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The vanadium nitrogenase of *Azotobacter chroococcum*

Purification and properties of the Fe protein

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1. Nitrogenase activity of a strain of *Azotobacter chroococcum* lacking the structural genes of Mo-nitrogenase (*nifHDK*) was associated with a V+Fe-containing protein and an Fe-containing protein [Robson, Eady, Richardson, Miller, Hawkins & Postgate (1986) Nature (London) **322**, 388–390; Eady, Robson, Richardson, Miller & Hawkins (1987) Biochem. J. **244**, 197–207]. 2. The Fe protein was purifed to homogeneity by the criterion of Coomassie Blue staining after electrophoresis in 10% or 17% (w/v) polyacrylamide gels in the presence of SDS. One type of subunit, of M_r 32000±2000, was found. 3. The native protein had an M_r of 62500±2500 and contained approximately 4 Fe atoms and 4 acid-labile sulphide groups per molecule. The amino acid composition was similar to those of other purified Fe proteins, and, characteristically, tryptophan was absent. The specific activities (nmol of protein/min per mg of protein) when assayed under optimum conditions with the VFe protein from this strain were 1211 for H_2 evolution under Ar, 337 for NH_3 from N_2 formation and 349 for C_2H_2 reduction. Activity of the Fe protein was O_2-labile with a $t_{\frac{1}{2}}$ of 36 s in air. At low temperatures the dithionite-reduced protein exhibited e.p.r. signals consistent with the presence of both $S = \frac{1}{2}$ and $S = \frac{3}{2}$ spin states. These signals were similar to those given by other nitrogenase Fe proteins, as were the changes in their line shape that occurred in the presence of MgATP or MgADP. The absorbance spectra showed that an increase in absorption occurred in the visible range on reversible oxidation of the dithionite-reduced protein. The oxidized-minus-reduced ϵ_{420} was 6000 $M^{-1} \cdot cm^{-1}$.

INTRODUCTION

The obligately aerobic diazotrophs *Azotobacter chroococcum* (Robson, 1986) and *Azotobacter vinelandii* (Bishop *et al.*, 1986) have been shown to have a V-containing nitrogenase that is encoded by different genes from those for the well-characterized Mo-containing nitrogenase (Robson *et al.*, 1986*a*; Hales *et al.*, 1986*a*,*b*; Eady *et al.*, 1987). Like Mo-nitrogenase, V-nitrogenase is a two-component system in which a VFe protein replaces the MoFe protein and a second Fe protein replaces that normally associated with Mo-nitrogenase (for review see Eady *et al.*, 1988). There is genetic evidence that *A. vinelandii* (Bishop *et al.*, 1982; Joerger *et al.*, 1986) but not *A. chroococcum* (R. L. Robson & P. Woodley, unpublished work) has a third nitrogenase system that is independent of Mo or V for activity. This system has recently been purified from *A. vinelandii* and been shown to be a two-protein component system in which Fe is the only metal present in significant amounts (Chisnell *et al.*, 1988). The Fe proteins of the two Mo-independent alternative nitrogenases of *A. vinelandii* have been purified (Hales *et al.*, 1986*b*; Chisnell *et al.*, 1988) and shown to be similar in their overall biochemical properties to the Fe proteins of Mo-nitrogenase (for review see Eady, 1986). In the present paper we report the purification and characterization of the Fe protein of the V-nitrogenase of *A. chroococcum* and compare its properties with those of the Fe proteins of the Mo-independent nitrogenases of *A. vinelandii*. A preliminary account of part of this work has been reported previously (Robson *et al.*, 1986*b*).

MATERIALS AND METHODS

Organism

The strain used, *Azotobacter chroococcum* MCD1155, is one in which the cluster of genes, *nifHDK*, encoding the polypeptides of Mo-nitrogenase has been deleted by a gene-replacement technique (Robson, 1986). It was grown at 30 °C in a 400-litre all-glass fermenter on the N-free medium of Robson (1986) modified by including $VOSO_4$ (40 μM) and decreasing the Na_2SO_4 to 200 μM. Organisms were harvested and cell extracts prepared as described by Eady *et al.* (1987).

Assay of nitrogenase activity

Nitrogenase activity was assayed by complementation with Ac1*. N_2 reduction, C_2H_2 reduction and H_2 evolution were measured as described by Eady *et al.* (1987).

Abbrevations used: nitrogenase components are abbreviated in accordance with the notation of Eady *et al.* (1972), in which Ac1 denotes the MoFe protein (component 1) and Ac2 the Fe protein (component 2) of nitrogenase of *Azotobacter chroococcum* grown under Mo-sufficient conditions; Ac1* and Ac2* are used for the VFe protein and Fe protein components of the V-nitrogenase isolated from *A. chroococcum* strain 1155; Av2′ is the corresponding protein of the V-nitrogenase of *Azotobacter vinelandii*; dinitrogenase reductase-3 is the Fe protein associated with the third Fe-containing nitrogenase of *A. vinelandii* (Chisnell *et al.*, 1988).

