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Original Contribution

Imidazole catalyzes chlorination by unreactive primary chloramines

Margo D. Roemeling [a], Jared Williams [a], Joseph S. Beckman [a,b,c], James K. Hurst [a,*,1]

[a] Department of Biochemistry and Biophysics, Oregon State University, Corvallis OR, USA
[b] Environmental Health Sciences Center, Oregon State University, Corvallis OR, USA
[c] Linus Pauling Institute, Oregon State University, Corvallis OR, USA

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ABSTRACT

Hypochlorous acid and simple chloramines (RNHCl) are stable biologically derived chlorinating agents. In general, the chlorination potential of HOCl is much greater than that of RNHCl, allowing it to oxidize or chlorinate a much wider variety of reaction partners. However, in this study we demonstrate by kinetic analysis that the reactivity of RNHCl can be dramatically promoted by imidazole and histidyl model compounds via intermediary formation of the corresponding imidazole chloramines. Two biologically relevant reactions were investigated—loss of imidazole-catalyzed chlorinating capacity and phenolic ring chlorination using fluorescein and the tyrosine analog, 4-hydroxyphenylacetic acid (HPA). HOCl reacted stoichiometrically with imidazole, *N*-acetylhistidine (NAH), or imidazoleacetic acid to generate the corresponding imidazole chloramines which subsequently decomposed. Chloramine (NH_2Cl) also underwent a markedly accelerated loss in chlorinating capacity when NAH was present, although in this case *N*-α-acetylhistidine chloramine (NAHCl) did not accumulate, indicating that the catalytic intermediate must be highly reactive. Mixing HOCl with 1-methylimidazole (MeIm) led to very rapid loss in chlorinating capacity via formation of a highly reactive chlorinium ion ($MeImCl^+$) intermediate; this behavior suggests that the reactive forms of the analogous imidazole chloramines are their conjugate acids, e.g., the imidazolechlorinium ion ($HImCl^+$). HOCl-generated imidazole chloramine (ImCl) reacted rapidly with fluorescein in a specific acid-catalyzed second-order reaction to give 3′-monochloro and 3′,5′-dichloro products. Equilibrium constants for the transchlorination reactions $HOCl + HIm = H_2O + ImCl$ and $NH_2Cl + HIm = NH_3 + ImCl$ were estimated from the dependence of the rate constants on [HIm]/[HOCl] and literature data. Acid catalysis again suggests that the actual chlorinating agent is $HImCl^+$; consistent with this interpretation, MeIm markedly catalyzed fluorescein chlorination by HOCl. Time-dependent imidazole-catalyzed HPA chlorination by NH_2Cl was also demonstrated by product analyses. Quantitative assessment of the data suggests that physiological levels of histidyl groups will react with primary chloramines to generate a flux of imidazole chloramine sufficient to catalyze biological chlorination via $HImCl^+$, particularly in environments that generate high concentrations of HOCl such as the neutrophil phagosome.

Introduction

It is generally accepted that neutrophils activated in aerobic environments generate microbicidal levels of HOCl through the concerted action of a phagosomal NADPH oxidase (NOX-2) and myeloperoxidase (MPO) [1–3]. Hypochlorous acid is sufficiently reactive toward biological targets [4,5] that it is not thought to persist in the phagosomal environment much beyond cessation of the NOX-2 respiratory burst (at about 20 min postactivation) [6,7]. However, chloramines formed from endogenous amines are among the immediate reaction products [5,8–11]; these can be equally bactericidal [9,10], but are considerably less reactive than HOCl [11] and *de facto* more selective in their reactions with potential reductants. As a consequence, chloramines can accumulate and extend the duration of the chlorinating capacity of activated neutrophils. Evidence that a long-lasting pool of chlorinating agents is generated within the neutrophil phagosome following the respiratory burst includes chlorination of tyrosyl rings to form stable 3-monochloro- and 3,5-dichloro products [12–14] and bleaching of green fluorescent protein (GFP) expressed within the cytosol of phagocytosed bacteria [15,16]; both of these

Abbreviations: HPA, 4-hydroxyphenylacetic acid; IAACl, 4-imidazoleacetic acid; ImCl, imidazole chloramine; MeIm, 1-methylimidazole; MPO, myeloperoxidase; NAH, *N*-acetylhistidine; NAHCl, *N*-α-acetylhistidine chloramine; NH_2Cl, chloramine; NOX-2, NADPH oxidase

* Corresponding author.
E-mail address: hurst@wsu.edu (J.K. Hurst).
[1] Visiting Professor, Department of Biochemistry and Biophysics.

