Biochimica et Biophysica Acta, 305 (1973) 445–454

BBA 46545

NITROGENASE

IV. SIMPLE METHOD OF PURIFICATION TO HOMOGENEITY OF NITROGENASE COMPONENTS FROM *AZOTOBACTER VINELANDII*

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(Received December 5th, 1972)

SUMMARY

Extracts of *Azotobacter vinelandii* have been fractionated by simple techniques to obtain highly purified components of nitrogenase. The yield of each component is greater than 60%. Purified Component I has a specific activity of 1638 nmoles ethylene formed/min per mg protein. The spectrum of Component I exhibits a broad absorption between 300 and 600 nm, with no distinctive peaks or shoulders. Addition of sodium dithionite or exposure to air has no effect on the absorption spectrum. Component I, examined at 4.2 °K has EPR signals at $g=4.2$, 3.65 and 2.01. Addition of sodium dithionite does not produce additional resonances nor does it alter the signals already present. Crystals of Component I are dark brown and needle-shaped.

Purified Component II has a specific activity of 1815 nmoles ethylene formed/min per mg protein. The absorption spectrum has no peaks or shoulders between 390 and 650 nm. Upon exposure of Component II to air, absorption increases between 400 and 650 nm. Treatment of oxidized Component II with dithionite causes this absorption to fall below that of the native Component II. EPR spectra of Component II has signals at g values of 2.05, 1.94, and 1.88. Upon inactivation by O_2, these signals disappear.

Neither component by itself has detectable acetylene-reducing or N_2-fixing activity. The ratio of acetylene reduced to N_2 fixed is 3.86 with different ratios of the components. Both components form aggregated species upon exposure to air. Dithionite does not reverse this effect.

INTRODUCTION

The N_2-fixing enzyme systems of several organisms[1–10] have been separated and partially purified into Component I (Fe–Mo protein) and Component II (Fe protein). Besides N_2 fixation, nitrogenase catalyzes ATP-dependent H_2 evolution[11] as well as reduction of various other substrates[12–19]. These nitrogenase-catalyzed reactions require an ATP supply, a source of reducing power and presence of both the components[4–6,8,9,20,21]. Acetylene is frequently used as an alternate substrate for assays of nitrogenase[22–24] because of the sensitivity and convenience of the assay.

Contradictory reports on the EPR[25,26] and visible spectra, substrate and ATP

binding[4,27], and component ratio for optimum activity[10,28,29], are perhaps due to studies carried out with insufficiently pure preparations. In this paper, we describe a method of purification that yields both components in a more purified form than previously described for *Azotobacter vinelandii*. Recently, EPR spectroscopy with components purified by these techniques has yielded information that indicates the role of each component in N_2 fixation[30].

MATERIALS AND METHODS

A. vinelandii OP[31] was used throughout these studies. The organism was grown in a modified Burk's N-free medium as described by Strandberg and Wilson[32]. Large-scale cultures were grown in a 150-l pilot plant fermentor aerated with 7–10 ft^3 of air per min. Growth was followed by measuring turbidity with a Klett–Summerson photoelectric colorimeter with a No. 64 filter. Cells were harvested in a Sharples centrifuge and the cell paste was stored frozen at -20 °C until use.

ATP, creatine phosphokinase (ATP:creatine phosphotransferase, EC 2. 7.3.2.), deoxyribonuclease I (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5), dithiotreitol and Tris base were obtained from Sigma Chemical Co., St. Louis, Mo. Creatine phosphate was obtained from Pierce Chemical Co., Rockford, Ill. Acrylamide (from Eastman Kodak Co., Rochester, N.Y.) was purified by double recrystallization. Nessler's reagent was obtained from Paragon C and C Co., New York, N.Y. All other chemicals and gases were of analytical grade available commercially.

Frozen cells were thawed and washed by suspension in 0.025 M Tris–HCl buffer at pH 7.4, followed by centrifugation. Extracts were prepared by a modification[33] of the osmotic shock disruption method of Robrish and Marr[34]. All buffers were thoroughly sparged with N_2, H_2 or He before use and all manipulations were performed anaerobically at room temperature under ultra-pure N_2, H_2 or He. Gases were purified by passing them through a heated copper catalyst (Sargent-Welch Scientific Co., Skokie, Ill.) and then through a freshly prepared sodium dithionite solution.

