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INTERACTION OF THE NITROGENASE COMPONENTS OF ANABAENA CYLINDRICA WITH THOSE OF CLOSTRIDIUM PASTEURIANUM

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SUMMARY

The two components of the nitrogenase, the MoFe protein and the Fe protein, of Anabaena cylindrica were separated and partially purified. In the reconstituted enzyme, acetylene reduction of 400 nmole/min and ATP'ase activity of 1900 nmole/min based on MoFe protein were observed. Acetylene reduction based on Fe protein was 346 nmole/min. The MoFe protein of nitrogenase from A. cylindrica forms an active complex with the Fe protein of C. pasteurianum. The rate of acetylene reduction and hydrogen evolution by this heterologous complex based on MoFe protein is about 50% of that of the homologous system from A. cylindrica. The combination of the MoFe protein of C. pasteurianum and the Fe protein of A. cylindrica gave acetylene reduction of only 6 nmole/min/mg MoFe protein and an ATP'ase of only 50 nmole/min/mg MoFe protein. No N_2 reduction was detected.

INTRODUCTION

Nitrogenase, the enzyme responsible for nitrogen fixation, has been isolated from several microorganisms (1-3) and always consists of two oxygen-sensitive iron-sulphur proteins, the MoFe protein and Fe protein. In several previous reports, (3-9) the interchange of the two components of nitrogenase from different sources demonstrated enzymatic activity. Nitrogenase components from the strictly anerobic bacterium Clostridium pasteurianum when combined with nitrogenase components from other nitrogen fixing bacteria demonstrated a low degree of interchangeability (4, 5, 6, 8); two positive cross reactions were observed in ten heterologous combinations. No information is available for the complementation of nitrogenase components from anaerobes and blue-green algae. For this reason, nitrogenase components of C. pasteurianum were examined for their ability to cross react with nitrogenase components of Anabaena cylindrica.

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MATERIALS AND METHODS

Culturing conditions

The alga, Anabaena cylindrica Lemm. (Indiana Culture Collection No. B629) was grown under nitrogen fixing conditions by the method previously described (10). No bacterial contamination could be detected at any stage of the culturing procedure.

Closteridium pasteurianum W5 cells were grown, harvested and dried as described previously by Mortenson (11).

Preparation of cell free extract and enzyme purification

The cell paste was suspended (1:4 w/v) in anaerobic 0.05 M Tris-HCl buffer (pH = 7.5). $Na_2S_2O_4$ was added to give a final concentration of 1 mM and the cells were broken anaerobically by treatment for 5 min with a Branson Sonifier. The broken cell suspension was then centrifuged anaerobically for 30 min at 18,000 g. The supernatant was used as crude extract.

The first step is similar to that described by Haystead et al. (10). The crude extract (Acetylene reduction activity 3 nmole/min/mg protein) was mixed anaerobically with an equal portion of a 50% (w/v) slurry of Whatman DEAE-52 in 0.05 M anaerobic Tris-HCl buffer at pH 7.5. The mixture was stirred under argon at 10 C for 30 min and then poured into an empty anaerobic column. After settling the nitrogenase components were eluted from the DEAE cellulose with a linear $MgCl_2$ (0-0.5 M) gradient in 0.05 M Tris-HCl, pH 7.5. The nitrogenase fractions collected were combined and concentrated to 25 mg protein/ml by ultrafiltration through an Amicon Diaflo Ultrafilter PM-10. The concentrated enzyme was stored as pellets in liquid nitrogen. Several preparations similar to the above were made in order to obtain enough enzyme at this state of purification to allow further purification. The stored samples were thawed quickly in an argon atmosphere and were then loaded on a Sephadex g-100 column (7.5 x 50 cm). Nitrogenase was eluted with a 0.05 M Tris-HCl (pH 7.5) buffer containing 0.1 M NaCl and 1mM $Na_2S_2O_4$. The fractions of nitrogenase obtained from the first brown band from the Sephadex column were adsorbed on a DEAE-52 column (2.5 x 30 cm) from which the nitrogenase components were eluted by a linear gradient of NaCl (0.1 - 0.6 M) in 0.5 M Tris-HCl (pH 7.5) containing 1 mM $Na_2S_2O_4$. The MoFe protein eluted in the first brown band after the blue phycocyanin band and the Fe protein eluted in the second brown band after the phycocyanin. Further purification of the MoFe protein was performed anaerobically at 5 C. Fractions from the DEAE column containing the MoFe protein were concentrated to a protein concentration of 20 mg/ml by ultrafiltration and loaded on a Sephadex G-200 column (2.5 x 70 cm) with the same buffer used for the first gel filtration. During elution the protein was easily followed by its brown color. Fractions of MoFe protein of high specific activity from the Sephadex column were adsorbed on a third DEAE-52 column (1.5 x 25 cm); elution was achieved by a linear gradient of NaCl (0.1 - 0.3 M) in 0.05 M Tris-HCl (pH 7.5) containing 1 mM $Na_2S_2O_4$. Fractions of 5 ml each were collected when the light brown band eluted.[2] High activity fractions of MoFe protein from the third DEAE-52 column were concentrated and loaded on a second Sephadex G-200 column (1.5 x 60 cm). The MoFe protein, eluted in 0.05 M Tris-HCL buffer, reached an acetylene reduction activity as high as 400 nmole/min/mg MoFe protein.[1]

The Fe protein was separated from the MoFe protein by the second DEAE-52 column, where it eluted as the second brown band after the blue phycocyanin band. Further purification was performed by filtering the Fe protein through a Sephadex G-200 column (1.5 x 60 cm). All purification steps were performed anaerobically at room temperature. The Fe protein of nitrogenase at this stage of purification had an acetylene reduction activity of 360[2] nmole/min/mg of iron protein.

