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A study on the NO-myeloperoxidase complex by absorption and electron paramagnetic (spin) resonance spectroscopies

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Abstract : The nitric oxide (NO) complexes of native myeloperoxidase and a modified myeloperoxidase (a cytochrome oxidase-like derivative) were investigated by absorption and electron paramagnetic (spin) resonance (ESR) spectrometries. The ferrous native myeloperoxidase formed two different complexes with NO, at low and high NO concentrations, while the ferrous modified myeloperoxidase formed only one complex regardless of the concentration of NO. The ESR spectrum of the NO complex of native myeloperoxidase (ferrous form) showed a characteristic triplet-triplet hyperfine structure, indicating a six-coordinate heme, and that of the modified myeloperoxidase (ferrous form) showed a single triplet hyperfine structure, indicating a five-coordinate heme. These results reveal that the native myeloperoxidase has two binding sites to NO, probably heme iron and the formyl group (-CHO) on the porphyrin ring of heme, and that the modified myeloperoxidase has only one such binding site, probably heme iron. Thus, the modification caused the loss of one binding site, probably that corresponding to the formyl group in the native myeloperoxidase. The ESR spectra of the native and modified myeloperoxidase complexes also indicated that the heme iron in native myeloperoxidase bonds with a fifth (proximal) ligand, probably the N nucleus of a histidine residue in the protein moiety of this enzyme, and that the heme iron in modified myeloperoxidase interacts weakly or does not bond with a fifth (proximal) ligand, probably the N nucleus of an amino residue in the protein moiety of this enzyme. The myeloperoxidase *in vivo* may scavenge NO, resulting in elimination of the NO molecule's paramagnetism.

抄録：一酸化窒素 (NO) と、還元型ミエロペルオキシダーゼ (MPO) および還元型修飾 MPO (シトクロムオキシダーゼ様誘導体) との結合物について、吸収スペクトルと電子スピン共鳴 (ESR) スペクトルを測定した。吸収スペクトルから MPO は低濃度、高濃度の NO の存在下で 2 種類の異なる NO 結合物を生成し、修飾 MPO は NO の濃度にかかわらず結合物は 1 種であることが明らかになった。NO に対する結合は MPO ではヘム鉄とポルフィリン環のホルミル側鎖の 2 カ所、修飾 MPO では修飾によりホルミル側鎖の NO に対する反応性を失い、ヘム鉄 1 カ所であることが示唆された。ESR スペクトルは MPO の NO 結合物ではヘムの 6 配位状態に特徴的な 3 組の 3 本線の超微細構造を示し、修飾 MPO の NO 結合物ではヘムの 5 配位状態に特徴的な 1 組の 3 本線の超微細構造を示した。第 5 配位子はタンパク質部分のヒスチジン残基の N が考えられる。MPO が NO と結合する生物学的意義は、常磁性を有する NO を捕捉し、その不対電子を消去する生体防御系としての役割が考えられる。

Introduction

The prosthetic group of the myeloperoxidase that is naturally present in neutrophils has been examined previously¹⁻⁴). We reported that when myeloperoxidase was acidified and gradually neutralized, a derivative was formed (named modified myeloperoxidase) that showed a comparable peroxidase activity to the untreated enzyme and a similar absorption spectrum to cytochrome oxidase⁵). We also reported that the myeloperoxidase heme was an *a*-type heme analog having a formyl group (-CHO) on the porphyrin ring, similar to the heme *a* existing in cytochrome oxidase, from examination of the reactivity of the enzyme with carbonyl reagents such as borohydride and hydrazine, and with nucleophiles such as cyanide, by using absorption and electron paramagnetic (spin) resonance (ESR) spectrometries⁵⁻⁷). It has been reported that two iron atoms were present in one myeloperoxidase molecule^{1,3}). We have also reported that two iron atoms were present in the myeloperoxidase molecule⁸), of which one existed in a formyl-heme moiety similar to heme *a* and the other existed as a non-heme iron⁷). The myeloperoxidase heme may be a proto-heme IX derivative^{9,10}) and its carbonyl side chain may interact with Asp²⁶⁰ residue⁹) of the protein moiety. However, the chemical entity of the myeloperoxidase heme is still to be identified, because of the difficulty in splitting intact heme from myeloperoxidase.

