EPR Spectral Evidence for a Binuclear Mn(II) Center in Dinitrogenase Reductase-Activating Glycohydrolase from *Rhodospirillum rubrum*

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Bacterial nitrogen fixation is catalyzed by the two oxygen-labile proteins dinitrogenase and dinitrogenase reductase, which compose the nitrogenase enzyme complex.[1] The nitrogenase enzyme complex catalyzes the reduction of atmospheric nitrogen to ammonium, which is a very energy-intensive reaction, in that at least 16 MgATP molecules are hydrolyzed per nitrogen molecule reduced. Therefore, *Rhodospirillum rubrum*[2,3] post-translationally inactivates nitrogenase by reversible ADP-ribosylation of Arg-101 on a single subunit of the dinitrogenase reductase homodimer under the conditions of energy stress or nitrogen sufficiency. The transfer of the ADP-ribose moiety from NAD^+ onto dinitrogenase reductase is catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DRAT).[4] Activation via glycohydrolysis of the ADP-ribosyl protein linkage is catalyzed by dinitrogenase reductase-activating glycohydrolase (DRAG).[5−7]

DRAG is a 32-kD monomeric enzyme which requires MgATP and free divalent metal for its activity with ADP-ribosylated dinitrogenase reductase as substrate,[6] and thus the binding site for the divalent metal is thought to be on the DRAG protein.[8,9] Mn^{2+} is thought to be the physiological cofactor, although activation of the DRAG enzyme by Fe^{2+}, Mg^{2+}, and Co^{3+} has been reported.[10] We report here EPR evidence that DRAG isolated from *Rhodospirillum rubrum* contains a spin-coupled Mn(II)−Mn(II) center.

DRAG was purified from an overexpressing *R. rubrum* strain UR 276 following the published procedure.[6] Activity was measured by coupling the activity of dinitrogenase reductase with the reduction of acetylene by dinitrogenase,[11] and protein concentration was determined using bicinchoninic acid following the published procedure.[12] Samples for EPR spectroscopy were prepared by concentrating the enzyme with a Pall Filtron microconcentrator to 12 mg/mL in 50 mM MOPS, 0.2 M NaCl, 2mM DTT, pH 7.0. Then $MnCl_2$ was added to a concentration of 0.3 mM to activate the enzyme. Low-temperature EPR spectra were recorded with a Varian E-15 EPR spectrometer equipped with an Oxford Instruments cryostat. The stoichiometry of Mn(II) binding to the protein was determined by EPR using a Varian E-3 spectrometer.[13]

The Heisenberg exchange coupling constant J and the deconvoluted spectra of the triplet and quintet states were obtained by a singular value decomposition (SVD) analysis of the spectra at different temperatures using a Boltzmann distribution.[14,15] Prior to SVD analysis, the spectra were smoothed, truncated to 4000 G, the number of field points reduced, and the region from 2438 to 3024 G containing signals from free Mn(II) removed.

EPR quantitation of Mn(II) binding revealed two equivalent binding sites for Mn(II) per enzyme monomer with a K_d of $27 \pm 5\ \mu M$.[16] X-band (9GHz) EPR spectra of Mn-activated DRAG at temperatures from 4.5 K to 30 K are shown in Figure 1. These spectra contain three sets of 11 ^{55}Mn hyperfine lines centered at 1400 G, 2800, and 3850 G with the 45-G spacing typical of exchange-coupled Mn(II) pairs.[17] The strong six-line pattern centered at 3200 G is due to residual free Mn(II) in the sample. An analysis of the data available in the literature shows that the spectral features are characteristic of a ligand-bridged binuclear cluster in the Mn^{2+}, Mn^{2+} state.[18] Treatment of the DRAG sample with 6 M guanidine hydrochloride at room temperature (overnight) eliminated the spectral features characteristic of the binuclear manganese cluster, indicating that the cluster is protein-based.

SVD analysis of 10 EPR spectra acquired between 4.5 K and 30 K indicates that the Mn(II) ions couple antiferromagnetically with $J = -3 \pm 1\ cm^{-1}$. The deconvoluted spectra for the triplet and quintet states of the pair are shown in Figure 2A. The set of signals centered at 1400 G in the triplet spectrum is due to a double quantum ($\Delta M_S = 2$) transition. The well-resolved 11-line hyperfine patterns at 2800 and 3850 G in the raw spectra are associated with the quintet state. Figure 2B shows the temperature dependence of the populations of triplet and quintet states. The peak-to-trough height of the first hyperfine line (at ∼1200 G) of the $\Delta M_s = 2$ transition of the triplet state is also plotted as a check on the SVD analysis.

DRAG is inhibited by sodium borate,[19] by free ADP-ribose, and by α-NAD, but not by β-NAD.[20] Addition of 1 mM ADP-ribose resulted in the loss of the 11-line signal spectrum, indicating that the ADP-ribose may bind to the catalytic site, perhaps directly to the Mn_2 site. Addition of 20 mM sodium borate to the sample lowered the intensity of the exchange-coupled signals relative to the rest of the spectrum but did not eliminate the coupling. Competition experiments with Fe^{2+} indicated that the addition of 1.5 mM Fe^{2+} can replace the bound Mn^{2+} from the 55% Mn^{2+} saturated enzyme.

