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Two Forms of Nitrogenase from the Photosynthetic Bacterium *Rhodospirillum rubrum*

ROBERT P. CARITHERS, DUANE C. YOCH,† AND DANIEL I. ARNON*
Department of Cell Physiology, University of California, Berkeley, California 94720

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Acetylene reduction by nitrogenase from *Rhodospirillum rubrum*, unlike that by other nitrogenases, was recently found by other investigators to require an activation of the iron protein of nitrogenase by an activating system comprising a chromatophore membrane component, adenosine 5'-triphosphate (ATP), and divalent metal ions. In an extension of this work, we observed that the same activating system was also required for nitrogenase-linked H_2 evolution. However, we found that, depending on their nitrogen nutrition regime, *R. rubrum* cells produced two forms of nitrogenase that differed in their Fe protein components. Cells whose nitrogen supply was totally exhausted before harvest yielded predominantly a form of nitrogenase (A) whose enzymatic activity was not governed by the activating system, whereas cells supplied up to harvest time with N_2 or glutamate yielded predominantly a form of nitrogenase (R) whose enzymatic activity was regulated by the activating system. An unexpected finding was the rapid (less than 10 min in some cases) intracellular conversion of nitrogenase A to nitrogenase R brought about by the addition to nitrogen-starved cells of glutamine, asparagine, or, particularly, ammonia. This finding suggests that mechanisms other than de novo protein synthesis were involved in the conversion of nitrogenase A to the R form. The molecular weights of the Fe protein and Mo-Fe protein components from nitrogenases A and R were the same. However, nitrogenase A appeared to be larger in size, because it had more Fe protein units per Mo-Fe protein than did nitrogenase R. A distinguishing property of the Fe protein from nitrogenase R was its ATP requirement. When combined with the Mo-Fe protein (from either nitrogenase A or nitrogenase R), the R form of Fe protein required a lower ATP concentration but bound or utilized more ATP molecules during acetylene reduction than did the A form of Fe protein. No differences between the Fe proteins from the two forms of nitrogenase were found in the electron paramagnetic resonance spectrum, midpoint oxidation-reduction potential, or sensitivity to iron chelators.

Investigations of the components of the nitrogenase (N_2ase) enzyme systems from *Clostridium pasteurianum*, *Azotobacter vinelandii*, and *Klebsiella pneumoniae* have shown that two and only two components are required for the enzymatic activity of N_2ase, namely, the iron protein and the molybdenum-iron protein (MoFe protein) (24, 27). Reports of a possible third component needed for N_2ase activity lack confirmation (9, 11, 20).

Recent studies (10, 14; S. Nordlund and H. Baltscheffsky, Int. Cong. Biochem. Abstr. **9**:240, 1973) have indicated that the enzymatic activity of N_2ase isolated from the photosynthetic bacterium *Rhodospirillum rubrum* has special requirements not observed in N_2ase isolated from any of the several heterotrophs just mentioned. The isolated *R. rubrum* N_2ase had little or no enzymatic activity unless its Fe protein component was activated (before reconstitution with the MoFe protein) by an activating system comprising a chromatophore membrane-bound activation factor, ATP, and divalent metal cations (10, 14).

Our earlier work on the differential effectiveness of the two soluble *R. rubrum* ferredoxins (26) as electron carriers for N_2ase activity (25) gave no indication that N_2ase isolated from *R. rubrum* cells required the recently described activating system (10, 14). Our current investigation was undertaken to resolve this discrepancy. We have now found that N_2ase isolated from *R. rubrum* cells can exist in two forms. One form, isolated from *R. rubrum* grown in a me-

† Present address: Department of Biology, University of South Carolina, Columbia, SC 29208.

dium in which the supply of nitrogen (in any form) had been exhausted before harvest of the cells, showed no dependence on an activating system. The other form of N_2ase, isolated from cells grown in a medium supplied with glutamate or N_2 as the nitrogen source, did show a dependence of enzymatic activity on an activating system. The physical and chemical differences between the two forms of N_2ase are described.

A brief account of this work has appeared (D. C. Yoch, R. P. Carithers, and D. I. Arnon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, K23, p. 130).

MATERIALS AND METHODS

Cell culture and cell extracts. *R. rubrum* S-1 was grown photosynthetically at pH 6.8 in a nutrient solution described by Arnon et al. (2), supplemented with 20 mM malate and biotin (15). The cells were grown in 14-liter Pyrex carboys filled completely to exclude air. A constant ambient temperature of about 20°C was maintained, and illumination was provided as described elsewhere (2).

Comparisons were made between the properties of N_2ases isolated from cells grown under several different conditions of nitrogen supply: (i) nitrogen starvation, after depletion of ammonium chloride (2 mM) that was initially included in the nutrient medium; (ii) nitrogen supplied as glutamate (5 mM); and (iii) nitrogen supplied as N_2 by sparging the nutrient medium with a CO_2/N_2 gas mixture (5:95, vol/vol).

N_2ase extracts were prepared from *R. rubrum* cells harvested in a refrigerated Sharples centrifuge and suspended in 1.5 volumes of chilled, argon-saturated, 0.3 M Tricine buffer (pH 8.8) containing 2 mM sodium dithionite and 1 mg of DNase. The cells were disrupted in a Ribi cell fractionator under a stream of argon or N_2 gas and centrifuged at 50,000 × *g* for 10 min in capped centrifuge tubes that had been preflushed with argon. The residue was discarded, and the supernatant solution was further fractionated by centrifugation at 250,000 × *g* for 90 min. The supernatant fraction contained the soluble N_2ase and the pellet contained the chromatophores.

Preparation of activating factor and N_2ase components. The chromatophore fraction was suspended anaerobically in 20 mM Tricine buffer (pH 8.0) containing 4 mM dithionite, and it was used for the preparation of the activating factor, as described by Ludden and Burris (10). Specifically, the activating factor was solubilized under anaerobic conditions by treatment of the chromatophores with 0.5 M NaCl followed by centrifugation at 250,000 × *g* for 1 h. Solid polyethylene glycol was added to the supernatant fluid, and the material that precipitated between 10 and 35% polyethylene glycol contained the bulk of the activating factor activity. This material was suspended anaerobically in 20 mM Tricine buffer (pH 8.0) and used as the source of crude activating factor. In several experiments, chromatophores were used directly as the source of activating factor.

