Notes

The Existence of Two Oxidized Mn(III)Mn(III) Forms of *Thermus thermophilus* Manganese Catalase

Isabelle Michaud-Soret,[†] Lilian Jacquamet,[†] Noëlle Debaecker-Petit,[†] Laurent Le Pape,[†] Vladimir V. Barynin,[‡] and Jean-Marc Latour*,[†]

Département de Recherche Fondamentale sur la Matière Condensée, Service de Chimie Inorganique et Biologique, Laboratoire de Chimie de Coordination (Unité de Recherche Associée au CNRS 1194), CEA-Grenoble, F-38054 Grenoble, France, Institute of Crystallography, Russian Academy of Sciences, Moscow, Russia, and Krebs Institute, Department of Molecular Biology & Biotechnology, University of Sheffield, Sheffield S10 2TN, U.K.

Received September 24, 1997

Catalases are ubiquitous enzymes that protect living cells against the deleterious effects induced by hydrogen peroxide by effecting its disproportionation into dioxygen and water. The catalases present in some bacteria[1] such as *Thermus thermophilus* and *Lactobacillus plantarum* possess a dimanganese active site, revealed by preliminary X-ray diffraction analyses[2] as well as by EPR studies.[3−5] Up to now four different redox states of the dimanganese center have been observed: Mn(II)Mn(II), Mn(II)Mn(III), Mn(III)Mn(III) and Mn(III)Mn(IV). Mechanistic studies[6] have shown that during catalysis the dimanganese center shuttles between Mn(II)Mn(II) and Mn(III)Mn(III) forms. No EPR spectrum has yet been detected for the Mn(III)Mn(III) state, which exhibits a broad absorption ($\lambda = 470-510$ nm; $\epsilon = 300$ $M^{-1}\cdot cm^{-1}$/monomer) in the visible region; the latter feature has been taken as an indication from analogy with model complexes[7] that in this form of the protein the two manganese(III) ions are bridged by an oxide and carboxylate ligands.

To derive structural information about the catalytically active Mn(III)Mn(III) state of the *T. thermophilus* catalase, we have used variable-field, variable-temperature saturation magnetization. Indeed, this technique proved very informative in the study of the reduced active form of the enzyme.[8] The results obtained in the present study show that two different Mn(III)Mn(III) forms exist in a pH-dependent equilibrium.

Experimental Section

All samples were checked by UV−visible and EPR spectroscopies which allowed quantitative analysis of the Mn(II)Mn(III), Mn(III)Mn(III), and Mn(III)Mn(IV) forms as previously described.[8]

Two samples of the autoxidized enzyme were studied in 5 mM Tris−HCl buffer at pH 8.4. The initial sample (3.4 mM in dimanganese center) was studied as isolated in aerobic conditions and contained 25(5)% of mixed-valence states: Mn(II)Mn(III) and Mn(III)Mn(IV). The second sample (2.1 mM) was studied after reduction to the Mn(II)Mn(II) state by addition of hydroxylamine (up to 10 equiv per manganese ion) followed by three dilution/concentration cycles against 5 mM Tris−HCl buffer at pH 8.4 and incubation in air at room-temperature overnight.

The UV−visible experiment was performed on a 0.45 mM sample containing the mixture of the two Mn(III)Mn(III) forms in 5 mM Tris−HCl buffer, at pH 7.7 initially. The pH was then switched from 6.6 to 9.8 by successive additions of HCl or NaOH. The measurements were performed on a Lambda 9 Perkin-Elmer spectrophotometer. Two other samples were autoxidized at the two extreme pHs (6.5 and 9.5) in a 20 mM bis-tris/propane buffer according to the above procedure. EPR controls showed that the low-pH sample (0.98 mM) contained ∼12% of the Mn(II)Mn(III) state while the high-pH sample (0.39 mM) contained ∼2% of the Mn(II)Mn(III) state and ∼10% of the Mn(III)Mn(IV) state.

Two last samples (0.77 and 0.95 mM) of the KIO_4-oxidized enzyme obtained as described in the literature[6] were studied in 10 mM phosphate buffer at pH 6.8.

The magnetization measurements were performed as described previously.[8,9]

Results

When reduced catalase was autoxidized at pH 8.4, the resulting sample showed less than 5% of the EPR-active mixed-valent species. On the other hand, the magnetization experiment showed that the sample contained a weakly antiferromagnetically coupled species, since the product of the molar susceptibility by temperature is almost independent of temperature over the 100−200 K temperature range and decreases at lower temperatures. Nevertheless, the quantity of manganese estimated from these high-temperature data was only ∼50% of the manganese

[†] Laboratoire de Chimie de Coordination.
[‡] Russian Academy of Sciences and University of Sheffield.

