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A [4Fe-4S] CLUSTER WITH SPIN S = 3/2: STUDIES OF THE NITROGENASE Fe-PROTEIN FROM *A. VINELANDII* IN THE PRESENCE OF 0.4 *M* UREA

The Fe protein of nitrogenase contains a [4Fe-4S] cluster which exhibits an EPR signal with g-values at 1.88, 1.94 and 2.05 in the reduced state. Several groups (see ref. [1]) have quantitated this "g = 1.94" signal and have consistently obtained a spin concentration of approximately 0.3 spins/4Fe, substantially less than the 1 spin/4Fe expected. ZUMFT *et al.* [2] noted that the g = 1.94 signal declines sharply when the sample contains 0.5 M urea. We noticed that their spectra exhibit feeble resonances between g = 6 and g = 4, the intensity of which seemed to be inversely related to that of the g = 1.94 feature. HAAKER *et al.* [3] reported that the addition of ethylene glycol to the *A. vinelandii* Fe-protein (denoted Av2) increases the g = 1.94 signal intensity. Using EPR, Mössbauer spectroscopy and magnetic susceptibility, we have investigated the solvent dependence of the electronic states of Av2. The details of these studies are reported elsewhere [4]. Here we focus on Mössbauer and EPR studies of reduced Av2 in 0.4 M urea (Av2/urea) and give evidence for the presence of a $[4Fe\text{-}4S]^{1+}$ cluster with a novel S = 3/2 ground state.

RESULTS

Sample preparations and experimental methods are described elsewhere [4]. Fig. 1 shows EPR spectra of dithionite-reduced Av2 in Tris HCl buffer (A) or 0.4 M urea (B). Both spectra display a g = 1.94 signal. This signal for the Tris HCl sample (Av2/Tris HCl) accounts for 0.35 spins/4Fe

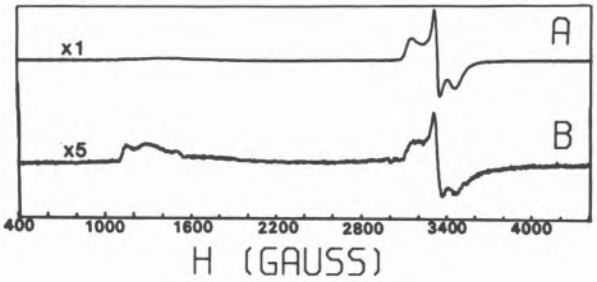

Fig. 1
X-band EPR spectra of reduced Av2/Tris HCl (Fig. 1A) and reduced Av2/urea. The spectra were recorded at 9 K; microwave power, 0.2 mW

and that of the urea sample for 0.1 spins/4Fe. Fig. 2 shows an expanded scan of the g = 5 region. Both samples exhibit resonances in that region but those of the urea sample are more intense and sharper. For Av2/urea the intensity at g = 5.8 increases relative to that at g = 5.1 as the temperature is lowered from 20 K to 5 K. This suggests that these two resonances arise from different doublets within an S ≥ 3/2 multiplet, with the

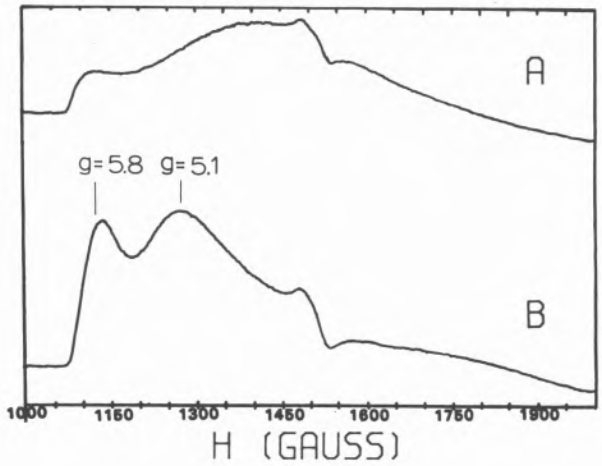

Fig. 2
Low-field features of EPR spectra of reduced Av2/Tris HCl (A) and reduced Av2/urea (B). Spectra recorded at 9 K; microwave power, 5 mW

