

A transient kinetic study on the reactivity of recombinant unprocessed monomeric myeloperoxidase

Paul Georg Furtmüller^a, Walter Jantschko^a, Günther Regelsberger^a, Christa Jakopitsch^a,
Nicole Moguilevsky^b, Christian Obinger^{a,*}

^aInstitute of Chemistry, University of Agricultural Sciences, Muthgasse 18, A-1190 Vienna, Austria

^bDepartment of Applied Genetics, Free University of Brussels, rue des professeurs Jeener et Brachet 12, B-6041 Gosselies, Belgium

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Abstract Spectral and kinetic features of the redox intermediates of human recombinant unprocessed monomeric myeloperoxidase (recMPO), purified from an engineered Chinese hamster ovary cell line, were studied by the multi-mixing stopped-flow technique. Both the ferric protein and compounds I and II showed essentially the same kinetic behavior as the mature dimeric protein (MPO) isolated from polymorphonuclear leukocytes. Firstly, hydrogen peroxide mediated both oxidation of ferric recMPO to compound I ($1.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, pH 7 and 15°C) and reduction of compound I to compound II ($3.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, pH 7 and 15°C). With chloride, bromide, iodide and thiocyanate compound I was reduced back to the ferric enzyme ($3.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $1.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, respectively), whereas the endogenous one-electron donor ascorbate mediated transformation of compound I to compound II ($2.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) and of compound II back to the resting enzyme ($5.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$). Comparing the data of this study with those known from the mature enzyme strongly suggests that the processing of the precursor enzyme (recMPO) into the mature form occurs without structural changes at the active site and that the subunits in the mature dimeric enzyme work independently. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Recombinant myeloperoxidase; Compound I; Compound II; Halide; Ascorbate; Stopped-flow spectroscopy

1. Introduction

Myeloperoxidase (MPO) is a major neutrophil protein and is also present in monocytes. In neutrophils, it is stored in azurophilic granules and released during phagocytosis [1]. It is a heme enzyme that uses hydrogen peroxide generated by the neutrophil oxidative burst to produce hypochlorous acid and other reactive intermediates [1]. Since the myeloperoxidase system has been demonstrated to be strongly bactericidal [2], the enzyme is considered to be an important component of

the neutrophil's antimicrobial armory. It has also been implicated in promoting tissue damage in numerous inflammatory diseases.

Mature MPO is an ~150 kDa symmetric, glycosylated homodimer ($(\alpha\beta)_2$), each identical half composed of heavy (α) and light (β) subunits of 59 kDa and 13.5 kDa, and the two halves covalently linked by a disulfide bond [3]. MPO is encoded by a single gene on chromosome 17 and expressed exclusively during the promyelocytic stage of myeloid differentiation [4]. The primary translation product of MPO undergoes cotranslational cleavage of the signal peptide, N-linked glycosylation, and limited deglycosylation of high mannose oligosaccharide side chains to generate the enzymatically inactive precursor apoproMPO [4]. Within the endoplasmic reticulum apoproMPO interacts transiently and reversibly with molecular chaperones with subsequent heme incorporation and generation of the enzymatically active ~90 kDa pro-MPO. Most proMPO undergoes dimerization, modification of oligosaccharide side chains, and proteolytic processing including excision of the N-terminal 125 amino acid propeptide, a small peptide between light and heavy subunits, and the carboxy-terminal serine residue [4].

Moguilevsky et al. have been successful in the massive production and purification of recombinant unprocessed monomeric myeloperoxidase (recMPO) from Chinese hamster ovary (CHO) cell lines [5]. Secreted recMPO is a single-chain precursor of 84 kDa. Amino-terminal amino acid analysis showed that the secreted recMPO starts at residue 49 in the protein [5]. The protein fails to undergo proteolytic processing into mature subunits, is expressed in the monomeric form and has oligosaccharide side chains slightly different from those of native MPO [5]. Physicochemical investigations indicate that this single-chain enzyme behaves essentially in the same way as the natural two-chain molecule [5,6]. In order to test whether the processing of the precursor enzyme (recMPO) into the mature form occurs with or without structural and functional consequences for the reactivity of its redox intermediate compounds I and II, the present stopped-flow study was performed.

