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The Role of Ergothioneine in the Oxidation of Reduced Nicotinamide Adenine Dinucleotide by Metmyoglobin or Methemoglobin

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In phosphate buffer (pH 6.8), reduced nicotinamide adenine dinucleotide (NADH) was oxidized by metmyoglobin or methemoglobin in the presence of Mn(II) ion and ergothioneine. The NADH oxidation was dependent on the concentrations of Mn(II) ion, ergothioneine and metmyoglobin (or methemoglobin). Various phosphorus compounds also influenced the oxidation. Orthophosphate was the most effective at concentrations of more than 50 mM. In contrast, nucleotides such as adenosine-5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine-5′-monophosphate (AMP) were less effective. Superoxide dismutase or catalase inhibited the present NADH oxidation, suggesting the participation of superoxide anion(O_2^-) and H_2O_2 in this metmyoglobin system as well as the peroxidase system. Metmyoglobin pretreated with H_2O_2, *i.e.*, ferrylmyoglobin, promoted the NADH oxidation reaction, but oxymyoglobin inhibited it. A mechanism is proposed for the oxidation of NADH by metmyoglobin or methemoglobin on the basis of the redox potential of ergothioneine, the formation of Mn-phosphate complex and the heme state of metmyoglobin or methemoglobin.

Keywords——NADH oxidation; ergothioneine; manganese phosphate complex; metmyoglobin; ferrylmyoglobin; methemoglobin; superoxide anion

Ergothioneine is widely distributed in biological materials.[1] In the red blood cells of animals, in particular, it is present in high concentration[1] and in the free form.[2] The compound is characterized by the peculiarities of imidazolethione[3] and the special properties of the betaine structure. However, its role in biological systems is uncertain, though several biological activities have been suggested.[1] We have studied the physical and chemical properties of ergothioneine[3–5] in order to facilitate the elucidation of its biological functions.

Klebanoff has observed that ergothioneine stimulates the *in vitro* oxidation of reduced nicotinamide adenine dinucleotide(NADH) by peroxidase[6] and metmyoglobin (or methemoglobin).[7] The oxidase reaction of peroxidase stimulated by phenols has been demonstrated by Yokota *et al.*,[8] who presented a possible mechanism and described the reactive oxidation states of peroxidase, compounds I, II and III. Metmyoglobin and methemoglobin have the same prosthetic and proximal groups,[9–11] but are unable to form compound I[12] as horseradish peroxidase does. Although it is known that metmyoglobin or methemoglobin shows an oxidase-like reaction in the presence of ergothioneine, the reaction mechanism has not previously been clarified. In the present work, we have investigated the effects of ergothioneine, Mn(II) ion and phosphorus compounds on the *in vitro* oxidation of NADH by metmyoglobin (or methemoglobin) and we propose a possible reaction mechanism of the oxidation. The possibility of *in vivo* NADH oxidation is also discussed on the basis of the concentrations of ergothioneine and methemoglobin in red blood cells and the *in vivo* form of ergothioneine unbound to proteins.

Materials and Methods

Materials——Equine muscle myoglobin (type I), bovine hemoglobin (type I), bovine-liver catalase (34000 units/mg) (EC 1.11.1.6), superoxide dismutase (SOD) (type I, 2650 units/mg) (EC 1.15.1.1) and NADH were obtained from Sigma. Oxymyoglobin was prepared by treating metmyoglobin with a small excess of sodium dithionite and obtained by eliminating dithionite through a Sephadex G-25 (medium) column

(Pharmacia, disposable column PD-10) pre-equilibrated with 50 mM phosphate buffer, pH 6.8. The concentration was calculated from the millimolar extinction coefficients at 542 nm ($\varepsilon=13.9$) and 580 nm ($\varepsilon=14.4$).[13] Copper analysis of SOD was performed by atomic absorption spectrophotometry with a graphite furnace atomizer (Shimadzu AA-620). Ergothioneine was purchased from Fluka. 2-Mercapto-1-methyl-imidazole (MMI) from ICN K & K was used after recrystallization from ethanol. NADH, ergothioneine and MMI were freshly dissolved in buffer solution. Adenosine-5′-diphosphate (ADP) was from P-L Biochemicals, and adenosine-5′-monophosphate (AMP) and adenosine-5′-triphosphate (ATP) were from Wako Pure Chemicals. 2,3-Diphosphoglycerate (DPG) was purchased from Boehringer. 2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was obtained from Dozindo Laboratories and used at the concentration of 50 mM and pH 7.0. The phosphate buffer was prepared to pH 6.8 from 50 mM KH_2PO_4–Na_2HPO_4. All other reagents were of the highest quality available.

