9704 *J. Am. Chem. Soc.* **1998,** *120,* 9704−9705

# Magnetic Circular Dichroism Study of the All-Ferrous [4Fe-4S] Cluster of the Fe-Protein of *Azotobacter vinelandii* Nitrogenase

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*Received May 29, 1998*

A recent Mössbauer and EPR study[1] of the Fe protein of nitrogenase from *Azotobacter vinelandi*i (*Av2*) has demonstrated that its [4Fe-4S] cluster attains the all-ferrous state, designated [4Fe-4S]$^0$, upon reduction with Ti(III) citrate. Parallel mode EPR spectra exhibited a sharp resonance at $g_{\mathrm{eff}} = 16.4$ that originates from a transition between the $M_S = \pm 4$ sublevels of an $S = 4$ spin multiplet. The electronic absorption spectrum of the [4Fe-4S]$^0$ cluster was found to be quite different from that observed for the [4Fe-4S]$^{1+}$ state, the former exhibiting a distinct band at ca. 500 nm.[1] Both the parallel mode EPR and visible absorption spectra were found to be independent of the reductant used to generate the all-ferrous state Thus, we obtained the same results employing Ti(III) citrate, deazariboflavin, or bisaquo(1,4,9,12-tetraazacyclopentadecanyl)-Cr(II).[2] Moreover, we observed the $g_{\mathrm{eff}} = 16.4$ resonance after radiolytic reduction (at 77 K) of the [4Fe-4S]$^{1+}$ cluster in a synchrotron beam.[2] Watt and Reddy[3] have reported that *Av*2 can be reduced to the all-ferrous state with methyl viologen; however, these authors also stated that only small spectral alterations occurred near 300 nm upon reduction of [4Fe-4S]$^{1+}$ to [4Fe-4S]$^0$, suggesting the possibility that they might have produced a state that differs from the one produced in our laboratories. We have studied a substantial number of Ti(III) citrate-reduced *Av*2 samples and found that the reaction is highly reproducible, leading to an all-ferrous cluster with high (>95%) spectral purity. Furthermore, with particular relevance to the present investigation, we found that the EPR spectrum of the [4Fe-4S]$^0$ cluster in 50% (v/v) water/glycerol solutions is identical to that observed without the glassing agent.

The magnetic circular dichroism (MCD) spectra of iron−sulfur clusters exhibit electronic transitions ranging from the ultraviolet to the near-infrared region.[4−6] The observed spectral features arise from d−d transitions, ligand-to-metal, metal-to-ligand, and intervalence charge-transfer processes. There is a good deal of spectral overlap between these different bands and, to date, there is probably no example of an iron−sulfur cluster for which any of the individual electronic transitions have been unambiguously assigned. The [4Fe-4S]$^0$ state of *Av2* is of interest because it provides a unique example of a paramagnetic iron−sulfur cluster for which all the iron sites are high-spin ferrous. This is expected to simplify the assignment of the electronic transitions, as the cluster lacks intervalence bands as well as the intense thiolate-to-ferric charge-transfer transitions[7,8] likely to be observed for [4Fe-4S]$^{1+,2+,3+}$ clusters.[5,6]

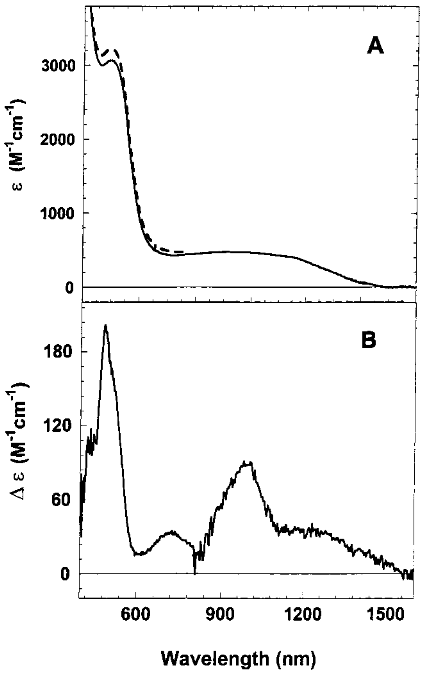

**Figure 1.** Visible-to-near-infrared electronic spectra of Ti(III) citrate-reduced *Av*2, pD 8.4, in 50 mM tris(hydroxymethyl)aminomethane buffer, 100 mM in NaCl (*before* glycerol addition). (A) Room-temperature absorption spectrum. Samples: 260 $\mu$M protein; 1.0 cm path length; aqueous buffer (solid line); 50% (v/v) $d_3$-glycerol (dashed line). (B) MCD spectrum at 4.2 K and 5.0 T. Sample: 1.25 mM protein; 0.5 mm path length; 50% (v/v) $d_3$-glycerol. The near-infrared region (~800−1600 nm) is the average of four spectral scans.