By X-ray crystallography, Kendrew¹¹), Perutz¹²) and Perutz *et al.*¹³) confirmed that the proximal ligand of hemoglobin was due to the histidine residue. In studies on the heme in hemoproteins, nitric oxide (NO) was proven to be an effective ESR probe for monitoring changes in the heme environment and identifying the nature of the axial ligand of the heme iron in the hemoproteins¹⁴⁻²⁹). According to these reports, the ESR spectra of NO-hemoprotein complexes were assigned to coordination spheres of the heme in the NO adduct : five-coordinate heme (exhibits a single triplet hyperfine structure), with a strong iron-NO bond and a weak or broken iron-proximal histidine bond ; and six-coordinate heme (exhibits a triplet-triplet hyperfine structure), with a strong iron-proximal

histidine bond. Ferric myeloperoxidase forms a complex with nitrite ($\text{Fe}^{3+}/\text{NO}_2^-$)³⁰), and in the case of lactoperoxidase the ferric-nitrite complex converts easily to NO-ferrous complex (Fe^{2+}/NO) in the presence of a sufficient amount of reducing agent such as sodium hydrosulfite³¹).

The aims of this study were (a) to determine whether native and modified myeloperoxidases equally and stoichiometrically react with NO, (b) to determine how many binding sites to NO the native and modified enzymes have, (c) to determine how the heme environment of the myeloperoxidase changes during the reaction with NO, (d) to identify the nature of the axial ligand of the iron in a myeloperoxidase having a formyl heme as a prosthetic group, by measuring the reactivity of the heme moiety with NO, and (e) to discuss the biological significance of the reaction between myeloperoxidase and NO.

We used absorption and electron paramagnetic (spin) resonance spectrometries to demonstrate that NO yields two different complexes of ferrous native myeloperoxidase and one complex of ferrous modified myeloperoxidase, and that the ESR spectra of NO-ferrous myeloperoxidases show characteristic triplet-triplet (native) and single triplet (modified) structures.

Materials and Methods

The myeloperoxidase (EC 1. 11. 1. 7) was prepared from polymorphonuclear leukocytes of normal pig blood⁷). The concentration of myeloperoxidase was calculated on the basis of a millimolar extinction coefficient of $95 \text{ mM}^{-1} \text{ cm}^{-1}$ at 430 nm. The peroxidase preparation typically exhibited an R. Z. value of more than 0.85 and was previously confirmed to give a single band on polyacrylamide gel electrophoresis. The modified myeloperoxidase, a cytochrome oxidase-like derivative, was prepared as described previously^{5,7}). The concentration of modified myeloperoxidase was calculated on the basis of a millimolar extinction coefficient of $111 \text{ mM}^{-1} \text{ cm}^{-1}$ at 428 nm. The myeloperoxidase and modified enzyme preparations were in ferric form. In the presence of sodium hydrosulfite, they were reduced to ferrous form

in 100% yield.

The spectrophotometric studies were carried out with a Hitachi instrument, type 200-20 or 557, or with a Shimadzu instrument, type MPS-5000, using a hand-made quartz cuvette (10 mm × 10 mm × height 90 mm) connected with a Thunberg tube modified for the experiments under anaerobic conditions at 25°C. The anaerobic conditions were made by passing argon gas (99.999%) over the surface of the solution at 0.2 kg/cm² from 30 min before and during the experiments. NO gas was used as a saturated solution in water, with NO at 0°C equivalent to 0.0984 g/kg water, i. e., 3.28 mM. Stock solutions of sodium hydrosulfite (saturated in 0.1 M NaOH), sodium nitrite (saturated in water) and 0.127 M potassium cyanide (in water) were prepared anaerobically in a Thunberg tube filled with nitrogen gas (99.9995%). The experiments were carried out in 0.01 M acetate (acetic acid/sodium acetate) buffer (pH 5.5).

In the present study, a nitric oxide-generating system (sodium hydrosulfite + sodium nitrite)²⁵⁾ was used to generate a continuous and adequate supply of NO in order to produce a uniform NO-myeloperoxidase complex and to avoid foaming of the concentrated myeloperoxidase that would be caused by the bubbling of NO gas.

