FEBS Letters 443 (1999) 290–296 FEBS 21468

Kinetics of oxidation of aliphatic and aromatic thiols by myeloperoxidase compounds I and II

Ursula Burner, Walter Jantschko, Christian Obinger*

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

Received 13 November 1998; received in revised form 16 December 1998

Abstract **Myeloperoxidase (MPO) is the most abundant protein in neutrophils and plays a central role in microbial killing and inflammatory tissue damage. Because most of the non-steroidal anti-inflammatory drugs and other drugs contain a thiol group, it is necessary to understand how these substrates are oxidized by MPO. We have performed transient kinetic measurements to study the oxidation of 14 aliphatic and aromatic mono- and dithiols by the MPO intermediates, Compound I (k_3) and Compound II (k_4), using sequential mixing stopped-flow techniques. The one-electron reduction of Compound I by aromatic thiols (e.g. methimidazole, 2-mercaptopurine and 6-mercaptopurine) varied by less than a factor of seven (between $1.39\pm0.12\times10^5$ M^{-1} s^{-1} and $9.16\pm1.63\times10^5$ M^{-1} s^{-1}), whereas reduction by aliphatic thiols was demonstrated to depend on their overall net charge and hydrophobic character and not on the percentage of thiol deprotonation or redox potential. Cysteamine, cysteine methyl ester, cysteine ethyl ester and α-lipoic acid showed k_3 values comparable to aromatic thiols, whereas a free carboxy group (e.g. cysteine, *N*-acetylcysteine, glutathione) diminished k_3 dramatically. The one-electron reduction of Compound II was far more constrained by the nature of the substrate. Reduction by methimidazole, 2-mercaptopurine and 6-mercaptopurine showed second-order rate constants (k_4) of $1.33\pm0.08\times10^5$ M^{-1} s^{-1}, $5.25\pm0.07\times10^5$ M^{-1} s^{-1} and $3.03\pm0.07\times10^3$ M^{-1} s^{-1}. Even at high concentrations cysteine, penicillamine and glutathione could not reduce Compound II, whereas cysteamine ($4.27\pm0.05\times10^3$ M^{-1} s^{-1}), cysteine methyl ester ($8.14\pm0.08\times10^3$ M^{-1} s^{-1}), cysteine ethyl ester ($3.76\pm0.17\times10^3$ M^{-1} s^{-1}) and α-lipoic acid ($4.78\pm0.07\times10^4$ M^{-1} s^{-1}) were demonstrated to reduce Compound II and thus could be expected to be oxidized by MPO without co-substrates.**

Key words: Myeloperoxidase; Compound I; Compound II; Thiol oxidation; Thiyl radical; Transient-state kinetics

1. Introduction

Myeloperoxidase (MPO, EC 1.11.1.7) is a member of the homologous mammalian peroxidase family that also includes lactoperoxidase, eosinophil peroxidase, and thyroid peroxidase. MPO is the most abundant protein of neutrophils (polymorphonuclear leukocytes) [1]. It is stored in their azurophilic or primary granules and it is involved in killing invading pathogens [2]. These granulocytic cells ingest microorganisms into phagosomes where they kill them by generating an array of reactive oxidants including superoxide and hydrogen peroxide. MPO is unique amongst the mammalian peroxidases in its ability to utilize hydrogen peroxide in the oxidation of chloride to hypochlorous acid. The potent cytotoxic action of hypochlorous acid [3,4] and the demonstration that it is produced inside phagosomes [5,6] underline the role of MPO in killing invading pathogens. Hypochlorous acid is also likely to contribute to the tissue damage caused by neutrophils at sites of inflammation by inactivating enzymes, cross-linking proteins, oxidizing susceptible amino acids, and chlorinating lipids and tyrosyl residues [7]. MPO also catalyzes the formation of hypothiocyanite at physiological concentrations of thiocyanate and chloride [8,9], and readily oxidizes numerous phenols, anilines and β-diketones to free radicals [7]. In addition, it is capable of hydroxylating aromatic compounds [10].

With increasing frequency, MPO is being implicated in neutrophil-mediated tissue damage that occurs in inflammatory diseases such as rheumatoid arthritis, reperfusion injury, asthma, inflammatory bowel disease and cancer [11]. Interestingly, most of the non-steroidal anti-inflammatory drugs and other drugs contain a thiol group (e.g. D-penicillamine and tiopronin) and are widely used in the treatment of rheumatoid arthritis [12,13] and related diseases. *N*-Acetylcysteine, mercaptopropionylglycine and cysteamine were shown to act as scavengers of hypochlorite in vivo [14–16], whereas the antiarthritic drug penicillamine and the antithyroid drug propylthiouracil were shown also to act as inhibitors of MPO [17,18]. But their mode of action still remains uncertain. Moreover, thiols are known to function as substrates for peroxidases [19–31]. Eqs. 1 and 3 and Eq. 4 summarize the conventional peroxidase cycle with thiols (RSH) and/or thiolates (RS^-) as potential electron donors of the MPO intermediates Compounds I and II:

$$PrxPHFe^{III} + H_2O_2 \xrightarrow{k_1} PrxPH^{\bullet+}Fe^{IV}=O + H_2O \quad (1)$$

$$PrxPH^{\bullet+}Fe^{IV}=O + X^- + H^+ \xrightarrow{k_2} PrxPHFe^{III} + HOX \quad (2)$$

$$PrxPH^{\bullet+}Fe^{IV}=O + RSH(RS^-) \xrightarrow{k_3} PrxPHFe^{IV}=O + RS^{\bullet} \quad (3)$$

$$PrxPHFe^{IV}=O + RSH(RS^-) \xrightarrow{k_4} PrxPHFe^{III} + RS^{\bullet} + H_2O \quad (4)$$

