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# Peroxynitrite efficiently mediates the interconversion of redox intermediates of myeloperoxidase ☆

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**Abstract**

Nitric oxide-derived oxidants (e.g., peroxynitrite) are believed to participate in antimicrobial activities as part of normal host defenses but also in oxidative tissue injury in inflammatory disorders. A similar role is ascribed to the heme enzyme myeloperoxidase (MPO), the most abundant protein of polymorphonuclear leukocytes, which are the terminal phagocytosing effector cells of the innate immune system. Concomitant production of peroxynitrite and release of millimolar MPO are characteristic events during phagocytosis. In order to understand the mode of interaction between MPO and peroxynitrite, we have performed a comprehensive stopped-flow investigation of the reaction between all physiological relevant redox intermediates of MPO and peroxynitrite. Both iron(III) MPO and iron(II) MPO are rapidly converted to compound II by peroxynitrite in monophasic reactions with calculated rate constants of $(6.8 \pm 0.1) \times 10^6\ M^{-1}\ s^{-1}$ and $(1.3 \pm 0.2) \times 10^6\ M^{-1}\ s^{-1}$, respectively (pH 7.0 and 25 °C). Besides these one- and two-electron reduction reactions of peroxynitrite, which produce nitrogen dioxide and nitrite, a one-electron oxidation to the oxoperoxonitrogen radical must occur in the fast monophasic transition of compound I to compound II mediated by peroxynitrite at pH 7.0 [$(7.6 \pm 0.1) \times 10^6\ M^{-1}\ s^{-1}$]. In addition, peroxynitrite induced a steady-state transition from compound III to compound II with a rate of $(1.0 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$. Thus, the interconversion among the various oxidation states of MPO that is prompted by peroxynitrite is remarkable. Reaction mechanisms are proposed and the physiological relevance is discussed.


*Keywords:* Myeloperoxidase; Iron(II) peroxidase; Iron(III) peroxidase; Compound I; Compound II; Compound III; Peroxynitrite; Nitrite; Nitrogen dioxide

Peroxynitrite [systematic name: oxoperoxonitrate(1−)] is a strong oxidizing and nitrating agent generated from the diffusion-limited reaction of nitrogen monoxide, ·NO, with the superoxide anion, $O_2^{\cdot -}$ [1]. Peroxynitrite, $ONOO^-$, promotes both nitration and hydroxylation reactions of biological targets, like tyrosine, tryptophan, cysteine, methionine, nucleic acids, and membrane lipids [2–7]. Metalloproteins can also be modified by peroxynitrite. In particular, hemoproteins like peroxidases [8], cytochrome *c* [9], nitric oxide synthase [10], cytochrome *c* oxidase [11], and myoglobin and hemoglobin [12] have been reported to react with peroxynitrite.

Production of ·NO and $O_2^{\cdot -}$ is a characteristic feature at sites of inflammation. There is substantial evidence to suggest that leukocyte peroxidases may interact with peroxynitrite and may also serve as enzymatic participants in generation of reactive nitrogen species [8,13–17]. Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are some of the most abundant proteins in neutrophils, monocytes, and eosinophils, which are the terminal effector cells of the innate immune system and are abundant cellular con-

☆ *Abbreviations:* MPO, myeloperoxidase; $E'^{\circ}$, standard reduction potential.

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stituents at sites of inflammation [18,19]. These heme enzymes utilize hydrogen peroxide and a variety of organic and inorganic low molecular weight substrates to generate an array of reactive oxidants and diffusible radical species by both one- and two-electron oxidation reactions [20].

In stimulated neutrophils, phagocyte respiratory burst oxidase, a multi-component enzyme system that plays a critical role in antimicrobial host defenses [21], catalyzes the electron transport from cytosolic NADPH to molecular oxygen, producing superoxide in the phagosome [22], which then serves as an intermediate in the formation of other reactive oxygen species, e.g., $H_2O_2$, or react with nitrogen monoxide, $^{\cdot}NO$, produced from inducible nitric oxide synthase (iNOS) [23]. Under physiological conditions, one of the main targets of peroxynitrite is thought to be $CO_2$, present in millimolar concentrations in most fluids and tissues. Reaction of $ONOO^-$ with $CO_2$ leads to $ONOOCO_2^-$ a stronger nitrating agent than peroxynitrous acid. However, MPO is also present in millimolar concentrations in the phagosome [24] and has been shown to react with peroxynitrite in a pH-dependent manner [8]. On mixing MPO with $ONOO^-$ the formation of a ferryl-type compound II was observed [8]. By contrast, upon mixing of horseradish peroxidase (HRP) with peroxynitrite compound I formation was observed [8] and nitrite, $NO_2^-$, was postulated to be the reaction product. However, a detailed mechanistic study that involves the reactivity of all relevant redox intermediates of MPO with peroxynitrite is still missing. This is important because the redox chemistry of MPO is peculiar. The midpoint potential ($E'^{\circ}$) for the iron(III)/iron(II) couple is +25 mV [25], which is similar to globins and much more positive than any other peroxidase [20]. Similarly, the standard reduction potentials of the redox couples compound I/native MPO (1.16 V) and compound I/compound II (1.35 V) of MPO are more positive than those of other peroxidases [20,26]. A consequence of these redox properties is that (i) the iron(II) form plays a prominent role in catalysis of MPO and (ii) compound I is an extremely potent oxidant regarding both two-electron and one-electron oxidation reactions. But the reaction of neither iron(II) MPO nor compound I with peroxynitrite is known. Moreover, the presence of compound III (being outside of both the halogenation and the peroxidation cycles) in stimulated intact neutrophils has been shown spectroscopically [27]. Compound III is an iron(II)-dioxygen/iron(III)-superoxide complex similar to oxyhemoglobin or oxymyoglobin. Peroxynitrite has been shown to diffuse into erythrocytes and oxidize oxyhemoglobin to methemoglobin in a fast and efficient manner [28]. Thus, it could be reasonable to assume that MPO compound III could act also as a sink of peroxynitrite with the consequence that peroxynitrite is scavenged and MPO can re-enter its catalytic cycle.

