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Reactions of Superoxide with Myeloperoxidase and Its Products

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SUMMARY: Myeloperoxidase (MPO) uses hydrogen peroxide to oxidize chloride to hypochlorous acid. It also converts numerous substrates to reactive free radicals. When released by neutrophils, the enzyme operates in the presence of a flux of superoxide. We show that superoxide has a profound influence on oxidative reactions catalysed by MPO. It reacts directly with the enzyme to modulate production of hypochlorous acid. Within neutrophil phagosomes, where MPO functions to kill micro-organisms, it may be the preferred substrate for the enzyme. Superoxide also reacts rapidly with radicals generated by MPO, e.g. from tyrosine and tyrosyl peptides. Initial products are organic peroxides. These species are likely to be toxic and contribute to the pathophysiological actions of MPO.

Myeloperoxidase (MPO) plays an important role in the microbicidal activity of neutrophils (1). It also contributes to tissue injury in inflammatory diseases. When neutrophils release MPO, they also undergo a burst of NADPH oxidase activity that converts oxygen to superoxide radicals. Thus, MPO acts in an environment where there is ongoing superoxide generation. Yet few studies have considered the influence of superoxide on reactions of the enzyme. MPO uses hydrogen peroxide to oxidize halides and classical peroxidase substrates, and most of its enzymology has been studied with hydrogen peroxide added to the enzyme. However, as well as providing a source of hydrogen peroxide via dismutation, superoxide interacts directly with MPO and its products (2). These reactions should have a major impact on how the enzyme functions in the phagosome and at inflammatory sites.

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Interactions of superoxide with MPO: Odajima and Yamazaki were the first to demonstrate that superoxide reacts with ferric MPO to form oxymyeloperoxidase (compound III) (3). Subsequently, it was shown that compound III is the predominant from of MPO in stimulated neutrophils. The reaction of superoxide with MPO is fast ($k_{MPO/O2}$- 2×10^6 M^{-1} s^{-1}) and therefore should influence the activity of the enzyme. Indeed, we have shown that superoxide can modulate the chlorination activity of purified MPO (2).

More recently we have investigated how superoxide affects extracellular production of HOCl by stimulated human neutrophils. When cells were stimulated with phorbol ester (PMA), superoxide dismutase did not affect the production of HOCl (data not shown). However, with opsonized zymosan, the production of HOCl increased by almost 2.5 fold. This result indicates that superoxide is capable of damping the formation of HOCl. Catalase inhibited production of HOCl regardless of the stimulus.

It is unlikely that superoxide was reacting with HOCl and thereby preventing its accumulation because superoxide dismutase should have had the same effect with both stimuli. Also HOCl would be expected to react more rapidly with taurine in the buffer than with superoxide (4). An alternative explanation for the effect of superoxide dismutase is that superoxide reacted directly with MPO and influenced its chlorination activity. To investigate this possibility, we determined the effect of superoxide on production of HOCl by purified MPO and xanthine oxidase. Both the flux of superoxide (Fig. 1) and concentration of MPO (not shown) were varied. At high fluxes of superoxide and low concentrations of MPO, superoxide dismutase inhibited the production of HOCl. However, at low fluxes of superoxide and high concentrations of MPO, superoxide dismutase enhanced chlorination activity. We propose that superoxide inhibits by converting the enzyme to compound III whereas it enhances activity by preventing the accumulation of inactive compound II (Scheme 1).

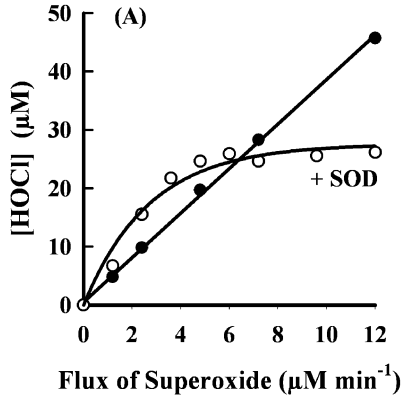
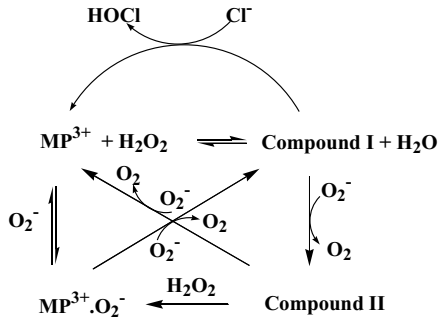


Fig. 1. The effect of superoxide dismutase on production of HOCl by purified myeloperoxidase.
Reactions were started by adding 10 mM acetaldehyde to varying concentrations of xanthine oxidase and 10 nM myeloperoxidase in 10 mM phosphate buffer pH 7.4 containing 140 mM NaCl, 100 μM DTPA and 5 mM taurine. After five minutes, reactions were stopped by adding 20 μg/ml of catalase and the amount of accumulated taurine chloramine was measured. When added, superoxide dismuatse (SOD) was present at 20 μg/ml. Results are typical of three independent experiments.



