SEPARATION OF THE NITROGENASE SYSTEM OF AZOTOBACTER INTO THREE COMPONENTS AND PURIFICATION OF ONE OF THE COMPONENTS

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Summary: The nitrogenase system of Azotobacter vinelandii is separated into three components by column chromatographies on Sephadex G-50 and DEAE-cellulose. The components, when all of them are present together, exhibit the nitrogenase activity. One of the components is purified to a state, homogeneous ultracentrifugally and electrophoretically. The sedimentation coefficient and molecular weight are respectively 5.8 S and 93,000. The component has an absorption maximum at 416 mμ, which is shifted to 418 mμ upon reduction with dithionite, and contains Fe and Zn atoms.

Separation of the nitrogenase system into its constituent components and purification of them are considered to be one of the most important approaches for elucidating the mechanism of the nitrogen fixation of bacteria. Bulen and LeComte(1966) first succeeded in separating the nitrogenase system of Azotobacter vinelandii into two components, and showed that each component exhibits no nitrogenase activity by itself. Following by the success with Azotobacter, it has also been shown that two components are involved in the nitrogenase systems of Clostridium pasteurianum (Mortenson, Morris and Jeng, 1967) and Bacillus polymyxa(Witz and Wilson, 1967). The components thus separated by these investigators were not purified satisfactorily. Each component, therefore, may further be separated into more components. In the present study it has been shown that the nitrogenase system of Azotobacter vinelandii is separated into at least three components.

Furthermore, one of the components has been obtained in a purified state, homogeneous ultracentrifugally and electrophoretically. The present communication deals with the separation procedures of the three components and purification and some properties of one of the components.

METHODS

Azotobacter vinelandii(IAM 1078) was cultured in a medium of the following composition per liter; 20 g sucrose, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 80 mg $CaSO_4 \cdot 1/2H_2O$, 5 mg $Fe_2(SO_4)_3 \cdot 9H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl and 0.1 mg Na_2MoO_4. The cells, after 16 hr of pre-incubation, were cultured aerobically in 10-liter medium for 16 hr at 30°C. The cells harvested by centrifugation were suspended in 0.025 M Tris-Cl buffer(pH 7.2) containing 5 mM $MgCl_2$ at a rate of 30 g cell paste to 60 ml buffer, subjected to cell-disruption in a French pressure cell, and then centrifuged at 100,000 x g for 40 min to obtain cell-free extracts.

Column chromatography was conducted at room temperature(10-15°C) under 1 atm H_2. A fraction collector was placed in a sealed box replaced by 1 atm H_2 and to each tube was added 2-3 ml of liquid paraffin. The column was previously rendered anaerobic by washing with a H_2-saturated buffer, and the buffer used for elution was also saturated with H_2. The buffer was 0.025 M Tris-Cl buffer(pH 7.2) containing 5 mM $MgCl_2$ with or without NaCl.

Nitrogenase activity was estimated by determining the ammonia formation from N_2. Ammonia was separated by the Conway method and determined colorimetrically with Nessler's reagent(Johnson, 1941). Protein was determined according to the Folin-Lowry method(Lowry et al., 1951).

RESULTS AND DISCUSSION

The cell-free extracts were heated at 50°C for 15 min under 1 atm H_2 and centrifuged at 15,000 x g for 30 min. On Sephadex G-50 column chromatography of the resulting supernatant the nitrogenase system was obtained as a single fraction. The fraction was concentrated by dialyzing against a H_2- and sucrose-saturated 0.025 M Tris-Cl buffer(pH 7.2) containing 5 mM $MgCl_2$ under 1 atm H_2 for 3 hr at room temperature, and then subjected to DEAE-

