Evidence for Rapid Inter- and Intramolecular Chlorine Transfer Reactions of Histamine and Carnosine Chloramines: Implications for the Prevention of Hypochlorous-Acid-Mediated Damage†

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ABSTRACT: Hypochlorous acid (HOCl) is a powerful oxidant generated from H_2O_2 and Cl^- by the heme enzyme myeloperoxidase, which is released from activated leukocytes. HOCl possesses potent antibacterial properties, but excessive production can lead to host tissue damage that is implicated in a wide range of human diseases (e.g., atherosclerosis). Histamine and carnosine have been proposed as protective agents against such damage. However, as recent studies have shown that histidine-containing compounds readily form imidazole chloramines that can rapidly chlorinate other targets, it was hypothesized that similar reactions may occur with histamine and carnosine, leading to propagation, rather than prevention, of HOCl-mediated damage. In this study, the reactions of HOCl with histamine, histidine, carnosine, and other compounds containing imidazole and free amine sites were examined. In all cases, rapid formation (k, 1.6×10^5 M^{-1} s^{-1}) of imidazole chloramines was observed, followed by chlorine transfer to yield more stable, primary chloramines (R-NHCl). The rates of most of these secondary reactions are dependent upon substrate concentrations, consistent with intermolecular mechanisms (k, 10^3-10^4 M^{-1} s^{-1}). However, for carnosine, the imidazole chloramine transfer rates are independent of the concentration, indicative of intramolecular processes (k, 0.6 s^{-1}). High-performance liquid chromatography studies show that in all cases the resultant R-NHCl species can slowly chlorinate *N*-α-acetyl-Tyr. Thus, the current data indicate that the chloramines formed on the imidazole and free amine groups of these compounds can oxidize other target molecules but with limited efficiency, suggesting that histamine and particularly carnosine may be able to limit HOCl-mediated oxidation in vivo.

Hypochlorous acid (HOCl) is a strong oxidant that plays an important role in the human immune system (*1*). It is generally accepted that the only source of HOCl in vivo is via the heme enzyme myeloperoxidase (MPO),[1] which catalyzes the oxidation of chloride ions by hydrogen peroxide (*1*, *2*). MPO is released into phagosomal vacuoles or the extracellular space by phagocytes (neutrophils, monocytes, and some macrophages) in response to the activation of the immune system by invading pathogens. In addition to its potent antibacterial properties, HOCl-mediated damage to host tissues (particularly proteins) has been observed in and linked to numerous diseases (e.g., atherosclerosis, kidney disease, and some cancers) (*3*−*8*).

HOCl reacts rapidly with a wide variety of biological materials (reviewed in ref *9*), but because of the high abundance of amino acids, peptides, and proteins in extracellular fluid, these are likely to be the major targets. The most reactive sites on proteins with HOCl are the sulfur-containing amino acid side chains of Met and Cys (both $k > 10^7$ M^{-1} s^{-1}) and cystine ($k \sim 10^5$ M^{-1} s^{-1}) (*10*−*13*). The sulfoxides and oxy acids produced in these reactions are stable products that do not retain the oxidizing ability of HOCl (reviewed in ref *9*). Amine functions such as those on His ($k \sim 10^5$ M^{-1} s^{-1}) and Lys ($k \sim 5 \times 10^3$ M^{-1} s^{-1}) side chains or α-amino groups ($k \sim 10^5$ M^{-1} s^{-1}) also react rapidly with HOCl (*10*, *11*, *14*). These reactions result primarily in the formation of chloramines (RNHCl and RR′NCl) that retain the oxidizing equivalents of HOCl and can induce further damage. Dichloramines can also be formed with high molar excesses of HOCl (*9*, *15*, *16*). The high relative concentration of amino groups compared with sulfur-containing residues in proteins, biological fluids such as plasma, and cells results in chloramine formation being a major process, with these reactions often accounting for more than 50% of added HOCl in in vitro experiments (*17*, *18*).

Because chloramines retain the oxidizing capacity of HOCl, are longer lived, and can penetrate (in some cases) into cells where they can initiate secondary reactions, they are widely believed to be crucial intermediates in HOCl-mediated damage. Most chloramines are much milder, more selective oxidants than HOCl (*15*, *19*−*22*). Thus, the rate constants for reactions of taurine, Gly, and Lys side-chain chloramines with Met and Cys have been reported to be ca. 10^5 times lower than those for HOCl (*21*, *22*). Reactions with other substrates such as amines are also slow ($k < 1$ M^{-1} s^{-1}) (*20*). In contrast, our recent studies on imidazole-

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[1] Abbreviations: Cl-Tyr, 3-chlorotyrosine; Cl_2-Tyr, 3,5-dichlorotyrosine; HPLC, high-performance liquid chromatography; MPO, myeloperoxidase; *N*-Ac-Cl-Tyr, *N*-α-acetyl-3-chlorotyrosine; *N*-Ac-Cl_2-Tyr, *N*-α-acetyl-3,5-dichlorotyrosine; TNB, 5-thio-2-nitrobenzoic acid.

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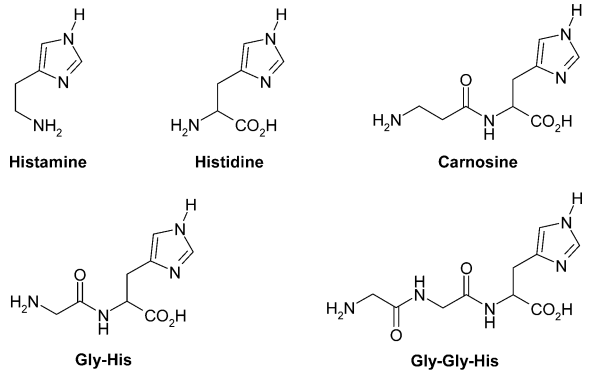
Scheme 1: Chemical Structures of Compounds Containing Both a Free Amine Group and an Imidazole Ring (Histamine, Histidine, Carnosine, Gly-His, and Gly-Gly-His) that Were Used for the Kinetic Studies



derived chloramines, formed on His side chains, have shown that these species are much less selective than other chloramines and that they react with rate constants only 5−30 times slower than HOCl itself (*23*). This is consistent with data for other cyclic chloramines such as those on pyrimidine bases (*19*).

