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The vanadium nitrogenase of *Azotobacter chroococcum*

Purification and properties of the VFe protein

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1. Nitrogenase activity of a strain of *Azotobacter chroococcum* lacking the structural genes for conventional nitrogenase (*nifHDK*) was separated into two components: an Fe-containing protein and a vanadoprotein. 2. The larger protein was purified to homogeneity by the criterion of electrophoresis of 10% (w/v) acrylamide gels in the presence of SDS. Two types of subunit, of M_r 50000 and 55000, were present in equal amounts. 3. The protein had an M_r of 210000 and contained 2 V atoms, 23 Fe atoms and 20 acid-labile sulphide groups per molecule. The Mo content was less than 0.06 g-atom/mol. All the common amino acids were present, with a predominance of acidic residues. Ultracentrifugal analysis gave a maximum sedimentation coefficient of 9.7 S and a symmetrical boundary at 5 mg of protein·ml^{-1}; dissociation occurred at lower concentrations. The specific activities (nmol of product/min per mg of protein), when assayed under optimum conditions with the complementary Fe protein from this strain, were 1348 for H_2 evolution, 350 for NH_3 formation and 608 for acetylene reduction. Activity was O_2-labile, with a $t_{\frac{1}{2}}$ of 40 s in air. At low temperatures the dithionite-reduced protein showed e.p.r. signals at $g = 5.6$, 4.35, 3.77 and 1.93, consistent with an $S = 3/2$ ground state with an additional $S = 1/2$ centre giving rise to the feature at $g = 1.93$. The u.v. spectra of dithionite-reduced and thionine-oxidized protein were very similar. Oxidation resulted in a general increase in absorbance in the visible region. The shoulder at 380 nm in the spectrum of reduced protein was replaced with shoulders near 330 nm and 420 nm on oxidation.

INTRODUCTION

Nitrogenase, the well-characterized Mo-containing enzyme system responsible for biological nitrogen fixation, consists of an MoFe protein (an $\alpha_2\beta_2$ tetramer) encoded by the nitrogen-fixation genes *nifD* and *nifK*, and an Fe protein encoded by the *nifH* gene. This enzyme system is both genetically and biochemically highly conserved in a large number of diazotrophs which have been investigated (see Dixon, 1984; Eady, 1986). The occurrence of Mo in an Fe- and Mo-containing cofactor (FeMoco) of the MoFe protein, probably at the site of N_2 reduction (Hawkes *et al.*, 1984) explained the Mo requirement for biological nitrogen fixation first reported by Bortels (1930). However, the observation that several classes of mutants of *Azotobacter vinelandii* defective in nitrogen fixation (*nif*$^-$) underwent phenotypic reversal under conditions of Mo deficiency led to the proposal that this organism possessed an alternative system for nitrogen fixation (Bishop *et al.*, 1980, 1982). The construction of strains of A. *vinelandii* (Bishop *et al.*, 1986) and *A. chroococcum* (Robson, 1986) deleted for the structural genes for nitrogenase (*nifHDK*), yet which retain the capability of diazotrophic growth, has clearly established that both these organisms have two systems for nitrogen fixation. One is the well-characterized and intensively studied nitrogenase involving Mo; the other is a system which functions under conditions of Mo deficiency and which does not require the structural genes for the Mo nitrogenase.

We have recently reported (Robson *et al.*, 1986) that growth and nitrogenase activity of the *nifHDK* deletion strain of *A. chroococcum* MCD1155 is stimulated by the addition of nanomolar levels of V (vanadium) to the medium, and that the conventional molybdoprotein of nitrogenase is replaced by a vanadoprotein. In the present paper we describe the purification and properties of the vanadoprotein from this strain and compare it with the MoFe nitrogenase protein from this organism.

MATERIALS AND METHODS

Organisms

The strain used for most of this work, *Azotobacter chroococcum* MCD1155, is a derivative of *A. chroococcum* N.C.I.B. 8003, in which the cluster of genes, *nifHDK*, encoding the polypeptides of nitrogenase has been deleted by a gene-replacement technique (Robson, 1986). Strain MCD1155 is defective in uptake hydrogenase, which allows nitrogenase activity *in vivo* to be measured from the rate of H_2 evolution. This strain is also resistant to 5 mM-tungstate, so that trace levels of molybdenum do not inhibit growth by preventing expression of the V nitrogenase activity (Robson, 1986). Organisms were grown at 30 °C in air in 400-litre all-glass fermenters on

Abbreviations used: nitrogenase components are abbreviated by using 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 corresponding activities of components isolated from *A. chroococcum* strain 1155.

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the medium described by Robson (1986), except that the Na_2SO_4 was decreased to 200 μM and 40 μM-$VOSO_4$ was added. During growth, dissolved O_2 was maintained at 0.8 to 1.6% of air saturation by manual manipulation of the aeration rate. Under these conditions cultures grew exponentially with a doubling time of 6.5 h, to a yield of 1.5 g wet wt. $\cdot$ litre^{-1} and had a specific activity for nitrogenase of 22 nmol of H_2 evolved $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}. The tungstate-resistant strain, *A. chroococcum* MCD-50 (Robson, 1986), was grown on Mo-sufficient media lacking added vanadium, and used to prepare extracts for comparison of the properties of nitrogenase in crude extracts.

Assay of nitrogenase

The assay conditions were those described by Eady *et al.* (1972), except that the liquid volume was decreased to 1 ml and the dithionite concentration was decreased to 10 mM. The reduction of acetylene to ethylene and H^+ to H_2 was determined by gas chromatography (Eady *et al.*, 1972). Rates of substrate reduction were linear for up to 10 min, when up to 1.2 μmol of products had been formed.

Determination of NH_3

NH_3 was determined directly in portions of nitrogenase assay mixtures by reaction with *o*-phthalaldehyde/mercaptoethanol reagent prepared by the method of Corbin (1984). After incubation of nitrogenase assay mixtures at 30 °C for 8 min, 0.1 or 0.2 ml was removed and added to an equal volume of *o*-phthalaldehyde/mercaptoethanol reagent in 1.5 ml capped polyethylene tubes. Fluorescence was allowed to develop in the dark for 60 min. Fluorescent products were separated by reversed-phase h.p.l.c. The mobile phase was acetonitrile/0.01 M-potassium phosphate buffer, pH 7.3 (1:1, v/v). A Milton–Roy variable-speed metering pump in series with a Whatman Solvecon precolumn and a 10 cm Whatman guard column packed with Partisil 10 ODS-3 C_{18} medium was used. The flow rate of the system was 1.0 ml/min. The small column allowed individual NH_3 determinations to be made in 2.0 min with minimum column flushing. Sample injections (4 μl) were made through a Valco manual-injection valve. Fluorescent products were quantified from peak heights with a Milton–Roy Fluoromonitor having a 390 nm excitation filter and a 455 nm long-pass emission filter.