1 s.p. Activity measured in the presence of saturating amounts of Fe protein of A. cylindrica.

2 s.p. Activity measured in the presence of saturating amounts of MoFe protein of A. cylindrica.

Preparation of crude extracts and anerobic purification of the two nitrogenase components of C. pasteurianum has previously been described (12).

Enzyme assays

Acetylene reduction was performed at 30 C in 8 ml vials fitted with a serum stopper and containing a gas atmosphere of 0.15 atm C_2H_2 and 0.85 atm argon. The reaction mixture contained in a final volume of 2 ml: 50 mM TES (N-tris(hydroxymethyl)methyl 2-aminoethanesulfonic acid)/NaOH, pH 7.5, 10 u mole $MgCl_2$, 2.4 umole ATP, 10 umole $Na_2S_2O_4$, an ATP-generating system (23 umole creatine phosphate, 20 units creatine phosphokinase), and either a crude extract or purified nitrogenase components as indicated in the Results section. Once the reaction began at 2 min intervals 30 ul of the gas atmosphere was removed and analyzed in a Varian 1520 gas chromatograph for ethylene produced.

Hydrogen evolution was measured by Warburg respirometry. The centerwell of the Warburg flasks contained 0.1 ml of 40% KOH. The reaction mixture was the same as above but with an argon gas phase.

Dinitrogen reduction by nitrogenase was measured by NH_3 formation. The reaction mixture was similar to that for acetylene reduction except that the gas phase was 1 atm of N_2. At each interval of time, as described in the results, 1 ml of each reaction mixture was removed for the analysis of ammonia (13).

ATP hydrolysis by nitrogenase was determined by measuring the inorganic phosphate released from ATP. In 2 ml volumes, the reaction mixture contained: 50 mM TES buffer, pH 7.5, 20 umole $MgCl_2$, 20 umole ATP, and 10 umole $Na_2S_2O_4$. Nitrogenase was added as described in the Results section. The gas phase consisted of 1 atm argon. The reaction was initiated by the addition of ATP. At 1 min intervals, 0.5 ml reaction mixture was added to 0.5 ml of a cold 25% solution of TCA. This mixture was then analyzed for Pi according to Taussky and Shorr (14).

Total protein determination

Protein was measured either by the Biuret (15) or by the Lowry (16) method. Total N x 6.25 was used as a measure of the protein in crude extracts of A. cylindrica. Total N was determined by Kjeldahl digestion followed by distillation of the NH_3 and nesslerization (17).

RESULTS

Cross reactions between Fe protein of C. pasteurianum and MoFe protein of A. cylindrica.

When the purified Fe protein from C. pasteurianum was combined with the purified MoFe protein from A. cylindrica, an active enzyme complex was formed which carried out the reduction of acetylene, dinitrogen, and H^+ as well as the hydrolysis of ATP. The specific activity of each assay is presented in the Table. A time course study of substrate reduction by the enzyme complex formed by the Fe protein of C. pasteurianum and the MoFe protein from A. cylindrica showed a linear rate of hydrogen evolution, acetylene reduction, dinitrogen reduction and ATPase activity after initiation of the reaction by addition of the protein to the assay system (Fig. 1).

