Biochimica et Biophysica Acta, 1020 (1990) 25–33
Elsevier

BBABIO 43270

Manganese-containing catalase from *Thermus thermophilus* peroxide-induced redox transformation of manganese ions in presence of specific inhibitors of catalase activity

S.V. Khangulov [1], V.V. Barynin [2] and S.V. Antonyuk-Barynina [1]

[1] *Institute of Chemical Physics and* [2] *Institute of Crystallography, Academy of Sciences of the USSR, Moscow (U.S.S.R.)*

(Received 2 May 1990)

Key words: Manganese; Bacterial catalase; Catalase; ESR; Oxygen evolution; Photosynthesis

The mechanism of peroxide decomposition involves: interconvertion of two equally active redox states: (Mn^{2+}, Mn^{2+}) and (Mn^{3+}, Mn^{3+}) states. Various inorganic anions such as Cl^- or HPO_4^- inhibit catalase activity by binding to the (Mn^{2+}, Mn^{2+}). This inhibition can also be observed following reduction of the (Mn^{3+}, Mn^{3+}) states to (Mn^{2+}, Mn^{2+}) by peroxide. The (Mn^{3+}, Mn^{4+}) state, which can be formed by periodate oxidation, does not interact with these anions and is inactive in peroxide decomposition. The (Mn^{2+}, Mn^{3+}) state, which forms in a minority of centers by auto-oxidation, also appears not to participate in a major way in peroxide decomposition.

Introduction

Clusters of manganese ions play an essential role in the process of photosynthetic decomposition of water [1–3]. At present, there is increasing interest in the study of enzymes whose active center is represented by a cluster of manganese ions. It is believed that one such enzyme is the pseudocatalase from *Lactobacillus plantarum* obtained by Kono and Fridovich [4,5]. Indeed, each subunit of this protein contains about two manganese ions [6]. Direct evidence for a dimeric Mn site in Mn-catalase from *L. plantarum* was obtained from an ESR investigation of the enzyme [7].

The presence of a binuclear cluster of manganese ions as the active center has been unambiguously established in the manganese-containing catalase from the extremely thermophilic bacterium *Thermus thermophilus* [8–13].

General

Subunit structure [8]. The manganese-containing catalase from *Thermus thermophilus* HB-8 is composed of six identical subunits. The molecular weight of native catalase is 210 000 ± 2000. The molecular weight of a single chain is 35 000 ± 2000.

X-ray structure analysis [9,10]. Transparent, as a rule, pinkish protein crystals are grown from concentrated protein solutions at 4 °C in the presence of ammonium sulfate (1.1 M). The crystals have a rhombododecahedral shape. The secondary and tertiary structures of catalase have been studied and each subunit was shown to contain two closely spaced (3.6 ± 0.3 Å) manganese ions.

Determination of manganese ion content [11]. The acidification of the protein to pH 1.5–2.0 produced denaturation accompanied by the appearance of the six-component ESR signal characteristic of free aquo-Mn^{2+} ions. Prolonged dialysis (24 h) versus 5 mM EDTA (pH 6.8) of the native enzyme failed to remove any manganese, as was assayed by subsequent acidification of released aquo-Mn^{2+}. Hence, all manganese is tightly bound. To determine the manganese content in Mn catalase by ESR we have used Mn^{2+} standards. It was shown that there are about two manganese ions per protein subunit of Mn-catalase.

Catalase contains a binuclear cluster of manganese ions [12,13]. At low temperatures (8–100 K), the preparations of catalase exhibited a complex multicomponent ESR signal. The ESR spectrum represents a superposition of three components: signals A, B and C. It was shown that the paramagnetic centers responsible for the appearance of these signals are represented by various

Correspondence: V. Khangulov, Department of Chemistry, Princeton University, Princeton, NJ 08544, U.S.A.

redox states of a binuclear cluster in the active center of the enzyme:

$$\underset{\text{Signal A}}{(Mn^{2+}, Mn^{2+})} \cdots\cdots\rightarrow \underset{\text{Signal B}}{(Mn^{2+}, Mn^{3+})} \cdots\cdots\rightarrow \underset{?}{(Mn^{3+}, Mn^{3+})}$$

$$\cdots\cdots\rightarrow \underset{\text{Signal C}}{(Mn^{3+}, Mn^{4+})} \quad (1)$$

Complex formation [13,14]. A number of exogenous ligands, including chloride, fluoride, azide and inorganic phosphate, are capable of forming complexes with the centers in the state (Mn^{2+}, Mn^{2+}). The shape of ESR signals from the reduced preparations depends, in general, on the exogenous ligand in the medium. The signals of reduced preparations will be denoted in the following by the subscript L (e.g., signal A_L), where L indicates the conditions under which the particular signal was observed. For example, signal A_{Cl^-} denotes the signal from a reduced catalase preparation in the presence of excess chloride. The changes in the shape of ESR signals produced by exogenous ligands are reversible and can be described by the following generalized scheme:

$$\underset{\text{Signal } A_{L_1}}{(Mn^{2+}, Mn^{2+})\cdot L_1} \underset{\text{dialysis against } L_2 \text{ solution}}{\overset{\text{dialysis against } L_1 \text{ solution}}{\rightleftharpoons}} \underset{\text{Signal } A_{L_2}}{(Mn^{2+}, Mn^{2+})\cdot L_2} \quad (2)$$

The shape of signals B and C does not depend on the presence of excess of inorganic phosphate and chloride. We suggest that the transformation from the (Mn^{2+}, Mn^{2+}) state to (Mn^{2+}, Mn^{3+}) or (Mn^{3+}, Mn^{4+}) is accompanied by the formation of oxo- (or hydroxo-) bridges between the manganese ions, whereby the possible bonding sites of the exogenous ligands are tenaciously occupied by bridging oxygen atoms. This concept can be illustrated by the following diagram:

$$(Mn^{2+}, Mn^{2+})L \xrightarrow{\text{oxidation}} Mn^{3+}\begin{matrix}O\\O\end{matrix}Mn^{4+} + L$$