The cycle starts with the reaction of MPO with hydrogen peroxide (H_2O_2) producing a two-electron oxidized species known as Compound I in which the ferric iron (Fe^{III}) is oxidized to a ferryl species ($Fe^{IV}=O$) and the porphyrin (PH) to a porphyrin radical cation ($PrxPH^{\bullet+}$) (Eq. 1) [32]. This redox intermediate oxidizes chloride and thiocyanate via a single

*Corresponding author. Fax: (43) (1) 36006-6059.
E-mail: cobinger@edv2.boku.ac.at

PII: S0014-5793(98)01727-X

two-electron reaction to produce the respective hypohalous acids and regenerate the native enzyme (Eq. 2). Alternatively, stepwise reduction of Compound I by two thiol-derived electrons produces Compound II, in which the porphyrin radical cation has been quenched ($PrxPHFe^{IV}=O$), and subsequently the resting ferric state ($PrxPHFe^{III}$). The formation of thiyl radicals has been demonstrated by electron spin resonance spectroscopy for thiol oxidation by horseradish peroxidase (HRP) [33,34], lactoperoxidase [35] and MPO [21].

Thiyl radicals show a very complex behavior in aqueous solutions undergoing conjugative reactions [30,31]. These non-enzymatic free radical reactions have the consequence that thiol oxidation is accompanied by oxygen consumption, superoxide formation and regeneration of hydrogen peroxide [30,31]. Characterization of the overall reaction makes it necessary to differentiate between the enzymatic (Eqs. 1 and 3 and Eq. 4) and the non-enzymatic free radical reactions, because the fate of thiyl radicals is strongly influenced by the kinetics of reactions that produce and remove them.

To fully appreciate the mode of action of thiol containing (anti-inflammatory) drugs it will be necessary to understand how these substrates are oxidized by MPO. In this investigation we have carried out transient-state kinetic studies to elucidate relationships between the structure of thiols and their abilities to react with MPO. For the first time a comprehensive sequential-stopped-flow investigation is presented comparing 14 aliphatic and aromatic mono- and dithiols for their ability to function as electron donors for Compound I (k_3) and Compound II (k_4) of human MPO. Moreover, the pH dependence of both k_3 and k_4 is presented.

2. Materials and methods

2.1. Materials

MPO was purified from human neutrophils to a purity index (A_{430}/A_{280}) of at least 0.86 as described by Kettle and Winterbourn [36]. Its concentration was calculated using $\varepsilon_{430}=91\,000\ M^{-1}\ cm^{-1}$ per heme [37] and was given in mol per heme per liter. Hydrogen peroxide, obtained from a 30% solution from Sigma Chemical Co., was diluted and the concentration determined by absorbance measurement at 240 nm where the extinction coefficient is $39.4\ M^{-1}\ cm^{-1}$ [38]. The other chemicals (L-cysteine, L-cysteamine, DL-penicillamine, DL-homocysteine, *N*-acetyl-L-cysteine, L-cysteine methyl ester, L-cysteine ethyl ester, glutathione, DL-α-lipoic acid, 2,3-dimercaptopropanol, 4-thiouridine, 2-mercaptopurine, 6-mercaptopurine, methimidazole and homovanillic acid) were also purchased from Sigma Chemical Co. at the highest grade available. All other chemicals were of analytical grade.

Thiol solutions were always prepared fresh and bubbled with nitrogen before use. All reactions were performed in buffers containing 100 μM of the metal ion chelator diethylene-triaminepentaacetic acid (DTPA) which is known to prevent thiol autoxidation [39]. For control, actual aliphatic thiol concentrations were determined according to [31], but this method has been established to be not applicable to aromatic thiols.

2.2. Methods

Sequential stopped-flow measurements were performed with an Applied Photophysics (UK) instrument (model SX-18MV). 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 approximately 1.5 ms. Multi-mixing analysis was used for kinetic measurements of both the conversion of Compound I to Compound II and the conversion of Compound II to the native enzyme. Compound I is inherently unstable [40]. With 0.5 μM MPO, 5 μM was the minimum hydrogen peroxide concentration required for complete formation of Compound I (characterized by a 50% hypochromicity in the Soret band at 430 nm). Under these conditions, Compound I was completely formed within 20 ms and was stable for at least a further 30 ms. In a typical experiment, 2 μM ferric MPO was premixed in the ageing loop with 20 μM hydrogen peroxide for 25 ms. Compound I was then allowed to react with varying concentrations of thiols. Reactions were carried out in 100 mM sodium/potassium phosphate buffer (pH 7.0) containing 100 μM DTPA and monitored at 456 nm. To ensure first-order kinetics, final thiol concentrations were at least 10 times that of the enzyme. At least three determinations of k_{obs}, the pseudo-first-order rate constant, were performed for each substrate concentration. Second-order rate constants were calculated from the slope of the plot of the mean k_{obs} values versus substrate concentration.

Reduction of Compound II by the thiols was measured as described in [41]. For its determination, 2 μM ferric MPO was premixed with 20 μM hydrogen peroxide and 1.8 μM homovanillic acid (HVA) in the ageing loop. Under these conditions, Compound II was stable for at least 100 s. 40 s after the initial mixing, Compound II was allowed to react with a substrate. To ensure first-order kinetics, the final concentrations of the thiols were at least 10 times that of the enzyme. Reactions were carried out in 100 mM sodium/potassium phosphate buffer (pH 7.0) containing 100 μM DTPA and were monitored by recording the loss in absorbance at 456 nm. At least three determinations of the pseudo-first-order rate constants, k_{obs}, were performed for each substrate concentration and the mean value was used to calculate k_4 as described above.

