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ESR spectroscopy of the binuclear cluster of manganese ions in the active center of Mn-catalase from *Thermus thermophilus*

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Our previous data of low-temperature ESR spectroscopy indicate that the active center of Mn-catalase from *Thermus thermophilus* is a binuclear manganese complex. The ESR spectra from initial preparations of Mn-catalase in the temperature range from 8 to 100 K are a superposition of three signals, A, B and C. These were attributed to a binuclear manganese site in the (Mn^{2+},Mn^{2+}), (Mn^{2+},Mn^{3+}) and (Mn^{3+},Mn^{4+}) states, respectively. In the present work we have studied the redox transformations of the manganese cluster and its interaction with exogenous ligands. Hydroxylamine reduces centers with signals B and C to give signal A. The (Mn^{2+},Mn^{2+}) centers easily form complexes with phosphate buffer, Cl^-, N_3^- and F^-. This state autooxidizes in air with the appearance of ESR signals B and C for states (Mn^{2+},Mn^{3+}) and (Mn^{3+},Mn^{4+}). Periodate (KIO_4) oxidizes all centers to the (Mn^{3+},Mn^{4+}) state. In the absence of KIO_4 this state undergoes spontaneous reduction to form the (Mn^{2+},Mn^{2+}) state. On the other hand, addition of KI reduces the (Mn^{3+},Mn^{4+}) state to the (Mn^{2+},Mn^{3+}). In contrast to the (Mn^{2+},Mn^{2+}) state, the (Mn^{3+},Mn^{4+}) state does not undergo ligand exchange with anions. We believe that in the (Mn^{3+},Mn^{4+}) state the manganese ions are connected by oxo- (or hydroxo-) bridges and that all coordination sites for the binding exogenous ligands are strongly occupied by oxygen atoms.

Introduction

The interest in enzymes whose active center is represented by a cluster of manganese ions arise due to the important role played by the four manganese ions in photosynthetic water oxidation system [1–4]. A manganese active center has been found in catalases from the extremely thermophilic bacterium *Thermus thermophilus* [5–10] and the nonthermophilic bacterium *Lactobacillus plantarum* [11–14]. The protein from lactic acid bacterium *L. plantarum* [11,12] has a molecular mass of 172000 Da and is composed of five subunits based on sedimentation studies. The active redox state was attributed to the Mn(III) oxidation state. Later investigations [13] revealed that each subunit contained about two manganese ions and that these were organized as a binuclear manganese center containing the Mn(III)...Mn(IV) state [14]. The latter report also attributes this state to the catalytically active state.

The catalase obtained from *T. thermophilus* HB 8, which we called Mn-catalase, exhibits unique thermal stability, retaining 85% activity after 10 min heating at 95°C and, like heme catalases, is inhibited by sodium azide. The Mn-catalase has a molecular mass of 210000 Da and consists of six identical subunits on the basis of X-ray diffraction [5]. The atomic structure has also been studied by X-ray diffraction and revealed the presence of two metal ions in each subunit separated by 3.6 ± 0.3 Å which were proposed to be manganese [6,7]. The metal ions in the active center were first identified as a binuclear manganese center by ESR [8]. A solution of Mn-catalase from *T. thermophilus* exhibits ESR signals from three oxidation states in the temperature range from 8 to 100 K [8–10]: signals A, B and C. Based on both spectral and thermal similarities we have previously suggested that signals A, B and C are due to the following redox states [8–10].

$$\underset{\text{Signal A}}{(Mn^{2+},Mn^{2+})} \text{---} \underset{\text{Signal B}}{(Mn^{2+},Mn^{3+})} \text{---} \underset{?}{(Mn^{3+},Mn^{3+})} \text{---} \underset{\text{Signal C}}{(Mn^{3+},Mn^{4+})} \quad (1)$$

Two of these, B and C, possess complex multicomponent spectra which are characteristic of manganese ions

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have mixed valencies of (Mn^{2+},Mn^{3+}) and (Mn^{3+},Mn^{4+}) [15–19]. The A-type signals are characteristic of binuclear clusters in the (Mn^{2+},Mn^{2+}) oxidation state [20–22].

To further identify the valence states responsible for the appearance ofsignals B and C we have studied various chemical treatments which interconvert signals A, B and C and the susceptibility of these states to complex formation with various anions.

