Antiferromagnetic Coupling in the Binuclear Metal Cluster of Manganese-Substituted Phosphotriesterase

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The phosphotriesterase from *Pseudomonas diminuta* catalyzes the hydrolysis of a wide range of organophosphates.[1] The monomeric enzyme has a molecular weight of 36 000 and contains two divalent metal ions that are required for catalytic activity.[2,3] Zn^{2+} is present in the native enzyme, but this metal can be replaced with Mn^{2+}, Cd^{2+}, Co^{2+}, or Ni^{2+} without loss of enzymatic activity.[2] The ^{113}Cd-NMR spectrum of the Cd/Cd-substituted enzyme exhibits resonances at 212 and 116 ppm downfield from Cd-$(ClO_4)_2$.[2] The separation in chemical shift between the two signals indicates that the ligand environment of the two metal ion sites is dissimilar.[2] Furthermore, the position of the two ^{113}Cd-NMR resonances suggests that the coordination environment of the two metal sites consists of a mixture of nitrogen and oxygen ligands while direct ligation to cysteine is excluded. However, little is known of the roles these metal ions play in the catalytic mechanism. To address whether the binding sites for the two required metal ions are structurally independent of each other, we have conducted an EPR investigation of the Mn/Mn-substituted enzyme. In this communication, we report that the two Mn^{2+} ions are present as an antiferromagnetically-coupled binuclear center.

A sample of Mn/Mn-substituted phosphotriesterase[4] displayed a complex EPR spectrum (Figure 1A) at the X-band (9.4 GHz). The predominant features were near $g = 2$ and exhibited what appeared to be more than 26 Mn hyperfine splittings at ~45-G intervals. To confirm that these features originated from Mn^{2+} ions that were enzyme-bound, a sample of the Mn/Mn-phosphotriesterase was digested with 2 M perchloric acid for 3 h. The resulting spectrum (Figure 1B) exhibited six Mn hyperfine lines separated by ~90 G, virtually identical to the spectrum of Mn^{2+} ions in 50 mM HEPES buffer. Thus, the unusual spectral features of Figure 1A undoubtedly arise from Mn^{2+} ions bound to the enzyme. The large number of hyperfine splittings, separated by approximately half the magnitude expected for a mononuclear Mn^{2+} center, suggests the presence of two spin-coupled Mn^{2+}

(1) Donarski, W. J.; Dumas, D. P.; Heitmeyer, D. P; Lewis, V. E.; Raushel, F. M. *Biochemistry* **1989**, *28*, 4650–4655.
(2) Omburo, G. A.; Kuo, J. M.; Mullins, L. S.; Raushel, F. M. *J. Biol. Chem.* **1992**, *267*, 13278–13283.
(3) Dumas, D. P.; Caldwell, S. R.; Wild, J. R.; Raushel, F. M. *J. Biol. Chem.* **1989**, *264*, 19659–19665.
(4) The phosphotriesterase was purified from an *Escherichia coli* strain containing the plasmid pJK01.[5] Cells were grown in a TB medium consisting of 12 g/L bactotryptone, 24 g/L yeast extract, 4 mL/L glycerol, and 100 mM potassium phosphate, pH 7.4, supplemented with 1 mM $CoCl_2$ and purified as previously described.[2] Protein concentration was determined spectrophotometrically from the absorbance of 280 nm using $\epsilon_{280} = 2.93 \times 10^4$ $M^{-1}cm^{-1}$.[2] Apoprotein was prepared by incubating the phosphotriesterase (~1.0 mg/mL) with 2 mM 1,10-phenanthroline overnight (the enzymatic activity dropped to less than 1% of its original value). The solution was then diafiltered to remove the metal chelator complex and excess chelator. The process of diafiltration was followed by monitoring the disappearance of absorbance at 327 nm. The Mn/Mn-activated form of phosphotriesterase was prepared by incubating the apoenzyme at ~1 mg/mL in 50 mM HEPES–KOH,[6] pH 8.3, with 2 equiv of $MnCl_2$ at 4 °C for at least 40 h. Samples for EPR spectroscopy were concentrated by utilization of an Amicon diafiltration device (PM10 membrane) and then by an Amicon Centricon-10 concentrator. The Mn/Mn-enzyme had a specific activity of 1800 s^{-1} for the hydrolysis of paraoxon at pH 9.0.
(5) Kuo, J. M.; Raushel, F. M. In preparation.
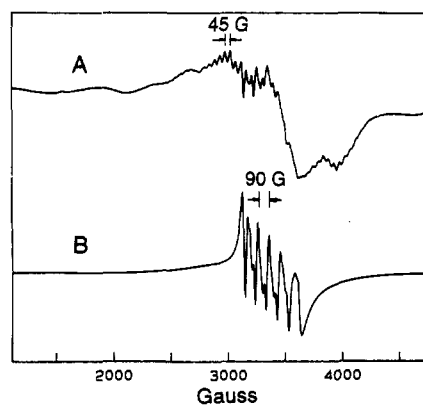(6) Abbreviation: HEPES, 4-(2-hydroxyethyl)piperazineethanesulfonic acid.