To determine the pH dependence of the reduction of Compound I, the sequential stopped-flow experiments were performed at different pH values from 4.0 to 9.0. In a typical pH jump experiment 2 μM

Table 1
Summary of bimolecular rate constants for reactions of myeloperoxidase Compound I (k_3) and Compound II (k_4) with aliphatic and aromatic thiols at pH 7.0

Thiol	pK_{a,NH_3^+}	$pK_{a,SH}$	% [RS^-] at pH 7.0[a]	Net charge at pH 7.0	k_3 [$M^{-1}\ s^{-1}$]	k_4 [$M^{-1}\ s^{-1}$]
Cysteine	10.8	8.3	4.8	−0.05	4.10 ± 0.10 E+3	< 1 E+1
Cysteamine	10.8	8.6	2.5	+0.98	1.65 ± 0.04 E+5	4.27 ± 0.05 E+3
Penicillamine	10.4	7.9	11.2	−0.11	2.64 ± 0.18 E+3	< 1 E0
N-Acetylcysteine	–	9.5	0.3	−1.00	4.56 ± 0.04 E+2	4.76 ± 0.26 E+2
Homocysteine	10.9	8.9	1.2	−0.01	1.38 ± 0.11 E+3	6.00 ± 0.32 E+2
Cysteine methyl ester	9.0	6.6	71.5	+0.27	2.49 ± 0.04 E+5	8.14 ± 0.08 E+3
Cysteine ethyl ester	9.2	6.7	66.6	+0.33	2.15 ± 0.04 E+5	3.76 ± 0.17 E+3
Glutathione	9.7	8.8	1.6	−1.02	7.21 ± 0.40 E+1	< 1 E0
Methimidazole	–	–	–	–	9.16 ± 1.63 E+5	1.33 ± 0.08 E+5
4-Thiouridine	–	–	–	–	6.79 ± 0.22 E+3	4.05 ± 0.10 E+2
2-Mercaptopurine	–	–	–	–	9.02 ± 1.26 E+5	5.25 ± 0.07 E+5
6-Mercaptopurine	–	7.8	13.7	−0.14	1.39 ± 0.13 E+5	3.03 ± 0.07 E+3
2,3-Dimercaptopropanol	–	–	–	–	–	5.77 ± 0.04 E+5
α-Lipoic acid	–	10.7	0.0	−1.00	7.75 ± 0.24 E+4	4.78 ± 0.07 E+4

For details concerning sequential-stopped-flow spectroscopy see Section 2. Data for pK_{a,NH_3^+} and $pK_{a,SH}$ are taken from [43]. Calculation of percentage thiolate at pH 7.0 is indicated.
[a]The fraction of RSH in the thiolate form (RS^-) has been calculated by $[RS^-]/([RS^-]+[RSH])=[1+10^{(pK-pH)}]^{-1}$.

MPO in 5 mM sodium/potassium phosphate buffer (pH 7.0) was premixed with 20 μM H_2O_2 in distilled water. After a delay time of 25 ms, Compound I was allowed to react with varying concentrations of thiols in 200 mM phosphate/citrate buffer (pH 4–7) or HEPES/Tris (pH 7–9), both containing 200 μM DTPA. For reduction of Compound II, 2 μM MPO in 5 mM phosphate buffer (pH 7.0) was premixed with 20 μM H_2O_2 in distilled water containing 1.8 μM HVA. After a delay time of 40 s, Compound II was allowed to react with varying concentrations of thiols in 200 mM phosphate/citrate buffer (pH 4–7) or HEPES/NaOH (pH 7–9), both containing 200 μM DTPA. All stopped-flow investigations were performed at 15°C.

Rapid spectral scans of MPO and its intermediates during reaction were conducted by taking time-dependent spectra from single-wavelength shots. The spectra generated from the rapid scans were reconstructed using the GLint application software from Applied Photophysics.

3. Results and discussion

In the present paper, structure-activity relationships of 14 thiols as MPO substrates have been investigated. In previous studies [21–24,26,28,29] only indirect methods (i.e. disulfide formation, ESR techniques and superoxide radical formation) were employed to measure thiol oxidation. But they are problematic in interpreting structure-activity relationships, because during thiol oxidation oxygen activation by thiyl radical reactions takes place leading to the formation of superoxide and hydrogen peroxide [30,31] which themselves are involved in both the enzyme activity (i.e. Compound I formation) and inactivation [7]. Therefore, it is necessary to know the actual rates of thiyl radical formation which influence both the nature and rate of the thiyl radical reactions [30]. Here we present for the first time actual bimolecular rates of the reaction between thiols and both higher oxidation states of MPO, namely Compound I (k_3) and Compound II (k_4).

In contrast to HRP [42], Compound I of human MPO is very unstable and spontaneously decays to its one-electron reduction product, Compound II. Fig. 1A shows the spectra of the relevant enzyme intermediates formed upon addition of a 10-fold excess of hydrogen peroxide to the native enzyme. The spectra were generated from a sequence of kinetic records collected at a series of monochromator wavelengths between 400 and 480 nm at 2 nm intervals. In Fig. 1A the points in time at which the slices across these records were taken are at 0 ms (spectrum 1, representing native enzyme), 20 ms (spectrum 2, Compound I) and 20 s (spectrum 3, Compound II) after the flow had stopped.

With our MPO preparations ($RZ > 0.86$) a minimum of 10-fold excess of hydrogen peroxide was necessary for complete formation of Compound I. A larger excess brought a stronger interference with Compound I reduction by H_2O_2, which is known to function as electron donor for Compound I [40]. The conversion of native MPO to Compound I displaying a monophasic exponential character was completed within 20 ms (Fig. 1B). Before its decay it was mixed (arrow in Fig. 1B) with varying concentrations of thiols.