The MoFe protein and Fe protein components of N_2ase were prepared by fractionation of the supernatant fluid from the 250,000 × *g* centrifugation of DEAE-cellulose columns, as described by Ludden and Burris (10). After the N_2ase was adsorbed on DEAE-cellulose, the column was washed with 50 mM triethanolamine buffer (pH 7.5) containing 4 mM dithionite to remove loosely bound contaminants. MoFe protein was eluted from the DEAE-cellulose column with 50 mM triethanolamine buffer (pH 7.5), containing 130 mM malate and 4 mM dithionite. As the MoFe protein was being eluted from the chromatography column with 130 mM malate, the Fe protein moved slowly down the column as a brown band. Before the Fe protein was finally eluted from the column with 50 mM triethanolamine buffer containing 450 mM NaCl, the DEAE-cellulose layer that was above the Fe protein band was removed to avoid unintended elution of adsorbed *R. rubrum* ferredoxins along with the Fe protein component of N_2ase. All manipulations of N_2ase and its components were performed anaerobically under an argon atmosphere.

N_2ase assays. N_2ase activity was assayed by acetylene reduction. Activity in whole cells was measured by transferring (with a gastight syringe) a 1.5-ml sample of the cell culture to a 5-ml Fernbach flask containing, in the gas phase, 86.5% argon and 13.5% acetylene. The flasks were shaken in a water bath (30°C) while they were being illuminated with incandescent lamps. The ethylene produced was measured by gas chromatography, using a 10-m alumina column operated at 160°C and equipped with a flame ionization detector.

N_2ase activity in cell-free extracts was also assayed in 5-ml Fernbach flasks with the same gas phase as that used for whole cells. The liquid phase (1.5 ml) contained 33.3 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.4), 25 mM phosphocreatine, 30 μg of creatine phosphokinase, 13.3 mM dithionite, 5 mM ATP, and the N_2ase extract. In addition, the assay flask contained either 5 mM Mg^{2+}, for assays under "nonactivating" conditions, or 15 mM Mg^{2+} plus 0.5 mM Mn^{2+} and activating factor, for assays under "activating" conditions.

Hydrogen evolution by N_2ase was measured when the gas phase consisted either of the argon/acetylene gas mixture or of argon alone. A 4-m chromatography column filled with molecular sieve 5A (Varian Associates) separated the gases; N_2 was the carrier gas; a thermal conductivity detector was used for measurement of hydrogen gas.

Other procedures. All gel chromatography procedures were performed with argon-saturated buffers and in an argon atmosphere. For the gel chromatography experiments represented by Fig. 8 and 9, N_2ase was concentrated by adsorption of the crude N_2ase fraction on a bed of DEAE-cellulose (equilibrated with 50 mM triethanolamine, pH 7.5, plus 4 mM hydrosulfite) and elution of the N_2ase with the same buffer containing 450 mM NaCl. The molecular weights of the Fe protein and MoFe protein components were estimated by the gel filtration method of Andrews (1). The reaction of Fe protein with α,α'-dipyridyl was performed as described by Walker and Mortenson (23) for the Fe protein of *C. pasteurianum*.

Total protein was determined by a modification of the Folin phenol method (17).

Potentiometric titrations of Fe protein were performed with an Aminco DW-2 spectrophotometer, using the anaerobic cuvette system described by Dutton (6), which made possible simultaneous measurement of absorbance changes and oxidation-reduction potential changes. The latter were measured with a calibrated platinum-Ag/AgCl electrode (Metrohm AG-911) and Metrohm pH meter (model 102). The oxidation-reduction mediator dyes used during the titrations are described in the legend to Fig. 7. The protein was oxidatively titrated with 50 mM potassium ferricyanide under an argon atmosphere.

The samples for electron paramagnetic resonance (EPR) analysis (see Discussion) were prepared in stoppered argon-flushed tubes and transferred anaerobically with a gastight, long-needle syringe to quartz EPR tubes (preflushed with argon) and then frozen to 77°K. The EPR spectrum was recorded with a Bruker Instruments EPR spectrometer equipped with a 20-cm ("8-inch") magnet (model ER 200tt) and provided with an Oxford Instruments liquid helium cooling system.

RESULTS

Influence of nitrogen nutrition on the formation of two forms of *R. rubrum* N_2ase. The first indication of different forms of *R. rubrum* N_2ase came from a comparison of the cation requirements for activity of the enzymes isolated from cells that differed in nitrogen nutrition but were assayed alike, with unfractionated chromatophores serving as the source of activating factor. N_2ase isolated from nitrogen-starved cells showed optimal activity at an Mg^{2+} concentration between 5 and 10 mM; added Mn^{2+} had no effect (Fig. 1a). By contrast, N_2ase isolated from cells grown on glutamate (Fig. 1b) or N_2 (Fig. 1c) required for maximum activity not only higher Mg^{2+} concentrations (about 15 mM) but also the addition of Mn^{2+}.

Further evidence that N_2ase isolated from nitrogen-starved cells was different from N_2ase isolated from glutamate-grown cells came from a comparison of the kinetic behaviors of the two enzymes. The activity of N_2ase from nitrogen-starved cells was linear with time and did not depend on the presence of activating factor (Fig. 2, A). However, the activity of N_2ase from glutamate-grown cells showed a strong dependence on activating factors (Fig. 2, compare C and D); when assayed under activating conditions (see Materials and Methods), the rate of N_2ase activity increased with time (Fig. 2, C). After preincubation under activating conditions, the activity of the enzyme was linear with time (Fig. 2, B) and comparable to that of N_2ase from nitrogen-starved cells (Fig. 2, A).