In light of the increasing evidence for a key role of HOCl and chloramines in cell and tissue damage (*22*, *24*, *25*), there is a growing interest in the prevention of such damage and a number of studies have suggested that materials, such as histamine and carnosine, which contain both a free amine and an imidazole ring (see Scheme 1 for structures), can be protective against HOCl-mediated damage. Histamine, which is present in the granules of mast cells at concentrations as high as 100 mM (*22*, *26*), is released at sites of inflammation and might be expected to undergo rapid and extensive chlorination. In support of this hypothesis, it has been reported that the addition of histamine to activated neutrophils in cell culture inhibits the formation of chloramines on endogenous amines (*27*). Carnosine (*β*-alanyl-L-histidine; Scheme 1), which is present at high concentrations in skeletal (ca. 20 mM) and cardiac muscle (low millimolar) (reviewed in refs *28* and *29*), has been postulated to possess anti-aging properties, because it can delay the onset of senescence in cultured human fibroblasts (*30*). A correlation has also been reported between the concentration of carnosine in muscle, which decreases with age, and the expected lifespan of various species (reviewed in ref *31*). Carnosine has also been postulated to be a metal ion chelator, free-radical scavenger, antiglycation agent, and antioxidant (*28*, *29*, *31*). The potential protective effects of carnosine against HOCl-mediated damage have received considerable attention (e.g., refs *28* and *32*−*34*), with mixed results (*33*, *34*). It has been reported that carnosine can reduce HOCl-mediated cross-linking of the lens protein, α-crystallin, in vitro (*33*), but no protective effect was observed against HOCl-mediated inhibition of the protease inhibitor α_1-antiproteinase (*34*), unless carnosine was preincubated with HOCl before the addition to the α_1-antiproteinase.

In the current study, the kinetics of the reaction of HOCl with histamine, carnosine, and other model compounds (Scheme 1) have been investigated by stopped-flow methods. The propensity of formation of chloramines on the free amine or imidazole groups has been assessed, together with subsequent inter- and intramolecular chlorine transfer between such sites and the ability of these species to chlorinate *N*-α-acetyl-Tyr. The latter reaction might be expected to yield 3-chlorotyrosine (Cl-Tyr), which has been used as a specific marker of HOCl-mediated protein oxidation in vivo.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were obtained from Sigma/Aldrich/Fluka (Castle Hill, NSW, Australia) and used as received, with the exception of sodium hypochlorite (in 0.1 M NaOH, low in bromine; BDH Chemicals, Poole, U.K.) and Gly-Gly-His (Bachem, Bubendorf, Switzerland). The HOCl was standardized by measuring the absorbance at 292 nm at pH 12 [$\epsilon_{292}(^{-}OCl) = 350\ M^{-1}\ cm^{-1}$] (*35*). All studies (unless otherwise stated) were performed in 0.1 M phosphate buffer (pH 7.4), which was prepared using Milli-Q-treated water and treated with Chelex resin (BioRad, Hercules, CA) to remove contaminating transition-metal ions. The pH values of solutions were adjusted, where necessary, to pH 7.4 using 100 mM H_2SO_4 or 100 mM NaOH.

Stopped-Flow Studies. All of the stopped-flow studies reported were carried out on an Applied Photophysics SX.18MV system in a 10 mm path-length cell as described in detail previously (*11*, *23*). The monitoring light was provided by a Xe arc lamp with the desired wavelength selected using a two-stage monochromator and subsequent detection by a photomultiplier. The temperature of the reaction chamber and solutions were maintained at 22 °C by circulating water from a thermostated water bath.

Kinetic data were accumulated at 10 nm wavelength intervals between 230 and 320 nm (0.1 M phosphate buffer baseline) and combined to give time-dependent spectral data. In all experiments, HOCl was kept as the limiting reagent with at least a 4-fold excess of substrate (typically 250 μM HOCl and 1.2−6.0 mM substrate). Data were processed by global analysis methods, using Specfit software (version 3.0.36, Spectrum Software Associates; see http://www.bio-logic.fr/rapid-kinetics/specfit.html for further details). A series of kinetic models describing both the initial reaction of HOCl with the substrates and subsequent chlorine transfer processes by intra- or intermolecular mechanisms were fitted to the data to assess the best fit. All reported rate constants are averages of at least six determinations, with errors specified as 95% confidence limits.

Quantification of Chloramines. Chloramine concentrations were quantified by the reaction with 5-thio-2-nitrobenzoic acid (TNB) as described previously (*15*). Briefly, a TNB solution (35−40 μM) was prepared from the disulfide, 5,5′-dithio-2-nitrobenzoic acid (1 mM), by stirring in NaOH (50 mM) for 5 min prior to 40-fold dilution in 0.1 M phosphate buffer (pH 7.4). The loss of TNB (in a 1 mL solution) following incubation with the chloramines (50 μL) for 15 min was quantified at 412 nm, using $\epsilon = 13\,600\ M^{-1}\ cm^{-1}$ (*15*).

Preparation of Samples for Measuring Chlorine Transfer to N-α-Acetyl-Tyr by High-Performance Liquid Chromatography (HPLC). For the HPLC studies determining the extent of chlorination of *N*-α-acetyl-Tyr, the samples were prepared by one of two methods. First, the chloramines of histamine, histidine, and carnosine were preformed by mixing HOCl (1.0 mM) with the parent amine (2.0 mM; both in 0.1 M phosphate buffer at pH 7.4) in equal volumes. The resulting

solutions were left for 2 min to allow for complete conversion to the corresponding terminal chloramine, before dilution into *N*-α-acetyl-Tyr-containing solutions, to give final concentrations of ca. 125 μM chloramine and 1 mM *N*-α-acetyl-Tyr; the resulting solutions were kept at 22 °C. Alternatively, a mixture of histamine or carnosine (250 μM) with *N*-α-acetyl-Tyr (1 mM) was prepared, and HOCl (125 μM) was added directly to this mixture. In both methods, the final concentrations of amine, *N*-α-acetyl-Tyr, and added active chlorine (either as HOCl or chloramine) were identical. For both methods of sample preparation, aliquots were removed from the bulk sample at specific time intervals after mixing (1 h, 24 h, 48 h, 72 h, and 9 days) and diluted 2-fold with a cold (4 °C) solution of *N*-α-acetyl-Cys (500 μM; a 4-fold excess over the initial chloramine concentration) to quench any remaining oxidant. The samples were mixed thoroughly, and the temperature was maintained at 4 °C until HPLC analysis (typically within 24 h).

Chloramine Stability Studies. The chloramines of histamine, histidine, and carnosine were prepared by mixing HOCl (5.5 mM) with histamine, histidine, or carnosine (11.0 mM) in equal volumes. After a few seconds, the solutions were diluted 10-fold with phosphate buffer (0.1 M at pH 7.4) to yield a final chloramine concentration of ca. 270 μM. The solutions were left at 22 °C for 24 h, with 50 μL aliquots removed at various time intervals (2, 15, 30, 45, 60, 90, and 120 min) for chloramine quantification by the TNB assay.

HPLC Instrumentation and Methods. Analysis and quantification of *N*-α-acetyl-Tyr, *N*-α-acetyl-3-chlorotyrosine (*N*-Ac-Cl-Tyr), and *N*-α-acetyl-3,5-dichlorotyrosine (*N*-Ac-Cl_2-Tyr) were carried out on a Shimadzu LC-10A HPLC system (Shimadzu, South Rydalmere, NSW, Australia), as described previously (*23*). Briefly, reaction mixtures were separated on a Zorbax reverse-phase HPLC column (Agilent Technologies, North Ryde, NSW, Australia) packed with octadecylsilanized silica and maintained at 30 °C. The mobile phase comprised of a gradient of solvent A (10 mM phosphoric acid with 100 mM sodium perchlorate at pH 2.0) and solvent B [80% (v/v) methanol in nanopure H_2O] eluting at 1 mL min^{-1} (*23*). The eluent was monitored by a UV detector at 280 nm. *N*-α-Acetyl-Tyr, *N*-Ac-Cl-Tyr, and *N*-Ac-Cl_2-Tyr eluted with retention times of ca. 9, 15, and 20 min, respectively, under these conditions.