The assay was standardized by adding aliquots of 10 mM-NH_4Cl (20–2000 nmol of NH_4^+) to reaction mixtures lacking enzyme proteins but containing dithionite and the complete ATP-regenerating system described for determination of H_2 evolution under argon (Eady *et al.*, 1972). The headspace of the assay bottles contained either purified dinitrogen or mixtures of dinitrogen and argon as specified for a given experiment. Controls containing non-functioning nitrogenase proteins in the complete assay mixture were also run and corrections to HPLC detector readings were made by subtracting values obtained in the presence of the complete reaction mixture plus the individual proteins under conditions of no enzymatic activity for reduction of dinitrogen. The *o*-phthalaldehyde/mercaptoethanol method produced a linear fluorescence response up to 2000 nmol of NH_4/ml of assay mixture. Within this range the response was 0.15 arbitrary fluorescence units/nmol of NH_4^+ per ml of assay mixture at a recorder sensitivity of 5 mV/cm. Blank fluorescence values corresponded to 0.2 nmol of NH_4^+/4 μl sample injected.

ATP hydrolysis

The rate of ATP hydrolysis was determined by estimation of creatine formed from phosphocreatine, the phosphate-donating component of the ATP-regenerating system of the nitrogenase assay. Assays were stopped by the injection of 0.1 ml of 20% NaOH, and samples (0.05 ml) were removed and added to 0.9 ml of 2 M-NaOH containing 50 mM-*p*-hydroxymercuribenzoate. Samples were then assayed for creatine by the method of Ennor (1957). Internal creatine standards (0–300 nmol) were included in replicate sample aliquots. In order to determine the ratio of ATP hydrolysis to H_2 evolution, the gas headspace was analysed for H_2 by gas chromatography.

Specific activity

One unit of enzyme activity is defined as the amount of nitrogenase required to produce 1 nmol of product/min under the conditions given above. In crude extracts, specific activities refer to the total protein in the extract, whereas with purified components they refer to the concentration of the nitrogenase component limiting the assay.

Iron analyses

Protein samples containing 100–300 nmol of iron were digested with 0.1 ml of conc. H_2SO_4 on a heating manifold. H_2O_2 was added to discharge any colour formed, and, after heating to constant volume, the sample digests were diluted to 1.0 ml with water. Aliquots were diluted to 0.65 ml for the determination of iron. Saturated ammonium acetate (0.07 ml) was added and the mixtures were vortex-mixed. Sodium metabisulphite (0.1 ml, 0.1 M) and bathophenanthroline sulphonate (0.01 ml, 0.1 M) were added and the mixture was vortex-mixed and warmed at 37 °C for 10 min. Absorbance was read at 540 nm and iron content was calculated from a standard curve. The determination was linear over the range 0–50 ng-atoms of iron per aliquot. Internal standards were run in some protein samples to ensure that iron in the digests was available for reaction with bathophenanthroline; recovery was generally better than 95%. The millimolar absorption coefficient for iron was 23 mM $\cdot$ cm^{-1} for this assay procedure.

Determination of molybdenum

Molybdenum was determined in aliquots of the H_2SO_4 digests of nitrogenase proteins by the colorimetric toluene/dithiol method as previously described (Eady *et al.*, 1972). A standard curve was run with ammonium heptamolybdate. Internal standards of vanadium were also run to ensure that there was no interference with colour development.

Determination of vanadium

Digestion reagents were Aristar grade from BDH Chemicals, Poole, Dorset, U.K. Samples containing 3–6 mg of protein were diluted to 0.6 ml with double-distilled water to which was added 0.03 ml of 70% (w/v) HNO_3. Water was removed by heating at 95 °C and the samples cooled. H_2O_2 (0.2 ml at 30% (v/v)) and 0.4 ml

of water were added. Water was again removed by controlled heating in order to minimize foaming. After cooling, further additions of peroxide and water were made and the heating procedure repeated until no colour, foam or particulate material was evident in the samples. Final heating was carried out at 100 °C until brown fumes of NO_2 began to form. The samples were cooled, diluted with 0.3 ml of water and quantitatively transferred to weighed 7 ml polystyrene screw-cap vials.

Aliquots of a standard vanadium solution were added to some protein samples before digestion and carried through the entire procedure. Also, internal standards were added to aliquots of the digested samples where insufficient protein sample was available for a complete digested-sample standard curve. The HNO_3 content of the diluted digests did not exceed 3% (w/v). This was important, as higher levels of HNO_3 interfered with the analysis, as did H_2SO_4 or phosphate ions.

Vanadium was determined by flameless atomic absorption with programmable pyrolysis (1050 °C) and vaporization (2850 °C) routines (Instrumentation Laboratory, model 151). The hollow cathode vanadium emission source was operated at 10 mA, and the photomultiplier was operated at 460 V. The sensitivity of the detector was 1.0 mA/unit. The method produced a linear response of approx. 80 arbitrary units/ng of V in the range 2.5–20 ng of V/10 μl sample analysed. Recovery of vanadium from digests was 60–80% on the basis of the internal standards added.

Acid-labile sulphide

This was determined colorimetrically by the method of King & Morris (1967). In order to minimize quenching interference by the oxidation products of dithionite (Chen & Mortenson, 1977), a range of small volumes of solutions were assayed and the results extrapolated to infinite dilution.

U.v. and visible absorbance spectra of Ac1* and Ac1

Reduced Ac1 and Ac1* proteins were 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). The proteins were collected and diluted to 3.0 ml in a 1-cm-light-path anaerobic quartz cuvette. Reduced proteins were oxidized with thionine bound to Dowex-1 8AG ion-exchange resin. A 1.5 cm layer of the thionine–resin was placed above a second Dowex-1 layer lacking thionine which in turn was located above a 0.7 cm × 15 cm column of Bio-Gel P6-DG. The proteins were allowed to remain in contact with the thionine layer for 5 min before washing through the gel-filtration column with buffer. The oxidized proteins were free of thionine and were diluted to 3.0 ml under strictly anaerobic conditions in the glove box. Spectra were recorded with a Perkin–Elmer λ5 spectrophotometer against a buffer blank.

E.p.r. measurements

E.p.r. spectra were run on a Bruker ER200D spectrometer with an Oxford Instruments ESR9 liquid-helium cooling system. The spectrometer was interfaced with a PDP1134 computer for recording and processing spectra.

Amino acid composition

Tris/HCl and dithionite were removed from protein samples before analysis by anaerobic chromatography on a column of Bio-Gel P6DG equilibrated with 10 mM-phosphate buffer, pH 7.5, containing 40 mM-NaCl. Samples (200 μg) were hydrolysed with 6 M-HCl for 16, 24, 46 and 70 h at 110 °C in evacuated sealed tubes as described by Moore & Stein (1963). Duplicate samples were analysed commercially [Torry Research Station (Ministry of Agriculture, Fisheries and Food), Aberdeen, Scotland, U.K.] and the results analysed as described by Eady *et al.* (1972). Half-cysteine and methionine residues were determined as cysteic acid and methionine sulphone respectively in performic acid-oxidized samples. Tryptophan was determined by the method of Spies & Chambers (1948).

