Formation of a Hydroxyl Radical by the Myeloperoxidase–NADH–Oxygen System

Sadaki FUJIMOTO,* Naoko KAWAKAMI, and Akira OHARA

Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607, Japan.

Received October 1, 1992

When phenylalanine was incubated with myeloperoxidase (MPO) and NADH in citrate buffer (pH 4.5), *o*-, *m*-, and *p*-tyrosines were identified as hydroxylated products. Tyrosine formation was dependent on the reaction time and MPO concentration. No significant quantities of tyrosines were formed on if MPO was omitted and inactivated MPO was added instead of active MPO. The tyrosine formation by the MPO–NADH system was greatly reduced under anaerobic conditions, and significantly inhibited by hydroxyl radical scavengers. Superoxide dismutase was a potent inhibitor, but catalase was less effective. Even though the superoxide radical (O_2^-)-producing ability of the MPO–NADH system was about 29% of that of the hypoxanthine–xanthine oxidase system, under the experimental conditions employed, the rate of tyrosine formation from phenylalanine by two systems was found to be a similar. The above results suggest that the formation of a hydroxyl radical ($OH\cdot$) may occur in the MPO–NADH system under aerobic conditions and a superoxide radical may be involved in the $OH\cdot$ formation, with MPO promoting the $OH\cdot$ formation from O_2^- .

Keywords myeloperoxidase; NADH; hydroxyl radical; superoxide radical; phenylalanine; tyrosine isomer

Introduction

Upon exposure to appropriate stimuli, neutrophils generate superoxide (O_2^-) through a single electron transfer reaction mediated by a membrane-bound NADPH oxidase.^{1–3)} The O_2^- undergoes rapid dismutation to hydrogen peroxide (H_2O_2).⁴⁾ The Harber–Weiss or transition metal-catalyzed Harber–Weiss reaction of O_2^- and H_2O_2 results in the formation of a hydroxyl radical ($OH\cdot$),⁵⁾ which may play a significant role in the bactericidal activity of neutrophils⁶⁾ and the pathology of tissue injury.^{7,8)}

It has been known that non-specific peroxidases (PO)-including horseradish peroxidase, an iron protoporphyrin enzyme, oxidize the substrate (SH_2) in the presence of H_2O_2 as follows.^{9–11)}

In addition, the substrate radical ($SH\cdot$) can also react with O_2 to produce O_2^- .¹¹⁾ A non-specific peroxidase of neutrophils, myeloperoxidase (MPO) (EC 1.11.1.7), is also an iron protoporphyrin enzyme. Therefore, it was thought that the above series of reactions with horseradish peroxidase might occur also in the oxidation of substrate by MPO in the presence of H_2O_2 .

It is interesting to examine the generation of $OH\cdot$ in the MPO– H_2O_2 – SH_2 system, in connection with the bactericidal ability of NADPH oxidase-deficient neutrophils and neutrophil-related tissue injury. We have previously reported that the hydroxylation of phenylalanine by MPO in the presence of a high concentration of H_2O_2 (1 mM) occurs, and that this hydroxylation is caused by $OH\cdot$.¹²⁾ In the present study, we examined the $OH\cdot$ generation by MPO in the presence of an electron-donor and a lower concentration of H_2O_2 by using phenylalanine as the $OH\cdot$ -trapping agent; the formation of tyrosines was monitored by HPLC. As electron donor, we employed NADH, because

it acts as an electron donor of peroxidase¹¹⁾ and its reactivity with O_2^- is very poor.¹³⁾