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Specific activity

After resolution of the nitrogenase components, 1 unit of enzyme activity is defined as the amount of either nitrogenase component required to produce 1 nmol of product/min when complemented with the other at an optimum molar ratio. In crude extracts specific activities refer to total protein in the extract, whereas with purified components they refer to the concentration of the nitrogenase component limiting the assay.

Analytical methods

Iron, acid-labile sulphide, ammonia and amino acid analyses were carried out as described by Eady *et al.* (1987).

Spectroscopic measurements

For absorbance spectra reduced Ac2* protein was prepared in 50 mM-Tris/HCl buffer containing 50 mM-$MgCl_2$. Dithionite was removed in an anaerobic glove-box by passage through a 0.7 cm × 10 cm gel-filtration column of Bio-Gel P6-DG (Bio-Rad Laboratories). The proteins were collected and diluted to 3 ml in a quartz 1 cm-light-path anaerobic cuvette. The dithionite-free reduced protein was oxidized with Indigo Carmine bound to Dowex-1 8AG ion-exchange resin. A 1.5 cm layer of the dye-saturated resin was placed above a second layer of Dowex alone, which was in turn layered above a 0.7 cm × 15 cm column of Bio-Gel P6-DG. The protein remained in contact with the dye layer for 5 min and was then eluted with the column buffer as specified above. Spectra were recorded with a Perkin–Elmer λ5 spectrophotometer against a buffer blank.

E.p.r. spectra were obtained with a Bruker ER200D spectrometer having an Oxford Instrument ESR9 liquid-He cooling system. The spectrometer was interfaced with a PDP1134 computer for recording and processing spectra.

RESULTS AND DISCUSSION

Purification of Fe protein (Ac2*)

Unless stated to the contrary, purification was at room temperature in a buffer system of 50 mM-Tris/HCl, pH 8.0, containing 0.4 g of $Na_2S_2O_4$/l. Chromatography columns were run at room temperature with standard anaerobic techniques as described by Eady (1980). The elution of coloured proteins was followed by using an LKB Uvicord S monitoring system with a narrow-bandpass 365 nm filter. Fractions were collected in Ar-filled conical flasks capped with rubber closures. Gel-filtration and ion-exchange materials were obtained from Pharmacia (U.K.) unless otherwise stated.

Step 1: preparation of crude extracts. Freshly harvested organisms were suspended at 5 °C in an equal volume of 50 mM-Hepes buffer, pH 8.0, and disrupted under N_2 by passage through a Manton–Gaulain homogenizer at 28 MPa (4000 lbf/in^2). The resulting suspension was collected on ice before being centrifuged at 25000 *g* at 5 °C for 1 h to remove cell debris and unbroken organisms. The clear brown supernatant fluid, referred to as the crude extract, was then frozen by dripping into liquid N_2, a procedure used throughout as a means of storage of nitrogenase components.

Step 2: separation of nitrogenase components. Crude extract (400 ml, typically containing 32 g of protein) was loaded on to an anaerobic 11 cm × 5 cm column of DEAE-Sephacel. The column, flowing under gravity at 250 ml/h, was washed with 300 ml of buffer containing 0.1 M-NaCl, which eluted membrane and particulate material. Subsequently the column was eluted in stepwise fashion with NaCl/buffer solutions: 150 ml of 0.15 M-NaCl, 200 ml of 0.2 M-NaCl, 200 ml of 0.35 M-NaCl and finally 400 ml of 0.5 M-NaCl. During this procedure a number of discrete brown–yellow bands were eluted. The elution profile was reproducible under these conditions, and a typical pattern is shown in Fig. 1. The nitrogenase activity was partially resolved into two components (Fig. 1), which initially were assayed by complementation with purified components of the conventional Mo-nitrogenase of *A. chroococcum*, Ac1 and Ac2. At a later stage in this work, as is described here, purified components of nitrogenase of strain MCD1155 were used for assays. Complementation assays (Table 1) showed that the bulk of the component 2 activity (Ac2*) was eluted as a golden-brown band following the addition of 0.35 M-NaCl. The three fractions that together contained 68% of the initial Ac2* activity were combined for further purification. A major band of Ac1* activity was well resolved from Ac2* and was eluted at higher NaCl concentration.

