Nitrogenase of *Sesbania Rhizobium* Strain ORS571: Purification, Properties and ‘Switch-off’ by Ammonia

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Nitrogenase from the *Rhizobium* strain ORS571, which forms both root and stem nodules on the tropical plant *Sesbania rostrata*, was purified from free-living diazotrophically grown organisms. The enzyme complex was resolved into two protein components resembling those obtained from other diazotrophs. Both components were purified to homogeneity as judged by SDS-gel electrophoresis. Component 1, a Mo-Fe protein, had a M_r of 219000 and contained 1.2 g-atoms Mo mol⁻¹, 22.5 g-atoms Fe mol⁻¹ and 20.5 g-atoms acid-labile S mol⁻¹. It consisted of two types of subunit of M_r 56000 and 59000. The specific activity [nmol product formed min⁻¹ (mg protein)⁻¹] of component 1, when assayed in the presence of an optimum concentration of component 2 (molar ratio 1:40), was 1250 for acetylene reduction and 1090 for hydrogen evolution. Component 2, an Fe protein, had a M_r of 74000 and contained 3.1 g-atoms Fe mol⁻¹ and 3.1 g-atoms acid-labile S mol⁻¹. It consisted of a single type of subunit of M_r 36000. The specific activity of component 2, when assayed in the presence of an optimum concentration of component 1 (molar ratio 1:1) was 1700 for acetylene reduction and 1305 for hydrogen evolution. Nitrogenase activity of strain ORS571 was subject to ‘switch-off’ when ammonia was added to a N₂-fixing culture. This effect was independent of protein synthesis and was reversible. Nitrogenase components 1 and 2 isolated from ‘switched-off’ organisms were purified to homogeneity. Both components had the same mobility on SDS-gels as those isolated from active cultures. ‘Switch-off’ resulted in a decrease in the specific activity of component 2 from 1700 to 280. Component 1 remained fully active. The addition of Mn²⁺ to a crude extract containing inactivated nitrogenase did not restore activity.

INTRODUCTION

The fast-growing *Rhizobium* strain ORS571, isolated from stem nodules of the tropical legume *Sesbania rostrata*, forms nodules on both stems and roots of the host plant (Dreyfus & Dommergues, 1981), and can grow in the free-living state at the expense of N₂ as sole nitrogen source (Elmerich *et al.*, 1982; Dreyfus *et al.*, 1983). This latter property suggested that the genetics and biochemistry of nitrogen fixation in this strain could be investigated using techniques developed in free-living diazotrophs such as *Klebsiella pneumoniae*. Isolation of Nif⁻ mutants of strain ORS571, cloning of a DNA sequence homologous to *K. pneumoniae* nifHDK and genetic complementation of a mutant with impaired nitrogenase activity have been described by Elmerich *et al.* (1982), but no data on the enzyme have as yet been published.

Nitrogenase, the enzyme complex that catalyses the reduction of N₂ to NH₃, has been isolated from a variety of diazotrophs (Eady & Smith, 1979). In all cases, it has been fractionated into two protein components, a Mo-Fe protein or component 1, a tetramer comprised of two different subunits, and an Fe protein or component 2, a dimer made of a single type of subunit (Eady & Smith, 1979). The structure of both components appears to have been highly conserved during evolution (Eady & Smith, 1979).

We report here a purification procedure and some properties of the nitrogenase of *Rhizobium* strain ORS571 grown in the free-living state, and also on the regulation of its activity *in vivo* by added NH_4^+ . A preliminary report of these results has been presented (Norel *et al.*, 1984).

METHODS

Media and bacterial growth. The minimal nitrogen-free LSO medium and complete YLS medium were described by Elmerich *et al.* (1982). Bacteria for studies of the 'switch-off' effect *in vivo* were grown at 30 °C in a 1.5 litre Biolafitte fermenter (Poissy, France) containing 1 litre of LSO medium as described by Elmerich *et al.* (1983). Bacteria for purification of nitrogenase were grown in a 20 litre Chemap fermenter (Zürich, Switzerland) containing 15 litres of LSO medium inoculated to an OD_{570} of 0.3 with exponentially growing bacteria in YLS medium under air. The pH was maintained at 7.0 by the addition of 10 M-HCl. The concentration of dissolved O_2 was maintained at about 15 μM (see Results). Bacteria were harvested under argon after 22 h (OD_{570} 3.5) in a cooled Sharples centrifuge. The paste (about 50 g, wet weight) was frozen and stored in liquid nitrogen until use. For enzymic studies of nitrogenase isolated from 'switched-off' organisms, the same procedure was used but $(\text{NH}_4)_2\text{SO}_4$ was added at 10 mM final concentration to the growing culture 1 h before centrifugation.