Scheme 1. Reactions of superoxide with the redox intermediates of myeloperoxidase.
Myeloperoxidase is a ferric heme enzyme (MP^{3+}) that reacts with hydrogen peroxide to form an Fe^{V} π-cation radical called Compound I. This redox intermediate undergoes one-electron reduction to give the Fe^{IV} compound II form. Superoxide also reacts with the native enzyme to form oxymyeloperoxidase (compound III), which will react again with superoxide to generate compound I. Compound I is the only form of the enzyme that oxidizes chloride to HOCl.

We have used steady state kinetics and computer modeling to investigate reactions of superoxide with all the redox intermediates of MPO (unpublished results). Reaction between superoxide and compound II was observed spectrophotometrically and its rate constant was calculated to be 4×10^6 M^{-1} s^{-1}. By monitoring the steady state concentrations of hydrogen peroxide in systems containing MPO and xanthine oxidase we were able to demonstrate that superoxide also reacts with compound I and compound III. Estimates of the rate constants for these reactions were 1×10^8 M^{-1} s^{-1} and 1×10^5 M^{-1} s^{-1}, respectively. These values indicate that superoxide is an excellent substrate for MPO. Indeed, the magnitude of these rate constants suggests that within phagosomes, superoxide will be a preferred substrate for MPO. It must react with the enzyme and compete with chloride for oxidation by compound I.

Interactions of superoxide with MPO products: Superoxide reacts with HOCl to produce hydroxyl radicals. While this is a potential source of hydroxyl radicals, it is only one of many reactions that HOCl could undergo in the neutrophil environment. These alternative reactions are likely to be much more favored and if hydroxyl radicals are formed by neutrophils they should only be minor products (1).

Reactions of superoxide with radicals are much more favorable, and would be expected to occur with many of the radicals generated in the peroxidase activity of MPO (5). For example, it is well established that MPO plus hydrogen peroxide converts tyrosine to tyrosyl radicals which, as shown in Fig. 2, can dimerize to form dityrosine. However, if superoxide is present, they should react preferentially with the superoxide as the rate constant for this reaction is about 3-fold higher than for dimerization (5). The two radicals react primarily by addition, to produce a tyrosine peroxide as in Fig. 2. Thus tyrosine oxidation by MPO and a superoxide generating system gives rise to dityrosine but even greater amounts of tyrosine peroxide (5). Tyrosine peroxide is also the predominant product of tyrosine oxidation by stimulated neutrophils (Fig. 3) (6).

Tyrosine peroxide, like other amino acid peroxides, is a metastable oxidant that could diffuse away from its site of formation and undergo injurious reactions with a variety of biological targets (7). Furthermore, similar reactions could give rise to peptide or protein peroxides. These peroxides could form directly on peptides that are substrates for MPO or via radical transfer to

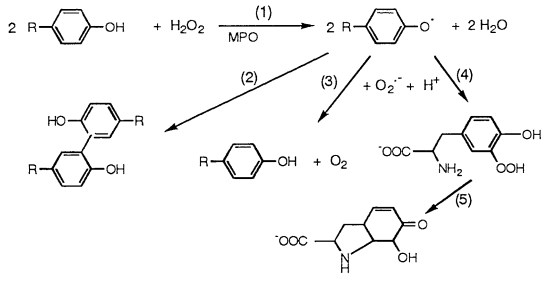


Fig. 2. Generation of tyrosyl and other phenoxyl radicals by myeloperoxidase (1) and subsequent reactions of the radicals with each other to form dimers (2) or with superoxide to repair the original substrate (3) or form an organic peroxide (4).
For tyrosine [where R is -$CH_2CH(COOH)NH_2$]; the peroxide can add at the ortho (shown) or para position and undergo a secondary cylization (5). See (9,6) for details.