Step 3: gel filtration on Sephacryl S-300. The pooled Ac2* fractions from step 2 were chromatographed in 30–40 ml portions on a 45 cm × 5 cm column of Sephacryl S-300 equilibrated with buffer containing 50 mM-$MgCl_2$. The column was developed with a downward flow of buffer at 60 ml/h. This gave good resolution of Ac2* from a small amount of contaminating Ac1* and also from a dark-coloured high-M_r material that was eluted first. Fractions containing Ac2* from several such columns were combined, and diluted 3-fold with buffer

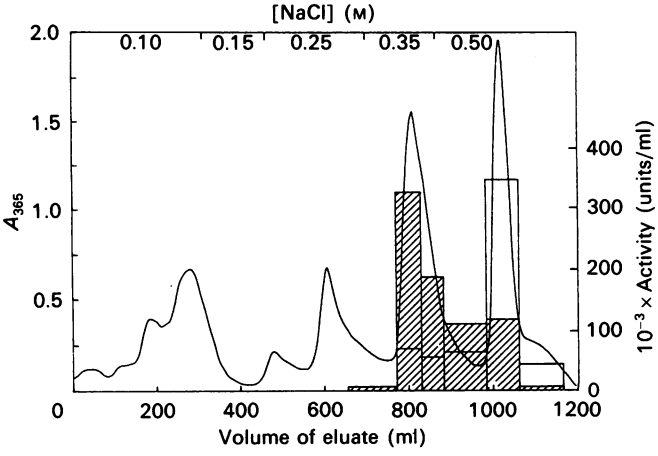

Fig. 1. Elution profile of nitrogenase components from *A. chroococcum* MCD1155 on DEAE-Sephacel

The continuous line is a typical protein elution profile when crude extracts are fractionated on a column of DEAE-Sephacel with stepwise increases in NaCl concentration (step 2). Elution of Ac2* (hatched bars) and Ac1* (open bars) activities is shown by the histogram. Overlap of these two activities (as determined by complementation assay) is indicated.

Table 1. Purification of the Fe protein (Ac2*) of *A. chroococcum* V-nitrogenase

For experimental details see the text.

Step no.	Fraction	Total volume (ml)	Concn. of protein (mg/ml)	Total protein (g)	10^{-3} x Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
1	Supernantant obtained from crude extract after centrifugation	290	73	21.1	457 assayed alone 1050 plus Ac2* 855 plus Ac1*	21.5 49.5 40.3	– – 100
2	DEAE-Sephacel, combined	440	37.5	8.6	248 assayed alone 707 plus Ac1* 546 plus Ac2*	39.4 105 88	82
3	Sephacryl S-300 effluents, combined and concentrated	33	28.5	0.942	633 plus Ac1*	818	74
4	Sephacryl S-200 effluents, combined and concentrated	22.2	44	0.983	502 plus Ac1*	558	52
5	Gradient elution from DEAE-Sephacel	11.5	25.4	0.292	266 plus Ac1*	909	31

before adsorption on a small (2.5 cm x 5 cm) column of DEAE-cellulose. The Ac2*, which bound as a dark-brown band at the top of the column, was eluted with buffer containing 0.5 M-NaCl in a small volume (typically 30 mg/ml in 30 ml).

Step 4: gel filtration in Sephacryl S-200. The concentrated Ac2* protein from step 3 was chromatographed in a 45 cm x 5 cm column of Sephacryl S-200 equilibrated with buffer containing 50 mM-$MgCl_2$. One symmetrical coloured peak containing the Ac2* activity was collected.

Step 5: gradient elution from DEAE-Sephacel. Ac2* from step 4 was diluted 2-fold before being loaded on to a 14 cm x 2.5 cm column of DEAE-Sephacel equilibrated with the standard buffer. The column was developed with a linear NaCl gradient (300 ml total volume) extending from 0.18 M- to 0.35 M-NaCl at a flow rate of 90 ml/h. A major peak eluted at an NaCl concentration range of 0.27–0.3 M was collected in 20 ml fractions. The fractions from this stage that had the highest activities, and that showed no contaminating proteins on gel electrophoresis in 10% and 17% polyacrylamide gels in the presence of SDS, were combined and concentrated on DEAE-cellulose as described in step 3. The concentrated protein was equilibrated with buffer containing 10 mM-$MgCl_2$ by gel filtration through Bio-Gel P6-DG before the data presented here were obtained. The purification is summarized in Table 1.

The procedure described here gave Ac2* of specific activity 900–1400 nmol of H_2 evolved/min per mg of protein when complemented with Ac1*, and an overall 31% recovery of units of activity present in the crude extract. Compared with the purification of Ac2 (Yates & Planque, 1975), the total amount of Ac2* obtained (292 mg) was about 50% that of Ac2. In contrast with the behaviour of Ac1* on DEAE-Sephacel (Eady *et al.*, 1987), Ac2* was eluted at a similar NaCl concentration to Ac2 (R. R. Eady, unpublished work) or Av2 (Burgess *et al.*, 1980), behaviour that contrasts with Av2′, which is eluted earlier than Av2 at 0.17 M-NaCl (Hales *et al.*, 1986*b*).

