Myeloperoxidase oxidation states involved in myeloperoxidase–oxidase oxidation of thiols

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The changes in the oxidation state of the leucocyte enzyme myeloperoxidase, induced by buffer and thiols, were studied with visible-light-absorption spectroscopy. It was concluded that phosphate buffer contains small amounts of H_2O_2 and that thiols, when added to buffer, induce the generation of minute amounts of superoxide radical anion. These minute amounts of reduced oxygen species are suggested to account for the initiation of myeloperoxidase–oxidase oxidation of thiols. Myeloperoxidase was found to be in its Compound III oxidation state during myeloperoxidase–oxidase oxidation of thiols. However, myeloperoxidase-mediated oxidation of thiols with concomitant O_2 consumption can also occur with myeloperoxidase in its Compound II oxidation state. These studies indicate that the ferro and Compound III oxidation states may not be essential intermediates in myeloperoxidase–oxidase oxidation of thiols, but rather that the formation of the Compound III oxidation state retards the reaction.

INTRODUCTION

Peroxidase–oxidase reactions are characterized by their use of O_2 , instead of H_2O_2 , as external co-substrate for the oxidation of compounds. Some aminothiols have recently been shown to be substrates in myeloperoxidase (MPO)–oxidase reactions (Svensson & Lindvall, 1988; Svensson, 1988). The mechanism for the MPO–oxidase oxidation of these thiols was shown to involve classical peroxidatic oxidation of the thiols, and the necessary regeneration of H_2O_2 was proposed to occur during the further non-enzymic oxidation of the thiols to disulphides. Thus MPO was suggested to function as a pseudo-oxidase (see left part of Scheme 1). However, the initiation of the loops was not explained, since the metal-ion-chelator diethylenetriaminepenta-acetic acid (DTPA) was added to the reaction mixtures at concentrations where no auto-oxidation of the thiols could be detected.

Scheme 1. Proposed scheme of reactions for MPO acting as a pseudo-oxidase (—) and as a genuine oxidase (----) during thiol oxidation

The numbers in parentheses stand for the oxidizing equivalents retained in the enzyme (Yamazaki & Yokota, 1973).

In the peroxidase–oxidase catalysis peroxidases have been observed in their ferro, Compound II (cII) and Compound III (cIII) oxidation states (Yamazaki & Yokota, 1973; Olsen & Davis, 1976; Wefers *et al.*, 1985). However, the functions, if any, of the ferro and cIII oxidation states in this reaction have not been unequivocally determined (Mottley & Mason, 1986). Theoretically, peroxidases can also function as genuine oxidases. Peroxidases can, in their ferro oxidation states, bind O_2 to form cIII (Wittenberg *et al.*, 1967; Odajima & Yamazaki, 1970), and cIII can, in turn, oxidize compounds (Tamura & Yamazaki, 1972). Thus, provided that the substrate can reduce ferric peroxidase to its ferro oxidation state, this mechanism does not need the preformation of H_2O_2 (Scheme 1). This latter mechanism has, for example, been proposed for the horseradish peroxidase–oxidase oxidation of indol-3-ylacetic acid at high substrate/enzyme ratio (Smith *et al.*, 1982).

To gain further insight in the mechanism of MPO–oxidase oxidation of thiols and especially the roles of the ferro-MPO (MPO^{2+}) and cIII in this reaction, the changes in the oxidation state of MPO induced by buffer and thiols under various conditions were studied.

EXPERIMENTAL

The purification of chronic-myeloid-leukaemia MPO (donor: H_2O_2 oxidoreductase, EC 1.11.1.7; cation-exchange peak II, dialysed against distilled water, A_{430}/A_{280} ratio 0.81, 648 kat/mol) has previously been described (Svensson *et al.*, 1987). Bovine catalase (H_2O_2 : H_2O_2 oxidoreductase, EC 1.11.1.6; dialysed, 31000 units/mg) and human SOD ($\text{O}_2^{\cdot-}$: $\text{O}_2^{\cdot-}$ oxidoreductase, EC 1.15.1.1; dialysed, 1800 units/mg) were bought from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Visible-light-absorption spectra were scanned (1200 nm/min) at about 30 °C in a Beckman DU-7HS spectrophotometer equipped with a kinetics accessory by using a cell with 5 cm light-path. Unless otherwise stated, other materials,

