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ORIGINAL ARTICLE

Kinetics of 3-nitrotyrosine modification on exposure to hypochlorous acid

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Abstract
The markers 3-nitrotyrosine and 3-chlorotyrosine are measured as surrogates for reactive nitrogen species and hypochlorous acid respectively, which are both elevated in inflamed human tissues. Previous studies reported a loss of 3-nitrotyrosine when exposed to hypochlorous acid, suggesting that observations of 3-nitrotyrosine underestimate the presence of reactive nitrogen species in diseased tissue (Whiteman and Halliwell, *Biochemical and Biophysical Research Communications*, **258**, 168–172 (**1999**)). This report evaluates the significance of 3-nitrotyrosine loss by measuring the kinetics of the reaction between 3-nitrotyrosine and hypochlorous acid. The results demonstrate that 3-nitrotyrosine is chlorinated by hypochlorous acid or chloramines to form 3-chloro-5-nitrotyrosine. As 3-nitrotyrosine from *in vivo* samples is usually found within proteins rather than as free amino acid, we also examined the reaction of 3-nitrotyrosine modification in the context of peptides. The chlorination of 3-nitrotyrosine in peptides was observed to occur up to 700-fold faster than control reactions using equivalent amino acid mixtures. These results further advance our understanding of tyrosine chlorination and the use of 3-nitrotyrosine formed *in vivo* as a biomarker of reactive nitrogen species.

Keywords: 3-chlorotyrosine, chloramine, biomarker, reactive species, protein damage

Abbreviations: 3ClY, 3-chlorotyrosine; AcHis, N-α-acetylhistidine; AcHisCl, N-α-acetyl-N-δ-chlorohistidine; AcLys, N-α-acetyllysine; AcLysCl, N-α-acetyl-N-ε-chlorolysine; AcTyr, N-acetyltyrosine; Ac3ClTyr, N-acetyl-3-chlorotyrosine; $AcNO_2Y$, N-acetyl-3-nitrotyrosine; $AcClNO_2Y$, N-acetyl-3-chloro-5-nitrotyrosine; $ClNO_2Y$, 3-chloro-5-nitrotyrosine; Cl_2Y, 3,5-dichlorotyrosine; HOCl, hypochlorous acid; NO_2Y, 3-nitrotyrosine; RNS, reactive nitrogen species; V_0, initial reaction rate

Introduction

Reactive chemicals are implicated in the pathology of human diseases associated with aberrant or chronic inflammation including cancer, neurodegenerative diseases, and cardiovascular disease [1–4]. Myeloperoxidase catalyzes the oxidation of chloride ions by hydrogen peroxide to form hypochlorous acid (HOCl) that damages biological molecules including tyrosine, which is free in solution and tyrosine residues in proteins, to form 3-chlorotyrosine (3ClY) [5]. Reactive nitrogen species formed when nitric oxide is oxidized, including peroxynitrite and nitrogen dioxide, damage biological molecules including free or protein-bound tyrosine, to produce 3-nitrotyrosine (NO_2Y) [4,6]. The markers NO_2Y and 3ClY are measured as surrogates, respectively, of reactive nitrogen species and HOCl formed *in vivo* because these products of tyrosine damage are chemically stable and readily measured with existing analytical methods [6,7]. Whiteman and Halliwell, however, demonstrated *in vitro* that NO_2Y is lost in the presence of HOCl, suggesting that observed NO_2Y underestimates the levels of reactive nitrogen species in tissues that also produce HOCl [8].

Coincident formation of reactive halogen and reactive nitrogen species occurs when the enzymes myeloperoxidase and nitric oxide synthase are both present. Myeloperoxidase can consume nitric oxide in the presence of hydrogen peroxide and generate reactive nitrogen species capable of nitrating tyrosine residues to form NO_2Y [9,10]. Resting human neutrophils isolated from peripheral blood do not normally express inducible nitric oxide synthase [11]. However, activated neutrophils exposed to cytokines can express inducible nitric oxide synthase, produce nitric oxide, and increase the level of reactive nitrogen species, suggesting that recruitment of neutrophils during inflammation can increase both tyrosine nitration and chlorination [12]. The observation of both 3ClY and NO_2Y in diseased tissues of patients with atherosclerosis, cardiovascular disease or neurodegeneration, confirms the presence of active MPO in tissues with reactive nitrogen species [13–15]. The kinetics of NO_2Y reacting with HOCl or HOCl-derived chloramines is therefore important for estimating the loss of the marker NO_2Y in these diseased tissues when both reactive nitrogen species and active myeloperoxidase are present.