Ultracentrifugation

Sedimentation rates were determined at 20 °C and 50000 rev./min on an MSE Centriscan 75 ultracentrifuge with absorption optics. Ultracentrifuge cells were filled under N_2 in a glove box containing less than 1 p.p.m. of O_2.

RESULTS AND DISCUSSION

Properties of nitrogenase activity in crude extracts

Nitrogenase activity in crude extracts of strain MCD1155 (measured as acetylene reduction or H_2 evolution), required MgATP, $S_2O_4^{2-}$ and the absence of air, similar to extracts of the parental strain MCD50 grown under Mo-sufficient conditions (results not shown). The specific activities obtained for strain MCD1155 were in the range 20–30 nmol of H_2 evolved·min^{-1}·mg of protein^{-1} for different preparations. This activity was comparable with those observed *in vivo*. Activity was stimulated (2-fold) when assays were complemented with purified Ac1* or purified Ac2* (see Table 1). A detailed comparison of the reactivity of Ac2 and Ac2* with purified Ac1* is presented below.

In contrast with the activity of extracts of strain MCD50, activity of strain MCD1155 was not sedimented by centrifuging at 110000 *g* ($r_{av.}$ 11.9 cm) for 90 min, and was not stable on exposure to air. Under comparable conditions of centrifugation, 44% of the activity for MCD50 was recovered in the pellet compared with 2% for MCD1155. When the supernatant obtained after centrifuging for 90 min was shaken in air at 30 °C, no loss of activity occurred over 25 min with material from MCD50, but MCD1155 nitrogenase was completely inactivated. These observations are not consistent with the formation of an O_2-tolerant high-M_r complex of nitrogenase components with a protective protein, characteristic of the Mo-nitrogenase of *Azotobacter* (see Robson & Postgate, 1980). The addition of purified Ac1* to extracts which had been inactivated by exposure to air restored activity, consistent with our observations, reported below, that this protein is markedly more sensitive than Ac1 to inactivation by O_2.

Purification of component 1 (Ac1*, summarized in Table 1)

Unless stated to the contrary, the buffer system was 50 mM-Tris/HCl, pH 8.0, containing $S_2O_4^{2-}$ (0.4 g·l^{-1}). Chromatography columns were run at room temperature

Table 1. Purification of the vanadium-containing component (Ac1*) of *A. chroococcum* nitrogenase

Step no.	Fraction	Volume (ml)	[Protein] (mg/ml)	Total protein (g)	Total activity (kunits), assay conditions	Specific activity (units/mg of protein)	Yield (%)
1	Supernatant obtained from crude extract after centrifugation	290	73	21.1	457, assayed alone 855, plus Ac1* 1050, plus Ac2*	21.5 40.3 49.5	– – 100
2	DEAE-Sephacel, combined active fractions (see Fig 1)	120	17.8	2.13	95.7, assayed alone 114.7, plus Ac1* 325.4, plus Ac2*	44.8 53.7 152	– – 31
3	Sephacryl S-300 effluents, combined and concentrated	19	23.6	0.448	438, plus Ac2*	977	41
4	Sephacryl S-200 effluents, combined and concentrated	68	4.8	0.326	438, plus Ac2*	1337	41
5	Gradient elution from DEAE-Sephacel, combined active fractions (see Fig. 2)	4.7	13.1	0.061	88.5, plus Ac2*	1426	8.4

by using the standard anaerobic techniques described by Eady (1980). The elution pattern of the proteins was monitored with a narrow-band-pass 365 nm filter in a LKB Uvicord S monitoring system. Coloured fractions were collected in argon-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 in an equal volume of 50 mM-Hepes buffer, pH 8.0, and disrupted under N_2 by passage through a Manton–Gualain homogenizer at 27.6 MPa (4000 lbf/in^2). The resulting suspension was collected on ice before centrifuging at 25000 ***g*** at 5 °C for 1 h to remove cell debris and unbroken organisms. The clear brown supernatant fluid, referred to as 'crude extract', was then frozen by allowing it to drip 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 thawed and loaded on to an anaerobic 11 cm × 5 cm column of DEAE-Sephacel. The column, flowing under gravity at 250 ml·h^{-1}, was washed with 300 ml of buffer containing 0.1 M-NaCl, which eluted membrane and particulate material. Subsequently the column was developed by stepwise elution with NaCl buffer solution, 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. At this stage 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. Complementation assays (Table 1) showed that the bulk of the component 1 activity (Ac1*) was eluted as a dark-brown band after the addition of 0.5 M-NaCl. When complemented with purified Ac2* this fraction contained approx. 30% of the total units present initially in the crude extract (Table 1). Fractions which were eluted at lower ionic strength contained most of the Ac2* activity and also accounted for approx. 26% of the total Ac1* units. These fractions in which Ac1* tended to be less stable were processed as Ac2*. The Ac1* obtained as a by-product of this procedure is not considered here. The total Ac2* activity recovered from Step 2 was usually half the units present in the crude extract.

Step 3: gel filtration in Sephacryl S-300. The Ac1* obtained as described above was chromatographed on a 45 cm × 5 cm column of Sephacryl S-300 equilibrated with buffer containing 50 mM-$MgCl_2$ in a Pharmacia K50/60 column. The material was chromatographed in 30–40 ml portions by downward flow at 90 ml·h^{-1}. Good resolution of two major coloured species present was obtained. Ac1*, which was eluted first, was resolved from a small amount of residual contaminating Ac2*, but the spectral properties of the yellow, second, major peak were consistent with this being a low-M_r

