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Mechanism of reaction of chlorite with mammalian heme peroxidases

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ABSTRACT

This study demonstrates that heme peroxidases from different superfamilies react differently with chlorite. In contrast to plant peroxidases, like horseradish peroxidase (HRP), the mammalian counterparts myeloperoxidase (MPO) and lactoperoxidase (LPO) are rapidly and irreversibly inactivated by chlorite in the micromolar concentration range. Chlorite acts as efficient one-electron donor for Compound I and Compound II of MPO and LPO and reacts with the corresponding ferric resting states in a biphasic manner. The first (rapid) phase is shown to correspond to the formation of a MPO-chlorite high-spin complex, whereas during the second (slower) phase degradation of the prosthetic group was observed. Cyanide, chloride and hydrogen peroxide can block or delay heme bleaching. In contrast to HRP, the MPO/chlorite system does not mediate chlorination of target molecules. Irreversible inactivation is shown to include heme degradation, iron release and decrease in thermal stability. Differences between mammalian peroxidases and HRP are discussed with respect to differences in active site architecture and heme modification.

1. Introduction

Chlorite ($^{-}$OClO) reacts with a variety of different heme containing enzymes. For example, it was shown to induce the formation of methemoglobin [1] and might act as hydroxylating agent in cytochrome P450 [2]. However, detailed mechanistic studies are only available for two classes of heme enzymes, namely chlorite dismutases and peroxidases. Chlorite dismutases are found in bacteria and archaea [3,4] and convert chlorite to chloride (Cl^{-}) and dioxygen (O_2) with hypochlorous acid (HOCl) as proposed reaction intermediate [5]. During this conversion a covalent O–O bond is formed, a reaction which was so far only observed at photosystem II of oxygenic phototrophic organisms.

Among heme peroxidases, chlorite was shown to be utilized by cytochrome *c* peroxidase [6], chloroperoxidase [7] and horseradish peroxidase (HRP) [8]. Upon reaction with chlorite the enzymes produced halogenating agents but not O_2. Detailed studies were carried out by Hager and co-workers on HRP [9–11]. Recently, by using sequential-mixing stopped-flow spectroscopy, we have reinvestigated this reaction and could demonstrate that HRP mixed with chlorite follows the whole peroxidase cycle [12]. Chlorite mediates the two-electron oxidation of ferric HRP [Fe(III)…Por] to Compound I [Fe(IV) = O…Por$^{\bullet+}$] thereby releasing hypochlorous acid (Reaction 1). Furthermore, chlorite acts as one-electron reductant of both Compound I and Compound II [Fe(IV) = O…Por] forming chlorine dioxide ($^{\bullet}ClO_2$) (Reactions 2 & 3). The HRP mediated reactions are strongly pH dependent, suggesting that chlorous acid (HOClO) is the reacting species [12]. In addition both reaction products also mediate the two-electron oxidation of ferric HRP to Compound I (Reaction 4 & 5) but cannot serve as electron donors for Compounds I or II.

$$[Fe(III)\ldots Por] + {}^{-}OClO \rightarrow \left[Fe(IV) = O\ldots Por^{\bullet+}\right] + HOCl/{}^{-}OCl, H^{+} \quad \text{(Reaction1)}$$

$$[Fe(IV) = O\ldots Por^{\bullet+}] + {}^{-}OClO \rightarrow [Fe(IV) = O\ldots Por] + {}^{\bullet}ClO_2 \quad \text{(Reaction2)}$$

$$[Fe(IV) = O\ldots Por] + {}^{-}OClO \rightarrow [Fe(III)\ldots Por] + {}^{\bullet}ClO_2 + H_2O \quad \text{(Reaction3)}$$

$$[Fe(III)\ldots Por] + HOCl \rightarrow \left[Fe(IV) = O\ldots Por^{\bullet+}\right] + Cl^{-} \quad \text{(Reaction4)}$$

$$[Fe(III)\ldots Por] + {}^{\bullet}ClO_2 \rightarrow [Fe(IV) = O\ldots Por^{\bullet+}] + {}^{\bullet}ClO. \quad \text{(Reaction5)}$$

In this work we have extended this study to human myeloperoxidase (MPO) and bovine lactoperoxidase (LPO). The fact that MPO is involved in the regulation of immune functions [13] and that commercially

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available wound healing and immunomodulatory drug solutions like Oxoferin and WF10 contain chlorite as the active principle [14], prompted us to this study.

