullet}$ generated by pulse radiolysis of 310 μM CANCl (●) or 300 μM $(Gly)_2Cl$ (■) in 10% *tert*-butyl alcohol solutions (under N_2) in the presence of increasing $[QH_2]$.

constant, $k_2 = (1.1 \pm 0.1) \times 10^9$ M^{-1} s^{-1}, in good agreement with previous studies, where $k = 1.7 \times 10^9$ M^{-1} s^{-1} at pH 9.2.[36] The addition of QH_2 to these solutions did not result in QH_2 oxidation, which is consistent with the formation of non-oxidising, cyclic and ring-opened, carbon-centred radicals (Scheme 3) as



Scheme 3 Structures of the carbon-centred radicals previously proposed to be generated by e^-_{aq} reduction of $(Gly)_2$.[36,37]

reported previously.[36,37] Thus, the lower yield of QH_2 oxidation for the cyclic chloramides, compared to the chloramines, can be explained by reaction of e^-_{aq} with any excess $(Gly)_2$ (or $(Ala)_2$) remaining in solution, and with the non-chlorinated amide site within $(Gly)_2Cl$ (or $(Ala)_2Cl$).

The yield data obtained from the Trolox quenching experiments follow a similar pattern to that for QH_2, but the maximum yield for the chloramines is $G = 3.1$–3.4, and for $(Gly)_2Cl$ it is slightly lower, with $G = 2.9$. These yields are slightly higher than the QH_2 data, but can still be attributed to chloramine reduction primarily by e^-_{aq}, with a small contribution from $H^\bullet$-mediated reduction and possibly $^\bullet CH_2C(CH_3)_2OH$ radicals.

Quenching of reducing radicals

It is well established that nitrogen-centred radicals can undergo further reactions, such as inter- or intra-molecular hydrogen atom abstraction, β-scission and decarboxylation processes.[12,17,18,20] The radicals produced by these reactions are typically carbon-centred radicals, and have reducing properties (Scheme 2).[12,17,18,20] Quenching experiments were carried out in the presence of methyl viologen (MV^{2+}), which reacts rapidly with strongly reducing radicals [eqn. (2)], to determine whether

$$\text{Reducing radicals} + MV^{2+} \longrightarrow MV^{+\bullet} + \text{product} \quad (2)$$

reducing radicals could be detected following the reduction of chloramines/amides by e^-_{aq}. The chloramine/amide concentration was increased to 10–20 mM for these experiments, in order to maximise reduction of the chloramines, and minimise direct reduction of MV^{2+} by e^-_{aq}. Due to the high chloramine/amide concentration the solutions were more basic (pH 9–10) for these studies than for the quenching of oxidising radicals described above.

Studies with CANCl and GlyCl yielded predominantly direct MV^{2+} reduction by e^-_{aq}. However, a slow growth due to formation of $MV^{+\bullet}$ was also observed, with a maximum yield of $G = 0.15$, but this signal was too small to allow accurate kinetic data to be determined. Addition of excess 6-aminohexanoic acid to the solutions did not alter the data. This suggests that the small growth is not due to intermolecular reactions of the nitrogen-centred radicals to yield reducing radicals. The process responsible for this small growth could not be determined, but may be due to one (or more) of the previously characterised intramolecular reactions that yield carbon-centred radical species.[12]

Similar experiments with $(Gly)_2Cl$ yielded larger signals and the observed rate of growth due to reaction of MV^{2+} with secondary radicals varied linearly with the MV^{2+} concentration, to yield a second-order rate constant, $k_2 = (8.5 \pm 0.9) \times 10^8$ M^{-1} s^{-1}. The maximum yield due to secondary radical reactions was determined as $G = 0.45$. However, experiments with parent $(Gly)_2$ yielded similar data, thus it is likely that the species responsible for MV^{2+} reduction with $(Gly)_2Cl$ are derived from the reactions of e^-_{aq} with excess $(Gly)_2$ or the non-chlorinated amide groups of $(Gly)_2Cl$.

Thus, despite conclusive evidence for the formation of reducing radicals during chloramine decomposition by EPR spectroscopy and other techniques,[12] it has not been possible to determine the kinetics of these rearrangement reactions in these pulse radiolysis studies.

Conclusions

These studies have shown that chloramines/amides are rapidly reduced by hydrated electrons (k, $6 \times 10^9 - 1.5 \times 10^{10}$ M^{-1} s^{-1}) to yield oxidising radicals and chloride ions. The oxidising radicals undergo rapid reactions (k, $2 \times 10^7 - 1 \times 10^8$ M^{-1} s^{-1}) with QH_2 and Trolox, which are models of the common biological antioxidants, ubiquinol-10 and α-tocopherol. These results suggest that ubiquinol-10 and α-tocopherol may provide protection in biological systems against damage induced by nitrogen-centred radicals derived from chloramine/amide degradation.