Assay of nitrogenase activity. Nitrogenase activity was measured either in whole cells or in crude extracts by acetylene reduction; the ethylene produced was determined in an Intersmat IGC 120 FB gas chromatograph (Delsi Instruments, Suresnes, France) fitted with a flame ionization detector. Gas samples (0.25 ml) were injected into a 40 $\times$ 0.2 cm Porapak T column at 55 °C with N_2 as carrier at a flow rate of 20 ml min^{-1} . With whole cells, a suspension of exponentially N_2 -growing bacteria in LSO medium was directly injected into a 50 ml rubber-sealed Erlenmeyer flask containing LSO medium and filled with Ar/O_2 (97:3, v/v). The final volume was 10 ml and the final OD_{570} was 0.3. Flasks were equilibrated by shaking at 30 °C for 10 min and acetylene was injected (10%, v/v) at zero time. With extracts, the technique described by Eady *et al.* (1972) was used, with minor modifications in the composition of the assay mixture (see Results). Except in time course studies, the reaction was stopped after 10 min by injecting 0.1 ml 30% (w/v) TCA. One unit (U) of activity was defined as 1 nmol ethylene produced min^{-1} . Hydrogen evolution was measured under the same conditions as acetylene reduction, except that the gas phase was Ar alone. Hydrogen formed was determined in a Girdel series-30 gas chromatograph (Delsi Instruments) fitted with a thermal conductivity detector. Gas samples (0.4 ml) were injected into a 2.0 m $\times$ 0.3 cm Porapak R column at 60 °C with Ar as carrier at a flow rate of 45 ml min^{-1} .

Purification procedure. All operations were done at 4 °C under strictly anaerobic conditions by working under an argon atmosphere, and by using de-aerated buffers containing 1 mM-sodium dithionite and 0.65 mM-dithiothreitol. In all purification steps by chromatography or gel filtration, fractions were eluted with an upward flow of buffer driven by a peristaltic pump.

Preparation of crude extract. Frozen cell paste (10 g) was thawed in 30 ml 0.1 M-Tris/HCl buffer pH 8.0. Bacteria were disrupted by passage through a French pressure cell at 15000 lbf in^{-2} (103.5 MPa). The resulting suspension was centrifuged at about 50000 g for 30 min. The supernatant is referred to as the crude extract.

Separation of nitrogenase components.

Step 1. DEAE-Trisacryl chromatography. The crude extract was adsorbed onto a 8.0 $\times$ 2.5 cm column of DEAE-Trisacryl (LKB) previously equilibrated with 25 mM-Tris/HCl buffer pH 7.4. The column was washed with one bed volume of buffer before development by stepwise additions of one bed volume each of 0.05 M- and 0.10 M-NaCl, two bed volumes of 0.15 M-NaCl, then two bed volumes of 0.1 M- MgCl_2 in the same buffer. The flow rate was approximately 25 ml h^{-1} . Component 1 was eluted as a broad brown band with 0.15 M-NaCl. Component 2 was eluted as a brown band with 0.1 M- MgCl_2 . Fractions of about 10 ml were collected and stored as beads in liquid nitrogen. Fractions were tested for activity alone or by complementation with the other component. When assayed alone, a residual activity was found only in fractions containing component 2.

Step 2. Concentration. Fractions with specific activity greater than 200 U (mg protein) $^{-1}$ were pooled. Fractions containing component 1 were diluted with an equal volume of 25 mM-Tris/HCl buffer pH 7.4, adsorbed onto a 4.0 $\times$ 0.9 cm column of DEAE-Trisacryl and eluted with 0.15 M-NaCl. Fractions containing component 2 were diluted with three volumes of 25 mM-Tris/HCl buffer pH 7.4, adsorbed onto a 4.0 $\times$ 0.9 cm column of DEAE-cellulose (Whatman DE52) and eluted with 0.1 M- MgCl_2 .

Step 3. Gel filtration. Gel filtration of both components was done in 80 $\times$ 0.9 cm columns, filled with Sephadex G-200 for component 1 and Sephadex G-100 for component 2. Columns were eluted with 25 mM-Tris/HCl buffer pH 7.4, at a flow rate of 15 ml h^{-1} .

Protein determination. Protein concentration was assayed by the Coomassie brilliant blue G-250 technique (Sedmak & Grossberg, 1977) with BSA as a standard.

