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Original Contribution

A sensitive and selective assay for chloramine production by myeloperoxidase

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Abstract

We describe a new assay for the chlorination activity of myeloperoxidase and detection of chloramines. Chloramines were detected by using iodide to catalyze the oxidation of either 3,3′,5,5′-tetramethylbenzidine (TMB) or dihydrorhodamine to form strongly absorbing or fluorescent products, respectively. With TMB as little as 1 μM taurine chloramine could be detected. The sensitivity of the dihydrorhodamine assay was about 10-fold greater. The chlorination activity of myeloperoxidase was measured by trapping hypochlorous acid with taurine and subsequently using iodide to promote the oxidation reactions of the accumulated taurine chloramine. A similar approach was used to detect hypochlorous acid production by stimulated human neutrophils. Iodide-dependent catalysis distinguished *N*-chloramines from *N*-bromamines. This allows for discrimination between heme peroxidases that generate either hypochlorous acid or hypobromous acid. The assay has distinct advantages over existing assays for myeloperoxidase with regard to sensitivity, specificity, and its ease and versatility of use.

Keywords: Neutrophil; Myeloperoxidase; Hypochlorous acid; Chloramine; Assay; Free radical

Introduction

In this investigation we sought to develop a sensitive and simple assay that measures the chlorination activity of myeloperoxidase. This green heme enzyme of neutrophils is the focus of much attention because of its involvement in many inflammatory pathologies [1–3]. It uses hydrogen peroxide to oxidize innumerable substrates either to hypohalous acids or reactive free radicals. Its ability to oxidize chloride to hypochlorous acid is unique among mammalian enzymes and is considered to be the dominant activity of myeloperoxidase in vivo [4,5]. Consequently, its chlorination activity is ideal for investigating the kinetics of the enzyme, production of hypochlorous acid by neutrophils, and for distinguishing myeloperoxidase from other peroxidases.

Current assays that measure hypochlorous acid production by myeloperoxidase lack sensitivity, interfere with enzyme activity, or are not specific [6]. Direct monitoring of hydrogen peroxide loss with a hydrogen peroxide electrode is extremely useful for continuous measurement of activity but is suitable only for defined kinetic experiments [7]. The classical peroxidase assays, in which a phenol or aniline is oxidized to a chromophore or fluorophore, are highly sensitive but do not distinguish myeloperoxidase from other heme proteins that have peroxidase activity.

Hypochlorous acid can be trapped with taurine to form a stable chloramine, which can then be detected to reveal enzyme activity. Taurine chloramine is normally assayed by measuring the bleaching of yellow 5-thio-2-nitrobenzoic

Abbreviations: TNB, 5-thio-2-nitrobenzoic acid; TMB, 3,3′,5,5′-tetramethylbenzidine; DHR, dihydrorhodamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid; DPI, diphenyliodonium; PMA, phorbol-myristate acetate; PBS, phosphate- buffered saline; BSA, bovine serum albumin.

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acid (TNB) [8]. This method is widely used but has limitations. Prior knowledge of the concentration of chloramines is required to get accurate and sensitive measurements, oxidants other than chloramines bleach this compound, and TNB has limited stability in the light. Hence, there is a need for a simple and robust assay that measures the chlorination activity of myeloperoxidase with sensitivity and specificity.

We have investigated the possibility of using iodide to catalyze oxidation reactions of taurine chloramine to develop a suitable assay for the chlorination activity of myeloperoxidase. Iodide has been shown to catalyze oxidation of NADH, cytochrome *c*, methionine, and thiols by chloramines [9,10]. It has also been used to detect chloramines formed in the chlorination of water [11]. However, until now its catalytic action has not been investigated to assess its suitability for the detection of biological chloramines or the chlorination activity of myeloperoxidase. The following reactions ((1)–(4)) explain how iodide catalyzes oxidation reactions of chloramines (RNHCl) [9].

