Biochemistry (Moscow), Vol. 70, No. 4, 2005, pp. 467-472. Translated from Biokhimiya, Vol. 70, No. 4, 2005, pp. 568-574.
Original Russian Text Copyright © 2005 by Lisov, Leontievsky, Golovleva.

Oxidase Reaction of the Hybrid Mn-Peroxidase of the Fungus *Panus tigrinus* 8/18

A. V. Lisov*, A. A. Leontievsky, and L. A. Golovleva

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, 142290 Pushchino, Moscow Region, Russia; fax: (7-095) 956-3370; E-mail: alex-lisov@rambler.ru

Received April 12, 2004
Revision received June 22, 2004

Abstract—The hybrid Mn-peroxidase of the fungus *Panus tigrinus* 8/18 oxidized NADH in the absence of hydrogen peroxide, this being accompanied by the consumption of oxygen. The reaction of NADH oxidation started after a period of induction and completely depended on the presence of Mn(II). The reaction was inhibited in the presence of catalase and superoxide dismutase. Oxidation of NADH by the enzyme or by manganese(III)acetate was accompanied by the production of hydrogen peroxide and superoxide radicals. In the presence of NADH, the enzyme was transformed into a catalytically inactive oxidized form (compound III), and the latter was inactivated with bleaching of the heme. The substrate of the hybrid Mn-peroxidase (Mn(II)) reduced compound III to yield the native form of the enzyme and prevented its inactivation. It is assumed that the hybrid Mn-peroxidase used the formed hydrogen peroxide in the usual peroxidase reaction to produce Mn(III), which was involved in the formation of hydrogen peroxide and thus accelerated the peroxidase reaction. The reaction of NADH oxidation is a peroxidase reaction and the consumption of oxygen is due to its interaction with the products of NADH oxidation. The role of Mn(II) in the oxidation of NADH consisted in the production of hydrogen peroxide and the protection of the enzyme from inactivation.

Key words: hybrid Mn-peroxidase, NADH oxidation, absorption spectra

Peroxidases catalyze the oxidation of various electron donors by hydrogen peroxide according to the following scheme:

$$E + H_2O_2 \longrightarrow EI + H_2O,$$

$$EI + S \longrightarrow EII + P^{\cdot},$$

$$EII + S \longrightarrow E + P^{\cdot},$$

Scheme 1

where E is the native enzyme, EI and EII are its oxidized forms that are called compound I and compound II, and S and $P^{\cdot}$ are the substrate and its radical product, respectively. In addition to the peroxidase reaction, peroxidases have been shown to oxidize various substrates without hydrogen peroxide with the consumption of oxygen, i.e., to catalyze the peroxidase-oxidase reaction. A classic subject for investigation of peroxidases is horseradish peroxidase. It is capable of oxidizing without hydrogen peroxide such substrates as dihydroxyfumarate, indole-3-acetic acid, NADH, NADPH, and naphthohydroquinones, being activated by Mn(II) and phenols [1-3]. The suggested mechanism implies the formation of traces of hydrogen peroxide due to the autooxidation of the substrates, this triggering the usual peroxidase reaction with the oxidation of the substrates. The products of the oxidation reduce oxygen to yield hydrogen peroxide and O_2^- [4]. In fact, the reaction is the usual peroxidase reaction and can be called the "oxidase-like" reaction. During the reaction, a catalytically inactive peroxidase is formed (compound III) that plays a regulatory role in the oxidase-peroxidase reaction [5].

Mn-peroxidase is an extracellular peroxidase involved in the degradation of lignin and is produced by lignin-degrading fungi. This enzyme possesses a unique feature: it is capable of catalyzing the oxidation of Mn(II) by hydrogen peroxide yielding Mn(III), which can subsequently oxidize various compounds [6]. The catalytic cycle of the enzyme is analogous to that of other peroxidases except that compound II of MnP can be reduced to the native form only in the presence of Mn(II) [7]. In the case of so-called hybrid Mn-peroxidases (hMnP), com-

Abbreviations: hMnP) hybrid Mn-peroxidase; SOD) superoxide dismutase; Cat) catalase.