Gel electrophoresis. SDS-gel electrophoresis was done according to Laemmli & Favre (1973) with slab gels run at 8 mA for 18 h. After fixation in 10% (w/v) TCA for 1 h, gels were stained with 0.2% Coomassie blue R-250 in 45:10:45 (v/v) methanol/acetic acid/water for 2 h and destained in 15:7.5:77.5 (v/v) methanol/acetic acid/water

overnight. To determine the M_r of polypeptides, phosphorylase-b (92 500), BSA (66 200), ovalbumin (45 000), carbonic anhydrase (31 000) and soybean trypsin inhibitor (21 500) were used as standards.

Gel filtration. To determine the M_r of native nitrogenase components, 3.0 mg of each of component 1 and 2 contained in about 1 ml of buffer were chromatographed through Sephadex G-200 (23 × 2.6 cm) and Sephacryl S-200 (25 × 2.6 cm) respectively. Protein standards were ferritin (440 000), catalase (232 000), and aldolase (158 000) for component 1 and aldolase, BSA and ovalbumin (M_r values given above) for component 2.

Isoelectric focusing. The basic technique of O'Farrell (1975) was modified according to the LKB 2117 Multiphor operational manual. Ampholines of pH range 3.5–10 (LKB) and 5–8 (Pharmacia) were used. Pre-focusing was done aerobically for 1 h at 15 °C by maintaining the current at 20 mA (200 W). Isoelectric focusing of 5 µg samples of pure nitrogenase components 1 and 2 was done under similar conditions for 2 h. After removing ampholines by washing the gels for 1 h with ethanol/acetic acid/water 60:5:35 (v/v), proteins were stained by the silver nitrate technique (Merril *et al.*, 1982).

Metal and acid-labile sulphur content. To remove non-specifically bound metal, the protein samples were treated with Chelex 100 (Bio-Rad) according to Eady *et al.* (1972). Dithionite and dithiothreitol were removed by anaerobic gel filtration through a Sephadex G-10 (20 × 1.5 cm) column, equilibrated with a 25 mM-Tris/HCl buffer pH 7.4 containing 10% (w/v) glycerol. Iron and molybdenum were determined colorimetrically using 4,7-diphenyl-1,10-phenanthroline (Doeg & Ziegler, 1962) and toluene-3,4-dithiol (Bulen & Le Compte, 1966) respectively. For calibration, an iron standard solution (Sigma) was used. Acid-labile sulphur was determined with *N,N'*-dimethyl-*p*-phenylenediamine by the method of Lovenberg *et al.* (1963) as modified by Suhara *et al.* (1975).

RESULTS

Optimization of culture conditions for nitrogenase biosynthesis

When *Rhizobium* strain ORS571 is placed in nitrogen-free medium under microaerobic conditions, nitrogenase activity is derepressed (Elmerich *et al.*, 1982; Dreyfus *et al.*, 1983). For liquid cultures or cultures on solid medium, a 3% O₂ concentration in the gas phase was found to be optimal. For cultures in a fermenter containing 1 litre of medium, with the pH maintained at 7.0, a 1 litre min⁻¹ flow of N₂/O₂ (97:3, v/v) was satisfactory. Scaling up to a 15 litre fermenter, flushed by a 15 litres min⁻¹ flow of the same gas mixture, led to a lower nitrogenase activity and to less reproducible results. This problem was solved by bubbling the fermenter with a permanent flow of 7 litres min⁻¹ of N₂/O₂ (97:3, v/v) and a discontinuous flow of N₂/O₂ (90:10, v/v) regulated by an O₂ electrode so that the dissolved oxygen concentration in the medium was maintained at about 15 µM. Under these conditions, a specific activity for nitrogenase of 40 U (mg protein)⁻¹ in whole cells was regularly obtained.

Optimization of the assay conditions for nitrogenase activity in crude extracts

Preparation of active crude extracts required absolute anaerobic conditions. The most active extracts were obtained by disrupting a suspension of bacteria (1 g wet weight bacteria in 3 ml 0.1 M-Tris/HCl buffer pH 8.0 containing 1 mM-sodium dithionite and 0.65 mM-dithiothreitol) in a French pressure cell. The crude extract (about 14 mg protein ml⁻¹) had a pH of 7.4. Centrifugation for 30 min at 50 000 *g* or for 1 h at 200 000 *g* resulted in the same activity in the supernatant. At room temperature, about 60% of nitrogenase activity was lost after 1 d. For this reason, purification was done at 4 °C, and all the fractions were stored in liquid nitrogen where activity was stable for months.

