Purification and Properties of the Nitrogenase of *Azospirillum amazonense*

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The nitrogenase of the free-living, microaerobic, N_2-fixing bacterium *Azospirillum amazonense* (strain Y1) was purified by chromatography on DEAE-52 cellulose, by heat treatment, and by preparative polyacrylamide gel electrophoresis. The specific nitrogenase activities were 2,400 nmol of C_2H_4 formed per min per mg of protein for dinitrogenase (MoFe protein) and 1,800 nmol of C_2H_4 formed per min per mg of protein for dinitrogenase reductase (Fe protein). The MoFe protein was composed of a minimum of 1,852 amino acid residues, had an isoelectric point of 5.2, and contained 2 atoms of Mo, 24 atoms of Fe, and 28 atoms of acid-labile sulfide per molecule. The Fe protein had 624 amino acid residues and an isoelectric point of 4.6 and contained four atoms of Fe and six atoms of acid-labile sulfide per molecule. The purified MoFe protein showed two subunits with molecular weights of 55,000 and 50,000. The purified Fe protein revealed two polypeptides on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with apparent molecular weights of 35,000 and 31,000. The two Fe protein polypeptides were demonstrated with immunological techniques in the purified, highly active enzyme as well as in extracts. Also, *Azotobacter vinelandii* Fe protein showed two closely migrating polypeptides that migrated differently from the Fe protein polypeptides of *Azospirillum brasilense* or *Rhodospirillum rubrum*. The nitrogenase activity of *Azospirillum amazonense* Y1 was independent of Mn^{2+}, and the addition of activating enzyme had no effect. No activating enzyme could be found in *Azospirillum amazonense*. Obviously, the nitrogenase system of *Azospirillum amazonense* Y1 is different from that of *Azospirillum brasilense* Sp7 and resembles the *Azotobacter* system.

Nitrogen fixation of *Azospirillum* spp., as free-living organisms and in association with the roots of a variety of nonleguminous plants, has been studied intensively since rediscovery of *Azospirillum* spp. as N_2 fixers (for reviews, see references 1, 7, 22, 32). The organisms can grow and fix N_2 under microaerobic conditions. *Azospirillum brasilense* can use organic acids (e.g., malate) and fructose as carbon and energy sources for growth and N_2 fixation, whereas *Azospirillum lipoferum* can also use glucose (31). Recently, *Azospirillum amazonense* was described as a microaerobic, acid-tolerant, root-colonizing bacterium that can use sucrose to support growth and N_2 fixation (8, 19).

Biological N_2 fixation is effected by the nitrogenase enzyme system, and its components have been isolated and purified from a variety of microorganisms. Nitrogenase consists of two O_2-sensitive proteins, a molybdenum-iron protein (dinitrogenase) with two kinds of subunit and an iron-sulfur protein (dinitrogenase reductase) with a single type of subunit (9). Both proteins are essential for enzymic activity together with ATP (which is hydrolyzed to ADP + P_i), a bivalent metal ion (usually Mg^{2+}), a source of electrons (usually $Na_2S_2O_4$ in vitro), and an anaerobic environment (for a review, see reference 26).

Information on the nitrogenase systems of *Azospirillum* spp. has been reported only for partially purified preparations of *Azospirillum brasilense* Sp7, formerly *Spirillum lipoferum* (18, 24). This nitrogenase system was separated into three components, an MoFe protein, an inactive Fe

protein, and an enzyme that activated the inactive Fe protein. The process of activation was similar to that found in *Rhodospirillum rubrum* (13, 17). The inactive Fe protein also could be activated in vitro by treatment with an activating enzyme isolated from the chromatophores of *R. rubrum* (29). In *R. rubrum*, the regulation of nitrogenase activity is mediated via the covalent modification (ADP ribosylation) of the iron protein (25).

It was deemed important to isolate and characterize the nitrogenase system of *Azospirillum amazonense* Y1, because the organism differs from the other azospirilla in acid tolerance, optimal pO_2, and ready growth and N_2 fixation with sucrose (19). We report here the purification and molecular characterization of the nitrogenase components and provide evidence that they are more closely related to nitrogenase components of *Azotobacter vinelandii* than to those of *Azospirillum brasilense*.