A typical kinetic time trace for Compound I reduction by cysteamine is shown in the inset of Fig. 2A. It displays a single-exponential character. The corresponding pseudo-first-order rate constants were obtained from these traces and plotted against the concentration of cysteamine. From the slope of this secondary plot the apparent second-order rate constant (k_3) was calculated to be $1.65 \pm 0.04 \times 10^5$ M^{-1} s^{-1} at pH 7.0 and 15°C. The corresponding k_3 values of the other thiols

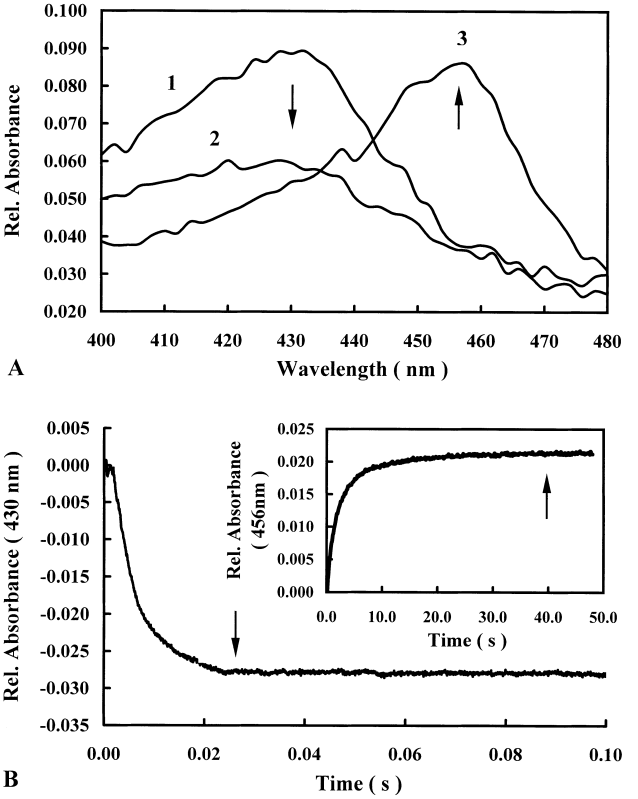


Fig. 1. A: Rapid-scan spectra of native myeloperoxidase (1) and after adding 10-fold excess H_2O_2. The spectra were taken (2) 20 ms and (3) 20 s after the flow had stopped. Arrows show the direction of absorbance changes with time. Final enzyme concentration: 1 μM in 100 mM phosphate buffer, pH 7.0. B: Typical time traces of this reaction followed at 430 nm (Compound I formation). The inset shows Compound II formation at 456 nm upon adding 10-fold H_2O_2 and substoichiometric homovanillic acid to native MPO. The arrows indicate the delay time used in the sequential mixing stopped-flow experiments.

investigated are summarized in Table 1 and demonstrate the effect of thiol structure on Compound I reduction.

On insertion of a carboxy group into cysteamine (cysteine), k_3 decreased dramatically ($4.10 \pm 0.10 \times 10^3$ M^{-1} s^{-1}). The cysteine homologue, homocysteine, showed 34% of the activity of cysteine, whereas insertion of methyl groups at the carbon atom close to the mercapto group in cysteine (penicillamine) led to a 36% decrease of k_3 compared with cysteine. *N*-Acetylation of cysteine further decreased k_3 ($4.56 \pm 0.04 \times 10^2$ M^{-1} s^{-1}) and cysteine as part of the tripeptide glutathione proved to be an extremely poor electron donor for Compound I. However, upon esterification of cysteine a dramatic increase of k_3 was observed. The reaction of Compound I with cysteine methyl ester and cysteine ethyl ester was even faster than with cysteamine, namely 51% and 30%, respectively. We have also investigated the electron donor capacity of two aliphatic dithiols. Compared with cysteine, α-lipoic was shown to react 19 times faster. Interestingly, reduction of Compound I by 2,3-dimercaptopropanol was biphasic and in contrast to its reaction with Compound II, which displayed a monophasic behavior, extremely slow. Since we do not understand this behavior at the moment, we have not included k_3 for 2,3-dimercaptopropanol in Table 1.

Aromatic thiols proved to be good electron donors for Compound I. With the exception of thiouridine, the magni-