Figure 1 shows the visible-to-near-infrared electronic absorption (A) and MCD (B) spectra of Ti(III) citrate-reduced *Av*2. The system is fully magnetized under the MCD conditions as witnessed by the observation that lowering the temperature to 1.7 K and raising the applied magnetic field to 7.0 T did not lead to any measurable change in the spectrum. Within experimental uncertainty the ratio of the band intensities $\Delta A_{490}/\Delta A_{990}$ was reproducible for different samples. This observation suggests that the spectrum of the all-ferrous cluster is not distorted by underlying spectral contaminants. Moreover, control experiments with Ti(III) citrate solutions showed that this reductant, variable excesses of which were present in different samples, does not cause spectral interference.

Studies of the variation in MCD signal intensity as a function of the applied magnetic field and sample temperature have been shown to facilitate the deconvolution of electronic spectral features which arise from chromophores with distinct ground-state magnetic properties (e.g. refs 8 and 9). Our data strongly suggest that all the observable bands in Figure 1B magnetize in essentially the same fashion and are, consequently, associated with the same ground-state spin system. As an example, we show in Figure 2A isofield MCD magnetization curves of the 490 nm band. In general, analysis of such curves involves a multiparameter fit to the data,[5,6,8,9,10] and the results may not necessarily be unique.

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S0002-7863(98)01867-8 CCC: $15.00

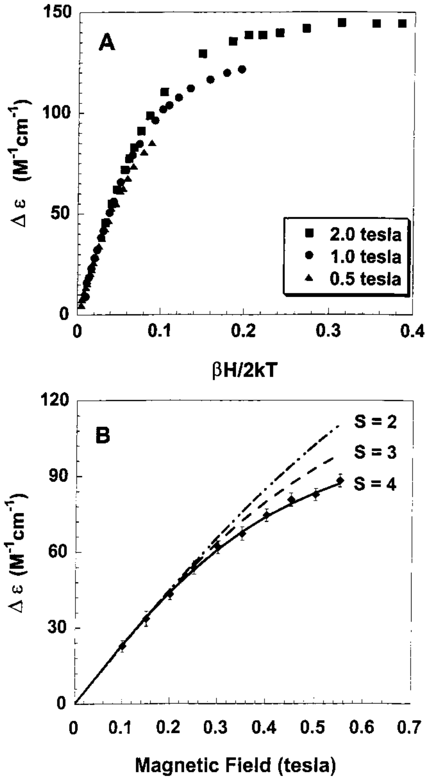

**Figure 2.** MCD magnetization curves recorded at 490 nm. Same sample as in Figure 1B. (A) Isofield curves recorded at 2.0 (■), 1.0 (●), and 0.5 (▲) T. (B) Isothermal data recorded at 1.7 K (◆). Theoretical curves were calculated with eq 1 with $A_{\text{lim}} = 465$, $g_z = 16$ (solid line); $A_{\text{lim}} = 610$, $g_z = 12$ (dashed line); and $A_{\text{lim}} = 900$, $g_z = 8$ (dots and dashes). The particular simulations for $g_z = 12$ and 8 shown in (B), matched to the initial slope of the data, are not necessarily the best overall fits. However, this presentation is useful because it illustrates systematic deviations for inappropriate *g*-values.