Figure 1. X-band EPR spectra of Mn(II) samples at 10 K. (A) Mn/Mn-substituted phosphotriesterase (24 mg/mL protein) in 50 mM HEPES, pH 8.3. (B) Mn/Mn-substituted enzyme (7.1 mg/mL) after digestion with 2 M perchloric acid for 3 h. EPR conditions: 9.4-GHz spectrometer frequency, 2-mW microwave power, 10-G modulation amplitude, 100-kHz modulation frequency, two accumulated scans.
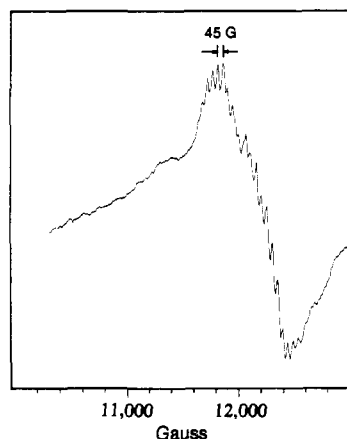

Figure 2. Q-band EPR spectrum of Mn/Mn-substituted phosphotriesterase at 150 K. The sample contained 24 mg/mL protein in 50 mM HEPES, pH 8.3. EPR conditions: 33.8-GHz spectrometer frequency, 200-mW microwave power, 10-G modulation amplitude, 100-kHz modulation frequency, five accumulated scans.

ions.[7,8] Further support for the spin-coupled Mn^{2+} complex was obtained from the Q-band (33.8 GHz) EPR spectrum of the Mn/Mn-substituted enzyme (Figure 2). At least 20 Mn hyperfine lines, also separated by ~45 G, are readily apparent.

The temperature dependence of the X-band EPR signal exhibits further evidence for a spin-coupled binuclear center. Although the temperature dependence followed the Curie law above 20 K, the product of the signal intensity and the temperature decreased as the temperature was lowered from 20 K to 5 K (Figure 3). To account for this behavior, the two Mn ions were assumed to be spin-coupled in accordance with an isotropic Heisenberg exchange interaction ($H = 2JS_1S_2$ where $S_1 = {}^5/_2$, $S_2 = {}^5/_2$), and the signal was assumed to arise from the $S = 1$ state of the resulting multiplet. The temperature dependence was adequately simulated with these assumptions, using the method and equations presented by Khangulov *et al.*[9] and $J = 5 \pm 1$ cm^{-1}. In summary, the X- and Q-band EPR results clearly demonstrate that the two Mn^{2+} ions in bacterial phosphotriesterase comprise an antiferromagnetically-coupled binuclear center.

Spin-coupled Mn(II) binuclear centers have been documented in a limited number of other enzymes, including arginase,[10]