Experimental procedures

Materials

N-Acetyl-L-tyrosine (AcTyr), N-α-acetyl-L-lysine (AcLys), and N-acetyl-L-histidine (AcHis) were purchased from Novabiochem (San Diego, CA). FMOC-amide resin was

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purchased from Applied Biosystems, Inc. (Foster City, CA). FMOC-OSu, FMOC-Lys(Boc)-OH, FMOC-His (Trt)-OH, and all other FMOC amino acids were purchased from Advanced ChemTech (Louisville, KY), with the exception of FMOC-Tyr(NO_2)-OH which was synthesized as described below. All other laboratory chemicals including 3-chloro-L-tyrosine, 3-nitro-L-tyrosine, L-methionine, sodium hypochlorite, N-acetylglycine, N-acetyl-L-alanine, and N-acetyl-L-glutamic acid were purchased from Sigma-Aldrich (St. Louis, MO).

Synthesis of N-acetyl-3-nitrotyrosine

3-Nitro-L-tyrosine was acetylated with excess acetic anhydride in acetone at room temperature and left to react overnight. The product formed was N-acetyl-3-nitrotyrosine ($AcNO_2Y$) and it was found soluble in acetone. The acetone was evaporated using a rotary evaporator and any remaining acetic anhydride was hydrolyzed by adding water. The solution was added to a SupelClean, LC-18 packing, solid phase extraction (SPE) column (Supelco, Bellefonte, PA). The column was washed with 0.1% trifluoroacetic acid (TFA) and subsequent washes were composed of increasing concentrations of methanol (5% increments) in 0.1% TFA. The aliquots were analyzed by analytic HPLC to determine the purity. Fractions, which were more than 98% pure, were combined and lyophilized. The compound gave the expected UV/Vis maximum absorption at 279 and 360 nm [16]. The pK_a value determined by UV/Vis spectroscopy was 7.13 (see Supplementary Material available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954110). The ^1H NMR spectra were consistent with $AcNO_2Y$. ^1H NMR (500 MHz, DMSO): 12.72 (s, 1H, -CO_2H), 10.79 (s, 1H, -O*H*), 8.18 (d, $J = 8.1$ Hz, 1H, N*H*), 7.74 (d, $J = 2.0$ Hz, 1H, H2), 7.41 (dd, $J = 2.0$, 8.5 Hz, 1H, H6), 7.05 (d, $J = 8.5$ Hz, 1H, H5), 4.38 (m, 1H, *H*α), 3.01 (dd, $J = 4.7$, 13.9 Hz, 1H, *H*β), 2.80 (dd, $J = 9.4$, 13.9 Hz, *H*β), 1.78 (s, 3H, CH_3).

Synthesis of N-acetyl-3-chloro-5-nitrotyrosine

3-Chloro-L-tyrosine was acetylated as described above. The crude product, N,O-diacetyl-3-chlorotyrosine, was produced after an overnight reaction and found soluble in acetone. The acetone was evaporated and the crude compound was then dissolved in water. Excess sodium nitrate (approximately 3-fold) was added, the solution was placed on ice, and then concentrated sulfuric acid was added dropwise to hydrolyze the phenol acetate ester and nitrate the phenol, giving N-acetyl-3-chloro-5-nitrotyrosine ($AcClNO_2Y$). The solution was allowed to react for 2 h before being purified by SPE as described above. Analytical HPLC showed that the final purity was more than 98%. The UV/Vis spectrum showed absorbance maximums at 285 nm and 358 nm under acidic conditions. The pK_a value determined by UV/Vis spectroscopy was 5.39 (see Supplementary Material available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954110). The ^1H NMR was consistent with $AcClNO_2Y$. ^1H NMR (500 MHz, DMSO): 12.77 (s, 1H, -CO_2H), 10.91 (s, 1H, -O*H*), 8.21 (d, $J = 8.3$ Hz, 1H, N*H*), 7.79 (d, $J = 2.0$ Hz, 1H, H6), 7.70 (d, $J = 2.0$ Hz, 1H, H2), 4.43 (m, 1H, *H*α), 3.05 (dd, $J = 4.8$, 13.8 Hz, 1H, *H*β), 2.83 (dd, $J = 9.7$, 13.8 Hz, 1H, *H*β), 1.80 (s, 3H, CH_3).

Synthesis of FMOC-3-nitrotyrosine

An FMOC protecting group was added to the N-terminus of NO_2Y by reacting with equimolar FMOC-OSu and a 3-fold excess sodium bicarbonate in a 50:50 mixture of water and acetone. After stirring overnight at room temperature, the aqueous layer was extracted four times with diethyl ether. The combined diethyl ether solution was back-extracted twice with 5% sodium bicarbonate. The combined aqueous layers were acidified by adding concentrated HCl until a precipitate formed. The product was extracted with ethyl acetate twice, combined, and dried using anhydrous magnesium sulfate. Finally, the solvent was removed by rotary evaporation, leaving a golden powder for use in peptide synthesis.