Methods——The oxidation of NADH was initiated by adding metmyoglobin (or methemoglobin) and determined by measuring the decrease in the absorption at 340 nm. The spectrophotometric measurements were made at 25°C with a Hitachi 320 recording spectrophotometer. The blank contained all components except NADH.

Results

In the presence of Mn(II) ion and ergothioneine, metmyoglobin oxidized NADH in 50 mM phosphate buffer. In the absence of Mn(II) ion, ergothioneine or metmyoglobin, no NADH oxidation occurred (Fig. 1). Methemoglobin had the same effect as metmyoglobin under

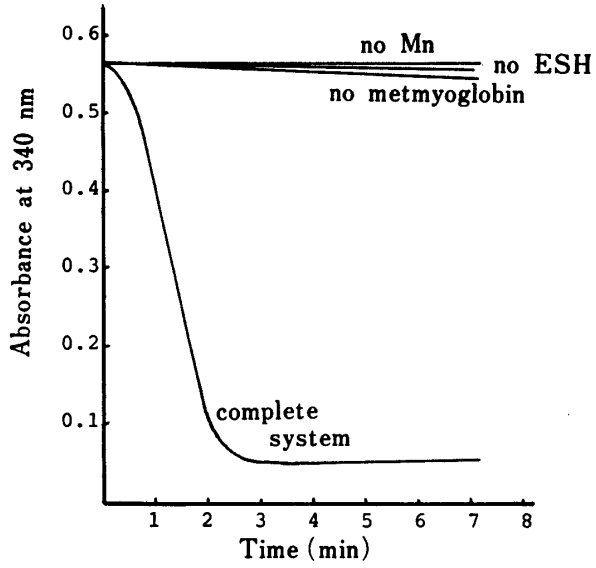

Fig. 1. Oxidation of NADH by Metmyoglobin or Methemoglobin

The complete system contained 0.1 mM NADH, 0.17 mM $MnCl_2$, 0.33 mM ergothioneine and 1.2 μM metmyoglobin or 0.3 μM methemoglobin in 50 mM phosphate, pH 6.8.

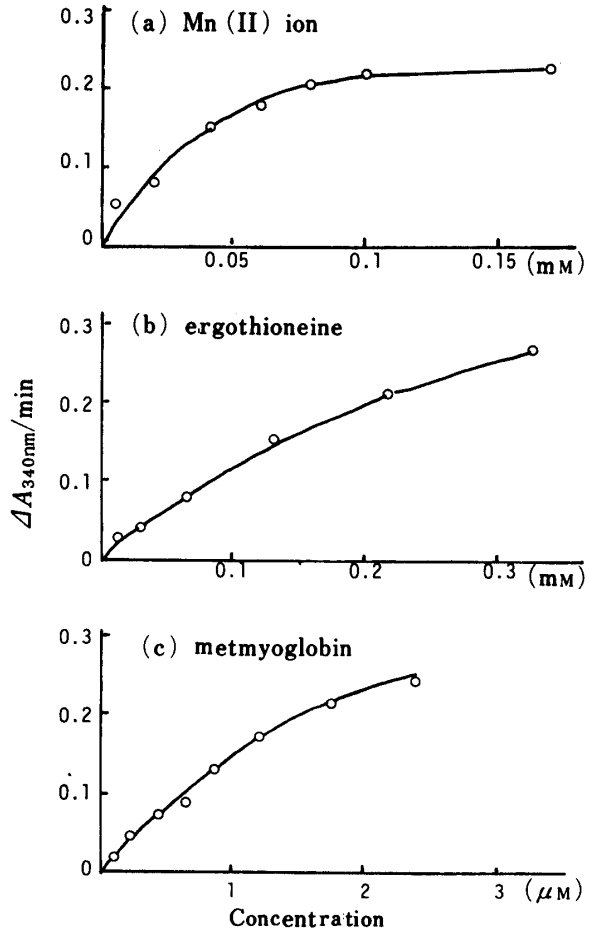

Fig. 2. Effects of Mn(II) Ion, Ergothioneine and Metmyoglobin on the Oxidation of NADH

The complete system contained 0.1 mM NADH, 0.17 mM $MnCl_2$, 0.33 mM ergothioneine and 1.2 μM metmyoglobin in 50 mM phosphate, pH 6.8. Manganese ion, ergothioneine or metmyoglobin was added as indicated in (a), (b) or (c), respectively. $\Delta A_{340\ nm}$/min indicates absorbancy change per min at 340 nm of NADH.

