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Thiols as myeloperoxidase–oxidase substrates

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Nine low-M_r thiols were compared with regard to their ability to function as myeloperoxidase–oxidase substrates under conditions where no auto-oxidation of the thiols could be observed. The methyl and ethyl esters of cysteine were found to be about twice as active as cysteamine at pH 7.0, in terms of increased O_2 consumption. Cysteine itself was poorly active, whereas glutathione, *N*-acetylcysteine and penicillamine were completely inactive as myeloperoxidase–oxidase substrates under these conditions. The structure–activity relationships indicated that both a free thiol and free amino group were required for peroxidase–oxidase activity, and also that a free carboxy group abolished activity. In analogy with cysteamine, the activities of both cysteine esters were inhibited by superoxide dismutase (< 5 μg/ml) and by catalase and not by the hydroxyl-radical scavenger mannitol. In contrast with cysteamine, the activities of both cysteine esters were stimulated more than 2-fold by high concentrations (> 5 μg/ml) of superoxide dismutase. The activities of both cysteine esters exhibited broad pH optima at pH 7. A mechanism for the myeloperoxidase–oxidase oxidation of the cysteine esters is proposed, which is partly different from that previously proposed for cysteamine.

INTRODUCTION

Dithiothreitol, cysteine, glutathione and cysteamine have been reported to be peroxidase–oxidase substrates (Olsen & Davies, 1976; Harman *et al.*, 1984; Wefers *et al.*, 1985; Svensson & Lindvall, 1988), i.e. compounds oxidized by peroxidase in an O_2-consuming reaction without H_2O_2 addition (Yamazaki & Yokota, 1973). The thiol oxidation has been found to be accompanied by the generation of reduced oxygen species such as superoxide radical anion ($O_2^{\cdot-}$) and H_2O_2. If occurring *in vivo*, the concomitant thiol oxidation and generation of reduced oxygen species may be predicted to show a synergistic toxicity to the surroundings *in situ*. Generation of reduced oxygen species has been shown to have deleterious effects on the surroundings, and is believed to play an important part in host defence mediated by phagocytes (Babior, 1978) as well as in a growing number of 'free radical diseases' (Armstrong *et al.*, 1984; Pryor, 1986). Thiols are generally considered to confer protection against the toxic effects of free radicals in living organisms (Meister, 1983).

The MPO–oxidase oxidation of cysteamine and the concomitant generation of reduced oxygen species may not normally be of any physiological importance since the cysteamine concentration is low *in vivo* (Ziegler *et al.*, 1983), although MPO may be present in sufficient concentration. However, other thiols, especially glutathione, are found in tissues at higher concentrations (Halliwell & Gutteridge, 1985). Also, other thiols, such as penicillamine and *N*-acetylcysteine, are used pharmacologically. Therefore it seemed of great importance to investigate further the behaviour of thiols in peroxidase–oxidase reactions and to elucidate the structural requirement for thiols being MPO–oxidase substrates.

EXPERIMENTAL

Materials

L-Cysteine and its hydrochloride salt, L-cystine dimethyl ester dihydrochloride, L-glutathione (reduced form) and 2-mercaptoethanol were bought from Fluka (Buchs, Switzerland). D-Mannitol, D-penicillamine and its hydrochloride salt, D-penicillamine disulphide, L-glutathione (oxidized form), L-cysteine methyl ester hydrochloride, L-cysteine ethyl ester hydrochloride, L-cystine dihydrochloride, thioglycollic acid sodium salt, xanthine sodium salt, (−)-adrenaline (epinephrine), bovine milk xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.1.3.22) (1.1 units/mg as measured by the manufacturer), bovine liver catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) (dialysed against distilled water, 31000 units/mg, SOD-free) and human superoxide dismutase (SOD, $O_2^{\cdot-}$:$O_2^{\cdot-}$ oxidoreductase, EC 1.15.1.1) (2800–3250 units/mg) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-Acetylcysteine [99.5% (w/w)] was from Astra (Södertälje, Sweden). Cation-exchange peak IV from chronic-myeloid-leukaemia MPO (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) (dialysed against distilled water) with a specific enzyme activity of 677 kat/mol and A_{430}/A_{280} ratio of 0.83 (Table 1) and from normal MPO [with (Table 3 and Figs. 2 and 5) and without (Table 2 and Figs. 1, 4 and 6) dialysis] with specific enzyme activities of 544 kat/mol and 749 kat/mol and A_{430}/A_{280} ratios of 0.85 and 0.83 were used (Svensson *et al.*, 1987). The sources of other materials have been described previously (Svensson & Lindvall, 1988).

Methods

U.v.-absorption increase at 270 nm or decrease at 235 nm (thiol oxidation), oxygen-electrode measurements (O_2 consumption), Nitro Blue Tetrazolium (NBT) reduction ($O_2^{\cdot-}$ generation, as indicated by the inhibition with SOD) and other methods and procedures were carried out as described previously (Svensson & Lindvall, 1988). The difference in ϵ_{235} between cysteine methyl ester and its disulphide form in the reaction mixture was determined to be 510 $M^{-1} \cdot cm^{-1}$. The differences in ϵ_{270} between cysteine, penicillamine, cysteine methyl ester and glutathione in their disulphide forms and in their

Abbreviations used: MPO, myeloperoxidase; SOD, superoxide dismutase; DTPA, diethylenetriaminepenta-acetic acid; NBT, Nitro Blue Tetrazolium.