Table 2. Amino acid composition of Ac2* and Ac2

Experimental conditions for hydrolysis and analysis of Ac2* were as in Eady *et al.* (1987). Data for Ac2 are from Yates & Planque (1975), and data for *nifH** are from Robson *et al.* (1986*b*).

Amino acid	Amino acid composition (residues/molecule)		
	From *nifH** sequence	Ac2*	Ac2
Asx	26	28	29
Thr	9	9	12
Ser	13	11	11
Glx	35	37	41
Pro	9	8	11
Gly	32	31	29
Ala	32	39	32
Val	22	24	28
Met	13	12	15
Ile	22	21	21
Leu	22	23	24
Tyr	9	9	9
Phe	6	6	7
His	4	4	4
Lys	17	17	19
Arg	11	11	5
Cysteic acid	7	8	7
Tyr	–	–	–
Proportion of Gly+Asx (%)	21.1	21.81	22.54
Proportion of Lys+Arg (%)	9.6	9.40	10.29
Total residues	289	306	304

Stability

Purified Ac2* in 50 mM-Tris/HCl buffer, pH 8, containing 10 mM-$MgCl_2$ was stable for up to 24 h at room temperature in an anaerobic glove-box. Exposure to air at 30 °C in the absence of dithionite (essentially as described by Eady *et al.*, 1987) caused an irreversible

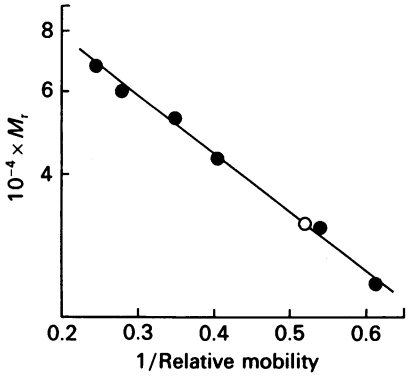

Fig. 2. Determination of the M_r of Ac2* polypeptide by SDS/polyacrylamide-gel electrophoresis

Samples and gels were prepared as described in the Materials and methods section. Standard proteins (●) were run simultaneously as listed in ascending M_r: trypsin (23300), Ac2 (31000), creatine kinase (40000), ovalbumin (43000), glutamate dehydrogenase (53000), catalase (60000) and bovine serum albumin (68000). The ○ symbol is Ac2*.

rapid loss of activity ($t_{\frac{1}{2}}$ 36 s), behaviour that is a characteristic of all Fe proteins that have been investigated.

Amino acid composition

The amino acid composition of Ac2* was determined and is compared with that of Ac2 (Table 2). Acidic residues are approximately twice as abundant as basic residues, and tryptophan is absent, both features that are characteristic of the Fe proteins associated with Mo-nitrogenase. From a best value for molar proportions of amino acids determined by the method of Thornber & Olson (1968) a minimum M_r of 30844 was calculated for Ac2*.

In wild-type *A. chroococcum* there are two copies of the gene encoding Fe proteins of nitrogenase, *nifH* and *nifH** (Jones *et al.*, 1984), and in the *nifHDK*-deletion strain MCD1155 used in this study only one, *nifH** (Robson, 1986). The correlation coefficient of the amino acid composition of Ac2* with the composition of the gene product of *nifH** (see Table 2) was 0.98, and with *nifH* it was 0.96. This small difference is probably a reflection of the marked degree of conservation of sequence and hence amino acid composition between Fe proteins (see Eady, 1986; Lowe *et al.*, 1985), but in itself this homology does not allow Ac2* to be assigned as the *nifH**-gene product. However, since *nifH* is lacking in strain MCD1155, and mRNA transcripts of *nifH** are only detected under N-free Mo-deficient conditions, Ac2* is very probable to be the *nifH**-gene product, as suggested by Robson *et al.* (1986*b*).

Subunit composition

Electrophoresis of Ac2* in 10% or 17% polyacrylamide gels after SDS treatment by the method of Weber & Osborn (1969) resulted in a single band on staining with Coomassie Blue R-250 (see Fig. 2 of Robson *et al.*, 1986*a*). Comparison of the rates of migration of protein standards of known M_r gave a value of 32000 ± 2000 for the M_r of the Ac2* peptides (Fig. 2). Similar values have been reported for Ac2′ (Hales *et al.*, 1986*b*) and dinitrogenase reductase-3 (Chisnell *et al.*, 1988).

M_r

Anaerobic thin-layer gel filtration on Sephadex G-150 equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl and 5 mM-$Na_2S_2O_4$ showed that Ac2* migrated at a rate similar to, but slightly faster than, that of Ac2. With Ac2, bovine serum albumin,

Table 3. Comparison of the physicochemical properties of Fe proteins of Mo-independent nitrogenases

Abbreviation: N.D., not determined.