$g = 5.8$ doublet being lower in energy. We describe the multiplet with the spin Hamiltonian

$$H = D[S_z^2 - S(S+1)/3 + \frac{E}{D}(S_x^2 - S_y^2)] + 2\,\beta\vec{H}\cdot\vec{S} \quad (1)$$

For $S = 3/2$, $D \simeq -2$ cm^{-1}, $E/D = 0.22$ and $\beta H \ll |D|$, EPR resonances would occur at $g = 1.4$, 1.1, 5.7 for the ground doublet and at $g = 2.6$, 5.1, 1.7 for the excited state [5]. (This is the only obvious choice for S which explains the data.) According to a method developed by AASA and VANNGARD [6], the intrinsic intensity of the absorption type peak at $g = 5.8$ is 20 times less than that at $g = 2.05$. Hence, the roughly equal areas of the resonances at $g = 5.8$ and $g = 2.05$ in Fig. 1B indicate that the concentration of the $S = 3/2$ center is large compared with that of the $g = 1.94$ species.

The Mössbauer spectra of Fig. 3 show that the majority of the iron in Av2/urea belongs to an unusual [4Fe-4S] cluster. They also provide strong evidence that this cluster is the EPR-active $S = 3/2$ center. Fig. 3A shows spectra of Av2 in 50% ethylene glycol (Av2/ethylene glycol). These spectra are typical of those observed for all previously studied $[4Fe\text{-}4S]^{1+}$ clusters which have $S = 1/2$, and which yield the $g = 1.94$ signal. Such spectra can be fitted by assuming two pairs of iron sites; one pair with negative internal fields, H_{int}, and one with positive internal fields. A positive H_{int} signifies that the magnetic splittings of the Mössbauer spectrum increase as the applied field H is increased. The outermost lines in Fig. 3A do move outward with increasing H.

Fig. 3B shows a 6.0 T spectrum of Av2/urea. The spectrum of Fig. 3A, scaled to 15% of the total absorption, is plotted over the data. There is no evidence for adventitiously bound Fe^{2+} or Fe^{3+} which would be expected if any cluster conversions or decompositions with iron release had occurred upon addition of urea. Approximately 80% of the iron belongs to the rather featureless paramagnetic component between -1 mm/s and $+2$ mm/s.

Fig. 3C shows 0.06 T and 6.0 T difference spectra of Av2/urea sample obtained by subtracting from the raw data 15% of the corresponding spectra of Av2/ethylene glycol (and 5% of oxidized Av2). The striking feature of Fig. 3C is that no spectral