For example, it has been shown that oxidation of low density lipoproteins (LDL) by HOCl (300 fold excess) results primarily in protein oxidation (> 80% of HOCl consumption) to form predominantly Lys chloramines, with some lipid peroxidation.[38] Extensive loss of ubiquinol-10 was also observed.[38] The results presented here suggest that some ubiquinol-10 may be consumed by reaction with nitrogen-centred radicals from Lys chloramines, as well as by lipid peroxide radicals. Similarly, the extent of lipid peroxidation induced by HOCl in phosphatidylcholine liposomes is attenuated by the presence of α-tocopherol.[39] The authors commented that the peroxidation is probably mediated by free radical formation, *via* an unknown mechanism. One possible explanation for this phenomenon is that chloramine formation occurs at the choline head group[40] and α-tocopherol acts as an antioxidant against the nitrogen-centred radicals formed upon chloramine degradation. Furthermore, α-tocopherol and ubiquinol-10 might be expected to play a role in protection against chloramine-induced damage in cellular systems, particularly as a role for nitrogen-centred radicals has recently been demonstrated in chloramine-mediated lysis of red blood cells.[41]

Finally, evidence for rearrangement reactions of these nitrogen-centred radicals to form reducing carbon-centred radicals was not observed in these experiments, despite the well-documented formation of these species in EPR spin-trapping studies.[12]

Experimental

Materials

All chemicals were of the highest purity available from Aldrich or Sigma and were used without further purification. Solutions were prepared with water purified by Serv-A-Pure Co. or Micropore Milli-Q systems. The pH was adjusted by addition of appropriate quantities of NaOH or $HClO_4$ for the pulse radiolysis and ion chromatography experiments, or was con-

trolled with Chelex-treated phosphate buffer (100 mM, pH 7.4) for all other experiments.

Pulse radiolysis

Pulse radiolysis experiments were carried out at the University of Notre Dame using an 8 MeV Titan Beta model TBS-8/16-1S linear accelerator. Pulse lengths were typically 2–10 ns with absorbed doses of 2–8 Gy (J kg^{-1}). Dosimetry was carried out using N_2O-saturated KSCN solutions as described previously.[17] A full description of the pulse radiolysis setup and the data acquisition system has been detailed elsewhere.[17–19]

All solutions were prepared with 10% *tert*-butyl alcohol and thoroughly degassed with N_2. Under these conditions the oxidising $HO^{\bullet}$ radicals were converted to relatively unreactive $^{\bullet}CH_2C(CH_3)_2OH$ radicals (G = 2.7), leaving the strongly reducing hydrated electrons (e^-_{aq}) as the major primary product (G = 2.7). In addition to e^-_{aq}, about 20% of the primary species comprised reducing $H^{\bullet}$ atoms (G = 0.6). The contributions of e^-_{aq} and $H^{\bullet}$ atoms to the observed kinetics were investigated by degassing with N_2O instead of N_2, as this converts e^-_{aq} to the unreactive $^{\bullet}CH_2C(CH_3)_2OH$ radicals (*via* $HO^{\bullet}$ formation), leaving $H^{\bullet}$ atoms as the primary reactive species.

Stock chloramine solutions were prepared in H_2O and the concentrations were determined by UV/visible spectroscopy using the molar absorption coefficients given in Table 1. Typically a small molar excess (1.1–1.2) of the substrate (amine or amide) was added over the HOCl to ensure complete consumption of the HOCl without the formation of dichlorinated products (which are known to be formed at higher HOCl : amine ratios).[10] Stock solutions were kept in the dark at 4 °C and aliquots were added *via* syringe to the bulk 10% *tert*-butyl alcohol solution. The quenchers (hydroquinone (QH_2) and methyl viologen (MV^{2+})) used to study the reactivity of the secondary radicals were also prepared as concentrated stock solutions and kept in the dark at 4 °C (under N_2 or Ar) before stepwise addition to the bulk sample. When Trolox was used as the quencher, individual samples (in 10% *tert*-butyl alcohol) were prepared for each Trolox concentration, as the low solubility of Trolox prevented preparation of a concentrated stock solution. A fixed concentration of chloramine was added to each Trolox-containing solution *via* syringe, and the samples were purged with N_2.

