EPR Spectral Evidence for a Dinuclear Active Site in the *Lactobacillus Plantarum* Manganese Catalase

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Mn-containing catalases have recently been isolated from *Lactobacillus plantarum*,[1] *Thermus thermophilus*,[2] and *Thermoleophilum album*.[3] The active sites in these enzymes are unknown, although the efficient disproportionation of hydrogen peroxide most likely requires an active site capable of undergoing 2-electron chemistry. The model originally put forward for the *L. plantarum* enzyme involved a single Mn cycling between Mn(III) and Mn(V).[1,4] In light of the more recent finding that this enzyme contains two atoms of Mn per subunit,[5] alternate mechanisms in which a dinuclear Mn site cycles between, e.g., Mn(III)/Mn(III) and either Mn(II)/Mn(II)[6] or Mn(IV)/Mn(IV) should also be considered. We report here EPR evidence that Mn catalase isolated from *L. plantarum* contains a mixed valence dinuclear Mn cluster.

Mn catalase was isolated following the procedure of Fridovich.[5] Mn was quantitated both by neutron activation analysis and by EPR analysis of the Mn(II) released from the acid-denatured protein. Protein concentration was determined spectrophotometrically from the absorbance at 280 and 260 nm.[7] Activity was determined spectrophotometrically[8,9] by following the decrease in H_2O_2 absorption at 240 nm. Purified protein had the same activity/Mn ratio as reported previously.[5] Polyacrylamide gel electrophoresis under denaturing conditions visualized by silver staining showed only a single band of ca. 30 kDa molecular weight.

The EPR spectrum[10] for the as-isolated protein at 6 K is shown in Figure 1. This 16-line spectrum with amplitude ratios of approximately (1:1:2:2:3:3:3:3:3:3:3:3:2:2:1:1) is characteristic of exchange-coupled mixed-valence Mn dimers. In the present case, the spectrum can be adequately simulated with use of the Hamiltonian[11]

$$g_{\parallel}\beta H_z S_z + g_{\perp}\beta(H_x S_x + H_y S_y) + (A_1 I_1 + A_2 I_2)S - 2JS_1 S_2$$

The simulation in Figure 1 was obtained with $g = 2.0075$, $A_1 = 144 \times 10^{-4}$ cm^{-1}, $A_2 = 76 \times 10^{-4}$ cm^{-1}. An M_I-dependent Lorentzian line width $\Delta = \Delta_0 + A|M_1| + B|M_2|$ was used, with $\Delta_0 = 15$ G, $A = 2B = 2$ G, where M_1 and M_2 are the projections of the nuclear spin corresponding to A_1 and A_2, respectively.

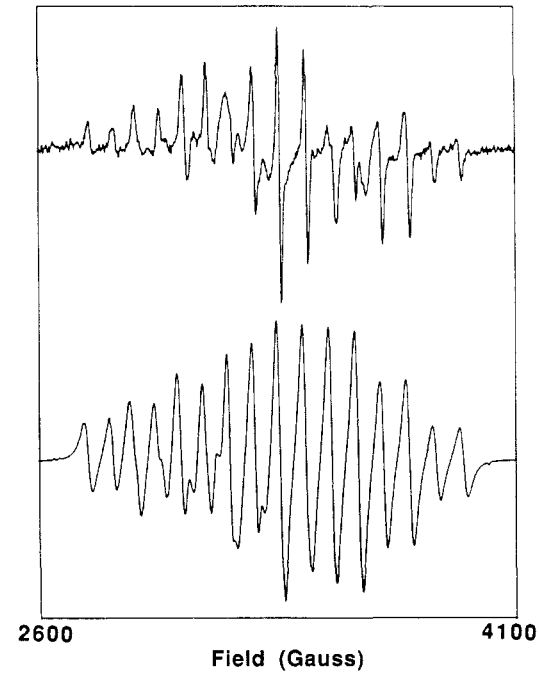

Figure 1. EPR spectrum for *L. plantarum* Mn catalase at 6 K. (Top) Experimental spectrum.[10] (Bottom) Simulation of catalase spectrum, using parameters described in the text.