Mass Spectral Analysis. Positive ion electrospray ionization was carried out on a LCQ Deca XP Max spectrometer (Thermo Electron Corporation, Rydalmere, NSW, Australia). Samples were prepared in H_2O, pH-corrected to pH 7.4 for the reaction, then diluted 2-fold with methanol containing 2% formic acid (to give 50:50 H_2O/MeOH with 1% formic acid), and run immediately. Samples were introduced into the spectrometer by infusion from the syringe pump at 10 μL min^{-1}. The instrument conditions were source voltage, 4.5 kV; N_2 sheath gas, 5.0 units; and heated capillary, 300 °C. For MS/MS studies, the normalized collision energy was set as 28%.

Statistical Analysis. Statistical analyses of the HPLC data comparing the extent of chlorine incorporation into *N*-α-acetyl-Tyr by the various chloramines at each time point were analyzed using one-way ANOVA with Tukey's post-hoc test. All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

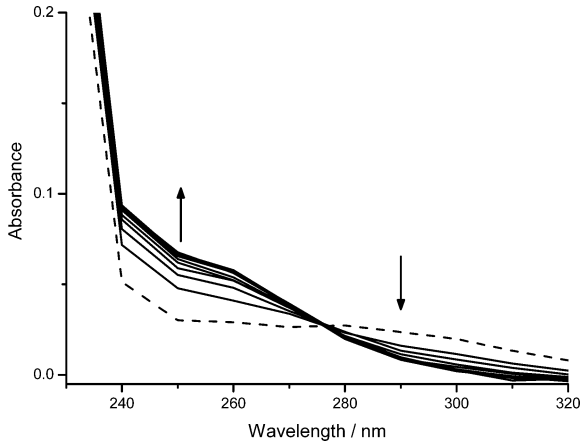


FIGURE 1: Graph showing the time-dependent spectral changes upon the reaction of HOCl (250 μM) with histamine (1.4 mM). The dotted spectrum indicates that obtained 2.5 ms after mixing, and subsequent spectra are given at 2.5 ms intervals up to a final time of 50 ms. The arrows show the direction of the absorbance change in each spectral region, consistent with HOCl loss (λ_{max}, 290 nm) and chloramine formation (λ < 270 nm).

RESULTS

Kinetics of the HOCl Reaction with Histamine, Histidine, Carnosine, and Other His-Containing Peptides. The reactions of HOCl with a variety of targets containing both imidazole rings and free amino groups were investigated by stopped-flow methods at 22 °C. In all cases, the initial reaction of HOCl (250 μM) with the target compound (typically at a range of concentrations between 1.0 and 5.8 mM) was observed by absorbance changes across the spectral range from 230 to 320 nm (Figure 1). These absorbance changes occurred over time scales from 20 to 50 ms depending upon the identity of the target and the concentration. The largest absorbance changes were detected at or close to the characteristic maximal absorbance wavelengths for HOCl (290 nm) and chloramines (250 nm) (*11*, *15*). At 290 nm, rapid consumption of HOCl was detected by the loss in absorbance (parts a, c, and e of Figure 2), whereas at lower wavelengths (<270 nm), rapid increases in absorbance (parts b, d, and f of Figure 2) were observed consistent with the formation of imidazole and/or free amine chloramines that absorb in this region (*11*, *15*, *23*).

To assess whether secondary chlorine transfer processes occur in these systems, the reaction mixtures were monitored for up to 20 s. Over this extended time scale, further increases in absorbance were detected with all target compounds, particularly in the region from 240 to 270 nm. This is consistent with rapid conversion of imidazole chloramines to terminal amine chloramines, because the extinction coefficient at 250 nm is typically greater for primary chloramines than imidazole chloramines (*23*). Representative single wavelength data at 250 nm (10 shot averages) for histamine (250 μM HOCl and 1.1 or 2.8 mM histamine) and carnosine (250 μM HOCl and 1.1 or 2.7 mM carnosine) are shown in Figure 3. Similar studies with Gly-His and Gly-Gly-His gave smaller absorbance changes at 250 nm, suggesting that chlorine transfer is less significant with these compounds.

Analysis of Kinetic Data. It should be noted that all of the rate constants calculated in the following section are apparent rate constants, incorporating both acid dissociation constants

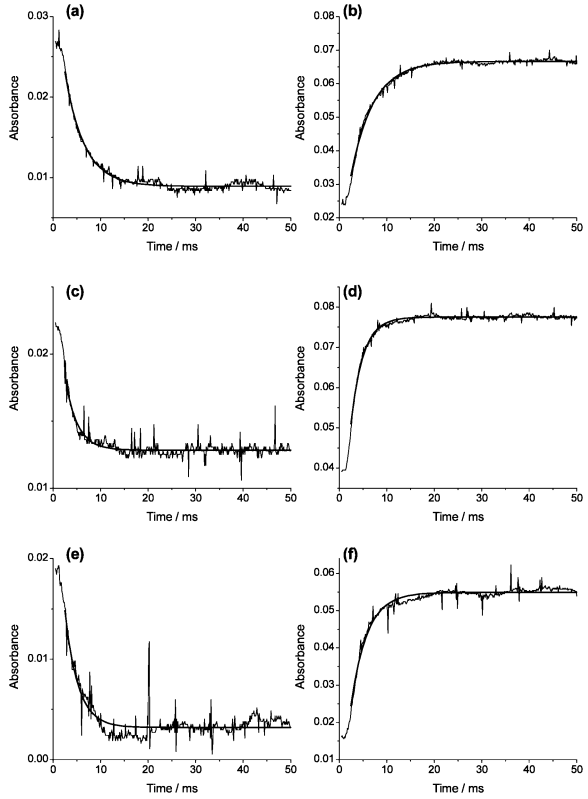


FIGURE 2: Graphs showing the changes in absorbance over short time scales (t = 50 ms) during the reaction (at 22 °C) of HOCl (250 μM) with (a and b) histamine (1.4 mM), (c and d) histidine (1.5 mM), and (e and f) carnosine (1.2 mM). The data in a, c, and e monitor the loss of HOCl at 290 nm, and the data in b, d, and f show the concomitant formation of chloramine species measured at 250 nm. In a−f, the experimental data are shown together with the fit obtained from global analysis for t > 2.5 ms (to avoid interference from the apparatus deadtime).