Materials and Methods

Phenylalanine, NADH, catalase (from bovine liver, 42000 units/mg), superoxide dismutase (SOD) (from bovine erythrocytes, 3000 units/mg) and cytochrome c were purchased from Sigma Chemical Co., mannitol, sodium formate, sodium benzoate, 1,4-diazabicyclo[2.2.2]octane (DABCO) and hypoxanthine (HX) from Wako Pure Chemicals, and alcohol dehydrogenase (from yeast) from Oriental Yeast Co. Xanthine oxidase (XO) (from cow's milk) was obtained from Boehringer Mannheim GmbH. The XO preparation was passed through a Sephadex G-25 column, washed with water before use. MPO (from human neutrophils) was purchased from Green Cross Co. The MPO preparation was electrophoretically homogeneous and its ratio of absorbance at the Soret maximum to the absorbance at 280 nm (*RZ* value) was not less than 0.8. The enzyme activity was determined by the guaiacol test.¹⁴⁾ The XO activity was assayed by measuring the absorption of uric acid at 290 nm at 35°C. One unit of activity was defined as the production of 1 nmol of uric acid per min at pH 7.5. Generation of O_2^- in the XO and MPO systems was assayed in terms of cytochrome c reduction. Reaction mixtures containing 2 mM phenylalanine, 1 mM NADH and MPO, in the presence and absence of other additives, in 0.5 ml 50 mM citrate buffer (pH 4.5), were incubated with shaking at 35°C. Reaction mixtures containing 2 mM phenylalanine, 1 mM HX and XO, in the presence and absence of other additives, in 0.5 ml 50 mM citrate buffer (pH 4.5), were also incubated with shaking at 35°C. After incubation for the times indicated, 50 μ l of catalase (1 mg/ml) was added to stop the reaction. One hundred μ l of the mixture was directly injected into the HPLC. The *o*-, *m*- and *p*-tyrosines derived from phenylalanine were measured by HPLC under the conditions described previously.¹⁵⁾ Determination of NAD^+ , formed in the reaction mixture, was carried out by assaying its coenzyme activity.¹⁶⁾

Results and Discussion

Formation of Tyrosines from Phenylalanine by the MPO–NADH– H_2O_2 System When phenylalanine was incubated with MPO, NADH, and H_2O_2 , significant amounts of *o*-, *m*- and *p*-tyrosine isomers, were observed (Table I). The tyrosine formation by the MPO–NADH– H_2O_2 system ceased when MPO was removed and was greatly reduced by the omission of NADH. Interestingly, the system without the external addition of H_2O_2 and the MPO–NADH system was also found to cause

TABLE I. Hydroxylation of Phenylalanine by the MPO-NADH-H₂O₂ System

Reaction systems	Tyrosines formed (pmol/0.5 ml/5 min)		
	<i>p</i> -Tyrosine	<i>m</i> -Tyrosine	<i>o</i> -Tyrosine
Complete system	67.4	95.7	97.9
-MPO	0.28	2.48	2.86
-NADH	8.5	12.1	10.7
-H ₂ O ₂	45.7	52.8	51.2

The complete system consisted of phenylalanine (2 mM), MPO (2 units/0.5 ml), NADH (1 mM), and H₂O₂ (0.01 mM) in 50 mM citrate buffer (pH 4.5). Samples were incubated for 5 min, and then assayed as described in Materials and Methods.

Fig. 1. Time Courses of Tyrosine Formation from Phenylalanine by the MPO-NADH System

Phenylalanine was incubated with MPO (2 units/0.5 ml) and NADH (●) or with heat-treated MPO and NADH (○) under the conditions described in Materials and Methods. Samples were incubated for the indicated times, and then assayed as described. Heat-treated MPO was prepared by incubation of MPO (20 units/ml) in a boiling water bath for 5 min, and 0.1 ml of the resulting solution was added to the reaction mixture to give a final volume of 0.5 ml.

a significant hydroxylation of phenylalanine. These results suggest that an excess of H₂O₂ was not necessary for the hydroxylation of phenylalanine by the MPO-NADH system, and that the presence of traces of H₂O₂ is enough to start the hydroxylation of phenylalanine if an electron-donor is present. Traces of H₂O₂ in the reaction mixture might come from the NADH solution used, as it has been recognized that traces of H₂O₂ are always present in NADH solutions.¹¹⁾ So, in the following experiments, H₂O₂ was not added to the reaction system, and the hydroxylation of phenylalanine was expressed as the total amount of *o*-, *m*-, and *p*-tyrosines. We observed that oxidation of NADH to NAD⁺ and O₂⁻ production were produced significantly by MPO in 50 mM citrate buffer (pH 4.5), but not by heat-inactivated MPO (data not shown). Figure 1 shows the time course of tyrosine formation from phenylalanine and NADH with MPO or heat-treated MPO. With active MPO, significant amounts of tyrosines were formed and the formation increased with the passage of time. Tyrosine formation by the MPO-NADH system was greatly reduced on addition of heat-inactivated MPO, instead of active MPO. It was observed that significant oxidation of NADH to NAD⁺ and O₂⁻ production were