Barynin and co-workers[12] recently reported EPR spectra for the Mn catalase from *T. thermophilus*. The spectra for this enzyme are quite complicated. At temperatures below 50 K, the dominant feature is an 18-line signal attributed to a {Mn(II)-Mn(III)} dimer. This signal is strongly temperature dependent and is not detectable above 50 K. At 50 K the spectrum is a mixture of a 22-line signal and a 16-line signal, attributed respectively to {Mn(II)Mn(II)} and {Mn(III)Mn(IV)} forms of the enzyme. The latter signal was modeled by using parameters essentially identical with those in Figure 1. We have seen no evidence for the 18-line or the 22-line signals in the *L. plantarum* catalase.

In order to determine the relationship between the observed 16-line signal and the catalytic activity, the EPR spectrum was measured for several samples prepared under slightly different conditions. The multi-line signal was observed for all samples, although in some cases (e.g., a sample heated to 40 °C for several hours) the 6-line signal characteristic of free Mn(II) was also observed. In all cases, the 6-line signal could be removed by dialysis and/or ultrafiltration using a membrane having a 10 kDa molecular weight limit. The intensity of the 16-line signal increases as catalytic activity increases; this dependence is linear within the noise level of the data. Taken together, these observations demonstrate that the 16-line signal is associated with active enzyme and suggest that this signal arises from the active site.

The intensity of the 16-line signal decreases by a factor of 2.5 between 3.9 and 9.3 K. Over this temperature range, the intensity exhibits Curie-law behavior, indicating that this signal arises from a spin $-^1/_2$ ground state and that there are no low-lying excited-spin states. The 16-line signal becomes undetectable at temperatures above ca. 110 K. This may account for the earlier report[5b] that the *L. plantarum* catalase does not show an EPR signal.

Acidification of the protein solution results in loss of the 16-line signal and appearance of a 6-line signal characteristic of free Mn(II). Double integration of these signals shows that the species giving rise to the 16-line signal accounts for at least 60% of the

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(9) Assay conditions: 13.0 mM H_2O_2, 50 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 25 °C, $\epsilon_{240}(H_2O_2) = 43.6$ M^{-1} cm^{-1}.
(10) EPR parameters: 9.43171 GHz spectrometer frequency; 300 μW microwave power; 100 kHz modulation frequency; 6.3 G peak-to-peak modulation amplitude; 5-10 μM [Mn].
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acid-labile Mn in the sample.[13]

Although Mn catalase was originally identified on the basis of its insensitivity to azide and cyanide,[1,14] it has since been shown[2,3,15] that all three Mn catalases are weakly inhibited by these anions. We observe no change in the EPR spectrum following addition of 25-fold excess of either NaCN or NaN_3. However, addition of 250-fold excess of NaN_3 results in loss of the 16-line signal and formation of the Mn(II) 6-line EPR spectrum. The enzyme shows no activity following treatment with 250-fold excess NaN_3, and activity is not recovered following dialysis.

There is substantial interest in mixed-valence Mn complexes because of their potential relevance to the photosynthetic oxygen-evolving complex. Many mixed valence Mn(II)/Mn(III)[16] and Mn(III)/Mn(IV)[11,17] dimers have been prepared in recent years. These complexes characteristically show a 16-line EPR signal similar to that observed for Mn catalase, with Mn(III)/Mn(IV) dimers showing the closest similarity.[18] On the basis of similarities in the optical spectra, it has been proposed[17c,f] that the Mn catalase contains a μ-oxo, di-μ-carboxylato bridged Mn(III) dinuclear core. One-electron oxidation of a model system having this core[17c] gives a species having an EPR spectrum similar to that for Mn catalase.

In conclusion, we have presented the first direct evidence for a dimeric Mn active site in the *L. plantarum* Mn catalase. This site is a mixed valence Mn cluster having spectroscopic properties similar to one of the forms of the *T. thermophilus* catalase. A low-resolution crystal structure of the latter enzyme[19] is consistent with a dinuclear Mn site; however, the resolution is not sufficient to provide additional structural details. The simplicity of the *L. plantarum* EPR spectra suggest that this enzyme will be amenable to more detailed spectroscopic investigation. Experiments along these lines are in progress.[20]