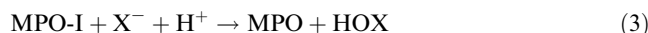
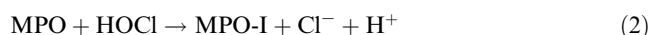
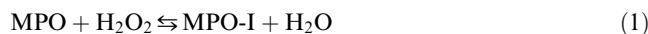
Of the oxidized intermediates of myeloperoxidase, compound I (MPO-I) is the only species that takes part in both the peroxidatic cycle (Reactions 1, 4 and 5) and the chlorination activity (Reactions 1 and 3) of myeloperoxidase. It is formed by oxidation of the ferric enzyme (MPO) by hydrogen peroxide (Reaction 1) or hypochlorous acid (Reaction 2) and is capable of oxidizing halides (X^-) to their corresponding

*Corresponding author. Fax: (43)-1-360 06 60 59.

E-mail address: cobinger@edv2.boku.ac.at (C. Obinger).

Abbreviations: recMPO, recombinant unprocessed monomeric myeloperoxidase; MPO, mature dimeric myeloperoxidase; MPO-I, compound I; MPO-II, compound II; X^- , halide; HOX, hypohalous acid; CHO, Chinese hamster ovary

hypohalous acids, HOX (Reaction 3). Alternatively, compound I is converted in two one-electron steps via compound II to the ferric enzyme (Reactions 4 or 6 and 5) [7].



In this work bimolecular rate constants for Reactions 1–5 catalyzed by recMPO have been determined. Comparing these data with those already published for mature MPO purified from human blood, they unequivocally suggest that the redox intermediates of the two protein species are functionally indistinguishable and that the maturation process is a conservative phenomenon with respect to the active site structure.

2. Materials and methods

2.1. Materials

Recombinant myeloperoxidase was prepared in the Department of Applied Genetics of the Free University of Brussels, Belgium [5] to a purity of 99% and the purity index (A_{430}/A_{280}) is ~ 0.6 . Determination of its concentration and that of hydrogen peroxide was performed as reported previously [8]. Hydrogen peroxide and other chemicals were purchased from Sigma Chemical Co. at the highest grade available. Peroxide and HOCl stock solutions were prepared freshly half-daily. The HOCl stock solutions were prepared in 5 mM NaOH and stored in the dark. The HOCl concentration was determined spectrophotometrically shortly before the experiments ($\epsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$ at 292 nm in 5 mM NaOH) [9].

2.2. Methods

2.2.1. Transient-state kinetics. The sequential stopped-flow apparatus (model SX-18MV) and the associated computer system were from Applied Photophysics (UK). For a total of 100 μl /sample transported into a flow cell with 1 cm light path the fastest time for mixing two solutions and recording the first data point was about 1.3 ms. Conventional stopped-flow analysis was used to monitor the reaction of native ferric recMPO with hydrogen peroxide or hypochlorous acid [8,10] and to establish the conditions for the multi-mixing experiments.

Because of the inherent instability of recMPO compound I, sequential stopped-flow (multi-mixing) analysis was used for determination of rates of the reaction of compound I with one- and two-electron donors. Principally, the conditions were the same as described recently for the mature enzyme purified from human blood [8,10]. Reactivity of compound II was investigated according to Burner et al. [11] starting with pre-formed compound II or, alternatively, by following the reaction of compound I with ascorbate to compound II and back to the ferric enzyme. In the latter case the resulting biphasic curves showed the initial formation of compound II and then its subsequent reaction with ascorbic acid causing an exponential decrease in absorbance. The kinetic traces were fitted using the single-exponential equation of the Applied Photophysics software. At least three determinations (2000 data points) of pseudo-first-order rate constants (k_{obs}) were performed for each substrate concentration 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 that of the enzyme. All reactions were performed at 15°C and followed both at single wavelengths as well as with a diode-array detector

(PD1, Applied Photophysics). Typically, the heme concentration used in these experiments was 2 μM .

3. Results and discussion

3.1. Reaction of recMPO with hydrogen peroxide and hypochlorous acid

Recombinant myeloperoxidase exhibits spectral features similar to those of the mature enzyme with the Soret peak at 429 nm and a small absorption band at 570 nm (first spectrum in Fig. 1). The purity index (Reinheitszahl, A_{429}/A_{280}) was ~ 0.6 , which differs from that of highly purified mature MPO (~ 0.85) and represents the difference in heme to protein ratio in the unprocessed and processed species. Similar to the mature enzyme [10,12–14] addition of hydrogen peroxide led to the sequential formation of compound I and compound II. The corresponding spectral transitions are shown in Figs. 1A and 2A. RecMPO compound I is characterized by both peak broadening in the Soret region and a decrease in the extinction coefficient at 429 nm. A good spectrum of compound I was formed within 20 ms when 4 μM recMPO was mixed with 40 μM hydrogen peroxide in the conventional