Fig. 2. Effect of MPO on Tyrosine Formation from Phenylalanine in the Presence of NADH

Phenylalanine was incubated with NADH and the indicated amount of MPO under the conditions described in Materials and Methods. Samples were incubated for 15 min, and then assayed as described.

Fig. 3. Effect of pH on Tyrosine Formation from Phenylalanine by the MPO-NADH System

Phenylalanine was incubated with MPO (2 units/0.5 ml) and NADH under the conditions described in Materials and Methods, except for using 50 mM citrate buffer (pH 3–6) (●) and 50 mM phosphate buffer (pH 6–8) (○). Samples were incubated for 15 min, and then assayed as described.

produced by active MPO, but not by heat-inactivated MPO, under the conditions in Fig. 1 (data not shown). Tyrosine formation by the MPO-NADH system increased with increasing amount of MPO at the concentrations employed (Fig. 2). The pH-dependency of hydroxylation by the MPO-NADH system showed that this was favored by acidic conditions (Fig. 3). This pH-dependency was similar to that of NADH oxidation by MPO (data not shown).

Factors Modifying Tyrosine Formation from Phenylalanine by the MPO-NADH System To obtain evidence that the hydroxylation of phenylalanine by the MPO-NADH system was actually caused by OH·, the effects of molecular oxygen and some radical scavengers were examined. Under anaerobic conditions produced by replacement of oxygen with nitrogen gas, tyrosine formation was approximately

TABLE II. Effect of Radical Scavengers on Tyrosine Formation from Phenylalanine by the MPO-NADH System

Scavengers added	Concentration (mM)	Tyrosines (<i>o</i> -+ <i>m</i> -+ <i>p</i> -) formed
		% of control
None (control)		100
Mannitol	1.0	78
	10	49
Sodium formate	1.0	95
	10	27
Sodium benzoate	1.0	91
	10	26
Thiourea	2.5	79
	10	10
Urea	10	100
DABCO	10	102
SOD	0.04 µg/ml	60
	0.2 µg/ml	34
	0.4 µg/ml	11
Catalase	10 µg/ml	87
	100 µg/ml	31

Phenylalanine was incubated with MPO (2 units/0.5 ml), NADH and the specified amount of various compounds in 50 mM citrate buffer (pH 4.5) under the conditions described in Materials and Methods. Samples were incubated for 15 min, and then assayed as described. When heat-treated SOD (0.4 µg/ml) or catalase (100 µg/ml), prepared by incubation in a boiling water bath for 10 min, was added to the above reaction mixture containing phenylalanine, MPO, and NADH, the amount of tyrosines formed was 97% of the control.

8% of that under aerobic conditions. This finding suggests the participation of molecular oxygen in the hydroxylation of phenylalanine by the MPO-NADH system. The effect of radical scavengers is summarized in Table II. Hydroxyl radical scavengers such as mannitol, sodium formate, sodium benzoate, and thiourea effectively prevented tyrosine formation. When the action of the hydroxyl radical scavengers on MPO activity was examined by the guaiacol test¹⁴⁾ in 50 mM citrate buffer (pH 4.5), significant effects on MPO activity were not observed with all the hydroxyl radical scavengers listed in Table II. The enzyme exhibited activities of 104, 101, 97, and 100% of control values in the presence of mannitol (10 mM), sodium formate (10 mM), sodium benzoate (10 mM), and thiourea (10 mM), respectively. DABCO, employed as a scavenger of singlet oxygen, had no effect on the hydroxylation of phenylalanine. These results suggest that OH· production occurs in the MPO-NADH system under aerobic conditions. The role of O₂⁻ and H₂O₂ in the OH· formation by the MPO-NADH system was also examined by observing the effect of SOD and catalase on the hydroxylation of phenylalanine. SOD strongly inhibited tyrosine formation, while catalase was less effective, and these inhibitory effects were abolished by heat treatment. These results suggest the involvement of O₂⁻ in OH· formation by the MPO-NADH system.