Materials and Methods

Protein preparations. Protein was isolated and purified by the procedure described in Ref. 5. The separated protein had the form of a fine-crystalline precipitate in 50%-saturated ammonium sulfate. The preparation was stored at 5 °C. Before the experiments the excess ammonium sulfate was removed by dialysis against the 10 mM potassium phosphate buffer (pH 6.8) to yield concentrated protein solutions (20–50 mg/ml). The protein concentrations were determined by spectrophotometry ($\epsilon_{280\,nm} = 0.95\ mg^{-1} \cdot cm^{-1}$).

Treatments. The reduced and oxidized Mn-catalase preparations were obtained by dialysis against the 10 mM potassium phosphate buffer (pH 6.8) containing the reductant (1 mM $(NH_2OH)_2H_2SO_4$) or oxidant (5 mM KIO_4), respectively. The reductant or oxidant was removed by dialysis against the potassium phosphate buffer. Complexes with N_3^-, Cl^- and F^- were produced by dialysis against 5 mM NaN_3, 50 mM NaCl and 100 mM KF, respectively. For complexes with potassium phosphate, protein was dialyzed against 100 mM KH_2PO_4 buffer (pH 6.8).

In the general case the form of the ESR spectra for signal A (Mn^{2+},Mn^{2+}) depends on the composition of buffer in which the protein is dissolved. The signals of reduced preparations will be denoted in what follows by subscript 'L', e.g., signal A_L, where 'L' will indicate the presumed ligand which associates with the reduced enzyme. For example, the signal from reduced samples in the presence of chloride is called signal A_{Cl^-}.

Catalase activity. The catalase activity was measured by oxygen evolution using a Clark-type electrode [23]. The rate constant of hydrogen peroxide decomposition at 20 °C in 5 mM potassium phosphate buffer (pH 7.0) for freshly prepared Mn-catalase was about $10^7 \cdot M^{-1} \cdot s^{-1}$ (at a H_2O_2 concentration of 6 mM).

ESR measurements. The X-band ESR spectra were measured, as in Ref. 8, using an EPR-V spectrometer with a liquid helium cryostat [24]. The maximum amplitude (for 0 dB) of the magnetic component of the microwave field (H_1) was about 0.02 mT, the modulation frequency was 100 kHz, and the modulation amplitude was 0.5 mT. For signals A, B and C the optimal temperatures for ESR detection are 50–100, below 20 and 50–80 K, respectively as previously determined [8]. The value of the exchange integral, J, results from a least-squares fit of the experimental data in Fig. 2(I) to the Eqn. 1. The amplitude, A, of signal A_p as a function of temperature in the absence of microwave saturation was used to estimate the population of ESR active states (the position of the peak studied is indicated by a

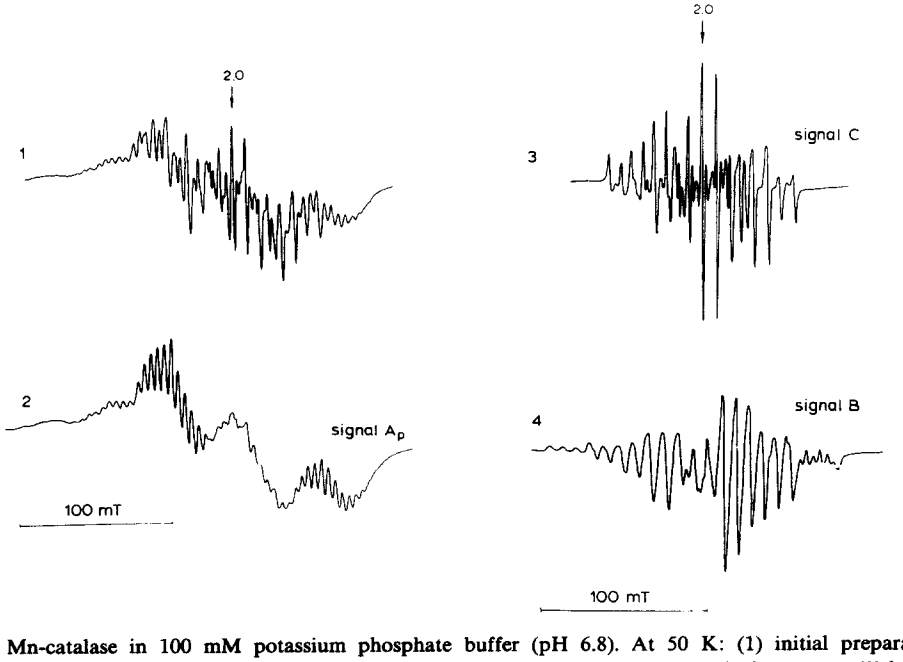


Fig. 1. ESR signals of Mn-catalase in 100 mM potassium phosphate buffer (pH 6.8). At 50 K: (1) initial preparations; (2) reduced by hydroxylamine (see 2.2); (3) oxidized by KIO_4 (the gain was reduced 8-times in comparison with (2)); (4) the same as (1) but at 12 K. The amount of the protein in the sample 2.7 mg. EPR conditions: microwave frequency, 9440 mHz; modulation frequency, 100 kHz; microwave power 25 mW; modulation amplitude, 0.5 mT.