The ESR experiments were carried out with a Varian E-4 spectrometer equipped with an automatic temperature controller or with a Varian V-4500 x-band instrument under the following conditions : field modulation, 100 kcycles ; modulation amplitude, 4 gauss ; microwave power, 20 mW ; microwave frequency, 9.023 GHz or 9.118 GHz ; time constant, 1 or 0.3 sec ; scan speed, 50 or 125 gauss per min ; and centering at 3.2 kgauss. A variable temperature assembly was employed for measurements at low temperatures using liquid nitrogen as a refrigerant. The *g* values were determined by measuring magnetic field strengths²²⁾ and/or by using a standard, 2, 2-diphenyl-1-picrylhydrazyl, and hyperfine coupling constants were estimated directly from charts. Ferrous nitrosyl myeloperoxidase derivative was prepared in a Thunberg tube, provided with a 1-cm quartz optical cell, in which ferric native or modified enzyme solution in 0.01 M acetate buffer (pH 5.5)

was placed, and two arms, in which solid sodium hydrosulfite (final concentration of 50 mM) and sodium nitrite (final concentration of 40 mM) were respectively placed. Under an argon atmosphere, sodium hydrosulfite and sodium nitrite, from the side arms of Thunberg tube, were added to the enzyme solution in the Thunberg tube and the tube was shaken gently and incubated for 25 min at 2°C. The nitric oxide compound formed was anaerobically transferred to ESR tube (quartz tube ; internal diameter 3.6 mm × length 190 mm) and immediately frozen in liquid nitrogen. The nitrosyl native and modified myeloperoxidase derivative samples left in the Thunberg tube were diluted with 0.01 M acetate buffer (pH 5.5) gassed with argon, and the light absorption spectra were determined.

Argon and NO gases were purchased from Takachiho Chemical Co. (Machida, Tokyo). Sodium hydrosulfite (Na₂S₂O₄), sodium nitrite (NaNO₂) and potassium cyanide (KCN) were purchased from Wako Pure Chemical Industries (Osaka). Other reagents were obtained from commercial sources in the highest available states of purity, unless otherwise stated.

Results

Absorption spectra of two different NO-myeloperoxidase complexes are shown in Figure 1. A specific complex, having absorption peaks at 465 and 630 nm (termed NO complex I), was formed in the reaction between ferrous myeloperoxidase and its equivalent amount of NO and by further bubbling of excess NO, complex I was converted into a new complex with absorption peaks at 450 and 628 nm (termed NO complex II). When the ferrous myeloperoxidase was titrated with NO, the change in absorbance was proportional to the amount of NO added up to the amount equivalent to the ferrous enzyme (inset of Fig. 1). From this, it was concluded that NO complex I is a compound formed from the reaction of ferrous myeloperoxidase with 1 mole NO and that NO complex II probably bonds a further 1 mole NO to NO complex I, i. e., it bonds a total of 2 moles NO. These two NO complexes were also observed in the presence of a NO-generating system (15 mM sodium hydrosul-

Fig. 1 Absorption spectra of the complexes between ferrous myeloperoxidase and NO.

Curve a, 4.6 μM ferric myeloperoxidase under anaerobic conditions; curve b (ferrous myeloperoxidase), 15 mM $\text{Na}_2\text{S}_2\text{O}_4$ added to the solution (a); curve c (NO-ferrous myeloperoxidase complex I) was recorded after the addition of 9.4 μM NO to the solution (b); and curve d (NO-ferrous myeloperoxidase complex II) was obtained after bubbling NO gas through the solution (c). The inset shows stoichiometry of the reaction between ferrous myeloperoxidase and NO. Aliquots of the saturated NO solution were added stepwise to the ferrous myeloperoxidase solution in the presence of hydrosulfite (as in curve b) under anaerobic conditions. Total volume of the reaction mixture was 2.5 ml. The experiments were carried out at 25°C in 0.01 M acetate buffer (pH 5.5).

fite+12.5 mM sodium nitrite), as in Table 1. In the process of changing from NO complex I to complex II, an isobestic point was observed at 455 nm in the absorption spectra, and completion of the reaction was observed in 20 min. When 1.27 mM potassium cyanide was added to NO complex I, its absorption peaks were shifted to 463 and 615 nm. On the other hand, treatment of NO complex II with 1.27 mM potassium cyanide caused the appearance of a compound having light absorption peaks at 454 and 634 nm, which was very similar to cyanide complex II⁸⁾ of the ferric form of native myeloperoxidase. A NO

Fig. 2 Absorption spectrum of the complex between ferrous modified myeloperoxidase and NO.