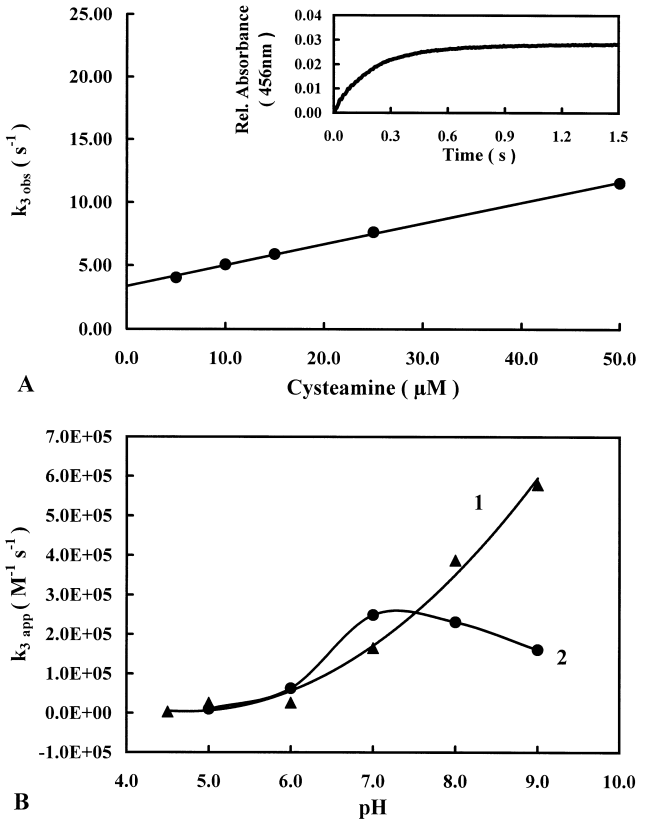


Fig. 2. A: Pseudo-first-order rate constants for Compound I reduction by cysteamine. The inset shows a typical time trace followed at 456 nm using sequential mixing mode. Final concentrations were 0.5 μM MPO, 5 μM H_2O_2 and 10 μM cysteamine in 100 mM phosphate buffer (pH 7.0) containing 100 μM DTPA at 15°C. B: The pH profile for reduction of Compound I by cysteamine (1) and cysteine methyl ester (2). Conditions were as described in A except that the buffers used were phosphate/citrate buffer (pH 4–7) or HEPES/Tris (pH 7–9), both containing 100 μM DTPA.

tudes of the corresponding k_3 values varied by less than a factor of seven (1.4–9.2×10^5 M^{-1} s^{-1}) and are comparable with tyrosine as electron donor [44].

There were finite intercepts for the secondary plots of k_{obs} for Compound I reduction versus the concentration of thiols (Fig. 2A). Their magnitudes were dependent on the nature of the substrate (not shown) and indicated the existence of reactions which also contribute to absorbance changes at 456 nm, i.e. both the spontaneous reduction of Compound I by excess hydrogen peroxide and/or the reduction of Compound II by a second substrate molecule. With the exception of methimidazole, the intercepts of aromatic thiols varied between 1.6 and 4.8 s^{-1} (not shown) which is in good agreement with those determined previously for aromatic electron donors [41,44]. Methimidazole showed a relatively high intercept of about 30 s^{-1}. It is known to be an irreversible, mechanism-based inhibitor of thyroid peroxidase and lactoperoxidase [45]. Since MPO is a better catalyst than thyroid peroxidase and lactoperoxidase with most of the substrates investigated so far [25,44], it is very likely that a reaction, which was part of the MPO inactivation mechanism, was responsible for this high intercept value. With aliphatic thiols the intercept values varied between 0.8 and 3.4 s^{-1} (not shown).

Formation of Compound II and measuring of its reduction is problematic. When Compound II formation occurs by excess hydrogen peroxide [40], the enzyme is likely to cycle when the reducing substrate is added. Recently, we have described a method for measuring Compound II reduction under pre-steady-state conditions [41]. We premixed a 10-fold excess of hydrogen peroxide and sub-stoichiometric concentrations of homovanillic acid to generate Compound II (inset of Fig. 1B), and then followed its reactions by adding the thiol after a delay time of 40 s (arrow in the inset of Fig. 1B). With each thiol, the loss of absorbance at 456 nm displayed single exponential character. A typical time trace for the reaction of cysteamine and methimidazole with Compound II is shown in the insets of Figs. 3A and 4A, respectively. The apparent second-order rate constants for the reduction of Compound II (k_4) were obtained from secondary plots (Figs. 3A and 4A) and were calculated for cysteamine and methimidazole to be 4.27±0.05×10^3 M^{-1} s^{-1} and 1.33±0.08×10^5 M^{-1} s^{-1}, respectively, at pH 7.0 and 15°C. Reduction of Compound II by thiols is far more constrained by the nature of the thiols than Compound I reduction. Even at high thiol concentrations, cysteine, penicillamine and glutathione were incapable of reducing Compound II. *N*-Acetylcysteine and homocysteine reacted poorly with Compound II, but cysteamine, cysteine methyl ester (8.14±0.08×10^3 M^{-1} s^{-1}) and cysteine ethyl

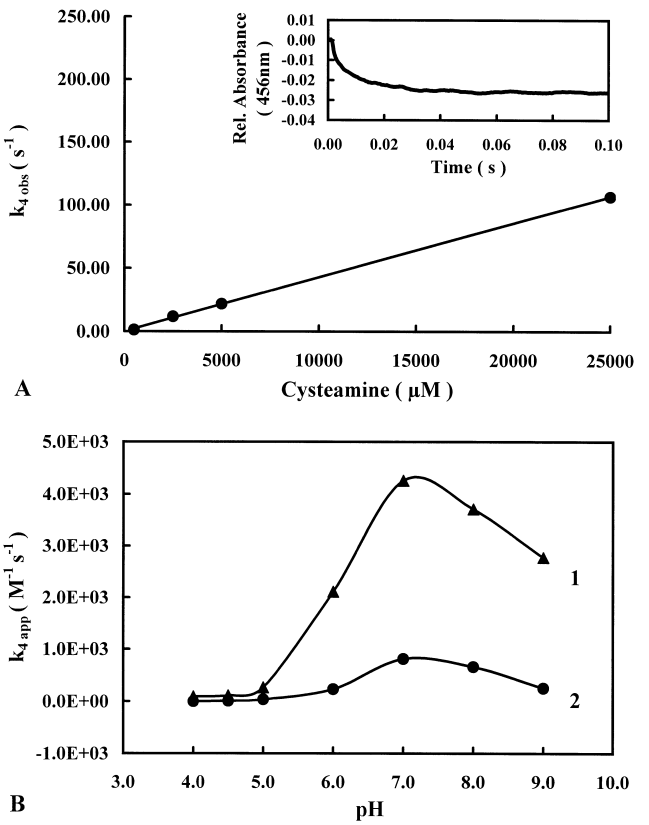
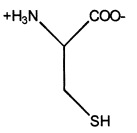
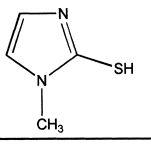
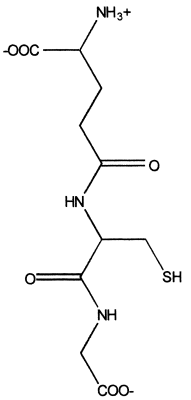
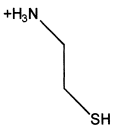
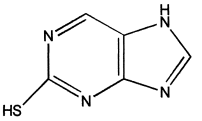
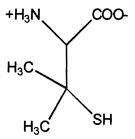
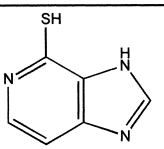
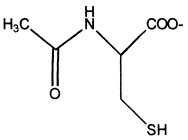
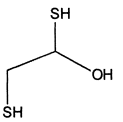
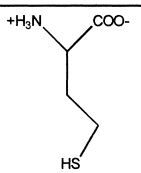
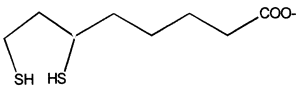
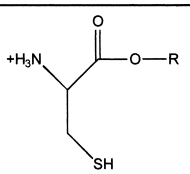
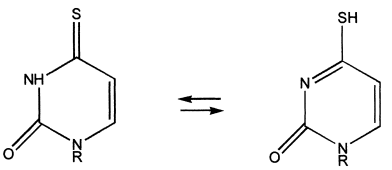