Acetylene-reducing activity of the crude extract was assayed in 40 mM-HEPES buffer, pH 7.4, containing 4 mM-ATP, 8 mM-creatine phosphate, 80 µg creatine kinase ml⁻¹ and 15 mM-sodium dithionite. Under these conditions, nitrogenase activity had an absolute requirement for Mg²⁺. Maximum activity was obtained with 10 mM-Mg²⁺. No activity was observed when Mg²⁺ was replaced by Mn²⁺. The optimum pH for activity was around 7.3. Under optimum conditions, nitrogenase activity in crude extracts was about 70 U (mg protein)⁻¹.

The nitrogenase components

Purification. A summary of the purification of the two components of nitrogenase from 10 g (wet weight) bacteria is given in Table 1. Both components were purified from the crude extract with a yield of approximately 25%. After purification, neither of the two components had any residual activity when assayed alone. After electrophoresis in SDS-gels, component 1 produced

Fig. 1. Electrophoresis of samples containing component 1 of *Rhizobium* strain ORS571 nitrogenase at each step of purification. Electrophoresis was done on 10% polyacrylamide gel in the presence of SDS. Lane (a), standard proteins of M_r indicated; lane (b), crude extract of bacteria grown under conditions of N_2 -fixation (65 μ g protein); lane (c), crude extract of bacteria grown under conditions of ammonia-assimilation (67 μ g protein); lane (d), DEAE-Trisacryl effluent fraction (40 μ g protein); lane (e), DEAE-Trisacryl concentration fraction (26 μ g protein); lane (f), Sephadex G-200 effluent fraction (10 μ g protein).

Table 1. *Purification of the components of Rhizobium strain ORS571 nitrogenase*

Step	Vol. (ml)	Total activity (U)	Total protein (mg)	Specific activity* [U (mg protein) ⁻¹]	Purification factor	Yield (%)
Crude extract	24	24400	336	73	1	100
Component 1						
DEAE-Trisacryl effluent	45	17200	58	296	4.1	70
DEAE-Trisacryl concentration	3.1	13000	28	465	6.3	53
Sephadex G-200 effluent	11.1	5800	4.4	1318	18	23.7
Component 2						
DEAE-Trisacryl effluent	45	21400	49.5	432	5.9	87.7
DEAE-cellulose concentration	6	18200	30	607	8.3	74.5
Sephadex G-100 effluent	20	6800	7	971	13.3	27.8

* After DEAE-Trisacryl chromatography, the activity was determined by complementation of one component by the other. As titration curves were not established, the specific activity does not necessarily represent the maximum value (see Results).

Fig. 2. Electrophoresis of samples containing component 2 of *Rhizobium* strain ORS571 nitrogenase at each step of purification. Electrophoresis was done on 10% polyacrylamide gel in the presence of SDS. Lanes (a), (b) and (c), as in Fig. 1; lane (d), DEAE-Trisacryl effluent fraction (37 µg protein); lane (e), DEAE-cellulose concentration fraction (20 µg protein); lane (f), Sephadex G-100 effluent fraction (7 µg protein).

two bands of equal intensity and of M_r 56000 and 59000 respectively, and component 2 produced a single band of M_r 36000 (Figs 1 and 2). It also appears that these bands, present in the crude extracts of bacteria grown under conditions of N_2 fixation, are absent from crude extracts of NH_4^+ -repressed organisms (Figs 1 and 2). This was confirmed by immuno-assays with specific antisera against purified components 1 and 2 (results not shown). As previously reported for component 1 of other diazotrophs (Kennedy *et al.*, 1976), resolution of *Rhizobium* strain ORS571 component 1 into two distinct protein-staining bands depended on the commercial source of the SDS used. Two bands were observed with Koch-Light SDS whereas a single diffuse band was formed with Sigma SDS (results not shown).

Molecular weight of the native components. The M_r determined by gel filtration was 219000 for component 1 and 74000 for component 2 (Fig. 3). This suggests that, as for other nitrogenases, component 1 is a tetramer made up of two different types of subunits ($\alpha_2\beta_2$) and component 2 is a dimer made up of a single type of subunit.

Metal and acid-labile sulphur content. Component 1 contained (mean $\pm$ SE) 1.16 ± 0.05 g-atoms Mo mol $^{-1}$, 22.5 ± 2.1 g-atoms Fe mol $^{-1}$ and 20.5 ± 1.6 g-atoms acid-labile S mol $^{-1}$, while component 2 contained 3.1 ± 0.4 g-atoms Fe mol $^{-1}$, 3.1 ± 0.4 g-atoms acid-labile S mol $^{-1}$ and no molybdenum (Table 2). Component 1 thus appears to be a typical Mo-Fe protein and component 2 appears to be a typical Fe protein of the nitrogenase complex.