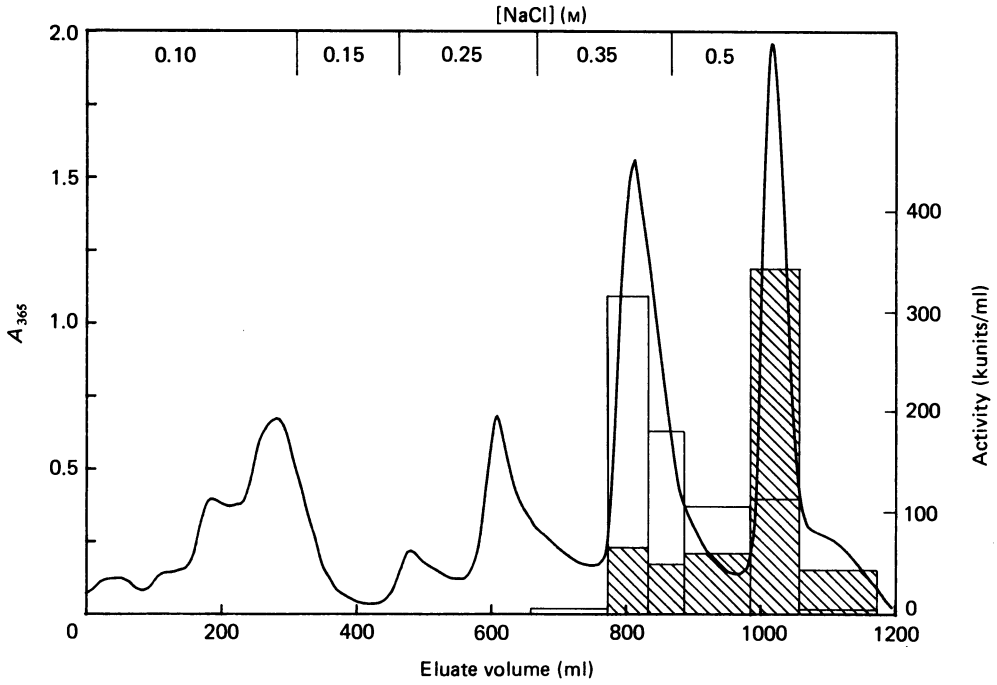

Fig. 1. Elution profile of nitrogenase components from *A. chroococcum* MCD-1155

The data (continuous line) show a typical protein elution profile when crude extracts are fractionated on a column of DEAE-Sephacel using stepwise increases in NaCl concentration (Step 2 of the purification procedure). Elution of Ac2* (open bars) and Ac1* (cross-hatched bars) activity is shown by the histogram. Overlap of the nitrogenase proteins (as determined by complementation assay) is indicated.

flavoprotein, probably flavodoxin. Fractions containing Ac1* from several columns were combined, and diluted 2-fold with buffer before absorption on to a small (2.5 cm × 5 cm) column of DEAE-cellulose. The Ac1*, which bound as a dark band at the top of the column, was eluted with buffer containing 0.5 M-NaCl in a small volume (typically 25 mg·ml^{-1} in 20 ml).

Step 4: gel filtration in Sephacryl S-200. The concentrated Ac1* protein from Step 3 was chromatographed in a 45 cm × 5 cm column of Sephacryl S-200 that had been equilibrated with buffer containing 50 mM-$MgCl_2$. One symmetrical major coloured peak containing the Ac1* activity was collected.

Step 5: gradient elution from DEAE-Sephacel. Ac1* from Step 4 was diluted 2-fold with buffer before loading on to a 14 cm × 2.5 cm column of DEAE-Sephacel equilibrated with the standard buffer. The column was developed at a flow rate of 90 ml·h^{-1} with a linear NaCl gradient extending from 0.225 M-NaCl to 0.5 M-NaCl in a total volume of 300 ml. The major peak containing Ac1*, which was eluted within an NaCl concentration range 0.34 M–0.44 M-NaCl, was collected in 10 ml fractions. Although SDS/10%-(w/v)-polyacrylamide electrophoresis showed only two major large polypeptides over this peak (Fig. 2), the specific activity of different fractions was not constant, but increased on the descending side of the absorption profile. This suggested that the procedure is not completely resolving species of Ac1* which have different activities, a problem which is encountered in the purification of Ac1 and other MoFe proteins (see Eady, 1980). Fractions from Step 5 which had the highest specific activities and which showed no polypeptides other than the doublet on electrophoresis in 10%-acrylamide gels were combined and concentrated on a small DEAE-cellulose column essentially as described in Step 3. The concentrated protein was equilibrated with

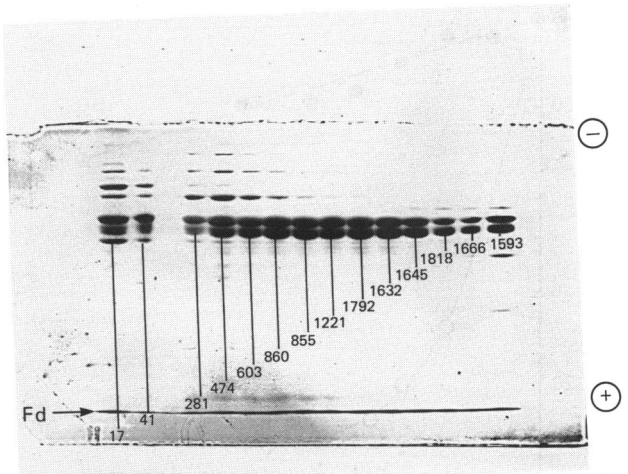

Fig. 2. SDS/polyacrylamide-gel-electrophoretic profile of Ac1* after gradient elution from DEAE-Sephacel

The data show a typical pattern of fractions obtained from Step 5 of the purification procedure. Fractions were subjected to electrophoresis in the presence of SDS in a 10%-(w/v)-acrylamide gel under the conditions described by Weber & Osborn (1969). The position of the weakly staining low-M_r material, which is resolved in 20% acrylamide gels, is marked 'Fd'. The specific activities of the fractions shown below each track are expressed as nmol of H_2 evolved·min^{-1}·mg of protein^{-1}.

buffer containing 10 mM-$MgCl_2$ by gel filtration through Bio-Gel P6 DG (Bio-Rad) before the results presented here were obtained. Concentration of the protein and removal of NaCl resulted in a 10–20% increase in the specific activity of Acl*.

The procedure described above gave Acl* of specific activity 1426 nmol of H_2 evolved·min^{-1}·mg of $protein^{-1}$, when complemented with Ac2*, with an overall recovery of 8.4%; typically a purification took 6 working days. A sample (1 mg) of Acl* was completely inactive when assayed alone. When compared with the purification of Acl (Yates & Planque, 1975), the yield of Acl* was approx. one-third that of Acl, although the specific activities are comparable. However, the yield was sufficient to allow physicochemical studies to be made on Acl*. A significant difference in the behaviour of Acl* compared with Acl and other MoFe proteins was the finding that it was eluted from DEAE Sephacel after the Fe protein was eluted. Although significant loss of total units of activity occurred during chromatography on DEAE-Sephacel (Steps 2 and 5), this technique was retained because of its high capacity and the satisfactory degree of purification from contaminating proteins.

The chromatographic behaviour of Acl* also contrasted with data reported for a comparable deletion strain of *A. vinelandii*, where elution of Avl* (the *A. vinelandii* equivalent of Acl*) from DEAE Sephacel at pH 8.0 occurred at 0.15 M-NaCl (Hales *et al.*, 1985; Chisnell & Bishop, 1985).

Stability

Purified Acl* was stable at room temperature for up to 48 h in 50 mM-Tris/HCl buffer, pH 8.0, under argon. Exposure to air at 30 °C in the absence of dithionite resulted in a rapid loss of activity ($t_{\frac{1}{2}} = 40$ s), in contrast with Acl, which was considerably more stable under similar conditions with a $t_{\frac{1}{2}}$ of 8 min (Fig. 3). The sensitivity of Acl* to inactivation by O_2 was observed also in crude extracts of MCD1155 (see above).