Curve a, 3.8 μM ferric modified myeloperoxidase in 0.01 M acetate buffer (pH 5.5) under anaerobic conditions; curve b (ferrous modified myeloperoxidase), 15 mM $\text{Na}_2\text{S}_2\text{O}_4$ added to the solution (a); curve c (NO-ferrous modified myeloperoxidase complex) was recorded after the addition of 15 μM NO to the solution (b). The inset shows stoichiometry of the reaction between ferrous modified myeloperoxidase and NO. Aliquots of the saturated NO solution were added stepwise to the ferrous modified myeloperoxidase solution in the presence of sodium hydrosulfite (as in curve b) under anaerobic conditions. Total volume of the reaction mixture was 2.5 ml. The experiments were carried out at 25°C in 0.01 M acetate buffer (pH 5.5).

complex having light absorption peaks at 424 and 600 nm was produced from the reaction between ferrous modified myeloperoxidase and its equivalent amount of NO (Fig. 2). When the ferrous modified myeloperoxidase was titrated with NO, the change in absorbance was proportional to the amount of NO added up to the amount equivalent to the ferrous modified enzyme (inset of Fig. 2). Further bubbling of excess NO did not convert the NO-complex into a new one. The complex was also formed from the ferrous modified myeloperoxidase in the presence of sodium hydrosulfite and sodium nitrite. In both reaction systems, only one species of NO complex was found, and

Table 1 The number of NO in NO-ferrous myeloperoxidase complexes, the number of CN in CN-myeloperoxidase complexes and the absorption peaks of these complexes and related derivatives

Enzyme form	System	Derivatives	Mole (ligand)	Wavelength (nm)	
Native	$\text{Fe}^{3+} + \text{Na}_2\text{S}_2\text{O}_4 + \text{NO}$	Fe^{2+}/NO complex I	1 (NO)	465	630
	$\text{Fe}^{3+} + \text{Na}_2\text{S}_2\text{O}_4 + \text{NaNO}_2$	Fe^{2+}/NO complex I	1 (NO)	465	630
	$\text{Fe}^{3+} + \text{Na}_2\text{S}_2\text{O}_4 + \text{NO}$	Fe^{2+}/NO complex II	2 (NO)	450	628
	$\text{Fe}^{3+} + \text{Na}_2\text{S}_2\text{O}_4 + \text{NaNO}_2$	Fe^{2+}/NO complex II	2 (NO)	450	628
	$\text{Fe}^{3+} + \text{NO}_2^-$	$\text{Fe}^{3+}/\text{NO}_2^-$ complex ³⁰⁾		447	573 628
	Fe^{2+}/NO complex I + KCN			463	615
	Fe^{2+}/NO complex II + KCN			454	634
	$\text{Fe}^{3+} + \text{KCN}$	Fe^{3+}/CN complex II	2 (CN) ⁸⁾	454	634
	$\text{Fe}^{3+} + \text{Na}_2\text{S}_2\text{O}_4 + \text{KCN}$	Fe^{2+}/CN complex	1 (CN) ⁵⁾	463	615
Modified	$\text{Fe}^{3+} + \text{Na}_2\text{S}_2\text{O}_4 + \text{NO}$	Fe^{2+}/NO complex	1 (NO)	424	600
	$\text{Fe}^{3+} + \text{Na}_2\text{S}_2\text{O}_4 + \text{NaNO}_2$	Fe^{2+}/NO complex	1 (NO)	424	600

Fe^{3+} , ferric myeloperoxidase; Fe^{2+} , ferrous myeloperoxidase. The conditions were the same as in the experiments in Figs. 1 and 2. The concentrations of $\text{Na}_2\text{S}_2\text{O}_4$, NaNO_2 and KCN were 15 mM, 12.5 mM and 1.27 mM, respectively

both complexes exhibited essentially the same light absorption spectra (Table 1).