Table 1. Comparison of thiols as MPO–oxidase substrates

Incubation mixtures contained 400 μM-thiol (200 μM in the case of cystamine), 56 nM-MPO (677 kat/mol), 200 μM-DTPA and 50 mM-potassium phosphate buffer, pH 7.0, and 25 μM-NBT (in the assay of NBT reduction). Experiments without enzyme are indicated. Mean values and ranges of indicated number of experiments (*n*) are shown.

pK_{a,NH_3}^{+}*	$pK_{a,SH}$*	Net charge at pH 7.0	Compound	Initial thiol oxidation rate ($10^4 \times \Delta A_{270}$/min) Mean	Range	Initial thiol oxidation rate (μM/min) Mean	Range	*n*	Initial O_2 consumption rate (μM/min) Mean	Range	*n*	NBT reduction ($10^4 \times \Delta A_{560}$/2.5 min) Mean	Range	*n*
10.8	8.6	+1	$H_2N-CH_2-CH_2-SH$ Cysteamine,HCl†	19	(17–20)	20	(19–22)	7	4.2	(3.4–5.2)	8	106	(104–110)	3
			−MPO†	0	(−1–1)	0	(−1–1)	2	0.1	(0.0–0.2)	2	−4	(−4–−4)	2
–	–	–	$H_2N-CH_2-CH_2-S$ / $H_2N-CH_2-CH_2-S$ Cystamine,2HCl (200 μM)	0	(−1–0)	–		2	0.0	(0.0–0.1)	2	2	(1–4)	2
–	9.5	0	$HO-CH_2-CH_2-SH$ Mercaptoethanol	−1	(−1–−1)	–		2	0.0	(0.0–0.0)	2	−2	(−4–−1)	2
–	10.3	−1	HO_2C-CH_2-SH Thioglycollic acid, sodium salt	0	(−1–0)	–		2	0.0	(0.0–0.0)	2	1	(0–3)	2
			CO_2H / $H_2N-CH-CH_2-SH$ Cysteine	2	(2–3)	2	(2–3)	3	0.3	(0.0–0.5)	2	25	(14–44)	6
10.8	8.3	0	−MPO	0	(0–0)	0	(0–0)	3	–			−3	(−6–1)	2
–	9.5	−1	$CH_3-CO-NH-CH(CO_2H)-CH_2-SH$ *N*-Acetylcysteine	0	(−1–0)	–		2	0.1	(0.0–0.4)	3	7	(6–9)	2
10.4	7.9	0	$HO_2C-CH(NH_2)-C(CH_3)_2-SH$ Penicillamine	0	(0–0)	0	(0–0)	2	0.2	(0.1–0.3)	2	0	(0–0)	2
9.0	6.6	+0.3	$OC-O-CH_3$ / $H_2N-CH-CH_2-SH$ Cysteine methyl ester,HCl	39	(37–43)	48	(46–53)	3	11.0	(10.7–11.3)	3	593	(544–660)	3
			−MPO	1	(1–1)	1	(1–1)	2	0.3	(0.2–0.4)	2	0	(−3–2)	2
9.2	6.7	+0.3	$OC-O-CH_2-CH_3$ / $H_2N-CH-CH_2-SH$ Cysteine ethyl ester,HCl	28	(27–29)	–	–	3	7.2	(6.0–8.0)	3	424	(343–539)	4
			−MPO	1	(1–1)	–	–	2	0.2	(0.2–0.3)	2	−2	(−3–1)	2
9.7	8.8	−1	$HN-CH_2-CO_2H$ / CO / $HN-CH-CH_2-SH$ / CO / $CH_2-CH_2-CH(CO_2H)-NH_2$ Glutathione, reduced form	0	(−1–0)	0	(−1–0)	2	0.1	(0.1–0.2)	2	−1	(−1–−1)	2

* Values taken from Fasman (1976).
† Values taken from Svensson & Lindvall (1988)

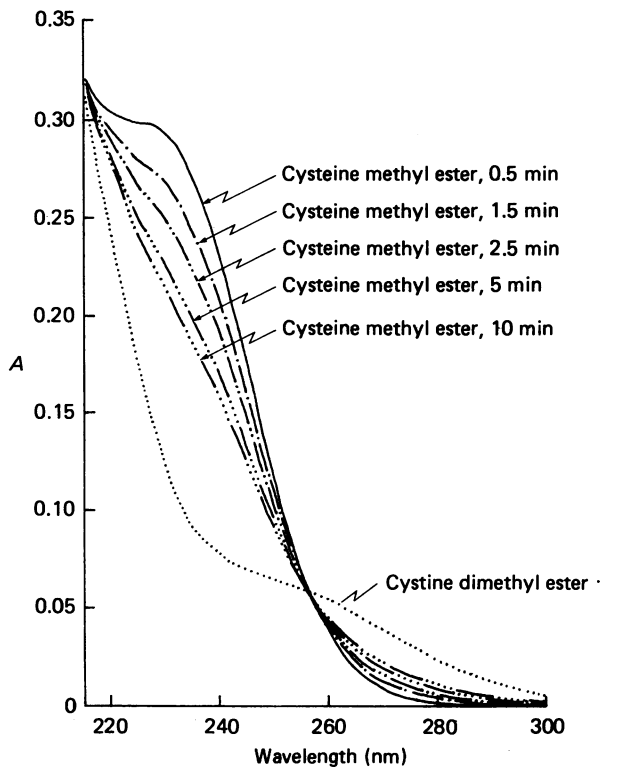


Fig. 1. Changes in the u.v.-absorption spectrum of cysteine methyl ester induced by MPO

Incubation mixtures contained 400 μM-cysteine methyl ester hydrochloride or 200 μM-cystine dimethyl ester dihydrochloride, 56 nM-MPO (749 kat/mol), 200 μM-DTPA and 50 mM-potassium phosphate buffer, pH 7.0. Typical results are shown.

reduced forms in the reaction mixture were determined to be 94, 50, 81 and 115 $M^{-1} \cdot cm^{-1}$ respectively. Inhibition of $O_2^{\cdot -}$-dependent oxidation of adrenaline to adrenochrome with SOD as reference was measured as described by Asada & Kanematsu (1976).

