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Reaction of ferrous lactoperoxidase with hydrogen peroxide and dioxygen: an anaerobic stopped-flow study

Walter Jantschko, Paul G. Furtmüller, Martina Zederbauer, Karin Neugschwandtner, Christa Jakopitsch, Christian Obinger*

Division of Biochemistry, Department of Chemistry, Metalloprotein Research Group, BOKU—University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria

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Abstract

Lactoperoxidase (LPO) is found in mucosal surfaces and exocrine secretions including milk, tears, and saliva and has physiological significance in antimicrobial defense which involves (pseudo-)halide oxidation. LPO compound III (a ferrous–dioxygen complex) is known to be formed rapidly by an excess of hydrogen peroxide and could participate in the observed catalase-like activity of LPO. The present anaerobic stopped-flow kinetic analysis was performed in order to elucidate the catalytic mechanism of LPO and the kinetics of compound III formation by probing the reactivity of ferrous LPO with hydrogen peroxide and molecular oxygen. It is shown that ferrous LPO heterolytically cleaves hydrogen peroxide forming water and oxyferryl LPO (compound II). The two-electron oxidation reaction follows second-order kinetics with the apparent bimolecular rate constant being $(7.2 \pm 0.3) \times 10^4\,M^{-1}\,s^{-1}$ at pH 7.0 and 25 °C. The H_2O_2-mediated conversion of compound II to compound III follows also second-order kinetics ($220\,M^{-1}\,s^{-1}$ at pH 7.0 and 25 °C). Alternatively, compound III is also formed by dioxygen binding to ferrous LPO at an apparent bimolecular rate constant of $(1.8 \pm 0.2) \times 10^5\,M^{-1}\,s^{-1}$. Dioxygen binding is reversible and at pH 7.0 the dissociation constant (K_D) of the oxyferrous form is 6 μM. The rate constant of dioxygen dissociation from compound III is higher than conversion of compound III to ferric LPO, which is not affected by the oxygen concentration and follows a biphasic kinetics. A reaction cycle including the redox intermediates compound II, compound III, and ferrous LPO is proposed, which explains the observed (pseudo-)catalase activity of LPO in the absence of one-electron donors. The relevance of these findings in LPO catalysis is discussed.

Keywords: Lactoperoxidase; Ferrous peroxidase; Compound II; Compound III; Dioxygen; Hydrogen peroxide; Stopped-flow spectroscopy

Together with myeloperoxidase (MPO),[1] eosinophil peroxidase (EPO), and thyroid peroxidase (TPO), lactoperoxidase (LPO; EC 1.11.1.7) constitutes the animal peroxidase superfamily II which is distinguished from the peroxidase superfamily I (enzymes from plants, fungi, and bacteria) in that the prosthetic heme group is covalently attached to the protein [1]. It is thought that the major physiological function of LPO, EPO, and MPO is to play a role in host defense mechanisms. EPO, and MPO are present in specialized white blood cells [2,3], which are recruited against invading pathogens, whereas LPO is produced by goblet cells and submucosal glands and is found in mucosal surfaces and exocrine secretions including milk, tears, and saliva [4,5]. Recently, it was shown that LPO is localized also in human bronchi and that a functional LPO system exists in human airways and may contribute to airway host defense against infection [6]. Generally, the role of these

* Corresponding author. Fax: +43 1 36006 6059.
E-mail address: christian.obinger@boku.ac.at (C. Obinger).

[1] *Abbreviations used:* LPO, lactoperoxidase; MPO, myeloperoxidase; EPO, eosinophil peroxidase; TPO, thyroid peroxidase; EPR, electron paramagnetic resonance, HOCl, hypochlorous acid.

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heme enzymes in antimicrobial processes seems to be the catalyzation of halide and thiocyanate oxidation thereby producing cytotoxic hypohalous acids and hypothiocyanite.

LPO has a single polypeptide chain and is a heme- and calcium-containing glycoprotein with one heme group per molecule. Until now crystallographic data are not available but there is evidence that the heme in LPO is covalently attached to the protein via two ester linkages, namely between the heme 1- and 5-methyl groups and a conserved glutamate and aspartate residue, respectively. The cross-linking process is thought to occur autocatalytically, with the 5-hydroxymethyl bond formed before the 1-hydroxymethyl bond [7–9]. By contrast, in myeloperoxidase—the only mammalian peroxidase for which a structure is available—three covalent links between the heme and the protein exist [10]. In addition to the two ester linkages a thioether sulfonium bond between the β-carbon of the 2-vinyl group and Met243 is present [10]. The existence of covalently linked heme and the differences in heme linkage within the animal peroxidase superfamily are responsible for the optical properties of these proteins and could be a major factor in the observed differences in substrate specificity. Principally, the mechanism of halide oxidation is similar (Fig. 1). It starts by reaction of the ferric enzyme with H_2O_2 to form compound I, which contains two oxidizing equivalents more than the resting enzyme (Reaction 1). Halides (X^-) or thiocyanate reduce compound I directly to native enzyme by a two-electron process (Reaction 2). Thereby, hypohalous acids are formed (HOX). Alternatively, substrates (AH_2) reduce compound I to native enzyme via compound II by two successive one-electron reductions (Reactions 3 and 4) releasing free radicals (·AH). At neutral pH, only MPO compound I is capable to oxidize chloride at a reasonable rate [11] and it is assumed that chloride and thiocyanate are competing substrates for MPO in vivo [12], whereas LPO cannot oxidize chloride but reacts extremely fast with thiocyanate [13].