Fig. 3. Molecular weight determination of native components 1 and 2. (a), Component 1 (arrowed). Protein standards were: 1, ferritin ($M_r = 440\,000$); 2, catalase ($M_r = 232\,000$); 3, aldolase ($M_r = 158\,000$). (b), Component 2 (arrowed). Protein standards were: 1, aldolase; 2, BSA ($M_r = 66\,200$); 3, ovalbumin ($M_r = 45\,000$).

Table 2. *Determination of metal and acid-labile sulphur content of components 1 and 2*

Element	Protein sample weight (μg)	Amount of element detected		Molar proportion of element* [mol (mol protein) ⁻¹]
		ng	n-atoms (mg protein) ⁻¹	
Component 1				
Molybdenum	55	27	5.11	1.12
	110	60	5.68	1.24
	220	109	5.16	1.13
Iron	55	332	108.0	23.6
	110	651	105.9	23.2
	220	1170	95.2	20.8
Acid-labile sulphur	55	175	99.2	21.7
	110	325	92.1	20.2
	220	627	88.9	19.5
Component 2				
Molybdenum	100	<2	—	—
Iron	50	125	44.8	3.31
	100	225	40.3	2.98
	200	462	41.4	3.06
Acid-labile sulphur	50	70	43.7	3.23
	100	125	39.0	2.89
	200	265	41.3	3.06

* M_r was estimated as 219000 for component 1 and 74000 for component 2.

Isoelectric point. When samples (5 μg) of components 1 and 2 were subjected to isoelectric focusing, single bands revealed by the silver nitrate technique were observed in both cases with pI values of 6.9 and 5.3 respectively.

Specific activity. The activity titration of component 2 with component 1 is shown in Fig. 4(a). Addition of increasing amounts of component 1 to a fixed amount (21 μg) of component 2 resulted in an almost linear increase of nitrogenase activity up to a maximum followed by a slight decrease. The maximum was reached at a ratio of 60 μg component 1 to 21 μg component 2. Taking the M_r values to be 219000 and 74000 respectively, this corresponds to a component 1/component 2 molecular ratio of 1:1.04. Under these conditions, the specific activity of component 2 was 1700 U (mg protein) $^{-1}$.

Fig. 4. Influence of nitrogenase component ratio on enzyme activity. (a), Titration of component 2 (21 µg) with component 1; (b), titration of component 1 (14 µg) with component 2.

Fig. 5. Effect of ammonia shock on nitrogenase activity *in vivo*. (a), Activity in the absence of chloramphenicol; (b), activity in the presence of chloramphenicol (50 µg ml⁻¹). After 20 min, ammonium sulphate was added to 10 ml bacterial suspension (OD₅₇₀ 0.3) in LSO medium to give a final NH₄⁺ concentration of 0.1 mM (○), 1 mM (▽) or 10 mM (■). ●, No NH₄⁺ added.

Titration of component 1 with component 2 is shown in Fig. 4(b). The maximum activity was reached at a ratio of 14 µg component 1 to 200 µg component 2. This corresponds to a component 1/component 2 molecular ratio of 1:42. Under these conditions, the specific activity of component 1 was 1250 U (mg protein)⁻¹.

Hydrogen evolution was measured with crude extracts and purified components. In both cases, hydrogen production was ATP-dependent. The specific activity of the crude extract was 79 nmol H₂ formed min⁻¹ (mg protein)⁻¹. Titration curves were not established with purified components, but it was assumed that maximum activity was reached at the same component ratios as for C₂H₄ production. The specific activity of component 1, in the presence of a 40-fold molar excess of component 2, was 1090 nmol H₂ formed min⁻¹ (mg protein)⁻¹; the specific activity of component 2, in the presence of an equimolar concentration of component 1, was 1300 nmol H₂ formed min⁻¹ (mg protein)⁻¹. As expected, components 1 and 2 had no H₂-evolving activity when assayed alone.