Fig. 1. Induction of cII by buffer

MPO (56 nM) was added to 50 mM-potassium phosphate buffer, pH 7.0 (with supplements and substitution as indicated below), and the visible-light-absorption spectrum was scanned after 30 s (—). The MPO^{3+} spectrum (·····) is included as reference (see *e*). (a) No supplement; (b) freshly deionized water with resistivity 16 $\text{M}\Omega \cdot \text{cm}$ (Milli-Q; Millipore Corp., Bedford, MA, U.S.A.) substituted for the buffer; (c) + 1 mM-DTPA; (d) + 1 mM-desferrioxamine; (e) + 16 μg of catalase/ml + 200 μM -DTPA; (f) + 5 μg of SOD/ml + 200 μM -DTPA. Typical results are shown.

methods and procedures were those previously described (Svensson & Lindvall, 1988; Svensson, 1988).

RESULTS AND DISCUSSION

Origin of H_2O_2 required for initiation

Previous investigations on the oxidation states of MPO have been carried out at high enzyme concentrations, and the isolation procedures have yielded MPO in its ferric oxidation state (MPO^{3+}) (e.g. Agner, 1958; Olsson *et al.*, 1978; Bakkenist *et al.*, 1978). Smith *et al.* (1982) have reported that horseradish peroxidase at low concentration in buffer exists in its cII oxidation state.

It was found that dilute and dialysed MPO in phosphate buffer, pH 7.0 (Fig. 1a) and pH 8.0, was mainly in its cII oxidation state. Although cII and cIII have similar absorption maxima, they can be differentiated by their relative absorption changes at 450 and 625 nm as well as by the width of the maximum at 625 nm (Agner, 1963; Svensson *et al.*, 1987), i.e. cII formation is characterized by a higher quotient $\Delta A_{450}/\Delta A_{625}$ and a lower quotient $\Delta A_{625}/\Delta A_{640}$ than is cIII formation. In freshly deionized water a typical MPO^{3+} spectrum was obtained (Fig. 1b). However, some highly

purified MPO preparations, after extensive dialysis against distilled water (40 h with eight to ten changes of water), gave some cII, as determined by their spectral characteristics, even at a concentration of 500 nM (results not shown). The differences in cII appearance between MPO preparations, at higher enzyme concentration, might be due to varying amounts of endogenous hydrogen donors (Tamura & Yamazaki, 1972).

Dilute MPO in buffer supplemented with 1 mM-DTPA (Fig. 1c) or 200 μM -desferrioxamine gave rise to a cII spectrum. In contrast, inclusion of 1 mM-desferrioxamine resulted in an MPO^{3+} spectrum (Fig. 1d). In the presence of catalase, with or without the simultaneous presence of SOD, the MPO^{3+} spectrum was also obtained (Fig. 1e), whereas SOD only yielded a cII spectrum (Fig. 1f). The abolition of cII by catalase indicates that buffer contains small amounts of H_2O_2 . The concentration of H_2O_2 can be estimated to be at least 50 nM, since this is the concentration necessary to convert the same concentration of MPO into its cI oxidation state, which is spontaneously and rapidly converted into cII (Harrison *et al.*, 1980). The prevention of cII formation by 1 mM-desferrioxamine may indicate that iron is involved in the H_2O_2 formation in the buffer. Buffers and glass inevitably

Fig. 2. Changes in the visible-light-absorption spectrum of MPO induced by cysteamine

Cysteamine hydrochloride ($400\ \mu\text{M}$) was added to $56\ \text{nM}$ -MPO/ $200\ \mu\text{M}$ -DTPA/ $50\ \text{mM}$ -potassium phosphate buffer, pH 7.0 (with supplements and substitutions as indicated), and the visible-light-absorption spectrum was scanned after the times indicated (—). The MPO^{3+} spectrum (·····) is included as reference (see Fig. 1e). (a) No supplement (0.5 min); (b) $10\ \text{mM}$ -cysteamine hydrochloride (instead of $400\ \mu\text{M}$) and $224\ \text{nM}$ -MPO (instead of $56\ \text{nM}$) (15 min); (c) + $16\ \mu\text{g}$ of catalase/ml (2.5 min); (d) + $5\ \mu\text{g}$ of SOD/ml (1.5 min); (e) + $5\ \mu\text{g}$ of SOD/ml + $5\ \mu\text{M}$ - H_2O_2 (0.5 min). Typical results are shown.

contain traces of iron (Wong *et al.*, 1981), and ferrous iron is known to reduce O_2 univalently to $\text{O}_2^{\cdot-}$, which may, in turn, dismutate to H_2O_2 (Bielski & Allen, 1977). Only traces of iron seem to be required, since neither $1\ \text{mM}$ -DTPA nor $200\ \mu\text{M}$ -desferrioxamine was able to prevent cIII formation. These small amounts of H_2O_2 in the buffer as well as minute thiol auto-oxidation, as detected by cIII formation (see below), may account for the initiation of MPO-oxidase oxidation of thiols.