Nevertheless, LPO and MPO share functional features that make them peculiar with respect to other peroxidases. An overview of open questions was presented recently by Ghibaudi and Laurenti [14] including the electronic structures of compound I and II, the reversibility of compound I formation and catalase activity as well as the role of compound III in enzyme turnover. LPO compound III, a ferrous–dioxy/ferric superoxide complex (similar to oxyhemoglobin or oxymyoglobin) is known to be formed by an at least 50-fold excess of hydrogen peroxide [15].

Recently, we analyzed those reactions of MPO which contribute to the interconversion of the ferrous protein, compound II, compound III, and the ferric form (Reactions 6, 7, 9, 10, and 11 in Fig. 1) [16,17]. Here, we report for the first time an anaerobic stopped-flow study of the:

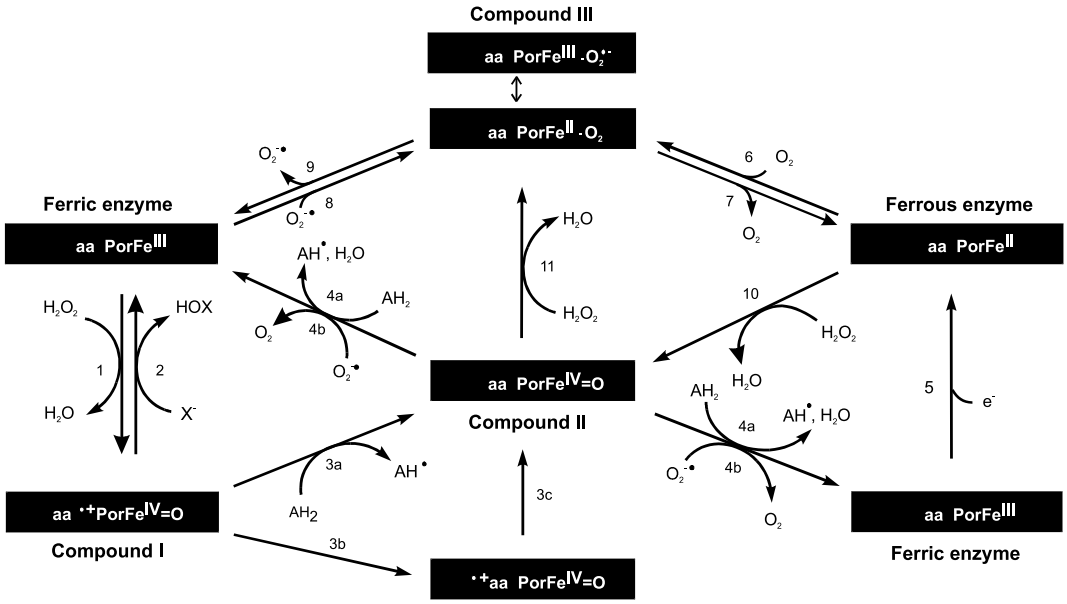

Fig. 1. Reaction scheme of lactoperoxidase. In the first step H_2O_2 is used for compound I formation (Reaction 1). Compound I is two oxidizing equivalents above that of the native enzyme with a porphyrin π-cation radical ($^{\cdot+}$Por) in combination with an iron(IV) center or an amino acid radical ($^{\cdot+}$aa) in combination with iron(IV). Compound I can react with halides or thiocyanate reducing the enzyme back to the ferric state (Reaction 2, halogenation activity). In the peroxidase reaction compound I is transformed in the first one-electron reduction to compound II, which contains an iron(IV) center (Reaction 3). Compound II is finally reduced back to ferric peroxidase in a second one-electron reduction (Reaction 4). Compound III (oxyperoxidase) is formed either from ferric peroxidase with superoxide (Reaction 8), from ferrous LPO with O_2 (Reaction 6) or from compound II with H_2O_2 (Reaction 11). It is a complex of ferrous–dioxygen in resonance with ferric-superoxide.