The ammonia 'switch-off' of nitrogenase

Studies with whole cells. The addition of NH₄⁺ to N₂-fixing cultures resulted in a rapid and almost total inhibition of nitrogenase activity (Fig. 5). Inhibition occurred both in the presence and in the absence of 50 µg chloramphenicol ml⁻¹. When the NH₄⁺ concentration was 0.1 mM or

Table 3. *Purification of the components of N₂ase R of Rhizobium strain ORS571*

Step	Vol. (ml)	Total activity (U)	Total protein (mg)	Specific activity* [U (mg protein) ⁻¹]
Crude extract	30	3300	390	8.5
Component 1				
DEAE-Trisacryl effluent	45	19650	73	269
DEAE-Trisacryl concentration	2.5	10945	26	421
Sephadex G-200 effluent	20	4980	6.0	830
Component 2				
DEAE-Trisacryl effluent	55	4290	55	78
DEAE-cellulose concentration	3.4	3635	31	117
Sephadex G-100 effluent	20	1550	6	258

* After DEAE-Trisacryl chromatography, the activity was determined by complementation with pure components of N₂ase A. As titration curves were not established the specific activity does not necessarily represent the maximum value (see Results).

lower (results not shown), nitrogenase activity in cells not treated with chloramphenicol reappeared after a period of inhibition (Fig. 5a). This phenomenon did not occur when chloramphenicol was present (Fig. 5b).

The addition of glutamine (0.1 to 10 mM) resulted in a 70% inhibition of nitrogenase activity. Surprisingly glutamate (0.1 to 10 mM), although a good N source for strain ORS571, did not inhibit activity.

The nitrogenase of ammonia-shocked bacteria. The inhibitory effect of NH₄⁺ on nitrogenase activity *in vivo* was reminiscent of the 'switch-off/on' effect first described, and now well documented, in photosynthetic bacteria (see e.g. Ludden *et al.*, 1984). We therefore decided to adopt the same terminology and to call the enzyme present in N₂-growing cells N₂ase A, and the enzyme present in ammonia-shocked cells N₂ase R.

Studies with crude extracts. Under standard assay conditions, the N₂ase R activity in crude extracts was between 1 and 10% of that found for N₂ase A. The 'activating conditions' (Ludden & Burris, 1976; Hallenbeck *et al.*, 1982), which restore activity to N₂ase R of photosynthetic bacteria and include addition of Mn²⁺ to the reaction mixture of the N₂ase R assay, were tested. Concentrations of Mn²⁺ ranging from 0.1 to 0.5 mM were added to reaction mixtures containing 5, 10 or 20 mM-Mg²⁺. Such additions did not modify the activity of N₂ase A (results not shown); neither was activation of N₂ase R observed. In contrast to photosynthetic bacteria (Gotto & Yoch, 1982a), the addition of extracts of ammonia-grown cells also failed to elicit activation.

Purification and properties of components 1 and 2 of N₂ase R. The same purification procedure as that described in Table 1 for N₂ase A was applied to a crude extract containing N₂ase R: both components were purified with approximately the same protein yield as the components of N₂ase A (Table 3). They were homogeneous as judged by SDS electrophoresis, and the *M_r* values of their subunits did not differ significantly from those of N₂ase A (Fig. 6). However, it appears from Table 3 that, as in photosynthetic bacteria, component 2 was impaired in its activity. This was confirmed by titration studies (Figs 7 and 8).

When N₂ase R component 2 was titrated with component 1 isolated from N₂ase A or N₂ase R (Fig. 7), approximately the same maximum activity was reached at the same molar ratio. The maximum specific activity of N₂ase R component 2 was 286 U (mg protein)⁻¹ when titrated with N₂ase A component 1, and 270 U (mg protein)⁻¹ when titrated with N₂ase R component 1. Taking the same *M_r* for components 1 and 2 of N₂ase A and R (Fig. 6), it appears from Fig. 7 that the maximum activity was reached in both cases for a component 1/component 2 molar ratio of about 1:10.

When N₂ase R component 1 was titrated with component 2 isolated from N₂ase A or N₂ase R (Fig. 8), maximum activity was similar in both cases but the amount of component 2 required to reach the maximum was four-fold higher when it originated from N₂ase R than from N₂ase A.

Fig. 6. Comparison of electrophoretic patterns of pure components 1 and 2 of N_2 ase A and N_2 ase R of *Rhizobium* strain ORS571. Electrophoresis was done on 10% polyacrylamide gel in the presence of SDS. Lane (a), standard proteins of M_r indicated; lane (b), N_2 ase A component 1 (7 μ g); lane (c), N_2 ase R component 1 (8 μ g); lane (d), N_2 ase A component 1 (3.5 μ g) and N_2 ase R component 1 (4 μ g); lane (e), N_2 ase A component 2 (5 μ g); lane (f), N_2 ase R component 2 (6 μ g); lane (g), N_2 ase A component 2 (2.5 μ g) and N_2 ase R component 2 (3 μ g).