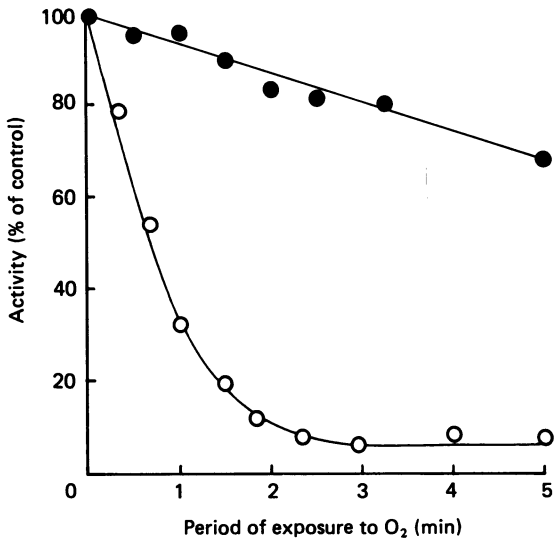

Fig. 3. Effect of exposure to air on the activity of Acl* and Acl

Acl* and Acl were shaken in air (60 strokes·min^{-1} over 2.5 cm) in serum bottles. Samples (0.02 ml) were removed and assayed for H_2 evolution at the time intervals indicated. The activity was compared with controls shaken under argon. ○, Acl*, 6.13 mg/ml in 700 μl of 50 mM-Tris/HCl buffer, pH 8.0. ●, Acl, 1.94 mg/ml in 500 μl of 50 mM-Tris/HCl buffer, pH 8.0.

Table 2. Amino acid compositions of Acl* and Acl

The experimental conditions for hydrolysis and analysis of Acl* are given in the Materials and methods section. Data for Acl are from Yates & Planque (1975).

Amino acid	Composition (residues/molecule) Acl*	Acl
Asx	192	202
Thr	104	96
Ser	86	88
Glx	206	219
Pro	84	96
Gly	178	163
Ala	170	125
Val	124	148
Met	86	62
Ile	122	98
Leu	184	140
Tyr	40	62
Phe	96	88
His	66	62
Lys	118	152
Arg	104	108
Cysteic acid	40	44
Trp	35	47
Total	2035	1980
Proportion of Glx+Asx (%)	19.56	21.26
Proportion of Lys+Arg (%)	10.91	13.13

Amino acid composition

The amino acid composition of Acl* is shown in Table 2, together with data for Acl. There is a clear similarity in overall composition, and acidic residues are approximately twice as abundant as basic residues. As a group, the MoFe proteins of nitrogenase show a high degree of compositional relatedness when their amino acid compositions are compared (Lowe *et al.*, 1980). From the best value for the molar ratios of amino acids, a minimum M_r of 110000 was calculated for Acl*.

Subunit composition of Acl*

Electrophoresis of Acl* in 10%-acrylamide gels after treatment with SDS under the conditions of Weber & Osborn (1969) produced two bands which stained with equal intensity with Coomassie Blue R-250. Comparison of the rates of migration with proteins of known M_r (Fig. 4) gave values of 55000 ± 3000 and 50500 ± 1000 for the subunits of Acl*. These data are consistent with Acl* being composed of two types of subunit and having a minimum M_r of 105000 ± 4000. This estimate may be more reliable than that obtained when this method was applied to Acl (see Kennedy *et al.*, 1976), since the commercial source of the SDS (BDH, Serva, Koch–Light) used in the reservoir buffer did not change the migration pattern of Acl* polypeptides significantly. When 20%-acrylamide gels were used, an additional

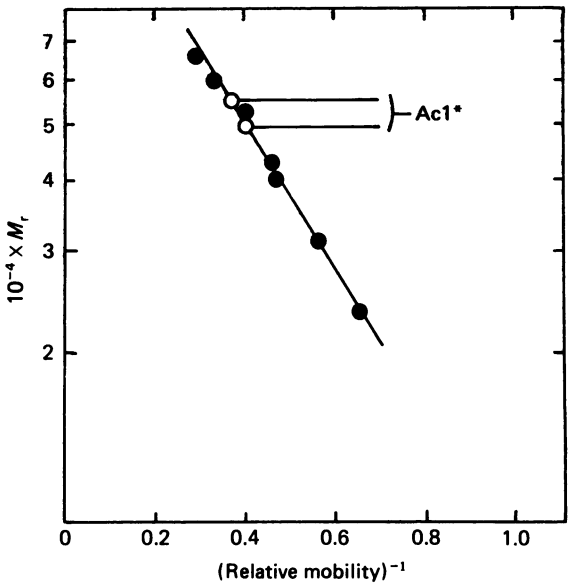

Fig. 4. Determination of the M_r of Ac1* polypeptides by SDS/polyacrylamide-gel electrophoresis

Samples and gels were prepared as described in the Materials and methods section. Standard peptides were run simultaneously as listed in order of ascending M_r ($\times 10^{-3}$): myoglobin (17.8), trypsin (23.3), Ac2 (32), creatine kinase (40), ovalbumin (43), glutamate dehydrogenase (53), catalase (60) and bovine serum albumin (68).

weakly staining band was observed with Ac1* but not with Ac1. The extent of migration was similar to that of ferredoxin from *Clostridium pasteurianum* (M_r 6000) (results not shown). The significance of this polypeptide is uncertain, but it was present in several different preparations of Ac1* and did not change in concentration ratio over fractions of an elution profile. It is an intriguing possibility that this may represent the ferredoxin encoded by the open reading frame located downstream from *nifH** (Robson, 1986), in which case it may be functionally significant, since although present as a minor proportion of the protein (< 5%), it could be stoichiometric with the other subunits. On the other hand this small peptide may be an unresolved contaminant in our Ac1* preparations, and further work is required to resolve this question.

Sedimentation coefficient

The dependence of the sedimentation coefficient of Ac1* on protein concentration in 50 mM-Tris/HCl buffer, pH 8.0, with or without 50 mM-$MgCl_2$ showed a maximum of 9.7 S at approx. 5 mg·ml^{-1} of Ac1*; extrapolation of the data to zero protein concentration gave an $s^0_{20,w}$ value of 10. At this concentration the absorption boundary was symmetrical. Sedimentation behaviour was more complex at lower protein concentrations, since the boundary broadened and the $s^0_{20,w}$ values decreased to 2.9 S at 0.7 mg of Ac1*·ml^{-1}. This decrease may be attributed to the dissociation of Ac1* into subunits under these conditions. Consistent with this, at intermediate protein concentrations, abrupt decreases in the rate of sedimentation occurred during ultracentrifugation, presumably as a consequence of radial dilution of the sample. No loss of activity occurred

Table 3. Metal and acid-labile-sulphide (S^{2-}) content of Ac1* and Ac1

The data for Ac1* were obtained as described in the Materials and methods section and have been calculated for an M_r of 210000. The analytical data for V, Mo, Fe and S^{2-} in ng-atoms·mg of protein^{-1} were 9.7±2.4; < 0.305; 97.76±5.04 and 92.7±1.28 respectively.