	Ac2* (present work)	Av2′ (Hales *et al.*, 1986*b*)	Dinitrogenase reductase-3 (Chisnell *et al.*, 1988)
M_r			
From amino acid composition	61500	N.D.	N.D.
From gel filtration	61000	63000	64000
From subunit composition	64000	62000	65000
Subunit composition	One type	One type	One type
M_r	32000	31000	32500
Fe content (g-atoms/mol)	3.7 ± 0.2	3.4 ± 0.4	3.5
Acid-labile S^{2-} (g-atoms/mol)	3.92 ± 0.31	N.D.	4.0
E.p.r., $S_2O_4^{2-}$-reduced	2.035, 1.941, 1.892	2.05, 1.94, 1.88	N.D.
Spin integration	0.46 ± 0.02	0.22 ± 0.04	
Specific activity (nmol of protein/min per mg of protein)			
NH_3 from N_2	337	N.D.	29
C_2H_4 from C_2H_2	341	1100–1400	22
H_2	1211	N.D.	503–650
H_2 under N_2	648	N.D.	488
H_2 under C_2H_2	435	N.D.	394
Activity with MoFe protein	Yes	Yes	Yes

ovalbumin and Ac1 as standards, an M_r value of 61000 was calculated for Ac2*, indicating that, like Ac2, Ac2* is a dimer of two identical subunits.

Iron and acid-labile sulphide content

The data for the iron and acid-labile sulphide content of Ac2* were obtained as described previously for Ac1* (Eady *et al.*, 1987). In calculating the values, which are given in Table 3, an M_r of 62500 was used. The iron content was determined by chemical assay of wet-ashed samples and also by the MgATP-enhanced chelation of Fe by bathophenanthrolinedisulphonate (Ljones & Burris, 1978). The latter technique is generally applicable to the Fe proteins of nitrogenase, and has been proposed as a criterion for assessing the proportion of catalytically competent Fe protein present. When applied to Ac2* this method gave values that were 10–15% lower than the chemically determined values given in Table 3. Since the reaction of Fe in Ac2* with the chelator was very slow in the absence of MgATP (results not shown), this suggests that, like Av2′ (Hales *et al.*, 1986*b*) and Fe proteins associated with Mo-nitrogenase (see Eady, 1986), the binding of MgATP results in a conformational change in Ac2*. This is consistent with the changes in the symmetry of the $S = \frac{1}{2}$ e.p.r. signal of Ac2* in the presence of both MgATP and MgADP (see Fig. 4).

U.v.- and visible-absorption spectra

U.v.- and visible-absorption spectra of dithionite-free reduced and dye-oxidized Ac2* are shown in Fig. 3. The reduced protein had a pronounced shoulder near 360 nm and an absorbance maximum at 276.7 nm with a shoulder at 285 nm. Oxidation as described in the Materials and methods section did not result in any loss of activity but resulted in a decrease in absorption in the far u.v. and an increase in the range 290–850 nm. Pronounced shoulders at 320 and 400 nm were present in the spectrum of oxidized protein. Re-reduction by the addition of $Na_2S_2O_4$ (60 μM) rapidly restored the spectrum characteristic of reduced Ac2*. For protein containing 3.7 mol of Fe/mol of Ac2* the oxidized-minus-reduced ϵ_{420} was 6000 $M^{-1} \cdot cm^{-1}$, a value at the upper end of the range for Fe proteins quoted by Ashby & Thorneley (1987). Reduced Ac2* (8 μM) catalysed the decomposition of $S_2O_4^{2-}$ at a rate of approx. 20 μM/h.

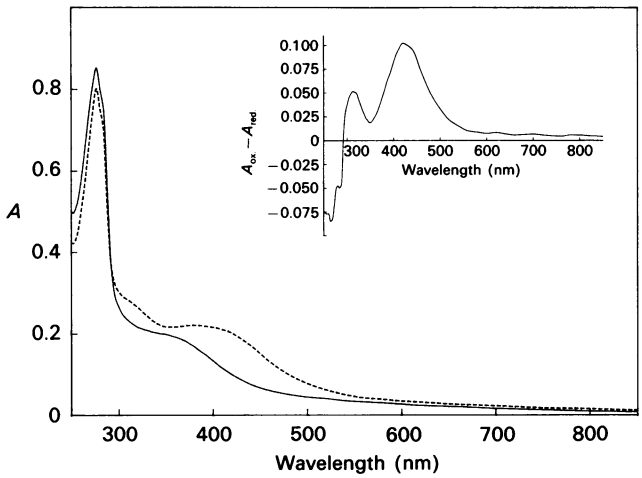

Fig. 3. U.v.- and visible-absorption spectra of oxidized and reduced Ac2*

Solutions of both reduced (——) and oxidized (----) proteins were obtained in 50 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-$MgCl_2$. Samples were prepared as described in the Materials and methods section, and spectra have been corrected to 1 mg of protein/ml. The inset shows the oxidized-minus-reduced difference spectrum.