MATERIALS AND METHODS

Growth of the organism. *Azospirillum amazonense* Y1 (kindly provided by N. R. Krieg) was grown in a 300-liter fermentor at a dissolved oxygen concentration of 0.6 to 1.0 kPa (controlled by an oxystat) and at a constant pH of 6.0. The minimal medium (19) contained 0.5% malate and an initial ammonium concentration of about 1 mM. The 300-liter fermentor was inoculated with a 15-liter culture on minimal medium that had been grown aerobically for 24 h with 20 mM ammonium chloride. The organic buffer 2-(N-morpholino)ethanesulfonic acid (MES) (pK_a = 6.2) had to be used at a concentration of 15 mM to stabilize the pH of this starter culture. After 48 h of growth at 30°C, the 300-liter culture had reached an optical density of 1.4 (A_{580}) and a nitrogenase

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activity of 33 nmol of ethylene produced from acetylene per min per mg of protein. Before harvest with a continuous Sharples centrifuge, the culture was flushed with N_2, and sodium dithionite (final concentration, 2 mM) was added to maintain anaerobic conditions. Tris hydrochloride buffer (pH 8; final concentration, 100 mM) was added to stabilize the pH during the harvest of the cells. The cell paste (about 500 g) was frozen immediately and stored in liquid nitrogen.

Purification of nitrogenase components. All procedures were performed anaerobically, and all solutions contained 2 mM sodium dithionite and 1 mM dithiothreitol. All buffers used were deoxygenated on a gassing manifold by repeated evacuation and flushing with argon purified by passage through a heated copper catalyst (BASF R3-11; Chemical Dynamics Corp., South Plainfield, N.J.). Solutions finally were sparged with prepurified N_2 before the addition of dithionite.

Approximately 100 g of frozen cell paste was thawed in 200 ml of 300 mM Tris acetate buffer (pH 8.5) containing 2 mM dithionite and 1 mM dithiothreitol. The cells were autolyzed at room temperature for 30 min with lysozyme (200 mg)–DNase (10 mg)–RNase (20 mg), and then they were sonicated (Heat Systems 350 Ultrasonic Cell Disrupter with 50% duty cycle at 14 W) for 2 min under anaerobic conditions. The broken cell preparation was centrifuged at 200,000 × g for 1 h to remove particulates. The dark brown supernatant (crude extract) was diluted to 1,800 ml with 50 mM Tris acetate buffer (pH 7.6) (column buffer) and applied to a DEAE-52 cellulose column (3.5 by 12 cm) equilibrated with the column buffer. The column was washed with 100 ml of the column buffer before the two nitrogenase components were eluted. The MoFe fraction was eluted as a dark brown band with column buffer containing 200 mM NaCl, and the Fe protein fraction was eluted as a yellow band with the same buffer containing 450 mM NaCl. The flow rate was approximately 150 ml/h.

Further purification of the MoFe protein. The MoFe protein fraction was heated for 5 min at 55°C and then cooled quickly. After centrifugation at 200,000 × g for 30 min, the supernatant was diluted with two volumes of column buffer to lower the NaCl concentration. It then was applied to a second DEAE-52 cellulose column (3.5 by 10 cm) and preequilibrated with column buffer, and the MoFe protein was eluted with 200 mM NaCl in column buffer. The MoFe protein then was precipitated with 30% polyethylene glycol 4000 and spun down at 200,000 × g for 1 h. The pellet was suspended in a small amount of column buffer, and insoluble material was removed by centrifugation. The supernatant, with 10% (wt/vol) sucrose added, was applied to a preparative polyacrylamide gel electrophoresis (PAGE) unit with 9% separating gel (4 cm), 4% stacking gel (1 cm), and 17% lower gel (4 cm). The electrophoresis unit consisted of a water-jacketed column (13 cm long) and an Ortec 4100 power supply (Ortec, Inc., Oak Ridge, Tenn.). Because the MoFe protein migrated very slowly, half the usual length of separating gel was used. The lower electrode reservoir was prepared aerobically and contained 40 mM Tris acetate (pH 8.5) plus 250 mM glycine. The upper electrode reservoir was anaerobic and contained 10 mM Tris acetate buffer (pH 8.3) plus 100 mM glycine. The gel was prerun with 300 mM Tris acetate buffer (pH 8.9) for 2 to 3 h at 60 V with 100 pulses per s and a discharge capacitance of 1.0 μF. About 100 mg (10 ml) of concentrated MoFe fraction was applied to the stacking gel, and electrophoresis was continued at the same voltage for 12 h. The voltage then was raised to 150 V with 200 pulses per s. The MoFe protein, visible as a dark brown

band, was eluted with 50 mM Tris acetate (pH 7.4)–5 mM magnesium acetate and adsorbed on line to a DEAE-52 cellulose column (1.5 by 8 cm). Finally, pure MoFe protein was eluted from the DEAE-cellulose column with 200 mM NaCl in column buffer.