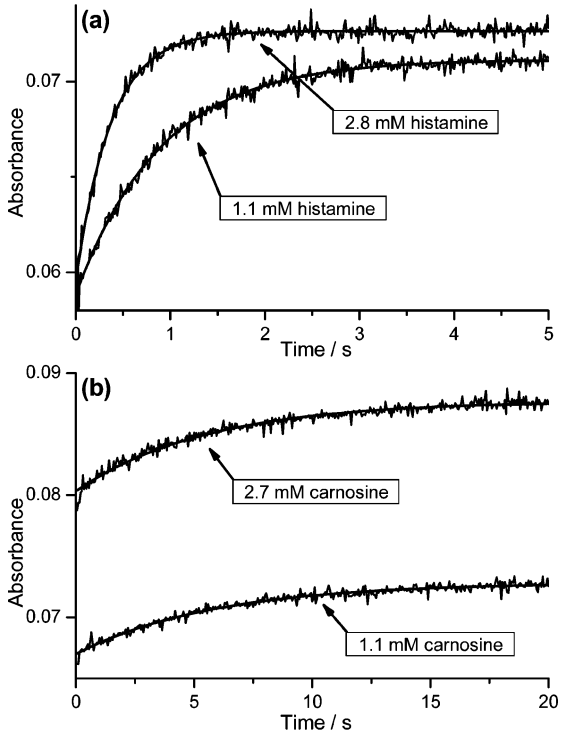


FIGURE 3: Graphs showing the changes in absorbance (at 250 nm) over long time scales during the reaction (at 22 °C) of HOCl (250 μM) with (a) histamine (1.1 or 2.8 mM; t = 5 s) and (b) carnosine (1.1 or 2.7 mM; t = 20 s). The experimental data shown in both graphs were obtained by averaging 10 traces and are shown together with the fit (—) obtained from global analysis for t > 100 ms (to avoid interference from the rapid absorbance changes detected with time scales < 50 ms).

and intrinsic rate constants for the reactions of HOCl or OCl^- with the substrates. Thus, the derived rate constants are highly pH-dependent and are specific to the physiological pH values used in this study.

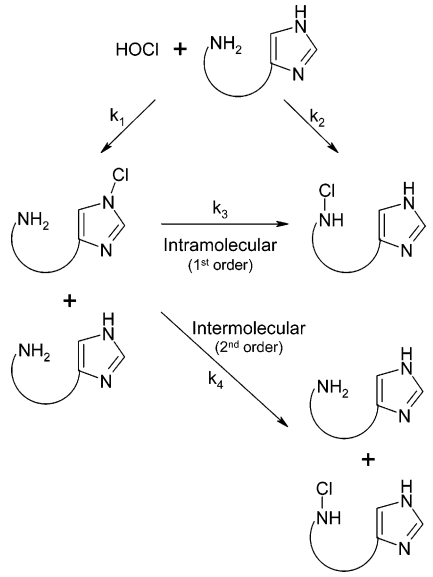
The initial reaction of HOCl with histamine, histidine, carnosine, and other His-containing peptides could occur at either the imidazole ring or the free amino group (Scheme 2; eqs 1 and 2). In the peptides (carnosine, Gly-His, and Gly-Gly-His), the amide nitrogen of the peptide bond is also a potential chlorination site by HOCl, but it has been previously shown that amide nitrogens are several orders of magnitude less reactive than either free amines or imidazole nitrogens (*11*); thus, this path has been disregarded in the data analysis. Consequently, the apparent second-order rate constant obtained from analysis of the short time-scale kinetic data obtained for HOCl consumption (typically < 50 ms) corresponds to the sum of the second-order rate constants for the reaction at both the imidazole (k_1) and free amine (k_2) sites.

$$\text{HOCl} + \text{target compound} \xrightarrow{k_1} \text{imidazole chloramine} \quad (1)$$

$$\text{HOCl} + \text{target compound} \xrightarrow{k_2} \text{terminal amine chloramine} \quad (2)$$

To fit the kinetic data and accurately assess the proportion of chlorination that occurs at the imidazole ring versus the free amine, the reactivity of these sites was determined independently. Previous studies have provided estimates for

Scheme 2: Postulated Reactions and Mechanisms that Occur Following the Addition of HOCl to Compounds Containing Both a Free Amine Group and an Imidazole Ring[a]



[a] Both intra- and intermolecular chlorine transfer mechanisms from the imidazole chloramine are displayed, consistent with the two kinetic models used to fit the experimental data. The arc between the imidazole ring and free amine group in these structures represents the carbon chain linking the two sites, as shown for histamine, histidine, carnosine, Gly-His, and Gly-Gly-His in Scheme 1.

Table 1: Rate Constants (with 95% Confidence Limits) Determined by Global Analysis (at 22 °C and pH 7.2−7.4) for the Reaction of HOCl with the Imidazole Rings (k_1) and Free Amine (k_2) Sites of Various Target Compounds, Together with Those Determined for the Subsequent Chlorine Transfer Reactions (Either Inter- or Intramolecularly) from Imidazole Chloramines to Terminal Amine Chloramines[a]

target compound	k_1 (HOCl + imidazole) (10^5 M^{-1} s^{-1})	k_2 (HOCl + amine) (10^5 M^{-1} s^{-1})	chlorine transfer rate constants	
			k_3 (intra) (s^{-1})	k_4 (inter) (10^5 M^{-1} s^{-1})
histamine	1.7 ± 0.1	0.086 ± 0.005		0.012 ± 0.001 (0.0100 ± 0.0003)
histidine	1.6 ± 0.2	1.6 ± 0.2		0.037 ± 0.006 (0.028 ± 0.002)
Gly-His	1.2 ± 0.8	6.3 ± 4.2		0.19 ± 0.03 (0.17 ± 0.02)
carnosine	1.65 ± 0.08	1.50 ± 0.07	0.6 ± 0.2	

[a] The values in parentheses show the corresponding k_4 values determined from plots of k_{obs} versus the substrate concentration (as in Figure 4).

the second-order rate constants (at 22 °C) for the reactions of HOCl with imidazole rings (e.g., in the His side chain) and free amine groups (e.g., in the Lys side chain) of $k \sim 1 \times 10^5$ and 5×10^3 M^{-1} s^{-1}, respectively (*11*). These values were used to fix the ratio of k_1/k_2 for histamine at 20. Similarly, for histidine, the rate constant determined previously for the α-amino group of Gly (k, 1×10^5 M^{-1} s^{-1}) (*11*), together with the rate constant for an imidazole ring, gave $k_1/k_2 = 1$. The second-order rate constants for the reaction of HOCl with the imidazole rings of histamine and histidine were determined (Table 1) by analyzing the short time-scale (<50 ms) data according to the mechanism in eqs 1 and 2 and fixing the k_1/k_2 ratio as detailed above.