It appeared, therefore, that, depending on their previous nitrogen nutrition, *R. rubrum* cells produced two kinds of N_2ase. One, to be referred to as the active form of N_2ase (N_2ase A), was produced by nitrogen-starved cells, and, like the well-characterized N_2ase from heterotrophic bacteria, it had no need of activating factor for maximum activity. The other form of N_2ase had considerably less and sometimes no activity at all when isolated from the cells; its activity was regulated by incubation with an activating factor, Mn^{2+}, high concentrations of Mg^{2+}, and ATP. This form of N_2ase was produced by cells grown with glutamate or N_2 as the source of nitrogen; it will be referred to here

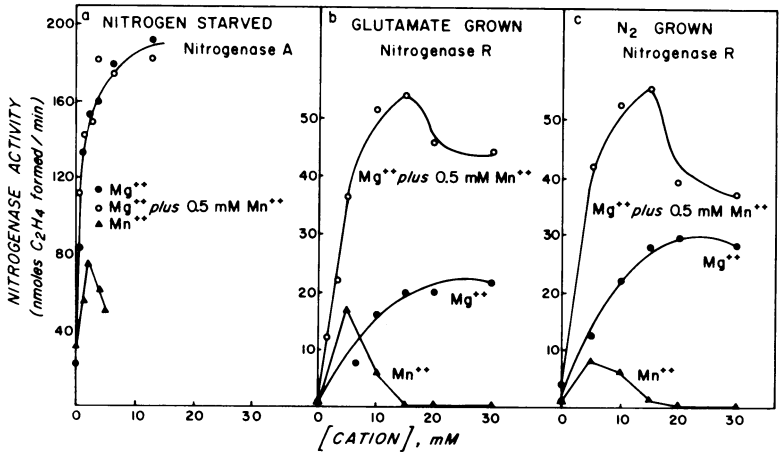

FIG. 1. *Cation requirements of N_2ases isolated from cells cultured under different conditions of nitrogen supply: (a) nitrogen starvation, (b) nitrogen supplied as glutamate, (c) nitrogen supplied as N_2. N_2ase activities were assayed for 20 min as described in the text except that variations in cation concentration were as indicated. Activating factor was supplied by the addition of chromatophores (0.07 mg of bacteriochlorophyll) to extracts from glutamate-grown and N_2-grown cells. Protein concentrations of the N_2ase extracts were: nitrogen starved, 3.6 mg; glutamate grown, 3.1 mg; N_2 grown, 2.8 mg.*

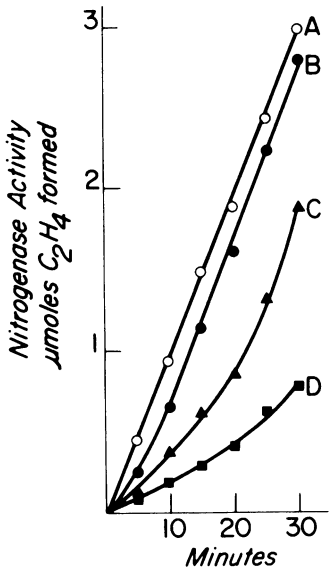

FIG. 2. *Effect of cations and preincubation on the time course of N_2ase activity of crude extracts from nitrogen-starved and glutamate-supplied cells. (A) Crude extract (3.7 mg protein) from nitrogen-starved cells assayed under nonactivating conditions (see text); (B) reaction mixture containing crude extract (3.7 mg protein) from glutamate-grown cells, 15 mM Mg^{2+}, and 0.5 mM Mn^{2+} preincubated for 15 min before acetylene was added at time zero (activating factor contained in chromatophores was included in the crude extract); (C) same as (B) except that the preincubation step was omitted; (D) same as (B) except that preincubation and Mn^{2+} were omitted and the concentration of Mg^{2+} was 5 mM.*

as the regulatory form of N_2ase (N_2ase R).

A detailed analysis of the influence of the nitrogen nutrition of cells on the form of N_2ase produced is given in Table 1. Again, N_2ase A was the predominant form of N_2ase in extracts from cells that were nitrogen starved, i.e., in cells from cultures in which the nitrogen supply was totally exhausted before harvest (treatments 1 and 2); N_2ase R was the predominant form in extracts from cells grown with N_2 or glutamate or in nitrogen-starved cells that were supplied with glutamate 24 h before harvest (treatments 3, 4, and 5). Aspartate (data not shown) had the same effect as added glutamate.

A very rapid formation of the N_2ase R (at the expense of the A form) was brought about by the addition of certain nitrogen compounds, specifically, glutamine, asparagine (data not shown), or, especially, ammonia, to nitrogen-starved cells. The effects of such a "shock" treatment of nitrogen-starved cells by the addition of glutamine or ammonia are shown in Table 1 (treatment 6). The first detectable effect of the

TABLE 1. *Influence of nitrogen nutrition of R. rubrum cells on the form of N_2ase produced*

Treatment	Nitrogen nutrition of cells	N_2ase activity (C_2H_4 formed, nmol/min per mg of protein)	
		Nonactivating conditions	Activating conditions[a]
1	Nitrogen starved	15.8	16.1
2	Glutamate grown, followed by 50 h of nitrogen starvation	30.6	25.4
3	Glutamate grown[b]	6.5	36.9
4	N_2 as nitrogen source	14.6	43.5
5	Glutamate fed[c]	0.0	25.1
6	Shock of nitrogen-starved cells		
	Glutamine, 30 min[d]	6.1	34.7
	Ammonia, 30 min[e]	2.6	8.9
	Ammonia, 10 min[f]	2.7	6.7

[a] Assayed with 15 mM Mg^{2+}, 0.5 mM Mn^{2+}, and activating factor.

[b] Cells grown on 1.5 mM sodium glutamate.

[c] Nitrogen-starved cultures fed 0.75 mM sodium glutamate and harvested about 24 h later.

[d] Nitrogen-starved cells shocked by the addition of approximately 5 mM glutamine 30 min before harvest.

[e] Nitrogen-starved cells shocked by the addition of 2.0 mM NH_4Cl 30 min before harvest.