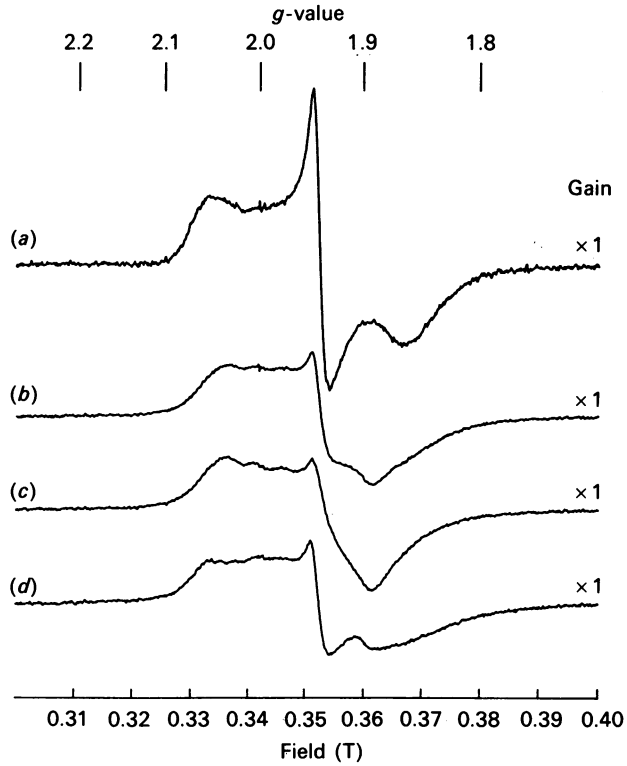

Fig. 4. E.p.r. spectra of the $S = \frac{1}{2}$ centre of Ac2 and Ac2*

The spectra were measured at 18 K with a microwave power of 20.3 mW at 9.567 GHz with a field modulation of 1.0 mT at 100 kHz. The samples, in 25 mM-Hepes buffer, pH 7.4, containing 1 mM-$Na_2S_2O_4$ and 10 mM-$MgCl_2$, were: (*a*) Ac2 (20.5 mg/ml); (*b*) Ac2* (20.5 mg/ml); (*c*) Ac2* (18.6 mg/ml)+9 mM-ATP; (*d*) Ac2* (18.6 mg/ml)+9 mM-ADP. The relative gains are shown at the right of the traces.

Redox potential

The decrease in absorbance at 420 nm on reduction of $Ac2^*_{ox.}$ (Fig. 3) has been utilized to measure the midpoint potential of the $Ac2^*_{ox.}(MgADP)_2/Ac2^*_{red.}(MgADP)_2$ couple (Bergström *et al.*, 1988). A stopped-flow redox equilibrium method using the $SO_2^{\cdot-}/SO_3^{2-}$ couple to set the potential showed that the reduction of $Ac2^*(MgADP)_2$ by $SO_2^{\cdot-}$ was a one-electron process with $E_m = -463$ mV. A similar value of $E_m = -450$ mV was obtained for $Ac2(MgADP)_2$ by using this technique (Bergström *et al.*, 1988).

E.p.r. spectra

At low temperature $S_2O_4^{2-}$-reduced Ac2* shows signals at $g = 2$ and $g = 4$–5 with similar form and relative intensity to those with Ac2 (Figs. 4 and 5). Although the detailed lineshapes are different and less well resolved in the $g = 4$–5 region, dependence of the intensity of these

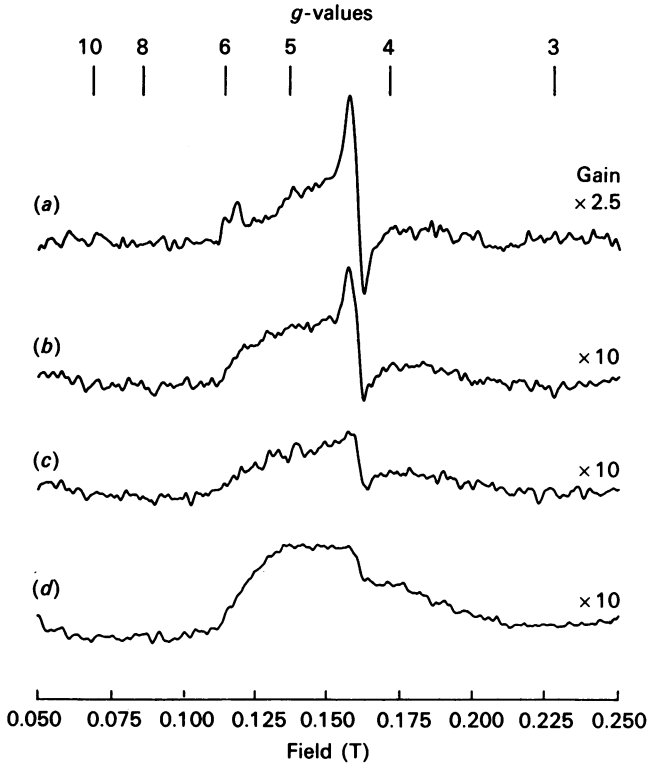

Fig. 5. E.p.r. spectra of the $S = \frac{3}{2}$ centre of Ac2 and Ac2*

The spectra were measured at 10 K and 51 mW microwave power with all other conditions as given in the legend to Fig. 4. The relative gains are not corrected for the temperature difference between these data and Fig. 4.