Peptide synthesis and purification

Peptides containing histidine or lysine residues in close proximity to the NO_2Y residue were synthesized on an ABI model 433A peptide synthesizer using fast FMOC chemistry. Following synthesis, the N-terminus was acetylated using acetic anhydride. The protecting groups and resin support were cleaved from the peptides using a mixture of TFA/phenol/water/triisopropylsilane (88:5:5:2). The resin was removed by filtration, the cleavage reaction was concentrated by rotary evaporation, and then the peptide was precipitated by adding cold diethyl ether to the cleavage mixture. The ether was removed following centrifugation to pellet the precipitated peptide. After washing the crude peptide with cold diethyl ether three times, the peptides were purified by reverse phase HPLC using a C18 column (Varian Dynamax, 250 × 21.4 mm, 300 Å, 5 μM) with a gradient of 20–60% mobile phase B (acetonitrile with 0.085% TFA), where mobile phase A contained aqueous 0.1% TFA. The sequence of the purified peptides was confirmed by MS/MS using a Thermo Finnigan LCQ Deca XP mass spectrometer, and a purity of more than 95% was verified by analytical HPLC (data not shown).

Methods

The purchased sodium hypochlorite stock was stored at 4°C. The pK_a of HOCl is 7.5 resulting in almost equal concentrations of hypochlorous acid and its conjugate base, hypochlorite, at physiological pH. The term "hypochlorous acid" is thus used to refer to both the acid and its conjugate base. The concentration of the sodium hypochlorite stock was determined daily using the absorbance at 290 nm ($\varepsilon_{290\ nm} = 350\ M^{-1}cm^{-1}$), for a fresh dilution of the stock into 0.1 M NaOH [17]. The stock

concentrations of chemicals containing NO_2Y or 3-chloro-5-nitrotyrosine ($ClNO_2Y$) were determined following dilution into 0.1 M HCl and using UV/Vis spectroscopy with molar absorptivity values of $\varepsilon_{360\,nm} = 2790\ M^{-1}cm^{-1}$ and $\varepsilon_{358\,nm} = 2496\ M^{-1}cm^{-1}$, respectively [16]. The molar absorptivity value of $ClNO_2Y$ was determined as a ratio of the published value for NO_2Y by mixing $AcClNO_2Y$ and $AcNO_2Y$ stock solutions with known UV spectra, vacuum centrifuging to remove water, dissolving in deuterated DMSO, collecting a 1D NMR spectrum, and calculating the ratio of both compounds based on pairs of integrated peaks corresponding to the same resonance.

Reactions of $AcNO_2Y$ with HOCl or chloramines

All reactions contained 200 μM HOCl, 350–1000 μM $AcNO_2Y$, and 100 mM phosphate buffer at the indicated pH. In some previous studies, the chloramine was formed first, followed by the addition of the tyrosine analog [18]. Preliminary reactions with $AcNO_2Y$ showed no statistically significant differences between adding the tyrosine analog or HOCl last. For reactions with lysine or histidine chloramines, a 5-fold excess AcLys or AcHis was added to the $AcNO_2Y$ solution before the addition of HOCl, as described in our previous publication [19]. All reactions with HOCl or chloramines were quenched with 10-fold excess methionine or cysteine, respectively, at time points ranging between 5 s and 2 h.

UV/Vis spectroscopy of $AcClNO_2Y$ chlorination by HOCl

The UV-spectra of $AcClNO_2Y$ reacting with HOCl was measured on a Varian (Palo Alto, CA) Cary 300 Bio UV/Vis spectrophotometer in a 1-cm micro quartz cell. $AcClNO_2Y$ (185 μM) was reacted with 720 μM HOCl at 37°C and the spectra between 350 and 550 nm were measured every 24 s.

Reaction of peptides with HOCl

HOCl was reacted with excess peptide at 20–240 μM peptide and 10–120 μM HOCl in 10 mM phosphate buffer at the indicated pH at 37°C and quenched with 10-fold excess cysteine at time points between 5 s and 2 h.