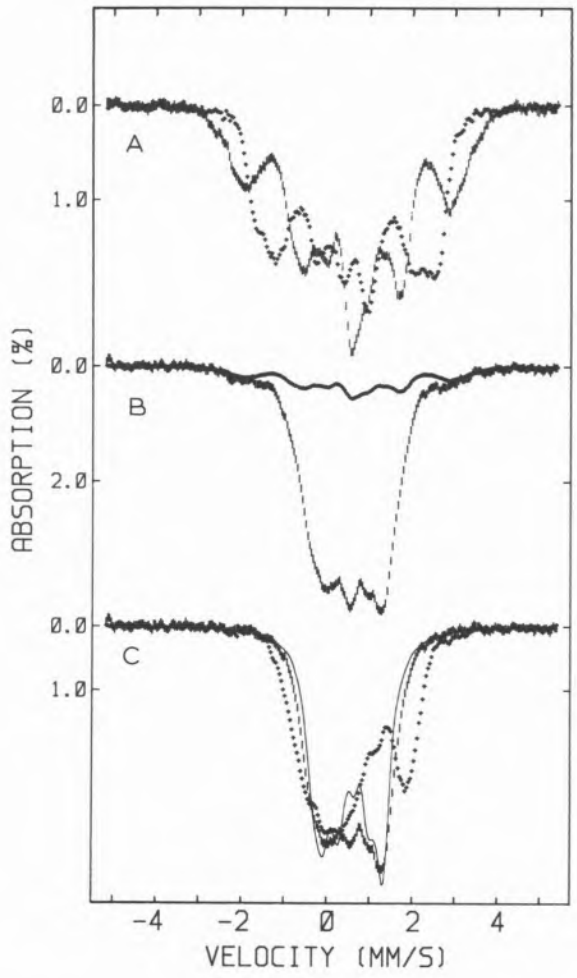

Fig. 3

4.2 K Mössbauer spectra of reduced Av2 in 50% ethylene glycol (A) and 0.4 M urea (B). The spectra in (A) were recorded in applied field of 0.06 T (full circles) and 6.0 T (hash marks). The spectrum in (B) was recorded in a 6.0 T field (hash marks). Indicated is also a 15% contribution of a species as observed in (A). In (C) the 6.0 T spectrum (hash marks) of (B) is compared with data recorded in a 0.06 T field (full circles). The solid line in (B) is a theoretical spectrum based on Eq. (1) with $S = 3/2$, $D = -2$ cm^{-1}, $E/D = 0.22$, $H = 6.0$ T, assuming two sites with magnetic hyperfine coupling constants $A_1 = -4$ MHz and $A_2 = -8$ MHz. Further details and computer analyses of all spectra displayed here are given in [4]

component of the 6.0 T data has a splitting larger than that of the 0.06 T data. That is, all the irons have negative (or unusually small) internal fields. Clearly, this cluster has an electronic structure very distinct from those of the clusters with the familiar $S = 1/2$ ground state. However, the Av2/urea spectra clearly exhibit magnetic hyperfine structure in zero applied field and, therefore,

must belong to a Kramers system. The only EPR-active Kramers system in this sample, other than the $g = 1.94$ species, is the $S = 3/2$ system with $g = 5.1$ and $g = 5.8$.

DISCUSSION

Our data establish a $S = 3/2$ ground state for the [4Fe-4S] cluster of Av2/urea. The EPR resonances at $g = 5.8$ and 5.1 and the unique Mössbauer spectra (with all components having negative internal fields) characterize this unusual state. The observed quadrupole splittings and isomer shifts (spectra not shown here) are similar to those of the more familiar $S = 1/2$ clusters, suggesting that the new state primarily arises from changes in the exchange interactions among the irons and not from a significant rearrangement of charge.

As indicated by the EPR data of Figs. 1 and 2, Av2/Tris HCl contains a mixture of $S = 1/2$ and $S = 3/2$ clusters. Saturation magnetization data (not shown) are dominated by the spin $S = 3/2$ cluster and rule out significant concentrations of any higher spin. Combined saturation magnetization, Mössbauer and EPR data show that the mixture of $S = 1/2$ and $S = 3/2$ clusters is roughly half and half. This observation explains the low concentration of the $g = 1.94$ EPR center ($S = 1/2$) that had perplexed many investigators, including us, for a long time.

The observation of negative internal fields of all Mössbauer spectral components of Av2/urea places a severe restriction on any theoretical model of the Av2/urea electronic structure. For all iron sulfur clusters studied thus far the iron sites are intrinsically high-spin. H_{int} of an isolated high-spin iron is dominated by the negative contribution of the Fermi contact term. In an exchange-coupled Fe-S cluster, the sign of H_{int} at a particular iron nucleus is opposite to the sign of the projection of the local spin of that iron onto the system spin (see for instance, ref. [7]). In Av2/urea all local spins must have positive projections onto the $S = 3/2$ system spin. Therefore, a successful theoretical model must yield local spin expectation values that are parallel, not antiparallel, to the system spin.