Here, for the first time a comprehensive multi-mixing stopped-flow analysis of the reactions between peroxynitrite and iron(II) and iron(III) MPO, compound I, compound II, and compound III was performed and apparent bimolecular rate constants are presented.

## Materials and methods

*Materials.* Highly purified myeloperoxidase of a purity index ($A_{430}/A_{280}$) of at least 0.85 was purchased from Planta Natural Products (http://www.myeloperoxidase.com). Its concentration was calculated using $\varepsilon_{430} = 91000\ M^{-1}\ cm^{-1}$ [29]. Hydrogen peroxide, obtained as a 30% solution from Sigma, was diluted and the concentration was determined using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$ [30].

Iron(II) MPO was produced by dithionite under anaerobic conditions as described recently [31]. Shortly, iron(II) MPO was made in the absence of molecular oxygen in a glove box (Meca-Plex, Neugebauer), with a positive pressure of nitrogen (250 mbar, oxygen <3 ppm). In addition, all solutions were prepared and flushed with nitrogen in the glove box. Sodium dithionite was purchased from Aldrich and was used either in solid form or from a freshly prepared anaerobic stock solution. Excess dithionite and bisulfite ion were removed by passing the solution through an anion exchange column (DEAE Sepharose Fast Flow, Pharmacia Biotech) and the solutions were proven to be dithionite free by measuring the absorbance at 315 nm. Iron(II) MPO was transported from the glove box to the stopped-flow apparatus in gas-tight syringes. The use of a diode array detector attached to the stopped-flow machine enabled us also to control the absence of dithionite at 315 nm. A good spectrum of iron(II) MPO had its peak maxima in the Soret region at 473 nm ($\varepsilon_{473} = 91700\ M^{-1}\ cm^{-1}$) and in the visible region at 638 nm with a shoulder around 600 nm.

Procedure for preparation of peroxynitrite solutions was followed according to Latal et al. [32] to minimize nitrite formation. All solutions and dilutions therefore have been kept on ice during work. Frozen stock solutions of peroxynitrite were prepared by thawing 1 mL aliquots in ice-cold water and immediately diluted with 0.02 M NaOH. The concentration of the resulting solutions was determined spectrophotometrically prior to each experiment by measuring their absorbance at 302 nm ($\varepsilon_{302} = 1705\ M^{-1}\ cm^{-1}$).

*Stopped-flow spectroscopy.* The multi-mixing stopped-flow measurements were performed with the Applied Photophysics (UK) instrument SX-18 MV. When 100 μL was shot into a flow cell having a 1 cm light path, the fastest time for mixing two solutions and recording the first data point was 1.3 ms. Kinetics were followed both at single wavelength and by using a diode-array detector. At least three determinations (2000 data points) of pseudo-first-order rate constants ($k_{obs}$) were performed for each substrate concentration (pH 7.0, 25 °C) and the mean value was used in the calculation of the second-order rate constants, which were calculated from the slope of the line defined by a plot of $k_{obs}$ versus substrate concentration. To allow calculation of pseudo-first-order rates, the concentrations of substrates were at least 10 times in excess of the enzyme.

The conventional stopped-flow technique was used in probing the reaction between iron(II) and iron(III) MPO and peroxynitrite, whereas the sequential-mixing technique was used in the investigation of the reactivities of compound I, compound II, and compound III. The conditions of MPO compound I formation were described recently [29]. Typically, 4 μM MPO was premixed with 40 μM $H_2O_2$ (both in 200 mM phosphate buffer, pH 6.8) and, after a delay time of 20 ms, compound I was allowed to react with varying concentrations of peroxynitrite in 20 mM NaOH. The reactions were followed at the Soret maximum of compound II (456 nm) and at 442 nm, the isosbestic point between compound II and the iron(III) protein. Reduction of compound II was measured as described in [33]. In a typical experiment, 8 μM MPO was premixed with 80 μM $H_2O_2$ and 7.2 μM homovanillic acid in 200 mM phosphate buffer, pH 6.8, and after a delay time of 40 s, compound II was allowed to react with varying concentrations of peroxynitrite in 20 mM NaOH. Reactions were followed at 456 nm (disappearance of compound II) and 430 nm (formation of iron(III) MPO). Alternatively, compound II was formed with peroxynitrite upon mixing of iron(III) MPO with peroxynitrite and following both compound II formation and reduction in one experiment.

Compound III reactivity (iron(II)-dioxygen/iron(III)-superoxide complex of MPO) was studied in two ways: (i) mixing iron(II) MPO