triangle in Fig. 2(I)). Eqn. 1 was used to fit the temperature dependence of the population of the first exited state $S=1$ (n_1) in the six-level system arising from strong antiferromagnetic exchange ($2J\ S_1S_2$) between two Mn^{2+} ions with $S_1 = S_2 = 5/2$ and $S = 0, \pm 1, \pm 2$.

$$A \approx n_S \approx (2S+1)\exp[-S(S+1)J/T] \Big/ \sum(2S+1)\exp[-S(S+1)J/T] \quad (1)$$

Results

In Fig. 1 the untreated catalase preparation dialyzed against the 100 mM potassium phosphate buffer (pH 6.8) exhibits at 50 K a multicomponent ESR spectrum which is a mixture of two major components, signals A_p and C (Fig. 1.2 and 1.3). As the temperature decreases to 8–15 K, an additional signal B appears in the untreated preparations (Fig. 1.4). Differences in the temperature dependences of signals B and C allow the choice of optimal conditions for separation of these signals [8].

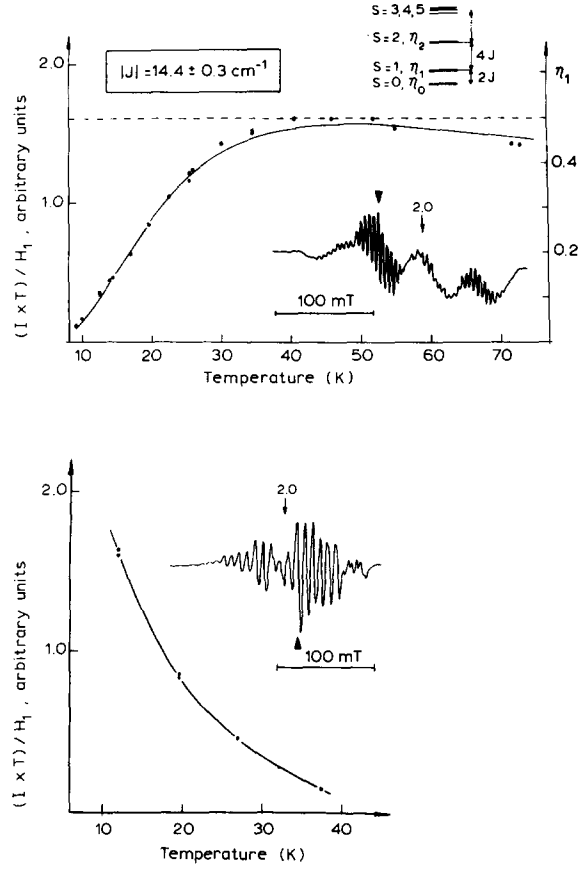


Fig. 2. Temperature dependence of A_p and B signals in absence of saturation. I - amplitudes of signals. The position of corresponding components are shown by triangles. The trivial dependences of the $1/T$ type and of the microwave power eliminated by using the product $I \times T/H_1$. In the case of temperature dependence of signal A_p (upper curve), the solid line represents theoretical curve for the temperature dependence of the population of the first excited state (see text, Eqn. 1).

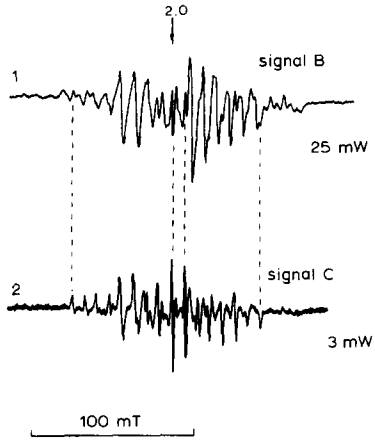


Fig. 3. Separation of signals B and C using the method of microwave saturation. (1) ESR signal of the initial catalase preparation measured at 25 mW power; (2) the same sample measured at 3 mV. The temperature of measurement 12 K. The preparation is the same as in Fig. 1 (1).