Acetylene-reduction and N_2-fixation assays were carried out as described by Shah *et al.*[33]. Ethylene production was measured with a Varian Aerograph 600-D gas chromatograph with a Porapak R column as described by Stewart *et al.*[22]. Quantitative determinations[32] of NH_4^+ in the N_2-fixation experiments were done using Nessler's reagent.

Specific activity of a component is defined as nmoles of ethylene formed/min per mg protein. Component activity is obtained upon titration with excess of the complementary component[33]. Protein concentrations were determined by the biuret method of Gornall *et al.*[35] with serum albumin as a standard. Fractions containing $Na_2S_2O_4$ were exposed to air for at least 1 h before the proteins were assayed to destroy the dithionite which otherwise interferes with the assay.

Spectra were recorded with a Cary Model 15 recording spectrophotometer with 1-cm (light path) cuvettes fitted with serum stoppers. Component I was diluted with 0.25 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4) and Component II was diluted with 0.025 M Tris–HCl buffer (pH 7.4).

Separation of Components I and II

Chromatography on DEAE-cellulose was performed by a method similar to that of Bulen and LeComte[1] with modifications as indicated. Whatman DE52

(microgranular) was equilibrated with 0.025 M Tris–HCl (pH 7.4), then thoroughly degassed and packed into a 2.5 cm × 17 cm column. The column was reduced with 400 ml of 0.025 M Tris–HCl buffer (pH 7.4), containing 0.5 mg/ml $Na_2S_2O_4$ and further washed with 200 ml of the same buffer containing 0.1 mg/ml $Na_2S_2O_4$. A volume of 100–125 ml of crude extract (2.5–3.0 g of protein) was applied to the top of the column with intermittent washing with 0.025 M Tris–HCl buffer (pH 7.4). The column was eluted successively with 2 bed vol. of 0.025 M Tris–HCl buffer (pH 7.4) and 3 bed vol. of 0.1 M, 1 bed vol. of 0.25 M and 1 bed vol. of 0.5 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4). All eluting buffers were sparged with H_2 and contained 0.1 mg/ml $Na_2S_2O_4$. Component I appears as a dark brown band in the 0.25 M NaCl fraction and Component II as a brown band in the 0.5 M NaCl fraction. Fractions were collected from the column into serum-stoppered 60-ml serum bottles kept anaerobic by flushing with H_2. More than 80% of each component was eluted in about 20 ml.

Further purification of Component I

Component I from the first DEAE-cellulose column was further purified by treatment at 52 °C for 5 min under H_2 with agitation followed by rapid cooling to room temperature and centrifugation at 20000 × *g* for 10 min. The supernatant solution was concentrated to about 1/3 the volume in an ultrafiltration cell (Amicon Corporation, Lexington, Mass.) using an XM 50 membrane. To this concentrated fraction, 14 ml of 0.025 M Tris–HCl buffer (pH 7.4) was added and this solution was rechromatographed on a 2.5 cm × 17 cm DEAE-cellulose column that had been washed with 400 ml of 0.15 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4) containing 0.3 mg/ml $Na_2S_2O_4$. The column then was washed with 2–3 bed vol. of 0.15 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4) before Component I was eluted in 18.5 ml of 0.25 M NaCl in the same buffer.

Crystallization of Component I

Attempts to crystallize Component I by a previously published method[36] were unsuccessful. Component I from the second DEAE-cellulose column was concentrated to about 4 ml in an ultrafiltration cell with an XM 50 membrane and then diluted with 20 ml degassed 0.025 M Tris–HCl buffer (pH 7.4) and concentrated again. The NaCl concentration for optimal crystallization is 0.04 M. Component I, which begins to crystallize in the ultrafiltration cell, is transferred anaerobically to a serum-capped centrifuge tube flushed with H_2 and kept at 38 °C for 1 h. Microscopic examination at this stage shows a dense population of needle-shaped crystals. The preparation was centrifuged at 20000 × *g* for 10 min. The supernatant liquid was transferred anaerobically to another serum-capped centrifuge tube and the dark brown pellet was suspended in 2–3 vol. of 0.042 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4) followed by centrifugation at 20000 × *g* for 20 min. The pellet was dissolved in 3 ml of degassed 0.25 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4), followed by centrifugation. Component I is highly soluble in 0.25 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4), whereas a small amount of white amorphous material is not soluble in this buffer. Usually, the buffers contain 0.1 mg/ml $Na_2S_2O_4$, but this was omitted when Component I was required for some studies like EPR, visible and ultraviolet spectra and O_2 sensitivity. A small additional crop of crystalline Component I can

be obtained by concentrating the supernatant solution and repeating the technique. Component I can also be crystallized after the heat-treatment step following the first DEAE-cellulose column, but the yields are lower because of interfering proteins.