HPLC quantitation of reaction products

The concentration of products and reactants in kinetic samples were measured using a Thermo Finnigan (Waltham, MA) Surveyor HPLC system with a MS Pump, Autosampler, and PDA detector (200–600 nm). Standard curves were generated using stocks (>98% pure) of $AcNO_2Y$ and $AcClNO_2Y$ ($r^2 > 0.999$). Peptides with NO_2Y and $ClNO_2Y$ residues were quantitated using the $AcNO_2Y$ and $AcClNO_2Y$, respectively, along with standard curves and the same HPLC method, assuming that the different retention time and small change in solvent composition would not affect analyte absorbance at 360 nm. The separation of each analyte was accomplished using a Restek Ultra IBD column (C18, 3 μm, 150 × 2.1 mm). The initial solvent mix was 95% mobile phase A (0.1% trifluoroacetic acid in water) and 5% mobile phase B (0.085% trifluoroacetic acid in acetonitrile) for 5 min, followed by a linear gradient to 50% mobile phase B over 20 min at a flow rate of 100 μL/min. The gradient stayed constant for 2.5 min, followed by a linear decrease to 5% B in 2.5 min. $AcNO_2Y$ and $AcClNO_2Y$ were quantitated at 360 nm.

Determination of initial reaction rate and reaction order

The peptides were reacted with HOCl and quenched with excess cysteine at 37°C in 10 mM phosphate buffer at pH 7.4. The reported initial reaction rate of NO_2Y chlorination, V_0, was determined using only data points where product formation was a linear function of time. The V_0 values of 12–15 reactions with varying initial concentrations of peptide and HOCl were used to solve for the reaction order using the equation $V_0 = k * [Peptide]_0^A * [Chloramine]_0^B$, where A and B are the rate order of peptide and chloramine, respectively. Because HOCl reacts with lysine forming a chloramine significantly faster than any measurable formation of $ClNO_2Y$, the initial concentration of chloramine peptide is equivalent to the initial concentration of HOCl [20].

Analysis of kinetic data and determination of rate constants

Microsoft Excel was used to analyze all kinetic data using a model comparison method as previously described [19]. A time interval of 0.1 s was used to numerically model the differential equations. The chemical models for chlorination of NO_2Y by HOCl or chloramines are presented in the Results section. The sum of the squared differences (SSD) was calculated between the experimental and modeled concentrations for time points in a kinetics experiment. The Solver tool in Excel was used to determine the optimized second order rate constants that minimized the SSD, giving SSD_{opt}. The error in each rate constant was estimated by determining the rate constant that gave $SSD = SSD_{opt} * (F\ (P/(N-P)) + 1)$, where F is the critical value of the F distribution, P is the number of model parameters, and N is the number of data points. We chose a value of F corresponding to the 95% confidence level. The rate constant values are reported as middle value ± 1/2*range; the middle value was calculated from the high and low SSD values that defined the range of certainty (95% confidence level) and were within 1% of the optimized value in all cases.

Results

HOCl reacts with NO_2Y to form $ClNO_2Y$

The published chlorination of 3-substituted tyrosine analogs by HOCl suggested that the loss of 3-nitrotyrosine

observed by Whiteman and Halliwell [8] resulted in the formation of 3-chloro-5-nitrotyrosine [19]. To avoid the competing reaction of HOCl with the amino group of 3-nitrotyrosine, the acetylated analogs of NO_2Y and $ClNO_2Y$ were synthesized [21]. The reaction of $AcNO_2Y$ with HOCl to form $AcClNO_2Y$ was monitored by HPLC (see Supplementary Material available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954110). The observed product peak had the same retention time and UV spectrum as observed for the authentic standard of $AcClNO_2Y$.

We monitored the reactions between $AcNO_2Y$ and HOCl, N-acetylhistidine chloramine (AcHisCl), or N-α-acetyllysine chloramine (AcLysCl) at pH 7.4 and 37°C between 5 s and 120 min. $AcNO_2Y$, at concentrations of approximately 500, 750, and 1000 μM, was reacted with 200 μM HOCl, both in the presence and absence of excess AcHis or AcLys. The reaction of 750 μM $AcNO_2Y$ with 200 μM HOCl (Figure 1a) or AcHisCl (Figure 1b) results in $AcClNO_2Y$ production. The reaction rate is pH sensitive with a maximum rate at 7.4 (see Supplementary Materials available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954110). The reaction with AcLysCl was too slow to produce any measureable $AcClNO_2Y$ after 2 h but $AcClNO_2Y$ was observed a day later (data not shown).

HOCl reacts with $AcClNO_2Y$ to form unknown products

We investigated the reaction of $AcClNO_2Y$ with HOCl by HPLC and UV/Vis spectroscopy. No product peaks were identified by UV/Vis-detected HPLC and the measured loss of $AcClNO_2Y$ was less than the initial HOCl introduced. This data indicates that an unknown product forms with no significant UV/Vis absorbance between 200 and 600 nm and that it reacts more readily with HOCl than $AcClNO_2Y$. We followed the loss of $AcClNO_2Y$ in the presence of HOCl by UV/Vis spectroscopy at 37°C, pH 7.4 (Figure 2). The rate of $AcClNO_2Y$ loss decreases as the reaction progresses, suggesting that the unknown products successfully compete with $AcClNO_2Y$ for reaction with HOCl. We also determined that an approximately 6-fold excess of HOCl is required to completely degrade $AcClNO_2Y$ as determined by UV/Vis spectroscopy (data not shown).