* To whom correspondence should be addressed.

0006-2979/05/7004-0467 ©2005 Pleiades Publishing, Inc.

pound II can be reduced by Mn(II) as well as by some organic substrates [8, 9]. Peroxidase of the fungus *Panus tigrinus* 8/18 is a hybrid Mn-peroxidase [10].

Like horseradish peroxidase, hMnP is capable of oxidizing some substrates, such as glyoxylate, quinones, NADH, and glutathione, without hydrogen peroxide and with the consumption of oxygen and the subsequent formation of hydrogen peroxide [6, 11-14]. The oxidase-peroxidase reaction catalyzed by hMnP completely depends on the presence of Mn(II). The mechanism of the reaction was investigated only for the oxidation of glyoxylate [11]. In this case, as well as in the case of horseradish peroxidase, the usual peroxidase reaction proceeds with the use of the hydrogen peroxide formed during the autooxidation of glyoxylate. The role of different intermediates of the catalytic cycle of either classic or hybrid Mn-peroxidase in the oxidase reaction has not been investigated.

The goal of this work was to investigate the mechanism of the oxidase reaction using the reaction of NADH oxidation by hybrid Mn-peroxidase and elucidation of the role of different intermediates of the catalytic cycle of the enzyme in this reaction.

MATERIALS AND METHODS

Cultivation of the fungus and purification of the enzyme. Cultivation of the fungus *P. tigrinus* 8/18 and purification of the enzyme was performed as described previously [10]. The purity number of the enzyme R_z (A_{406}/A_{280}) was 4.9.

Spectrophotometric measurements. The rate of oxidation of the substrate and the absorption spectra were recorded using a Shimadzu UV-2501PC spectrophotometer (Japan) in 20 mM sodium-tartrate buffer, pH 4.0. Oxidation of NADH was monitored by a decrease in the absorption of the reaction mixture at 340 nm. The concentration of the enzyme was calculated from the absorption at 406 nm, using $\varepsilon_{406} = 131\ mM^{-1}\cdot cm^{-1}$ (determined by the pyridine-hemochrome method) [15].

Measurement of oxygen consumption. Oxygen consumption was measured at 30°C using a Clark electrode and a YSI 5300 oxygen monitor (USA). The electrode was calibrated using a buffer solution saturated with oxygen (the concentration of oxygen was taken as 225 μM after 15 min of stirring without the electrode) and a solution without oxygen (containing an excess of sodium dithionite). Manganese(III)acetate was dissolved in methanol, undissolved particles were removed by centrifugation, and then the concentration of Mn(III) was determined by the oxidation of ferricyanide using $\varepsilon_{420} = 1.02\ mM^{-1}\cdot cm^{-1}$ [16].

Chemicals. The following chemicals were used: NADH (Fluka, Germany); superoxide dismutase (SOD) from bovine erythrocytes (Sigma, USA); catalase (Cat) from bovine liver (Serva, Germany); and manganese(III)acetate (Aldrich, USA). Other chemicals were from Reakhim (Russia).

RESULTS

Oxidation of NADH. hMnP of the fungus *P. tigrinus* 8/18 oxidized NADH in the absence of H_2O_2. The oxidation was preceded by a period of induction that can be removed by the addition of a low concentration (10 μM) of hydrogen peroxide (data not shown). The induction time decreased with increasing starting concentration of NADH (Fig. 1a). The induction time also depended on the amount of hMnP (Fig. 1b), the rate of the reaction increasing with increase in concentration of hMnP. The rate of the reaction increased with increasing Mn(II) concentration, but the induction time was constant in the presence of different concentrations of Mn(II). NADH was not oxidized in the absence of Mn(II) (Fig. 1c).