Enzymatic activity [15,16]. The initial preparations are characterized by a high enzymatic activity. It was shown that with the chosen concentration intervals of enzyme (0.05–3 mM) and peroxide (0.5–10 mM) the O_2 evolution rate was a linear function of both concentrations. The rate constant for the decomposition of H_2O_2 at 20 °C and pH 7.0 is about 10^7 $M^{-1} \cdot s^{-1}$. The enzyme is inhibited at pH below 6, with a $pK_a = 5.5$, and is independent of pH in the range 6–10. The catalase activity is effectively suppressed by hydroxylamine, nitrite, chloride, azide, fluoride and inorganic phosphate. The efficiency of such inhibitors as chloride and azide decreases strongly with pH between 6 and 10 as is explained by the following formal scheme:

$$\text{Cat } OH^- + L^- \rightarrow \text{Cat } L^- + OH^- \quad (4)$$

Cat OH^- is the active (i.e., catalysing the decomposition of H_2O_2) form of catalase, while the Cat L^- complex is unable to catalyse H_2O_2 decomposition.

We have also shown that active centers in the (Mn^{3+}, Mn^{4+}) state produced with periodate, KIO_4, possess no catalytic activity. However, the inactivation was reversible and treatment of the oxidized preparations with hydroxylamine resulted in a practically full reactivation of catalase. It should be noted that hydroxylamine acts not only as a reducing agent, but also as an effective inhibitor of catalase activity. For this reason, the observation of the reactivation effect is possible only after removal of hydroxylamine by dialysis.

Optical properties [8]. The electronic spectrum of catalase in the visible range is characterized by a wide, slightly split band with maxima at 460 and 500 nm ($\epsilon_{500nm} = 1.35 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$). The preparations have a pink or pink-brown color. In the ultraviolet region, there is an intense band at 280 nm ($\epsilon_{280nm} = 2.05 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$). As a whole, the optical spectrum of the enzyme is very similar to that of the pseudocatalase from *Lactobacillus plantarum* [4].

Problem formulation. In the interpretation of previous results [15,16], we suggested that the catalase activity was a property of the (Mn^{2+}, Mn^{2+}) state. This proposition was based on the experimentally observed correlation between the efficiency of some ligands (inorganic phosphate, chloride, fluoride and azide) for inhibition of catalase activity and their ability to coordinate to centers in the (Mn^{2+}, Mn^{2+}) state.

It was naturally assumed that the more centers which are present in the (Mn^{2+}, Mn^{2+}) state, the higher would be the catalase activity. In order to check this assumption, we previously performed experiments [15,16] comparing the catalase activities of initial and reduced preparations. These preparations vary considerably in the content of centers in the (Mn^{2+}, Mn^{2+}) state. However, the catalase activity of reduced preparations did not differ noticeably from the initial level.

Materials and Methods

Protein preparation. Mn-catalase was obtained as described in Ref. 8. and stored at 5 °C as a fine crystalline precipitate in half-saturated ammonium sulfate solution. Before each experiment 100 μmol of this suspension was dialysed for 24 h against 10 mM potassium phosphate buffer (pH 6.8). The concentration of Mn-catalase was determined spectrophotometrically, by the absorption at 280 nm ($\epsilon_{280nm} = 0.95$ $Mg^{-1} \cdot cm^2$) [8] and was found to be in the range 20–50 mg/ml.

Sample treatments. Unless otherwise stated, the protein preparations were solutions of Mn-catalase in 10 mM potassium phosphate buffer (pH 6.8) ('potassium phosphate buffer') The reagents used were also dissolved in the potassium phosphate buffer. All reagents

were analytical grade, while KCl and NaH_2PO_4 were additionally purified by recrystallization. The preparations of reduced and oxidized catalase were obtained by dialysis against 1 mM $(NH_2OH)_2H_2SO_4$ (for 1 h), or 5 mM KIO_4 (for 2 h) respectively. The excess reducer or oxidizer was removed by dialysis against the potassium phosphate buffer. Autoxidized preparations were obtained by prolonged (for 24 h) dialysis of reduced preparations against 20 mM borate buffer (pH 9.2) followed by dialysis against 10 mM potassium phosphate buffer (pH 6.8). The complexes of catalase with chloride and inorganic phosphate were produced by dialysis against 50 mM NaCl and 100 mM NaH_2PO_4 (pH 6.8), respectively.

Treatment of Mn-catalase preparations with peroxide. At this stage of the work we encountered some experimental difficulties. The problem was that, on the one hand, the catalase preparations possess high enzymatic activity and, on the other hand, the optical or ESR monitoring of the redox state of the centers requires a high concentration of enzyme (about 10 mg/ml) in the sample. As a result, peroxide in such concentrated preparations decomposes violently and the sample splashes around. For this reason we studied the effect of peroxide mainly on catalase preparations in the presence of inhibitors of catalytic activity. It has been shown [16] that Cl^-, NO_2^-, NH_2OH, inorganic phosphate and some other agents are effective inhibitors of Mn-catalase activity. In the present study we chose chloride and inorganic phosphate, because these substances are capable of forming complexes with centers in the (Mn^{2+}, Mn^{2+}) state. These complexes give rise to pronounced ESR signals A_{Cl^-} and A_p [14]. Thus it is possible to investigate not only peroxide-induced changes of the (Mn^{2+}, Mn^{3+}) and (Mn^{3+}, Mn^{4+}) states (signals B and C), but also the peroxide-induced changes of the (Mn^{2+}, Mn^{2+}) state (signal A). The following nomenclature designates the sample buffers and treatments:

I. Red (K-buffer – NH_2OH reduced catalase dialysed against 10 mM potassium phosphate buffer (pH 6.8);
II. Aut(K-buffer) – autoxidized catalase dialysed against 10 mM potassium phosphate buffer (pH 6.8);
III. Red(NaH_2PO_4) – reduced catalase dialysed against 100 mM NaH_2PO_4 (pH 6.8);
IV. Aut(NaH_2PO_4) – autoxidized catalase dialysed against1 00 mM NaH_2PO_4;
V. Red(Cl^-) – reduced catalase dialysed against 50 mM NaCl;
VI. Aut(Cl^-) – autoxidized catalase dialysed against 50 mM NaCl.