[f] Nitrogen-starved cells shocked by the addition of 2.0 mM HN_4Cl 10 min before harvest.

ammonia shock was the apparent loss of N_2ase activity in the intact cells. When the cells were broken and tested for N_2ase activity, it was discovered that the ammonia shock had converted the A form of N_2ase, normally found in nitrogen-starved cells, to the N_2ase R form within about 10 min (Table 1), a time span too short to permit de novo protein synthesis (see Discussion).

In contrast to the rapid conversion of N_2ase A to N_2ase R when ammonia, glutamine, or asparagine was added to nitrogen-starved cells, there was a much slower conversion brought about by the addition of glutamate. In cell-free extracts, the N_2ase R form did not begin to predominate until about 8 h after the addition of glutamate (Fig. 3).

It is to be noted that the conversion of the N_2ase A form into the N_2ase R form could be detected only when the N_2ase was isolated from the cells. Unlike the rapid effect of ammonia shock, which stopped N_2ase activity in whole cells, the addition of glutamate was not followed by a cessation of N_2ase activity in whole cells (Fig. 3, top curve; also unpublished observations of vigorous hydrogen evolution by the culture). Nevertheless, cell-free assays of N_2ase activity

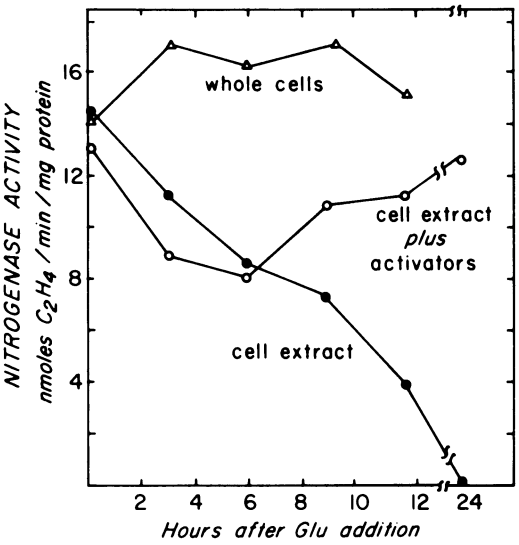

FIG. 3. *Effect of added glutamate on N_2ase activity of nitrogen-starved cells. At time zero, sodium glutamate (0.77 mM final concentration) was added to each of six 14-liter nitrogen-starved cultures. At the time indicated, a sample from one of the cultures was assayed for N_2ase activity in whole cells, the remaining cells were harvested, and a crude extract was prepared (see text). Symbols: △, whole-cell activity; ●, assays under nonactivating conditions; ○, assays under activating conditions, i.e., when the reaction mixture contained 15 mM Mg^{2+} and 0.5 mM Mn^{2+}. Activating factor was provided by chromatophores contained in the crude extract.*

showed that a conversion of N_2ase A to N_2ase R had occurred after the addition of glutamate: the activity of isolated N_2ase was enhanced by activating conditions. For reasons not yet understood, in intact cells treated with glutamate, N_2ase R appeared to be in an activated form, whereas the isolated N_2ase R was not.

Although an ammonia shock was the most rapid method for obtaining the R form of N_2ase, operationally our most frequent procedure for producing this form of N_2ase involved supplying nitrogen-starved cells with glutamate for 24 h before cell harvest. This procedure gave high yields of N_2ase R free from remaining N_2ase A.

Activation of H_2 evolution in N_2ase R. The difference between the A and R forms of N_2ase was not limited to the reduction of acetylene. The pattern of H_2 evolution paralleled that of acetylene reduction (Table 2). The activation of N_2ase R by cations and activating factor that was required for acetylene reduction was also required for H_2 evolution.

Once N_2ase R was activated by cations and activating factor, it behaved like N_2ase A with respect to inhibition of H_2 evolution by acetylene and to inhibition of acetylene reduction by carbon monoxide (Table 2).

TABLE 2. *Activation of H_2 evolution by R. rubrum N_2ase*

N_2ase type	Gas treatment	H_2 evolved (nmol/min)	C_2H_4 formed (nmol/min)
A	Ar	116.9	
	Ar + CO	144.2	
	Ar + C_2H_2	10.8	215.5
	Ar + C_2H_2 + CO	109.5	2.4
R[a]	Ar	7.7	
	Ar + CO	7.7	
	Ar + C_2H_2	0.0	0.8
	Ar + C_2H_2 + CO	5.6	0.0
R[b]	Ar	90.6	
	Ar + CO	113.1	
	Ar + C_2H_2	22.7	149.2
	Ar + C_2H_2 + CO	110.0	9.0

[a] Assayed under nonactivating conditions (see text).
[b] Assayed under activating conditions (see text).

Some properties of the *R. rubrum* Fe protein component of N_2ase. The unique properties of the Fe protein component of *R. rubrum* N_2ase fall into two categories: first, there are the properties that distinguish the Fe protein of N_2ase A from that of N_2ase R; second, there are the properties of the *R. rubrum* Fe protein that differ from those of other N_2ases.

The most striking property of the Fe protein from N_2ase R is its requirement for activation before it is able to support N_2ase activity. Although Ludden and Burris (10) and Nordlund et al. (14) found that the Fe protein could be activated in the absence of MoFe protein, we sought other evidence to this point by combining Fe protein from N_2ase R with FeMo protein from N_2ase A and, conversely, Fe protein from N_2ase A with FeMo protein from N_2ase R. Activating conditions were stimulatory only when the cross-combined N_2ase complex contained Fe protein from N_2ase R; otherwise, activating conditions were actually inhibitory (Fig. 4). These findings support the view (10, 14) that activation is a unique property of the Fe protein; the MoFe protein, whether from N_2ase A or N_2ase R, does not determine whether the N_2ase is of the A or the R form.