Fig. 3. A: Pseudo-first-order rate constants for Compound II reduction by cysteamine. The inset shows a typical time trace followed at 456 nm using sequential mixing mode. Final concentrations were 0.5 μM MPO, 5 μM H_2O_2, 0.45 μM HVA and 25 mM cysteamine in 100 mM phosphate buffer (pH 7.0) containing 100 μM DTPA at 15°C. B: The pH profile for reduction of Compound II by cysteamine (1) and cysteine methyl ester (2). Conditions were as described in A except that the buffers used were phosphate/citrate buffer (pH 4–7) or HEPES/Tris (pH 7–9), both containing 100 μM DTPA.

Table 2
Schematic formulas of the thiol compounds

Cysteine 	Methimidazole 	Glutathione 
Cysteamine 	2-Mercaptopurine 	
Penicillamine 	6-Mercaptopurine 	
N-Acetylcysteine 	2,3-Dimercaptopropanol 	
Homocysteine 	α-Lipoic acid 	
Cysteine ester 	4-Thiouridine 	

ester ($3.76 \pm 0.17 \times 10^3$ M^{-1} s^{-1}) reacted at acceptable rates and could be expected to be oxidized by the enzyme. This is consistent with the findings of Svensson and coworkers [25,28] who investigated the ability of MPO to oxidize aliphatic aminothiols in the absence of hydrogen peroxide using steady-state spectroscopy and polarographic measurements of oxygen consumption. They found that only cysteamine and cysteine esters could be oxidized by MPO and consequently, by the reactions of thiyl radicals, generate hydrogen peroxide in order to maintain the peroxidatic cycle. Interestingly, both aliphatic dithiols proved to be very efficient in reduction of Compound II. Their corresponding k_4 values were of the same order of magnitude as those calculated for the aromatic thiols and for tyrosine [44].

Our study further revealed, that at physiological pH MPO is an active catalyst of thiol oxidation. Fig. 2B shows the pH-dependence of the apparent second-order rate constants for the reaction of Compound I with cysteamine and cysteine methyl ester. Reaction of Compound I with cysteine methyl ester was favored between pH 7 and 7.5, whereas, in the pH range investigated, the corresponding k_3 values for cysteamine increased with increasing pH. Considering the different $pK_{a,SH}$ values of cysteamine and cysteine methyl ester (i.e. 8.6, and 6.6, respectively) [43], a possible explanation for the observed differences in pH dependence between these thiols could not be that the thiolate anion form is the actual substrate for MPO. This is underlined by the similar pH profiles of Compound II reduction by cysteamine and cysteine methyl ester showing a maximum of activity between pH 7 and 7.5 (Fig. 3B). On the contrary, with methimidazole k_4 slightly increased by decreasing pH (Fig. 4B).

In conclusion, both Compound I and Compound II of MPO are able to react with aliphatic and aromatic thiols via one-electron oxidations and therefore generate thiyl radicals. Compared with phenols (e.g. tyrosine [44]) aromatic thiols were shown to have similar electron donor capacities, whereas the structural requirements for aliphatic thiols to function as electron donors for MPO are more restricted. Compared with the reaction rate of Compound I reduction by chloride [9], the major physiological substrate of MPO, aromatic thiols, cysteamine, both cysteine esters and α-lipoic acid proved to be better electron donors than the halide. The differences in the redox potentials between the thiols are too low to be used to answer the questions concerning the differing reactivity of aliphatic thiols [46] and our experiments about the pH dependence of k_3 and k_4 demonstrated unequiv-