Treatment of inactive preparations by peroxide was realized by dialysis (for 1 h) against 10 mM H_2O_2 with 100 mM NaH_2PO_4 (for Red(NaH_2PO_4), Aut(NaH_2PO_4)) or 50 mM NaCl (for Red(Cl^-), Aut(Cl^-)). To avoid difficulties accompanying the investigation of the influence of peroxide upon preparations with high catalytic activity, we used the well-known method of low-temperature inhibition. The experimental set-up for trapping kinetic intermediates involves rapid mixing of peroxide-containing buffer and catalase preparation, followed by rapid transfer to cooled pentane (143 K). The diameter of the drops is about 0.1 mm. The delay time (which was no more than 0.1 s) was estimated by the reaction of azide with hemoglobin as in Ref. 17.

Enzymatic activity. Enzymatic activity of the solutions containing protein at concentrations of 1–3 μg/ml was determined (as in Ref. 18) for a peroxide concentration of 2–4 mM by measuring the extent of O_2 evolution using a Clark electrode and an OH-106 polarograph. The concentration of H_2O_2 was determined spectrophotometrically by absorption at 230 nm ($\epsilon_{230} = 60$ $M^{-1} \cdot cm^{-1}$). Oxygen evolution was initiated by addition of 10 μl of assayed protein solution (0.8 μg/ml) into a thermostatic cell (20 °C), which contained 2.7 ml of 3 mM H_2O_2 in 10 mM potassium phosphate buffer (pH 6.8) with a suitable inhibitor of catalase activity. The measurements of catalytic activity was performed together with the study of the optical properties of Mn-catalase.

Optical measurements. The optical spectra were measured on Specord UV-Vis and Specord M40 spectrophotometers. All spectra presented in this work were obtained using two 1 mm quartz cells and show the differential curves of 'protein-minus-buffer'. The protein concentrations were about 10 mg/ml. After the measurement of optical spectra, the protein solutions were used for the preparation of samples intended for ESR studies.

ESR measurements. The ESR spectra were measured on an EPR-V spectrometer equipped with a low-temperature attachment [19]. The maximum amplitude (for 0 dB) of the magnetic component (H_1) of the microwave field was about 0.02 mT, the modulation frequency was 100 KHz, and modulation amplitude was 0.5 mT.

The ESR spectrum of Mn-catalase in general is represented by superposition of signals A, B and C. Investigation of the temperature dependence of these signals and the dependence on microwave power was as performed previously [12,14]. The appropriate conditions have been chosen here taking these data into account. For the signals A, C and B optimal temperatures are 50–110, 50–80 and below 20 K, respectively.

Evaluation of relative contents of centers in various redox states. The relative amounts of centers (N_{ij}) in the (Mn^{2+}, Mn^{2+}), (Mn^{2+}, Mn^{3+}), and (Mn^{3+}, Mn^{4+}) states were estimated by the ESR signals A, B and C. The method used for determining the relative content of centers in the various redox states is based on the following suggestions:

(i) The set of redox states achieved in our experiments is

represented by (Mn^{2+}, Mn^{2+}), (Mn^{2+}, Mn^{3+}), (Mn^{3+}, Mn^{3+}) and (Mn^{3+}, Mn^{4+}) states. This means that $N_{22} + N_{23} + N_{33} + N_{34} = N_o$ where N_o is the full concentration of binuclear clusters in the sample, and N_{ij} is the concentration of binuclear clusters in (ij) state.

(ii) The concentrations of chloride and inorganic phosphate employed are sufficiently high to transfer all the (Mn^{2+}, Mn^{2+}) centers to form complexes with these ligands. This means that the number of (Mn^{2+}, Mn^{2+}) centers in preparations may be estimated by signal A_p (for Red(NaH_2PO_4) and Aut(NaH_2PO_4) and signal A_{Cl^-} (for preparation Red(Cl^-) and Aut(Cl^-)); in other words, $N_{22} \propto$ signal A;

(iii) In reduced preparations (see peroxide treatment in Materials and Methods) all the centers are in the (Mn^{2+}, Mn^{2+}) state, $N_0 = N_{22}$;

(iv) the addition of KIO_4 transfers all the centers to the (Mn^{3+}, Mn^{4+}) state: $N_0 = N_3$.

These suggestions are based on our previouse data [12–14] and on supplementary investigations performed in the present study. As was shown previously, the addition of phosphate and chloride to the initial preparations induced the signals A_p and A_{Cl^-}. We studied the dependence of these signals on the concentration of the exogenous ligands. It was shown that the concentration of the exogenous ligands used in the present studies (50 mM NaCl and 100 mM NaH_2PO_4) were saturating and further addition of chloride and inorganic phosphate, respectively, did not increase the signals A_{Cl^-} and A_p.

Addition of hydroxylamine (1 mM) to initial preparations (dialysed against chloride or inorganic phosphate) increased (by 2- to 3-times) signals A_{Cl^-} and A_p. Thus, only a small portion of the active centers of the initial preparations of catalase are in the (Mn^{2+}, Mn^{2+}) state. The further addition of hydroxylamine (20 mM) did not lead to any further increase in the signals A_{Cl^-} and A_p.

The investigation of the effects of KIO_4 on catalase has shown that the amplitude of the KIO_4-induced signal C did not change over a wide range of KIO_4 concentrations (3–15 mM). Moreover, the amplitude of the signal C induced by the prolonged dialysis (for 5 h) after 5 mM KIO_4 was the same as after 1 h dialysis. Therefore, we conclude that further oxidation to the (Mn^{4+}, Mn^{4+}) state does not occur. This is consistent with the lack of oxidation of (Mn^{3+}, Mn^{4+}) complexes which are functional analogues of Mn-catalase [20].