Oxygen consumption during NADH oxidation and effect of catalase and SOD on NADH-dependent activity. To elucidate the role of oxygen in the oxidation of NADH, oxygen consumption during the reaction was studied. Incubation of reaction mixture containing NADH and Mn(II) did not result in the consumption of oxygen. The addition of hMnP into the reaction mixture resulted in the consumption of oxygen after some period of induction (Fig. 2a). The addition of catalase resulted in the release of oxygen, this indicating the formation of hydrogen peroxide during the reaction (Fig. 2a). The addition of catalase into the reaction mixture before the addition of hMnP completely prevented the consumption of oxygen. Mn(III) is a product of the reaction catalyzed by hMnP, so we checked its influence on the consumption of oxygen. The presence of Mn(III) in the reaction mixture did not result in oxygen consumption, the reaction starting instantly after the addition of NADH. As in the case of hMnP, the addition of catalase into the reaction mixture resulted in the release of oxygen (Fig. 2b).

The formation on hydrogen peroxide indicates the involvement of reactive oxygen species in the reaction, so we investigated the effect of SOD on the oxygen consumption. Figure 2 demonstrates that the addition of SOD into the reaction mixture decreased the oxygen consumption both in the case of hMnP (c), and in the case of Mn(III) (d). The addition of SOD before the enzyme or before Mn(III) did not prevent the oxygen consumption, but decreased it (result not shown).

Considering the formation of hydrogen peroxide and O_2^- during the oxidation of NADH, we investigated the effect of catalase and SOD on the oxidation of NADH. As in the case of oxygen consumption, catalase completely inhibited NADH oxidation. SOD only decreased the rate of NADH oxidation, this indicating the interaction of

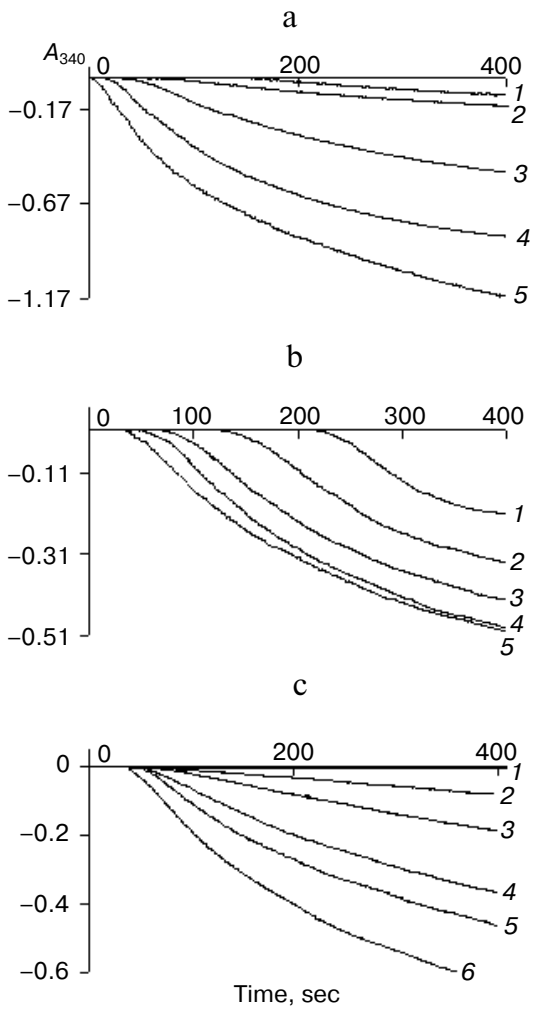

Fig. 1. Oxidation of NADH. The reaction mixture contained: a) 0.3 mM NADH, 100 μM $MnSO_4$, and 0.75 (*1*), 1.5 (*2*), 3 (*3*), 5 (*4*), or 7 nM hMnP (*5*); b) 100 μM $MnSO_4$, 3 nM hMnP, and 0.061 (*1*), 0.075 (*2*), 0.22 (*3*), 0.3 (*4*), or 0.5 mM NADH (*5*); c) 3 nM hMnP, 0.3 mM NADH, and 0 (curve *1* coincides with the abscissa), 10 (*2*), 40 (*3*), 70 (*4*), 100 (*5*), or 130 μM $MnSO_4$ (*6*) in 20 mM sodium-tartrate buffer, pH 4.0. The reaction was started by the addition of the enzyme.