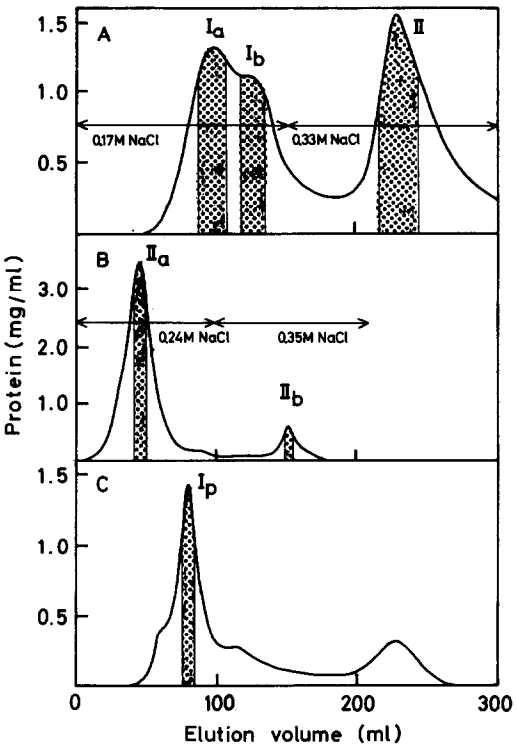

Figure 1. Separation of the nitrogenase system of Azotobacter vinelandii by column chromatographies.
A; Elution pattern of fractions I_a, I_b and II from DEAE-cellulose column. The column(4.0 x 7.0 cm) was equilibrated with the Tris buffer containing 0.17 M NaCl and eluted with the buffer used for equilibration and then with 0.33 M NaCl in the Tris buffer.
B; Elution pattern of components II_a and II_b from DEAE-cellulose column. The column(2.2 x 11.0 cm) was equilibrated with the Tris buffer containing 0.24 M NaCl. Elution was performed with the buffer used for equilibration and then with 0.35 M NaCl in the Tris buffer. C; Elution pattern of component I_p from Biogel P-150 column. The column(2.2 x 65.0 cm) was equilibrated with the Tris buffer.

cellulose column chromatography. As shown in Figure 1A, the nitrogenase system was separated into three fractions(I_a, I_b and II). Each fraction, as shown in Table I, exhibited no nitrogenase activity by itself. However, fractions I_a and I_b exhibited remarkable activities respectively when fraction II was present together.

Table I: Nitrogenase Activities of Fractions I_a, I_b and II

Fraction	Nitrogenase Activity*
$I_a + I_b$	0.0
II	0.05
I_a + II	1.8
I_b + II	2.8

Reaction mixtures contained in 2 ml: 50 µmoles Tris; 100 µmoles cacodylic acid; 15 µmoles $MgCl_2$; 18 µmoles ATP; 14 µmoles dithionite; and pH was adjusted to 7.0. 1.2 mg protein of fractions I_a and I_b and 4.4 mg protein of II were added. Reaction was carried out for 60 min at 30°C under 1 atm N_2 or 1 atm He as a control.

* µmoles NH_3 formed in 60 min per mg protein of fractions I_a or I_b

Table II: Nitrogenase Activities of Components II_a and II_b

Component or Fraction	Nitrogenase Activity*
$I_b + II_a$	0.9
$I_b + II_b$	0.0
$I_b + II_a + II_b$	5.2

Reaction mixtures as given in Table I. 1.5 mg protein of fractions I_b, 2.5 mg of components II_a and 0.2 mg of II_b were added.

* µmoles NH_3 formed in 60 min per mg protein of fraction I_b

As shown in Figure 1B fraction II was further separated into two components, II_a and II_b, by column chromatography on DEAE-cellulose after concentration by dialysis. Ultracentrifugal

analyses revealed that components II_a and II_b were still contaminated with other proteins. The components, therefore, may further be separated into more components indispensable for the nitrogenase system. Components II_a and II_b were very labile, and even when they were stored under 1 atm H_2 at 0-15°C the activities were almost completely lost upon 12 hr of storage. Attempts so far examined to purify these components or separate them into more components have been unsuccessful.

When considered together with the elution pattern(Figure 1A) and nitrogenase activities(Table I) of fractions I_a and I_b the nitrogenase activity of fraction I_a is suggested to be due to contamination of I_b, although the possibility that I_a is another fraction can not be excluded. Further purification, therefore, was undertaken with fraction I_b. Gel filtration of I_b on Biogel P-150 after concentration by dialysis resulted in separation into many components(Figure 1C). The nitrogenase activity measured with fraction II, however, was found to be **localized** in only the fraction of most sharp protein peak. The fractions shadowed in the figure were collected and designated as component I_p. Ultracentrifugal and electrophoretic analyses revealed that component I_p is completely homogeneous(Figure 2). Summerizing the results so far obtained it may be considered that the nitrogenase system of Azotobacter vinelandii consists of at least three components, I_p, II_a and II_b.

The sedimentation coefficient($s_{20,w}$) of component I_p was 5.8 S and molecular weight was estimated to be 93,000 by meniscus depletion method(Yphantis, 1963). The component was shown to contain about 3 moles Fe and 1 mole Zn per mole of the component by measuring the atomic absorption spectrum. The nitrogenase activity of I_p measured with fraction II was 4.8-5.4 μmoles NH_3 formed in 60 min