Further purification of Component II

Component II from the first DEAE-cellulose column was concentrated to 9 ml in an ultrafiltration cell with a UM 20E membrane, diluted with 1 vol. of 0.025 M Tris–HCl buffer (pH 7.4) and applied to a fully reduced 2.5 cm × 18 cm DEAE-cellulose column equilibrated with 0.25 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4). The column was eluted with 2 bed vol. of 0.25 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4) and Component II was eluted with 0.35 M NaCl in the same buffer.

Preparative gel electrophoresis

Anaerobic preparative gel electrophoresis in 8% (w/v) polyacrylamide separation gel (4 cm) and 6% (w/v) stacking gel (1 cm) in a water-jacketed column (13 mm internal diameter) was performed with a Fractophorator (Buchler Instruments, Fort Lee, N.J.) using the Ortec 4100 power supply (Ortec Incorporated, Oak Ridge, Tenn.). Both the lower and upper chambers contained 0.065 M Tris–borate (pH 9.0) with 0.3 mg/ml $Na_2S_2O_4$; while the eluting buffer was 0.065 M Tris–HCl (pH 7.4) containing 0.1 mg/ml $Na_2S_2O_4$ and 0.1 mg/ml dithiothreitol. The gel was pre-run for 4–6 h at 5 mA (75 V), 100 pulses per s and a discharge capacitance of 1.0 μF. About 15–20 mg of Component II from the second DEAE-cellulose column was applied to the gel and electrophoresis continued at the same current for 10–12 h. The current then was raised to 10 mA (150 V), 200 pulses per s. Component II, which is visible as a dark brown band, was eluted in 1.5–2.0 ml of buffer. The buffers were degassed before use, and during electrophoresis all the regions of the equipment normally open to the atmosphere were continuously flushed with ultra-pure N_2.

The time of electrophoresis can be reduced to 6 h by operating at 12 mA (200 V), 250 pulses per s with similar results. We prefer the former method because it allows for the convenience of overnight operation.

Analytical acrylamide gel electrophoresis using the Ortec electrophoresis system was carried out anaerobically as described by Davis *et al.*[26]. The gel was pre-run for 5 min at 75 pulses per s, 5 min at 150 pulses per s and 50 min at 225 pulses per s using 325 V and discharge capacitance of 1.0 μF. The operation was continued as stated above after applying the samples, except that the pulses were raised to 300 per s after the first 15 min and the electrophoresis continued for 45 min to give a total running time of 1 h. The gel was stained for protein with 0.25% Buffalo Black NBR (Allied Chemical, Morristown, N.J.) in 7% acetic acid and was destained by washing with 7% acetic acid.

Unless otherwise specified, both components were quick-frozen in an ethanol–solid CO_2 bath and then stored at −20 °C. The components were rapidly brought to room temperature before use.

RESULTS AND DISCUSSION

The results of a representative purification of Component I and Component II are shown in Table I. The procedure is striking in its simplicity and both the compo-

TABLE I

PURIFICATION OF NITROGENASE COMPONENTS OF *A. VINELANDII*

Activity of a component (in all fractions including crude extract) is obtained upon titration with the complementary component.

Fraction	*Vol.* (*ml*)	*Total activity* (*units* × *10*—4)	*Total protein* (*mg*)	*Spec. act.**	*Recovery*
Component I					
Crude extract	100	12.0	2560	47	100
1st DEAE-cellulose column	22	11.0	563	196	92
52 °C supernatant	21	11.0	396	278	92
2nd DEAE-cellulose column	18.5	9.3	163	569	77
Crystallized	3.3	7.8	48	1638	65
Component II					
Crude extract	100	8.8	2560	35	100
1st DEAE-cellulose column	18.5	7.2	285	255	82
2nd DEAE-cellulose column	10.2	6.0	64	942	68
Polyacrylamide gel	4.8	5.6	31	1815	63**

* nmoles ethylene formed/min per mg protein.