Figure 3 shows the ESR spectra of NO-ferrous native myeloperoxidase and NO-ferrous modified myeloperoxidase. These ESR samples were characterized respectively as NO-ferrous native myeloperoxidase and NO-ferrous modified myeloperoxidase complexes by spectroscopy, i. e., the former had the same light absorption peaks at 450 and 628 nm as those of the NO complex II observed in the experiment of Figure 1 (curve d) and the latter had the same light absorption peaks at 424 and 600 nm as those of the NO complex observed in the experiment of Figure 2 (curve c). The ESR signal of NO-ferrous native myeloperoxidase clearly showed rhombic symmetry, exhibiting three well-resolved g -values at $g_x = 2.081$, $g_z = 2.006$ and $g_y = 1.965$, and the hyperfine structure of the central resonance signal (g_z) was composed of nine very regularly spaced lines as a triplet of triplets (curve B) with 7 G splitting (Table 2), indicating a six-coordinate heme. The ESR signal of NO-ferrous modified myeloperoxidase (curve D) had a characteristic single triplet hyperfine structure at $g = 2.022$, 2.011 and 2.001 with 20 G splitting (Table 2), indicating a five-coordinate heme.

Discussion

The results in Figure 1 and Table 1 revealed that the myeloperoxidase molecule had two binding sites to NO. These binding sites may be two iron atoms, both sides of a single heme iron, or heme iron and a porphyrin ring side chain, for example, a formyl group ($-\text{CHO}$), which is well known to have a high affinity for nucleophiles. The latter case is suggested by the fact that the heme group of myeloperoxidase is a formyl heme, heme *a* or its analogue^{5,7)}. Several findings indicate that heme iron and the formyl group are the binding sites of the NO molecule. Of special importance are the facts that, in the presence of cyanide, NO complex I converts into cyanide-ferrous myeloperoxidase complex, and that NO complex II converts into another compound having a light absorption spectrum similar to that of cyanide-ferric myeloperoxidase complex II⁸⁾, suggesting the oxidation of the heme iron from the ferrous to ferric state. The former can be explained by the replacement of NO with cyanide. However, it is difficult to explain the latter finding, because the amount of reductant, sodium hydrosulfite, present in the reaction solution, may be sufficient to countervail the back-donating

Fig. 3 The ESR spectra of NO-ferrous native and modified myeloperoxidases.

Curve A was obtained after the additions of 50 mM $\text{Na}_2\text{S}_2\text{O}_4$ and 40 mM NaNO_2 to 0.2 mM native myeloperoxidase at 93°K; curve B was obtained after the additions of 50 mM $\text{Na}_2\text{S}_2\text{O}_4$ and 40 mM NaNO_2 to 0.35 mM native myeloperoxidase at 77°K; curve C, 0.25 mM ferric modified myeloperoxidase at 93°K; and curve D was obtained after the additions of 50 mM $\text{Na}_2\text{S}_2\text{O}_4$ and 40 mM NaNO_2 to 0.25 mM modified myeloperoxidase at 93°K. The experiments were carried out in 0.01 M acetate buffer (pH 5.5). Modulation amplitude, 4 gauss; microwave power, 20 mW; microwave frequency, 9.023 GHz (A, C and D), 9.118 GHz (B); time constant, 1 sec (A, C and D), 0.3 sec (B); scan speed, 125 gauss per min (A, C and D), 50 gauss per min (B); and centering at 3.2 kgauss.

Table 2 The g-values and superfine coupling constants (in gauss) of NO-ferrous myeloperoxidases

Complex	g-value (signal)			Coupling constant
NO-ferrous native MPO	2.081 (g_x)	2.006 (g_z)		
		1.965 (g_y)		
		2.023 (a-1)	2.010 (b-1)	1.995 (c-1)
		2.019 (a-2)	2.005 (b-2)	1.991 (c-2)
		2.014 (a-3)	2.001 (b-3)	1.987 (c-3)
NO-ferrous modified MPO		2.022 (a)	2.011 (b)	2.000 (c)
				20.0