Another difference between the Fe proteins from N_2ase R and N_2ase A is the utilization of ATP during enzyme turnover. The utilization of ATP is considered to pertain specifically to the Fe protein, as other investigators have shown that ATP binding occurs on this component of N_2ase (24, 27). Figure 5 shows a comparison of the ATP requirements of N_2ase A and N_2ase R in which N_2ase activity (derived from the linear

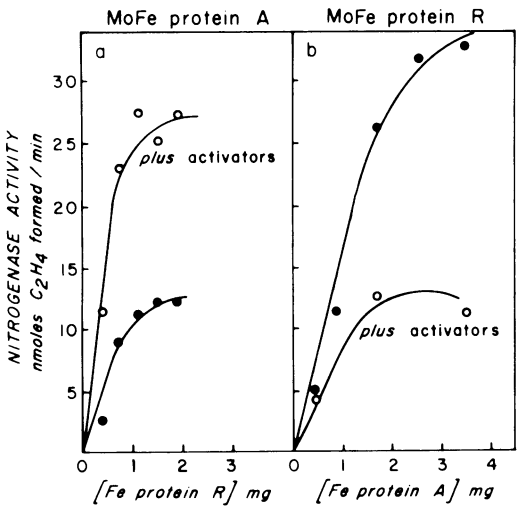

FIG. 4. *Response to activators by N_2ase reconstituted by cross-combinations of Fe proteins and MoFe proteins derived from N_2ase A and N_2ase R. The concentration of MoFe protein was 0.88 mg in all assays; the concentration of Fe protein was varied as indicated. (a) N_2ase reconstituted with Fe protein from the R form of the enzyme and with MoFe protein from the A form; (b) N_2ase reconstituted with Fe protein from the A form of the enzyme and with MoFe protein from the R form. ("Plus activators" corresponds to activating conditions as described in the text.)*

portion of the time course after N_2ase R was fully activated) was recorded as a function of ATP concentration. The ATP concentration was maintained at the specified levels by "buffering" the initial addition of ATP with phosphocreatine and phosphocreatine kinase, which regenerated ATP from the newly formed ADP.

In the presence of Mn^{2+} and activating factor, N_2ase R from glutamate-grown cells had an ATP optimum for enzyme turnover of between 1 and 2 mM; this response to ATP appeared to be sigmoidal. On the other hand, N_2ase A from nitrogen-starved cells required about 5 mM ATP for maximum activity (Fig. 5). Higher levels of ATP (not shown here) were inhibitory in both cases. A Hill plot (Fig. 5, inset) showed the binding of ATP to N_2ase R from glutamate-grown cells to be highly cooperative ($n \simeq 8$). By contrast, the Hill plot for N_2ase A from nitrogen-starved cells gave a value ($n = 1.4$) that indicated a much lower degree of cooperativity. Apparently, more ATP molecules are bound or utilized during a rate-limited step in N_2ase R than in N_2ase A.

The *R. rubrum* Fe proteins differ in several respects from the more thoroughly studied Fe proteins from other organisms. The Fe protein of *C. pasteurianum* has been shown to be relatively stable in the presence of the (ferrous) iron chelator α,α'-dipyridyl (23). However, in the presence of Mg · ATP, α,α'-dipyridyl rapidly attacked the iron-sulfur center of the clostridial Fe protein (23). We tested the reactivity of α,α'-dipyridyl with respect to *R. rubrum* Fe proteins from the R and A forms of N_2ase. The formation of the iron-chelator complex, monitored by absorbance changes at 520 nm, was different from that of the clostridial protein. The iron atoms of Fe proteins from both N_2ase A and N_2ase R of *R. rubrum* were rapidly complexed by α,α'-dipyridyl, but in neither case was the reaction influenced by addition of Mg · ATP (Fig. 6).

Another unusual feature of the *R. rubrum* Fe

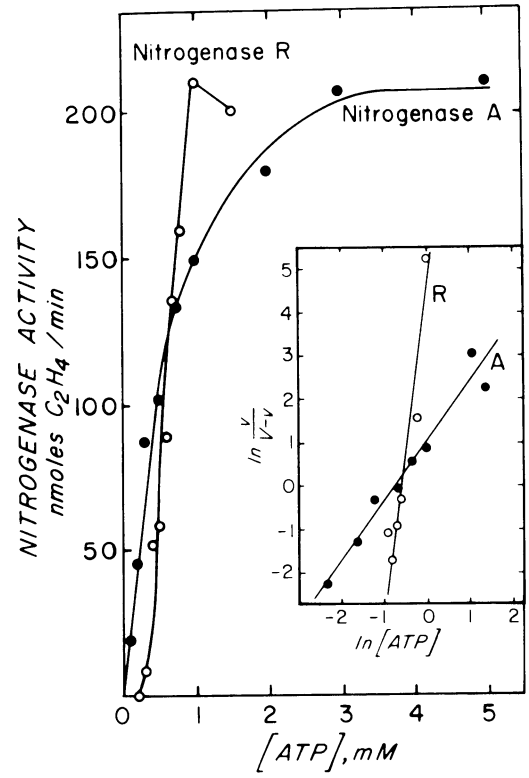

FIG. 5. *Dependence of rate of acetylene reduction by A and R forms of N_2ase on the concentration of ATP. The reaction mixture included the indicated initial amount of ATP, an ATP-regenerating system (see text), either 5 mM Mg^{2+} (N_2ase A) or 15 mM Mg^{2+} (N_2ase R) and, in the case of N_2ase R, also Mn^{2+} (0.5 mM) and activating factor (approximately 1 mg of protein). The rate of N_2ase R activity was recorded only after the rates of acetylene reduction became linear with time. (Inset) Hill plot of ln ATP concentration versus ln $[v/(V_{max} - v)]$ of the A and R forms of N_2ase.*