Because no appropriate literature data exists for the reactivity of the free β-alanyl amine group, stopped-flow studies were carried out, at 22 °C, for the reactions of HOCl (250 μM) with both β-Ala (from 1 to 4.5 mM; $t = 50-200$ ms) and β-Ala-Ala (from 1.0 to 2.7 mM; $t = 20-50$ ms). The data showed clean conversion of HOCl (by the loss of absorbance at 290 nm) into a monochloramine (characteristic absorbance increase at 250 nm) with an isosbestic point at ca. 280 nm. The data were analyzed using a simple mechanism (HOCl + amine → chloramine) and yielded second-order rate constants at 22 °C of k_2(HOCl + β-Ala) = $(5.0 \pm 0.1) \times 10^4$ M^{-1} s^{-1} and k_2(HOCl + β-Ala-Ala) = $(1.53 \pm 0.04) \times 10^5$ M^{-1} s^{-1}. The value for k_2(HOCl + β-Ala-Ala) was used in conjunction with that determined for the imidazole ring in histamine and histidine, to estimate a k_1/k_2 ratio of 1.1 for carnosine. Subsequent analysis of the short time-scale data for carnosine yielded the second-order rate constants given in Table 1.

Similarly, the rate constant for Gly-Ala was determined at 22 °C to provide an estimate of the reactivity of the free amine group in Gly-His. The reaction of HOCl (250 μM) with Gly-Ala (540 μM−2.2 mM, $t = 20-50$ ms) resulted in the expected formation of a monochloramine with a second-order rate constant of k_2(HOCl + Gly-Ala) = $(5.7 \pm 0.4) \times 10^5$ M^{-1} s^{-1}. These data were used to determine second-order rate constants (Table 1) for the reaction of HOCl with Gly-His, as described above for other compounds. Similar studies with Gly-Gly-His gave rise to absorbance changes that occurred over time scales (<5 ms) that were very close to the dead time of the apparatus (ca. 2 ms), thereby precluding accurate analysis.

The data obtained on longer time scales, where potential chlorine transfer from the initial imidazole chloramine might occur, were analyzed using two kinetic models describing intra- and intermolecular transfer reactions. Equations 1 and 2 were incorporated into both models to provide estimates of the concentration of imidazole chloramine generated, and residual free terminal amine available as a target for chlorine transfer. Equation 3 was used to define the intramolecular transfer reaction, and eqs 4 and 5 were used to define the intermolecular transfer. For the intermolecular mechanism (eqs 1, 2, 4, and 5), it was assumed that the rate of chlorine transfer from an imidazole chloramine to a free amine was identical for any specific target compound, irrespective of whether the imidazole ring was chlorinated, thus the rate constants for eqs 4 and 5 were constrained to be identical, in addition to constraining the k_1/k_2 ratio as described above. To achieve a unique fit, the UV/vis spectra of HOCl and the target compound were included as "known spectra", thus reducing the number of independent variables.

$$\text{imidazole chloramine} \xrightarrow{k_3} \text{terminal amine chloramine} \quad (3)$$

$$\text{imidazole chloramine} + \text{imidazole chloramine} \xrightarrow{k_4} \text{terminal amine chloramine} \quad (4)$$

$$\text{imidazole chloramine} + \text{target compound} \xrightarrow{k_5} \text{terminal amine chloramine} \quad (5)$$

Global analyses of the long time-scale data using these reaction mechanisms showed conclusively that chlorine transfer from the imidazole chloramines of histamine, histidine, and Gly-His occur via an intermolecular pathway, because the reaction rates are clearly dependent upon the concentration of the target (see Figure 3a for histamine data and Table 1 for second-order rate constants). This was confirmed by fitting the data obtained at 250 nm to a single-exponential function. The observed rate constants (k_{obs}) obtained from this fitting process were plotted against the substrate concentration (0−7 mM, Figure 4). The gradients of the resulting straight lines are equivalent to the second-order rate constants for the chlorine transfer reactions and agree well with the second-order rate constants obtained by global analysis (Table 1). For carnosine, the rate of absorbance change at 250 nm showed no dependence with carnosine concentrations up to 14 mM (see Figures 3b and 4), consistent with intramolecular chlorine transfer; the derived first-order rate constant is given in Table 1.

Mass Spectrometric Characterization of Histamine, Histidine, and Carnosine Chloramines. Positive ion electrospray mass spectrometry yielded $[M + H]^+$ ions for histamine,

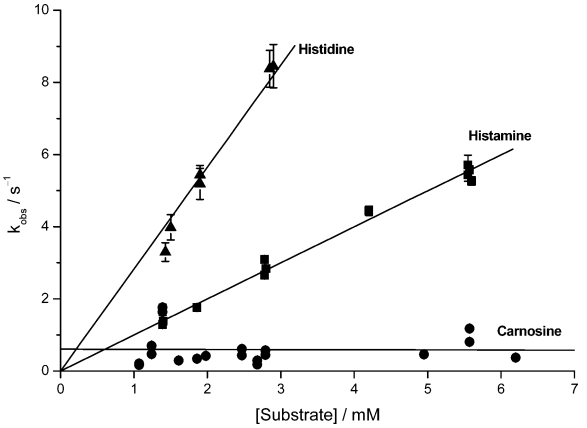


FIGURE 4: Graph showing plots of the observed rate constants (k_{obs}) for the long time-scale processes (2−20 s; as shown in Figure 3) versus substrate concentrations for histidine (▲), histamine (■), and carnosine (●). k_{obs} was determined by fitting the absorbance changes at 250 nm to a single-exponential growth. The linear fits to the data are shown, with clear concentration dependence for histidine and histamine but not carnosine. Kinetics were also measured with 12.4 mM carnosine, but no increase in k_{obs} (0.5 s^{-1}) was observed relative to the data shown at lower concentrations.

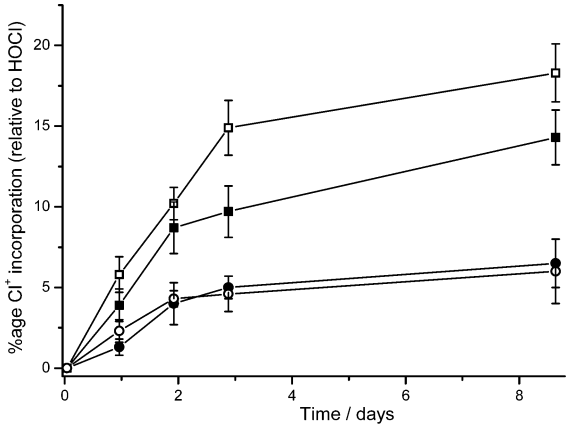


FIGURE 5: Graph showing the time course (over 9 days) of the extent of chlorination of *N*-α-acetyl-Tyr upon incubation of *N*-α-acetyl-Tyr (1.0 mM) with preformed histamine (■) or carnosine (●) chloramines (ca. 125 μM) or following the addition of HOCl (ca. 125 μM) to mixtures of *N*-α-acetyl-Tyr (1.0 mM) with 250 μM histamine (□) or carnosine (○). The formation of *N*-Ac-Cl-Tyr was assessed by HPLC with UV detection (280 nm), and yields are normalized to data from parallel experiments with HOCl. All data are represented as the mean ± 1 standard deviation ($n = 3$ experiments).

histidine, and carnosine at *m*/*z* 112.1, 156.1, and 227.2, respectively. Upon the addition of HOCl to the parent amines (5:1 amine/HOCl), new pairs of peaks corresponding to $[M + 34 + H]^+$ and $[M + 36 + H]^+$ were observed at *m*/*z* 146.2 and 148.2, 190.1 and 192.1, and 261.2 and 263.2 for histamine, histidine, and carnosine, respectively. These increases in mass are consistent with replacement of an H atom by a ^{35}Cl or ^{37}Cl atom, with the expected 3:1 intensity ratio for the Cl isotope pattern. The addition of *N*-α-acetyl-Cys to the chlorinated samples prior to analysis resulted in the loss of the product peaks. This observation confirms that the products were chloramines, because N−Cl bonds are rapidly quenched by thiols to regenerate the parent amine (*21*, *22*).