Thiol-induced cIII formation

Addition of $400\ \mu\text{M}$ -cysteamine to MPO in phosphate buffer, pH 7.0 (Fig. 2a) and pH 8.0, supplemented with DTPA induced an instant cIII spectrum. A progressive decrease in the Soret band occurred, which indicates that the enzyme is destroyed during the reaction. The cIII spectrum was also formed after addition of $100\ \mu\text{M}$ -cysteamine. However, in this case the changes developed more slowly, i.e. in about 2 min (results not shown). With $10\ \text{mM}$ -cysteamine a typical MPO^{2+} spectrum was found (Fig. 2b).

cIII was partly formed also in the presence of catalase (Fig. 2c). When $5\ \mu\text{g}$ of SOD/ml (Fig. 2d) or both $5\ \mu\text{g}$ of SOD/ml and $16\ \mu\text{g}$ of catalase/ml were present in the reaction mixture, MPO^{3+} instead of the cIII spectrum was obtained after cysteamine addition.

In analogy with cysteamine, cysteine methyl ester (also in the presence of $16\ \mu\text{g}$ of catalase/ml) induced the cIII spectrum on addition to MPO. In the simultaneous presence of $16\ \mu\text{g}$ of catalase/ml and $5\ \mu\text{g}$ of SOD/ml, MPO^{3+} was formed (results not shown).

Cysteine, dithiothreitol and GSH have previously been reported to induce cII, cIII and a sulphhaem derivative on addition to peroxidases (Schultz & Rosenthal, 1959; Olsen & Davis, 1976; Nakamura *et al.*, 1984; Wefers *et al.*, 1985). Cuperus *et al.* (1987) have found that addition of metal-ion chelator prevented induction of the MPO cIII by penicillamine.

A pathway for the formation of cIII by cysteamine may be generation of $\text{O}_2^{\cdot-}$ (Svensson & Lindvall, 1988), which may react with MPO^{3+} to form cIII (Odajima & Yamazaki, 1970, 1972). In the presence of catalase (Fig. 2c), whereby the MPO-oxidase reaction is inhibited (Svensson & Lindvall, 1988), a source of $\text{O}_2^{\cdot-}$ may be the reduction by cysteamine of contaminating metal ions (Wong *et al.*, 1981), which may, in turn, reduce O_2 to $\text{O}_2^{\cdot-}$. The extent of formation of the radical seems to be very small in the presence of DTPA, since it cannot be detected by Nitro Blue Tetrazolium reduction (Svensson & Lindvall, 1988). The formation of cIII has previously been reported to be a very sensitive method for determination of $\text{O}_2^{\cdot-}$ generation (Nakamura & Yamazaki,

1969; Sawada & Yamazaki, 1973; Cuperus *et al.*, 1986).

Another pathway is that involving formation of cIII from the addition of O_2 to ferro-MPO (Odajima & Yamazaki, 1970). The latter may form by the reduction of MPO^{3+} by cysteamine. This pathway seems, however, unlikely to occur with $400 \mu M$ -cysteamine, since this pathway is not likely to be inhibited by SOD, and SOD inhibited cIII formation (Fig. 2d). This pathway may be operative only at higher cysteamine concentration, since by the addition of 10 mM -cysteamine to MPO a typical MPO^{2+} spectrum was obtained (Fig. 2b). A third pathway for horseradish peroxidase cIII formation has been reported, namely through the reaction of H_2O_2 with cII (Chance, 1952; George, 1953; Nakajima & Yamazaki, 1987). But this also seems unlikely to occur under these conditions, since such a formation is probably inhibited by catalase, and cIII was formed also in the presence of catalase (Fig. 2c). On the basis of these considerations it is proposed that $400 \mu M$ -cysteamine cannot reduce MPO^{3+} and that cIII is formed by the reaction of $O_2^{\cdot -}$ with MPO^{3+} . Similarly, cIII may also be formed with the cysteine esters by the same reaction.