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reactions appear to be specific for HOCl, but both are observed to occur as late as 1–2 h postactivation of the neutrophil.

These reactions pose something of a puzzle since, under physiological conditions, tyrosine and GFP are moderately reactive toward HOCl, but virtually unreactive toward simple chloramines, i.e., NH_2Cl or RNHCl (where R is an alkyl or aminoacyl substituent). Furthermore, the amount of HOCl present in equilibrium with endogenous chloramines under physiological conditions is expected to be in the low nanomolar range, which is far too low to account for observed rates of tyrosine formation and GFP bleaching.[2] This conclusion is confirmed by the experimental design of the extracellular studies themselves, in which the chloramines are typically formed by reaction of HOCl with the amines, in essence achieving the equilibrium distribution of HOCl and chloramine before introduction of the HOCl-sensitive reactant. The absence of detectable reaction under these conditions therefore precludes the possibility that reaction in cellular environments arises from residual HOCl.

If not HOCl or simple chloramines, how then does one account for the relatively slow, continued chlorinating capacity demonstrated within the neutrophil phagosome? Work from the Davies group has shown that imidazole chloramines formed by reaction of imidazole-containing compounds with HOCl retain a chlorinating capacity that is much greater than simple chloramines [4,19,20]; for these compounds, relative chlorination rate constants approach within one or two orders of magnitude that of HOCl itself. One attractive possibility therefore is that endogenous imidazole groups may catalyze transchlorination reactions from the chloramine pool to receptive molecules or functional groups within the phagosomal microenvironment (e.g., as envisioned in Fig. 1).

In the present study, we investigate further the nature of imidazole catalysis of several reactions involving nominally unreactive NH_2Cl as the chlorination source. Focus is placed on the reactions of fluorescein, which undergoes facile ring chlorination by HOCl but reacts only very sluggishly with NH_2Cl. This molecule was chosen because it has numerous advantages for mechanistic studies, including spectroscopic properties that make it amenable to detailed kinetic analyses, as well as easy identification of chlorinated reaction products. Imidazole-catalyzed ring chlorination of phenolic compounds is also demonstrated; this reaction could be portentous to interpretations of *in vivo* chlorination since chlorotyrosines are now finding widespread use as stable markers of MPO-mediated oxidative stress in both intraphagosomal and extracellular environments [13,14,21].

Experimental

Materials

Chloramine (NH_2Cl) solutions were prepared by reacting millimolar HOCl obtained from dilution of commercial bleach solutions with > 5-fold excess phosphate-buffered ammonia; ice-cold reactant solutions were flow-mixed using a 12-jet tangential mixer attached to a Harvard Apparatus 2000 syringe-drive unit. Hypochlorous acid and chloramine concentrations were routinely determined spectrophotometrically using $\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$ for OCl^- in strongly alkaline media [22] and $\varepsilon_{244} = 429\ M^{-1}\ cm^{-1}$ for NH_2Cl [23]. The chloramine reagent concentrations were confirmed by analyzing their oxidizing capacity using Ellman's reagent, in which 2-nitro-5-thiobenzoate (NTB) is oxidized to 5,5′-dithio(2-nitrobenzoate); $\varepsilon_{405} = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$ was used to determine the amount of NTB consumed in the reaction [23]. These analyses indicated > 95% conversion of HOCl to NH_2Cl. Imidazole (ImCl), *N*-α-acetylhistidine (NAHCl) and 4-imidazoleacetic acid (IAACl) chloramines, and the 1-methyl-3-chloroimidazolium cation ($MeImCl^+$) were similarly prepared by reacting the parent amine with HOCl and their concentrations determined with Ellman's reagent. However, since these chloramines (particularly $MeImCl^+$) were relatively unstable and lost chlorinating capacity on incubation at 37 °C, it proved convenient for kinetic studies with fluorescein to prepare them immediately before reaction by using the 4-syringe mixing capability of the stopped-flow instrument. All other reagents were best-available materials obtained from commercial suppliers and were used as received. Reagent solutions were prepared from 18.1 MΩ water purified by passage through a Millipore Milli-Q Model Elix-S reverse osmosis/deionization unit.