(1) (a) Penner-Hahn, J. E. *Structural Properties of the Manganese Site in the Manganese Catalases*; VCH: New York, 1992; pp 29−45. (b) Dismukes, G. C. *Chem. Rev.* **1996**, *96*, 2909−2926.
(2) (a) Barynin, V. V.; Vagin, A. A.; Melik-Adamyan, V. R.; Grebenko, S. V.; Khangulov, S. V.; Popov, A. N.; Andrianova, M. E.; Vainshtein, B. K. *Sov. Phys. Dokl.* **1986**, *31*, 457−459. (b) Barynin, V. V.; Hempstead, P. D.; Vagin, A. A.; Antonyuk, S. V.; Melik-Adamyan, W. R.; Lamzin, V. S.; Harrison, P. M.; Artymiuk, P. J. *J. Inorg. Biochem.* **1997**, *67*, 196.
(3) Khangulov, S.; Sivaraja, M.; Barynin, V. V.; Dismukes, G. C. *Biochemistry* **1993**, *32*, 4912−4924.
(4) Zheng, M.; Khangulov, S. V.; Dismukes, G. C.; Barynin, V. V. *Inorg. Chem.* **1994**, *33*, 382−387.
(5) Khangulov, S. V.; Pesseki, P. J.; Barynin, V. V.; Ash, D. E.; Dismukes, G. C. *Biochemistry* **1995**, *34*, 2015−2025.
(6) Khangulov, S. V.; Barynin, V. V.; Antonyuk-Barynina, S. V. *Biochim. Biophys. Acta* **1990**, *1020*, 25−33.
(7) (a) Sheats, J. E.; Czernuszewicz, R. S.; Dismukes, G. C.; Rheingold, A. L.; Petrouleas, V.; Stubbe, J.; Armstrong, W. A.; Beer, R. H.; Lippard, S. J. *J. Am. Chem. Soc.* **1987**, *109*, 1435−1444. (b) Wieghardt, K.; Bossek, U.; Nuber, B.; Weiss, J.; Bonvoisin, J.; Corbella, M.; Vitols, S. E.; Girerd, J. J. *J. Am. Chem. Soc.* **1988**, *110*, 7398−7411. (c) Vincent, J. B.; Tsai, H. L.; Blackman, A. G.; Wang, S.; Boyd, P. D. W.; Folting, K.; Huffman, J. C.; Lobkovsky, E. B.; Hendrickson, D. N.; Christou, G. *J. Am. Chem. Soc.* **1993**, *115*, 12353−12361.
(8) Jacquamet, L.; Michaud-Soret, I.; Debaecker-Petit, N.; Barynin, V. V.; Zimmermann, J. L.; Latour, J. M. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 1626−1628.
(9) Day, E. P. *Methods Enzymol.* **1993**, *227*, 437−463.

S0020-1669(97)01217-2 CCC: $15.00
Published on Web 07/01/1998

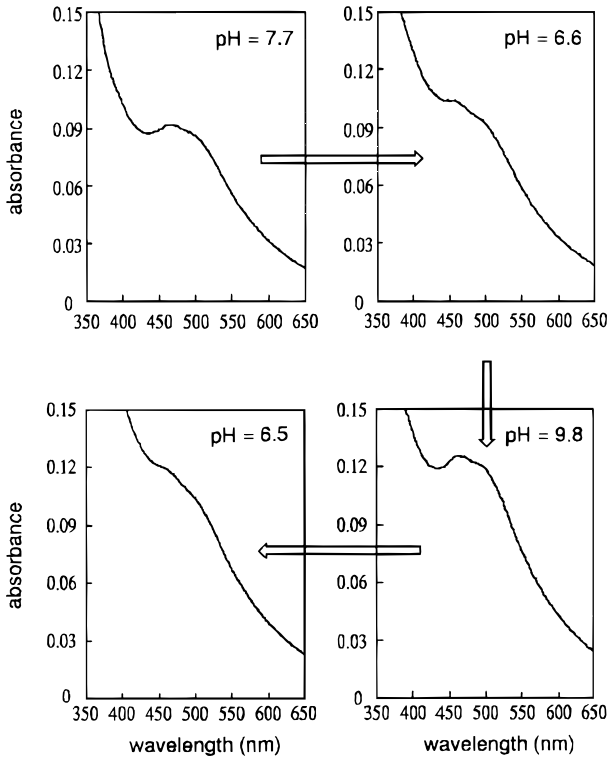


Figure 1. Electronic absorption spectra of the mixture of Mn(III)Mn(III) forms as a function of pH (see Experimental Section).