Thiol oxidation and O_2 consumption with MPO in its cII oxidation state

The addition of catalytic amounts of H_2O_2 to MPO and inhibitory concentration of SOD caused a rapid MPO-dependent oxidation of cysteamine (Fig. 3, curve B), and this oxidation was accompanied by an MPO-dependent consumption of O_2 (Fig. 3, curve F). During these conditions MPO was found mainly in its cII oxidation state (Fig. 2e).

H_2O_2 , by itself, can oxidize cysteamine to cystamine, but the bimolecular rate constant was calculated to be less than $5 \text{ M}^{-1} \cdot \text{s}^{-1}$ (from curve A in Fig. 3). No Nitro Blue Tetrazolium reduction ($\Delta A_{560}/2.5 \text{ min} < 0.0002$) was observed under these conditions, which indicates that the oxidation of cysteamine by H_2O_2 is not accompanied by $O_2^{\cdot -}$ generation. Thus these results indicate that the direct H_2O_2 -mediated oxidation of cysteamine is not involved in the MPO-oxidase reaction. Rather, the direct oxidation of cysteamine by H_2O_2 might retard the MPO-oxidase reaction by not regenerating reduced oxygen species.

MPO-oxidase oxidation of cysteine methyl ester has previously been found to be stimulated by $50 \mu g$ of SOD/ml (Svensson, 1988). Under these conditions MPO was found to be mainly in its cII oxidation state (results not shown). These results suggest that cIII formation is not necessary for the MPO-oxidase oxidation of the thiols.

Inhibition by cIII

With the assumption that each peroxidatic cycle is accompanied by the oxidation of 4 molecules of cysteamine (Svensson & Lindvall, 1988), the bimolecular rate constant for the oxidation of cysteamine by cII was calculated to be about $2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ by using the initial rate of curve B in Fig. 3. In contrast, the cIII formation in the presence of catalase without any detectable thiol oxidation, O_2 consumption or Nitro Blue Tetrazolium reduction indicates that cysteamine and cysteine methyl ester are sluggish substrates for MPO cIII, i.e. rate constants less than $100 \text{ M}^{-1} \cdot \text{s}^{-1}$ by assuming that the reduction of cIII to MPO^{3+} is accompanied by the

Fig. 3. H_2O_2 - and MPO/ H_2O_2 -mediated oxidation of cysteamine

Incubation mixtures contained $400 \mu M$ -cysteamine hydrochloride / $200 \mu M$ -DTPA / 50 mM -potassium phosphate buffer, pH 7.0 (with supplements as indicated below.) Cysteamine oxidation was monitored by scanning the u.v.-absorption spectrum and A_{270} is plotted in the Figure. The changes in the u.v.-absorption spectrum of cysteamine were consistent with cystamine formation. Cysteamine oxidation: curve A, + $100 \mu M$ - H_2O_2 ; curve B, + 56 nM -MPO + $5 \mu g$ of SOD/ml + $5 \mu M$ - H_2O_2 ; curve C, + $5 \mu M$ - H_2O_2 ; curve D, + $5 \mu g$ of SOD/ml + $5 \mu M$ - H_2O_2 . Mean values and ranges of duplicate experiments are shown. O_2 consumption: curve E, + 56 nM -MPO (heat-inactivated) + $5 \mu g$ of SOD/ml + $5 \mu M$ - H_2O_2 ; curve F, + 56 nM -MPO (native) + $5 \mu g$ of SOD/ml + $5 \mu M$ - H_2O_2 . Typical results are shown.

oxidation of 6 molecules of thiol (Tamura & Yamazaki, 1972; Svensson & Lindvall, 1988; Svensson, 1988).

The MPO-oxidase oxidation of cysteamine has previously been found to be inhibited by high concentrations of MPO and to show an exponential-like dependence on cysteamine concentration (Svensson & Lindvall, 1988). However, the MPO/ H_2O_2 -mediated oxidation of cysteamine in the presence of SOD (as in Fig. 3, curve B) was linearly dependent on MPO concentration (up to 150 nM) and showed a classical dependence on cysteamine concentration (results not shown). SOD has also been shown to convert the exponential-like dependence on cysteine ester concentration of MPO-oxidase activity into a linear dependence (Svensson, 1988).

These results support the view that cIII formation inhibits MPO-oxidase oxidation of the thiols and that SOD may stimulate MPO-oxidase oxidation of cysteine esters by inhibiting cIII formation. The formation of cIII has previously been found to inhibit the horseradish peroxidase-oxidase oxidation of NAD(P)H and dihydroxyfumaric acid (Yamazaki & Yokota, 1973, and references cited therein).

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