NADH with O_2^-, but did not affect the induction time of the reaction.

Participation of intermediates of the catalytic cycle of the enzyme in oxidation of NADH. To elucidate the role of different forms of the enzyme in the oxidation of NADH, we studied the changes in the absorption spectra of hMnP in the presence of NADH. The addition of NADH resulted in the formation of the oxidized form of the enzyme (compound III), as seen from the appearance of the absorption maxima at 419, 548, and 580 nm (Fig. 3). These spectral characteristics are close to those of usual Mn-peroxidase of the fungus *Phanerochaete chrysosporium* [7]. Destruction of the heme group of peroxidases by excess of hydrogen peroxide results in the inactivation of the enzyme with the disappearance of the absorption maxima in the visible region of the spectrum (heme bleaching) [16]. As seen from Fig. 3, the enzyme was inactivated quickly with the heme bleaching, the maxima of compound III at 548 and 580 nm being shifted to 530 and 558 nm, i.e., compound III was transformed into an

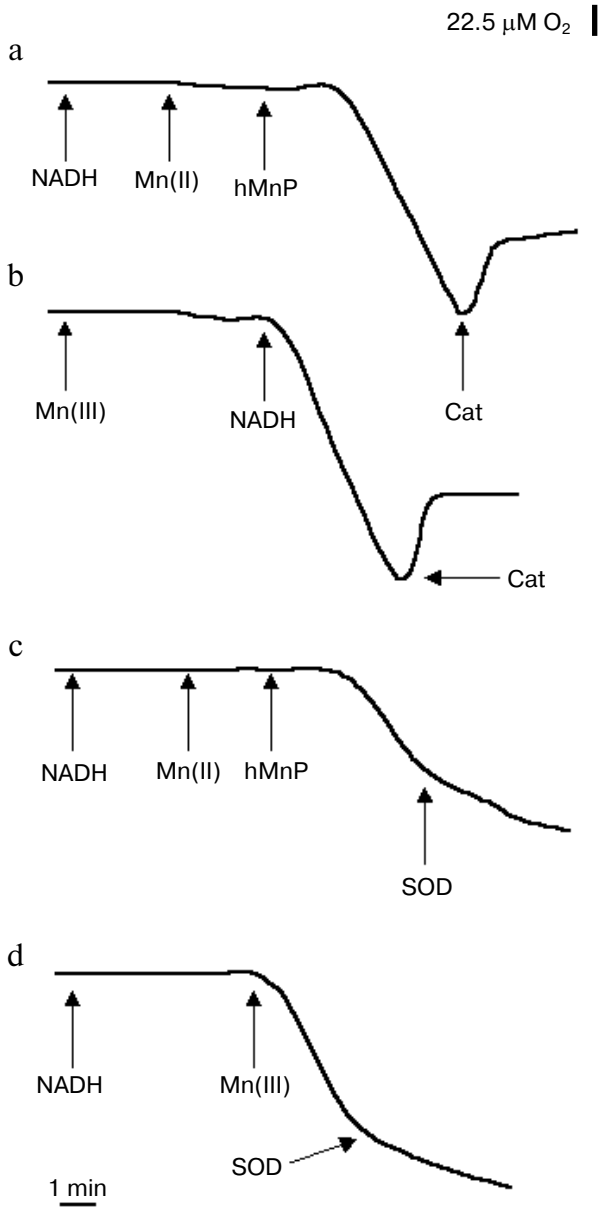

Fig. 2. Oxygen consumption during NADH oxidation and effect of catalase (a and b) and SOD (c and d) on this reaction. The reaction was performed at 30°C in 20 mM sodium-tartrate buffer, pH 4.0; final volume 7 ml. a) 100 μM $MnSO_4$, 0.3 mM NADH, 3 nM hMnP, and 4200 U of catalase; b) 100 μM Mn(III)acetate, 0.3 mM NADH, and 4200 U of catalase; c) 100 μM $MnSO_4$, 0.3 mM NADH, 3 nM hMnP, and 1400 U of SOD; d) 100 μM Mn(III)acetate, 0.3 mM NADH, and 1400 U of SOD. The time of the addition of the components is indicated by arrows.