(7) Reed, G. H.; Markham, G. D. *Biol. Magn. Reson.* **1984**, *6*, 73–142.
(8) Owen, J.; Harris, E. A. *Electron Paramagnetic Resonance*; Geschwind, S., Ed.; Plenum: New York, 1972; pp 427–492.
(9) Khangulov, S. V.; Barynin, V. V.; Voevodskaya, N. V.; Grebenko, A. I. *Biochim. Biophys. Acta* **1990**, *1020*, 305–310.
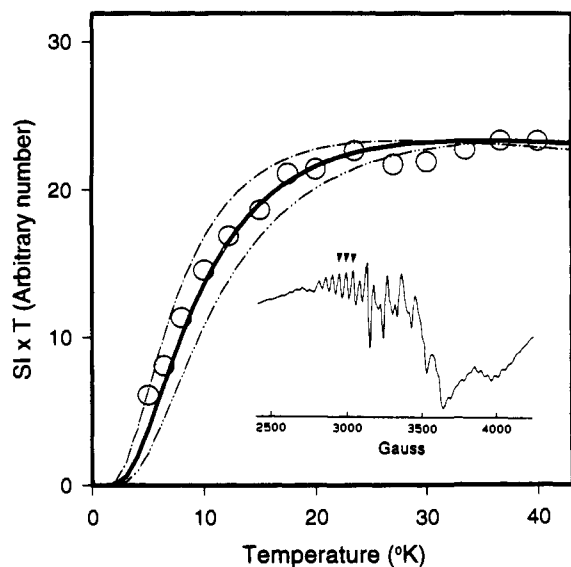
Figure 3. Temperature dependence of the X-band EPR signal intensities of the Mn/Mn-substituted phosphotriesterase. The signal intensity (SI) was obtained from an average of three peak intensities shown by the triangles. The solid line is a fit of the experimental data as described in the text with a calculated $|J| = 5$ cm^{-1} for the temperature dependence of the population of the first excited state $(S = 1)$. The dashed lines illustrate the calculated temperature dependence for $|J|$ values of 4 and 6 cm^{-1}. The half saturation power at 3 K was approximately 30 mW.

enolase,[11,12] concanavalin A,[13] and catalase.[14] The EPR spectra and calculated J values (0.9 cm^{-1} for concanavalin A[13] and 14 cm^{-1} for catalase[9]) are quite similar to those of the Mn-substituted phosphotriesterase. The distances between the two metals bound to concanavalin A and catalase have been reported as 4.3 Å[15] and

3.6 Å,[16] respectively, and we expect a similar range of separation for the two metal ions in the Mn/Mn-substituted phosphotriesterase. The likely candidates for bridging ligand(s) in the binuclear metal center include water, histidine, or a carboxyl group from either glutamate or aspartate. The precise role for the binuclear metal complex during the hydrolysis of phosphotriesters is unknown. However, the second metal ion may be postulated to modulate the reactivity of the hydrolytic water molecule coordinated to the other metal ion, or it may act independently in assisting the delocalization of the developing charge of the departing leaving group. It is interesting to note that purple acid phosphatase (which hydrolyzes phosphomonoesters) contains a spin-coupled diiron center with an oxo bridge.[17] Experiments to more precisely determine the role of the metal ions in the phosphotriesterase as well as the identity of the coordinating ligands to the metal ions are currently underway.

(10) Reczkowski, R. S.; Ash, D. E. *J. Am. Chem. Soc.* **1992**, *114*, 10992–10994.

(11) Chien, J. C. W.; Westhead, E. W. *Biochemistry* **1971**, *10*, 3198–3203.

(12) Poyner, R. R.; Reed, G. H. *Biochemistry* **1992**, *31*, 7166–7173.

(13) Antanaitis, B. C.; Brown, R. D., III; Chasteen, N. D.; Freeman, J. H.; Koenig, S. H.; Lilienthal, H. R.; Peisach, J.; Brewer, C. F. *Biochemistry* **1987**, *26*, 7932–7937. The $|J|$ value reported by Antanaitis et al. was obtained using $H = JS_1S_2$ rather than $H = 2JS_1S_2$, the Hamiltonian used in this paper for phosphotriesterase and by Khangulov et al. for catalase. To readily compare J values between the three binuclear Mn proteins we have divided the reported $|J|$ value by a factor of 2 (1.8/2).

(14) Khangulov, S. V.; Barynin, V. V.; Antonyuk-Barynina, S. V. *Biochim. Biophys. Acta* **1990**, *1020*, 25–33.

(15) Hardman, K. D.; Agarwal, R. C.; Freiser, M. J. J. *Mol. Biol.* **1982**, *157*, 69–86.

(16) Barynin, V. V.; Khangulov, S. V.; Popov, A. N.; Andreanova, M. E.; and Vanstein, B. K *Dokl. Acad. Sci. USSR* **1986**, *288*, 877–880.

(17) Davis, J. C.; Averill, B. A. *Proc. Natl. Acad. Sci. U. S. A.* **1982**, *79*, 4623–4627.