Archives of Biochemistry and Biophysics 570 (2015) 14–22

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Inactivation of myeloperoxidase by benzoic acid hydrazide ☆

Jiansheng Huang [a], Forrest Smith [a], Jennifer R. Panizzi [b], Douglas C. Goodwin [c], Peter Panizzi [a,*]

[a] Department of Drug Discovery and Development, Harrison School of Pharmacy, Auburn University, Auburn, AL 36849, United States
[b] Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849, United States
[c] Department of Chemistry and Biochemistry, College of Science and Mathematics, Auburn University, Auburn, AL 36849, United States

ARTICLE INFO

Article history:
Received 25 August 2014
and in revised form 20 January 2015
Available online 14 February 2015

Keywords:
Myeloperoxidase
Inflammation
Benzoic acid hydrazide

ABSTRACT

Myeloperoxidase (MPO) is expressed by myeloid cells for the purpose of catalyzing the formation of hypochlorous acid, from chloride ions and reaction with a hydrogen peroxide-charged heme covalently bound to the enzyme. Most peroxidase enzymes both plant and mammalian are inhibited by benzoic acid hydrazide (BAH)-containing compounds, but the mechanism underlying MPO inhibition by BAH compounds is largely unknown. Recently, we reported MPO inhibition by BAH and 4-(trifluoromethyl)-BAH was due to hydrolysis of the ester bond between MPO heavy chain glutamate 242 ($^{HC}Glu^{242}$) residue and the heme pyrrole A ring, freeing the heme linked light chain MPO subunit from the larger remaining heavy chain portion. Here we probed the structure and function relationship behind this ester bond cleavage using a panel of BAH analogs to gain insight into the constraints imposed by the MPO active site and channel leading to the buried protoporphyrin IX ring. In addition, we show evidence that destruction of the heme ring does not occur by tracking the heme prosthetic group and provide evidence that the mechanism of hydrolysis follows a potential attack of the $^{HC}Glu^{242}$ carbonyl leading to a rearrangement causing the release of the vinyl-sulfonium linkage between $^{HC}Met^{243}$ and the pyrrole A ring.

Introduction

Myeloperoxidase (MPO)[1] is a heme-dependent peroxidase, but it is the only one capable of consuming hydrogen peroxide (H_2O_2) to mediate chloride oxidation to hypochlorous acid (HOCl). In its resting state, MPO contains ferric heme (MPO-Fe(III)). Upon reaction with H_2O_2 the MPO heme is oxidized to a short-lived intermediate termed Compound I (half life ~100 ms; [1]), which contains a ferryl porphyrin π cation radical (MPO-Fe(IV)=O$^{\cdot+\pi}$) (Eq. (1)) [2,3]. In the absence of Cl^- and in the presence of classical peroxide electron donor (AH_2), MPO follows a typical peroxidase catalytic cycle where Compound I is reduced back to the ferric state in two sequential one-electron steps (Eqs. (2) and (3)). In the first of these, the porphyrin radical is reduced leaving a ferryl heme known as Compound II (Eq. (2)). A second equivalent of AH_2 then reduces Compound II to ferric enzyme (Eq. (3)). In the process two equivalents of electron donor are oxidized to the corresponding free radical product (AH$^{\cdot}$) [4,5].

$$\text{MPO-Fe(III)} + H_2O_2 \rightarrow \underset{\text{“Compound I”}}{\text{MPO-Fe(IV)}{=}O^{\cdot+\pi}} + H_2O \quad (1)$$

$$\text{Compound I} + AH_2 \rightarrow \underset{\text{“Compound II”}}{\text{MPO-Fe(IV)}{=}O} + AH^{\cdot} \quad (2)$$

$$\text{Compound II} + AH_2 \rightarrow \text{MPO-Fe(III)} + AH^{\cdot} + H_2O \quad (3)$$

$$\text{Compound I} + Cl^- \rightarrow \text{MPO-Fe(III)} + HOCl \quad (4)$$

$$\text{MPO-Fe(III)} + O_2^- \rightarrow \underset{\text{“Compound III”}}{\text{MPO-Fe(III)}{\cdot}O_2^-} \quad (5)$$

In the presence of Cl^-, MPO Compound I is uniquely able to oxidize Cl^- to HOCl, and in the process Compound I is reduced directly to the ferric state (Eq. (4)). Neither Compound II (Eq. (3)) nor superoxide-inactivated Compound III (Eq. (5)) participates in

☆ Supported by the National Institutes of Health through grants provided by the National Heart, Lung, and Blood Institute, R00HL094533 (to P.P.) and R01HL114477 (to P.P.), the National Institute of Allergy and Infectious Diseases grant 2R44AI085840-02 (to P.P.) and Auburn University Research Initiative Cancer Graduate Fellowship (to J.H.)