To obtain information on the mechanism of OH· formation by the MPO-NADH system, the inhibitory effects of SOD and catalase on tyrosine formation by the MPO-NADH system were compared with those of the HX-XO system, which has been well characterized as a system for generating O₂⁻¹⁷⁾ (Table III). Both catalase and SOD significantly inhibited tyrosine formation by the MPO-NADH system. In addition, tyrosine formation by the MPO-NADH system was strongly inhibited by SOD, but not by catalase. Also, the O₂⁻ forming and hydroxylating

TABLE III. Effect of SOD and Catalase on Tyrosine Formation from Phenylalanine by the MPO-NADH and HX-XO Systems

Additions	Reaction systems	
	MPO-NADH	HX-XO
	% of control	
None (control)	100 (232) ^{a)}	100 (239) ^{a)}
Catalase (2.5 µg/0.5 ml)	93	5
SOD (2 µg/0.5 ml)	6	28

Phenylalanine was incubated with MPO (1.6 units/0.5 ml) and NADH or with HX and XO (4.2 units/0.5 ml) in the presence and absence of the indicated amount of catalase and SOD in 50 mM citrate buffer (pH 4.5) under the conditions described in Materials and Methods. Samples were incubated for 15 min, and then assayed as described. a) The figures in parentheses represent the amount of tyrosines (*o*-+*m*-+*p*-) formed in pmoles in 0.5 ml of reaction mixture in 15 min.

TABLE IV. Determination of O₂⁻ and Tyrosine Formation by the MPO-NADH and HX-XO Systems

	O ₂ ⁻ formed (pmol/0.5 ml/min)	Tyrosines (<i>o</i> -+ <i>m</i> -+ <i>p</i> -) formed (pmol/0.5 ml/min)
MPO-NADH system	83.3 ± 6.3	22.3 ± 5.2
HX-XO system	283.3 ± 37.8	21.0 ± 4.7

Phenylalanine was incubated with MPO (0.8 units/0.5 ml) and NADH or with HX and XO (1.6 units/0.5 ml) in 50 mM citrate buffer (pH 4.5) under the conditions described in Materials and Methods. O₂⁻ and tyrosine formed were assayed as described. Each value represents the mean ± S.D. of at least three separate experiments.

abilities of the MPO-NADH system were compared with those of the HX-XO system. Although O₂⁻ formation by the MPO-NADH system was only about 29% of that by the HX-XO system, under the experimental conditions employed, tyrosine formation by the two systems was similar (Table IV). To determine whether contamination by traces of free metal ions had an effect in the above tyrosine formation or not, the action of desferrioxamine (1 mM), a chelating agent of free iron ions, on the hydroxylation of phenylalanine by above the MPO-NADH system, was examined. In addition, the effect of neocuproine (1 mM), a chelating agent of Cu(I) ions, was also examined. Because Cu(I) ions, formed by the reduction of Cu(II) ions by O₂⁻, are powerful electron-donors the reaction with H₂O₂ to give OH· was highly probable. No inhibitory effect with the both chelating agents, however, was observed (data not shown). The results suggest that the formation of OH· from O₂⁻ may occur more effectively in the MPO-NADH system than that in the HX-XO system.