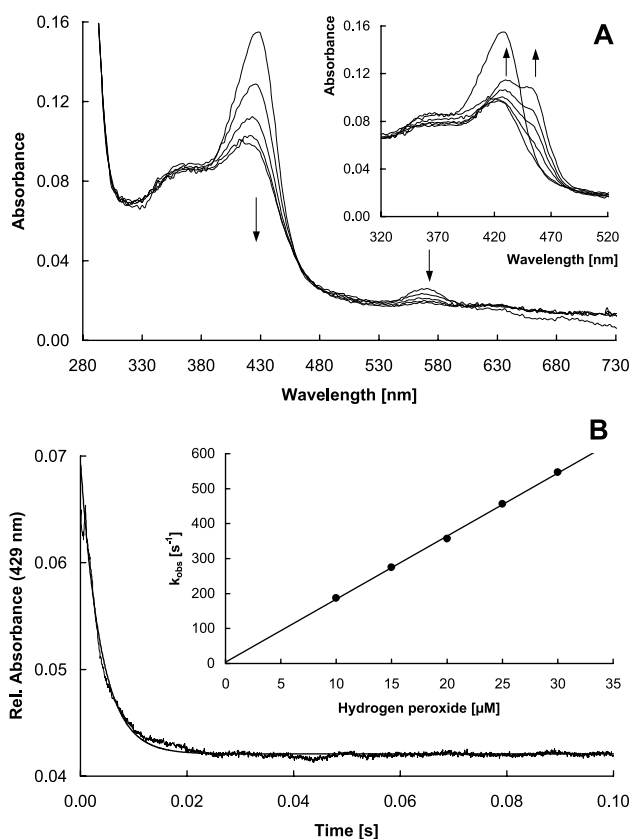


Fig. 1. The reaction of recombinant myeloperoxidase with hydrogen peroxide at pH 7. A: RecMPO (4 μM heme) was mixed with 40 μM hydrogen peroxide. The first spectrum shows the native enzyme, the first scan was taken 1.3 ms after mixing, subsequent spectra were taken at 3.8, 8.9 and 19 ms, respectively. The inset shows the native enzyme, compound I and subsequent spectra at 195, 500, 1000 and 5000 ms. The reaction was carried out in 100 mM phosphate buffer at 15°C. B: Time trace (429 nm) and single-exponential fit for the reaction of 2 μM recMPO with 30 μM H_2O_2 in 100 mM phosphate buffer at 15°C. The inset shows calculated pseudo-first-order rate constants plotted against hydrogen peroxide concentration.

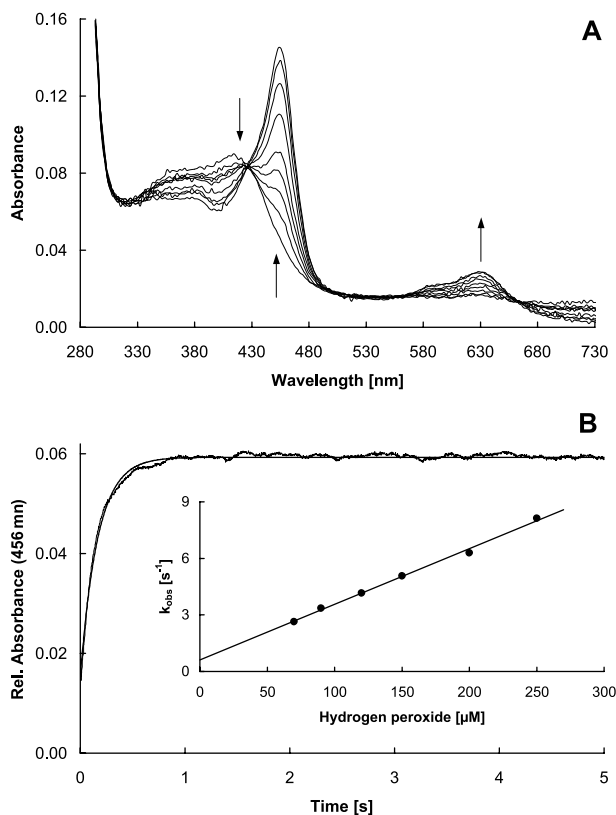


Fig. 2. The reaction of recombinant myeloperoxidase compound I with hydrogen peroxide at pH 7. A: Spectral changes upon addition of 400 μM hydrogen peroxide to recMPO (4 μM heme). The first spectrum was taken 3.8 ms after adding H_2O_2 , subsequent spectra were taken at 24, 44, 65, 90, 170, 303, 536 and 2900 ms, respectively. The reaction was carried out in 100 mM phosphate buffer, pH 7, and 15°C. B: Typical time trace (456 nm) and single-exponential fit for the reaction between 2 μM recMPO and 400 μM H_2O_2 in 100 mM phosphate buffer and 15°C. The inset shows calculated pseudo-first-order rate constants for this reaction plotted against peroxide concentration.