(13) Samples were denatured by acidifying to pH 0 with concentrated HCl and heating at 50 °C for 15 min. The concentration of Mn(II) released, as determined by comparison with standard Mn(II) solutions, was similar to that determined by neutron activation analysis, demonstrating that these conditions are efficient in converting protein bound Mn to the $Mn^{II}(H_2O)_6$ complex. Quantitation was estimated by double integration of the 16-line and the 6-line spectra measured at 6 K. Integrals were calculated over 2722–4722 G. The dominant feature in the EPR spectrum of the denatured protein is the 6-line signal arising from the $m_s = -1/2$ to $1/2$ transition, with some additional contribution from the other allowed transitions. The experimental ratio of double integrated intensities was 10:1 (6-line:16-line). The expected ratio ranges from 6:1 (assuming only $m_s = -1/2$ to $1/2$ transitions contribute to 6-line spectrum) to 23:1 (assuming all allowed transitions contribute to 6-line spectrum), thus at least 60% of the acid-labile protein-bound manganese is present in the $S = 1/2$ dinuclear cluster.

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(18) If the experimental dinuclear A_1 and A_2 are converted[17b] to mononuclear Mn hyperfine coupling constants assuming a Mn(III)/Mn(IV) formulation, we obtain 72 and 76 G for Mn(III) and Mn(IV). A Mn(II)/Mn(III) formulation gives 62 and 57 G for Mn(II) and Mn(III). The former is most consistent with literature values.[16,17]

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π-Localization in Aromatic Ligands: Formation of Mixed-Metal η^2:η^2-μ-Arene Complexes of Ruthenium(II) and Osmium(II) Ammines

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Recently we reported the synthesis of a novel class of pentaammineosmium(II) compounds in which an arene is coordinated η^2 to the metal center.[1] Relative to others reported,[2] these complexes offer unusual kinetic stability, allowing their convenient manipulation and study at room temperature. Both theoretical calculations[3] and crystallographic data[4] indicate that the η^2 mode of ligation disrupts the aromaticity of the arene. Bearing this in mind, it does not seem surprising that $[Os(NH_3)_5(\eta^2\text{-}C_6H_6)](OTf)_2$, (**1**), is inherently unstable with respect to the liberation of benzene and formation of $[\{Os(NH_3)_5\}_2(\eta^2{:}\eta^2\text{-}\mu\text{-}C_6H_6)](OTf)_4$.[1] This shows that the affinity of pentaammineosmium(II) for an η^2-coordinated arene is greater than for the unperturbed ligand and suggests that the loss in stabilization by π electron localization is greater in the first stage of metal binding than in the second. These considerations led us to investigate the reaction of **1** with other metal centers in order to evaluate the ability of pentaammineosmium(II) to activate arenes through the localization of their π electrons.

Though our attempts to form an arene complex of $\{[Ru(NH_3)_5]^{2+}\}$ with either benzene or naphthalene have been unsuccessful,[5] we find that when a MeOH solution of $[Ru(NH_3)_5(CH_3OH)]^{2+}$ is treated with 1 equiv of **1** the formation of the mixed-metal μ-arene complex **2** is observed.[6]

$$[Ru(NH_3)_5]^{2+} + [Os(NH_3)_5(\eta^2\text{-}C_6H_6)]^{2+} = [Os(NH_3)_5Ru(NH_3)_5(\eta^2{:}\eta^2\text{-}\mu\text{-}C_6H_6)]^{4+}$$
2

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(5) The affinity of olefins for $\{[Ru(NH_3)_5]^{2+}\}$ and other π acids has been widely demonstrated. See: Ford, P. C. *Coord Chem. Rev.* **1970**, 75. Also see ref 14. Reducing $Ru(NH_3)_5(OTf)_3$ in the presence of excess arene in either acetone or MeOH resulted solely in the corresponding (solvent)pentaammine complex.

(6) All reactions were carried out under argon. $Ru(NH_3)_5(OTf)_3$ (ref 18) (50 mg) and Zn/Hg (250 mg) were combined in MeOH (4.0 mL), and the solution was stirred 0.5 h. The clear yellow solution was pipetted away from reducing agent and added to 43 mg of $[Os(NH_3)_5(\eta^2\text{-}C_6H_6)](OTf)_2$ (see ref 1). After an additional 0.5 h, the reaction mixture was added slowly to Et_2O (20 mL) upon which an oily solid forms. The oil was worked into a solid from acetone and Et_2O and dried under vacuum. Alternatively, **2** can be isolated as a chloride salt by the addition of excess bis(triphenylphosphoranylidene)-ammonium chloride to the original reaction mixture.

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