	Content (g-atom/mol)			
	V	Mo	Fe	S^{2-}
Ac1*	2.0±0.37	0.064	20.8±1.3	19.4±0.24
Ac1	0.06	1.98±0.30	23±2†	20±2†

† Data from Yates & Planque (1975)

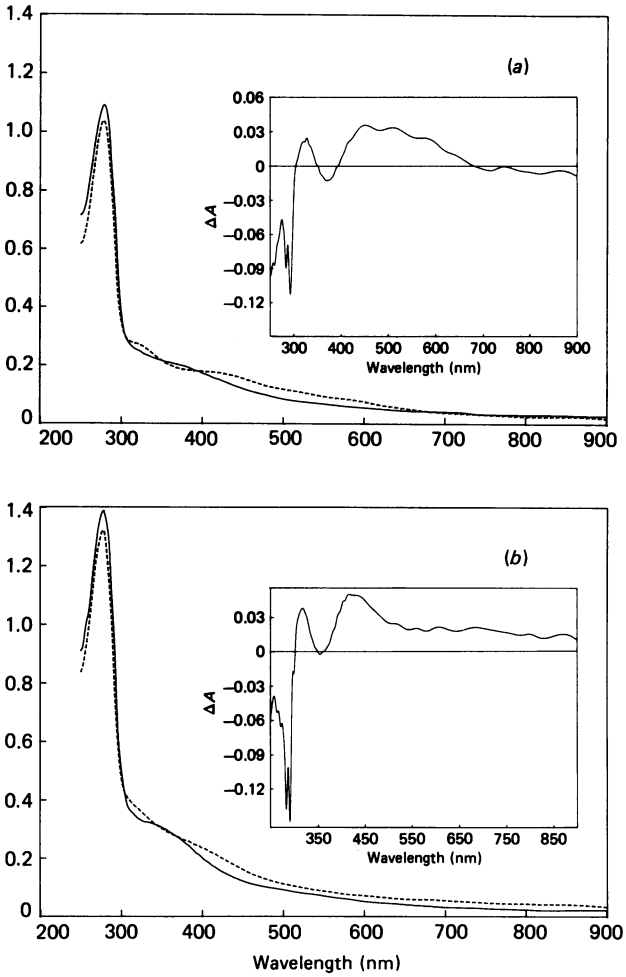

Fig. 5. U.v. and visible absorption spectra of oxidized and reduced Ac1* and Ac1 proteins

Solutions of both reduced (——) and oxidized (------) proteins were obtained in 50 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-$MgCl_2$. Preparation of the protein solutions was as described in the Materials and methods section. Spectra have been corrected to 1 mg of protein·ml^{-1}. (*a*) Ac1*; (*b*) Ac1. Insets show the oxidized-minus-reduced difference spectra.

Table 4. Spectral characteristics of thionine-oxidized and dithionite-reduced Ac1 and Ac1*

Duplicate determinations of absorbance ratios were made for spectra corrected to 1.0 mg/ml.

Protein	Absorbance ratio $A_{380}/A_{277.8}$	Absorbance ratio $A_{430}/A_{277.8}$
Ac1		
Reduced	0.179±0.004	0.100±0.005
Oxidized	0.197±0.004	0.150±0.004
Ac1*		
Reduced	0.178±0.002	0.125±0.002
Oxidized	0.178±0.004	0.154±0.004

in such experiments, so the dissociation must be reversible, but these properties make estimation of the M_r of Ac1* from sedimentation data impracticable.

Gel filtration

On anaerobic thin-layer gel filtration in Sephadex G-150 equilibrated with 50 mM-Tris buffer, pH 8.0, containing 100 mM-NaCl and 5 mM-$S_2O_4^{2-}$, Ac1* and Ac1 migrated to a similar extent, and, with Ac1, Ac2, Ac2*, bovine serum albumin and ovalbumin as M_r standards, an M_r value of 210000 was obtained for Ac1*. Together with our data for the subunit M_r values, an $\alpha_2\beta_2$ tetrameric structure for native Ac1* is indicated.

Metal and acid-labile sulphide content

The iron, vanadium, molybdenum and acid-labile-sulphide content of Ac1* are given in Table 3. When compared with the available data for Ac1 (Yates & Planque, 1975), it is apparent that the Fe and S^{2-} content of the two proteins are similar and approximately equivalent. The major differences arise in the Mo content, where Ac1 contains 1.98±0.3 g-atom of Mo/mol and Ac1* 0.064 g-atom of Mo/mol. Ac1* contained 2±0.37 g-atoms of V/mol compared with < 0.06 g-atom/mol for Ac2*.

U.v. and visible absorption spectra

The u.v. and visible absorption spectra of dithionite-free reduced and oxidized Ac1* (1 mg/ml) and Ac1 (1 mg/ml) are compared in Fig. 5. Both proteins retained full activity after oxidation with thionine, but Ac1* was extremely prone to damage by O_2 under these conditions. The dithionite-reduced proteins showed significant differences in absorbance in the region of 430 nm with the $A_{430}/A_{277.8}$ ratio being higher for Ac1* (Table 4). Both proteins exhibited protein absorbance maxima at 277.8 nm, with prominent shoulders in their spectra at 380 nm. The absorbance of Ac1 was greater than Ac1* between 250 and 450 nm at 1 mg/ml. On oxidation, absorbance of both proteins at 277.8 nm decreased, whereas the shoulder at 380 nm was replaced with shoulders near 330 nm and 420 nm. Oxidized − reduced difference spectra are shown as insets to Fig. 5. Spectra of air-oxidized Ac1* and Ac1 closely resembled the spectra of thionine-oxidized material. Isosbestic points occurred at 304 nm and at longer wavelengths in both sets of spectra. The shoulders appearing in the spectrum of thionine-oxidized Ac1* were more pronounced than those in the spectrum of oxidized Ac1. Spectral changes were reversible on re-reduction of thionine-oxidized proteins under rigorous anaerobic conditions. Both proteins in the reduced state catalysed the decomposition of dithionite (approx. 5 μM·h^{-1} for 4 μM-proteins). During this process, contributions to