In contrast to plant-type peroxidases the heme in MPO is covalently attached to the protein via two ester-linkages and a sulfonium ion linkage [15]. These posttranslational modifications significantly modify the spectral and redox properties of this oxidoreductase, e.g. allowing MPO to oxidize chloride to hypochlorous acid that acts as antimicrobial agent in the innate immune system [16]. Additionally, the covalent heme to protein bonds were proposed to protect the heme of MPO from its highly reactive reaction products [17].

Here we demonstrate that – in contrast to horseradish peroxidase (HRP) – human MPO and bovine LPO cannot utilize chlorite to form chlorinating species [6]. Moreover, we show that both peroxidases are very susceptible to chlorite resulting in fast loss of activity accompanied by (almost) complete heme bleaching. We report the first comprehensive study of the reactions of all biological relevant redox intermediates (ferric state, Compounds I & II) of MPO and LPO with chlorite and their potential reaction products. We propose a mechanism of interaction between chlorite and the metalloproteins, discuss the observed differences with plant-type peroxidases as well as the biological relevance of our findings.

2. Material and methods

2.1. Reagents

Standard chemicals and biochemicals were obtained from Sigma-Aldrich Handels GmbH (Vienna, Austria) at the highest grade available. Hydrogen peroxide concentration was determined spectrophotometrically using an extinction coefficient at 240 nm of 39.4 M^{-1} cm^{-1} [18]. The concentration of sodium chlorite (Sigma, 80%) and hypochlorite were determined using the extinction coefficient at 260 nm of 154 M^{-1} cm^{-1} [19] and at 292 nm of 350 M^{-1} cm^{-1} [20], respectively. Highly purified dimeric leukocyte myeloperoxidase of a purity index (A_{428}/A_{280}) of at least 0.85 was purchased as lyophilized powder from Planta Natural Products (http://www.planta.at) and the concentration was determined spectrophotometrically using ε_{428} = 91 000 M^{-1} cm^{-1} per heme [21]. Lactoperoxidase from bovine milk was purchased as a lyophilized powder (Sigma, purity index A_{412}/A_{280} = 0.9). Enzyme concentration was determined by using the extinction coefficient 112,000 M^{-1} cm^{-1} at 412 nm [22]. Horseradish peroxidase type VIa was obtained from Sigma and used without further purification. An extinction coefficient at 403 nm of 102,000 M^{-1} cm^{-1} [23] was used for the determination of protein concentration. Oxovasin was obtained from NUVO Manufacturing GmbH (Wanzleben, Germany). Essentially the same drug is also marketed as Oxoferin™, a term more frequently used in a scientific context, therefore we will also refer to Oxoferin instead of Oxovasin in this study.

2.2. Preparation of chlorine dioxide

Chemically pure, chlorine free chlorine dioxide ($^{\bullet}ClO_2$) solutions were prepared by acidification of sodium chlorite (NaOClO) with sulfuric acid (H_2SO_4) according to $4\,NaOClO + 2\,H_2SO_4 \rightarrow 2\,^{\bullet}ClO_2 + HCl + HOClO_2 + 2\,Na_2SO_4 + H_2O$ [12]. In detail, 100 mL sodium chlorite (2.5% $^{w}/_{w}$) was acidified with 10 mL sulfuric acid (10% $^{v}/_{v}$). Sulfuric acid was added in 1 mL portions with 5 min intervals. Formed gaseous $^{\bullet}ClO_2$ was removed from the reaction flask by a nitrogen flow, purified by bubbling through a gas scrubbing tower containing 100 mL sodium chlorite solution (2.5% $^{w}/_{w}$) and, finally, recovered by absorbing in 100 mL chilled deionized water in an amber reagent bottle. The concentration of $^{\bullet}ClO_2$ was determined using an extinction coefficient at 359 nm of 1230 M^{-1} cm^{-1} [24]. The obtained stock solution (≈42 mM) was immediately aliquoted in Eppendorf tubes and stored in the dark at −30 °C. After thawing the stock solution was kept in the dark on ice and was stable for about 4 h. More diluted solutions were prepared immediately before use and the concentrations were determined simultaneously to the measurements.