titrated by elemental analysis. This observation points to the presence of an additional species that does not contribute to the magnetization. This species must involve a strongly antiferromagnetically coupled homovalent dimanganese center. In view of the mildly oxidative operating conditions and the absence of an EPR signal, it is most likely that it is a dimanganese(III) center. Indeed, the magnetization data could be accounted for by considering that the sample contained a mixture of two Mn(III)Mn(III) centers: a weakly coupled form ($J \sim -2$ cm^{-1}) and a strongly coupled one with $J \sim -100$ cm^{-1}.

In addition, when the pH of the sample was varied from 6.6 up to 9.8, a significant increase in the absorbance around 500 nm was observed and the variation was reversible (Figure 1). It therefore appears that the two species are in a pH-dependent equilibrium. Moreover, their optical spectra differ only by the intensity of the visible bands and not by their energy. This indicates that the structures of the two chromophores are probably closely related. Magnetization experiments were repeated on two last samples which had been autoxidized at low and high pH (6.5 and 9.5, respectively). These experiments showed that the low-pH sample contained 51(6)% of the weakly coupled form and 37(6)% of the strongly coupled one in addition to 12% of the mixed-valent Mn(II)Mn(III) state. On the other hand, the high-pH sample contained 18(6)% of the weakly coupled form and 70(6)% of the strongly coupled one in addition to 12% of the mixed-valent Mn(II)Mn(III) and Mn(III)Mn(IV) states. These experiments indicate that the strongly coupled species is favored at high pH.

When reduced catalase was oxidized by KIO_4 as described in the literature,[6] quantitation of the EPR spectrum and elemental analysis of the sample revealed that about a third of the manganese is EPR-silent and therefore most probably in the Mn(III)Mn(III) state. The temperature dependence of the product of the molar susceptibility by temperature is illustrated for such a sample in Figure 2. Neither the height of the plateau

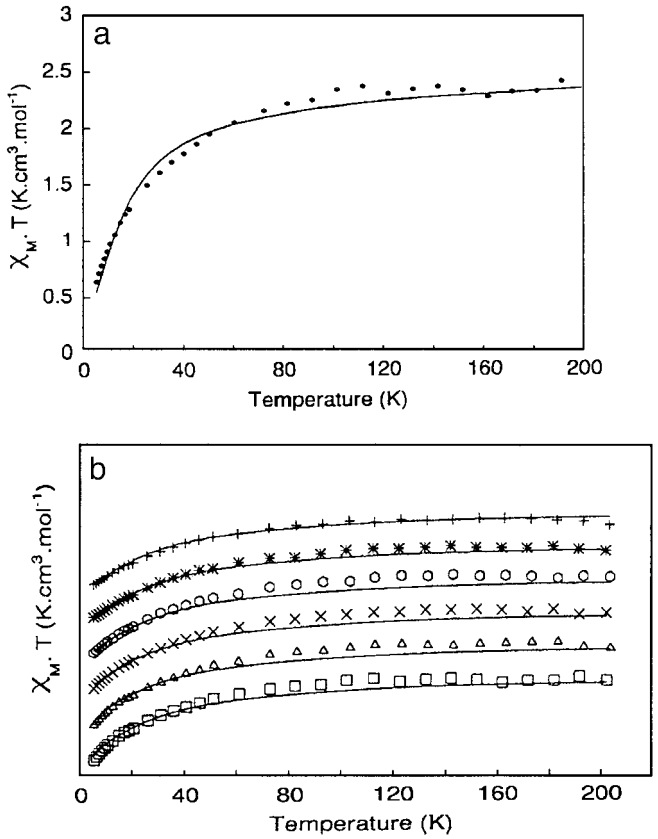


Figure 2. (a) Temperature dependence of the χ*T product of the $KMnO_4$-oxidized sample at 0.5 T: experimental data *, theoretical curve (see text) —. (b) Temperature dependence of the molar experimental data extracted for the Mn(III)Mn(III) form at 0.5, 1, 2, 3, 4, and 5 T and the theoretical curve obtained as detailed in the text; for clarity an offset of H*2 K·cm^3·mol^{-1} has been added to the data obtained at 1−5 T.