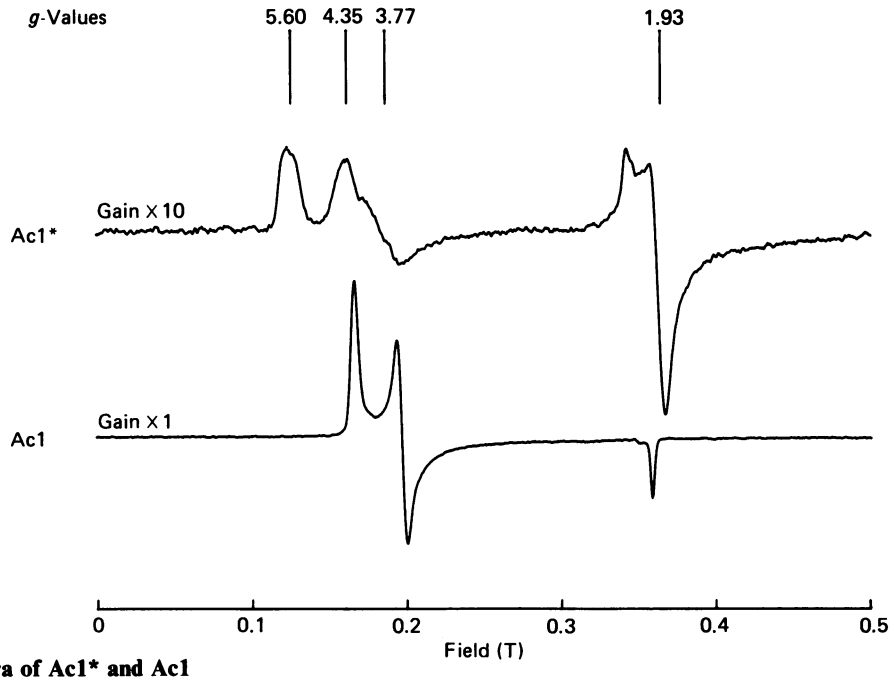

Fig. 6. E.p.r. spectra of Ac1* and Ac1

E.p.r. spectra were run at 10 K with 0.1 mT field modulation at 100 kHz. The microwave power was 20.6 mW at 9.85 GHz. The only signal that is significantly saturated under these conditions is that from Ac1* at $g = 1.93$. The protein samples Ac1* (17 mg·ml^{-1}) and Ac1 (12.4 mg·ml^{-1}) were in 50 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-MgCl and 2 mM-$S_2O_4^{2-}$.

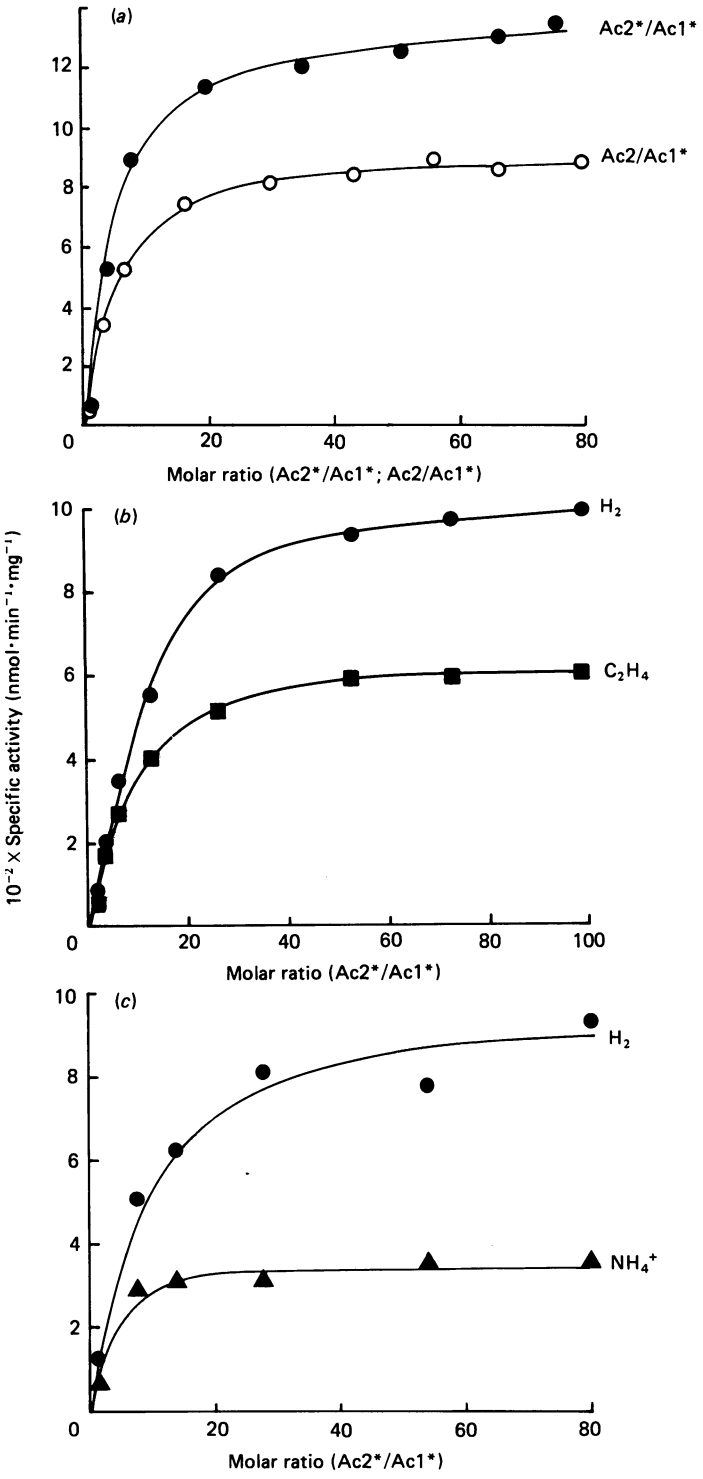

Fig. 7. Titration of Ac1* nitrogenase activity for various reducible substrates

(*a*) Hydrogen evolution under argon. ○, Titration with Ac2 (specific activity 1875 nmol of H_2 produced·min^{-1}·mg^{-1}); ●, titration with Ac2* (specific activity 1875 nmol of H_2 produced·min^{-1}·mg^{-1}. (*b*) Hydrogen evolution (●) and ethylene formation (■) under acetylene (C_2H_2) (10%, v/v, in argon). (*c*) H_2 evolution (●) and NH_4^+ formation (▲) under N_2. The Ac1* concentration was 81 μM in (*a*) and 48 μM in (*b*) and (*c*); Ac2* and Ac2 concentrations were varied as indicated. Assays were performed and products quantified as described in the Materials and method section.

absorbance due to 10 μM-dithionite in the region 250–380 nm became undetectable. The resulting Ac1* and Ac1 spectra were identical with those obtained with the reduced proteins from which dithionite had been removed by gel filtration.

E.p.r. spectrum

At low temperature dithionite-reduced Ac1* exhibited an e.p.r. spectrum with features of $g = 5.6 \pm 0.1$, $g = 4.35 \pm 0.05$, $g = 3.77 \pm 0.05$ and $g = 1.93 \pm 0.02$ (Fig. 6). The spectrum is significantly different from that of Ac1 (Fig. 6), but is still consistent with an $S = 3/2$ ground state (D. J. Lowe, unpublished work), with an additional $S = 1/2$ centre giving rise to the feature at $g = 1.93$. The possibility arises that this latter feature may be associated with the low-M_r polypeptide present in our preparations of Ac1*. The e.p.r. signal from the $S = 3/2$ centre of Ac1* is approximately 20-fold weaker in intensity than that of the $S = 3/2$ centre of Ac1.