RESULTS

Structure–activity relationships

Several thiol analogues were investigated for their ability to function as MPO–oxidase substrates. The methods used were u.v.-absorption increase at 270 nm (thiol oxidation), oxygen-electrode measurements (O_2 consumption) and NBT reduction ($O_2^{\cdot -}$ generation). The results are shown in Table 1, where previous results with cysteamine are included as reference. The corresponding disulphide of cysteamine (cystamine) was inactive as measured by u.v.-absorption changes, O_2 consumption and NBT reduction. The replacement of the amino group in cysteamine with a hydroxy group (2-mercaptoethanol), as well as the replacement of the aminomethylene group with a carboxy group (thioglycollic acid), also resulted in abolition of the peroxidase–oxidase activity. These results indicate that both the amino group and the thiol group of cysteamine are necessary for peroxidase–oxidase activity.

Insertion of a carboxy group into the cysteamine molecule (cysteine) abolished 70–90% of the peroxidase–oxidase activity as measured by all three methods. The slight activity observed with cysteine was completely abolished by *N*-acetylation of cysteine (*N*-acetylcysteine). Insertion of methyl groups at the carbon atom close to the mercapto group in cysteine (penicillamine) also abolished the substrate activity of cysteine. The hydrochloride salts of cysteine and penicillamine gave similar results to the parent amino acids as measured by u.v.-absorption change. However, upon esterification of cysteine the peroxidase–oxidase activity was regained. Both the methyl and the ethyl esters of cysteine exhibited even higher peroxidase–oxidase substrate activities than cysteamine. On omission of MPO the activities with cysteine and its esters were completely lost. Glutathione did not have any peroxidase–oxidase substrate activity.

Comparison of the u.v.-absorption changes during the MPO–oxidase oxidation of the cysteine methyl ester with the spectrum of cystine dimethyl ester indicates that the cysteine ester was oxidized to its disulphide form (Fig. 1). No changes were observed when MPO was omitted. With the same conditions but with cysteine and its

Table 2. Comparison of thiols as peroxidatic substrates for MPO

Incubation mixtures contained 100 μM-thiol, 56 nM-MPO (749 kat/mol), 50 μM-H_2O_2, 200 μM-DTPA and 50 mM-potassium phosphate buffer, pH 7.0, and 25 μM-NBT (in the assay of NBT reduction). Reaction was started by the addition of a 10 μl portion of thiol. The same results were obtained when 100 μM-NaCl was added to cysteine, penicillamine and glutathione. NBT reduction was completely inhibited by 1 μg of SOD/ml. No thiol oxidation (< 1 μM/min) or NBT reduction (ΔA_{560}/2.5 min $< 5 \times 10^{-4}$) was observed on omission of MPO or H_2O_2, except in the case of cysteine methyl ester without H_2O_2 [6 (5–7) μM/min and ΔA_{560}/2.5 min 70 (50–76) respectively]. Mean values and ranges of triplicate experiments are shown.

	Initial thiol oxidation rate (μM/min)		NBT reduction ($10^4 \times \Delta A_{560}$/2.5 min)	
	Mean	Range	Mean	Range
Cysteamine,HCl	16	(14–18)	553	(479–620)
Cysteine	1	(1–1)	9	(3–14)
Penicillamine	2	(0–4)	7	(3–13)
Cysteine methyl ester,HCl	20	(19–21)	762	(707–794)
Glutathione	0	(−2–2)	2	(−3–4)

Table 3. Effects of SOD, catalase and mannitol on MPO-oxidase activity with cysteine methyl ester as substrate

Incubation mixtures contained 400 μM-cysteine methyl ester hydrochloride, 56 nM-MPO (544 kat/mol), 200 μM-DTPA and 50 mM-potassium phosphate buffer, pH 7.0, and 25 μM-NBT (in the assay of NBT reduction). SOD, catalase and mannitol were added at the indicated concentrations. Mean values and ranges of duplicate experiments are shown.

	Initial thiol oxidation rate ($10^4 \times \Delta A_{270}$/min)		Initial O_2 consumption rate (μM/min)		NBT reduction ($10^4 \times \Delta A_{560}$/2.5 min)	
	Mean	Range	Mean	Range	Mean	Range
Cysteine methyl ester	31	(29–32)	8.3	(8.0–8.5)	348	(343–353)
+ SOD (1 μg/ml)	16	(14–18)	3.2	(3.1–3.2)	1	(0–3)
+ Catalase (16 μg/ml)	2	(1–3)	0.3	(0.2–0.3)	3	(3–4)
+ Mannitol (100 mM)	28	(27–28)	7.6	(6.7–8.5)	339	(338–341)

disulphide (cystine) substituted for the cysteine ester and its disulphide, the slight u.v.-absorption changes shown in Table 1 were also consistent with disulphide formation. U.v.-absorption-spectral analysis of the inactive MPO–oxidase substrates was also carried out, but no changes were observed. Thus these studies show that, as well as cysteamine, cysteine methyl and ethyl esters (and to a slight extent cysteine) are MPO–oxidase substrates and, in addition, that univalent reduction of O_2 to $O_2^{\cdot-}$ occurs during the MPO–oxidase oxidation of these substrates. The ratio cysteine methyl ester oxidized/O_2 consumed and the concomitant disulphide formation indicate that O_2 is ultimately reduced to water, although $O_2^{\cdot-}$ and H_2O_2 (see below) were detected as intermediates.

