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Original Contribution

Reevaluation of the rate constants for the reaction of hypochlorous acid (HOCl) with cysteine, methionine, and peptide derivatives using a new competition kinetic approach

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ABSTRACT

Activated white cells use oxidants generated by the heme enzyme myeloperoxidase to kill invading pathogens. This enzyme utilizes H_2O_2 and Cl^-, Br^-, or SCN^- to generate the oxidants HOCl, HOBr, and HOSCN, respectively. Whereas controlled production of these species is vital in maintaining good health, their uncontrolled or inappropriate formation (as occurs at sites of inflammation) can cause host tissue damage that has been associated with multiple inflammatory pathologies including cardiovascular diseases and cancer. Previous studies have reported that sulfur-containing species are major targets for HOCl but as the reactions are fast the only physiologically relevant kinetic data available have been extrapolated from data measured at high pH (> 10). In this study these values have been determined at pH 7.4 using a newly developed competition kinetic approach that employs a fluorescently tagged methionine derivative as the competitive substrate (k(HOCl + Fmoc-Met), $1.5 \times 10^8\ M^{-1}\ s^{-1}$). This assay was validated using the known k(HOCl + NADH) value and has allowed revised k values for the reactions of HOCl with Cys, *N*-acetylcysteine, and glutathione to be determined as 3.6×10^8, 2.9×10^7, and $1.24 \times 10^8\ M^{-1}\ s^{-1}$, respectively. Similar experiments with methionine derivatives yielded k values of $3.4 \times 10^7\ M^{-1}\ s^{-1}$ for Met and $1.7 \times 10^8\ M^{-1}\ s^{-1}$ for *N*-acetylmethionine. The k values determined here for the reaction of HOCl with thiols are up to 10-fold higher than those previously determined and further emphasize the critical importance of reactions of HOCl with thiol targets in biological systems.

Activated neutrophils and monocytes use myeloperoxidase to generate hypochlorous acid (HOCl)[1] and related species (HOBr, HOSCN) [1]. These oxidants are potent bactericides and disinfectants that play a key role in the human immune response to invading pathogens [1]. Inappropriate formation of these species has, however, been associated with multiple inflammatory pathologies including cardiovascular disease [2,3]. Second-order rate constants (k) for the reactions of these oxidants at physiological pH provide insight into their likely targets in vivo [4–6], with these values indicating that sulfur- and selenium-containing substrates are key targets for HOCl and HOSCN [7–10]. However, the high reactivity of these compounds with HOCl has made determination of accurate k values at pH 7.4 problematic and only estimates are available [11–16].

The relative rate constants for reactions of thiols with HOCl have been reported to show little variation with structure [11], whereas similar data for reduced glutathione (GSH), Cys, and Met at pH 7.4 indicate that k for the reaction of HOCl with thiols is greater than for the thioether of Met [12]. Direct stopped-flow kinetic studies of the reaction of HOCl with GSH at pH 7.4 have yielded a lower limit of k for this reaction of $10^7\ M^{-1}\ s^{-1}$ at physiological pH [14]. Further stopped-flow studies at pH 10–14 allowed much lower apparent rate constants to be determined, as OCl^- typically reacts 3–5 orders of magnitude slower than HOCl with the thiolate anion, RS^- [15,16]. These high-pH data, together with mechanistic information, have been used to predict rate constants at pH 7.4 and 22 °C of 3.2×10^7 and $3.8 \times 10^7\ M^{-1}\ s^{-1}$ for Cys and Met, respectively [13,15,16], but these apparent k values are subject to considerable potential error as the extrapolation is

Abbreviations: DTDPA, 3,3′-dithiodipropionic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; Fmoc-Met, 9-fluorenylmethyloxycarbonyl-tagged methionine; Fmoc-MetSO, 9-fluorenylmethyloxycarbonyl-tagged methionine sulfoxide; HOBr, the physiological mixture of hypobromous acid and its anion OBr^-; HOCl, the physiological mixture of hypochlorous acid and its anion OCl^-; HOSCN, the physiological mixture of hypothiocyanous acid and its anion $OSCN^-$; NAc-Cys, *N*-acetylcysteine; NAc-Met, *N*-acetylmethionine; NADH, reduced β-nicotinamide adenine dinucleotide; NMNH, reduced β-nicotinamide mononucleotide; UHPLC, ultrahigh-performance liquid chromatography; MeOH, methanol; THF, tetrahydrofuran

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critically dependent on the pK_a values used for HOCl and the substrates [13]. With thiols such as Cys and GSH, this extrapolation is complicated by the presence of two functional groups (the α-amino group and the thiol) that have similar ionization properties. In these cases the microscopic (rather than macroscopic) pK_a values indicate there are at least two reactive thiolate species (e.g., for Cys: $(^-SCH_2CH(COO^-)NH_3^+)^-$ and $(^-SCH_2CH(COO^-)NH_2)^{2-}$) present in the pH range that is being extrapolated. These species will each have different rate constants for reaction with HOCl and OCl^-, making accurate extrapolation from the high-pH data difficult [17,18].