As the temperature decreases from 70 to 10 K, the intensities of some hyperfine-structure components of signal A_p drops, provided microwave saturation is absent (arrow in Fig. 2 (I)). This fact explains the disappearance of signal A_p in the spectra obtained in temperature range from 8 to 15 K (Fig. 1.4). In contrast to the signal A_p, whose intensity drops as the temperature decreases, the low temperature range from 8 to 25 K favors detection of signal B. As the temperature grows, the amplitude of this signal in the absence of microwave saturation decreases at a greater rate than $1/T$ (Fig. 2). The C signal can be observed in a wide temperature range (from 8 to 120 K) and is easy saturated at lower tempratures. Below 77 K the temperature dependence of the intensity of the individual spectral components (in the absence of microwave saturation) obeys the Curie $1/T$ law (data not shown). Unlike signal C, the intensity of signal B exhibits practically no saturation at low temperatures. The components of signal B and C are strongly overlapping. In spite of this, the difference in relaxation properties allows a reliable separation of both signals. Fig. 3 shows the variation of the shape of the spectrum measured at 12 K, at two microwave powers. By choosing a sufficiently large microwave power and a sufficiently low temperature, it is possible to separate the pure B signal.

Complex formation; ESR spectra from the reduced preparations

Mn-catalase in the state (Mn^{2+},Mn^{2+}) is highly active in the ractions of complex formation with ligands (Fig. 4). Thus, the dialysis of the reduced preparations against 100 mM potassium phosphate buffer (pH 6.8) results in the appearance of signal A_p (see Figs. 1.2 and

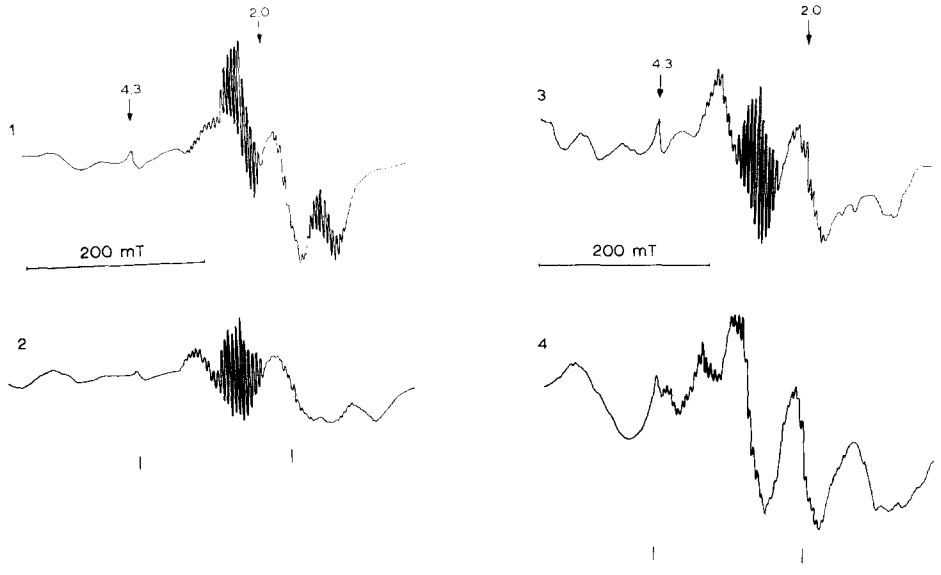


Fig. 4. ESR signals from (Mn^{2+},Mn^{2+}) preparations of Mn-catalase. The initial preparations were reduced by hydroxylamine; the excess reducing agent removed by dialysis against (1) 100 mM potassium phosphate buffer (pH 6.8); (2) 50 mM NaCl; (3) 5 mM NaN_3; (4) 100 mM KF. Temperature of measurements 50 K; the microwave power 25 mW.

4.1). Dialysis of the reduced preparations against 50 mM NaCl, 10 mM NaN_3 or 100 mM KF eliminate signal A_p and produces the new signals A_{Cl^-}, $A_{N_3^-}$, and A_{F^-} (Fig. 4, curves 2, 3, 4, respectively). In the absence of anions the ESR of the reduced preparations is weak and without characteristic structure. These spectral changes do not involve redox transformations and have the character of ligand substitution reactions, presumably involving the manganese coordination sphere.