HPLC Analysis of N-α-Acetyl-Tyr Chlorination by Histamine, Histidine, and Carnosine Chloramines. Chlorination of the Tyr side chain to form Cl-Tyr and 3,5-dichlorotyrosine (Cl_2-Tyr) is a classic marker of HOCl-mediated protein damage (*9*, *36*, *37*), because these products are stable and not produced by other endogenous oxidants. Both HOCl and a number of chloramines (e.g., those on His and Lys side chains) are known to generate Cl-Tyr and Cl_2-Tyr from Tyr side chains (*23*, *37*, *38*). Thus, to further assess whether histamine, carnosine, and related compounds are capable of acting as antioxidants, the ability of their chloramines to chlorinate *N*-α-acetyl-Tyr was investigated using previously developed HPLC methods (*23*).

N-α-Acetyl-Tyr (1.0 mM) was reacted with preformed histamine, histidine, or carnosine chloramines [i.e., the more stable free amine chloramines, all ca. 125 μM as quantified by the TNB assay (*15*)] over a period of 9 days, with samples removed for analysis at 1, 24, 48, 72, and 216 h, and the extent of chlorine incorporation into *N*-α-acetyl-Tyr to give the mono- and dichlorinated species was calculated. Previous studies (*23*) have shown that the total chlorine incorporation into *N*-α-acetyl-Tyr from HOCl is extremely efficient (>95%), and the extent of incorporation from these chloramines has been normalized to that observed for HOCl in parallel experiments.

The free amine chloramines of histamine and carnosine chlorinated *N*-α-acetyl-Tyr slowly, over a period of days, consistent with previous data for Lys side-chain chloramines (*23*). At the 1 h time point, no chlorine transfer was detected with either chloramine, but over 9 days, approximately 15% of the active chlorine available in histamine chloramines was incorporated into *N*-Ac-Cl-Tyr, with significantly lower values (ca. 6%, $p < 0.01$) observed with carnosine chloramines (Figure 5). Over this time, the chloramines had fully decomposed (as measured by the TNB assay); thus, the overall efficiency of chlorine transfer to *N*-α-acetyl Tyr is low.

The potential antioxidant properties of histamine and carnosine were also assessed in experiments where histamine, carnosine, or a buffer control were mixed with *N*-α-acetyl-Tyr (final concentrations: *N*-α-acetyl-Tyr, 1.0 mM; histamine or carnosine, 250 μM) before the addition of HOCl (final concentration of 125 μM). The active chlorine yields were assessed by TNB in the absence of *N*-α-acetyl-Tyr. The extent of chlorine incorporation into *N*-α-acetyl-Tyr in these experiments was not significantly different ($p > 0.05$) to that observed with preformed histamine or carnosine chloramines (Figure 5), except for the 72 h time point with histamine ($p < 0.01$).

In contrast to histamine and carnosine chloramines, no chlorination of *N*-α-acetyl-Tyr by the preformed chloramine of histidine was detected, even after 9 days. This is consistent with the relative stabilities of the chloramines. For histidine chloramines, less than 10% of the initial concentration remained after 2 h at room temperature (22 °C; Figure 6), whereas more than 60% of histamine and carnosine chloramines still remained after 24 h (Figure 6).

DISCUSSION

The reactions examined in this study between HOCl and compounds containing an imidazole ring consistently yield second-order rate constants for the reaction of HOCl with the imidazole ring of ca. 1.6×10^5 M^{-1} s^{-1} (at pH 7.4 and

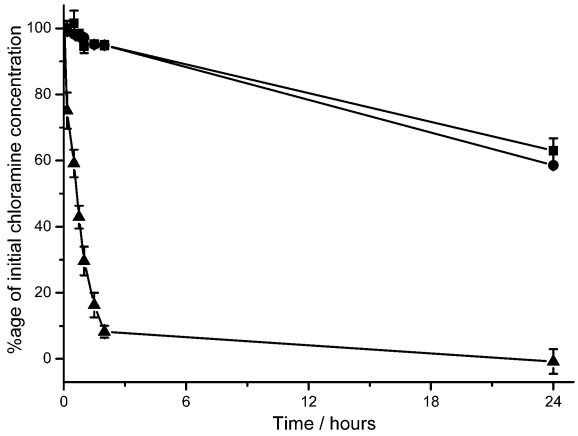


FIGURE 6: Graph showing the stability of histamine (■), histidine (▲), and carnosine (●) chloramines at 22 °C and pH 7.4 over 24 h. In each case, ca. 275 μM HOCl was added to a 2-fold excess of the parent compound. All data are represented as the mean ± 1 standard deviation ($n = 3$ experiments); all data points include error bars, but in some cases, these are smaller than the symbol size.

22 °C). This value is in close agreement to that predicted from experiments undertaken at 10 °C in previous studies (*11*). While the use of the previous value (*11*) was required to undertake accurate kinetic analysis of the current data, the excellent fits to the data and the close agreement between the two values confirms the validity of the earlier predictions (*11*). In the current study, no variation in the rate of the reaction of HOCl with the imidazole group was observed, showing that the substituents on the imidazole ring do not significantly affect its reactivity with HOCl. However, as discussed previously (*11*), the reactivity of the His side chain in proteins may vary considerably at pH 7.4 because of variations in the pK_a of the imidazole ring induced by interactions with nearby amino acid residues.

In contrast, the rate of the reaction of HOCl with the free amino groups varies considerably with these compounds, with second-order rate constants ranging from ca. 5×10^3 M^{-1} s^{-1} for the free amine group of histamine to ca. 10^6 M^{-1} s^{-1} for the amine group of Gly-Gly-His, although the latter rate constant could not be assessed accurately because the kinetics were heavily distorted by the dead time of the apparatus. This variability in reactivity is consistent with previous studies for other amine compounds (*10*, *11*, *13*, *14*, *16*). In particular, it is interesting to note that the rate constant for the reaction of HOCl with the amine group of β-Ala (k, 5×10^4 M^{-1} s^{-1}) is increased 3-fold simply by the addition of a further alanyl group (β-Ala-Ala). This is consistent with our previous studies (*11*), where the presence of a negatively charged group (e.g., R-COO^-) in the vicinity of an amide site reduced the rate of chloramide formation up to 10-fold compared to a neutral group (e.g., R-$COCH_3$); this is attributed to the unfavorable interaction between the negative charges of ClO^- and R-COO^-.