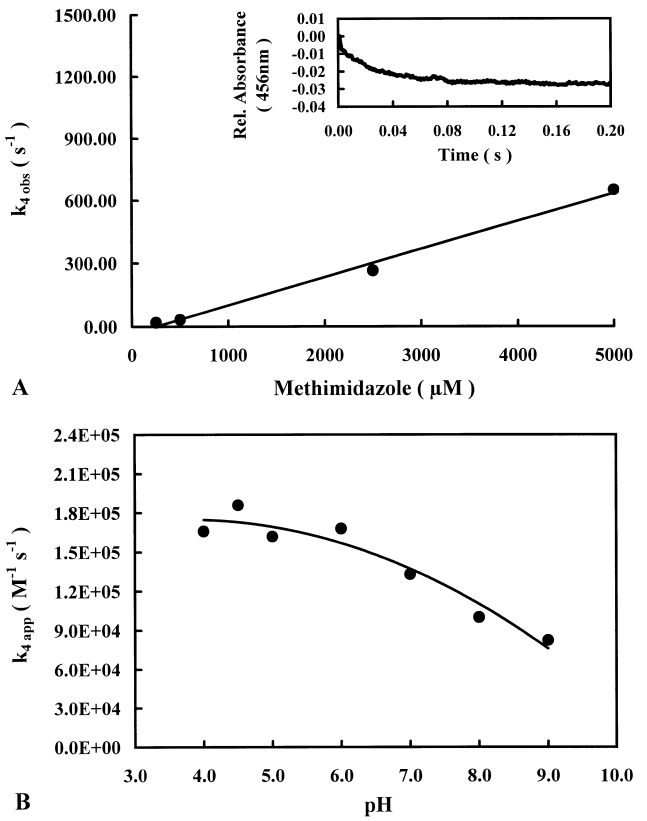


Fig. 4. A: Pseudo-first-order rate constants for Compound II reduction by methimidazole. The inset shows a typical time trace followed at 456 nm using sequential mixing mode. Final concentrations were 0.5 μM MPO, 5 μM H_2O_2, 0.45 μM HVA and 500 μM methimidazole in 100 mM phosphate buffer (pH 7.0) containing 100 μM DTPA at 15°C. B: The pH profile for reduction of Compound II by methimidazole. Conditions were as described in A except that the buffers used were phosphate/citrate buffer (pH 4–7) or HEPES/Tris (pH 7–9), both containing 100 μM DTPA.

ocally that the portion of thiolate at a defined pH did not have an impact on reactivity. Nevertheless, the overall net charges of the thiols are likely to be more important for reaction (see Table 2) than the actual size of the substrate. This was best demonstrated by appreciating the actual rates when going from cysteamine to cysteine and finally cysteine methyl ester. These structure-activity relationships of the aliphatic thiols suggest the existence of a negatively charged group (e.g. carboxy group) either in the substrate channel or at the active site of MPO, which hampers the reaction with substrates containing a negatively net charge (cysteine, homocysteine, *N*-acetylcysteine, penicillamine and glutathione). Increasing the hydrophobic character of the potential electron donor diminished these constraints (e.g. α-lipoic acid), which goes well together with the known overall hydrophobic character of the entrance to the distal cavity of MPO which favors interaction with aromatic molecules [47].

While all hemoprotein peroxidases are generally considered to catalyze oxidation reactions in a similar manner, a comparison of the rate constants for thiol oxidation between MPO and HRP [31] reflects differences in the effectiveness of reactivity. Compared with MPO Compound I, HRP Compound I is a less effective catalyst for thiol oxidation. This is consistent with the high oxidation potential of MPO Compound I [32] which enables the enzyme even to oxidize chloride. The bimolecular rate constants for the reaction of MPO Compound I are 1–2 orders of magnitude higher than the corresponding reactions catalyzed by HRP Compound I [31]. Thus MPO Compound I should be able to oxidize a wide range of organic substrates. In contrast, MPO Compound II will be far more constrained by the nature of the aliphatic thiols, when compared with the corresponding rates published for HRP [9]. With both peroxidases, cysteine, penicillamine and glutathione were unable to reduce Compound II. But in contrast to the corresponding reactions with HRP Compound II, the reactivity of cysteamine, 2,3-dimercaptopropanol, α-lipoic acid proved to be about 100 times higher, which was also the case when aromatic thiols functioned as electron donors of MPO Compound II.

Our results could help to understand the mechanism of action of thiol containing drugs used in the treatment of diseases where MPO is thought to be involved. Anti-inflammatory drugs of aromatic character are likely to be oxidized by the enzyme, whereas the oxidation of aliphatic drugs depends on structural requirements which have been demonstrated in the present work. Drugs that react poorly with Compound II (i.e. penicillamine) would not be expected to be oxidized by the enzyme. However, in the presence of suitable co-substrates that readily reduce Compound II, such as tyrosine or ascorbate [44,48], the high oxidation potential of Compound I could be exploited to catalyze the oxidation of even these thiols.

Acknowledgements: This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung (Projekt P10751-GEN).