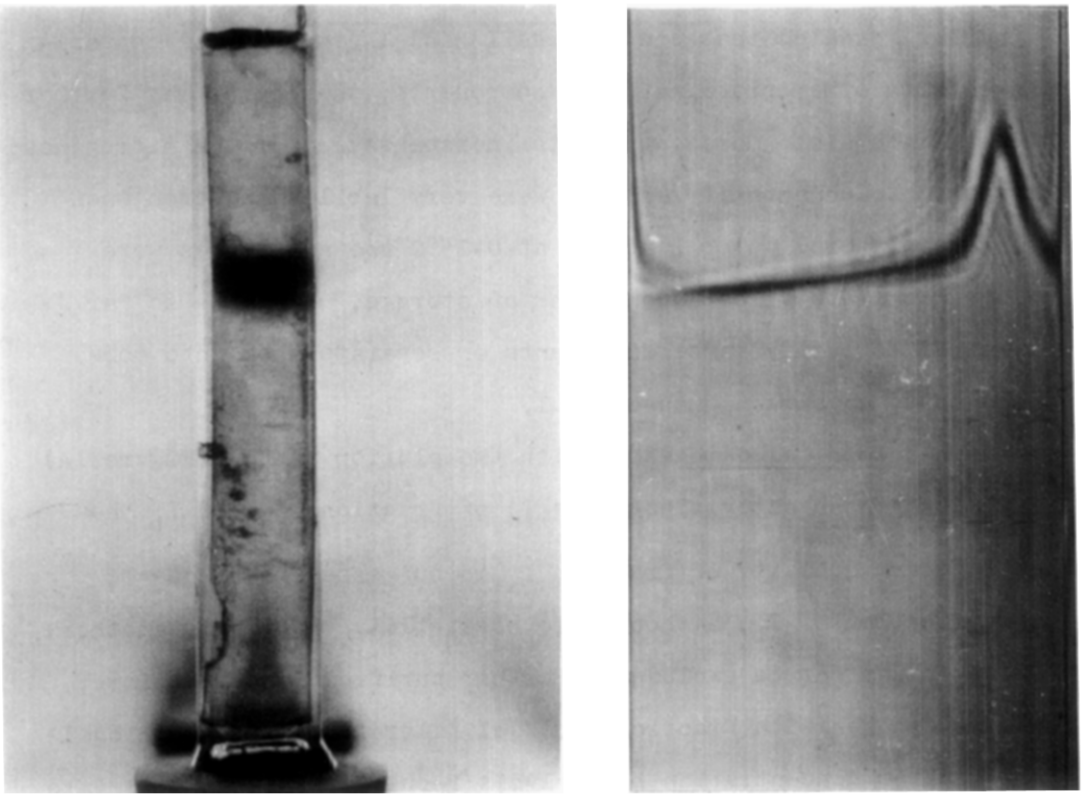

Figure 2. Homogeneity of component Ip.
A; Sedimentation pattern of Ip. Photograph was taken 15 min after reaching full speed, 60,000 rev/min in a Hitachi analytical ultracentrifuge, UCA-1. B; Disc electrophoretic pattern of Ip. Electrophoresis was carried out in a Tris-glycine buffer(pH 8.3) at 4°C for 45 min at 4 mA/cm.

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per mg protein of I_p. The visible absorption spectrum of I_p measured under 1 atm H_2 exhibited a maximum band at 416 mμ and broad shoulder in the 460 mμ region(curve A in Figure 3). When component I_p was shifted to 418 mμ and the 460 mμ shoulder disappear (curve B). This spectral character of component I_p, together with the iron content, may indicate that the component is a nonheme iron protein. Two components separated by Bulen and LeComte(1966) were nonheme iron, molybdoprotein(Mol. wt. = 100,000-120,000) and nonheme iron protein(Mol. wt. = 30,000-40,000). Hence it seems

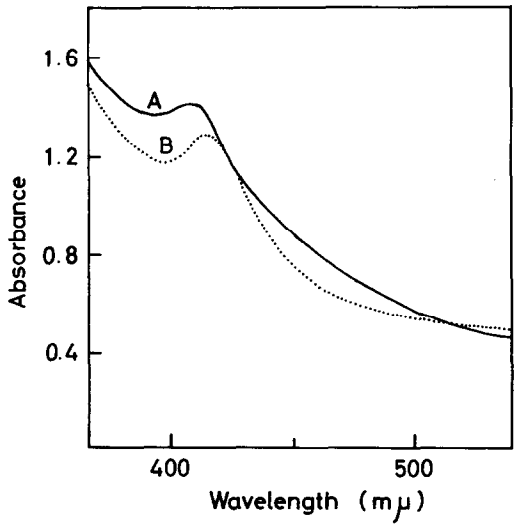

Figure 3. Visible absorption spectra of component Ip. A; Component as isolated and measured under 1 atm H_2 in 0.025 M Tris-Cl buffer(pH 7.2) containing 5 mM $MgCl_2$. B; Reduced with 7 mg/3 ml dithionite under 1 atm H_2.

unlikely that component I_p is identical to either of the two components of Bulen and LeComte.

When component I_p was left standing at 0-15°C in air or under 1 atm N_2 a considerable amount of precipitate was formed within 10-15 min. Even under 1 atm H_2 the nitrogenase activity of I_p was completely lost upon 18 hr of storage. As the cell-free extracts were stable for about 3 days under similar conditions the results indicate that three components became unstable when they were separated or purified.

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