The e.p.r. spectrum of Mo-containing nitrogenases arises from the iron- and molybdenum-containing cofactor (FeMoco), which is most likely the reducible substrate-binding site (see Eady, 1986; Hawkes *et al.*, 1984). The altered e.p.r. spectrum of Ac1* suggests that, in this protein, vanadium is incorporated into an analogous cofactor with a consequent change in substrate specificity compared with Mo-nitrogenase, resulting in acetylene being a poor substrate relative to H^+ or N_2 (see Fig.7 and Robson *et al.*, 1986).

Substrate reduction patterns

Ac1* was active when recombined with Ac2* or, to a lesser extent, when combined with Ac2. Titration curves for the complementation of Ac1* activity with increasing concentrations of Ac2* for H^+ reduction, N_2 and acetylene reduction are shown in Fig. 7. The general shape of the curves is similar for all substrates: activity increases rapidly up to an approx. 20-fold molar excess of Ac2* over Ac1*, and thereafter activity only increases slowly. Specific activities are given in Table 5. An unusual feature of these curves is the increase, at high Ac2* concentrations, in the rate of H^+ reduction, either under argon or in the presence of the reducible substrates N_2 or acetylene. As the Ac2*/Ac1* ratio increased, the total electron flux is increased. However, when activity was complemented with Ac2, the rate of H^+ reduction was approx. 30% lower over the entire titration curve and the stimulation in electron flux at high Ac2*/Ac1* ratios, noted above, was not observed. The inefficiency of the coupling of Ac2 to Ac1* was not overcome by increasing the Ac2 concentration further.

The substrate reduction patterns presented here are similar to those obtained with the components of Mo-nitrogenase from other organisms (Eady *et al.* 1972; Emerich *et al.*, 1981). A notable difference is the relatively high rate of H_2 evolution in the presence of the reducible substrates acetylene and N_2. Under 10 kPa (0.1 atm) of acetylene the rates of formation of the two-electron reduction products H_2 and ethylene are 998 nmol and 608 nmol·min^{-1}·mg of Ac1*$^{-1}$ respectively (61% going into H_2 evolution). For the Mo-nitrogenase of *Klebsiella pneumoniae* in similar titration experiments, only 25% of the electron flux resulted in H_2 evolution (Eady *et al.*, 1972).

As the acetylene concentration was increased above 10 kPa, the rate of acetylene formation increased but H_2

Table 5. Maximum observed specific activities of Ac1* for reducible substrates

Data are derived from titration curves presented in Fig. 7. Activities are expressed as nmol of product formed·min^{-1}·mg of Ac1*$^{-1}$.

Substrate	Product...	Activity			
		H_2	NH_4^+	Ethylene	Total two-electron flux
H^+ under argon		1348 (884)†	–	–	1348
H^+, N_2 under N_2		928	350	–	1403
H^+, C_2H_2 under argon		998	–	608	1606

† Titrated with Ac2.

evolution was only inhibited to 40% of its maximum rate. The stimulation of electron flow by increasing the acetylene concentration indicated that, at 10 kPa, a concentration used routinely in activity measurements, maximum activity for acetylene reduction was not observed.

Fe proteins of conventional nitrogenase function as specific electron donors to MoFe proteins. Our observation that Ac2 is 70% as effective as Ac2* in complementing Ac1* in proton reduction (Table 5) suggests that, although encoded by different genes from Ac1, the topology of the contact surface of Ac1* is sufficiently similar to that of Ac1 to permit the complex-formation that enables electron transfer to occur.

Substrate-concentration-dependence and electron allocation

Analysis of rates of H_2 evolution as [MgATP] was varied in assays optimal for Ac1* activity (20-fold molar excess of Ac2*) gave an apparent K_m value for MgATP of 0.43 ± 0.05 mM (results not shown). Linear Lineweaver–Burk plots were obtained in the range 0.1–2 mM-ATP. The dependence of the rate of ethylene formation on acetylene concentration was complex, indicating multiple binding sites, the weakest of which was not saturated under our assay conditions (20 kPa of acetylene). At this concentration, acetylene appeared to stimulate total electron flux by up to 15% compared with the flux under Ar or N_2 (Table 4).

The apparent K_m for N_2 determined in assays optimal for Ac1* activity (20-fold excess of Ac2*) was 14 kPa, a value the same as that determined for the Mo-nitrogenase of *A. vinelandii* (see Hardy, 1979). Under N_2 the electron allocation coefficient for the reduction of N_2 was 0.5 (Table 4) compared with 0.75 for Mo-nitrogenase (see Simpson & Burris, 1984), the remaining electrons being utilized for the reduction of H^+.

The efficiency of electron allocation to substrate reduction was assessed from the determination of the ratio of mol of ATP hydrolysed/mol of H_2 produced (ATP/2e ratio) with a 20-fold excess of Ac2* over Ac1*. The value obtained (5.5) was not significantly different from that found for Ac1 and Ac2 under these conditions.

The dependence of hydrogen production activity on total protein concentration at a 45-fold excess of Ac2* over Ac1* showed an optimum at 2.59 mg·ml^{-1}. This behaviour is similar to, although more pronounced than, that observed with Mo-nitrogenase. Disproportionately low activity at high dilution (the so-called 'dilution effect') and inhibition at high total protein concentrations have been explained (Thorneley *et al.*, 1975; Thorneley & Lowe, 1984) as being due to rates of association–dissociation of the Mo-nitrogenase components changing with total protein concentration as well as component ratio. However, in the case of the V-nitrogenase, dissociation of the subunits of the vanadoprotein could be an additional factor.

Commentary

The stimulation of growth of *A. vinelandii* and *A. chroococcum* on N_2 by V (Becking, 1962) prompted McKenna *et al.* (1970) and Burns *et al.* (1971) to investigate the properties of nitrogenase in V-grown organisms. Partially purified preparations showed a distribution of electrons to H^+, acetylene and N_2 similar to our purified V-nitrogenase. Since their preparations contained significant amounts of Mo, it is likely that these workers had isolated a mixture of the two types of nitrogenase. However, these findings were subsequently attributed to V exerting a sparing effect on the availability of low levels of Mo required for activity of conventional nitrogenase MoFe protein by Benemann *et al.* (1972). The work reported here by Robson (1986) provides definitive evidence that the alternative nitrogenase of *A. chroococcum*, which is expressed under conditions of Mo-deficiency, is a genetically distinct V-based nitrogenase system.

The substrate-reduction patterns of our preparation of V-nitrogenase are similar to those observed *in vivo* in Mo-deficient chemostat cultures of both wild-type and a *nifHDK*-deletion strain of *A. vinelandii* (Eady & Robson, 1984; Bishop *et al.*, 1986), suggesting an involvement of V in the alternative nitrogenase of *A. vinelandii* (see also Bishop *et al.*, 1982). A V-nitrogenase has recently been purified from a *nifHKD*-deletion strain of *A. vinelandii* and the VFe protein has similar physicochemical properties to those of Ac1* (Hales *et al.*, 1986).

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