As a comparison with the MPO–oxidase oxidations described above, the peroxidatic oxidation (i.e. with added H_2O_2) of some thiols was investigated (Table 2). To minimize MPO–oxidase oxidation, these studies were carried out with lower concentrations (100 μM) of the thiols (see below). Both cysteamine and cysteine methyl ester were oxidized but cysteine, penicillamine and glutathione were poorly oxidized. These results were confirmed by scanning the u.v.-absorption spectra during the reactions. The changes observed were consistent with disulphide formation. A possible $O_2^{\cdot-}$ generation was investigated by NBT-reduction experiments. Only with cysteamine and cysteine methyl ester was a reduction of NBT found (Table 2).

Involvement of reduced oxygen species

The influence of SOD, catalase and the hydroxyl-radical scavenger mannitol on MPO–oxidase oxidation of the cysteine esters was investigated and the results with cysteine methyl ester are shown in Table 3. Experiments with SOD at 1 μg/ml indicated a partial inhibition of the activity of the cysteine esters as measured by thiol oxidation and O_2 consumption. NBT reduction was completely inhibited, which indicates that NBT reduction measures $O_2^{\cdot-}$ generation. Also, the slight NBT reduction observed with cysteine as substrate (see Table 1) was completely inhibited by SOD (results not shown). Catalase (16 μg/ml) inhibited MPO–oxidase activity of both cysteine esters as measured by all three methods. A 100 mM concentration of the hydroxyl-radical scavenger mannitol did not inhibit the peroxidase–oxidase activity of either cysteine ester. Thus this indicates that both $O_2^{\cdot-}$ and H_2O_2 are formed in the reaction mixtures and that they are intermediates in the reaction. The H_2O_2- and MPO-dependence suggests an involvement of a peroxidatic oxidation step. Although hydroxyl radical might be formed, it does not seem to be an intermediate in the reaction mechanism.

The relative insensitivity to SOD of the peroxidase–oxidase activity with the cysteine esters as substrates is in contrast with the high sensitivity to SOD observed when cysteamine was used as substrate (Svensson & Lindvall, 1988). Therefore the concentrations of SOD were varied. However, complete inhibition was not observed at any concentrations of SOD. Surprisingly, a pronounced stimulation was found at higher concentrations of SOD. SOD at a concentration of 50 μg/ml increased the peroxidase–oxidase activity, as measured by O_2 consumption, 3-fold when the cysteine methyl ester was used as peroxidase–oxidase substrate (Fig. 2) and 4–5-fold when the cysteine ethyl ester was used as substrate (results not

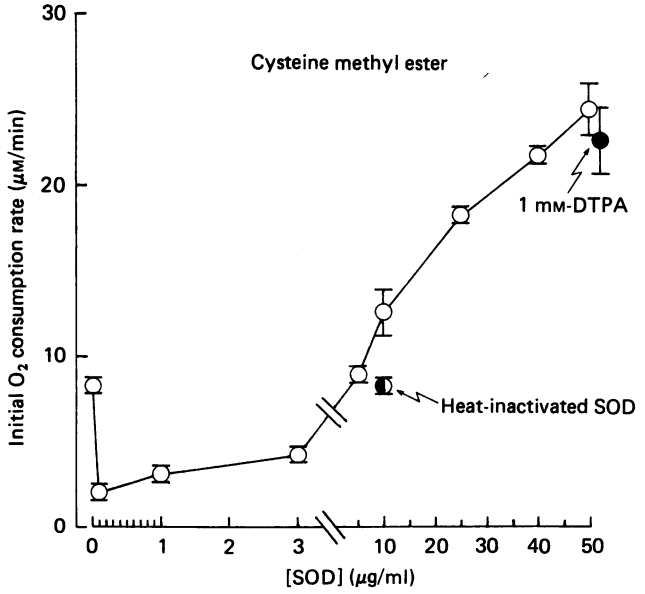


Fig. 2. Effect of SOD on O_2 consumption mediated by cysteine methyl ester and MPO

Incubation mixtures contained 400 μM-cysteine methyl ester hydrochloride, 56 nM-MPO (544 kat/mol), native or heat-inactivated (◐) SOD (at the indicated concentrations), 200 μM-DTPA or 1 mM-DTPA (●) and 50 mM-potassium phosphate buffer, pH 7.0. Mean values and ranges of duplicate experiments are shown.