The kinetic data presented here show that, for each of the substrates investigated, both the free amine and imidazole ring are competitive targets for chloramine formation by HOCl, with the product ratio primarily depending upon the variation in the reactivity of the free amine, rather than the imidazole ring. Thus, with histamine, where the free amine site is relatively unreactive, imidazole chloramine formation initially accounts for ca. 95% of the added HOCl. For His and carnosine, the yield of imidazole chloramines is around 50%, and for Gly-His and Gly-Gly-His, imidazole chloramine formation accounts for <20% of the HOCl added. Thus, the populations of imidazole and free amine chloramines and therefore the extent and rate of subsequent chlorine transfer reactions are markedly structure-dependent.

The kinetic data obtained for the reaction of HOCl with histamine, histidine, carnosine, Gly-His, and Gly-Gly-His over long time scales clearly indicate that chlorine transfer occurs from imidazole chloramines to free amine sites. The magnitudes of the observed absorbance changes are consistent with the extent of formation of imidazole versus free amine chloramines described above. For Gly-Gly-His, the chlorination of the α-amino group occurs very rapidly, thus only low yields of imidazole chloramine are formed. As a result, the secondary absorbance changes (because of secondary transfer reactions of the imidazole chloramine) are correspondingly small, thus preventing accurate analysis of the data. For histamine, histidine, and Gly-His, transfer was complete in less than 5 s and the rate of transfer was dependent upon the concentration of the target (Figure 4), consistent with intermolecular transfer (see Scheme 2). The second-order rate constants for these reactions follow the same pattern of reactivity as the initial reaction of the amines with HOCl, with Gly-His > His > histamine (data from this study and ref *11*). However, for both histamine and His, these rates are slightly faster (2−5-fold) than those for the reaction of *N*-α-acetyl-His chloramines with *N*-α-acetyl-Lys or Gly (*23*). In contrast, the transfer reaction observed with the imidazole chloramine of carnosine occurs over a longer time period (ca. 20 s) and is independent of the carnosine concentration (Figure 4), consistent with intramolecular transfer (see Scheme 2). It should be noted that the carnosine data were not fitted perfectly by global analysis by either mechanism, although analysis was more reproducible with the intramolecular process; it is likely that the observed kinetic traces are composites of both first- and second-order processes, with intramolecular transfer being the major pathway for carnosine. It was not possible to analyze these data using a mixed mechanism by global analysis because the model is over parametrized and does not converge to a unique solution.

The reason that intramolecular chlorine transfer occurs with carnosine but not the other compounds is unknown. Mass spectrometry studies confirmed the formation of semistable chloramines at the free amine site of histamine, His, and carnosine but did not provide any insight into the underlying reasons for the different chlorine transfer mechanisms. We postulate that the intramolecular transfer process observed with carnosine is due to an intrinsic property that is unique to the imidazole chloramine of carnosine, such as the formation of an internal hydrogen bond that disfavors intermolecular chlorine transfer, allowing intramolecular transfer to compete efficiently. However, why this does not occur with the almost identical compound Gly-His is unclear. Thus, the reasons for the intramolecular chlorine transfer observed with carnosine remain to be elucidated.

The reactivity of the terminal amine chloramines formed either directly or as a result of chlorine transfer from the imidazole ring is also important in any assessment of potential antioxidant properties of these compounds. Therefore, the ability of such chloramines to generate chlorinated products from Tyr side chains [a commonly used marker of

HOCl-induced damage (*9*, *36*, *37*)] was assessed. Previous studies (*23*) have shown that both HOCl and preformed imidazole chloramines (e.g., on *N*-α-acetyl-His) chlorinate *N*-α-acetyl-Tyr with 95−100% yield within 1 h. In contrast, the preformed terminal amine chloramines of histamine and carnosine examined in the current study chlorinated *N*-α-acetyl-Tyr only slowly (over days rather than minutes) and in low yield (ca. 15 and 5% incorporation of active chlorine, respectively). The time course of this chlorination is similar to that observed with chloramines formed on the terminal amine of Lys side chains or the α-amino groups of Gly (*23*). The extent of chlorination induced by carnosine and histamine chloramines is approximately half that observed with Lys side-chain chloramines (*23*), despite the similarity in stability of these chloramines (Figure 6 and ref *39*). Low yields of *N*-α-acetyl-Tyr chlorination are also observed when carnosine or histamine are added to *N*-α-acetyl-Tyr solutions prior to the addition of HOCl. Thus, histamine and carnosine can efficiently scavenge HOCl and prevent rapid initial *N*-α-acetyl-Tyr chlorination, although slow secondary processes do give *N*-Ac-Cl-Tyr in low yield. These secondary processes are sufficiently slow that in vivo the chloramines are likely to be quenched by other agents (e.g., thiols), therefore diminishing the extent of damage compared to that mediated by HOCl alone.

These data provide important insights into the potential protective role(s) of histamine and carnosine in vivo. Production of HOCl is known to occur at sites of inflammation, and the damage that it induces has been linked with the progression of inflammatory diseases such as atherosclerosis (*4*−*7*). Evidence has been presented for an elevated concentration (300−750 μM) of histamine in blood from patients with atherosclerosis (*40*). These elevated histamine levels have been suggested as a potential marker of atherosclerosis, although they may merely indicate the presence of ongoing inflammation and not be specific to this pathology. These measured histamine levels (300−750 μM) are relatively low compared to, for example, free amino acids (ca. 2.5 mM) and protein-bound Lys (ca. 59 mM, calculated from data in ref *41*) and His (ca. 15 mM, estimated from the data in ref *41*, using the amino acid composition of HSA to compare to the Lys concentration) residues in plasma. Thus, the major targets of HOCl in plasma are likely to be the protein-bound Lys and His residues because of their sheer abundance and to a lesser extent the α-amino groups of free amino acids; it would be interesting to undertake computational modeling studies [as undertaken in previous studies (*11*, *42*, *43*)] to verify this prediction, but the kinetic data required to generate a complete model are not yet available.

In contrast to the relatively low histamine concentrations measured in blood (*40*), the concentrations of histamine at sites of inflammation are likely to be significantly greater. Activated mast cells are preferentially found in the rupture-prone shoulder regions of atherosclerotic lesions and also in the erosion-prone fibrous caps (reviewed in refs *44* and *45*); degranulation of these cells is known to release a number of inflammatory mediators, including a range of cytokines and chemokines, together with matrix metalloproteinases, proteases, and histamine (*44*, *45*). With the histamine concentration in mast cell granules ca. 100 mM (*22*, *26*), it is likely that the local concentrations of histamine following mast cell degranulation could be sufficiently high for histamine to represent an important target for HOCl released from activated monocytes and macrophages in atherosclerotic lesions.