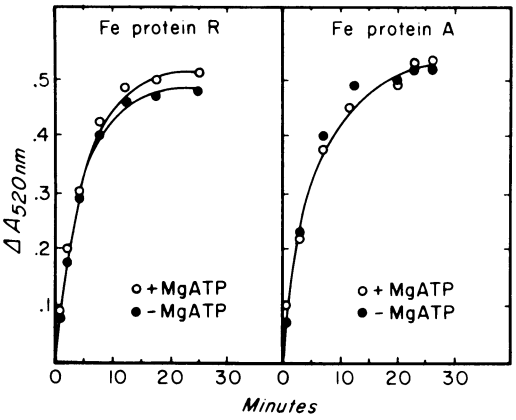

FIG. 6. *Reaction of Fe protein with α,α'-dipyridyl. R-type Fe protein (0.97 mg protein) or A-type Fe protein (1.0 mg of protein) was added anaerobically to a degassed, sealed cuvette containing 60 mM Tris-hydrochloride buffer (pH 8.0) and, where indicated, 2.0 mM each of Mg^{2+} and ATP. Fe protein was omitted in the reference cuvettes. The reaction was started by the anaerobic addition of 0.5 ml of 25 mM α,α'-dipyridyl (final concentration, 5 mM) to both the sample and the reference cuvettes. Absorbance at 520 nm (A_{520nm}; 1-cm light path) was measured in a Cary 14 spectrophotometer.*

proteins was the oxidation-reduction properties of their iron-sulfur centers. Oxidative titrations of the reduced *R. rubrum* Fe protein were performed under strictly anaerobic conditions with potassium ferricyanide as the oxidant; oxidation of the iron-sulfur centers was monitored by optical absorbance increases at 420 nm. The iron-sulfur cluster of the Fe protein from N_2ase A titrated indistinguishably from that of unactivated N_2ase R (Fig. 7). The iron-sulfur center of the Fe protein from N_2ase R showed a single $n = 1$ transition with a midpoint potential of about −480 mV, both in the presence and in the absence of Mg · ATP (Fig. 7). Our findings with the Fe proteins from *R. rubrum* are in contrast with studies of the Fe protein of *C. pasteurianum* (28), in which the addition of Mg · ATP shifted the midpoint potential from about −300 to −400 mV.

Molecular weights and association of N_2ase components. The question arose whether the differences between the A and R forms of N_2ase in *R. rubrum* discussed so far were accompanied by differences in such physical properties as molecular size. Differences in molecular size of the N_2ase complex could arise either because one or both constituent proteins differed in molecular weight or because they associated with each other in different proportions. To answer this question, N_2ase A and N_2ase R were isolated from the respective cultures, mixed, and then chromatographed on gel filtration columns (Fig. 8). The first gel filtration was performed in the absence of NaCl, i.e., under

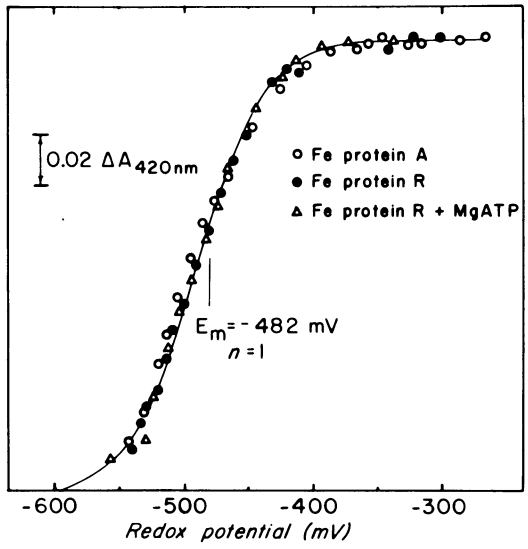

FIG. 7. *Oxidative titrations of Fe protein with potassium ferricyanide. The titration mixture contained (in 5.0 ml) 200 mM Tricine buffer (pH 8.8) and the following oxidation-reduction mediators (each 10 μM): 2-anthraquinone-sulfonate, 2-hydroxy-1,4-naphthaquinone, benzyl viologen, and methyl viologen. Symbols: ○, Fe protein from N_2ase A (4.1 mg of protein); ●, Fe protein from N_2ase R (4.0 mg of protein); △, Fe protein from N_2ase R plus 10 mM each Mg^{2+} and ATP. A_{420nm}, Absorbance at 420 nm; E_m, midpoint potential.*

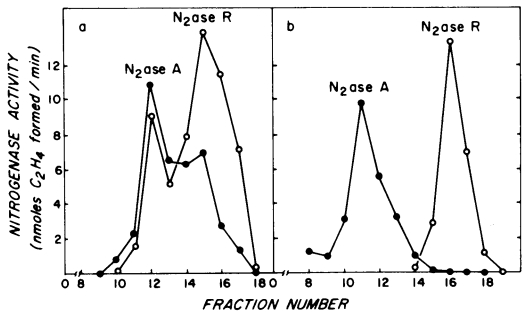

FIG. 8. *Separation of N_2ase A and N_2ase R by gel filtration. Equal volumes of N_2ases isolated from glutamate-fed and nitrogen-starved cells were mixed together and applied anaerobically to chromatography columns (3 by 35 cm) of Bio-Gel A 1.5m (a) or Sephacryl S-200 (b) preequilibrated and then eluted with argon-saturated 50 mM triethanolamine buffer (pH 7.5) containing 4 mM sodium dithionite. N_2ase fractions were collected anaerobically and assayed under nonactivating (●) or activating (○) conditions.*

conditions that minimized the dissociation of N_2ase into its component proteins. Elution fractions containing N_2ase R were identified by the stimulation of N_2ase activity under activating conditions, as compared with the activity measured under nonactivating conditions (see Materials and Methods). Fractions that were enzymatically active without added activating factor were classified as containing N_2ase A.

The elution profile from a Bio-Gel A 1.5-m column (Fig. 8a) showed two distinct, but incompletely resolved, peaks. N_2ase A eluted first and therefore appeared to have a higher molecular weight than N_2ase R. A better separation of the two forms was obtained by chromatography with Sephacryl S-200 (Fig. 8b). Again, the indication was that N_2ase A, which eluted first, was of a larger molecular size than N_2ase R. These findings indicate that the conversion of N_2ase A into N_2ase R (e.g., during ammonia shock) resulted not only in a modification of the Fe protein component (10, 14) but also in a decrease in the effective size of the entire N_2ase complex.