Fig. 7. Influence of nitrogenase component ratio on activity of a complex containing nitrogenase R component 2. (a), Titration of N_2 ase R component 2 (150 μ g) with N_2 ase A component 1; (b), titration of N_2 ase R component 2 (150 μ g) with N_2 ase R component 1.

Fig. 8. Influence of nitrogenase component ratio on activity of a complex containing nitrogenase R component 1. (a), Titration of N₂ase R component 1 (15 µg) with N₂ase A component 2; (b), titration of N₂ase R component 1 (15 µg) with N₂ase R component 2.

The maximum specific activity of N₂ase R component 1 was 1144 U (mg protein)⁻¹ when titrated with N₂ase A component 2 and 995 U (mg protein)⁻¹ when titrated with N₂ase R component 2. This maximum was reached at a component 1/component 2 molar ratio of 1:41 when N₂ase A component 2 was added and 1:162 in the case of N₂ase R component 2.

By comparing these results with those reported in this paper for N₂ase A components, it appears that the loss of nitrogenase activity in ammonia-shocked cells was due to a strong inactivation of component 2, whereas component 1 remained fully active.

DISCUSSION

The purified nitrogenase components of *Sesbania Rhizobium* strain ORS571 grown in the free-living state have very similar physicochemical properties to previously characterized nitrogenases (Eady & Smith, 1979).

The Mo-Fe protein is a tetramer of *M_r* 219000 made up of two different subunits of *M_r* 56000 and 59000 respectively. Early reports on the Mo-Fe protein of *R. japonicum* (Israel *et al.*, 1974) and *R. lupini* (Whiting & Dilworth, 1974) have described the protein as made up of a single type of subunit. Further studies showed that this finding was due to the commercial source of SDS used (Kennedy *et al.*, 1976). In addition, the existence of two types of subunits is in agreement with the characterization of genes equivalent to *nifK* and *nifD* in all diazotrophs which have been studied (see e.g. Robson *et al.*, 1983) including strain ORS571 (Elmerich *et al.*, 1982 and this laboratory unpublished). The Mo-content of about 1 g-atom mol⁻¹ is in agreement with many values reported (Eady & Smith, 1979). However the most active preparations from *Clostridium pasteurianum* and *Klebsiella pneumoniae* have been shown to contain 2 g-atoms mol⁻¹ (Eady & Smith, 1979) and it is possible that, as in other cases, our preparation was contaminated by inactive protein lacking molybdenum. The iron and acid-labile sulphur content of about 20 g-atoms mol⁻¹ is also compatible with other values reported (Eady & Smith, 1979). The pI of 6.9 is higher than that of *K. pneumoniae* Mo-Fe protein (Eady *et al.*, 1972). This is in agreement with the fact that the enzyme is eluted from a DEAE ion-exchanger at a lower concentration of NaCl (0.15 M instead of 0.23 M).

The Fe protein is a dimer of *M_r* 74000 made of a single type of subunit. The iron and acid-labile sulphur content is closer to 3 than to 4 g-atoms mol⁻¹, the usual value found for other Fe proteins (Eady & Smith 1979). This may be due to contamination of the preparation by an inactive fraction.

Titration of the Mo-Fe protein with the Fe protein and vice versa gave results similar to those found with other nitrogenase components (e.g. see Eady *et al.*, 1972; Hallenbeck *et al.*, 1982). A

maximum activity of 1250 units (mg Mo-Fe protein)⁻¹ was found in the presence of a 42-fold molar excess of Fe protein, whereas a maximum activity of 1700 units (mg Fe protein)⁻¹ was found in the presence of an equimolar concentration of Mo-Fe protein. As observed with other nitrogenases, e.g. *K. pneumoniae* (Eady *et al.*, 1972), the specific activity of components 1 and 2 for ATP-dependent H₂ production was of the same order of magnitude as for acetylene reduction. However, in contrast to *K. pneumoniae* nitrogenase, the specific activity of which is a little higher for H₂ evolution than for C₂H₂ reduction, the opposite situation was observed with *Rhizobium* ORS571 nitrogenase, particularly with component 2. Further studies are required to confirm that the values obtained, 1300 and 1700 for H₂ production and acetylene reduction respectively, are significantly different and reflect specific features of the Fe protein.