* Corresponding author at: Department of Drug Discovery and Development, Harrison School of Pharmacy, Auburn University, 3211-J Walker Building, Auburn, AL 36849, United States. Fax: +1 334 844 7941.
E-mail address: panizzi@auburn.edu (P. Panizzi).

[1] *Abbreviations used:* MPO, myeloperoxidase; LC, light chain of myeloperoxidase; HC, heavy chain of myeloperoxidase; Cy5-hydrazide, cyanine5 hydrazide; ADHP, 10 -acetyl-3,7-dihydroxyphenoxazine; ABAH, aminobenzoic acid hydrazide; 2-ABAH, 2-aminobenzoic acid hydrazide; 4-ABAH, 4-aminobenzoic acid hydrazide; BAH, benzoic acid hydrazide; 4-FBAH, 4-fluorobenzoic acid hydrazide; 4-NBAH, 4-nitrobenzoic acid hydrazide; 4-TFMBAH, 4-(trifluoromethyl) benzoic acid hydrazide; 3-DMABAH, 3-(dimethylamino) benzoic acid hydrazide; HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; glutathione, (GSH); HRP, horseradish peroxidase; LPO, lactoperoxidase, superoxide dismutase, (SOD); PMF, peptide mass fingerprinting.

http://dx.doi.org/10.1016/j.abb.2015.01.028
0003-9861/

Cl^- oxidation. These reactions (Eqs. (1)–(5)) occur through octahedral coordination of the active site Fe by the protoporphyrin IX heme and the proximal histidine 336 on the MPO heavy chain ($^{HC}His^{336}$).

MPO also auto-catalytically forms three covalent associations with the porphyrin macrocycle the sum of which is an arrangement found nowhere else in nature. An MPO light chain aspartate ($^{LC}Asp^{94}$) forms an ester with the methyl side chain of pyrrole C. Additionally, a heavy chain glutamate ($^{HC}Glu^{242}$) forms an ester with the methyl side chain of pyrrole A, and the immediately adjacent methionine ($^{HC}Met^{243}$) is involved in a vinyl-sulfonium linkage with pyrrole A [6]. Interestingly, these bonds establish, through the prosthetic group itself, a covalent link between MPO's light and heavy chains and may account for the distinct saddling observed in the MPO heme. The extent of covalent association between mammalian peroxidases and their heme varies. It is completely absent in all non-animal peroxidases including horseradish peroxidase (HRP) [7–9], lignin peroxidase [10], bacterial catalase-peroxidases (KatG) [11,12], and ascorbate peroxidase [13], indicating that this type of heme modification is not required for classical peroxidase activity. However, mammalian peroxidases like lactoperoxidase (LPO) have two ester linkages analogous to those observed in MPO but lack the vinyl-sulfonium adduct [14,15]. In LPO, the ester bonds are between the heme b and its single subunit via $^{LPO}Glu^{375}$ and $^{LPO}Asp^{225}$ to pyrrole rings A and C, respectively. It is thought that the covalent tethers between mammalian peroxidases and their heme cofactors afford them a certain level of resistance necessary to protect the heme from oxidation by the HOCl and HOBr, which they generate [16].

Recently, we reported that incubation of benzoic acid hydrazide (BAH) with MPO in the presence of H_2O_2 causes a disruption of the linkages that occurred between the heme b and MPO heterodimer subunits [17]. Analysis of H_2O_2/BAH-treated MPO by SDS–PAGE revealed the co-migration of heme with the LC, suggesting that cleavage of the $^{HC}Glu^{242}$ ester and vinyl- $^{HC}Met^{243}$ sulfonium preceded loss of the $^{LC}Asp^{94}$ ester bond. Indeed, H_2O_2/BAH- induced shifts in heme absorption were also consistent with the disruption of its vinyl-sulfonium linkage [17]. The molecular mechanism by which this cleavage takes place and the role of this cleavage in the inhibition of MPO remains to be elucidated. There also has been no study to our knowledge that reports correlation between the MPO heme liberation with any other inhibitors that did not involve concomitant Fe loss. A panel of BAH analogs were used here to probe structure and function (i.e. cleavage) relationship to better understand the underlying mechanism by which the disruption occurs. Furthermore, we tracked how a Cy5-hydrazide inhibitor was incorporated into the MPO protein to determine a key event in the reaction mechanism that should parallel the BAH analog mechanism of MPO inhibition. Using peptide mass mapping, we also identified three MPO lysine (Lys) residues ($^{HC}Lys^{138}$, $^{HC}Lys^{308}$, and $^{HC}Lys^{463}$) where benzoic acid radical form adducts following oxidation by Compound I. Additionally, we found a number of methionine (Met) residues ($^{LC}Met^{85}$, $^{LC}Met^{87}$, $^{HC}Met^{243}$, $^{HC}Met^{249}$, $^{HC}Met^{306}$, and $^{HC}Met^{385}$) that were differentially oxidized in the presence of BAH with a relatively low concentration of H_2O_2 compared to the native MPO protein. Oxidation of $^{HC}Met^{243}$, in particular, may be a direct result of inhibition by BAH but further studies are needed to refine the exact chemical reactions leading to release of the heme from the HC of MPO. Finally, we tested whether BAH could be used to liberate the heme b prosthetic group from the active site of the analogous ester linkages present in LPO. Taken together, these studies provide new insight into the molecular mechanism of MPO inhibition by BAH providing new avenues for future drug discovery efforts that aim to limit the production of peroxidase-derived oxidants in chronic inflammatory diseases [18].