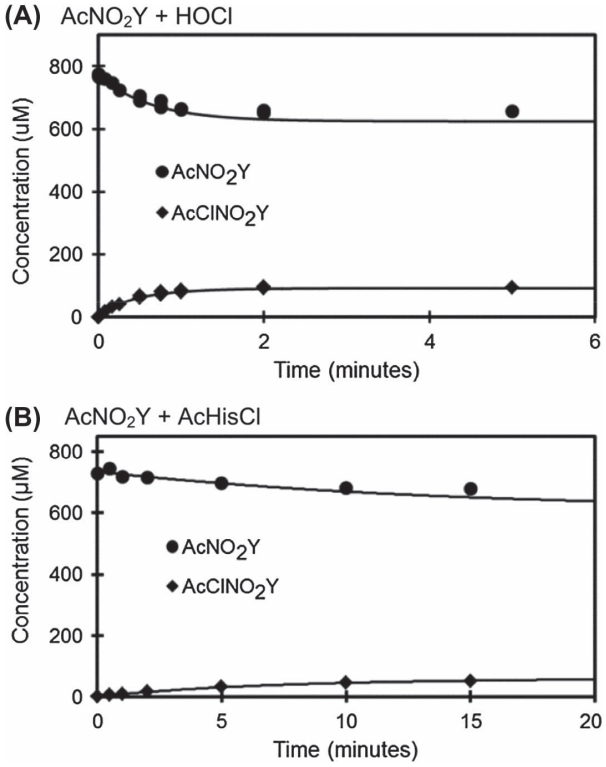

Figure 1. Kinetics of $AcNO_2Y$ chlorination by HOCl or AcHisCl. (A) 780 μM $AcNO_2Y$ or (B) 730 μM $AcNO_2Y$ and 1000 μM AcHis were reacted with 200 μM HOCl at 37°C for the indicated time; the reaction was stopped with excess methionine, and the products were quantitated by UV-detected HPLC. The solid lines in each panel represent the modeled reaction progress with optimized rate constants. The rate constant k_1 for $AcNO_2Y$ reacting with HOCl in panel A is 24.5 $M^{-1}s^{-1}$. The rate constant k_1 for $AcNO_2Y$ reacting with AcHisCl in panel B is 1.0 $M^{-1}s^{-1}$. Similar experiments were done for AcLysCl but no product was measured after 2 h of reaction.

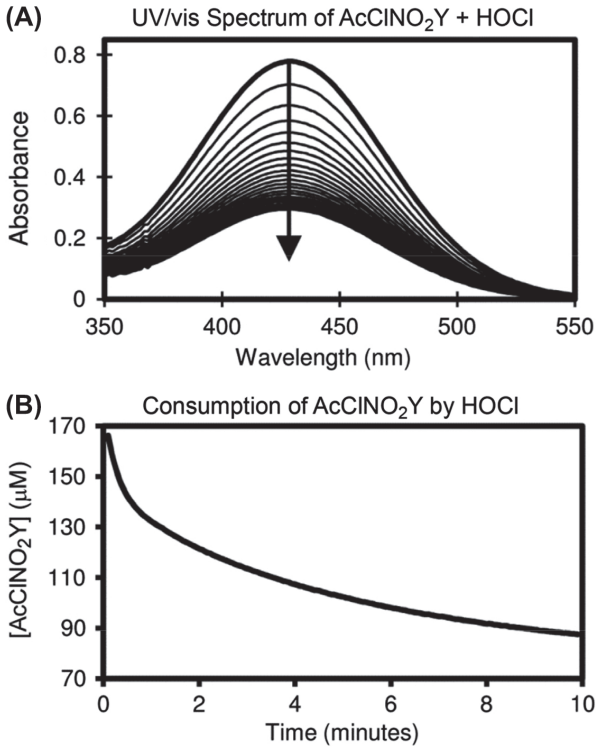

Figure 2. UV/Vis analysis of $AcClNO_2Y$ reacting with HOCl. (A) UV spectrum of 185 μM $AcClNO_2Y$ reacting with 720 μM HOCl at 37°C, pH 7.4. The initial spectrum is shown as a bold line while the arrow shows the changes in the spectra collected every subsequent 24 s. (B) The consumption of 185 μM $AcClNO_2Y$ by 720 μM HOCl at 37°C, pH 7.4. The concentration of $AcClNO_2Y$ was based on the absorbance at 430 nm and a molar absorptivity value of $\varepsilon_{430} = 4205\ M^{-1}cm^{-1}$. The change in the rate of $AcClNO_2Y$ degradation indicates multiple reactions going on, likely between HOCl and the product of $AcClNO_2Y$ chlorination. It takes approximately a 6-fold excess HOCl to completely degrade $AcClNO_2Y$ (data not shown).