These observations have prompted previous investigations into the cellular effects of histamine chloramines. The addition of HOCl to cells growing in media supplemented with histamine results in rapid irreversible uptake of histamine by the cells (*27*), with this attributed to the formation of neutral, membrane-permeable, histamine chloramines, which are subsequently reduced inside the cell by thiols (e.g., glutathione). It has also been reported that histamine chloramines are moderately cytotoxic (*22*), able to modulate apoptotic pathways (*25*), mutagenic to bacteria (*46*), promote bronchoconstriction in guinea pigs (*47*), and increase epithelial permeability in the rabbit small intestine (*48*).

The data obtained in the current study, together with that obtained by Peskin and Winterbourn for other small molecules containing terminal amine sites (e.g., taurine, glycine, and histamine) (*20*), suggest another possible explanation for some of these observations. It has already been demonstrated (*20*) that the addition of the terminal amine chloramines, of any of the above molecules, to a solution containing one or more of the other compounds results in slow equilibration to form a mixture of chloramines. The current data suggest that exposure of biological fluids rich in histamine to HOCl will initially give rise to high yields of the imidazole chloramine from histamine. However, subsequent rapid intermolecular chlorine transfer reactions will occur, resulting in the formation of a mixture of free amine chloramines on most, if not all, available amine sites. The resulting chloramine composition will depend upon both the concentration and the relative reactivity with the imidazole chloramine of the available amine sites. Because the free amine site of histamine is one of the less reactive amine groups, it is unlikely that chloramines formed on the free amine site of histamine will be a major contributor to the total chloramine pool, but chloramines on the α-amino groups of free amino acids are likely to be major products given their high reactivity (*11*) and abundance in biological fluids [e.g., 2.5 mM in plasma (*11*)]. Thus, the cellular and in vivo effects previously attributed to histamine chloramines (*22*, *25*, *27*, *46*−*48*) may result from a complex mixture of chloramines on all available amine sites.

The kinetic data obtained here suggest that carnosine may be a more efficient protective agent against HOCl-mediated damage than histamine in complex systems, because of its ability to undergo intramolecular chlorine transfer. Thus, production of HOCl in biological fluids that are rich in carnosine is likely to result in extensive initial formation of the imidazole chloramine on carnosine, followed by preferential intramolecular chlorine transfer, thus retaining the chloramine on the carnosine molecule rather than generating an equilibrium mixture of chloramines. Because this imidazole chloramine and the free amine chloramine formed by internal chlorine transfer react slowly and with low efficiency with *N*-α-acetyl-Tyr, the overall effect of formation of these chloramines will be to ameliorate the potential damage induced by HOCl. Furthermore, the long lifetime of these species will favor the reaction with (protective) thiol groups and hence removal of the oxidizing equivalents, rather than further chlorination reactions.

It is well-established that chloramines formed on the α-amino sites of amino acids undergo hydrolysis and deamination to form amino-acid-derived aldehydes (e.g., ref *49* and reviewed in ref *16*). These aldehydes are able to undergo further reactions with protein amine groups via the formation of Schiff bases with subsequent rearrangement mechanisms. The resulting adducts have been detected in low-density lipoprotein (LDL) oxidized in vitro by the MPO/H_2O_2/Cl^- system (*50*) and in LDL isolated from atherosclerotic lesions (*51*). The kinetic data presented here suggest that carnosine will inhibit the formation of chloramines at the α-amino sites of amino acids and therefore may also reduce the damage mediated by reactive aldehydes by preventing their formation via this pathway.

The data obtained also provide a rationale for the conflicting data in the literature on the protective properties of carnosine against HOCl-mediated damage. Hipkiss et al. found that adding physiological concentrations (0.1−20 mM) of carnosine to HOCl-treated bovine α-crystallin abolished protein cross-linking and aggregation (*33*). While the molar excesses of HOCl to α-crystallin used are unclear, the results from the current studies suggest that, with the concentrations of carnosine added, most of the added HOCl will have reacted with the carnosine, with a consequent sparing of damage to the protein. Because protein damage was assessed by gel electrophoresis only 2 min after the addition of HOCl (*33*), it is unlikely that extensive secondary damage arising from chlorine transfer from the relatively stable terminal amine chloramine would have occurred. The situation with longer incubation periods, where transfer would have been expected to occur on the basis of the data reported here, may have been very different. In contrast to the above reports, Halliwell et al. found that carnosine could not prevent the inhibition of α_1-antiproteinase by HOCl (*34*). In this study, the enzyme activity was assayed 30 min after the addition of HOCl, a time period that allowed for subsequent chlorine transfer from the carnosine chloramines to occur. It is known that α_1-antiproteinase contains a readily oxidized Met residue at its reactive site (*52*), and it has been shown that a number of chloramines react rapidly with Met residues (*21*, *22*); this is also likely to occur with the carnosine-derived chloramines.

These data can be extended to understanding the mechanisms of HOCl oxidation of small molecules and proteins and suggest that cascades of chlorine transfer and subsequent chloramine reactions are likely to occur. Imidazole chloramines are likely to be formed in high yield during the initial reactions of HOCl with proteins, because of the high reactivity of the imidazole ring. However, these species can rapidly transfer chlorine to other amine sites (such as Lys side chains), which can in turn chlorinate other residues such as Tyr. Thus, the production of the marker compound Cl-Tyr on proteins in vivo may arise from a large number of different HOCl- and chloramine-mediated pathways.

However, care should be taken when extrapolating these results to larger molecules such as proteins. Some of these limitations have been discussed in detail previously (*11*, *42*, *43*) but include factors such as the varying accessibility of residues within the three-dimensional protein structure and the effect of localized steric and charge environments on the reactivity of some amino acid side chains (notably His and Cys). It has also been demonstrated that the localized protein sequence can be extremely important. For example, -Tyr-Xxx-Xxx-Lys- (where Xxx = an amino acid residue that does not react with HOCl) motifs in apolipoprotein A-I can direct chlorination to specific Tyr residues via the generation of intermediate Lys chloramines (*38*). We have previously postulated that -Tyr-His- motifs (or three-dimensional structures that bring these residues into close proximity) may also direct chlorination of Tyr residues in proteins (*23*), but this remains to be established. It has also been shown that nearby Met residues (e.g., in -Tyr-Xxx-Xxx-Lys-Xxx-Xxx-Met- motifs) can act as localized antioxidants, which modulate Tyr chlorination, presumably by selective scavenging of HOCl or intermediate chloramines (*53*).

In conclusion, the studies presented here suggest that both histamine and carnosine, which contain both imidazole and free amine sites, may be able to limit HOCl-mediated oxidation in vivo, albeit with limited efficiency and with carnosine likely to be more efficient than histamine. This protective action is proposed to arise primarily from their ability to sequester HOCl in a form that is unable to mediate efficient further damage by chlorine transfer. With histamine, indiscriminate chlorine transfer occurs from the initially formed imidazole chloramine to other amine groups, resulting in a mixture of chloramine products. In contrast, with carnosine selective intramolecular chlorine transfer occurs to give a relatively long-lived chloramine, which is inefficient at inducing further chlorination (at least as assessed by chlorination of *N*-α-acetyl-Tyr).

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