$$\mathrm{RNHCl} + \mathrm{H^+} + \mathrm{I^-} \rightarrow \mathrm{RNH_2} + \mathrm{ICl} \quad (1)$$

$$\mathrm{ICl} + \mathrm{H_2O} \rightarrow \mathrm{HOI} + \mathrm{HCl} \quad (2)$$

$$\mathrm{HOI} + \mathrm{R'H_2} \rightarrow \mathrm{R'} + \mathrm{I^-} + \mathrm{H^+} + \mathrm{H_2O} \quad (3)$$

or

$$\mathrm{ICl} + \mathrm{R'H_2} \rightarrow \mathrm{R'} + \mathrm{I^-} + \mathrm{Cl^-} + 2\mathrm{H^+} \quad (4)$$

We have used 3,3′,5,5′-tetramethylbenzidine (TMB) as a chromophore because it is oxidized by hypoiodous acid to a strongly absorbing blue product [12]. Dihydrorhodamine was also investigated as a suitable detector because it is oxidized to rhodamine, which is highly fluorescent [13,14].

Materials and methods

Materials

3,3′,5,5′-Tetramethylbenzidine, dihydrorhodamine (DHR), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), diphenyliodonium (DPI), phorbol-myristate acetate (PMA), bovine erythrocyte catalase, bovine serum albumin, and taurine were purchased from the Sigma Chemical Company (St. Louis, MO). Domestic sodium hypochlorite was purchased as an approx 500 mM solution in 100 mM sodium hydroxide. The concentration of hypochlorite was determined by measuring its absorbance at 292 nm in a solution at pH 12 (ε_{292} 350 M^{-1} cm^{-1}) [15]. It was neutralized before use. Hypobromous acid was formed by reacting hypochlorous acid with a slight excess of bromide in phosphate buffer at pH 7.4. Its concentration was determined by measuring its absorbance at 329 nm in a solution at pH 12 (ε_{329} 332 M^{-1} cm^{-1}) [15]. Hydrogen peroxide was purchased from BDH as a 30% solution and its concentration was determined by measuring its absorbance at 240 nm (ε_{240} 43.6 M^{-1} cm^{-1}) [16]. Analytical grade dimethyformamide was from BDH. Myeloperoxidase was purified from human white blood cells as described previously [6] and its purity index (A_{430}/A_{280}) was at least 0.72. Its concentration per heme group was determined by measuring its absorbance at 430 nm (ε_{430} 89,000 M^{-1} cm^{-1}/heme) [17]. Neutrophils were isolated from human blood by density centrifugation through Ficoll-Hypaque, sedimentation of red blood cells with dextran, and hypotonic lysis of the remaining red blood cells [6].

Absorbances were recorded either in a Cary 1E UV-Vis spectrophotometer or a Spectra Max 190 microtiter plate reader (Molecular Devices, Sunnyvale CA). Fluorescence measurements were made with a Hitachi 4500 fluorescence spectrophotometer.

Detection of hypochlorous acid production by myeloperoxidase

The TMB method

Standard curves. Standard curves were constructed by adding known concentrations of hypochlorous acid to 5 mM taurine in 10 mM phosphate buffer, pH 7.4, containing 140 mM sodium chloride and 10 mM potassium chloride (PBS). The concentration of hypochlorous acid was always less than 100 μM to ensure that no taurine dichloramine was formed. The standards were kept on ice and assayed within 30 min. Taurine chloramine was detected by rapidly mixing 1 vol of standard with 0.25 vol of developing reagent. Formation of a blue product indicated the presence of chloramine. Five minutes after mixing, absorbances were recorded either at 645 nm in a spectrophotometer or at 650 nm in a plate reader. Standard curves were then plotted for the initial concentration of hypochlorous versus the recorded increase in absorbance of the blue product.

The developing reagent was composed of 2 mM TMB in 400 mM acetate buffer, pH 5.4, containing 10% dimethylformamide and 100 μM sodium iodide. This solution was prepared by dissolving TMB in 100% DMF, diluting with acetate buffer to get the desired final concentration of TMB, and then adding sodium iodide. When the developing reagent was mixed with the solutions of chloramines, the final concentration of TMB was always at least 10 times that of the concentration of chloramine. This prevented further oxidation of the blue product.

Hypochlorous acid production by isolated myeloperoxidase. Myeloperoxidase was incubated in 20 mM phosphate buffer, pH 6.5, containing 100 mM sodium chloride and 5 mM taurine. Reactions were started by adding hydrogen peroxide with rapid and complete mixing and stopped at the desired time by adding 20 μg/ml of catalase. An aliquot (200 μl) of this solution was then