Methods

Optical spectra were obtained with a Shimadzu UV-2401PC instrument. Stopped-flow kinetics were obtained using an Applied Photophysics SX20 instrument with data collection/analysis using their ProData SX (version 2.2.5.6) /ProData Viewer (version 4.2.0) software. Reagent concentrations were adjusted so that first-order conditions were met in all quantitative studies; recorded kinetic traces were exponential to greater than four half-lives. (Typical curves are given in Fig. 2, S2, S3, and S6.) The range of rate constants measured for repetitive runs on individual solutions was ≤ 5% for single-mixing experiments or ≤ 10% for multimixing experiments. Rate constants reported are the mean values of 5–10 runs whose error limits, expressed as average deviation from the mean, were ≤ 3%.

HPLC analyses were made using a Shimadzu LC10AD unit equipped with a SPD-M10A diode array detector and 250 × 4.6 mm 5 μm ultracarb ODS column; chromatograms were obtained by isocratic elution with 29% methanol/71% 20 mM P_i, pH 7.4, at a flow rate of 0.3 mL/min. Mass spectrometric analysis of chlorofluoresceins was made using a LTQ-FT Ultra mass spectrometer (Thermo, San Jose, CA) in LTQ mode, with a Finnigan Ion Max API source set up for electrospray ionization in positive ion mode. Conditions were 5 kV spray voltage, 200 °C capillary temperature, 40 V capillary voltage, and 240 V tube lens voltage, 100–2000 *m/z* detection range. Analyte samples were adsorbed onto a C4 Ziptip, desalted with water, and eluted inline to the mass spectrometer at 20 μL/min with 50% ethanol, 50% water, and 0.1% formic acid, as previously described [24]. Mass spectrometric analysis of chlorophenolic compounds was made using an ABSciex 4000 Q-trap LC/MS/MS system operated in the negative ion mode at 250 °C under the following conditions: CUR 30; tem 250; GS1 40; GS2 40; IS -4500; DP -45; EP -10. Sample was introduced into the mass spectrometer using a Shimadzu SIL-HTC liquid chromatograph equipped with a 3.5 μm Agilent Zorbax 300SB-C8 column (2.1 × 50 mm) operated at 0.2 mL/min total flow rate and 15 °C by applying a 5–90% gradient of H_2O/CH_3CN over 12 min; both eluants contained 0.1% formic acid.

Results

Chloramine decomposition reactions

When HOCl was mixed with 5-fold excess imidazole in phosphate buffers at pH 6–8, the OCl^- absorption band at 292 nm was immediately replaced by a broad featureless absorption tailing

[2] The equilibrium constant for chloramine formation, defined as $K_f = [NH_2Cl]/[NH_3][HOCl]$ for the reaction $HOCl + NH_3 \rightarrow NH_2Cl + H_2O$, is estimated to be $K_f \sim 10^{11}\ M^{-1}$ [17]. At pH 7, assuming an effective total amine concentration of 1–10 mM, the equilibrium ratio of chloramine to HOCl within the phagosome would be on the order of $[NH_2Cl]/[HOCl] = 10^5$–10^6. This ratio is also dependent on protic equilibria among the reactants; specifically, because only the acidic form of HOCl and the free base forms of the amines are reactive [18], the $[NH_2Cl]/[HOCl]$ ratio will depend on the solution pH and identities of endogenous amines. Acid dissociation constants do not vary greatly for amines with alkyl and aminoacyl substituents, however, so that their equilibrium ratios are expected to be similar to that determined for ammonia and HOCl.