these conditions. The oxidation was dependent on the concentration of Mn(II) ion, ergothioneine or metmyoglobin as shown in Fig. 2. Mn(II) ion no longer increased the NADH oxidation at levels above 0.1 mM. Ergothioneine and metmyoglobin promoted the oxidation increasingly with increase of their concentrations. MMI added in place of ergothioneine exhibited almost the same effect as ergothioneine. Indeed, ergothioneine and MMI were also effective in the peroxidase system. However, thyroxine, which is effective in the peroxidase system,[6] did not support the oxidation by the metmyoglobin system. Thiols such as cysteine and glutathione suppressed the oxidation by metmyoglobin.

SOD and catalase inhibited the oxidation of NADH in the metmyoglobin system (Fig 3). Catalase (of which one unit consumes 1 μmol H_2O_2 per min) inhibited about 90% of the NADH oxidation at 300 units, but slight oxidation still occurred even in the presence of 3000 units. SOD, which decomposes superoxide anion (O_2^-), effectively inhibited the NADH oxidation in a concentration-dependent manner, and the oxidation scarcely proceeded in the presence of 300 units. The concentration of SOD required to yield 50% inhibition in the metmyoglobin system was 4.7×10^{-8} M in terms of copper content.

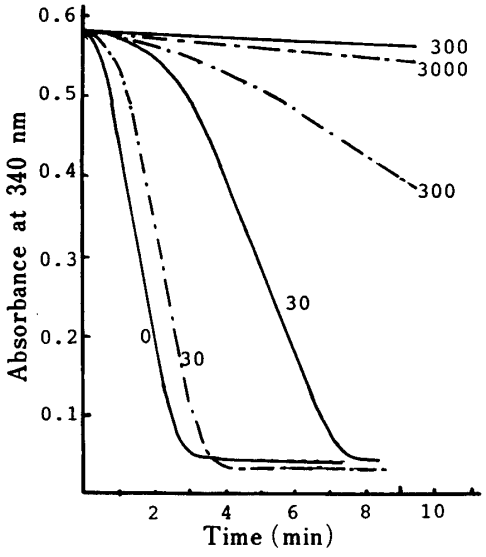

Fig. 3. Effects of Superoxide Dismutase and Catalase on the Oxidation of NADH by Metmyoglobin

The reaction mixture contained 0.1 mM NADH, 0.17 mM $MnCl_2$, 0.33 mM ergothioneine and 1.2 μM metmyoglobin in 50 mM phosphate, pH 6.8. Superoxide dismutase (——) and catalase (— — —) were added in the amounts (units) indicated in a volume of 3 ml.

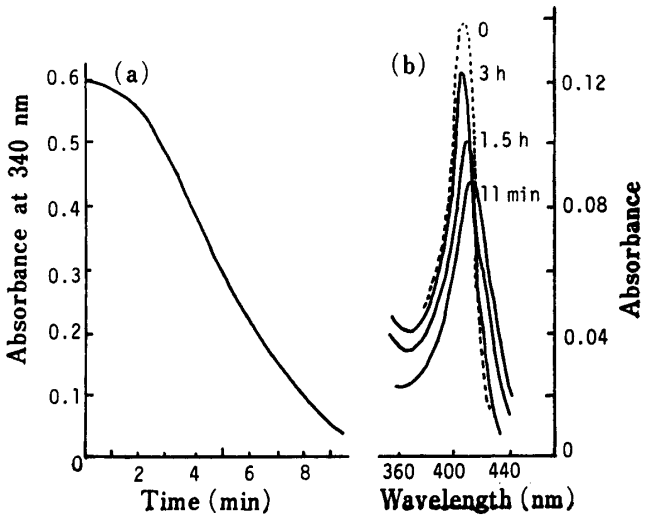

Fig. 4. Oxidation of NADH by Metmyoglobin (a) and Spectra of Soret Peaks of Metmyoglobin (b)

The reaction mixture contained 0.1 mM NADH, 0.17 mM $MnCl_2$, 0.67 μM ergothioneine and 1.2 μM metmyoglobin in 50 mM phosphate, pH 6.8.