Simple inspection of the raw data in Figure 2A, however, is of value for several reasons. First, in plots of MCD signal intensity against the dimensionless quantity *βH/(2kT)* a nested set of curves is obtained only for ground multiplets with $S > {}^1/_2$ that exhibit zero-field splittings. For our samples the measured signals clearly arise from a chromophore with $S > {}^1/_2$ and are not due to the presence of some contaminating $S = {}^1/_2$ species, such as the $S = {}^1/_2$ form of the *Av2* [4Fe-4S]$^+$ cluster. Second, the higher temperature data extrapolate through the origin at all applied fields, demonstrating that the MCD signal contains only *C*-term components and that it lacks significant contributions from temperature-independent *A* and *B* terms. Third, the observation that the system saturates at about 3 K in a field of only 2.0 T is unusual. Noting that the magnetization curve in the high-temperature region is expected to get steeper with increasing ground-state spin of the chromophore[10] and comparing the results of Figure 2A with those obtained for a variety of $S = 2$ systems (e.g. ref 11) suggests that the present set of magnetization data must arise from a system with spin $S > 2$.

The Mössbauer and EPR studies of the all-ferrous cluster have revealed that the $M_S = \pm 4$ ground doublet is split in zero field by $\delta \approx 0.03$ cm$^{-1}$ and that this quasidoublet has effective *g*-values $g_x \approx g_y < 0.1$ and $g_z = 16.4$.[1] Moreover, we have determined[2] that the zero-field splitting parameters of the $S = 4$ multiplet are $D = -0.75$ cm$^{-1}$ and $E/D = 0.33$. For the quoted value of $D$ more than 98% of the molecules will be in the $M_S = \pm 4$ states at 1.7 K. To avoid crossing of the $M_S = +4$ level with the $M_S = -3$ level, we kept the applied field well below 1.0 T. Under these conditions all MCD transitions originate from the $M_S = \pm 4$ doublet. The results of this study are shown in Figure 2B, in which the solid line represents a theoretical fit to the expression given by Solomon et al.[11] for *xy*-polarized transitions arising from an isolated doublet:[12]

$$\Delta\epsilon = A_{\text{lim}} \int_0^{\pi/2} \cos\theta \sin\theta \tanh\left[\frac{g_z \beta H \cos\theta}{2kT}\right] d\theta \qquad (1)$$

Allowing $A_{\text{lim}}$ and $g_z$ to vary as the free parameters, we obtained a good fit to the data with $g_z = 16$, in excellent agreement with the EPR-determined *g*-value and confirming our expectation that the 490 nm peak is associated with the $S = 4$ system. We did not obtain acceptable fits for effective *g*-values $g_z = 12$ or 8, clearly excluding possible ground states with $S = 3$ or 2 (e.g. broken lines in Figure 2B)

The MCD spectrum of the all-ferrous cluster in *Av*2 exhibits positive bands at 490, 720, 990, and 1250 nm. In contrast, the high-spin ferrous site of rubredoxin exhibits no transitions in this spectral range.[5,7] The known d−d transitions of reduced rubredoxin are between 1400 and 2500 nm[7] where the spectrum of the all-ferrous cluster has no detectable bands (not shown). Therefore, the present data are noteworthy because they demonstrate the existence of electronic transitions in a spectral region where none were previously identified for Fe$^{II}$S$_4$ sites.[13] A recent EXAFS study[15] suggests that the all-ferrous cluster has two Fe−Fe distances at 2.77 Å and four which are considerably shorter at 2.53 Å suggestive of, for example, a compressed structure (however, the Mössbauer data have revealed that one of the ferrous sites is distinct from the other three). The short Fe−Fe distances inferred from EXAFS suggest metal−metal interactions that may shift the electronic transitions away from those observed for monomeric ferrous sites. While we are presently comfortable attributing the low-energy bands (800−1600 nm) to d−d transitions ($\epsilon_{910} \approx 400$ M$^{-1}$ cm$^{-1}$), we feel that it is premature to speculate about the nature of the ca. 500 nm band which, in conjunction with an intense shoulder emerging from the ultraviolet region, gives the all-ferrous protein its distinctive pink color.

**Acknowledgment.** This research was supported by NSF grant MCB 9406224 (E.M) at Carnegie Mellon University and NIH grant GM 43144 (B.K.B) at the University of California, Irvine.

JA981867O

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[12] Equation 1 is a special case of the more general expression, eq 20 of ref 11, that takes rhombicity into account. However, because of the small value of $\delta \approx 0.03$ cm$^{-1}$, both equations give the same result.

[13] Similar bands have been observed at ca. 500 and 750 nm in the MCD spectra of some [4Fe-4S]$^+$ clusters,[14] but it is not clear whether they are associated with the diferrous pair or the mixed-valence pair.

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