The reactions were monitored by optical absorbance at the absorbance maxima of the species of interest: for e^-_{aq}, λ = 720 nm, ε = 19000 M^{-1} cm^{-1}; $Q^{-\bullet}$, λ = 427 nm, ε = 7200 M^{-1} cm^{-1}; Trolox-O$^{\bullet}$, λ = 440 nm, ε = 5400 M^{-1} cm^{-1}; $MV^{-\bullet}$, λ = 600 nm, ε = 12820 M^{-1} cm^{-1}.[19,42,43]

Analysis of the kinetic traces was carried out using Origin 4.1 (Microcal). A single exponential fitting procedure was used to analyse pseudo-first-order e^-_{aq} decay curves. However, for secondary quencher growth curves, a fitting expression that corrected for long-term decays due to radical–radical reactions was used. Absolute errors for kinetic radiation chemical experiments are generally considered to be about ±10%. These errors also apply to the current data unless otherwise stated. All experiments were carried out at approximately 22 °C.

Determination of Cl^- concentrations

Chloride ion concentrations were determined by means of high performance ion chromatography using a Dionex LC20 chromatography enclosure coupled with a Dionex GP40 gradient pump and a Dionex ED40 electrochemical detector. The Cl^- ions were separated by isocratic elution (retention time, 3.6 min) with a buffer containing 2.7 mM Na_2CO_3 and 0.3 mM $NaHCO_3$ (flow rate, 1.5 mL min^{-1}) on an IonPac AS12A (4 mm) analytical column with an IonPac AG12A (4 mm) guard column. An anion self-regenerating suppressor (ASRS-ULTRA (4 mm)) was used to minimise the background conductivity (<15 μS). Ion peaks were detected with a CD25 shielded conductivity detector. Standard curves were determined with Cl^- concentrations from 0–150 μM. Chloramine decomposition was achieved by steady state radiolysis experiments with a ^{60}Co source (dose rate, 37.5 Gy min^{-1}). Samples were prepared in 10% *tert*-butyl alcohol solutions and degassed with N_2 immediately before irradiation. The HOCl used for chloramine formation in these experiments was purified to reduce the initial Cl^- concentration by the method of Henderson *et al*.[44]

Determination of chloramine molar absorption coefficients and stability studies

The UV absorbance of chloramine solutions at 252 nm was determined on a Perkin Elmer Lambda 40 UV/visible spectrometer. The chloramine concentrations were determined by the use of 5-mercapto-2-nitrobenzoic acid (TNB) as described previously.[2,10] Experiments to investigate the stability of the chloramides showed 65% conversion of HOCl to chloramide for $(Gly)_2$, or 45% for $(Ala)_2$; these values were used to calculate the chloramide concentrations in the stock solutions used for pulse radiolysis. The molar absorption coefficients could not be determined accurately due to interfering absorbances from the amides themselves, and other products.

Acknowledgements

This work was supported by the Australian Research Council, Grants A00001441 and F00001444. David Pattison would like to thank the Sydney Free Radical Group for providing a travel grant to undertake this research. He is also grateful to the Radiation Laboratory of the University of Notre Dame and the U.S. Department of Energy for the opportunity to use their pulse radiolysis, and other laboratory, equipment. The authors would also like to acknowledge Mr Kurt Belting and Dr Igor Štefanic for technical assistance. K.-D. Asmus is currently on sabbatical leave from the Department of Chemistry and Biochemistry of the University of Notre Dame.