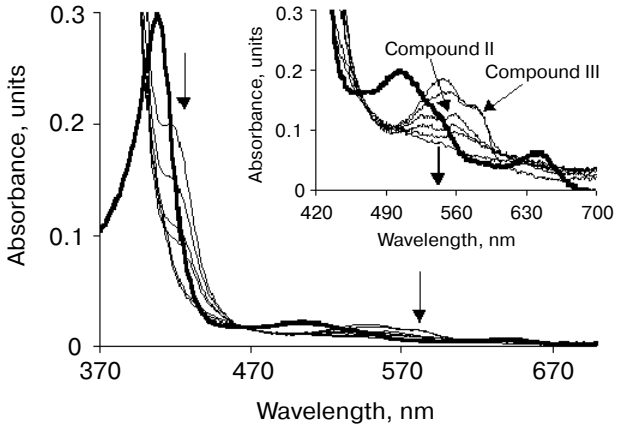

Fig. 3. Change in the absorption spectrum of hMnP after the addition of NADH. The reaction mixture (1 ml) contained 20 mM sodium-tartrate buffer, pH 4.0, 2.29 μM hMnP, and 3 mM NADH. The spectrum of the native enzyme is shown bold. The insert shows the region from 420 to 700 nm with 9-fold magnification. Arrows indicate the direction of the decrease in the absorption. The spectra were taken with an interval of 2 min.

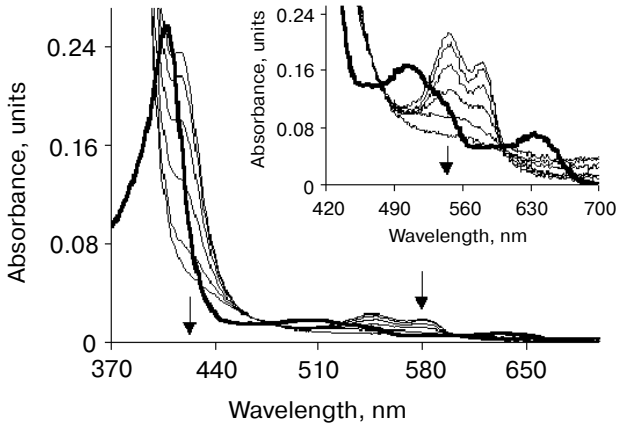

Fig. 5. Change in the absorption spectrum of hMnP in the presence of NADH and SOD. The reaction mixture (1 ml) contained 20 mM sodium-tartrate buffer, pH 4.0, 2 μM hMnP, 3 mM NADH, and 200 U SOD when indicated. The spectrum of the native enzyme is shown bold. The insert shows the region from 420 to 700 nm with 9-fold magnification. The spectra were taken with an interval of 5 min. Arrows indicate the direction of the changes in the absorption.

intermediate of the catalytic cycle of hMnP, compound II [10]. Similarly, the enzyme was inactivated through compound III in the presence of excess of hydrogen peroxide (500 equivalents), but compound II was not formed. The addition of 600 U/ml of catalase did not prevent the formation of compound III of the enzyme in the presence of NADH, but compound III existed for 6 min and then was transformed into the native enzyme (Fig. 4). Compound III also can be formed due to the interaction of the native peroxidase with O_2^- [17], so we tested the effect of SOD on the change in the absorption spectrum of the enzyme after the addition of NADH. In the presence of SOD, the enzyme was inactivated through compound III, but the inactivation was slower and without the formation of compound II (Fig. 5). As seen from Fig. 6, the addition of Mn(II) as the substrate of hMnP to compound III obtained in the reaction of the native enzyme with NADH returned hMnP to its native state. Consequently, Mn(II) prevented the inactivation of the enzyme. In the case of the addition of Mn(II) to the enzyme before NADH, compound III was not formed.