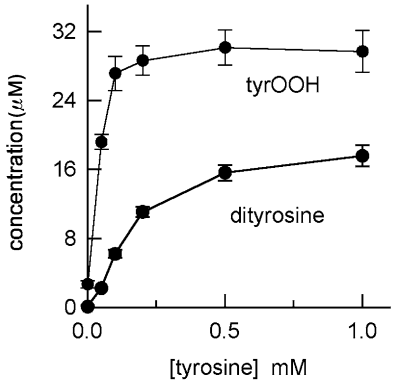


Fig. 3. Formation of tyrosine peroxide (tyrOOH) and dityrosine by neutrophils stimulated with PMA.
Data taken from (6).

the protein tyrosyl residues from other substrate radicals such as nitrogen dioxide. This could alter functional properties of the proteins as well as generating secondary reactive oxidants. Therefore, we investigated tyrosyl peroxide formation on peptides (8). We were particularly interested in whether free amino groups influence the reaction, because with tyrosine radical addition is followed by cyclization through the amine group to give the peroxide product (reaction 5, Fig. 2) (9). By comparing a series of peptides and analogues, we found that tyramine and peptides with N-terminal tyrosines formed peroxides whereas those with blocked amino groups did not (Table 1). Lysine residues adjacent to the tyrosine enabled some peroxide formation, as did high concentrations of free lysine or ethanolamine. Therefore, accessible amines in the vicinity of tyrosine are important to stabilize the peroxide. Otherwise, it appears that regeneration of tyrosine and oxygen is favored. These results suggest that certain tyrosyl residues in proteins should be more susceptible to peroxide formation than others.

A wide range of physiological compounds and xenobiotics are peroxidase substrates for MPO. The reaction of superoxide with radicals formed in these reactions could be a common route to organic peroxide formation during neutrophil activity and a possible toxicity mechanism (5). Although this concept has received little attention, there is indirect evidence, for example with the vitamin E analogue, Trolox, that radical reactions with superoxide do occur (10). Whether they form metastable organic peroxides or derived species, some of these products are likely to be toxic and must be considered as potential players in the pathophysiological actions of MPO.

We conclude that reactions of superoxide with the redox intermediates of myeloperoxidase will be pivotal to how neutrophils use oxidants to kill bacteria. Radical-radical reactions involving MPO and superoxide should be highly favored in the environment of neutrophils.

Table 1. Superoxide-dependent formation of peroxides of tyrosine and tyrosyl peptides incubated with a xanthine oxidase system and peroxidase

Peptide	Peroxide yield (μM)
Tyrosine (Y)	12.7
GY	0.5
AY	0.9
YG	24
KYK	6.2
GYK	2.6
KYG	2.9
N-acetyltyrosine	0.4
N-acetyltyrosine + lysine (10 mM)	6.6

Results are from (8). Measurements were obtained with the FOX assay relative to a hydrogen peroxide standard and have been corrected for the lower response of tyrosine peroxide compared with hydrogen peroxide in the assay. SOD blanks have been subtracted.

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REFERENCES

1. Hampton, M. B., Kettle, A. J. and Winterbourn, C. C. (1998): Inside the neutrophil phagosome: oxidants, myeloperoxidase and bacterial killing. Blood, 92, 3007-3017.
2. Kettle, A. J. and Winterbourn, C. C. (1997): Myeloperoxidase: A key regulator of neutrophil oxidant production. Redox Rep., 3, 3-15.
3. Odajima, T. and Yamazaki, I. (1972): Myeloperoxidase of the leukocytes of normal blood. III The reaction of ferric myeloperoxidase with superoxide anion. Biochim. Biophys. Acta, 284, 355-359.
4. Pattison, D. I. and Davies, M. J. (2001): Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds. Chem. Res. Toxicol., 14, 1453-1464.
5. Winterbourn, C. C. and Kettle, A. J. (2003): Radical-radical reactions of superoxide: a potential route to toxicity. Biochem. Biophys. Res. Commun., 305, 729-736.
6. Winterbourn, C. C., Pichorner, H. and Kettle, A. J. (1997): Myeloperoxidase-dependent generation of a tyrosine peroxide by neutrophils. Arch. Biochem. Biophys., 338, 15-21.
7. Fu, S., Gebicki, S., Jessup, W., Gebicki, J. M. and Dean, R. T. (1995): Biological fate of amino acid, peptide and protein hydroperoxides. Biochem. J., 311, 821-827.
8. Winterbourn, C. C., Parsons-Mair, H. N., Gebicki, S., Gebicki, J. M. and Davies, M. J. (2004): Requirements for superoxide-dependent tyrosine hydroperoxide formation in peptides. Biochem. J, 381, 241-248.
9. Jin, F., Leitich, J. and von Sonntag, C. (1993): The superoxide radical reacts with tyrosine-derived phenoxyl radicals by addition rather than by electron transfer. J. Chem. Soc. Perkin Trans. II, 1583-1588.
10. Jonsson, M., Lind, T., Reitberger, T. E., Eriksen, T. E. and Merenyi, G. (1993): Free radical combination reactions involving phenoxyl radicals. J. Phys. Chem., 97, 8229-8233.