The results obtained in the present study suggest that OH· production occurs effectively in the MPO-NADH system under aerobic conditions, and O₂⁻ is the main precursor of OH·. As described in the introduction, it is known that O₂⁻ formation occurs during the action of peroxidase in the presence of H₂O₂ and an electron donor.¹¹⁾ We believe that the mechanism of NADH oxidation by MPO in the presence of H₂O₂ is similar to that exhibited by horseradish peroxidase.¹¹⁾ That is,

Although the mechanism of $\text{OH}\cdot$ formation from O_2^- is obscure, possible reactions resulting in $\text{OH}\cdot$ formation may be dismutation of O_2^- and the Harber–Weiss reaction, as follows,

The inhibitory effect of catalase was much less in the MPO–NADH system than in the HX–XO system (Table II). $\text{OH}\cdot$ formation from O_2^- took place in the MPO–NADH system (Table III). Based on these facts, we believe that an additional mechanism of $\text{OH}\cdot$ formation from O_2^- and H_2O_2 in the MPO–NADH system is as follows: traces of H_2O_2 produced in the reaction medium may bind to MPO molecules to form $\text{MPO}(\text{Fe}^{3+})\cdot\text{H}_2\text{O}_2$ (compound I). Iron ion (Fe^{3+}) in MPO protoporphyrin is reduced to Fe^{2+} by O_2^- . Then, H_2O_2 may react with Fe^{2+} in the MPO molecule to form $\text{OH}\cdot$. Therefore, the so-called iron catalyzed Harber–Weiss reaction may occur in the MPO molecule as shown in the following equations.

Although further studies are necessary to clarify the mechanism of $\text{OH}\cdot$ formation by the MPO–NADH system and to obtain information about the nature of the electron donor of MPO *in vivo*, the present findings suggest the existence of an additional $\text{OH}\cdot$ generating system through

which MPO acts in neutrophils or in neutrophil-mediated tissue injury.

References

- 1) B. M. Babior, R. S. Kipnes, and J. T. Curnutte, *J. Clin. Invest.*, **52**, 741 (1973).
- 2) R. B. Johnston, B. B. Kelle, H. P. Misra, J. E. Lehmeyer, L. S. Webb, R. L. Baehner, and K. V. Rajagopalan, *J. Clin. Invest.*, **55**, 1357 (1975).
- 3) H. J. Cohen, P. E. Newburger, M. E. Chovaniec, J. C. Whitin, and E. R. Simon, *Blood*, **58**, 975 (1981).
- 4) R. K. Root, J. Metcalf, N. Oshino, and B. Chance, *J. Clin. Invest.*, **55**, 945 (1975).
- 5) F. Haber and J. Weiss, *Proc. R. Soc. London, A*, **147**, 332 (1934).
- 6) S. J. Klebanoff, *Semin. Hematol.*, **12**, 117 (1975).
- 7) S. J. Weiss, *Acta Physiol. Scand. Suppl.*, **548**, 9 (1986).
- 8) S. J. Weiss, *N. Engl. J. Med.*, **320**, 365 (1989).
- 9) H. S. Mason, *Adv. Enzymol.*, **19**, 79 (1957).
- 10) I. Yamazaki, H. S. Mason, and L. Piette, *J. Biol. Chem.*, **235**, 2444 (1960).
- 11) B. Halliwell and J. M. C. Gutteridge, "Free Radicals in Biology and Medicine," Oxford University Press, Oxford, 1987, p. 79.
- 12) S. Fujimoto, S. Ishimitsu, S. Hirayama, N. Kawakami, and A. Ohara, *Chem. Pharm. Bull.*, **39**, 1598 (1991).
- 13) P. C. Chan and B. H. J. Bielski, *J. Biol. Chem.*, **249**, 1317 (1974).
- 14) B. Chance and A. C. Maehly, "Methods in Enzymology," Vol. 2, ed. by P. S. Colowick and N. O. Kaplan, Academic Press, New York, 1955, p. 770.
- 15) S. Ishimitsu, S. Fujimoto, and A. Ohara, *J. Chromatogr.*, **378**, 222 (1986).
- 16) M. M. Ciotti and N. O. Kaplan, "Methods in Enzymology," Vol. 3, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, 1957, p. 891.
- 17) I. Fridovich, *J. Biol. Chem.*, **245**, 4053 (1970).