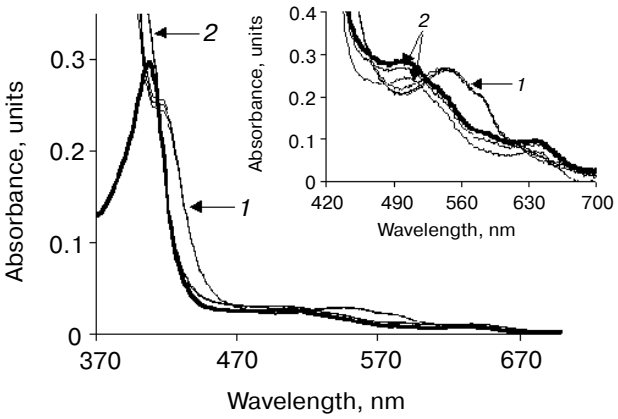

Fig. 4. Change in the absorption spectrum of hMnP in the presence of NADH and catalase. The reaction mixture (1 ml) contained 20 mM sodium-tartrate buffer, pH 4.0, 2.2 μM hMnP, 3 mM NADH, and 600 U catalase when indicated. Spectrum of the native enzyme is shown bold. The insert shows the region from 420 to 700 nm with 9-fold magnification. *1*) Compound III formed after the addition of NADH; *2*) native enzyme restored after the addition of catalase.

DISCUSSION

Mechanism of the oxidase oxidation of NADH. NADH was oxidized by hMnP in the presence of Mn without hydrogen peroxide, the oxidation starting after an induction period. On the addition of NADH, the enzyme was inactivated through compound III that could be reversed back to the native enzyme in the presence of Mn(II). It can be assumed that the induction period was due to the conversion of the enzyme to compound III and its gradual reactivation into the native enzyme. However, the latter assumption is unlikely, since Mn(II) prevented the formation of compound III and the induction time decreased with increase in NADH concentration (Fig. 1b).

NADH can be oxidized in the presence of oxygen yielding hydrogen peroxide [18], so we assumed that sim-

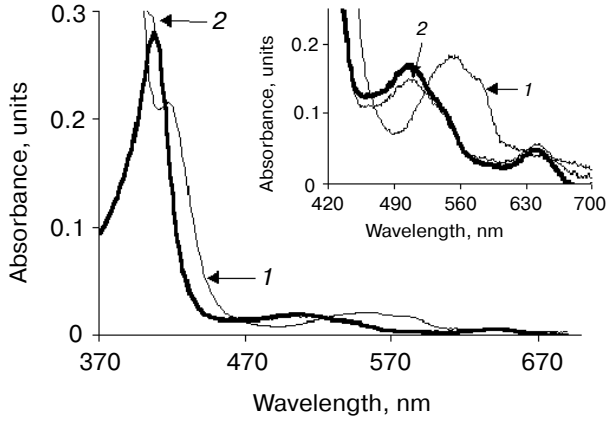

Fig. 6. Restoration of the spectrum of the native enzyme in the presence of Mn(II). The reaction mixture (1 ml) contained 20 mM sodium-tartrate buffer, pH 4.0, 2.1 μM hMnP, 3 mM NADH, and 100 μM $MnSO_4$. The spectrum of the native enzyme is shown bold. The insert shows the region 420-700 nm with 9-fold magnification. *1*) Compound III formed after the addition of NADH; *2*) the spectrum of the enzyme taken directly after the addition of $MnSO_4$.

ilarly to NADH oxidation by horseradish peroxidase [2, 4], the induction can be due to the accumulation of hydrogen peroxide. This is supported by the following facts: 1) the reaction is completely inhibited by catalase, and 2) the reaction proceeds only in the presence of Mn(II) (Fig. 1c) that is necessary for the normal peroxidase catalytic cycle of hMnP [7]. However, unlike horseradish peroxidase, hMnP used this hydrogen peroxide to catalyze the formation of Mn(III) from Mn(II). Oxidation of NADH to NAD^+ usually proceeds by two-electron transfer, but the coenzyme can also exist as the one-electron oxidized derivative $NAD^{\cdot}$ that is formed during the interaction of NADH with the one-electron oxidants O_2^- and $^{\cdot}OH$ [19]. Mn(III) formed in the reaction of hMnP with hydrogen peroxide and Mn(II) is a strong one-electron oxidant ($E_0' \sim 1500$ mV [6]), so in its presence NADH is oxidized yielding $NAD^{\cdot}$ ($E_0' = 282$ mV [20]):