Further purification of the Fe protein. The Fe protein fraction from the first DEAE-52 column was heat treated at 55°C for 5 min and cooled quickly, and denatured proteins were centrifuged down. The supernatant was diluted with three volumes of 50 mM Tris acetate buffer (pH 7.6) and loaded onto a second DEAE-52 cellulose column (1.5 by 12 cm) equilibrated with 50 mM Tris acetate buffer (pH 7.6). The Fe protein was eluted with 450 mM NaCl in 50 mM Tris acetate (pH 7.6), and a yellowish band was collected. After desalting by gel filtration on a column of Sephadex G-25, sucrose was added (10% wt/vol). The Fe protein was finally purified by preparative PAGE. The Fe protein appeared as a yellow band and was eluted much earlier than the MoFe protein. All purified proteins were frozen and stored in liquid nitrogen. The adequacy of the anaerobic technique applied during purification was checked routinely by injecting a small quantity of buffer into a solution of methyl viologen.

Assay for nitrogenase activity. Nitrogenase activity was determined by acetylene reduction with sodium dithionite as reductant (5, 12). The reaction mixture (1 ml) contained 40 μmol of phosphocreatine, 14.5 U of creatine phosphokinase, 25 μmol of magnesium acetate, 0.5 μmol of $MnCl_2$, 30 μmol of 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, 5 μmol of ATP, and 5 μmol of dithionite. The gas phase was 10% acetylene in argon. The reaction was initiated by the addition of enzyme. After 20 min at 30°C and rapid shaking (160 complete reciprocations per min), a 0.5-ml gas sample was taken for ethylene analysis by gas chromatography. Specific activity was calculated on the basis of the nitrogenase protein limiting the assay, and it is expressed as nanomoles of product formed per min per mg of protein. To determine the maximum specific activities of MoFe and Fe proteins, we performed assays with different levels of the complementary nitrognease protein.

SDS slab gel electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gels were prepared, and electrophoresis was performed as described by Laemmli (16) and modified by Kanemoto and Ludden (13); they used a lower cross-linker content (acrylamide-N,N'-methylenebisacrylamide ratio, 100:0.58 in a 10% acrylamide gel). After electrophoresis, the protein in the separating gel was visualized by staining with Coomassie brilliant blue G.

Proteins and amino acids. Protein concentration was determined by the microbiuret method with bovine serum albumin as the standard (11).

Nitrogenase components (MoFe and Fe proteins) were hydrolyzed with 6 N HCl for 24 h at 110°C. Amino acids were analyzed by Dan Omilianowski on a Durrum D-500 amino acid analyzer. Cysteine was determined as cysteic acid after oxidation with performic acid. Tryptophan was determined by the method of Opienska-Blauth et al. (23).

UV spectra. The UV spectra of nitrogenase components were determined with a Cary 14 recording spectrophotometer. The MoFe and Fe proteins were separated from UV-absorbing dithionite and dithiothreitol by precipitating them with 5 ml of 1% perchloric acid and sedimenting them by centrifugation. To record the spectrum, we suspended the pellet in 1 ml of 100 mM Tris hydrochloride (pH 8) and added 10 μl of 1 M NH_4OH solution.

Isoelectric point. Isoelectric focusing was performed by the methods of O'Farrell (21) and Wrigley (33). The electro-

phoresis of MoFe and Fe proteins with 2% [pH 3 to 10] ampholines on 3% acrylamide gel was performed at 350 V for 12 h followed by 800 V for 1 h. The proteins were visualized by staining with 0.1% Coomassie brilliant blue G in 50% trichloroacetic acid solution. The final pH gradient of the tube gel extended from pH 4.2 to 7.3 as measured with a pH meter on 5-mm slices of the gel.