** Overall yield; protein from 2nd DEAE-cellulose column was electrophoresed in three batches.

nents are obtained in homogeneous states in good yields. Crude extract from 25 g of a frozen cell paste gave 48 mg (65% yield) of crystalline Component I and 31 mg (63% yield) of homogeneous Component II.

Properties of Component I

Crystals of Component I obtained by the procedure described are dark brown. We did not observe the disappearance of brown color with the formation of white crystals reported earlier[36]. Microscopic examination (Fig. 1) reveals that the crystals are needle-shaped measuring 25–60 μm long and 1–4 μm wide as reported by Burns *et al.* [36]. The same preparation examined after 30 min exposure to air showed threads rather than needles. This may be due to the formation of aggregated species of Component I upon oxidation.

Component I behaves as a single molecular species at pH 9.0 (Fig. 2) and pH 7.5 in anaerobic analytical acrylamide gel electrophoresis. Up to the maximum concentration of Component I (2 mg) that can be applied to the gel, no trace of a second band was observed. On exposure to air for 10 min, Component I loses 51% of its activity (Table II) and begins to precipitate. Attempts to restore the activity by treatment with $Na_2S_2O_4$ were unsuccessful. Component I stored at 0–4 °C for 24 h does not lose any appreciable activity (Table II). Irreversible inactivation of Component I upon exposure to air has been observed in other organisms[9,28,37].

Preliminary studies on electrophoresis of Component I, after treatment with 1% sodium dodecyl sulphate and 1% β-mercaptoethanol, in gels containing 0.1% sodium dodecyl sulphate under the conditions of Weber and Osborn[38] showed two

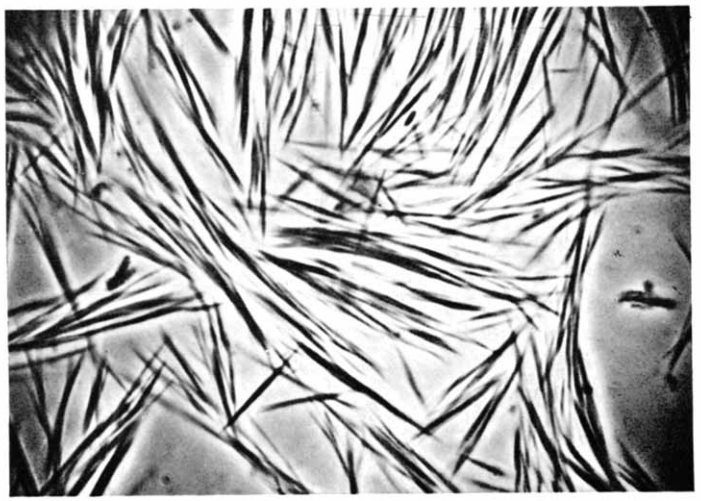

Fig. 1. Phase contrast photomicrograph of crystals of Component I.

types of subunits as observed for Component I from other N_2-fixing organisms[28,37]. Studies on the molecular weight, subunit composition and active sites are in progress.

The spectrum of Component I (Fig. 3) exhibits a broad absorption between 300 and 600 nm with no distinctive peaks or shoulders. Sodium dithionite has no effect on the spectrum. Sodium dithionite accounts for absorption below 390 nm. Burns *et al.*[36] observed a shoulder at 410–420 nm in their preparations of crystallized Component I, which upon treatment with $Na_2S_2O_4$ showed absorption peaks at 420, 525 and 557 nm. From those absorption peaks, we conclude that their[36] preparations of crystalline Component I have cytochrome c_5 contamination. Spectra of

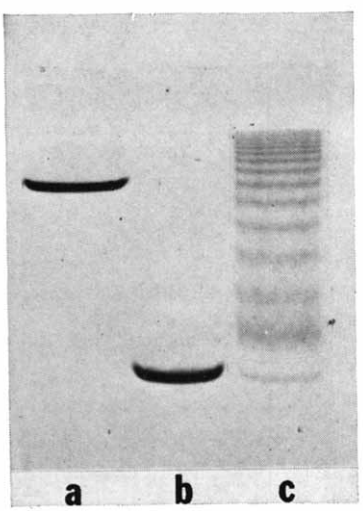

Fig. 2. Polyacrylamide gel electrophoresis of Components I and II. (a) Component I, 150 μg; (b) Component II, 200 μg; (c) Component II exposed to air for 5 min, 200 μg.