The A and R forms of N_2ase were next chromatographed on Sephacryl S-200 in the presence of 200 mM NaCl, i.e., under conditions that favored the dissociation of the enzyme into its component proteins and made possible the determination of their molecular weights. The MoFe protein from the two forms of N_2ase eluted as a single symmetrical peak, which was followed by a single Fe protein peak (Fig. 9). It was noted, however, that the single Fe protein peak included two kinds of Fe protein that had the same elution volume but were distinguishable on the basis of enzymatic activity. The Fe protein derived from N_2ase R supported high N_2ase activity (when combined with MoFe protein) only under activating conditions, whereas the Fe protein derived from N_2ase A required no activation (Fig. 9). Because only one species of MoFe protein was detected and because both species of Fe protein cochromatographed, it appeared that the molecular weights of both the MoFe and Fe proteins were the same in N_2ase R and N_2ase A.

The molecular weights of the Fe protein and the MoFe protein were estimated by elution of the N_2ase component proteins from a Sephacryl S-200 column calibrated with proteins of known molecular weight. This method gave molecular weights of approximately 60,000 and 200,000 for the Fe and MoFe proteins, respectively (Fig. 10). These values are similar to those of the Fe and MoFe proteins of N_2ases from other organisms (16, 24, 27).

When no difference was found between the molecular weights of the component MoFe and

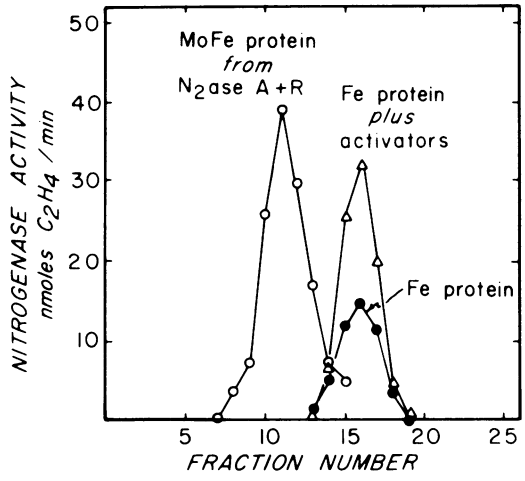

FIG. 9. *Chromatographic separation in Fe protein and MoFe protein from the A and R forms of N_2ase. A mixture of N_2ases from glutamate-fed and nitrogen-starved cells was applied anaerobically to a Sephacryl S-200 column (3 by 38 cm) that was equilibrated and eluted with argon-saturated 50 mM triethanolamine buffer (pH 7.5) containing 200 mM NaCl and 4 mM dithionite (flow rate, 15 ml/h). Samples (4 ml) were collected under a stream of argon. The MoFe protein components (○) were assayed by adding to the reaction mixture Fe protein (0.15 mg of protein) from N_2ase A. The Fe protein constituent of N_2ase was assayed with a fixed amount of MoFe protein (0.17 mg of protein) either under nonactivating (●) or under activating (△) conditions.*

Fe proteins, it became clear that the larger size of N_2ase A (Fig. 8) probably resulted from different proportions in which the component proteins associated in N_2ase A, as compared with their association in N_2ase R. The proportions or ratios in which the two component proteins need to be associated for optimal enzymatic activity are still not known with certainty for N_2ases from other organisms, despite much work in this area. Ratios of Fe protein to MoFe protein of 1:1 (7, 8, 19) and 2:1 (3, 12, 21, 22) have been reported.

Evidence that the larger size of the *R. rubrum* N_2ase A (Fig. 8) resulted from more Fe protein components than are present in N_2ase R was sought by purifying the component proteins and determining the relative proportions in which they recombined to restore maximal N_2ase activity. Curves of enzyme activity were obtained by titrations of a fixed amount of MoFe protein with variable amounts of Fe protein derived from either N_2ase R or N_2ase A.

Figure 11a shows two such titrations in which N_2ase activities were assayed under nonactivating conditions. The reconstituted N_2ase formed

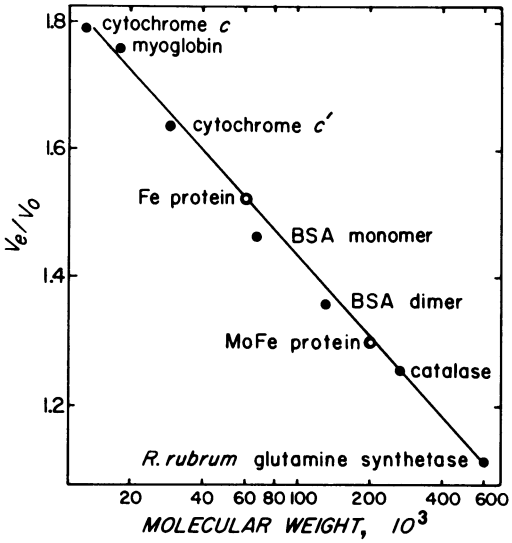

FIG. 10. *Estimation by gel filtration of the molecular weights of Fe protein and MoFe protein from the A and R forms of N_2ase. The column (described in the legend to Fig. 9) was calibrated with the following protein markers of known molecular weight: horse heart cytochrome c, 12,400; myoglobin, 17,800; R. rubrum cytochrome cc', 29,800; bovine serum albumin (BSA) monomer, 67,000; BSA dimer, 135,000; catalase, 240,000; R. rubrum glutamine synthetase, 600,000. The V_e/V_o ratios of Fe and MoFe proteins represent the averages of several chromatography experiments, as described in Fig. 9.*

