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

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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. First, hydrogen peroxide mediated both oxidation of ferric recMPO to compound I ($1.9 \times 10^7$ M$^{-1}$ s$^{-1}$, pH 7 and 15°C) and reduction of compound I to compound II ($3.0 \times 10^9$ M$^{-1}$ s$^{-1}$, pH 7 and 15°C). With chloride, bromide, iodide and thiocyanate compound I was reduced back to the ferric enzyme ($3.6 \times 10^4$ M$^{-1}$ s$^{-1}$), whereas the endogenous one-electron donor ascorbate mediated transformation of compound I to compound II ($2.3 \times 10^5$ M$^{-1}$ s$^{-1}$) and of compound II back to the resting enzyme ($5.0 \times 10^4$ M$^{-1}$ 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 ((αβ)$_2$), 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 apopMPO [4]. Within the endoplasmic reticulum apopMPO interacts transiently and reversibly with molecular chaperones with subsequent heme incorporation and generation of the enzymatically active ~90 kDa proMPO. 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...
hypohalous acids, HOCl (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].

\[
\begin{align*}
\text{MPO} + H_2O_2 &\rightleftharpoons \text{MPO-I} + H_2O \\
\text{MPO} + HOCl &\rightarrow \text{MPO-I} + Cl^- + H^+ \\
\text{MPO-I} + X^- + H^+ &\rightarrow \text{MPO} + \text{HOX} \\
\text{MPO-I} + AH_2 &\rightarrow \text{MPO-II} + \text{AH} \\
\text{MPO-II} + AH_2 &\rightarrow \text{MPO} + \text{AH} + H_2O \\
\text{MPO-I} + H_2O_2 &\rightarrow \text{MPO-II} + HO_2^- 
\end{align*}
\]

(1) (2) (3) (4) (5) (6)

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 (\(A_{280}/A_{360}\)) is \(\sim 0.6\). 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 Photophysxics (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 hypohalous 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 (\(k_{\text{obs}}\)) 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 \(k_{\text{obs}}\) 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, \(A_{430}/A_{390}\)) was \(\sim 0.6\), which differs from that of highly purified mature MPO (\(\sim 0.85\)) 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 two 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 3000 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.](image-url)
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 taken at 456 nm and 605 nm. Similar to mature MPO, at least a 10-fold excess of hydrogen peroxide was determined to be at 456 nm and 605 nm. The reaction was monophasic and the pseudo-first-order rate constants for this reaction plotted against peroxide concentration.

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 transformed to the protein in its ferric resting state.

Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mature MPO (M$^{-1}$s$^{-1}$)</th>
<th>recMPO (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO$\rightarrow$MPO-I</td>
<td>1.4$\times$10$^7$ [19]</td>
<td>1.9$\times$10$^7$</td>
</tr>
<tr>
<td>hydrogen peroxide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPO-I$\rightarrow$MPO-II</td>
<td>4.4$\times$10$^4$ [19]</td>
<td>3.0$\times$10$^4$</td>
</tr>
<tr>
<td>hydrogen peroxide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ascorbate</td>
<td>1.1$\times$10$^6$ [15]</td>
<td>2.3$\times$10$^3$</td>
</tr>
<tr>
<td>MPO-II$\rightarrow$MPO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ascorbate</td>
<td>1.1$\times$10$^4$ [15]</td>
<td>5.0$\times$10$^3$</td>
</tr>
<tr>
<td>MPO-I$\rightarrow$MPO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chloride</td>
<td>6.6$\times$10$^4$ [10]</td>
<td>3.6$\times$10$^4$</td>
</tr>
<tr>
<td>bromide</td>
<td>2.6$\times$10$^4$ [10]</td>
<td>1.4$\times$10$^4$</td>
</tr>
<tr>
<td>iodide</td>
<td>1.8$\times$10$^4$ [10]</td>
<td>1.4$\times$10$^4$</td>
</tr>
<tr>
<td>thiocyanate</td>
<td>2.0$\times$10$^4$ [10]</td>
<td>1.4$\times$10$^4$</td>
</tr>
</tbody>
</table>

MPO, ferric resting state.
formed by addition of HOCl to the protein in its resting state. From the plot an apparent bimolecular rate constant of $(1.4 \pm 0.2) \times 10^{4} \text{M}^{-1} \text{s}^{-1}$ 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$_2$O$_2$ 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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References