nor the shape of the curve is consistent with the sample comprising solely a strongly antiferromagnetically coupled Mn(III)Mn(IV) species, but rather they indicate the presence of a weakly antiferromagnetically coupled species. Taking this heterogeneity into account, it was possible to fit the experimental data as a mixture of 64(2)% of a strongly antiferromagnetically coupled Mn(III)Mn(IV) species ($S_1 = 2$, $S_2 = {}^3/_2$, $J < -175$ cm^{-1}, $H = -2JS_1 \cdot S_2$) and 36(2)% of a weakly antiferromagnetically coupled Mn(III)Mn(III) species ($S_1 = S_2 = 2$, $J \sim -1.7(2)$ cm^{-1}). The small magnetic contribution of the Mn(III)Mn(IV) center can be subtracted from the data to obtain molar data for the Mn(III)Mn(III) center. These data (Figure 2b) have been analyzed at six magnetic fields with an Hamiltonian taking into account both the exchange interaction (J) and the anisotropy of the two manganese(III) ions (D_1 and D_2).[10] The best fit was obtained with the following set of parameters: $g = 1.98$, $J = -1.8(2)$ cm^{-1}, $D_1 = +4.8(10)$ cm^{-1}, and $D_2 = -4.3$ (8) cm^{-1}. It is worth noting that the data could be simulated *only* with zero-field splitting (ZFS) parameters of opposite signs. Also noteworthy is the fact that the magnetic properties of this sample could be accounted for with only one Mn(III)Mn(III) center, the weakly antiferromagnetically coupled center; this observation points to the absence of the second form in the KIO_4-oxidized sample.

(10) Corbella, M.; Costa, R.; Ribas, J.; Fries, P.; Latour, J. M.; Öhrström, L.; Solans, X.; Rodriguez, V. *Inorg. Chem.* **1996**, *35*, 1857−1865.

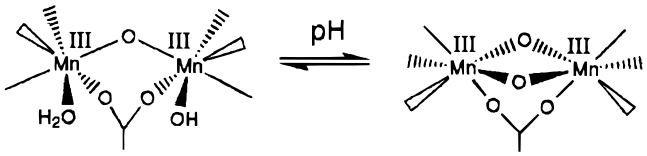
Discussion

Spectroscopic and magnetic techniques show that autoxidized catalase contains two dimanganese(III) species in equilibrium. The only form detected previously was postulated to be a (μ-oxo)(μ-carboxylato) species.[7] The antiferromagnetic exchange interaction found in the present magnetization experiments in the low-pH form of oxidized catalase ($J = -1.8$ cm^{-1}) is consistent with an oxo- and carboxylato-bridged Mn(III)Mn(III) center. Indeed, (μ-oxo)bis(μ-carboxylato)dimanganese(III) complexes exhibit exchange interactions in the range -5 cm^{-1} $< J <$ $+9$ cm^{-1}. Nevertheless an alternate structure of the low-pH form of oxidized catalase can be envisaged. Indeed Pecoraro[11] has shown that two successive protonations of the oxo bridges of the [Mn(IV)(μ-O)$_2$Mn(IV)]$^{4+}$ core lessen the exchange interaction from $J = -92$ cm^{-1} to $J = -6$ cm^{-1}. On the other hand, it is known[12] that the folding induced by a carboxylate bridging a [Mn(IV)(μ-O)$_2$Mn(IV)]$^{2+}$ core lessens the exchange interaction from $J = -92$ cm^{-1} to $J = -44$ cm^{-1}. There is no theoretical reason for what dimanganese(III) species would not behave as dimanganese(IV) ones. Therefore it cannot be totally excluded that protonation of a [Mn(III)(μ-O)$_2$(μ-carboxylato)Mn(III)]$^{+}$ entity brings the exchange interaction down to the low value that we observed for oxidized catalase at low pH. Nevertheless, such a [Mn(III)(μ-OH)$_2$Mn(III)] species is unprecedented.[13] Therefore, we favor the (μ-oxo)-(μ-carboxylato)dimanganese(III) formulation.