The g-values of NO-ferrous native myeloperoxidase complex were estimated from the ESR signals, which are numbered 1 to 3 in a set of signals a, b and c, presented in Fig. 3 (curve B). The g-values of NO-ferrous modified myeloperoxidase complex were estimated from the ESR signals, a, b and c, presented in Fig. 3 (curve D). MPO; myeloperoxidase.

effect of NO, which converts the heme iron in NO complex II to a nearly ferric state, as Figure 4 (A). But, the light absorption peaks of NO-ferrous native myeloperoxidase complex I (Fig. 1, curve c), and the light absorption peaks of NO complex II (Fig. 1, curve d), were both not in agreement with those of the nitrite-myeloperoxidase complex³⁰⁾, as in Table 1. From this, we concluded that there was no possibility that the replacement of NO with nitrite ion (NO_2^-), similar to the replacement with cyanide, occurs during the reaction of NO-ferrous native myeloperoxidase complex I or II molecules and the nitrite ion. However modified myeloperoxidase, like the ferric native enzyme, reacts with cyanide⁵⁾ and in the reaction between the ferric modified enzyme and cyanide two different cyanide complexes were formed⁷⁾. In the present study, however, only one NO complex, having an absorption spectrum similar to NO-cytochrome oxidase³²⁾, was formed between the ferrous modified enzyme and NO (Fig. 2 and Table 1). This difference indicated that the loss of one binding site to NO was caused by the modification of myeloperoxidase. The formyl group of heme *a* interacts with an amino acid residue such as $-\text{NH}_2$ of lysine at an alkaline pH^{33,34)} and or an SH group in the frozen state³⁵⁾, as in case of cytochrome oxidase. Similar interactions may occur in the modified myeloperoxidase (Fig. 4 B). Thus, it seems that NO can react only with the heme iron in the modified enzyme. Since native myeloperoxidase reacted with an equivalent amount of NO to form NO complex I, the native and modified myeloperoxidases were found to have comparable affinities to NO (insets of Figs. 1 and 2). This suggests that the first binding site to form NO-complex I in the native myeloperoxidase molecule is the iron of heme (Fig. 4 A).

The ESR spectra of NO complexes of native and modified myeloperoxidases in Figure 3 clearly show hyperfine structures of a triplet of triplets and a single triplet, respectively. These characteristic hyperfine structures are specific to the heme iron and are not found in the ESR spectra of non-heme irons. From the present ESR spectra, it was concluded that the fifth (proximal) ligand of the heme iron in native myeloperoxidase is a nitrogen atom, probably the N

nucleus of a histidine residue of the protein moiety, which bonds tightly with the iron of heme (Fig. 4 A), and the fifth (proximal) ligand of the heme iron in the modified myeloperoxidase may also be the nitrogen atom of a histidine residue in the protein moiety of this enzyme, although the bond between the iron and the proximal ligand is weakened (Fig. 4 B). This weakened bond in the modified enzyme may be due to a strong iron-NO bond and an increase in the strength of interaction between the formyl group and amino acid residue of the protein. The ESR spectrum of NO-native myeloperoxidase has the same profile as those of cytochrome *c* peroxidase^{16,17)}, cytochrome oxidase^{20,21)} and horseradish peroxidase^{16,17,23)}, and the ESR spectrum of NO-modified myeloperoxidase was the same as those of catalase^{16,17)}, lactoperoxidase^{16,17)}, cytochrome P-450^{22,23)} and L-tryptophan-2, 3-dioxygenase²⁵⁾. Furthermore, both the *g*-values and the hyperfine splitting parameters for NO-ferrous native myeloperoxidase complex II were in agreement with those reported for cytochrome *c* peroxidase^{16,17)}, horseradish peroxidase^{16,17,23)} and cytochrome oxidase^{20,21)}, and the *g*-values and the hyperfine splitting parameters for NO-modified myeloperoxidase were consistent with those of catalase^{16,17)}, lactoperoxidase^{16,17)}, cytochrome P-450^{22,23)} and L-tryptophan-2, 3-dioxygenase²⁴⁾. These enzymes could thus be classified into two groups : group I, consisting of native myeloperoxidase, cytochrome *c* peroxidase, cytochrome oxidase and horseradish peroxidase, in which the NO complex exhibits a triplet-of-triplets hyperfine structure and the heme iron bonds tightly with the fifth (proximal) ligand, i. e., six-coordinate heme ; and group II, consisting of modified myeloperoxidase, catalase, lactoperoxidase, cytochrome P-450 and L-tryptophan-2, 3-dioxygenase, in which the NO complex exhibits a single-triplet hyperfine structure and the heme iron does not bond or weakly interact with the fifth (proximal) ligand, i. e., five-coordinate heme. From this, it appears that the bonding of the heme iron in these enzyme molecules with the proximal ligand is not essential for the expression of their activities. It is also noteworthy that the bonding between the heme iron in the hemoproteins, especially in peroxidases, and the fifth (proximal) ligand may