These uncertainties prompted the development of a new method to investigate the rapid reactions of HOCl directly at pH 7.4, using a fluorescently tagged Met derivative (Fmoc-Met) as a competitive substrate. Upon reaction with HOCl, Fmoc-Met would be expected to react rapidly ($k > 10^7\ M^{-1}\ s^{-1}$ [4,13,15]) to generate the sulfoxide (Fmoc-MetSO) in a stoichiometric manner [19]. Unlike the labile products that are generated with other potential competitive substrates such as the thiol of GSH (reviewed in [5]), sulfoxides are more stable and oxidized relatively slowly by HOCl [20]. Furthermore, the sulfoxides are not readily reduced (e.g., by free thiols) except by specialized enzymes (e.g., by methionine sulfoxide reductases; reviewed in [21]). These redox properties of Met derivatives make them potential competitive kinetic substrates for HOCl as the formation of methionine sulfoxide is not easily perturbed and therefore can be readily assayed. Furthermore, in these studies the Fmoc derivative was used, as the hydrophobicity of the Fmoc group facilitates the separation by UHPLC, whereas its fluorescence properties allow ready detection of both the parent (Fmoc-Met) and the oxidized product (Fmoc-MetSO). These properties are important as they remove the need for postreaction derivatization that could lead to artifactual perturbation of the Fmoc-MetSO yields. Thus, a novel, robust competitive kinetic assay for monitoring the rate constants of the fast, physiologically relevant, reactions of HOCl is reported.

Materials and methods

Reagents

Cys and Met derivatives were all obtained from Sigma–Aldrich (Castle Hill, NSW, Australia) or Bachem (Bubendorf, Switzerland) and used as received. Fluorescently tagged Fmoc-Met and the corresponding sulfoxide (Fmoc-MetSO) were from Bachem. Reduced β-nicotinamide adenine dinucleotide (NADH; disodium salt) was from Roche (Sydney, Australia). Sodium hypochlorite was purchased from Ajax Finechem (Sydney, Australia). UV/visible spectroscopy was used to quantify HOCl at pH 12 using $\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$ as described previously [22]. All studies were performed in 10 mM phosphate buffers (pH 7.4) that were prepared using Nanopure water that had been filtered using a four-stage Milli-Q system (Millipore, North Ryde, Australia) and buffers were subsequently treated with washed Chelex resin (Bio-Rad, Gladesville, NSW, Australia) to remove contaminating transition metal ions.

UHPLC instrumentation and methods

Fmoc-Met and Fmoc-MetSO were separated using a Shimadzu Nexera UHPLC system (Shimadzu, South Rydalmere, NSW, Australia) on a Shim-Pack XR-ODS (Shimadzu, 100 × 4.6 mm, 2.2 μm) column at 40 °C. The column was eluted at a rate of 1.2 ml min^{-1} for 12 min with a gradient comprising solvent A (MeOH (20%, v/v), THF (2.5%), 1 M NaOAc, pH 5.3 (5%), and H_2O (72.5%)) and solvent B (MeOH (80%, v/v), THF (2.5%), 1 M NaOAc, pH 5.3 (5%), H_2O (12.5%)), with the gradient composition as follows: starting composition was 75% solvent B and 25% solvent A, with solvent B increasing to 87.5% over 5 min, then to 100% solvent B over 0.5 min and washing for 2.5 min, before a rapid (0.5 min) return to 75% solvent B and reequilibration for 3.5 min before the next injection. Samples were centrifugally filtered (0.2 μm; Pall Nanosep MF; 2 min, 9300*g*) to remove particulate matter before injection of 5 μl for each run. The Fmoc derivatives were detected by fluorescence detection (RF-20AXS; λ_{ex} 265 nm; λ_{em} 310 nm), with peak areas determined using Lab Solutions 5.32 SP1 software (Shimadzu) and compared to authentic standards. Under these conditions, Fmoc-MetSO eluted at ca. 2 min and Fmoc-Met at ca. 3.5 min.

Stopped-flow methodology

The rapid absorbance changes (< 100 ms) that occurred on mixing HOCl (0.5 mM) with 3,3′-dithiodipropionic acid (DTDPA; 0.5–2.5 mM) were monitored using an SX20 stopped-flow instrument (Applied Photophysics, Leatherhead, UK) fitted with a 1-cm-pathlength cell. The detection system consisted of an ozone-producing Xe light source (150 W; Osram GmbH, Munich, Germany) with wavelength selection achieved using a single monochromator (slit width, 0.5 mm; bandwidth, ± 1.2 nm) and photomultiplier detection. Time-dependent spectral data from 230 to 320 nm were obtained in a point-by-point manner by acquiring kinetic traces at 10-nm intervals with the photomultiplier over this wavelength region. The system was controlled by a personal computer running Pro-Data SX (version 2.2.12; Applied Photophysics). The sample chamber was maintained at 22 °C using circulating water from a thermostated water bath. Limiting concentrations of HOCl were used for all kinetic measurements to minimize the occurrence of secondary reactions. The resulting kinetic data were all fitted using the global analysis software Pro-Kineticist Mk IV (version 1.0.1.4; Applied Photophysics) to the simple mechanism HOCl + DTDPA → oxidized DTDPA.

Computational modeling studies

Computational models to calculate the theoretical product distributions for the various experimental conditions were run in Kintecus (Windows version 3.95, 2008, www.kintecus.com) [23]. The Kintecus simulation parameters were defined as follows: starting integration time, 1 μs; maximum integration time, 10 μs; accuracy, 1×10^{-12}; simulation length, 50 ms.

Statistics

Statistical analyses of the linear fits for the competition plots were carried out in either OriginPro 8.0 (OriginLab, Northampton, MA, USA) or Prism 5.0 (GraphPad, La Jolla, CA, USA). Standard error propagation methods were used to combine the experimental uncertainties in *k*(DTDPA + HOCl) and the slopes of the competition plots to provide the final confidence intervals for the reported *k* values.

Results

Development of an UHPLC assay for Fmoc-Met and Fmoc-MetSO

An UHPLC assay for the separation and quantification of Fmoc-Met and its oxidation product Fmoc-MetSO has been briefly reported previously [10]. The reactions of varying HOCl concentrations with Fmoc-Met (5 μM) were investigated at pH 7.4 (in 10 mM phosphate buffer) and 22 °C. Under these conditions, Fmoc-Met was quantitatively converted to Fmoc-MetSO (Fig. 1),

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