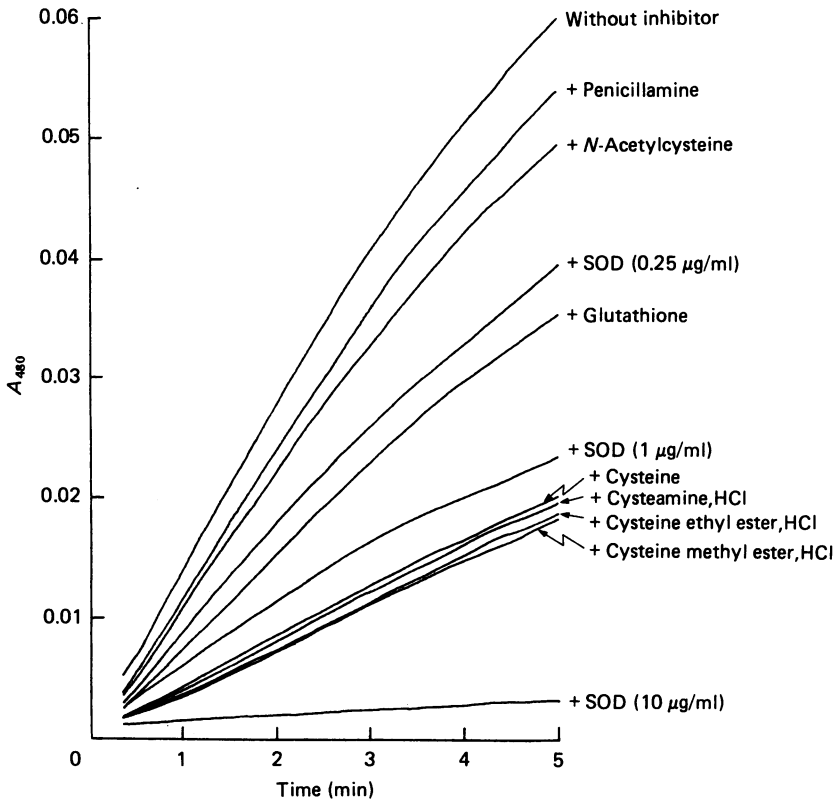


Fig. 3. Inhibition of $O_2^{\cdot-}$-dependent oxidation of adrenaline by thiols

Incubation mixtures contained 25 μM-thiol (or SOD at the indicated concentration), 500 μM-adrenaline, 100 μM-xanthine, 12.5 μg of xanthine oxidase/ml, 200 μM-DTPA and 50 mM-potassium phosphate buffer, pH 7.0. Typical results are shown.

shown). Since SOD might release Cu^{2+} ions (McCord & Fridovich, 1969) and since thiol auto-oxidation is catalysed by traces of Cu^{2+} (Jocelyn, 1972), the effect of an increased concentration of the metal-ion chelator diethylenetriaminepenta-acetic acid (DTPA) was investigated. The stimulation by SOD was, however, only to a small extent reversed by 1 mM-DTPA. Only a slight auto-oxidation (i.e. incubation without MPO) of the cysteine methyl ester was observed in the presence of 50 μg of SOD/ml (less than 1.0 μM/min). Furthermore, 10 μg of heat-inactivated SOD/ml did not induce a stimulation of the O_2-consumption rate with either of the cysteine esters. This indicates that the enzymic property of SOD is mainly responsible for the stimulation. Scanning of the u.v.-absorption spectrum indicated that, also in the presence of SOD, cysteine methyl ester was oxidized to its disulphide form and that the oxidation rate of the ester was higher (results not shown). Corresponding experiments with cysteamine or cysteine as substrate showed that 50 μg of SOD/ml did not stimulate thiol oxidation or O_2 consumption (rather, it inhibited the reactions).

Owing to the involvement of $O_2^{\cdot-}$ in the reactions, the interactions of $O_2^{\cdot-}$ with thiols were studied. The method used is based on the inhibition of $O_2^{\cdot-}$-dependent oxidation of adrenaline to adrenochrome with SOD as reference (Asada & Kanematsu, 1976). Typical results are shown in Fig. 3. Cysteamine, cysteine and its esters were all inhibitory to about the same degree. Some inhibition was observed with glutathione, but *N*-acetylcysteine and penicillamine exhibited only slight inhibition. The results with cysteine and glutathione are in good agreement with those obtained by Asada & Kanematsu (1976).

Influence of cysteine methyl ester concentration

MPO–oxidase activity with cysteine methyl ester as substrate showed an exponential-like dependence on thiol concentration at low thiol concentrations (Fig. 4). In the presence of low concentration of SOD (1 μg/ml) the inhibition by SOD was only observed at higher concentrations of the thiol. In contrast with that, stimulation by high concentrations of SOD (50 μg/ml) was observed at all concentrations of cysteine methyl ester used, and a linear dependence on thiol concentration of thiol oxidation was found.

Effects of pH and chloride

A broad optimum at about pH 7 was found both when cysteine methyl ester and cysteine ethyl ester was used as peroxidase–oxidase substrate (Fig. 5). Even at pH 8.0 no O_2 consumption (i.e. less than 0.2 μM/min) was observed in the absence of MPO in the reaction mixture, i.e. no auto-oxidation. This means that the pH–activity profiles shown in Fig. 5 reflect the enzyme-dependent O_2 consumption. The slight peroxidase–oxidase activity found at pH 7 with non-esterified cysteine was not further increased at higher pH, as measured by O_2 consumption.

The effect of Cl^- concentration on O_2 consumption mediated by MPO and cysteine methyl ester was studied. No difference in initial O_2 consumption rate could be