Fig. 4 Previsional mechanisms of the binding of NO to ferrous native (A) and modified (B) myeloperoxidases.

The Fe²⁺, -CHO, N and L indicate respectively the ferrous iron of heme, the formyl residue on the porphyrin ring, the proximal ligand (nitrogen atom of amino group of protein moiety) for heme iron, and the ligand (probably an amino group of the protein moiety) for the formyl group on the porphyrin ring. The weak interaction is represented by dotted bond. The outer frame shows the protein moiety of the heme environment.

participate in the formation of a hydrogen peroxide-peroxidase compound (compound I) as a reaction intermediate, which is the first enzyme-substrate complex having a two-equivalent oxidized level above that of the ferric enzyme, i.e., the peroxidases in group I form a relatively stable compound I with hydrogen peroxide. The change of the hyperfine structure of the ESR spectrum from a triplet-triplet to a single triplet which accompanies the modification of myeloperoxidase to the cytochrome oxidase-like derivative suggests the same shifting process of the heme environment as in hemoglobin when treated with salicylate¹⁴⁾, inositol hexaphosphate²⁵⁾, pH alterations²⁶⁾, dehydration²⁷⁾, endotoxin shock²⁸⁾ and tumor necrosis factor, interleukin-1 and/or *E. coli* lipopolysaccharide²⁹⁾.

Thus, it was ascertained by measuring the absorption spectra that the native myeloperoxidase had two binding sites to NO, and the modified myeloperoxidase had only one binding site to NO; and in their ESR spectra the NO-native and modified myeloperox-

idases revealed hyperfine structure of a triplet-triplet, which indicated a six-coordinate heme, a single triplet, which indicated a five-coordinate heme. From these observations, it was concluded that the two binding sites to NO in native myeloperoxidase were probably the iron of heme and the formyl group on the porphyrin ring of heme, and that the fifth (proximal) ligand of the heme iron was probably the N nucleus of a histidyl residue of the protein moiety, which bonds tightly with the iron of heme (Fig. 4 A). The interaction of a formyl group of heme with an amino acid residue such as Asp⁹⁾ of the protein moiety in the native myeloperoxidase molecule may be a relatively weak bond cleaved by NO. In the modified myeloperoxidase, the binding site to NO may also be the iron of heme, which in this case interacts weakly or does not bond with the fifth (proximal) ligand, probably the N nucleus of an amino residue in the protein moiety of this enzyme (Fig. 4 B).

NO *in vivo* is synthesized from L-arginine by NO synthases in various organs and cells, as reviewed by

Knowles and Moncada³⁶⁾ and Schmidt and Walter³⁷⁾. It is released from phagocytes (macrophages)^{38,39)} and endothelial cells in response to stimulatory substances^{40~43)}, and generated as a product of rejection following organ transplantation⁴⁴⁾. It also acts as a neurotransmitter³⁶⁾, an endothelium-derived relaxing factor⁴⁰⁾ or a relaxing substance of mammalian blood vessels³⁷⁾, it inhibits leukocyte adhesion⁴⁵⁾ and the migration of neutrophils and their infiltration into specific tissue sites³⁹⁾, and it participates in the nitration of protein tyrosines in human atherosclerosis lesions⁴⁶⁾, but it is potentially toxic and injures organs and cells³⁷⁾. The biological significance of ferrous myeloperoxidase binding to NO *in vivo* might relate to a possible role in the molecular immune system in the host-defence system : myeloperoxidase may act as a receptor for NO, which would scavenge the NO and/or eliminate the paramagnetism of NO molecules.

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