(a) (Mn^{2+}, Mn^{2+}). The fraction of centers occurring in the (Mn^{2+}, Mn^{2+}) state was found from comparison of amplitudes of signals A in the studied and reduced samples. For example, the relative content of the (Mn^{2+}, Mn^{2+}) state in the autooxidized preparations Aut(NaH_2PO_4) may be found by the equation:

$$n_{22} = \frac{N_{22}}{N_0} = \frac{\text{Signal } A_{Cl^-} \text{ (in preparation Aut}(NaH_2PO_4))}{\text{Signal } A_{Cl^-} \text{ (in preparation Red}(NaH_2PO_4))}$$

(b) (Mn^{2+}, Mn^{4+}). The same method was used for determination of (Mn^{3+}, Mn^{4+}) content. The fraction of centers present in the (Mn^{3+}, Mn^{4+}) state was estimated by comparison of the amplitudes of signals C:

$$n_{34} = \frac{N_{34}}{N_0} = \frac{\text{Signal C (of studied preparation)}}{\text{Signal C (after addition of the excess of } KIO_4 \text{ to studied preparation)}}$$

(c) (Mn^{2+}, Mn^{3+}). The fraction of centers in the (Mn^{2+}, Mn^{3+}) state was determined by the comparison of the integrated absorption of signal B in the studied sample and signal C arising after addition of KIO_4 to sample studied:

$$n_{23} = \frac{N_{23}}{N_0} = \frac{S(\text{signal B of studied preparation})}{S(\text{signal C arising after addition of } KIO_4 \text{ to studied preparation})}$$

where $S = \sigma T/\sqrt{P}$, and σ is the integrated adsorption of the suitable ESR signal, T the temperature of measurement, P the microwave power (unsaturated). The optimal conditions (unsaturated) for detection are 15 K and 200 mV for the signal B and 50 K and 15 mW for the signal C.

(d) Relative content. The relative content of the centers in the ESR-silent state $Mn^{3+} \ldots Mn^{3+}$ (n_{33}) was determined by the condition:

$$n_{22} + n_{23} + n_{33} + n_{34} = 1$$

Results

Initial preparations

The initial catalase preparations are pink in color, typical for the trivalent manganese complexes [22–24]. In the ultraviolet range, there is a pronounced band with a maximum at 280 nm attributed to the absorption of aromatic amino acids. In the visible range, these preparations are characterized by the presence of a slightly split band with maxima at 460 and 500 nm. It should be noted that the absorption intensity of different preparations in this range may vary considerably. However, the form of the spectrum of initial preparations is the same as in Fig. 1.2.

Reduced preparations

The reduction of preparations produced practically complete discoloration of the sample and abolished absorption between 450 and 550 nm. In the ultraviolet range, a slight discoloration at 250 nm is observed. Earlier it was shown that treatment of pseudocatalase from *L. plantarum* with hydroxylamine also resulted in discoloration of samples [5].

Autooxidation

The storage of reduced preparations for 2 or 3 days at room temperature resulted in the development of a pink color and the restoration of the absorption bands at 450–550 nm. This process was hindered if the samples were stored in a medium containing agents capable of forming complexes with centers in the (Mn^{2+}, Mn^{2+}) state. For example, no color was restored in the presence of 100 mM potassium phosphate buffer (pH 6.8) or 50 mM NaCl solution in 5 mM potassium phosphate buffer (pH 6.8). The coloration occurs due to autoxidation of preparations; no development of color is observed under anaerobic conditions (argon atmosphere).

The autoxidation process in transition metal complexes occurs more intensively in an alkaline medium. Thus, dialysis of the reduced preparations against 20 mM borate buffer (pH 9.2) accelerated the development of the pink color significantly and the absorption intensity at 500 nm reached maximum after only 12 h (Fig. 1).

Oxidation by periodate

The treatment of catalase by periodate was accompanied by the development of a yellow-brownish color. Fig. 2 shows the transformation of visible and ultraviolet spectra of the samples treated by periodate.

In the autoxidized catalase the active center occurs in the (Mn^{3+}, Mn^{3+}) state

The analysis of the ESR signals of the preparations autoxidized for 12 h has shown that the manganese centers of the autoxidized catalase are mainly in the (Mn^{3+}, Mn^{3+}) state. The ESR spectrum of preparation Aux(NaH_2PO_4) (measured at 50 K) is represented in Fig. 3.2. The presence of a significant amount of the

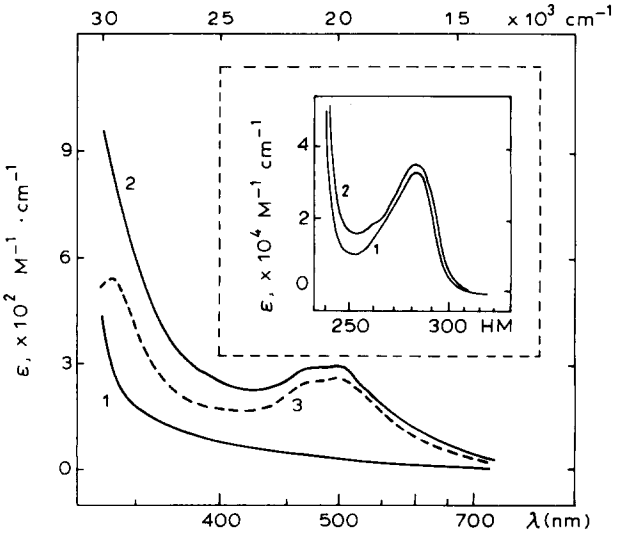


Fig. 1. Electronic spectra of manganese-containing catalase in visible and UV regions. (1) preparation Red(K-buffer); (2) preparation Aut(K-buffer); (3) difference spectrum, 2 – 1.

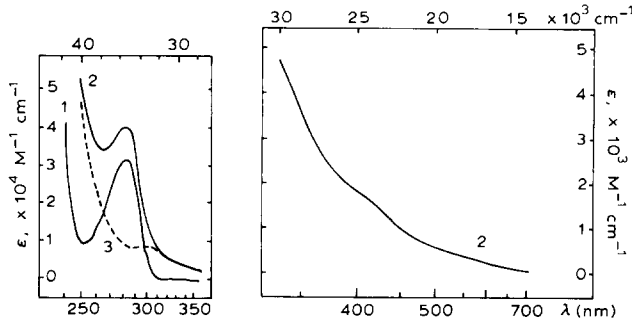


Fig. 2. Effect of periodate on the optical spectrum of catalase. (1) Preparation Red(K-buffer); (2) preparation oxidized by periodate; (3) difference spectrum, 2 – 1.