Signal transformation during the action of redox agents

Fig. 5 shows schematically the redox transitions of the enzyme. As has been noted above the spectra from initial preparations of the Mn-catalase represent a superposition of signals A, B and C. Treatment with reducing agents hydroxylamine, hydrazine or dithionite,

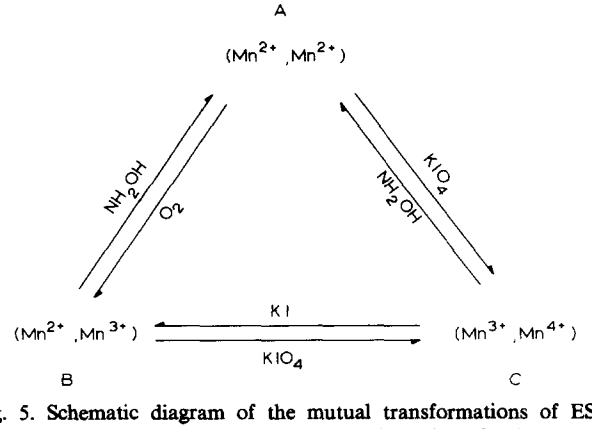


Fig. 5. Schematic diagram of the mutual transformations of ESR signals from Mn-containing catalase due to the action of redox agents. The letters A, B and C denote which type of signal is characteristic for each particular state (see text).

followed by dialysis against the 100 mM potassium phosphate buffer (pH 6.8), eliminates signals B and C, and induces the growth of signal A_p, along with loss of the visible absorption bands. The reduction resulted in discoloration of the samples which were initially pinkish in appearance.

The reduced enzyme (state (Mn^{2+},Mn^{2+})) undergoes auto-oxidation following prolonged dialysis (48 h) against the 5 mM potassium phosphate buffer (pH 6.8), as seen by the formation of the signals B (primary) and C. This treatment also restores the pinkish color associated with the visible absorption bands. The rate of auto-oxidation can be accelerated (3–4 times) by dialysing the reduced preparations against weak alkaline solutions (20 mM borate buffer (pH 8.5–9.0)). A significant retardation of the auto-oxidation process can be achieved by storing the reduced catalase preparations in a sufficiently concentrated potassium phosphate buffer (100 mM (pH 6.8)), or by addition of NaCl (5 mM potassium phosphate buffer (pH 6.8), 50 mM NaCl). This suppression of auto-oxidation appear to be due to the formation of specific coordination complexes between phosphate or chloride and manganese ions, as deduced from the ESR studies (Fig. 4). Treatment of both the initial and the reduced (Mn^{2+},Mn^{2+}) preparations with periodate, which is an effective oxidizer of divalent manganese ions [25], eliminates signals A and B and yields the 16-component signal C (Mn^{3+},Mn^{4+}) state. Compared to the initial preparations, the amplitude of signal C increased 50–80-times, and the sample became yellow-brown. The oxidation by periodate is reversible; in the presence of 100 mM KH_2PO_4 (pH 6.8) reduction by hydroxylamine eliminates signal C

and induces signal A_p. The preparations oxidized by periodate also exhibit spontaneous reduction after removal of the oxidizing agent; a gradual transition over 24 h at 20 °C to the reduced state occurs in 20–50% of centers, as manifested by the appearance of signal A_p.

The addition of KI (200 mM) to the oxidized catalase preparations in state (Mn^{3+},Mn^{4+}) eliminated signal C and induced and intense signal B. The iodide titration of oxidized Mn-catalase showed that an increase in the intensity of signal B was accompanied by a decrease of signal C. After the addition of iodide, the intensity of signal B was 50–100-times that observed in the initial preparations. Prior to interpretation of the iodide titration experiments, we sought to see if molecular iodine (I_2) in the concentrated KI solutions was the source of this reaction. Addition of water-saturated I_2 to the oxidized Mn-catalase preparations (1 : 1, v/v) produces no change in the intensity of signal B or C, thus establishing iodide as the source of the reaction. The line shape of signal C (Mn^{3+},Mn^{4+}), unlike that of signal A, does not depend on the composition of buffer in which the sample is oxidized, or used to simply measure after formation. Oxidized preparations dialyzed against distilled water, potassium phosphate buffer, Tris-HCl buffer, sodium acetate buffer, chloride or fluoride salts were indistinguishable. There appears to be no evidence for differences in coordination environment once the Mn-catalase is oxidized to the (Mn^{3+},Mn^{4+}) state.