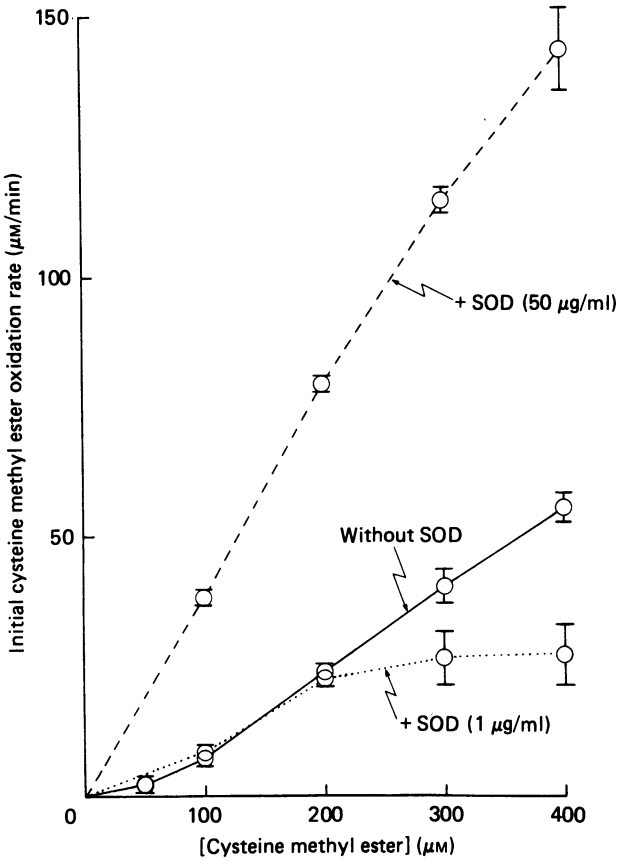


Fig. 4. Effect of thiol concentration on MPO–oxidase oxidation of cysteine methyl ester

Incubation mixtures contained cysteine methyl ester hydrochloride (at the indicated concentrations), 56 nM-MPO (749 kat/mol), 200 μM-DTPA and 50 mM-potassium phosphate buffer, pH 7.0, and SOD where indicated. Thiol oxidation was measured at 235 nm. Mean values and ranges of duplicate experiments are shown.

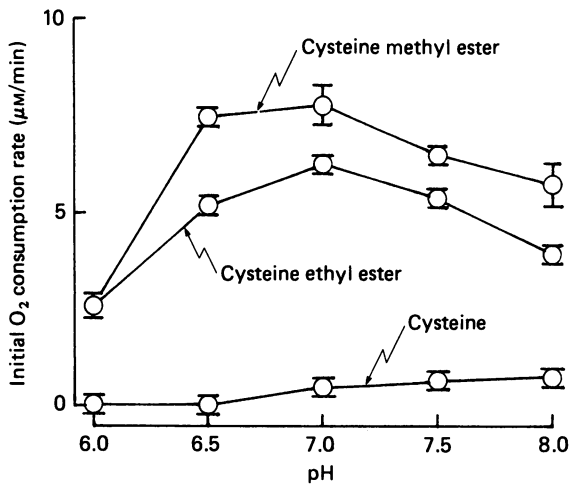


Fig. 5. Effect of pH on O_2 consumption mediated by cysteine and its esters and MPO

Incubation mixtures contained 400 μM-thiol, 56 nM-MPO (544 kat/mol), 200 μM-DTPA and 50 mM-potassium phosphate at the pH indicated. Mean values and ranges of duplicate experiments are shown.

observed between 0.4 mM- and 1 mM-NaCl (Fig. 6). Higher concentrations of NaCl gave inhibition, but complete inhibition was not obtained even at 100 mM-NaCl at pH 7.0. The MPO-oxidase oxidation of cysteine methyl ester (as measured by O_2 consumption) was less sensitive at pH 8.0 than at pH 7.0 to inhibition by Cl^-.

With the conditions in Fig. 6, it is possible that Cl^- is oxidized to HOCl (Zgliczyński, 1980). To investigate a potential reaction between HOCl and the cysteine esters, 200 μM-NaOCl was substituted for MPO in the reaction mixture used in Fig. 1. The resulting u.v.-absorption changes then observed were consistent with formation of the disulphide as the main product. However, in contrast with the MPO–oxidase oxidation of the ester, the HOCl-mediated oxidation does not seem to be accompanied by $O_2^{\cdot-}$ generation, since no NBT reduction was observed (ΔA_{560}/2.5 min $< 8 \times 10^{-4}$) when 200 μM-NaOCl was substituted for MPO in the reaction mixture used in Table 1.

DISCUSSION

To study MPO–oxidase reactions separate from auto-oxidation 200 μM-DTPA was included in the reaction mixture and thiols were used at 400 μM, at which concentration no auto-oxidation could be observed. With these conditions the two cysteine esters, in addition to cysteamine (Svensson & Lindvall, 1988), were found to be MPO–oxidase substrates. Other thiols, such as glutathione and cysteine, were poor MPO–oxidase substrates. The latter thiols, at millimolar concentrations, have previously been found to be peroxidase–oxidase substrates with micromolar concentrations of horseradish peroxidase, lactoperoxidase and microperoxidase (Olsen & Davis, 1976; Harman *et al.*, 1984; Wefers *et al.*, 1985).

Proposed mechanism for the MPO–oxidase oxidation of cysteine esters

The principal features of peroxidase–oxidase reactions have been found to be auto-oxidation of the substrate, yielding H_2O_2. This H_2O_2 is then used by the peroxidase to oxidize the substrate to an intermediate, which can reduce O_2, whereby H_2O_2 and/or organic peroxides are regenerated (Yamazaki & Yokota, 1973; Harman *et al.*, 1984; Wefers *et al.*, 1985). On the basis of the properties of the MPO–oxidase oxidation of the cysteine esters and because of some structural similarity of the cysteine esters and cysteamine, it is suggested that the same reactions occur during the the MPO–oxidase oxidation of cysteine esters as has previously been suggested to occur during the oxidation of cysteamine (Svensson & Lindvall, 1988). This reaction pathway, involving thiyl radical ($RS^{\cdot}$) and disulphide radical anion ($RS\dot{S}R^-$), is shown as the upper loop in Scheme 1 and can partly explain the propagation of the MPO–oxidase oxidation of cysteine esters, the concomitant O_2 consumption and $O_2^{\cdot-}$ generation as well as the inhibition by catalase and SOD.