Table I. Rate constants for chlorination of tyrosine, 3ClY, and NO_2Y.[a]

Chlorinating species	AcTyr[b] ($M^{-1}s^{-1}$)	Ac3ClTyr[b] ($M^{-1}s^{-1}$)	$AcNO_2Y$ ($M^{-1}s^{-1}$)
HOCl	71 ± 8	238 ± 27	24.5 ± 1.2
HisCl	3.0 ± 0.3	10.4 ± 1.0	1.1 ± 0.2
LysCl	0.004 ± 0.003	0.008 ± 0.002	ND[c]

[a]Reactions were performed at 37°C, pH 7.4, in 100 mM phosphate buffer.
[b]Rate constants were reported previously in reference [19].
[c]Product was not detected after 2 h of reaction.

Determination of kinetic rate constants

Only the initial 1 and 5 min, respectively, were used to determine the kinetics of $AcNO_2Y$ chlorination by HOCl and AcHisCl (Table I). At these initial time points, the total concentrations of $AcNO_2Y$ and $AcClNO_2Y$ are constant, indicating that the possible reaction of HOCl with $AcClNO_2Y$ is not sufficient to alter the observed concentrations or calculated rate constants. Including the decomposition of HOCl and/or the degradation of AcHisCl in the kinetic model did not result in significant changes to the calculated rate constants [18,22,23].

Rate of NO_2Y chlorination by HOCl increase in peptides

Previous studies indicate that the chlorination of tyrosine residues in polypeptides is facilitated by nearby histidine or lysine residues [18,19,24]. We reacted the synthetic peptide, Ac-HGNY(NO_2) AE-NH_2, with a 0.5-fold concentration of HOCl and followed product formation by HPLC (see Supplementary Materials available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954110), as shown in Figure 3 and summarized in Table II. An amino acid mixture of N-acetyl amino acids that match the residue composition of the peptide Ac-HGNY(NO_2)AE-NH_2 was also reacted with 0.5-fold concentration of HOCl as a control reaction. The production of $ClNO_2Y$ in the peptide was approximately 700-fold faster than in the amino acid mixture at the concentrations and reaction conditions examined.

The kinetics of NO_2Y chlorination by HOCl in peptides is a first order reaction

To probe the mechanism whereby neighboring lysine or histidine residues facilitate NO_2Y chlorination, we varied the peptide and HOCl concentrations to determine the order of the reaction kinetics. The initial reaction kinetics were measured to minimize the effect of $ClNO_2Y$ reacting with HOCl or chloramines. Initial experiments found that the reaction kinetics were independent of the initial peptide concentration and changed only with the HOCl concentration when excess peptide was present. Since HOCl rapidly reacts with the peptide amines to form a chloramine [20], the reaction is first order with respect to the peptide-chloramine concentration (Figure 4).

Table II. Rate constants for chlorination of NO_2Y within a peptide.[a]

Peptide	Rate constant (s^{-2})
Ac-**H**GN-**Y(NO_2)**-AE-NH_2	$2.60 \pm 0.53 \times 10^{-2}$
Ac-**K**GN-**Y(NO_2)**-AE-NH_2	$2.54 \pm 0.03 \times 10^{-5}$
Ac-KGN-**Y**-AE-NH_2[b]	$2.38 \pm 0.07 \times 10^{-4}$

[a]Reactions were performed at 37°C, pH 7.4, in 10 mM phosphate buffer.
[b]Data from reference [19].

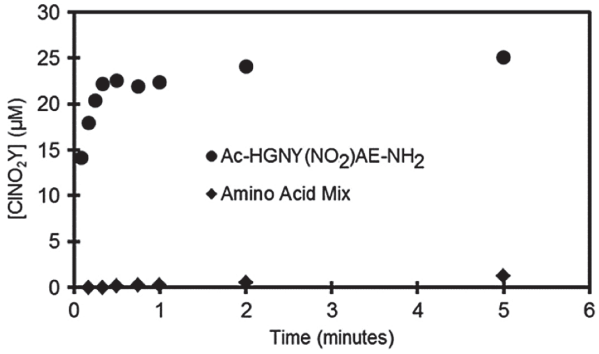

Figure 3. Acceleration of NO_2Y chlorination by His chloramine in a peptide. 75 μM Ac-HGNY(NO_2)AE-NH_2 or an analogous amino acid mixture were reacted with 37.5 μM HOCl at 37°C for the indicated times; the reaction was stopped with excess cysteine and the products were quantitated by UV-detected HPLC. At the concentrations indicated here, the rate of NO_2Y chlorination in the peptide is approximately 700-fold faster than in the analogous amino acid mixture.