References

[1] Klebanoff, S.J. (1988) in: Inflammation: Basic Principles and Clinical Correlates (Gallin, J.I., Goldstein, I.M. and Snyderman, R., Eds.), pp. 391–443, Raven Press, New York.
[2] Hampton, M.B., Kettle, A.J. and Winterbourn, C.C. (1996) Infect. Immun. 64, 3512–3517.
[3] Klebanoff, S.J. (1991) in: Peroxidases in Chemistry and Biology (Everse, J., Everse, K.E. and Grisham, M.B., Eds.), pp. 1–35, CRC Press, Boca Raton, FL.
[4] Hurst, J.K. (1991) Eur. J. Biochem. 202, 1275–1282.
[5] Hazen, S.L., Hsu, F.F., Mueller, D.M., Crowley, J.R. and Heinecke, J.W. (1996) J. Clin. Invest. 98, 1238–1289.
[6] Jiang, Q. and Hurst, J.K. (1997) J. Biol. Chem. 272, 32767–32772.
[7] Kettle, A.J. and Winterbourn, C.C. (1997) Redox Rep. 3, 3–15.
[8] van Dalen, C.J., Whitehouse, M.W., Winterbourn, C.C. and Kettle, A.J. (1997) Biochem. J. 327, 487–492.
[9] Furtmüller, P.G., Burner, U. and Obinger, C. (1998) Biochemistry (in press).
[10] Kettle, A.J. and Winterbourn, C.C. (1994) J. Biol. Chem. 269, 17146–17151.
[11] Weiss, S.J. (1989) New Engl. J. Med. 320, 365–376.
[12] Bonta, I.L., Parnham, M.J., Vincent, J.E. and Bragt, P.C. (1980) in: Progress in Medicinal Chemistry Vol. 17 (Ellis, G.P. and West, G.B., Eds.), pp. 185–273, Elsevier/North-Holland, Amsterdam.
[13] Pasero, G. and Ciompi, M.L. (1979) Arthritis Rheum. 22, 803–804.
[14] Aruoma, O.I., Halliwell, B., Hoey, B.M. and Butler, J. (1989) Free Radical Biol. Med. 6, 593–597.
[15] Puppo, A., Cecchini, R., Aruoma, O.I., Bolli, R. and Halliwell, B. (1990) Free Radical Res. Commun. 10, 371–381.
[16] Aruoma, O.I., Halliwell, B., Hoey, B.M. and Butler, J. (1988) Biochem. J. 256, 251–255.

[17] Cuperus, R.A., Muijsers, A.O. and Wever, R. (1985) Arthritis Rheum. 28, 1228–1233.
[18] Lee, E., Miki, Y., Katsura, H. and Kariya, K. (1990) Biochem. Pharmacol. 39, 1467–1471.
[19] Randall, L.O. (1946) J. Biol. Chem. 164, 521–526.
[20] Olsen, J. and Davis, L. (1976) Biochim. Biophys. Acta 445, 324–329.
[21] Cuperus, R.A., Hoogland, H., Wever, R. and Muijsers, A.O. (1987) Biochim. Biophys. Acta 912, 124–131.
[22] Svensson, B.E. and Lindvall, S. (1988) Biochem. J. 249, 521–530.
[23] Svensson, B.E. (1988) Biochem. J. 253, 441–449.
[24] Svensson, B.E. (1988) Biochem. J. 256, 751–755.
[25] Svensson, B.E. (1988) Biochem. J. 256, 757–762.
[26] Osswald, W.F., Schütz, W. and Elstner, E.F. (1989) Free. Radical Res. Commun. 5, 259–265.
[27] Marquez, L.A. and Dunford, H.B. (1990) Biochem. Biophys. Res. Commun. 169, 1158–1163.
[28] Svensson, B.E., Gräslund, A., Ström, G. and Moldeus, P. (1993) Free Radical Biol. Med. 14, 167–175.
[29] Lindvall, S. and Rydell, G. (1995) Chem.-Biol. Interact. 97, 53–62.
[30] Obinger, C., Burner, U., Vötsch, B., Hofstetter, W. and Ebermann, R. (1996) in: Plant Peroxidases: Biochemistry and Physiology (Obinger, C., Burner, U., Ebermann, R., Penel, C. and Greppin, H., Eds.), pp. 106–112, University of Geneva, Geneva.
[31] Burner, U. and Obinger, C. (1997) FEBS Lett. 411, 269–274.
[32] Hurst, J.K. (1991) in: Peroxidases in Chemistry and Biology (Everse, J., Everse, K.E. and Grisham, M.B., Eds.), pp. 37–62, CRC Press, Boca Raton, FL.
[33] Harman, L.S., Mottley, C. and Mason, R.P. (1984) J. Biol. Chem. 259, 5606–5611.
[34] Harman, L.S., Carver, D.K., Schreiber, J. and Mason, R.P. (1986) J. Biol. Chem. 261, 1642–1648.
[35] Mottley, C., Mason, R.P., Chignell, C.F., Sivarajah, K. and Elling, T.E. (1982) J. Biol. Chem. 257, 5050–5055.
[36] Kettle, A.J. and Winterbourn, C.C. (1988) Biochem. J. 252, 529–536.
[37] Odajima, T. and Yamazaki, I. (1970) Biochim. Biophys. Acta 206, 71–77.
[38] Nelson, D.P. and Kiesow, L.A. (1972) Anal. Biochem. 49, 474–478.
[39] Misra, H.P. (1974) J. Biol. Chem. 249, 2151–2155.
[40] Marquez, L.A., Huang, J.T. and Dunford, H.B. (1994) Biochemistry 33, 1447–1454.
[41] Burner, U., Obinger, C., Paumann, M., Furtmüller, P.G. and Kettle, A.J. (1998) J. Biol. Chem. (in press).
[42] Dunford, H.B. and Nadezhdin, A. (1982) in: Oxidases and Related Redox Systems (King, T.E., Mason, H.S. and Morrison, M., Eds.), pp. 653–670, Pergamon Press, Oxford.
[43] Fasman, G.D. (1976) in: Handbook of Biochemistry and Molecular Biology: Physical Data and Chemical Data, Vol. 1, 3rd edn., pp. 305–351, CRC Press, Cleveland, OH.
[44] Marquez, L.A. and Dunford, H.B. (1995) J. Biol. Chem. 270, 30434–30440.
[45] Petry, T.W. and Eling, T.E. (1987) J. Biol. Chem. 262, 14112–14118.
[46] Leu, A.-D. and Armstrong, D.A. (1986) J. Phys. Chem. 90, 1449–1454.
[47] Hori, H., Fenna, R.E., Kimura, S. and Ikeda-Saito, M. (1994) J. Biol. Chem. 269, 8388–8392.
[48] Marquez, L.A. and Dunford, H.B. (1990) Biochem. Biophys. Res. Commun. 169, 1158–1163.