Assays for metals and acid-labile sulfide. Mo (6), Fe (4), and acid-labile sulfide (14) were determined in both nitrogenase components by established methods after the proteins were desalted on Sephadex G-25.

Enzyme-linked immunoblotting. Crude extracts (2 or 10 μg of protein) or purified enzyme (0.1 μg of protein) were subjected to SDS-PAGE as described above. The proteins were electroblotted from the polyacrylamide gel onto a nitrocellulose filter for 3 h in Tris glycine buffer (pH 8.3; containing 20% [vol/vol] methanol, 25 mM Tris, and 192 mM glycine) with 250 mA of constant current. Then the filters were equilibrated for 1 h with the blotting buffer (20 mM Tris, 500 mM sodium chloride [pH 7.6], 5 g of skim milk per 100 ml). The filters were incubated with the antisera or preimmune sera in plastic bags overnight at room temperature with gentle agitation. After washing the filters in Tris sodium chloride buffer (pH 7.6) containing 0.05% Tween 20 for 10 min and then twice in Tris buffer without detergent, incubation with the second antiserum (goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate) was performed for 1 h. The filters were washed again as described above and finally stained for 15 to 45 min in 0.015% H_2O_2 in Tris sodium chloride buffer (pH 7.6) with 30 mg of horseradish peroxidase development reagent per 10 ml.

Chemicals. All chemicals and gases used were of analytical grade. Dithiothreitol and Tris base were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; phosphocreatine, ATP, ampholine, lysozyme, MES, DNase, RNase, bovine serum albumin, sodium dithionite, and creatine phosphokinase were obtained from Sigma Chemical Co., St. Louis, Mo.; acrylamide and *N,N'*-methylene bisacrylamide and the immunoblotting kit were purchased from Bio-Rad Laboratories, Richmond, Calif.; DEAE-52 cellulose was obtained from Whatman, Inc., Clifton, N.J.; Sephadex G-25 and polyethylene glycol 4000 were obtained from Pharmacia Inc., Piscataway, N.J.; and MOPS was purchased from United States Biochemical Corporation.

TABLE 2. Heterologous complementation of the nitrogenase components of *Azospirillum amazonense* Y1 with components of other species (nmol of C_2H_4 per min per mg)[a]

	Aa2	Av2	Kp2	Cp2	Rr1 + 2
Aa1	2,016	1,290	2,259	0	0
Av1	1,303				
Kp1	1,805				
Cp1	0				
Rr1 + 2	975				

[a] 1, MoFe protein; 2, Fe protein; Aa, *Azospirillum amazonense*; Av, *Azotobacter vinelandii*; Kp, *K. pneumoniae*; Cp, *C. pasteurianum*; Rr, *R. rubrum*; i, inactive.

RESULTS

Reconstitution of nitrogenase from purified components. A typical purification protocol is represented in Table 1. MoFe and Fe proteins were purified in a terminal step by preparative PAGE. The final preparations of MoFe and Fe proteins showed no residual activity when assayed alone for reduction of acetylene or for ATP hydrolysis. The maximum activities of Fe and MoFe proteins (1,800 and 2,400 nmol per min per mg, respectively) were obtained in the presence of optimal amounts of the other component. When nitrogenase was reconstituted by adding increasing amounts of MoFe or Fe protein to a given amount of Fe or MoFe protein, the activity per unit of Fe or MoFe protein increased, but with excess MoFe protein the specific activity of the Fe protein decreased. The optimal combination of the Fe and MoFe proteins for maximum nitrogen-fixing activity was at a molar ratio of approximately 1 Fe protein to 2 MoFe protein and 1 MoFe protein to 120 Fe protein, respectively.

Table 2 shows the interspecies reactivity of nitrogenase components (MoFe and Fe proteins) of *Azospirillum amazonense* with the nitrogenase components of *Azotobacter vinelandii*, *Klebsiella pneumoniae*, *Clostridium pasteurianum*, and *Rhodospirillum rubrum*. The nitrogenase components of *Azospirillum amazonense* had high reactivity with the nitrogenase components of *Azotobacter vinelandii*, *K. pneumoniae*, and *R. rubrum*, but they were not active combined with components from *C. pasteurianum*.