Discussion

Reactive nitrogen species (RNS) chemically damage tyrosine residues to form 3-nitrotyrosine (NO_2Y), a surrogate marker used to investigate the role of RNS in human diseases. However, Whiteman and Halliwell [8] reported that NO_2Y disappears when exposed to HOCl, suggesting that the observed NO_2Y underestimates the role of RNS in tissues when chlorinating species are also present. Whiteman and Halliwell [8] did not report the resulting product when NO_2Y reacts with HOCl, but subsequent studies [19,24–26] on the reaction of HOCl with 3ClY to form Cl_2Y suggested that the expected product should be $ClNO_2Y$. This expectation is now confirmed since the reaction of $AcNO_2Y$ with HOCl forms a new product that has the same HPLC retention time and UV-spectra as authentic $AcClNO_2Y$.

As observed with Cl_2Y [19], $ClNO_2Y$ also reacts with HOCl. The complex kinetics of $ClNO_2Y$ loss indicate that the unknown compound(s) formed upon exposure to HOCl also react with HOCl (Figure 2). Previous reports demonstrate that HOCl and chloramines chlorinate phenolic compounds, degrading the aromatic phenol and eventually producing chloroform [27,28]. As the focus of this report is the physiological significance of NO_2Y loss, we did not identify the unknown products or reaction kinetics when $AcClNO_2Y$ reacts with HOCl. The reported kinetic rates used excess NO_2Y and were

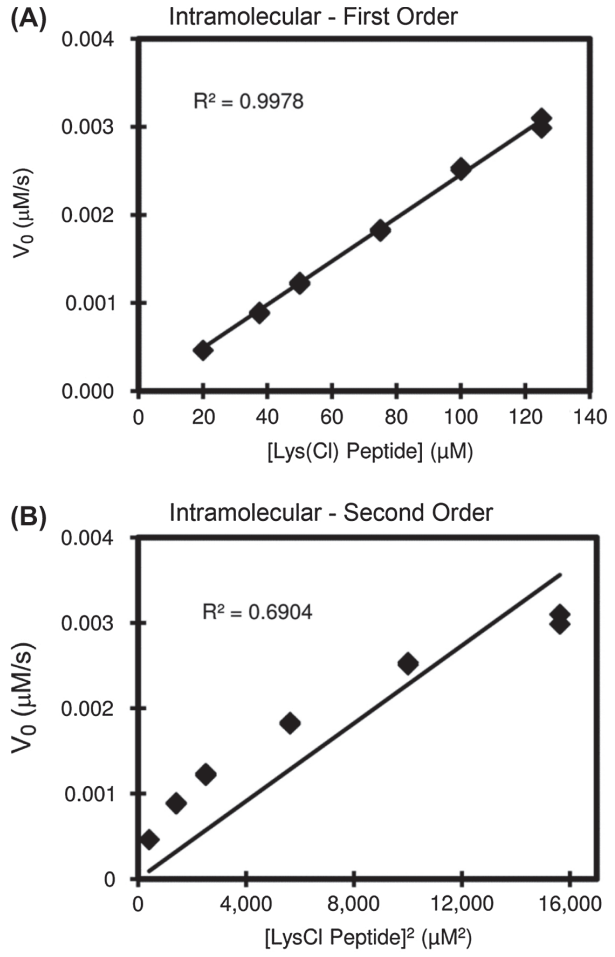

Figure 4. Determining the rate order of NO_2Y chlorination by chloramines within a peptide. Excess Ac-KGNY(NO_2)AE-NH_2 was reacted with HOCl at 37°C, pH 7.4. The initial reaction rate was determined by using HPLC to measure the formation of $ClNO_2Y$ at early time points where less than 20% of the reactant peptide was consumed. The samples were quenched with excess cysteine before HPLC analysis. The initial reaction rate was calculated by linearly fitting the early time points. Because the reaction of Lys with HOCl is significantly faster than the chlorination of NO_2Y, it was assumed that the concentration of peptide containing lysine chloramine was equal to the amount of HOCl added. The initial reaction rate was plotted to (A) the concentration of Ac-K(Cl)GNY(NO_2)AE-NH_2 to model a first-order reaction and to (B) the square of the concentration of this peptide to model a second-order reaction. The linearity of the first-order fit indicates that NO_2Y is chlorinated through an intramolecular reaction with the lysine chloramine.

calculated using reaction times where the loss of $ClNO_2Y$ was not observed.