signals on temperature is consistent with a mixture of spin $S = \frac{1}{2}$ and $S = \frac{3}{2}$ spin states, as has been reported for a number of Fe proteins associated with Mo-nitrogenases (Hagen *et al.*, 1985; Morgan *et al.*, 1986). The signal shapes are affected by the binding of MgATP or MgADP, consistent with a change in conformation of the Ac2* e.p.r.-active centre.

Hales *et al.* (1986*b*) reported e.p.r. spectra from their preparations of Av2′ that are very little different from those characteristic of Fe proteins of Mo-nitrogenases, such as Av2 and Ac2 (see Fig. 4*a*), both in the free form and that with MgATP bound. The reason for the much greater difference in lineshapes in the $g = 2$ region in our preparation of Ac2* is not clear, although such differences presumably reflect changes in the environment of the [4Fe-4S] cluster. The sharp feature at $g = 4.3$ in the $S = \frac{3}{2}$ spectra (Fig. 5) is thought to be due to adventitiously bound iron (Hagen *et al.*, 1985). The $S = \frac{1}{2}$ signals of Ac2*, Ac2*MgATP and Ac2*MgADP integrate to $46 \pm 2\%$ of an electron per molecule of protein, with the rest of the spin intensity presumably in the $S = \frac{3}{2}$ signal.

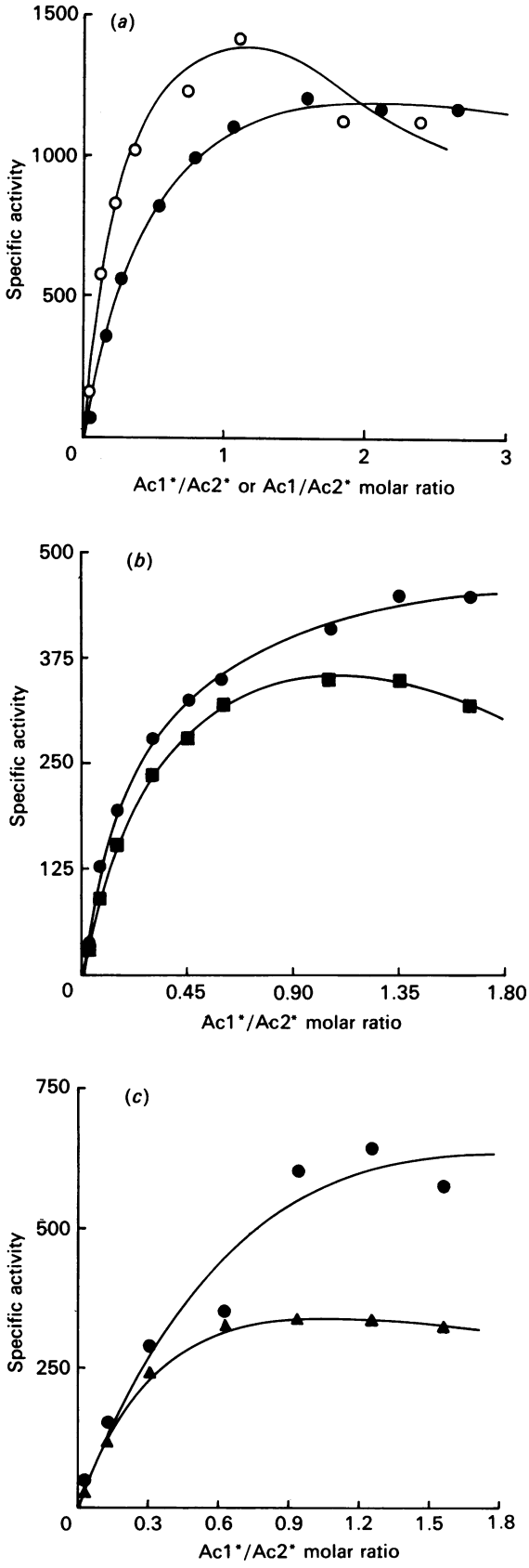

Fig. 6. Titration of Ac2* nitrogenase activity for various reducible substrates

(*a*) H_2 evolution under Ar: ○, titration with Ac1 (specific activity 1406 nmol of H_2 produced/min per mg); ●, titration with Ac1* (specific activity 1300 nmol of H_2 produced/min per mg). (*b*) H_2 evolution (●) and C_2H_4 formation (■) under C_2H_2 (10%, v/v, in Ar). (*c*) H_2 evolution (●) and NH_4^+ formation (▲) under N_2. The Ac2* concentration was 1.5 μM in (*a*) and (*c*) and 1.6 μM in (*b*); Ac1* and Ac1 concentrations were varied as indicated. Assays were performed and products quantified as described previously (Eady *et al.*, 1987).