In contrast with the MPO–oxidase oxidation of cysteamine, the oxidation with the cysteine esters were not completely inhibited by SOD and, in addition, SOD at high concentrations stimulated the oxidation of the esters. This may indicate that the mechanism of MPO-mediated oxidation of the cysteine esters involves an

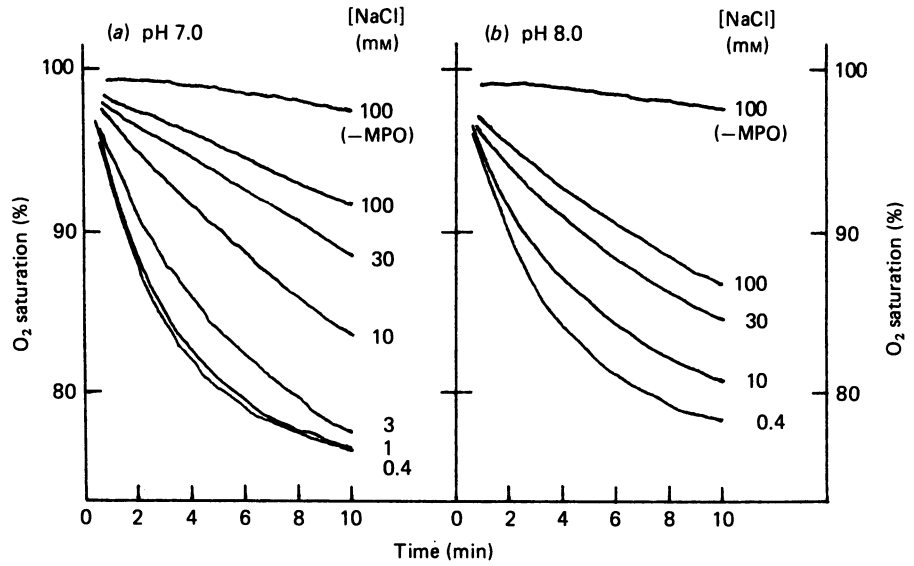
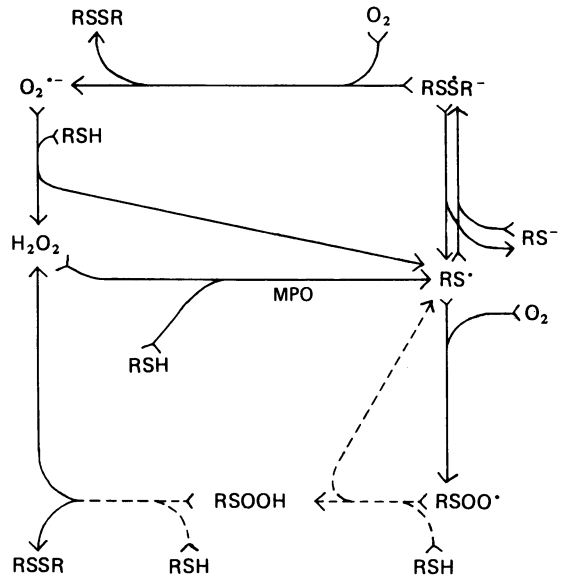


Fig. 6. Effect of chloride on O_2 consumption mediated by cysteine methyl ester and MPO

Incubation mixtures contained 400 μM-cysteine methyl ester hydrochloride, 56 nM-MPO (749 kat/mol), 200 μM-DTPA and 50 mM-potassium phosphate buffer, pH 7.0 (*a*) or pH 8.0 (*b*), supplemented with the indicated concentrations of NaCl. Typical time traces are shown.

$O_2^{\cdot-}$-independent reaction sequence that is not found in the mechanism of the MPO–oxidase oxidation of cysteamine.

Extensive investigations of the radiolysis of thiols have indicated that thiols may be oxidized to disulphides by another O_2-consuming reaction pathway with concomitant H_2O_2 generation (Lal, 1982, and references cited therein). Such a pathway is proposed to also occur during the MPO–oxidase oxidation of the cysteine esters and is shown as the lower loop in Scheme 1. The precise nature of the intermediates of this loop has not been proven, but the thiol peroxysulphenyl radical (RSOO·) or a radical derived from it (Sevilla *et al.*, 1987) may react with thiol to yield thiol sulphenyl hydroperoxide (RSOOH) and regenerate thiyl radical. The further reaction with thiol may then yield disulphide and regenerate H_2O_2. This reaction pathway generates a surplus of radical intermediates and may therefore contribute to the rapid burst of O_2 consumption observed. Moreover, this pathway is independent of $O_2^{\cdot-}$ and may therefore also explain why the MPO–oxidase oxidation of the cysteine esters was only partially inhibited by low concentrations of SOD.



Scheme 1. Proposed scheme of reactions for the propagation of the MPO-oxidase oxidation of the cysteine esters

The propagation of MPO–oxidase oxidation of the cysteine esters may be explained without any involvement of auto-oxidation of the esters (Scheme 1). This is consistent with the experimental finding, i.e. auto-oxidation was not observed. However, some auto-oxidation might still occur, although not detected by the methods used in the present paper. Such minute auto-oxidation might contribute to the initiation of the loops. However, auto-oxidation might not be necessary even for the initiation, as MPO, at a concentration of 56 nM in phosphate buffer, has been found to exist in its Compound II oxidation state (B. Svensson, unpublished work).