Comparison of different Fe proteins on SDS-PAGE. Figure 1 shows a comparison of purified Fe proteins separated by SDS-PAGE (with low cross-linker content) and stained with Coomassie blue. Two closely migrating bands could be
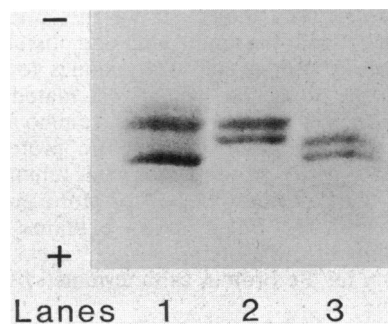
TABLE 1. Purification and yield of nitrogenase components from *Azospirillum amazonense*[a]

Nitrogenase component	Total protein (mg)	Total activity		Sp act (U/mg)	Purification (fold)
		U[b]	Yield (%)		
MoFe protein					
Crude extract	10,800	464,400	100	43	1
First DEAE-52	1,920	394,000	85	205	4.8
Second DEAE-52	672	338,000	73	503	11.7
PAGE	93	224,000	48	2,400	55.8
Fe protein					
Crude extract	10,800	464,400	100	43	1
First DEAE-52	1,044	193,000	42	185	4.3
Second DEAE-52	542	157,000	34	290	6.7
PAGE	66	119,000	25	1,800	41.9

[a] Data from 100 g of cell paste.
[b] One unit of activity is defined as the amount of nitrogenase enzyme required to cause the formation of 1 nmol of C_2H_4 per min from C_2H_2.

FIG. 1. SDS-PAGE (low cross-linker content) of purified Fe proteins (5 μg of protein) with Coomassie brilliant blue staining. Lanes: 1, *R. rubrum*; 2, *Azotobacter vinelandii*; 3, *Azospirillum amazonense*.
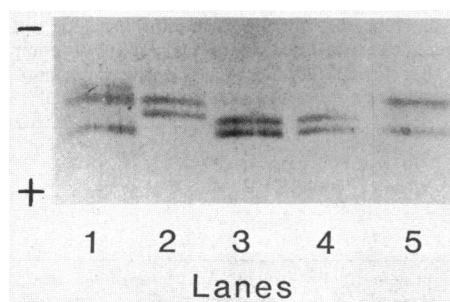
FIG. 2. Enzyme-linked immunoblotting for Fe protein. SDS-PAGE with low cross-linker content (0.58% C) was used. Lanes: 1, *R. rubrum* Fe protein (0.025 μg); 2, *Azotobacter vinelandii* Fe protein (0.2 μg); 3, *Azospirillum amazonense* Fe protein (0.2 μg); 4, *Azospirillum amazonense* extract (4 μg); 5, *Azospirillum brasilense* extract (4 μg).

demonstrated with active Fe proteins of *Azospirillum amazonense* and *Azotobacter vinelandii*, whereas the inactive modified Fe protein of *R. rubrum* clearly migrated differently. On SDS-PAGE with higher cross-linker content (Laemmli gels), the same results were obtained, but the separation was not as clear (data not shown).

In addition, purified Fe proteins and extracts of nitrogen-fixing cultures were evaluated with antiserum (provided by P. W. Ludden) against the Fe protein of *R. rubrum* by the enzyme-linked immunoblotting technique. Both Fe protein polypeptides of *Azospirillum amazonense* cross-reacted with the antiserum (Fig. 2, lane 3), and they were also present in highly active crude extracts of *Azospirillum amazonense* (lane 4). This demonstrated that their appearance cannot be attributed to an artifact of the purification process. The purified Fe protein of *Azotobacter vinelandii* exhibited the same type of double band as did *Azospirillum amazonense* (lane 4). *Azospirillum brasilense* crude extract and the purified Fe protein of *R. rubrum* (lanes 1 and 5) also showed two major bands, but these were much further separated than the bands of the *Azospirillum amazonense* Fe protein subunits.

Physical properties. The molecular weights of the nitrogenase components were determined by SDS-polyacrylamide slab gel electrophoresis with bovine serum albumin (65,400), alcohol dehydrogenase (40,000), and α-chymotrypsinogen (23,650) as standards. For MoFe protein a molecular weight of 210,000 daltons ([2 × 55,000] + [2 × 50,000]) and for Fe protein a molecular weight of 66,000 daltons (35,000 + 31,000) was calculated on the basis of assumed subunit composition.