TABLE II

STABILITY OF NITROGENASE COMPONENTS OF *A. VINELANDII*

Component I used for these studies was free of $Na_2S_2O_4$ while Component II contained 0.2–0.3 mg/ml $Na_2S_2O_4$ and 0.1 mg/ml dithiothreitol.

Treatment	*Specific activity*	*Activity (% of original)*
Component I		
As isolated	1638	100
Stored at 0–4 °C for 24 h	1625	99
Stored at −20 °C for 24 h	1637	100
Stored at −20 °C for 20 days	1528	93
Shaken in air for 10 min	799	49
Component II		
As isolated	1815	100
Stored at 0–4 °C for 24 h	1767	97
Stored at 0–4 °C for 96 h	1111	61
Stored at −20 °C for 24 h	1814	100
Stored at −20 °C for 20 days	1379	76
Shaken in air for 15 s	1270	70
Shaken in air for 5 min	0.0	0.0

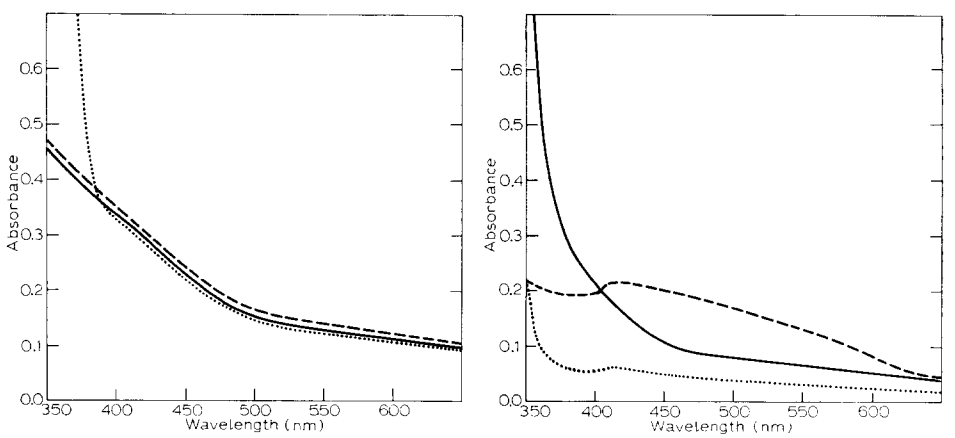

Fig. 3. Absorption spectra of Component I (1.16 mg/ml). ———, component I as isolated; – – – –, component I exposed to air for 5 min; · · · · ·, component I + sodium dithionite.

Fig. 4. Absorption spectra of Component II (1.62 mg/ml). ———, component II as isolated (contains small quantity of sodium dithionite); – – – –, component II exposed to air for 5 min; · · · · ·, component II exposed to air for 5 min + sodium dithionite.

Component I from *Clostridium pasteurianum*[37] and *Klebsiella pneumoniae*[28] exhibit a broad absorption between 300 and 600 nm, with no distinctive peaks in this region; and this agrees well with our observations with Component I from *A. vinelandii*. Component I that had been exposed to air for 5 min exhibited slightly higher absor-

bance throughout the visible region without any significant change in spectral pattern. The increase in absorbance may be due to the turbidity developed during exposure to air. The absorbance spectrum of Component I from *C. pasteurianum*[37] changes significantly, with the formation of a peak at 435 nm upon brief exposure to air, while that from *K. pneumoniae*[28] exhibits a slight increase in absorbance over the 360–600-nm region.

Our preparations of crystalline Component I at 4.2 °K showed [26] EPR signals at $g=4.32$, 3.65 and 2.01. Addition of $Na_2S_2O_4$ to Component I did not produce additional resonances nor did it alter the signals already present. Hardy *et al.*[25] reported electron spin resonances at g values of 2.01, 3.67 and 4.30 for native[36] Component I. They[25] observed an increase in the intensity of resonances at $g=4.30$ and 3.67, and the appearance of a new signal at $g=1.94$ on reduction of native protein with $Na_2S_2O_4$. As reported by others[28,37,39], O_2 denaturation of Component I leads to disappearance of some of the signals and the appearance of a large narrow signal at $g=2$. Partial O_2 inactivation of Component I during preparation can, of course, complicate the interpretation of the properties of impure preparations. The observation[25,37] of signals near $g=1.94$ in addition to the $g=2.01$ resonance may be caused by small amounts of contaminating Fe–S proteins.