The data in this report as well as our previous report [19] allow us to compare the chlorinating potential of HOCl and the chloramines of lysine or histidine reacting with tyrosine analogs (Table I). The chlorination of tyrosine analogs in Table I by HOCl is 23 ± 1 and $23{,}750 \pm 6{,}000$ times faster than HisCl and LysCl, respectively. The kinetics of 3ClY reacting with HOCl or a histidine chloramine are approximately 3 times faster than tyrosine chlorination while the kinetics of NO_2Y chlorination are 3 times slower. The kinetics of chlorination are affected by pH, with maximum rates at pH values of 7.4 and 8.0 for NO_2Y and 3ClY [19], respectively (see Supplementary Material available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954110). The effect of 3-nitro and 3-chloro groups on pK_a values and reaction kinetics is consistent with an electrophilic aromatic substitution reaction where phenolate is more reactive than a phenol and a chlorine substituent is less deactivating than a nitro substituent.

The rate of NO_2Y loss due to chlorination in the peptide Ac-HGNY(NO_2)AE-NH_2 is 700-fold faster than an equivalent amino acid mix (Figure 3 and Supplementary Material available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954110). We argue that this increased rate of chlorination is due to the close proximity maintained between the histidine chloramine and the NO_2Y residues in the peptide context. The rapid intermolecular reaction of HOCl with a polypeptide amine to form a chloramine followed by the slower intramolecular chlorination of a nearby tyrosine analog predicts that the kinetics of peptide tyrosine chlorination will follow first order kinetics (see Supplementary Material available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954110). To our knowledge, this report is the first to demonstrate that the kinetics of tyrosine analog chlorination in a peptide or protein with a nearby amine follows first order kinetics.

The relative kinetics of NO_2Y chlorination by histidine or lysine chloramines appear independent of reaction order. The second order kinetics of AcTyr or N-acetyl-3chlorotyrosine (Ac3ClTyr) chlorination by lysine chloramines are 1025 ± 275 times slower than chlorination by histidine chloramines [19]. Likewise, the first order kinetics of NO_2Y chlorination in a peptide by a lysine chloramine is 1024 times slower than a histidine chloramine (Table II). However, tyrosine chlorination by a HisCl is 9-fold faster in the peptide context (Table II) whereas the expectation was 3-fold faster given the data in Table I.

Measurements of 3ClY or NO_2Y from biological tissues usually measure these surrogate markers in the context of proteins instead of the free amino acid. The observation that tyrosine residues with histidine or lysine residues nearby are preferentially chlorinated is better explained by the intramolecular reaction with a nearby chloramine than the direct intermolecular chlorination by HOCl to form a protein-bound 3ClY [18,19,24,26,29]. The fast reaction of HOCl with protein amines ($\sim 10^5$ $M^{-1}s^{-1}$) better competes with the antioxidant glutathione ($\sim 10^8$ $M^{-1}s^{-1}$) than the direct chlorination of tyrosine analogs ($\sim 10^2$ $M^{-1}s^{-1}$) and explains the preferential chlorination of some protein-bound tyrosine residues, provided the intramolecular chlorination kinetics are sufficiently fast [20,30].

The loss of NO_2Y will depend on the quantity of HOCl although the likelihood of NO_2Y loss in biological tissues is still uncertain, given that most reports do not measure both NO_2Y and 3ClY. We expect that the formation of $3ClNO_2Y$ in biological tissues is less likely than the formation of Cl_2Y, given that HOCl reacts 9-fold

faster with 3ClY than with $3NO_2Y$. *In vitro* experiments that expose proteins to HOCl observe Cl_2Y formation when the 3ClY is observed at levels, relative to undamaged tyrosine, found *in vivo* [25,26]. In the context of proteins, the loss of NO_2Y due to HOCl likely requires a nearby lysine or histidine residue. For example, Tyr^{192} of apolipoprotein A-1 can be nitrated or chlorinated, suggesting that measurements of Tyr^{192} nitration will underestimate RNS when HOCl is present [31]. The loss of $3NO_2Y$ in proteins from biological tissues should also depend on the sequence dependence of tyrosine nitration and chlorination [32–35]. The results reported here can improve the estimation of NO_2Y loss in tissues where both NO_2Y and 3ClY are observed and may allow a clearer determination of the quantity of reactive nitrogen species present.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Material and Figures 1–5.