The amino acid composition of nitrogenase proteins is shown in Table 3. The molecular weights of 204,000 and 67,000 calculated from the amino acid compositions of 1,852 residues for MoFe protein and 624 residues for Fe protein were close to the molecular weights calculated from SDS-PAGE data. The predominance of acidic amino acids (Glx + Asx = 347 residues [18.7%] for MoFe protein and 137 residues [21.8%] for Fe protein) over basic amino acids (Lys + Arg + His = 261 residues [14.1%] for MoFe protein and 73 residues [12.1%] for Fe protein) explains the acidic isoelectric points of the proteins, which were 5.2 for MoFe protein and 4.6 for Fe protein as determined by isoelectric focusing (Table 4).

The UV spectrum of MoFe protein showed two minor peaks in the 282- to 285- and 290-nm ranges (Fig. 3). The Fe protein showed only one peak in the 275- to 278-nm range; this peak is similar to those of the Fe proteins from

Azotobacter vinelandii and *C. pasteurianum* (9). No evidence was found for an adenyl group.

The purified MoFe protein had two atoms of Mo per molecule. Analysis for Fe indicated 24 Fe atoms per molecule of MoFe protein and 4 Fe atoms per molecule of Fe protein. The acid-labile sulfide level was 28 and 6 atoms per molecule of the MoFe and Fe proteins, respectively (Table 4).

The optimal pH for nitrogenase activity was in the range of 6 to 7.5 (Fig. 4). The optimal temperature was 35°C. The stabilities of the enzymes at different temperatures are shown in Fig. 5. Purified MoFe protein was stable for more than 30 days at 4°C when precautions were taken to keep the preparation anaerobic, whereas at room temperature the activity lasted only about 4 days. The less stable Fe protein lost its activity in 10 days at 4°C and in only 5 h at room temperature.

Tests for AEs. To extend the comparison of the nitrogenase systems of *Azospirillum brasilense* and *Azospirillum amazonense* further, we tried to find evidence for functioning of an activating enzyme (AE). In contrast to *Azospirillum brasilense* and *R. rubrum*, the time course of in vitro nitrogenase activity in an extract from *Azospirillum amazonense* was linear after a lag of 1 min. The extract was prepared from a nitrogen-fixing culture that had been supplied ammonium initially. The addition of AE of *R. rubrum* or manganese or both did not increase activity. No AE could be demonstrated in the buffer wash or 0.1 M NaCl wash fractions from the first DEAE-cellulose column used in the purification of nitrogenase from *Azospirillum amazonense*; tests were made on the inactive Fe protein from *R. rubrum*. Also, no AE activity was found in the 0.5 M NaCl wash of *Azospirillum amazonense* membranes. AE is found in these fractions from *R. rubrum* (29). Finally, the enzyme-linked immunoblotting technique with antiserum against purified activating enzyme of *R. rubrum* (kindly provided by L. L. Saari) was used in the search for AE. In *Azospirillum brasilense*, a cross-reacting protein migrated on SDS-PAGE like the AE of *R. rubrum*. No immunologically cross-

TABLE 3. Amino acid compositions of nitrogenase components from *Azospirillum amazonense*

Amino acid(s)	No. of residues per molecule of:	
	MoFe protein	Fe protein
Asx (Asp + Asn)	171	61
Thr	103	30
Ser	94	31
Glx (Glu + Gln)	176	76
Pro	82	19
Gly	187	68
Ala	198	79
Val	116	31
Cys[a]	10	4
Met	42	23
Ile	96	36
Leu	144	58
Tyr	73	24
Phe	85	11
Lys	108	29
His	54	13
Arg	99	31
Trp[b]	14	0

[a] Determined as cysteic acid.
[b] Determined by colorimetric method.

TABLE 4. Properties of nitrogenase components of *Azospirillum amazonense*

Component	Mol wt $(\times 10^{-3})$	Subunit mol wt $(\times 10^{-3})$	Mo (g-atom/mol)	Fe (g-atom/mol)	Acid-labile sulfide (g-atom/mol)	Iso-electric point	Sp act (nmol of C_2H_4/min per mg)
MoFe protein	210	$2 \times 55; 2 \times 50$	2	24	28	5.2	2,400
Fe protein	66	35; 31	0	4	6	4.6	1,800

reactive protein was found in extracts from nitrogen-fixing *Azospirillum amazonense* (data not shown).