stopped-flow mode. Defined isosbestic points, derived from both normal data sets (Fig. 1A) and computer analysis using the Pro-K simulation program from Applied Photophysics (not shown), between ferric recMPO and compound I were determined to be at 456 nm and 605 nm. Similar to mature MPO, at least a 10-fold excess of hydrogen peroxide was necessary for maximum absorbance decrease at 429 nm. The reaction was monophasic and the pseudo-first-order rate constants, k_{obs} , could be easily obtained from the corresponding exponential time courses (Fig. 1B). In the inset of Fig. 1B these k_{obs} values are plotted against the H_2O_2 concentration and from the slope a rate constant of $(1.9 \pm 0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7 and 15°C was calculated. In contrast to mature MPO [14] the intercept of this plot was small ($(4.4 \pm 11.4) \text{ s}^{-1}$). It has been argued that the excess of hydrogen peroxide required for compound I formation of mature MPO (and also for recMPO) is necessary because Reaction 1 is reversible, meaning that compound I is able to oxidize water to hydrogen peroxide [14]. Assuming the intercept represents the reverse reaction (k_{-1}), the value for the equilibrium constant $K = k_{-1}/k_1$ is 0.23 μM , which is smaller than the value estimated with mature MPO [14].

RecMPO compound I is not stable. This is shown in the

inset of Fig. 1A for low and in Fig. 2A for high peroxide concentrations. In the first case both an absorbance increase at 429 and 456 nm, in the latter case a clear transition to compound II was observed with isosbestic points between compounds I and II at 426 and 661 nm (Fig. 2A). Compound II (last spectrum in Fig. 2A) exhibited a Soret band at 456 nm (with an extinction coefficient of about 95% of that at 429 nm) and a small absorbance at 632 nm. With ascorbate a similar transition was observed (not shown). Formation of compound II was monophasic (Fig. 2B) and strongly depended on H_2O_2 concentration (inset of Fig. 2B). The calculated rate constant was $(3.0 \pm 0.1) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7 and 15°C). The one-electron oxidation of hydrogen peroxide to superoxide radicals by compound I has been demonstrated in [13,14] and seems to be typical for MPO.

Myeloperoxidase also rapidly reacts with hypochlorous acid to form compound I (Reaction 2) [10]. It has been reported that compound I formation by hypochlorous acid offers some advantages when compound I is under investigation [10]. Generally, compound II formation mediated by H_2O_2 (Reaction 6) is absent and the observed absorbance changes mediated by halides are bigger. This was also the case with recMPO. With a 4-fold excess of HOCl, compound I was formed within 15 ms (not shown) and its reactivity with halides could easily be followed.

3.1.1. Reaction with ascorbate. Upon addition of ascorbate to recMPO compound I, compound II was formed immediately and finally was transformed to the protein in its resting state (not shown). The calculated bimolecular rate constants for compound I and compound II reduction are $(2.3 \pm 0.04) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $(5.0 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, respectively, which are very similar to those published for the mature enzyme [15]. Rates for compound II reduction were estimated by two ways, namely (i) starting with pre-formed compound II [11] or (ii) by following the reaction of compound I with ascorbate to compound II and back to the ferric enzyme. In this case a biphasic curve resulted and the second phase was fitted using the single-exponential equation. With both methods similar results were obtained within experimental errors (not shown). These findings indicate that both redox intermediates involved in the peroxidase cycle of recMPO are fully active with this endogenous one-electron donor.

3.1.2. Reaction with halides. In Fig. 3 both the spectral transition and a typical trace of the reaction of recMPO compound I with thiocyanate are shown. Compound I was

Table 1
Apparent second-order rate constants for the reactions of recMPO in comparison to mature MPO (pH 7, 15°C)

Substrate	Mature MPO ($\text{M}^{-1} \text{ s}^{-1}$)	recMPO ($\text{M}^{-1} \text{ s}^{-1}$)
MPO \rightarrow MPO-I		
hydrogen peroxide	1.4×10^7 [19]	1.9×10^7
MPO-I \rightarrow MPO-II		
hydrogen peroxide	4.4×10^4 [19]	3.0×10^4
ascorbate	1.1×10^6 [15]	2.3×10^5
MPO-II \rightarrow MPO		
ascorbate	1.1×10^4 [15]	5.0×10^3
MPO-I \rightarrow MPO		
chloride	6.6×10^4 [10]	3.6×10^4
bromide	2.6×10^6 [10]	1.4×10^6
iodide	1.8×10^7 [10]	1.4×10^7
thiocyanate	2.0×10^7 [10]	1.4×10^7

MPO, ferric resting state.