Nitrogenase activity had an absolute requirement for Mg²⁺; no residual activity was observed when Mg²⁺ was replaced by Mn²⁺ in contrast to other nitrogenases, e.g. *K. pneumoniae* nitrogenase (Eady *et al.*, 1972). Both the Mo-Fe protein and the Fe protein, either purified or in crude extracts were highly sensitive to inactivation by oxygen. Their instability when stored under anaerobic conditions at room temperature is unusual for nitrogenase preparations and necessitated their purification at 4 °C; however they could be stored for months in liquid nitrogen.

The absence of Mo-Fe protein and Fe protein in ammonia-grown cells suggests that their biosynthesis is repressed under these conditions, as is the case in all other nitrogenases studied. Regulation by oxygen was not examined in detail but no activity was detected in bacteria placed in a nitrogen-free medium in the presence of air. Gebhardt *et al.* (1984) reported that nitrogenase activity of *Rhizobium* strain ORS571 was strongly decreased when the bacteria were grown at dissolved O₂ levels above 9 µM. This result differs appreciably from our observations which showed that both maximum growth rate (about 6 h division time) and maximum nitrogenase activity were obtained at a dissolved O₂ concentration around 15 µM. It is possible that this apparent discrepancy is due to our use of batch cultures whereas Gebhardt *et al.* (1984) used continuous cultures at dilution rates of 0.1 or less. As there is apparently no specific system for protection of nitrogenase inactivation by O₂ in strain ORS571, the reduction of the metabolic rate in a chemostat may result in a greater sensitivity of nitrogenase to O₂. However, when strain ORS571 was grown under our optimum conditions, the specific activity of nitrogenase, 40 U (mg protein)⁻¹ in whole cells and 70 U (mg protein)⁻¹ in crude extracts, was similar to values obtained with the most efficient free-living diazotrophs.

The addition of NH₄⁺ to 10 ml samples or to a 15 litre fermenter culture of N₂-growing cells resulted in an immediate and practically total inhibition of nitrogenase activity *in vivo* and in crude extracts. As in the 'switch-off/on' effect which exists in photosynthetic bacteria (see e.g. Ludden *et al.*, 1984) the inhibition of nitrogenase in the presence of chloramphenicol (Fig. 5) suggests that the phenomenon is independent of protein synthesis. In addition, the phenomenon is also reversible since nitrogenase activity reappeared after 100 min when the NH₄⁺ inhibitory concentration was 0.1 mM (Fig. 5a) or earlier for lower concentrations (results not shown). In the presence of chloramphenicol (Fig. 5b) it is likely that nitrogenase activity did not reappear because the antibiotic, by preventing protein synthesis, also prevented the exhaustion of ammonia in the medium. It cannot be totally excluded, however, that protein synthesis is necessary to restore activity.

In photosynthetic bacteria, the Fe protein is inactivated by attachment of a modifying group consisting of phosphate, pentose and an adenine-like moiety (Ludden & Burris, 1978). Whereas the active Fe protein consists of two identical subunits of M_r 30000, the inactive form consists of two different subunits migrating in SDS-gels as two bands of M_r 30000 and 31500 (Gotto & Yoch, 1982b; Ludden *et al.*, 1982). An O₂-sensitive membrane-bound activating enzyme that requires Mn²⁺ for activity has been shown to remove the modifying group (Ludden & Burris, 1976; Hallenbeck *et al.*, 1982). This enzyme is present in cells grown on any nitrogen source, even ammonia (Gotto & Yoch, 1982a). In strain ORS571, NH₄⁺ 'switch-off' results in a specific inactivation of the Fe protein from 1700 U (mg protein)⁻¹ in N₂ase A to 280 U (mg protein)⁻¹ in N₂ase R. In contrast to *Rhodospirillum rubrum* (Gotto & Yoch, 1982b; Ludden *et al.*, 1982), SDS-gel electrophoresis failed to resolve the inactive Fe protein of strain ORS571 into two bands

(Fig. 6). This might be an artefact, or modification of the Fe protein might be different from that observed in photosynthetic bacteria and so be undetectable by this method.

Attempts to reactivate N_2 ase R of strain ORS571 *in vitro* are still too preliminary to be discussed in detail. It is, however, worth noting that the lack of response to Mn^{2+} in N_2 ase R containing crude extracts contrasts with the situation in photosynthetic bacteria.

To our knowledge, this is the first documented report of the existence of a NH_4^+ 'switch off' effect in a *Rhizobium* strain. Whatever the mechanism, one may speculate that this control of nitrogenase activity also occurs when strain ORS571 is living in symbiosis with the host plant, and may also be a general property of rhizobia.

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