At the end point of the oxidation, the Soret peak of metmyoglobin shifted from 409 to 413 nm with a decrease of the absorbance, but this was reversed with time (Fig. 4). Metmyoglobin is converted to ferrylmyoglobin by treatment with a small amount of H_2O_2.[14] Figure 5 shows the effect of H_2O_2 previously added to metmyoglobin on the oxidation of NADH. The addition of H_2O_2 to metmyoglobin shortened the lag time, but addition of more than 30-fold molar excess decreased the rate of the oxidation. The NADH oxidation by oxymyoglobin is shown in Fig. 6. Oxymyoglobin oxidized NADH more slowly than metmyoglobin.

The influence of phosphate on the oxidation of NADH was also investigated (Fig. 7). NADH was scarcely oxidized in the absence of phosphorus compounds. Phosphate, pyrophosphate and DPG oxidized NADH more effectively than nucleotides. Phosphate enhanced

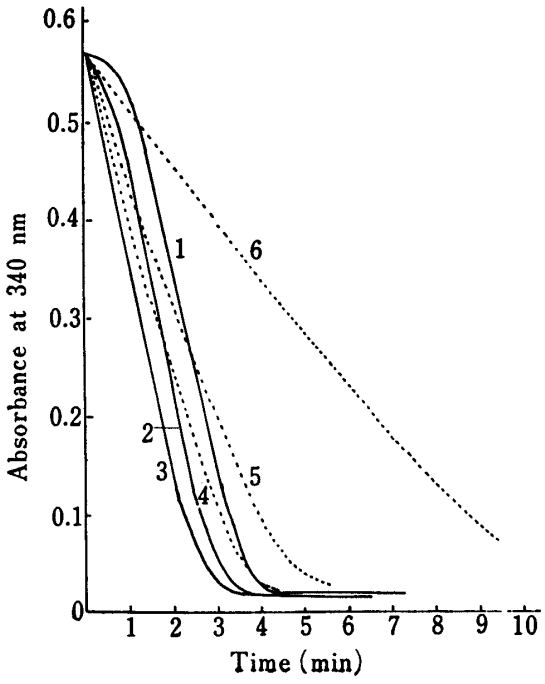

Fig. 5. Effect of H_2O_2 pre-added to Metmyoglobin on the Oxidation of NADH

The reaction mixture contained 0.1 mM NADH, 0.17 mM $MnCl_2$, 0.33 mM ergothioneine and 0.53 μM metmyoglobin with H_2O_2 in 50 mM phosphate, pH 6.8. H_2O_2 was added as follows; 1) none, 2) 0.33 μM, 3) 3.3 μM, 4) 17 μM, 5) 33 μM and 6) 170 μM.

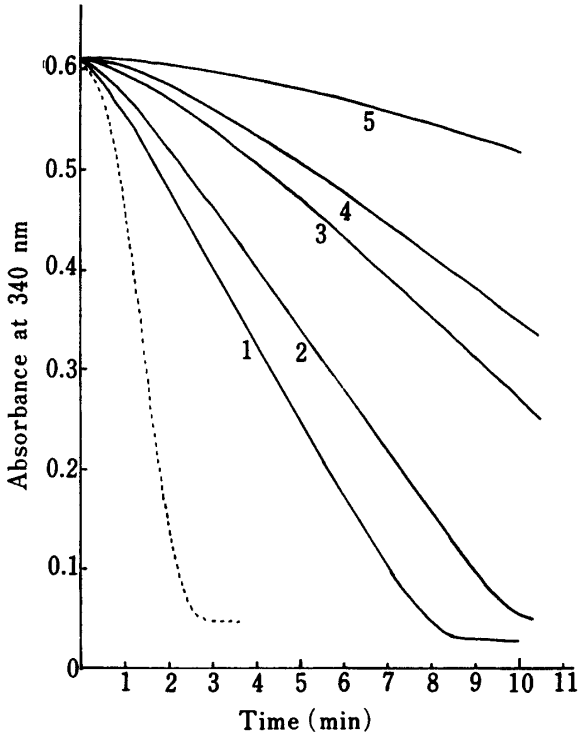

Fig. 6. Oxidation of NADH by Oxymyoglobin

The reaction mixture contained 0.1 mM NADH, 0.17 mM $MnCl_2$, 0.33 mM ergothioneine and oxymyoglobin in 50 mM phosphate, pH 6.8. Oxymyoglobin was added as follows; 1) 4.7 μM, 2) 2.8 μM, 3) 0.93 μM, 4) 0.56 μM, and 5) 0.19 μM. The broken line represents the addition of metmyglobin (2.8 μM) instead of oxymyoglobin.

the oxidation increasingly with increasing concentration, while pyrophosphate exhibited the highest oxidation in the range of 0.7 to 2.0 mM. DPG showed a constant oxidation rate at concentrations above 10 mM. The effect of AMP or ATP was independent of the concentration, but that of ADP slightly dependent. The NADH oxidation in the presence of nucleotides was not directly proportional to the number of their phosphate moieties.