$$Mn(III) + NADH \rightarrow Mn(II) + NAD^{\cdot} + H^+. \quad (1)$$

$NAD^{\cdot}$ can react with O_2 [21] according to the equation:

$$NAD^{\cdot} + O_2 \rightarrow NAD^+ + O_2^-. \quad (2)$$

O_2^- reacts with NADH [19] yielding hydrogen peroxide. The formed hydrogen peroxide can be used by hMnP to regenerate Mn(III), which continues the reaction chain. Thus, unlike classic peroxidases such as horseradish peroxidase, the main role in the formation of hydrogen peroxide during the oxidation of NADH by hMnP and in the maintenance of the reaction is played by Mn(III). The formation of hydrogen peroxide during NADH oxidation in the presence of the enzyme and Mn(III) coupled with the oxygen consumption is shown in Fig. 2. The oxidation of NADH is accompanied by formation of O_2^- (Fig. 2) that then interacts with NADH, this being concluded from the inhibition of the NADH oxidation by SOD. The fact that SOD does not affect the induction time of the reaction is in agreement with the described mechanism, since the reaction of O_2^- with SOD yields hydrogen peroxide. Besides, hydrogen peroxide can be formed from O_2^- in the superoxide dismutase reaction of the complexes of Mn(II) with organic acids [22]:

$$Mn(II) + O_2^- + 2H^+ \rightarrow Mn(III) + H_2O_2. \quad (3)$$

Formation of different enzyme forms. It is considered that the heme iron in the native peroxidase exists as Fe^{3+}. During the reaction of hMnP with NADH, the enzyme was converted into its oxidized derivative, compound III with oxidation level 6+. Compound III of peroxidases can be formed in three ways: 1) on the interaction of the native enzyme with O_2^- [1, 17]; 2) due to the reaction of intermediate compound II with excess of hydrogen peroxide [23]; and 3) due to the interaction of the reduced form of peroxidase with oxygen [24, 25]. In our case compound III could not be formed in the second way, since there was no excess of hydrogen peroxide. The interaction of O_2^- with the native enzyme can partially explain the formation of compound III since SOD decreased the rate of heme bleaching, but it did not prevent this reaction. The most probable way is the formation of compound III by oxidation with oxygen of the enzyme reduced by NADH. The reduction of peroxidases with $NAD^{\cdot}$ or hydroquinone radicals is known from the literature [4, 25]. It is more difficult to explain the formation of compound II during the bleaching of the heme. As it was shown [4], compound III of horseradish peroxidase decomposes yielding compound II.

$$\text{Compound III} \rightarrow \text{Compound II} + H_2O_2$$

Scheme 2

Compound II of hMnP is converted into the native enzyme only in the presence of Mn(II). Therefore, accumulation of compound II will result in its reaction with hydrogen peroxide yielding compound III with subsequent inactivation. The experiments with catalase indirectly support this hypothesis, since catalase, removing hydrogen peroxide, is capable of shifting the equilibrium indicated in Scheme 2 to the right, and compound II of hMnP from the fungus *P. tigrinus* can spontaneously revert to the native enzyme [10]. But hMnP from *P. tigrinus* is the hybrid peroxidase, i.e., its compound II can be reduced with both Mn(II) and some organic compounds.

It is complex to reveal whether NADH can reduce compound II, since in the presence of NADH the enzyme is converted into compound III. SOD did not prevent the bleaching of the heme, but the formation of compound II was not observed (Fig. 5). This is likely due to the fact that SOD yields additional amounts of hydrogen peroxide, this shifting the equilibrium between compound II and compound III to compound III.

Compound III plays a regulatory role in NADH oxidation by horseradish peroxidase, its decomposition leading to the fast oxidation of the substrate [5]. However, active formation of compound III during NADH oxidation by hMnP is unlikely because Mn(II) converts it into the native enzyme (Fig. 6), so compound III is likely a inactive side product and does not play any role in the oxidase reaction of hMnP.