(Mn^{2+}, Mn^{2+}) and (Mn^{3+}, Mn^{4+}) states in this preparation would lead to the appearance of the signals A_p and C. From the comparison of signals shown in Fig. 3 we conclude that the relative content of the (Mn^{2+}, Mn^{2+}) and (Mn^{3+}, Mn^{4+}) states is quite small and the sum $n_{22} + n_{34}$ (see Eqn. 8) is less than 1%. A decrease in the temperature leads to the appearance of the signal B in the autoxidized preparations (not shown). The estimation of the relative content of the centers in the (Mn^{2+},

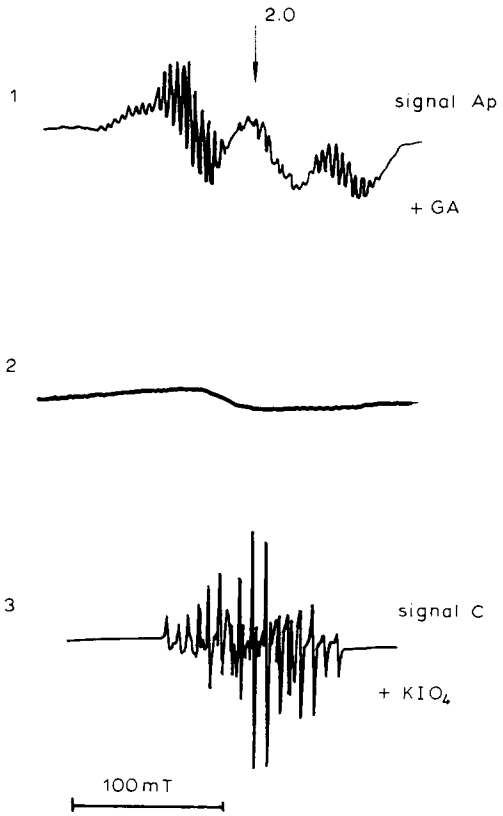


Fig. 3. Transformation of ESR signals from autoxidized preparations of catalase due to the action of various redox agents. (1) Preparation Red(NaH_2PO_4); (2) preparation Aut(NaH_2PO_4); (3) oxidation by periodate (signal C). For spectrum 3, the gain is reduced 8-times in comparison with spectra 1 and 2. $T = 50$ K and microwave power = 25 mW.

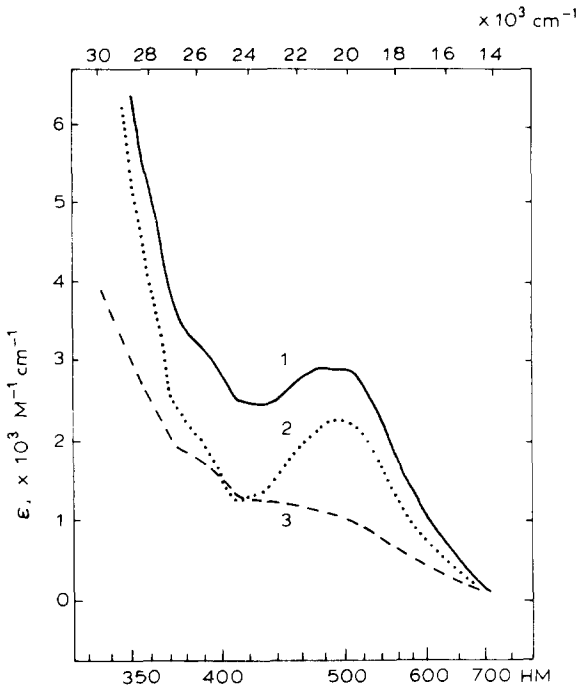


Fig. 4. H_2O_2-induced variations of the optical spectrum of autoxidized catalase. (1) Catalase Aut(K-buffer); (2) preparation Aut(NaH_2PO_4); (3) the same as in (2), but after H_2O_2 treatment.

Mn^{3+}) state performed by Eqn. 7 gives $n_{23} \approx 0.03$ and thus

$$n_{33} = 1 - n_{22} - n_{34} - n_{23} \approx 0.97$$

Prolonged (for 24 h) autoxidation is accompanied by the appearance of a small signal C (as in spectra presented in Fig. 6). In this case the n_{33} value may decrease to 0.94 due to the increase in $n_{34} \approx 0.03$.

Effect of H_2O_2 on the various redox states of the binuclear cluster

Effect of H_2O_2 on the (Mn^{3+}, Mn^{4+}) state. Earlier [16] we have shown that the catalase oxidized by KIO_4 to the state (Mn^{3+}, Mn^{4+}) exibits no activity in the H_2O_2 decomposition reaction and the ESR spectra of such preparations contain only signal C with no conversion to signal A or B [12,14]. This procedure also did not lead to any change in the electronic spectrum of the oxidized preparation.

Effect of H_2O_2 on the (Mn^{3+}, Mn^{3+}) state. Treatment of preparations Aut(NaH_2PO_4) and Aut(Cl^-) by peroxide drastically reduced the intensity of the band at 480–500 nm (Fig. 4) and caused the appearance of signals A_p and A_{Cl^-}, respectively (Figs. 5 and 6). The presence of the intense A_p (or A_{Cl^-} signals indicates that dialysis of the autoxidized preparations against the peroxide solutions transfers centers to the (Mn^{2+}, Mn^{2+}) state. Thus in these conditions peroxide has an effect like that of hydroxylamine and provides practically complete reduction of the oxidized centers to the (Mn^{2+}, Mn^{2+}) state.