References

1 C. L. Hawkins and M. J. Davies, *Biochem. J.*, 1998, **332**, 617.
2 C. L. Hawkins and M. J. Davies, *Biochem. J.*, 1999, **340**, 539.
3 J. Arnhold, S. Hammerschmidt, M. Wagner, S. Mueller, K. Arnold and E. Grimm, *Biomed. Biochim. Acta*, 1990, **49**, 991.
4 A. J. Kettle, *FEBS Lett.*, 1996, **379**, 103.
5 N. M. Domigan, T. S. Charlton, M. W. Duncan, C. C. Winterbourn and A. J. Kettle, *J. Biol. Chem.*, 1995, **270**, 16542.
6 S. Fu, H. Wang, M. J. Davies and R. T. Dean, *J. Biol. Chem.*, 2000, **275**, 10851.
7 S. L. Hazen and J. W. Heinecke, *J. Clin. Invest.*, 1997, **99**, 2075.
8 C. L. Hawkins and M. J. Davies, *Free Radical Biol. Med.*, 1998, **24**, 1396.
9 W. A. Prutz, *Arch. Biochem. Biophys.*, 1996, **332**, 110.
10 E. L. Thomas, M. B. Grisham and M. M. Jefferson, *Methods Enzymol.*, 1986, **132**, 569.
11 E. L. Thomas, *Infect. Immun.*, 1979, **23**, 522.
12 C. L. Hawkins and M. J. Davies, *J. Chem. Soc., Perkin Trans. 2*, 1998, 1937.
13 A. V. Peskin and C. C. Winterbourn, *Free Radical Biol. Med.*, 2001, **30**, 572.
14 W. A. Prutz, *Arch. Biochem. Biophys.*, 1999, **371**, 107.
15 W. A. Prutz, R. Kissner and W. H. Koppenol, *Arch. Biochem. Biophys.*, 2001, **393**, 297.
16 C. L. Hawkins, M. D. Rees and M. J. Davies, *FEBS Lett.*, 2002, **510**, 41.
17 M. Bonifacic, I. Stefanic, G. L. Hug, D. A. Armstrong and K.-D. Asmus, *J. Am. Chem. Soc.*, 1998, **120**, 9930.
18 M. Bonifacic, D. A. Armstrong, I. Carmichael and K.-D. Asmus, *J. Phys. Chem. B*, 2000, **104**, 643.
19 I. Stefanic, M. Bonifacic, K.-D. Asmus and D. A. Armstrong, *J. Phys. Chem. A*, 2001, **105**, 8681.
20 G. L. Hug, M. Bonifacic, K.-D. Asmus and D. A. Armstrong, *J. Phys. Chem. B*, 2000, **104**, 6674.

21 M. L. Canle, J. A. Santaballa and S. Steenken, *Chem. Eur. J.*, 1999, **5**, 1192.
22 J. Lind, M. Jonsson, T. E. Eriksen, G. Merenyi and L. Eberson, *J. Phys. Chem.*, 1993, **97**, 1610.
23 G. Merenyi, J. Lind and L. Eberson, *Acta Chem. Scand.*, 1998, **52**, 62.
24 J. A. Howard, *Adv. Free Radical Chem.*, 1972, **4**, 49.
25 R. S. Neale, *Synthesis*, 1971, **1**, 1.
26 B. C. Gilbert, R. G. G. Holmes, H. A. H. Laue and R. O. C. Norman, *J. Chem. Soc., Perkin Trans. 2*, 1976, 1047.
27 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.
28 P. E. Elford and B. P. Roberts, *J. Chem. Soc., Perkin Trans. 2*, 1996, 2247.
29 D. I. Pattison and M. J. Davies, *Chem. Res. Toxicol.*, 2001, **14**, 1453.
30 J. M. Antelo, F. Arce and M. Parajo, *Int. J. Chem. Kinet.*, 1995, **27**, 637.
31 L. Abia, X. L. Armesto, M. Canle, M. V. Garcia, M. Losada and J. A. Santaballa, *Int. J. Chem. Kinet.*, 1994, **26**, 1041.
32 P. O'Neill and S. E. Davies, *Int. J. Radiat. Biol.*, 1987, **52**, 577.
33 A. J. S. C. Vieira and S. Steenken, *J. Am. Chem. Soc.*, 1990, **112**, 6986.
34 G. S. Nahor, P. Neta and Z. B. Alfassi, *J. Phys. Chem.*, 1991, **95**, 4419.
35 P. Neta, R. E. Huie, P. Maruthamuthu and S. Steenken, *J. Phys. Chem.*, 1989, **93**, 7654.
36 E. Hayon and M. Simic, *J. Am. Chem. Soc.*, 1971, **93**, 6781.
37 M. D. Sevilla and R. Failor-Koszykowski, *J. Phys. Chem.*, 1977, **81**, 1198.
38 L. J. Hazell and R. Stocker, *Biochem. J.*, 1993, **290**, 165.
39 O. M. Panasenko, J. Arnhold, J. Schiller, K. Arnold and V. I. Sergienko, *Biochim. Biophys. Acta*, 1994, **1215**, 259.
40 D. I. Pattison and M. J. Davies, unpublished work.
41 C. L. Hawkins, B. E. Brown and M. J. Davies, *Arch. Biochem. Biophys.*, 2001, **395**, 137.
42 G. L. Hug, *Optical spectra of nonmetallic inorganic transient species in aqueous solution*, National Bureau of Standards (United States), 1981, Vol. 69.
43 R. H. Bisby, S. Ahmed and R. B. Cundall, *Biochem. Biophys. Res. Commun.*, 1984, **119**, 245.
44 J. P. Henderson, J. Byun and J. W. Heinecke, *J. Biol. Chem.*, 1999, **274**, 33440.