Discussion

Peroxidase is known to oxidize NADH by a chain reaction.[8] The reaction is stimulated by thyroxine and ergothioneine.[6] In the presence of ergothioneine, Mn(II) ion and phosphate, a similar reaction was produced by metmyoglobin or methemoglobin, which has the same protoheme as peroxidase. NADH was scarcely oxidized in the absence of any one of these three substances, and the NADH oxidation was evidently dependent on their concentrations (Figs. 1,2). The results indicate that they play an essential role in the present system.

The inhibition of the oxidation by SOD and catalase (Fig. 3) strongly indicates the participation of O_2^- and H_2O_2 in the reaction of the metmyoglobin system. The fact that SOD inhibited the oxidation more effectively than catalase suggests that O_2^- is the primary reactant, and H_2O_2 produced from O_2^- also stimulates the oxidation of NADH.

In the process of oxidation, the Soret band of metmyoglobin shifts to longer wavelength (Fig. 4). Metmyoglobin reacts with O_2^-[15] and H_2O_2[14] to form oxymyoglobin and ferrylmyoglobin, respectively. The addition of oxymyoglobin did not result in effective oxidation of NADH, even at high concentration (Fig. 6). If the O_2^- generated through the reaction predo-

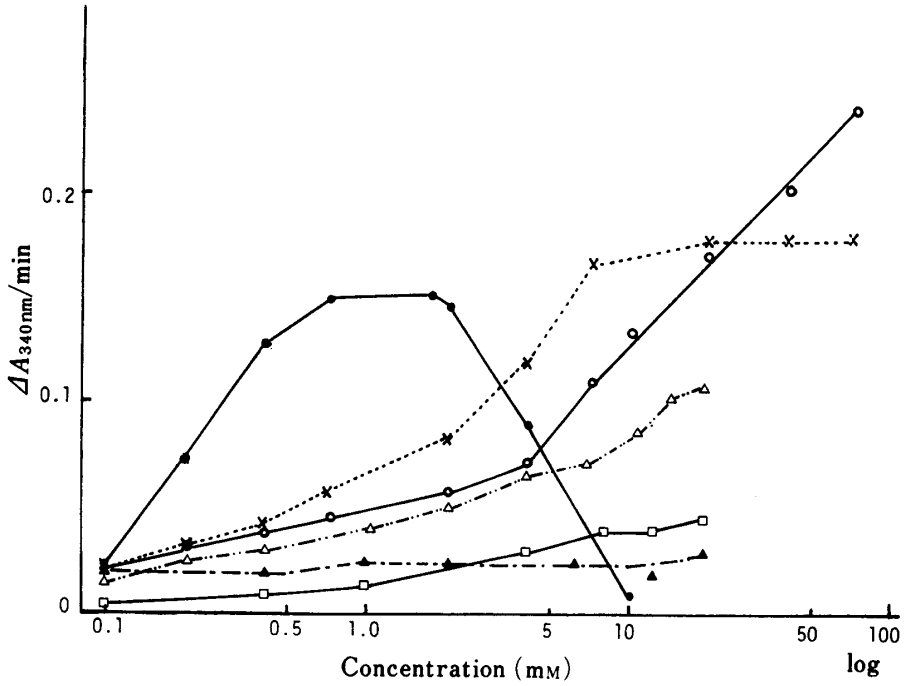

Fig. 7. Effects of Phosphorus Compounds on the Oxidation of NADH by Metmyoglobin

The reaction mixture contained 0.1 mM NADH, 0.17 mM $MnCl_2$, 0.33 mM ergothioneine and 1.2 μM metmyoglobin in 50 mM HEPES, pH 7.0. Phosphorus compounds were added as indicated. (—○—○—); phosphate, (—●—●—); pyrophosphate, (··· × ··· × ···); diphosphoglycerate, (—□—□); ATP, (---△---△---); ADP and (--▲---▲--); AMP. ΔA_{340nm}/min indicates absorbancy change per min at 340 nm of NADH.