Effect of H_2O_2 on the (Mn^{2+}, Mn^{3+}) state. The use of sufficiently low temperatures (below 20 K) allows the monitoring of signal B, which is related to the (Mn^+, Mn^{3+}) state. Dialysis of autoxidized preparations Aut(NaH_2PO_4) against H_2O_2 solution decreases the concentration of (Mn^{2+}, Mn^{3+}) centers. Since the relative contents of centers in the (Mn^{2+}, Mn^{3+}) state in these experiments is rather small, it is difficult to decide which process, oxidation or reduction, leads to the peroxide-induced decrease in the signal B amplitude (see Fig. 6, spectrum 3; note high-field hyperfine peaks). Fig. 6.I shows that the autoxidized catalase preparations

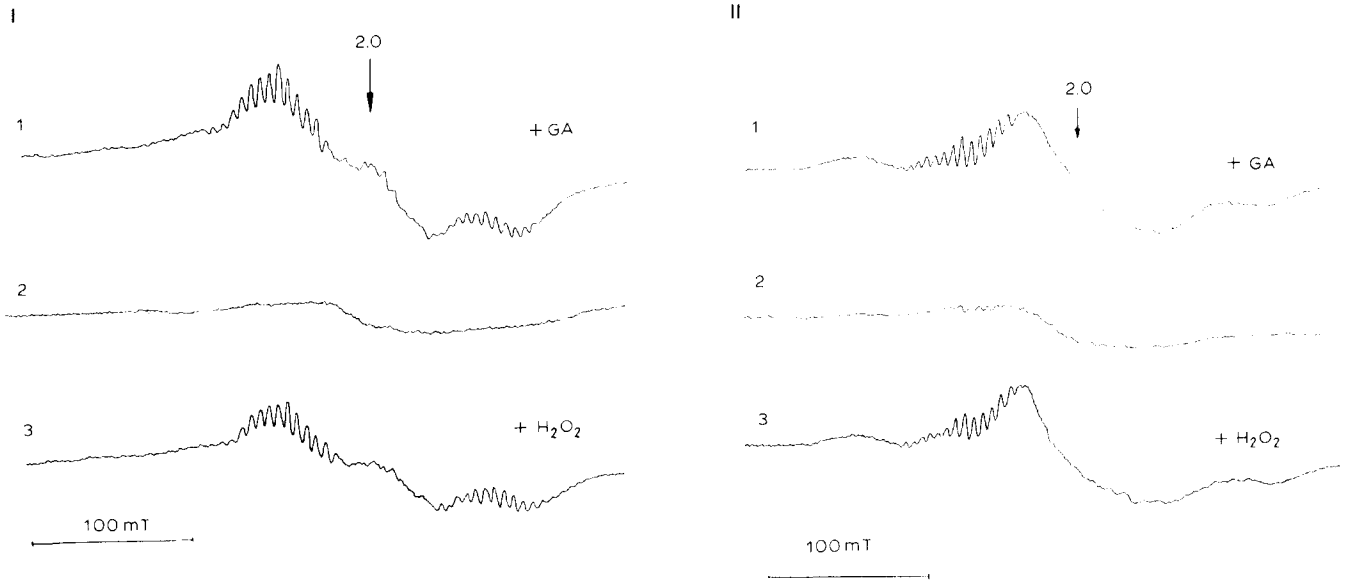


Fig. 5. Centers peroxide-reduced to the state $Mn^{2+}..Mn^{2+}$. I.(1) Preparation Red(NaH_2PO_4); (2) preparation Aut(NaH_2PO_4); (3) as (2) but after H_2O_2 treatment. II.(1) Preparation Red(Cl^-); (2) preparation Aut(Cl^-); (3) as (2) but after H_2O_2 treatment. The spectra were measured at 110 K with microwave power of 25 mW.

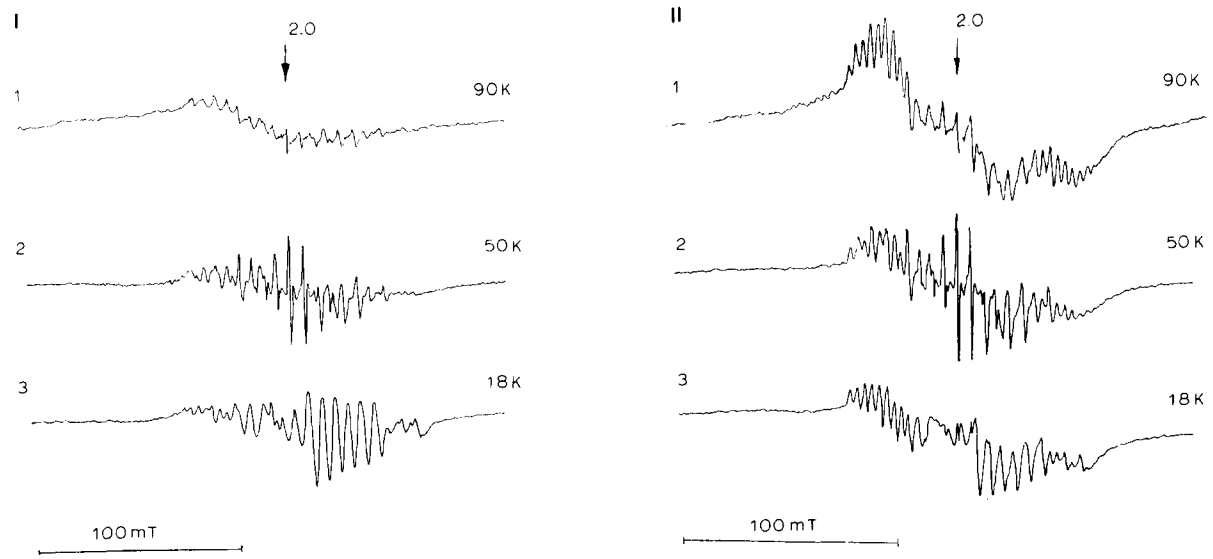


Fig. 6. H_2O_2-induced transformations of ESR spectrum of autooxidized catalase. (I) preparation Aut(NaH_2PO_4); (II) as (I) but after H_2O_2 treatment. The spectra were measured at (1) 90; (2) 50; and (3) 18 K. Microwave power of 25 mW.