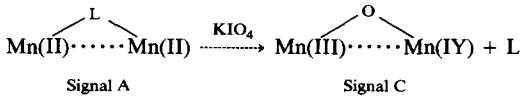
Discussion

A characteristic feature of the signal C is the presence of 16 main components. Separation between the components is about 7.3 mT and the linewidth of individual component is 1–2 mT. The total width of the signal is nearly 120 mT. No extra peaks have been observed in the range of lower and higher field strengths. Signal B extends over 156 mT, the distance between individual components in the central part of the spectrum is about 8 mT and the linewidth of such a component is about 3 mT. Line shapes of these signals are characteristic of both (Mn^{2+},Mn^{3+}) and (Mn^{3+},Mn^{4+}) complexes [15,19]. The interconversion of ESR signals induced by different chemical agents allow us to connect signals B and C with (Mn^{2+},Mn^{3+}) and (Mn^{3+},Mn^{4+}) states, respectively.

An analysis of the data available in the literature shows that the A-type signals are characteristic of ligand-bridged binuclear clusters in the (Mn^{2+},Mn^{2+}) state [22–24]. The signal A_p is characterized by the presence of several wide components in the range 250–450 mT arising from an excited spin state, a weak transition with g-factor in the range from 9–11 and two hyperfine-structured line groups separated by nearly 104 mT, with the individual linewidth about 2.2 mT. Each group contains 11 intense lines separated by 4 mT and a number of satellites. The major characteristic features responsible of signal A_p were modelled assuming that the paramagnetic center responsible for this signal is due to a triplet state $S = 1$ of the binuclear cluster (Mn^{2+},Mn^{2+}) coupled by an antiferromagnetic interaction ($2JS_1S_2$) [8]. The model explained the presence of both wide components in the central part of the spectrum and the 11-component groups. According to this model, the paramagnetic state is an excited triplet state which lies at $2J = 28 \pm 0.6$ cm^{-1} above the $S = 0$ ground state.

Investigation of the magnetic properties of binuclear manganese complexes of polydentate Schiff bases [26] has shown that the Mn(II) ions are coupled by a weak antiferromagnetic interaction with $J = 0.65$ cm^{-1}. Such a small J value (compared with that of Mn(II)–Mn(II) pair of Mn-catalase) may be explained either by a longer Mn...Mn distance or by an ineffective orbital-exchange pathway. A better fit can be obtained by comparison of our data with the results of investigation of dimanganese(II) complexes which are functional analogs of the Mn catalases [22]. Indeed, the temperature dependence of one ESR signal observed in such complexes (Fig. 4 in Ref. 22) practically coincides with that of the signal A_p. The optical spectrum of centers in auto-oxidized preparations has been shown to resemble that observed in dimanganese(III) complexes possessing the μ-oxo-di-μ-carboxylate bridging ligands [26].

We hypothesize that oxidation of Mn-catalase from the (Mn^{2+},Mn^{2+}) to the (Mn^{3+},Mn^{4+}) state, is accompanied by formation of oxo- (or hydroxo-) bridges between the manganese ions, which give a complete coordination shell that is inert to ligand substitution reactions. Upon reduction to the (Mn^{2+},Mn^{2+}) state, the oxo- (or hydroxo-) bridges are weakened of broken to form labile or vacant coordination sites available to exogenous ligands, as depicted in scheme 2:



(2)

By analogy, it has been shown that hydroxo- (oxo-) bridges form by chloride displacement and two-electron oxidation of a synthetic dimanganese(II) complex, yielding Mn(III)–O–Mn(III) during catalytic disproportionation of H_2O_2 [22].

We note the similarity of lineshapes of the ESR signals observed from auto-oxidized preparations of Mn-catalase from *L. plantarum* [14] and the signal C given here. On the other hand, the authors of Ref. 14 noted that Mn-catalase preparations from *L. plantarum* exhibited no other ESR signals analogous to our signals A and B. This is not a contradiction, but is presumably due to a lack of examination of the full range of

conditions needed to observed these weaker signals. We believe that reduction of the enzyme from *L. plantarum*, and a proper choice of exogenous ligands may lead to the appearance of both signals. The selection of suitable ligands is essential because it is difficult to predict what kind of anions will lead to the appearance of a pronounced ESR signal from the (Mn^{2+},Mn^{2+}) state.

The unique structure of the active center of Mn-catalase makes the enzyme a very convenient object for the investigation of various physico-chemical properties of binuclear cluster of manganese ions, such as the oxidation-reduction transformations, complex formation, reactivity, etc.

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