minantly reacts with metmyoglobin to produce oxymyoglobin, the NADH oxidation in the present metmyoglobin system would proceed more slowly. When metmyoglobin treated with a small excess of H_2O_2, *i.e.*, ferrylmyoglobin, was added, the rate of oxidation was faster (Fig. 5). The slowdown of the oxidation caused by the addition of more than 30-fold molar excess of H_2O_2 to metmyoglobin appears to be due to the damage to the heme and globin moieties of metmyoglobin caused by H_2O_2. Ōyanagi has reported that phosphates prompt the production of O_2^- in the NADH oxidation by macrophages and increase the oxidation rate.[16] In the present study, some phosphorus compounds appreciably enhanced the oxidation of NADH (Fig. 7). Recently, Archibald *et al.* indicated[17] that orthophosphate and pyrophosphate can influence the interaction of Mn(II) with O_2^-. In phosphate buffer, Mn(II) is first oxidized by O_2^- to Mn(III), and then H_2O_2 is formed. The Mn(III) thus formed is reduced to Mn(II) by H_2O_2 with generation of O_2. In pyrophosphate buffer, the Mn(III) is re-reduced to Mn(II) by a second O_2^-. The SOD-like dismutation action of the pyrophosphate–Mn complex may contribute to the suppression of the NADH oxidation observed in the presence of 10 mM pyrophosphate.

The standard redox potentials (E_0') of typical thiol compounds are generally in the range of −0.20 to −0.40 V. Ergothioneine, which predominantly exists as the thione form, shows E_0' of −0.06 V[18] and is resistant to oxidation. Ergothioneine disulfide is formed slowly in neutral solution even on addition of H_2O_2, and is quite unstable.[3,19] Thiols such as cysteine and glutathione reduce metmyoglobin to oxymyoglobin, but ergothioneine does not. These facts may account for the different effects of ergothioneine and typical thiols on the oxidation of NADH.

On the basis of the present results, we would like to propose the following mechanism for

the cyclic oxidation of NADH by metmyoglobin or methemoglobin (Fig. 8). Trace amounts of H_2O_2 existing in the reaction mixture oxidize ergothioneine to its disulfide and metmyoglobin to ferrylmyoglobin, respectively. The NADH· formed transfers electrons to ferrylmyoglobin and O_2 to form NAD. Ergothioneine acts as an accelerator of the formation of NAD. As the O_2^- generated is changed to H_2O_2 by reaction with a second NADH and Mn(II) in the presence of phosphate, NADH· and Mn(III) are produced. The NADH· is further used in the cyclic reaction. The Mn(III) is re-reduced to Mn(II) by reaction with the H_2O_2. Ergothioneine (*ca.* 0.35 mM) in human red blood cells exists predominantly in the free form, and approximately 2% (50—100 μM) of hemoglobin in human blood is always found as met-type. The present mechanism indicates that the oxidation of NADH may be induced by methemoglobin as well as peroxidase under physiological conditions. Such a role of ergothioneine can be substantially attributed to the unique properties of its thione form.

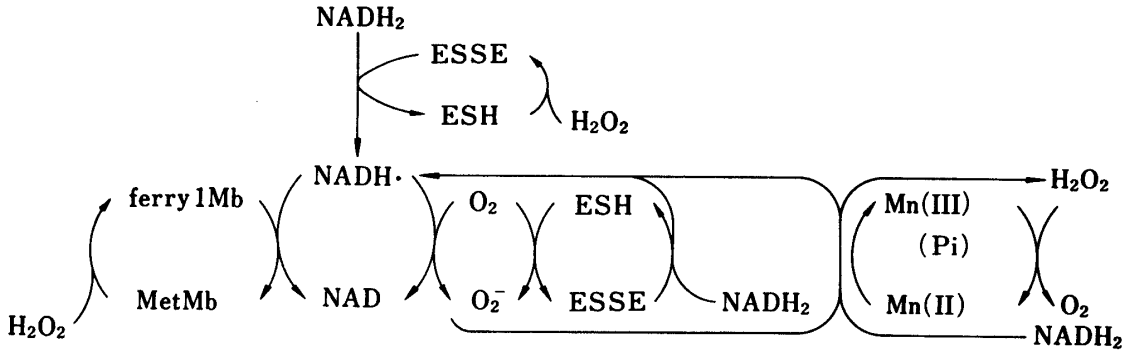

Fig. 8. Proposed Mechanism for Ergothioneine-dependent Oxidation of NADH by Metmyoglobin or Methemoglobin

FerrylMb, ferrylmyoglobin; MetMb, metmyoglobin; ESH, ergothioneine; ESSE, ergothioneine disulfide; Pi, phosphate.

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