Neither component by itself at concentrations up to 2 mg per assay has detectable acetylene reduction or N_2-fixation activity. Component I (36 μg) assayed with excess of Component II (138 μg) gave a specific activity of 1638 nmoles of acetylene formed/min per mg protein for Component I and this activity remained the same on recrystallization. A specific activity of 1488 nmoles of H_2 evolved/min per mg protein for Component I of *A. vinelandii* has been reported earlier[36]. The slopes of the activity curves for acetylene reduction and N_2 fixation are the same, giving the acetylene reduction:N_2 fixation ratio of 3.86 at different ratios of the components. Earlier reports[40–42] of the requirement of three components for nitrogenase activity and the requirement of an additional component for N_2 fixation *vs* acetylene reduction appear to be erroneous.

Properties of Component II

The purification procedure described gives preparations of Component II that behave as a single molecular species at pH 9.0 (Fig. 2) and pH 7.5 in anaerobic polyacrylamide gel electrophoresis. Upon exposure to air for 5 min, Component II completely loses its enzymatic activity (Table II) and multiple bands are formed (Fig. 2) with concomitant disappearance of the band corresponding to the native Component II. Treatment of air-inactivated Component II with $Na_2S_2O_4$ failed to restore the activity or reverse the multiple electrophoretic band pattern. Multiple bands, observed by Eady *et al.*[28], for Component II of *K. pneumoniae* are due to the aggregated species formed upon O_2 inactivation of Component II[43]. There is sufficient O_2 contamination in most commercial gases to partially inactivate Component II. 10–15 s exposure to air results in 25–30% loss of Component II activity. Therefore, it is extremely important that all traces of O_2 be excluded during purification and handling steps.

Component II stored at 0–4 °C for 24 h loses only 3% of its enzymatic activity while 96 h of storage causes 39% activity loss (Table II). Component II from many N_2-fixing organisms[9,44,45] is reported to be cold and O_2 labile, while that of *K.*

pneumoniae[28] is O_2 labile but not cold labile. As judged by spectral changes, the nature of cold inactivation is distinct from that of O_2 inactivation[45]. If cold inactivation causes only minor changes in the structure of Component II, then it may not be possible to separate cold-inactivated Component II from active Component II by conventional methods. Partial inactivation of the components by cold and/or O_2 during purification, storage and handling for assay might explain some of the contradictory reports[10,28,29] on the component ratio for maximal activity.

The spectrum of Component II (Fig. 4) exhibits a broad absorption between 390 and 650 nm with no distinctive peaks. On exposure to air, absorption increases in the region of 400–650 nm. Reduction of oxidized Component II with $Na_2S_2O_4$ bleaches the color and absorbance in the region of 400–650 nm falls below that of the native Component II. Sodium dithionite accounts for absorption below 390 nm. Similar spectral changes on oxidation have been reported for Component II from other organisms[28,45]. The EPR spectra of Component II examined at <20 °K with g values of 2.05, 1.94 and 1.88 is similar to that observed in Component II from *K. pneumoniae*[28] and *C. pasteurianum*[30,45]. Upon inactivation by O_2, the observed signals in Component II of *A. vinelandii* disappear completely.

Titration of Component I with Component II provides the sigmoidal activity curve and calculation from the initial linear portion of this curve gave the specific activity of 1815 nmoles of acetylene formed/min per mg protein of Component II. The specific activity of 2128 nmoles of acetylene formed/min per mg protein of Component II of *A. vinelandii*[4] was observed using cold-inactivated crude extract as a source of complementary component and hence the assay system is not comparable with that used in present studies. The reports [47,48] on 3–4-fold stimulation of activity of partially-purified nitrogenase by ferredoxin, NADH dehydrogenase and other proteins indicate that the results obtained with assays involving crude extracts must be interpreted with caution. Most of the original contaminating proteins are still present in the system used by some investigators[44–46] and hence characteristics of the system could be affected.

ACKNOWLEDGEMENTS

This research was supported by the Graduate Research Committee, the College of Agricultural and Life Sciences, University of Wisconsin, Madison, U.S.A., and N.S.F. Grant GB-36787. The authors thank Dr W. H. Orme-Johnson for his aid with the EPR studies and Mr M. Stieghorst and Mr H. Momont for their valuable assistance.

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