DISCUSSION

The nitrogenase components of *Azospirillum amazonense* Y1 were purified to high specific activities (2,400 and 1,800 nmol of C_2H_4 formed per min per mg of protein for MoFe and Fe proteins, respectively). The nitrogenase of *Azospirillum amazonense* Y1 is a two-protein system, consisting of dinitrogenase (MoFe protein) with a molecular weight of 204,000 and dinitrogenase reductase (Fe protein) with a molecular weight of 67,000. Thus, the nitrogenase components of *Azospirillum amazonense* Y1 have molecular weights similar to those of the nitrogenases of other organisms (9). Unlike *R. rubrum* (17, 29) or *Azospirillum brasilense* (18, 24), no evidence for an inactive Fe protein species or an activating enzyme could be found in *Azospirillum amazonense*. Only weak, short-term inhibition of nitrogenase activity and no inhibition of in vitro nitrogenase activity by ammonium chloride has been found in *Azospiril-*

lum amazonense Y1 (A. Hartmann, H. Fu, and R. H. Burris, unpublished data). Apparently, *Azospirillum amazonense* does not possess the inactivating-activating enzyme system for Fe protein that regulates the Fe protein of *R. rubrum* and *Azospirillum brasilense*.

The nitrogenase of *Azospirillum amazonense* again exhibits the high degree of conservation characteristic of these enzymes. The amino acid compositions of the MoFe and Fe proteins of *Azospirillum amazonense* were compared with those of other nitrogenases (Table 5). The difference index, $\Sigma|\Delta D| \times 50$ (where D is the difference in the fractional contents of each amino acid between two proteins) (20), showed closest relatedness between *Azospirillum amazonense* and *Rhizobium lupini* and *R. japonicum*. The least related Fe protein was that of *R. rubrum*. The nitrogenase components of *Azospirillum amazonense* functioned readily with the components of *Azotobacter vinelandii*, *K. pneumoniae*, and *R. rubrum* but not with those of *C. pasteurianum* (Table 2). Thus, *Azospirillum amazonense* fits in the heterologous complementation pattern described by Emerich and Burris (10).

Since *Azospirillum amazonense* is acid tolerant, the pH profile of its nitrogenase is of interest. As reported for other nitrogenases, the optimal pH range was from pH 6.0 to 7.5. After short exposures of isolated nitrogenase to pH 5.0, about 50% activity was retained.

The MoFe protein was relatively stable at room temperature if oxygen was excluded, but the Fe protein was inactivated in a few hours. This higher lability of Fe protein is characteristic of Fe proteins of nitrogenases (26) and may reflect easily damaged Fe-S centers. The Fe and S content of the highly active Fe protein fits the pattern of Braaksma et al. (3) for correlating the Fe and S content of *Azotobacter vinelandii* Fe protein with activity.

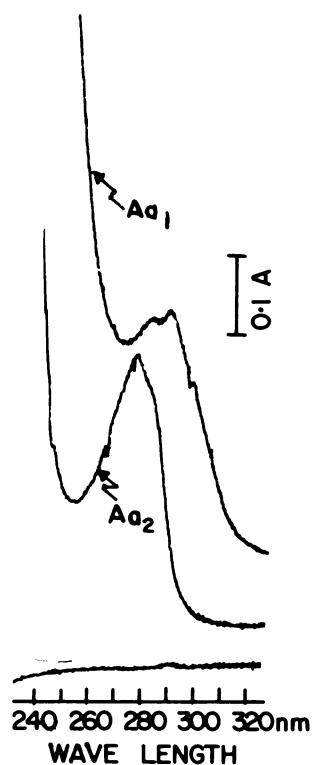The SDS-PAGE system used by Kanemoto and Ludden

FIG. 3. UV spectra of the MoFe (Aa1) and Fe (Aa2) proteins from *Azospirillum amazonense*. The spectra were recorded against a blank containing all components except the nitrogenase enzyme component. The buffer used was 1 ml of 100 mM Tris hydrochloride (pH 8) containing 10 μl of 1 M NH₄OH.
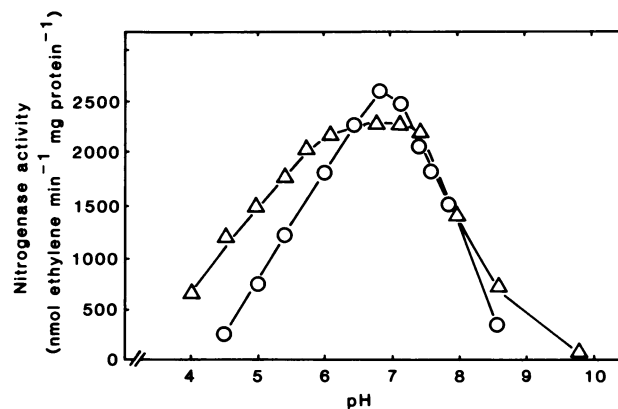