Experimental procedures

Reagent, proteins and chemicals

Ultra-pure myeloperoxidase (MPO) purified from human neutrophils was obtained from Lee Biosolutions Inc. (St. Louis, MO) and lactoperoxidase (LPO) of bovine and superoxide dismutase (SOD) were purchased from Worthington Biochemical Corporation (Lakewood, NJ). 4-aminobenzoic acid hydrazide (4-ABAH), benzoic acid hydrazide (BAH), 4-fluorobenzoic acid hydrazide (4-FBAH), 4-nitrobenzoic acid hydrazide (4-NBAH), sodium azide (NaN_3), Dimethyl sulphoxide (DMSO) from Alfa Aesar (Ward Hill, MA). Cyanine5 (Cy5) hydrazide was purchased from Lumiprobe Corporation (Hallandale Beach, FL). H_2O_2, glutathione (GSH), 2-aminobenzoic acid hydrazide (2-ABAH), 4-(trifluoromethyl) benzoic acid hydrazide (4-TFMBAH), 3-(dimethylamino) benzoic acid hydrazide (3-DMABAH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum were purchased from Innovative Research Inc. (Novi, MI). For protein staining, Gelcode Blue was purchased from Pierce (Rochford, IL), with tracking of the heme prosthetic group accomplished by use of the chemiluminescent Western Lightning ultra-reagent from PerkinElmer Inc. (Waltham, MA).

The acetate assay buffer was prepared to pH 5.6 by adjusting the pH of the sodium acetate with acetic acid. All working solutions of H_2O_2 were prepared fresh daily by diluting 30% H_2O_2 (BDH Chemicals, London, UK) and measuring the H_2O_2 concentration using the extinction coefficient for H_2O_2 at 240 nm of 39.4 M^{-1} cm^{-1} [19,20]. Cy5-hydrazide, 4-ABAH, 2-ABAH, BAH, 4-FBAH, 4-NBAH, 4-TFMBAH, 3-DMABAH, NaN_3 and isoniazid were dissolved in DMSO and subsequently diluted into acetate buffer. MEBSS buffer (144 mM NaCl, 5.4 mM KCl, 800 μM $MgSO_4$, 800 μM NaH_2PO_4, 1.2 mM $CaCl_2$, 5.6 mM glucose, 4 mM Hepes, pH 7.4 with 1% fetal bovine serum) was used in the luminescence assay for MPO activity, in accordance with the literature [21].

SDS–PAGE analysis of (heme b)-LC generation

To determine the effect that BAH analogs and the fluorescent analog Cy5-hydrazide have on the heme catalytic ability of MPO, MPO (1.2 μM) was incubated at room temperature with different BAH analogs following addition of H_2O_2 (20 μM). These samples of 20 μL were added to non-reducing sample loading buffer (Bio-rad), and loaded without prior heating onto a 4–15% gradient SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Of note, heat treatment was avoided to minimize the autocatalytic cleavage of MPO heavy chain at $^{HC}Met^{243}$–$^{HC}Pro^{244}$ bond that results in 39 kDa and 20 kDa bands [22]. Protein staining of elaborated gels was performed by GelCode Blue reagent (Pierce) followed by probing of the gel for the movement of the heme b through topical application of Western Lightning reagent (PerkinElmer Inc.). The light production generated as a result of this chemiluminescence reaction was collected using a Fujifilm LAS-1000 luminescence imager. For reactions examining incorporation of Cy5-hydrazide, an additional fluorescence scan was performed with fluorescent-labeled protein bands detected with a FLA-5100 imager with the 670 nM far-red laser and the Cy5 filter set (Fujifilm Inc., Tokyo, Japan). These scans were performed using excitation at λ_{640nm} and emission at λ_{670nm}. Total protein staining was done with Gelcode Blue reagent and imaged with Bio-Rad Gel Doc imager using Quantity One version 4.6.9. The luminescence signal of the gel was quantified using ImageJ 64 software (National Institutes of Health, Maryland, USA).