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NICKEL-IRON-SULFUR-SELENIUM CONTAINING HYDROGENASES ISOLATED FROM *DESULFOVIBRIO BACULATUS* STRAIN 9974

Hydrogenases from the periplasmic, cytoplasmic and membrane fractions of *Desulfovibrio baculatus* strain 9974 (DSM 1743) have been purified to apparent electrophoretic homogeneity.

PHYSICO-CHEMICAL DATA

Table I indicates the results of metal analysis as well as other physico-chemical data, namely the specific activity of the enzyme in respect to hydrogen evolution (μmoles H_2/min.mg.). Plasma emission metal analysis detects the presence of iron, and of nickel and selenium in equimolecular amounts. The U.V. and visible spectra show broad bands around 277 and 390-400 nm, typical of iron-sulfur containing proteins.

EPR DATA

The EPR spectra of the native ("as isolated") enzymes are shown in Fig. 1 A-C. All the enzymes show a weak isotropic EPR signal centered around g = 2.02 observable at low temperatures (below 20 K) that accounts for about 0.002 to 0.03 spins per molecule. The periplasmic and membrane bound enzymes also show additional

Table I
Physico-chemical data on D. baculatus *(9974) hydrogenases*

	Cytoplasmic	Periplasmic	Membrane bound
Specific Activity (μmoles H_2 evolved/min.mg.)	466	527	120
Molecular weight (kDa)	100[a] 81(54, 27)[b)c)]	110[a] 75(49, 26)[b)c)]	100[a] 89(62, 27)[b)c)]
Metal content			
Fe	7.7 (14.1)[d]	9.25(13.5)	10.3(11.4)
Ni	0.54(1.0)	0.69(1.0)	0.90(1.0)
Se	0.56(1.03)	0.66(0.96)	0.86(0.95)
Ratio $A_{390/280}$	0.28	0.25	0.10

a) Molecular mass determined by high pressure liquid chromatography.
b) Molecular mass determined in the presence of SDS.
c) Molecular mass of subunits are indicated between brackets.
d) Values in ( ) were converted per 1 nickel per minimal molecular weight.

EPR signals with g-values greater than 2.0 assigned to nickel(III), which are detectable up to 77 K. The periplasmic hydrogenase shows EPR features at 2.20, 2.06 and ~2.00 (Fig. 1-B); the signals of the membrane bound enzyme can be

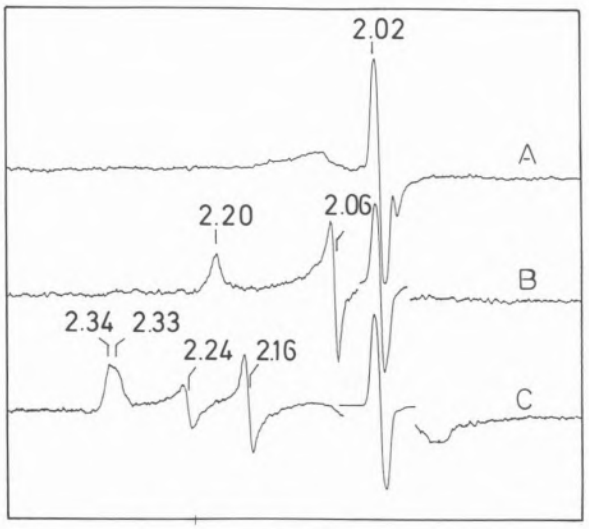

Fig. 1
EPR spectra of D. baculatus *(strain 9974) native hydrogenase: A — Cytoplasmic fraction; B — Periplasmic fraction; C — Membrane bound fraction.*
Experimental conditions: temperature 8 K; microwave power 2 mW; modulation amplitude 1 mT; microwave frequency 9.41 GHz