FIG. 4. pH profiles of activity of the purified nitrogenase enzyme from *Azospirillum amazonense*. Symbols: △, after 5 min of incubation; ○, after 30 min of incubation.
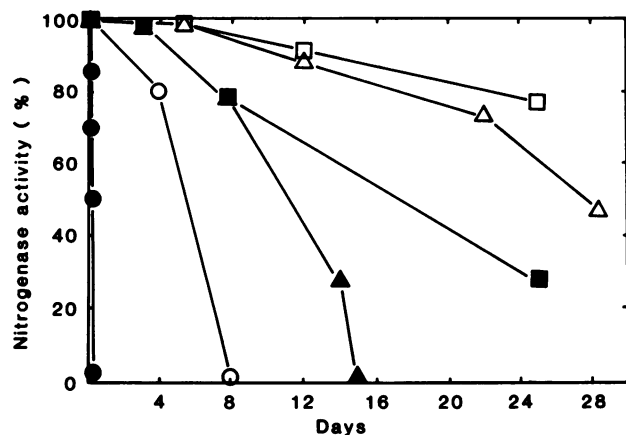
FIG. 5. Loss of activity of the MoFe (open symbols) and the Fe (closed symbols) proteins at different temperatures. The assays were performed with optimal molar ratios of MoFe and Fe proteins. Symbols: O and ●, 25°C; Δ and ▲, 4°C; □ and ■, -20°C.

(13) clearly revealed two electrophoretically distinguishable polypeptides of the Fe proteins of *Azospirillum amazonense* and *Azotobacter vinelandii*. The cross-reaction of proteins of these two bands with antiserum against Fe protein in the immunoblotting experiments identified them as Fe protein species. Because the crude extracts and the purified Fe protein of *Azospirillum amazonense* had very high nitrogenase activities, and because the slower migrating subunit did not comigrate with the modified Fe protein subunit from *R. rubrum* and *Azospirillum brasilense*, it appears that the Fe protein subunits do not represent inactivated species. There was no spectrophotometric evidence for a bound adenine residue in the purified Fe protein.

A separation of the Fe protein of *Azotobacter vinelandii* into two polypeptides on SDS-PAGE was reported by Klugkist et al. (15). The same effect was described for the Fe protein of *K. pneumoniae* (28). Since there is convincing evidence (e.g., DNA and protein sequence data) that the Fe protein subunits are identical, the different behaviors on SDS-PAGE could be caused by posttranscriptional alterations (processing) of the Fe protein subunit. In *nif* M mutants of *K. pneumoniae* (28), the slower-migrating subunit of Fe protein was diminished, indicating that this species might be a processed form. Alternatively, perhaps two different *nif* H genes from *Azospirillum amazonense* are present and are transcribed. Evidence for multiple, nonidentical copies of nitrogenase structural genes has been

found in *Azotobacter vinelandii* (2), *Anabaena* 7120 (27), and *Rhodopseudomonas capsulata* (30).

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TABLE 5. Comparison of the compositional relatedness[a] of the MoFe and Fe proteins of *Azospirillum amazonense* Y1 with other nitrogenases[b]

	Aa1	Rl2	Cp2	Kp2	Av2	Ac2	Rr2
Aa2		6.43	9.32	11.65	10.13	10.69	15.62
Rj1	5.39						
Rl1	6.29						
Cp1	10.27						
Kp1	8.35						
Ac1	9.93						
Av1	8.26						

[a] Determined by difference index ($\Sigma|\Delta D| \times 50$, where D is the difference in the fractional contents of each amino acid between two proteins).

[b] Ac, *Azotobacter chroococcaum*; Rl, *Rhizobium lupini*; Rj, *R. japonicum*; for other abbreviations, see the footnote to Table 2.

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