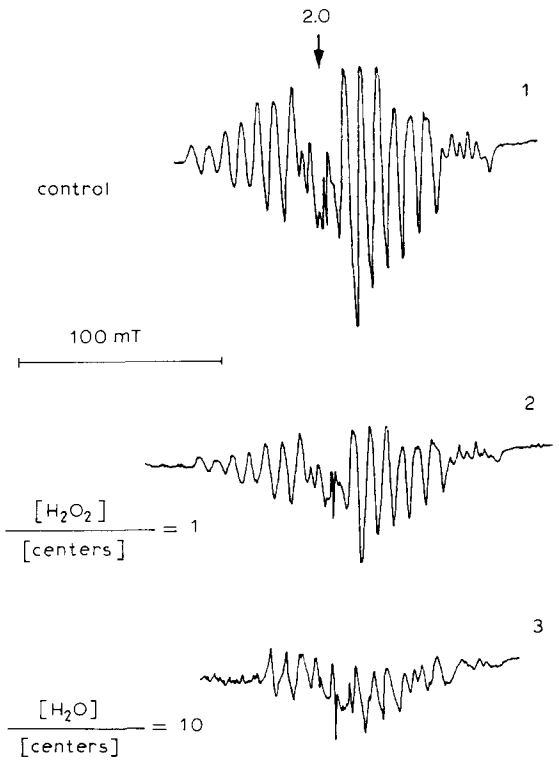


Fig. 7. Effect of H_2O_2 on the signal B in the experiments involving low-temperature inhibition. 0.2 mM of concentrated catalase solution (1 mg/ml) in 20 mM borate buffer (pH 9.2) was rapidly mixed with 0.2 ml of borate buffer containing peroxide solution. (1) Control sample (with peroxide-free borate buffer); (2) 0.3 mM peroxide solution (concentration equal to that of catalase subunits); (3) 3 mM peroxide solution (10-times the concentration of catalase subunits). Temperature for (1)–(3) is 18 K. Increasing the ESR measurement temperature results in the reversible disappearance of signal B (1–3) and in the appearance of signal C (see text). The intensity of the signal C at 50 K we used as an internal standard to determine the extent of filling of each samples. Microwave power of 25 mW.

exhibit weak ESR signals only from two states of Mn centers, (Mn^{2+}, Mn^{3+}) and (Mn^{3+}, Mn^{4+}). This established that the primary population is an ESR-silent state which we presume to be (Mn^{3+}, Mn^{3+}). The comparison of ESR spectra from preparations (I) and (II) measured at 90 K (spectrum 1) suggests that the treatment by H_2O_2 results in the appearance of signal A_p, reduction to state (Mn^{2+}, Mn^{2+}).

Effect of H_2O_2 on the (Mn^{2+}, Mn^{2+}) state. We have studied the effect of H_2O_2 on reduced preparations Red(NaH_2PO_4) and Red(Cl^-). A prolonged dialysis (1.5–2 h) of reduced preparations of both types (Red(NaH_2PO_4) and Red(Cl^-)) against 10 mM H_2O_2 produced no decrease in the intensity of signals A_p and A_{Cl^-} and, also, no noticeable increase in signals B and C. The dialysis did not result in the development of coloration of the catalase.

Study of effects of H_2O_2 on the active catalase preparations by the method of low-temperature inhibition

This method allows us to investigate the effects of H_2O_2 just on the preparations with high catalase activity Red(K-buffer) and Aut(K-buffer).

Reduced preparations I. The mixture of peroxide solution (20 mM H_2O_2 in 10 mM potassium phosphate (pH 6.8)) and reduced preparation with subsequent rapid freezing, using the low-temperature trapping set-up, produced no changes in the ESR signals. The experiments revealed neither signals B nor new ESR signals. It should be noticed that, although manganese clusters in this preparation are in the (Mn^{2+}, Mn^{2+}) state, the A-type signal is weak, so that the (Mn^{2+}, Mn^{2+}) state is practically ESR-undetectable. Indeed, it has been shown [13] that the (Mn^{2+}, Mn^{2+}) states give rise to pro-

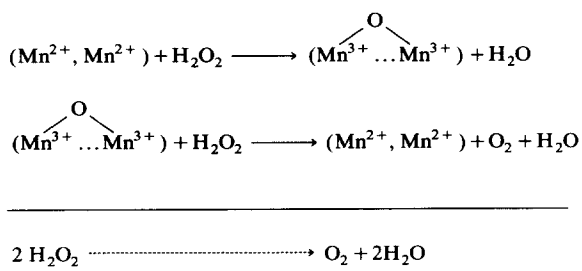
nounced ESR signals only if they are in the form of the complexes with suitable ligands (Cl^-, inorganic phosphate). Of course, it is possible to prepare Red(K-buffer) with a (Mn^{2+}, Mn^{2+}) state that is ESR detectable. After this procedure, however, the catalase activity of the preparations would be lost.

Autoxidized preparations. The mixing of peroxide and autoxidized preparations with subsequent rapid freezing was accompanied by a significant decrease in signal B (Fig. 7) which is present initially at very low concentration.

Discussion

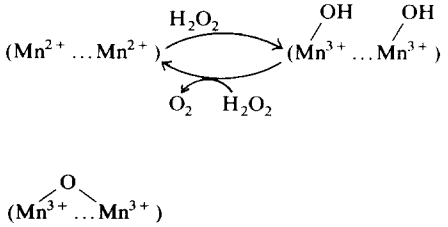
The experimental results of this work show that:
(a) Incubation of reduced catalase in oxygenated buffer results in the spontaneous oxidation of $Mn^{2+} \rightarrow Mn^{3+}$.
(b) The autoxidation occurs most effectively at alkaline pH;
(c) As a result of prolonged autoxidation, practically all centers are transformed into the (Mn^{3+}, Mn^{3+}) state. This state is not detectable by ESR, but can be determined spectrophotometrically by the absorption at 480–500 nm. In these preparations the content of centers in the (Mn^{2+}, Mn^{3+}) and (Mn^{3+}, Mn^{4+}) states does not exceed 7%.
(d) The addition of peroxide in the presence of such inhibitors as chloride and inorganic phosphate to the autoxidized catalase preparations reduces them to (Mn^{2+}, Mn^{2+}). In the present work we have obtained direct evidence that peroxide interacts effectively with manganese ions. Moreover, this experimental fact eliminates the confusion related to the result obtained earlier [16] of equal enzymatic activities of the reduced and autooxidized preparations. Indeed, in the experiments on amperoammetric determination of the decomposition rate of H_2O_2 using a Clark electrode the concentration of peroxide was 10^8-times that of enzyme molecules. Thus, immediately after the H_2O_2 addition an insignificant fraction of peroxide is consumed upon the reduction of the (Mn^{3+}, Mn^{3+}) centers to the (Mn^{2+}, Mn^{2+}) state. In other words, even if the catalase is initially present in the state (Mn^{3+}, Mn^{3+}), peroxide reduces the active (Mn^{2+}, Mn^{2+}) state, from which it instantly begins its normal catalytic redox cycle.