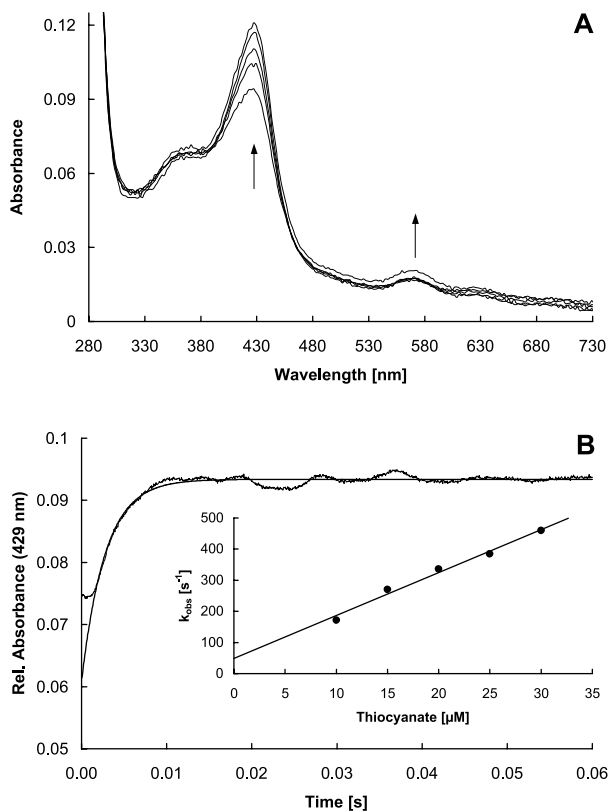


Fig. 3. The reaction of recombinant myeloperoxidase compound I with thiocyanate at pH 7. A: Spectral changes upon addition of 20 μM thiocyanate to compound I (3 μM) formed with HOCl. 6 μM recMPO was mixed with 18 μM HOCl and, after a delay time of 14 ms, 20 μM thiocyanate was added. The first spectrum was taken 1.3 ms after adding thiocyanate, subsequent spectra at 3.8, 6.4, 19 and 30 ms, respectively. The reaction was carried out in 100 mM phosphate buffer and 15°C. B: Typical time trace (429 nm) and single-exponential fit for the reaction between 2 μM compound I with 40 μM thiocyanate. The reaction was carried out in 100 mM phosphate buffer and 15°C. The inset shows pseudo-first-order rate constants of this reaction plotted against thiocyanate concentration.

formed by addition of HOCl to the protein in its resting state. From the plot an apparent bimolecular rate constant of $(1.4 \pm 0.11) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ was calculated. Similar plots were obtained for chloride, bromide and iodide ($(3.6 \pm 0.2) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $(1.4 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $(1.4 \pm 0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, respectively). As summarized in Table 1, both the hierarchy with these two-electron donors and the absolute values (within experimental errors) correspond to the data recently published for mature MPO [8,10].

3.1.3. Conclusion. Table 1 unequivocally demonstrates the functional identity of mature MPO and recMPO. It underlines that the maturation process does not alter the active site and that the MPO-specific features are already established at the monomeric stage of its biosynthesis. Several MPO-specific features cannot be found in the other members of the peroxidase superfamily II (enzymes from animals including MPO, eosinophil peroxidase, lactoperoxidase and thyroid peroxidase). All members of this superfamily share a heme group derived from protoporphyrin IX, although the coordinating

residues in the heme pocket differ. Animal peroxidases have covalent bonds between the heme group and the carboxylate groups of glutamate and aspartate residues that extend into the heme pocket. However, only MPO possesses a sulfonium linkage (M409) with the heme, which is made responsible for its unique spectral and functional properties [16,17]. It has been demonstrated that compound I of the homologous eosinophil peroxidase is not competent in oxidizing H_2O_2 to superoxide according to Reaction 6 and that it oxidizes chloride only very slowly [18]. In comparison, these reactions are catalyzed by recMPO and mature MPO in an identical manner, suggesting that heme binding and coordination to the protein are already established at this stage of biosynthesis and that the succeeding maturation process is a conservative phenomenon with respect to the active site structure.

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