Recently, a rationalization of the magnetic behavior of the (μ-oxo)(μ-carboxylato)dimanganese(III) compounds was provided,[10] which links the nature of the interaction (the sign of J) through the type of Jahn−Teller distortion of the Mn(III) ion (the sign of D) to the strength of donors located in the equatorial plane perpendicular to the Mn−O axis. For a strong donor ((N$_3^-$, OH$^-$), D and J are positive while they are negative for a weak donor (Cl$^-$, NO$_3^-$, ClO$_4^-$, H$_2$O). In this respect, whatever the actual structure of the center, observation of opposite values of the ZFS parameter D ($D_1 = +4.8$ cm^{-1} and $D_2 = -4.3$ cm^{-1}) indicates that the two manganese ions experience different distortions of their coordination sphere. One manganese is bound by a strong equatorial ligand, most probably a hydroxide, while the other has a weakly bound equatorial ligand (water molecule) or is pentacoordinated. It is worth noting that the presence of an hydroxide ligand has been proposed to explain the pH dependence of the inhibition of the enzyme by various anions.[14]

The high-pH form is structurally related to the preceding one but differs in that it is favored in basic media, has stronger absorption in the visible region, and is more strongly antiferromagnetically coupled. Moreover, it is more easily oxidized since it was not present in the Mn(III)Mn(IV) sample which was not completely oxidized by KIO$_4$. All of these observations suggest that the new species is a di(μ-oxo)(μ-carboxylato)-dimanganese(III) center. Such complexes do in fact exhibit strong antiferromagnetic coupling in the -80 to -100 cm^{-1} range and have enhanced absorption near 500 nm.[15] In addition Mukherjee et al.[16] have shown that a di(μ-oxo)(μ-carboxylato)-dimanganese(III) species is easier to oxidize to the (III,IV) state than a (μ-oxo)bis(μ-carboxylato) dimanganese (III,III) by 1.30 V. This fact explains the absence of the [Mn(III)(μ-O)$_2$Mn-(III)] form in the KIO$_4$-oxidized sample.

Scheme 1



low pH form high pH form

In summary, magnetization experiments have proven very helpful to supplement magnetic resonance techniques in the investigation of oxidized catalase. They reveal that oxidized catalase contains not one but two Mn(III)Mn(III) forms which are in a pH-dependent equilibrium as illustrated in Scheme 1. The structure deduced for the high-pH form from magnetization experiments is consistent with that of oxidized catalase obtained from preliminary X-ray crystallographic studies.[2b] This situation appears closely similar to the one of the methane monooxygenase hydroxylase component from *Methylosinus trichosporium* OB3b for which a μ-hydroxo- and di-μ-hydroxodiiron core have been proposed to coexist.[17] The discovery of a second oxidized form of the enzyme raises the question as to which of the two forms is actually involved in the catalysis. The poor lability of oxo groups militates against the bis-μ-oxodimanganese(III) form being the active one. The mono-μ-oxodimanganese(III) entity appears more suited to binding a hydroperoxide substrate since on one manganese it bears an easily exchangeable aqua ligand and on the other a hydroxide able to deprotonate a hydroperoxide bound to the former.

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(11) Baldwin, M. J.; Stemmler, T. L.; Riggs-Gelasco, P. J.; Kirk, M. L.; Penner-Hahn, J. E.; Pecoraro, V. L. *J. Am. Chem. Soc.* **1994**, *116*, 11349−11356.
(12) Reddy, K. R.; Rajasekkharan, M. V.; Padhye, S.; Dahan, F.; Tuchagues, J. P. *Inorg. Chem.* **1995**, *34*, 428−433.
(13) (a) Kitajima *et al.*[13b] reported the structure of a [Mn(II)(μ-OH)$_2$Mn-(II)] complex. (b) Kitajima, N.; Singh, U. P.; Amagai, H.; Osawa, M.; Moro-oka, Y. *J. Am. Chem. Soc.* **1991**, *113*, 7757−7758.
(14) Khangulov, S. V.; Goldfeld, M. G.; Gerasimenko, V. V.; Andreeva, N. E.; Barynin, V. V.; Grebenko, A. I. *J. Inorg. Biochem.* **1990**, *40*, 279−292.
(15) Goodson, P.; Oki, A.; Glerup, J.; Hodgson, D. *J. Am. Chem. Soc.* **1990**, *112*, 6248−6254.
(16) Mahapatra, S.; Lal, T. K.; Mukherjee, R. *Inorg. Chem.* **1994**, *33*, 1579−1580.
(17) Shu, L.; Liu, Y.; Lipscomb, J.; Que, L. *J. Biol. Inorg. Chem.* **1996**, *1*, 297−304.