(Mechanism I)



In such cases, the question of which of the two states, is active, becomes inappropriate. Mechanism I should be treated as one of the most probable mechanisms for the functioning of manganese-containing catalases. Indeed, this mechanism has been experimentally confirmed by the study of catalase activity of a binuclear manganese cluster, representing a functional analog of the active center of manganese pseudocatalase [20].

Because we did not succeed in detecting stable compounds of peroxide with the catalase, we cannot exclude the existence of alternative mechanisms of peroxide decomposition. For example, formation of a reactive bridging peroxide intermediate cannot be excluded. Recent synthetic studies have succeeded in isolation of a peroxide-bridged (Mn^{4+}, Mn^{4+}) dimer (Wieghardt, K., personal communication). A third possibility is given by Mechanism II:



In this case, the intermediate forms an unbridged dihydroxyl structure. This differs from Mechanism I only by the addition of water. Further work is needed to distinguish these possibilities.

Acknowledgement

The authors are grateful to Professor L.A. Blumenfeld and Dr. M.G. Goldfield for fruitful discussion of the results.

References

1 Cheniae, G.M. and Martin, I.F. (1970) Biochim. Biophys. Acta 197, 219–239.
2 Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274–278.
3 Hansson, O. and Andreasson, L.E. (1982) Biochem. Biophys. Acta 679, 261–268.
4 Kono, Y. and Fridovich, I. (1983) J. Biol. Chem. 258, 6015–6019.
5 Kono, Y. and Fridivich, I. (1983) J. Biol. Chem. 258, 13646–13648.
6 Beyer, W.F. and Fridovich, I. (1985) Biochemistry 24, 6460–6467.
7 Fronko, R.M., Penner-Hahn, J.E. and Bender, C.J. (1988) J. Am. Chem. Soc. 110, 7555–7556.
8 Barynin, V.V. and Grebenko, A.I. (1986) Dokl. AN SSSR 286, 461–464.
9 Vainshtein, B.K., Melik-Adamyan, V.R., Barynin, V.V. and Vagin, A.A. (1984) in Progress in Bioorganic Chemistry and Molecular Biology, (Ovchinnikov, Yu., ed.), pp. 117–132, Elsevier, Amsterdam.
10 Barynin, V.V., Vagin, A.A., Melik-Adamyan, W.R., Grebenko, A.I., Khangulov, S.V., Popov, A.I., Andreanova, M.E. and Vainshtein, B.K. (1986) Dokl. AN SSSR 228, 877–880.

11 Khangulov, S.V., Barynin, V.V., Voevodskaya, N.V., Grebenko, A.I. and Melik-Adamyan, W.R. (1987) Int. Conf. Chem. Phys. Enzyme Catalysis Tallinn, Sept. 21–24, 1988.
12 Khangulov, S.V., Barynin, V.V., Melik-Adamyan, W.R., Grebenko, A.I., Voevodskaya, N.V., Blumenfeld, L.A., Dobryakov, S.N. and Ilyasova, V.B. (1986) Bioorg. Khim. 12, 741–748.
13 Khangulov, S.V., Barynin, V.V., Voevodskaya, N.V., Grebenko, A.I. and Melik-Adamyan, V.R. (1987) Biofizika 32, 960–966.
14 Khangulov, S.V., Barynin, V., Voevodskaya, N.N. and Grebenko, A.I. (1990) Biochim. Biophys. Acta, submitted.
15 Khangulov, S.V., Andreevo, N.E., Gerasimenko, V.V., Goldfeld, M.G., Barynin, V.V. and Grebenko, A.I. (1990) Fizich Khimii 61, 17–27.
16 Khangulov, S.V., Andreeva, N.E., Gerasimenko, V.V., Goldfeld, M.G., Barynin, V. and Grebenko, A.I. (1990) J. Inorg. Biochem., in press.
17 Stepanov, S.V., Khangulov, S.V., Burbaev, D.Sh. and Blumenfeld, L.A. (1985) Biofizika 30, 703–704.
18 Goldstein, D.B. (1968) Anal. Biochem. 24, 431–437.
19 Asaturyan, K.A., Chernyak, I.V., Nekhoroshev, N.S., Kuzyaev, A.P. and Muromtsev, V.I. (1968) Pribiry i Tekhn. Eksperim. 6, 203–207.
20 Mathur, P., Cronder, M. and Dismukes, G.C. (1987) J. Am. Chem. Soc. 109, 5227–5233.
21 Cooper, S.R., Dismukes, G.C., Klein, M.P. and Calvin, M. (1978) J. Am. Chem. Soc. 100, 7248–7252.
22 Magers, K.D., Smith, S.G. and Sawyer, D.T. (1980) Inorg. Chem. 19, 492–496.
23 Bodini, M.E., Willis, L.A., Riechel, T.V. and Sawyer, D.T. (1976) Inorg. Chem. 15, 1538–1543.
24 Sheats, J.S., Czernuziewicz, R., Dismukes, G.C., Rheingold, A., Petrouleas, V., Stubbe, J., Armstrong, W.H., Beer, R. and Lippard, S.J. (1987) J. Am. Chem. Soc. 109, 1435–1444.
25 Sychov, A.Ya, Isak, V.G. and Chan Tkhi Fyong (1984) Zh. Fiz. Khim. 58, 2332–2334.