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(16) The stoichiometry of Mn(II) binding to the enzyme was determined by EPR[13] using a Varian E-3 spectrometer as follows: Mn(II) standard curve was determined for the concentration range 25−500 μM in 50 mM MOPS, 0.2 M NaCl, 2 mM DTT, pH 7.0. The enzyme was then titrated with different concentrations of Mn(II) (25−500 μM), and the concentration of free Mn(II), hence the concentration of bound Mn(II), was calculated. Finally, a Rosenthal−Scatchard plot was constructed by plotting ligand-bound $\{B_L = [Mn^{2+}]/[DRAG]\}$ V/S B_L/L[free Mn^{2+}] and the values of B_{max} and K_d were obtained.
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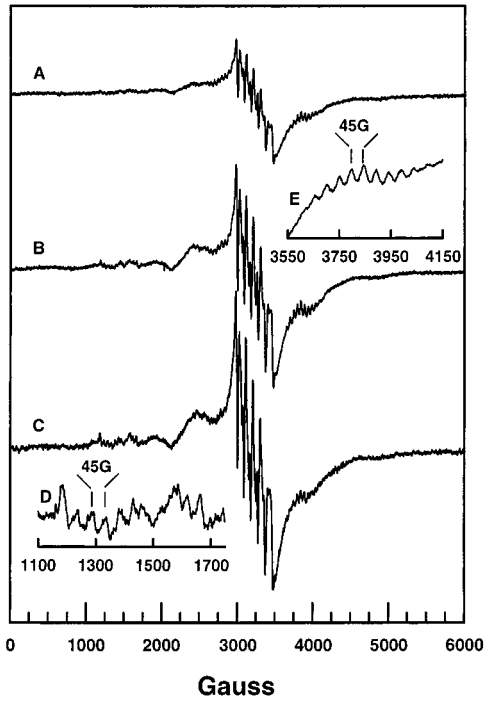

Figure 1. EPR spectra of the DRAG−Mn complex. Spectrometer settings: 9.25 GHz, 0.06 mW microwave power, 12.5 G modulation, 4 min scan time, 6000 G sweep width, 0.1 s time constant. (A) spectrum at 30.7 K. (B) spectrum at 13.5 K. (C) spectrum at 4.2 K. (D) expansion of 1100 to 1750 G region of C. (E) expansion of the 3550 to 4150 region of B. Variation of the microwave power indicated that none of the signals present were saturated under the conditions used.

Mn(II) binuclear centers have been documented in a limited number of other enzymes.[21] The crystal structure of rat liver arginase reveals that the metal cluster resides at the bottom of an active-site cleft and the arginine hydrolysis is achieved by a metal-activated solvent molecule which symmetrically bridges the two metal ions.[22] The two Mn(II) ions are separated by 3.3 Å. In catalase the two Mn ions are separated by 3.6 Å, and the amino acid residues that ligate the metal ions have not been identified by X-ray crystallography.[23,24] A comparison of the Heisenberg exchange interaction constant to catalase, arginase, and several dimanganese (II,II) compounds, [25] suggests a possible bridging structure with a μ-aqua ligand and one or two μ-carboxylates for DRAG at neutral pH.

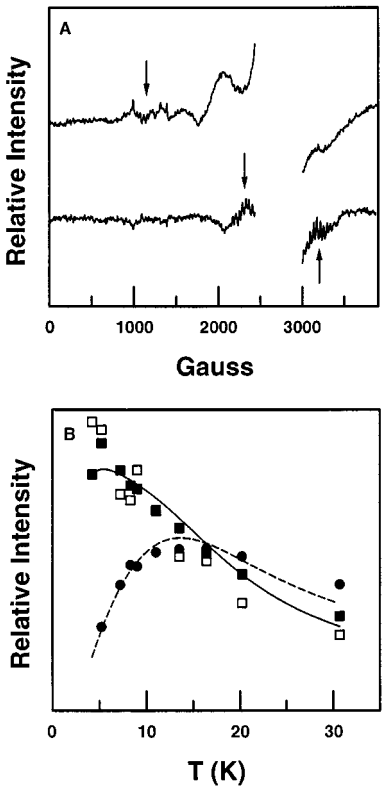

Figure 2. Results of SVD analysis of the temperature dependence of the EPR spectrum of the DRAG−Mn(II) complex. (A) The upper curve is the spectrum of the triplet state; the lower curve is the spectrum of the quintet state. (B) Relative populations of the triplet and quintet states as a function of temperature. The solid squares and solid line represent the triplet state. The solid circles and dashed line represent the quintet state. The open squares represent the peak-to-trough height of the peak at ∼1200 G (see Figure 1D).

In conclusion, we have presented direct evidence for a dimeric Mn^{2+} active site in DRAG from *R. rubrum.* The role of the binuclear Mn(II) center in the hydrolytic deribosylation reaction of the protein substrate L-arginine-ADP-ribosyl, remains to be established. Structure−function analysis of the binuclear Mn-(II) center of DRAG is the focus of our ongoing crystallographic studies.

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