The inhibition by Cl^- of the MPO–oxidase oxidation of cysteamine has previously been proposed to be due to HOCl formation and its oxidation of the thiol without the necessary formation of $O_2^{\cdot-}$ (Svensson & Lindvall, 1988). Since the loop via RSOO· is independent of $O_2^{\cdot-}$, it is logical that the MPO–oxidase oxidation of cysteine methyl ester is less sensitive to Cl^- (see Fig. 6) than is the oxidation of cysteamine. The relative frequencies of the two suggested loops during the MPO–oxidase oxidation of the cysteine esters can not be determined from the experimental data in the present paper. With the cysteine and glutathione thiyl radicals similar bimolecular rate constants have been determined for the addition reactions with O_2 and with their thiolate anions (Barton & Packer, 1970; Hoffman & Hayon, 1973; Quintiliani *et al.*, 1977; Schäfer *et al.*, 1978). The increased inhibition of cysteine

methyl ester oxidation by 1 μg of SOD/ml at higher thiol concentration may, however, indicate that the loop via $RS\dot{S}R^-$ may be more important at higher thiol concentration. This is logical, with $RS\dot{S}R^-$ formation (in contrast with RSOO$^\cdot$ formation) being dependent on thiol concentration.

A difference in the pH–activity profiles was found when cysteine esters (Fig. 5) and cysteamine (Svensson & Lindvall, 1988) were used as MPO–oxidase substrates. The activity with the cysteine esters have broad pH optima at about pH 7, whereas the activity with cysteamine increases between pH 6 and 8. This difference seems to coincide with the degrees of deprotonation of the thiol groups in the cysteine esters ($pK_{a,SH}$ 6.6–6.7) and cysteamine ($pK_{a,SH}$ 8.6). Hoffman & Hayon (1973) found that the thiolate anion forms react more avidly than their protonated forms with thiyl radicals (to form $RS\dot{S}R^-$). Therefore one possible explanation for the pH–activity profiles of cysteine esters and cysteamine may be their difference in pH-dependence of $RS\dot{S}R^-$ formation. The decreased O_2 consumption rate observed above pH 7 with the cysteine esters as substrates might be related to the low pK_{a,NH_3^+} value found for cystine dimethyl ester, i.e. deprotonation of the amino groups decrease their stabilizing effect on the $RS\dot{S}R^-$ (Hoffman & Hayon, 1972).

A possible explanation for the stimulation by SOD at high concentration may be that SOD prevents the generated $O_2^{\cdot-}$ from reacting with ferric MPO to form Compound III as described by Odajima & Yamazaki (1970), 1972). For all substrates investigated, horseradish peroxidase Compound III has been found to be much less reactive than the corresponding Compound I and Compound II (Yamazaki & Yokota, 1973). This implies that the Compound III formation may retard the peroxidase–oxidase reaction, and consequently SOD may show an apparent stimulation of the reaction by preventing Compound III formation. Compound III formation has also been suggested to retard horseradish-peroxidase-mediated NAD(P)H and dihydroxyfumaric acid oxidation (Yamazaki & Yokota, 1973; Van Steveninck *et al.*, 1987).

Thiol properties and their abilities to be MPO–oxidase substrates

The different abilities of the thiols to function as MPO–oxidase substrates (Table 1) raise new questions about possible differences in reactivity of the various thiols. The knowledge of any differences in redox potentials between the thiols is insufficient to be used to answer these questions (Leu & Armstrong, 1986, and references cited therein). In both active and inactive compounds containing carboxy and/or primary amino groups, these groups are ionized at pH 7.0. This means that the ionization of these groups cannot be the crucial difference between substrate and non-substrate thiols. Neither can the ionization of the thiol group by itself determine the ability of thiol compounds to function as peroxidase–oxidase substrates, since the thiol groups in cysteine and penicillamine are more ionized than is the thiol group in cysteamine and less ionized than the thiol groups in the cysteine esters (Table 1). Nevertheless, only cysteamine and the cysteine esters are good MPO–oxidase substrates.

On the basis of the structure–activity relationships (Table 1) it is concluded that both a free thiol and a free amino group are necessary for the compounds to be MPO–oxidase substrates. Moreover, insertion of a free carboxy group abolishes most of the peroxidase–oxidase activity. This might wholly or partly explain why a correlation between the net charge of the thiols and their MPO–oxidase activities seems to exist. Those thiols having a positive net charge are good MPO–oxidase substrates, whereas those with a negative net charge are inactive. Of those thiols with a zero net charge, only cysteine is slightly active as MPO–oxidase substrate (Table 1).

On the basis of visible-light-absorption spectroscopy of MPO, Stelmaszyńska & Zgliczyński (1974) have suggested that the active site of MPO contains a carboxy group, which means that compounds containing carboxy groups (i.e. cysteine, penicillamine and glutathione) might be repelled. This may be a possible explanation for these thiols being poor peroxidatic substrates for MPO (Table 2) and consequently poor MPO–oxidase substrates. The ability to form $RS\dot{S}R^-$ may also contribute to the structure–activity relationships found for thiols as substrates in MPO–oxidase reactions. Hoffman & Hayon (1972, 1973) found a correlation between the overall charge of the thiolate anion and $RS\dot{S}R^-$ formation.

According to the proposed scheme of reactions the oxidation of thiol by $O_2^{\cdot-}$ is essential for the generation of a surplus of intermediates in the loop via $RS\dot{S}R^-$. The slight inhibition of $O_2^{\cdot-}$-dependent adrenaline oxidation by penicillamine and *N*-acetylcysteine (Fig. 3) indicates that these thiols are poorly oxidized by $O_2^{\cdot-}$. Thus this property of penicillamine and *N*-acetylcysteine might therefore also contribute to these thiols not being MPO–oxidase substrates.

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