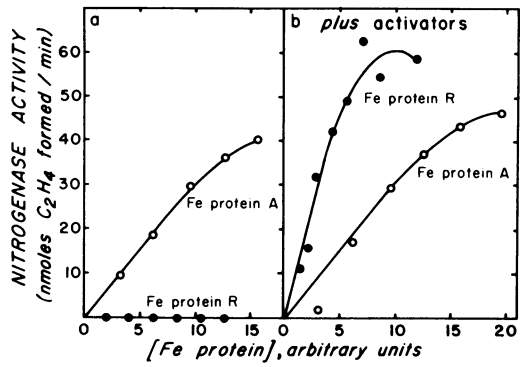

FIG. 11. *Titration of MoFe protein with Fe protein derived from either the A or the R form of N_2ase (a) Reconstituted N_2ases assayed under nonactivating conditions. ○, Enzyme reconstituted with Fe protein derived from the N_2ase A; ●, enzyme reconstituted with Fe protein derived from the N_2ase R. (b) Reconstituted N_2ases assayed under activating conditions (symbols have the same meanings as in [a]). The activity of reconstituted N_2ase R was measured during a 10-min period after activity became linear with time. The concentration of MoFe protein was the same in all samples. EPR spectrometry was used to determine the amounts of Fe protein and MoFe protein; Fe protein was measured by the amplitude of the g = 1.94 signal, the MoFe protein was measured by the amplitude of the g = 4.2 signal. EPR instrument settings: temperature, 15°K; power, 6 mW; frequency, 9.305 GHz.*

with Fe protein derived from N_2ase R was inactive, whereas the N_2ase complex reconstituted with Fe protein from N_2ase A was fully active; the increase in enzyme activity was proportional to the increase in added Fe protein until the MoFe protein was saturated, with higher levels of Fe protein. Enzymatic activity was not affected by the source of the MoFe; the activity was the same whether the MoFe protein was derived from N_2ase A or N_2ase R.

The same titrations were repeated when the activities of the reconstituted N_2ases were assayed under activating conditions; that is, in the presence of Mn^{2+} and activating factor (Fig. 11b). Under these conditions, the reconstituted N_2ase A required approximately twice as much Fe protein as did the reconstituted N_2ase R for optimal N_2ase activity. It seems reasonable to conclude, therefore, that the observed smaller molecular size of N_2ase R is the result of fewer Fe protein units per MoFe protein than is the case in N_2ase A.

DISCUSSION

It has been known for some time that N_2ase isolated from *R. rubrum* cells varies greatly in activity from preparation to preparation, to an extent not encountered with the more common N_2ases from heterotrophs (4). Recently, Ludden and Burris (10) and Nordlund et al. (14) demonstrated that *R. rubrum* N_2ase has special requirements for enzymatic activity. They found that, unlike N_2ases of the more thoroughly studied organisms, the *R. rubrum* N_2ase complex became enzymatically active only after its Fe protein component underwent special activation with a membrane-bound factor (contained in the chromatophores), ATP, Mn^{2+}, and high concentrations of Mg^{2+}. The MoFe protein component required no such activation (10, 14).

We confirmed and extended those observations by showing that similar activation of *R. rubrum* nitrogenase was required for N_2ase-linked H_2 production. Moreover, upon isolation of the N_2ase complex from *R. rubrum* cells, we unexpectedly found another kind of N_2ase, one that resembled the more common N_2ases of heterotrophs in that its enzymatic activity was not governed by activating conditions. This nitrogenase we designated as N_2ase A to distinguish it from the form of N_2ase described by Ludden and Burris (10) and by Nordlund et al. (14), which we have called N_2ase R.

We found that the production of the A and R forms of N_2ase by *R. rubrum* cells is determined by their nitrogen supply regime. Cells that were nitrogen starved, namely, cultures in which the nitrogen supply was totally exhausted before harvest, yielded predominantly N_2ase A. Cells supplied up to harvest time with glutamate or N_2 yielded predominantly N_2ase R. It is noteworthy that glutamate was the nitrogen source in the *R. rubrum* cultures of Ludden and Burris (10) and that N_2 was the nitrogen source in the *R. rubrum* cultures of Nordlund et al. (14).

Another unexpected observation was the rapid intracellular conversion of N_2ase A to N_2ase R, brought about by the addition to nitrogen-starved cells of glutamine, asparagine, or, particularly, ammonia. As shown in Table 1, the addition of ammonia (ammonia shock) resulted in the transformation of the A form of N_2ase into the R form in 10 min or less—a finding which suggests that mechanisms other than de novo protein synthesis were involved.

The inactivation of nitrogen fixation by additions of ammonia, glutamine, or asparagine to *R. rubrum* cells (13, 18) and by ammonia in other organisms (cf. 5) was previously observed in other laboratories. In light of the present results, it appears that, at least in *R. rubrum* cells, the observed loss of N_2ase activity after the addition of these nitrogen compounds probably reflects a conversion of N_2ase A into N_2ase R. These results suggest that within the cell N_2ase R is closely regulated.

Our investigation of some of the physical and chemical properties of *R. rubrum* N_2ase and its components disclosed that the N_2ase A complex was larger than N_2ase R. However, the molecular weights of the component Fe proteins and MoFe proteins were found to be the same in the two forms of N_2ase. This paradox seems to result from a difference in the proportions in which the Fe protein combines with the MoFe protein. Our evidence indicates that the N_2ase A complex has the larger molecular size because the N_2ase R complex contains fewer Fe protein units per MoFe protein.

As already stated, the differences between the N_2ase A form and the N_2ase R form are localized entirely in their Fe protein components. The ATP requirements for acetylene reduction by *R. rubrum* N_2ases differed, depending on the form of the Fe protein. The results shown in Fig. 5 support the conclusion that the R type of Fe protein, when combined with MoFe protein, requires a lower optimal concentration of ATP for substrate reduction but binds or utilizes more ATP molecules during the reaction than does the A type of Fe protein.

Other differences between the Fe proteins of N_2ase R and of N_2ase A are still poorly understood. They do not seem to involve alterations in molecular weight, EPR spectrum (unpublished observations), midpoint oxidation-reduction potential, or sensitivity to iron chelators. Indeed, at present the only method for detecting whether the Fe protein is of the A or the R type is to recombine it with the MoFe protein and to investigate the size of the complex and its enzymatic characteristics.

Further work will be needed to determine the nature of the modification of Fe protein during the conversion of N_2ase A to N_2ase R and the cellular mechanisms for regulating the activity of N_2ase R. Another unresolved question concerns the extracellular properties of N_2ase R when it was induced by the use of glutamate as the source of nitrogen. Here, N_2ase R appeared to be in an active state in the intact cells (before isolation), but upon isolation and purification the addition of activating factors became essential for acetylene reduction.

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