Thus, the reaction of NADH oxidation in the absence of hydrogen peroxide, as in the case of horseradish peroxidase, is the peroxidase reaction, but the main role in the formation of hydrogen peroxide and in the maintenance of the reaction is played by Mn(III). Oxygen consumption is due to its interaction with the products of the reaction of Mn(III) with NADH with subsequent formation of the reactive oxygen species. Mn(II) plays a role of substrate of hMnP in the peroxidase reaction and prevents the inactivation of the enzyme.

This work was supported by INTAS YSF 2002-0162.

REFERENCES

1. Yamazaki, I., and Piette, L. (1963) *Biochim. Biophys. Acta*, **77**, 47-64.
2. Yokota, K., and Yamazaki, I. (1965) *Biochim. Biophys. Acta*, **105**, 301-313.
3. Klappe, M. H., and Hackett, D. P. (1963) *J. Biol. Chem.*, **238**, 3736-3742.
4. Yamazaki, I., and Yokota, K. (1967) *Biochim. Biophys. Acta*, **132**, 310-321.
5. Yamazaki, I., and Yokota, K. (1973) *Mol. Cell. Biochem.*, **2**, 39-52.
6. Glenn, J. K., and Gold, M. H. (1985) *Arch. Biochem. Biophys.*, **242**, 329-341.
7. Wariishi, H., Akileswaran, L., and Gold, M. (1988) *Biochemistry*, **27**, 5365-5370.
8. Camarero, S., Sarkar, A., Ruis-Duenas, F. J., Martinez, M. J., and Martinez, A. T. (1999) *J. Biol. Chem.*, **274**, 10324-10330.
9. Mester, T., and Field, J. A. (1998) *J. Biol. Chem.*, **273**, 15412-15417.
10. Lisov, A. V., Leontievsky, A. A., and Golovleva, L. A. (2003) *Biochemistry (Moscow)*, **68**, 1027-1035.
11. Kuan, I.-Ch., and Tien, M. (1993) *Arch. Biochem. Biophys.*, **302**, 447-454.
12. Gomez-Toribio, V., Martinez, A. T., Martinez, M. J., and Guillen, F. (2001) *Eur. J. Biochem.*, **268**, 4787-4793.
13. Glenn, J. K., Akileswaran, L., and Gold, M. H. (1986) *Arch. Biochem. Biophys.*, **251**, 688-696.
14. Paszczyski, A., Huynh, V.-B., and Crawford, R. L. (1985) *FEMS Lett.*, **29**, 37-41.
15. Pajot, P., and Grondinsky, O. (1970) *Eur. J. Biochem.*, **12**, 158-164.
16. Timofeevskii, S. L., Reading, N. S., and Aust, S. D. (1998) *Arch. Biochem. Biophys.*, **361**, 287-295.
17. Odajima, T., and Yamazaki, I. (1972) *Biochim. Biophys. Acta*, **284**, 355-359.
18. Bernofsky, C., and Wanda, S.-Y. (1982) *J. Biol. Chem.*, **257**, 6809-6817.
19. Land, E. J., and Swallow, A. J. (1971) *Biochim. Biophys. Acta*, **234**, 34-42.
20. Anderson, R. F. (1980) *Biochim. Biophys. Acta*, **590**, 277-281.
21. Sipe, H. J., Corbett, J. T., and Mason, R. P. (1997) *Drug. Metab. Dispos.*, **25**, 468-480.
22. Archibald, F. S., and Fridovich, I. (1982) *Arch. Biochem. Biophys.*, **214**, 452-463.
23. Nakajima, R., and Yamazaki, I. (1987) *J. Biol. Chem.*, **262**, 2576-2581.
24. Kettle, A. J., and Winterbourn, C. C. (1992) *J. Biol. Chem.*, **267**, 8319-8324.
25. Tamura, M., and Yamazaki, I. (1972) *J. Biochem.*, **71**, 311-321.