2.3. In gel activity staining

For in-gel heme staining MPO samples (10 μg) were separated using SDS-PAGE electrophoresis under non-reducing conditions. Gels were incubated with a mixture of 6.3 mM 3,3,5,5,-tetramethylbenzidine (TMB) in methanol (3 parts) and 0.25 M sodium acetate at pH 5.0 (7 parts) for 1 h in the dark. Activity staining was started by adding hydrogen peroxide at a final concentration of 30 mM [25].

2.4. Activity assays

Monochlorodimedon (MCD) is a substrate often used to study halogenation reactions. Upon chlorination to dichlorodimedon (DCD) it loses its absorbance at 290 nm [26]. Monochlorodimedon (100 μM) and various amounts of chlorite were dissolved in 100 mM phosphate buffer, pH 5.0, and the reaction was started by addition of MPO or LPO.

For the determination of the inhibitory effect of chlorite on the chlorination activity of MPO assays contained additional hydrogen peroxide (100 μM) and chloride (100 mM). In this case the reaction was started either with MPO (100 nM) or hydrogen peroxide. The latter case resulted in a preincubation of MPO with both chloride and chlorite for 1 min.

Additionally, the inhibitory effect of chlorite on the chlorination activity was measured by continuously monitoring hydrogen peroxide concentration polarographically with a platinum electrode covered with a hydrophilic membrane and fitted to the Amperometric Biosensor Detector 3001 (Universal Sensors, Inc., U.S.A.). Again the reaction was started by adding either MPO (100 nM) or hydrogen peroxide (100 μM) after one minute. All reactions were performed at 25 °C.

As a further method to study the inhibitory effect of chlorite on MPO we used aminophenyl fluorescein (APF) which specifically detects the halogenating enzyme activity [27]. Briefly, 10 nM MPO was preincubated with 0–500 μM chlorite in phosphate buffered saline (PBS), pH 7.4, at 37 °C for 1 min. All samples also contained 10 μM APF. The enzymatic activity was started by adding 25 μM H_2O_2 via an injector device. The HOCl-derived oxidation of APF was followed for 30 min at 37 °C by measuring the fluorescence intensity at 522 nm (excitation at 488 nm). All measurements were performed in a fluorescence microplate reader Tecan Infinite 200 PRO, Switzerland.

2.5. Circular dichroism and electron paramagnetic resonance spectroscopy

Circular dichroism spectra (CD) were recorded using a Chirascan CD spectrophotometer (Applied Photophysics, UK). In the far-UV region an enzyme concentration of 2 μM and a cuvette with a path length of 1 mm was used. For measurements in the Soret region (300 nm–500 nm) the enzyme concentration was 5 μM and the path length of the cuvette was 1 cm.

Samples for electron paramagnetic resonance (EPR) measurements were prepared using 100 μM MPO in 100 mM phosphate buffer, pH 5. After addition of chlorite in 5- and 10-fold excess, respectively, 5% glycerol was added as cryoprotectant and 100 μL of the reaction solution were immediately transferred into a quartz EPR tube (4 mm OD) and frozen in liquid nitrogen. Before measurement, the tube was degassed with Ar while the sample was kept frozen on dry ice.

EPR measurements were carried out at 10 K on an EMX continuous wave (cw) spectrometer (Bruker, Germany), operating at X-band (9 GHz) frequencies, equipped with a high sensitivity resonator and a helium cryostat from Oxford Instruments (ESR900). EPR spectra were recorded under non-saturating conditions using 2 mW microwave power, 100 kHz modulation frequency, 1 mT modulation amplitude, 20 ms conversion time and 20 ms time constant and 4096 data points. Simulations of high-spin Fe(III) spectra were carried out using the