Substrate reduction pattern

Ac2* was active in the reduction of H^+, C_2H_2 and N_2 when recombined with Ac1 or Ac1*. Titration curves for the complementation of Ac2* with Ac1* and Ac1 for H_2 evolution are shown in Fig. 6(*a*). At ratios of Ac1 or Ac1* to Ac2 less than 1:1, activity for proton reduction was greater with Ac1 than with Ac1*. The ratio of the components of the nitrogenase systems giving maximal activity is approx. 1:1, above which there is a decline in activity when Ac1 is used to complement Ac2*. At a 2:1 molar ratio of Ac1* or Ac1 to Ac2* the proton-reducing activity of the system was the same regardless of which protein was used to complement Ac2*. C_2H_2 reduction also showed an optimum ratio of Ac1*/Ac2* near 1:1, with a slight decline in C_2H_4 formation at higher ratios. The decrease in the rate of reduction of C_2H_2 was balanced by an increase in the rate of concomitant proton reduction (Fig. 6*b*) such that there was no inhibition of total electron flux at protein ratios above 1:1. Fig. 6(*c*) shows that N_2 reduction and concomitant H_2 evolution were optimum at component ratios near to 1:1. As reported previously for Ac1* titrations (Eady *et al.*, 1987), even under optimum conditions H^+ remained an effective substrate in the presence of C_2H_2 or N_2 (Figs. 6*b* and 6*c*).

The pattern of the substrate-reduction curves presented here and those of Ac1* titrated with Ac2* (Eady *et al.*, 1987) are similar to those for the components of Mo-nitrogenase (Eady *et al.*, 1972; Emerich *et al.*, 1981), which in the case of *Klebsiella pneumoniae* have been simulated for H_2 evolution (Lowe & Thorneley, 1984) in terms of a comprehensive model for the mechanism of nitrogenase action (Thorneley & Lowe, 1985). The Mo-independent nitrogenase systems differ from Mo-nitrogenase in that C_2H_2 is a poor substrate compared with H^+ or N_2 (Robson *et al.*, 1986*a*; Hales *et al.*, 1986*a*; Eady *et al.*, 1987; Chisnell *et al.*, 1988) (see Table 3). In addition, C_2H_6 is a minor product of C_2H_2 reduction by V-nitrogenase (Dilworth *et al.*, 1987, 1988) and nitrogenase-3 of *A. vinelandii* (R. R. Eady & R. Pau, unpublished work). Despite these differences, the ability of components of Mo-independent nitrogenases to form active hybrid nitrogenases with components of Mo-nitrogenase is strongly suggestive of an overall similarity in their mechanism of action. Further work on V-nitrogenase is clearly needed to establish whether the Lowe–Thorneley mechanism developed for Mo-nitrogenase is more generally applicable.

Comparison of Ac2* with other Fe proteins of Mo-independent nitrogenases

The properties of Ac2* are compared with those of the Fe protein of V-nitrogenase and the Fe protein of the Fe-containing nitrogenase of *A. vinelandii* in Table 3. Available data for these Fe proteins show them to be very similar in their physicochemical properties to their more intensively studied counterparts in Mo-nitrogenases. In the case of Ac2* this may be expected, since the *nifH* and *nifH** genes have been sequenced (Robson *et al.*, 1986*b*) and their products differ in 32 of a total of 289 residues, but the five cysteine residues likely to be involved in ATP binding and FeS-cluster ligation (Hausinger & Howard, 1983) are conserved. An overall conservation of structure is shown by the formation of active hybrid nitrogenases with the MoFe proteins (up to 70% of the homologous activity in the case of Ac2* (Eady *et al.*, 1987). Similar active hybrids are not always formed in activity cross-reactions among Mo-nitrogenase components from different organisms. Ac2* and dinitrogenase reductase-3 show a similar pattern of substrate specificity, and the lower activity of dinitrogenase reductase-3 towards N_2 or C_2H_2 would not appear to be due to partial inactivation since the rates of H^+ reduction in the presence of N_2 or C_2H_2 are comparable with those of Ac2* (Table 3). The general differences in substrate specificity between Mo-dependent and Mo-independent nitrogenase systems were discussed above.

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