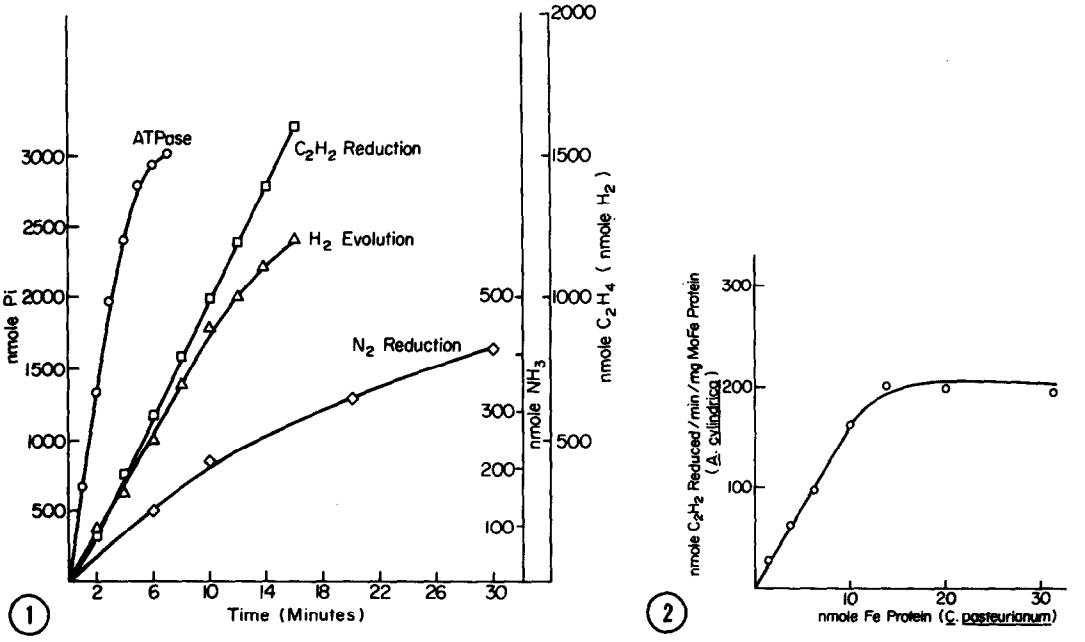

Figure 1: Time course study of acetylene reduction, hydrogen evolution, nitrogen reduction and ATP hydrolysis by the heterologous nitrogenase system (Fe protein of C. pasteurianum and MoFe protein of A. cylindrica). Details of the assays are given in the Material and Methods Section.

Substrate reduction	Ac MoFe protein	Cp Fe protein
C_2H_2 reduction	500 ug	1540 ug
N_2 reduction	600 ug	1980 ug
H^+ reduction	400 ug	1232 ug
ATP hydrolysis	500 ug	1560 ug

FIGURE 2: The effect of varying the molar ratio of Fe protein (C. pasteurianum to the MoFe protein (A. cylindrica) with MoFe protein constant 1 nmole based on MW of 250,000 and absolute purity) and Fe protein varied as indicated (based on MW of 57,675 and absolute purity). Assays are given in the Material and Methods Section.

Activity titration of MoFe protein from nitrogenase of A. cylindrica with Fe protein from nitrogenase of C. pasteurianum.

When increasing amounts of C. pasteurianum Fe protein were added to the assay system that contained a fixed amount of A. cylindrica MoFe protein (Fig. 2), the acetylene reduction activity increased rapidly and reached a plateau at an approximate molar ratio of C. pasteurianum Fe protein/A.

TABLE

Substrate Reduction by Homologous and Heterologous Nitrogenase Systems of A. cylindrica and C. pasteurianum. Assay conditions for C_2H_2 reduction, H^+ reduction, N_2 reduction and ATP hydrolysis are given in the Materials and Methods Section.

Nitrogenase System		C_2H_2 Reduction	H_2 Evolution	NH_3 Production	ATP Hydrolysis	C_2H_2 Red.	Pi/2e$^-$ based on H_2 Evol.	N_2 Red
Fe protein	MoFe protein	(Sp. activity in nmole/min/mg MoFe protein)						
A. cylindrica (864 ug)	A. cylindrica (450 ug)	400	388	102	1920	4.8	4.9	6.2
C. pasteurianum (663 ug)	C. pasteurianum (434 ug)	1100	1080	270	4900	4.5	4.5	6
C. pasteurianum (1540 ug)	A. cylindrica (500 ug)	200	206	36	1320	6.6	6.3	12
A. cylindrica (965 ug)	C. pasteurianum (465 ug)	6	---	0	50	8	---	---
A. cylindrica (800 ug)	----------	0	0	0	0	---	---	---
----------	A. cylindrica (400 ug)	0	0	0	0	---	---	---
C. pasteurianum (1200 ug)	----------	0	0	0	0	---	---	---
----------	C. pasteurianum (450 ug)	0	0	0	0	---	---	---

cylindrica MoFe protein of 14:1 at which a maximum acetylene reduction of 202 nmole/min/mg of MoFe protein was obtained. No inhibition was observed by excess C. pasteurianum Fe protein even at a molar ratio as high as 30:1 (C.p. Fe protein/A.c. MoFe protein).

Pi/2e$^-$ ratio based on substrate reduction by a homologous nitrogenase system and a heterologous nitrogenase complex.

The ATP'ase activity presented in the Table was obtained in the absence of an ATP-generating system. The reduction of dinitrogen, acetylene and H^+ by either homologous or heterologous nitrogenase systems was measured in the presence of an ATP-generating system. In each assay, the optimum ratio of Fe protein/MoFe protein was used. The Pi/2e$^-$ ratio obtained from the heterologous nitrogenase complex (6.6) was always higher than that obtained from either homologous system (4.5-4.8) showing that a higher efficiency of substrate reduction coupled to ATP utilization was present in the homologous nitrogenase systems.

Cross reaction between the Fe protein of A. cylindrica and MoFe protein of C. pasteurianum.

Combination of the Fe protein of A. cylindrica with the MoFe protein of Clostridium gave very low enzymatic activity as determined by C_2H_2 reduction (Table) and yet a linear rate was seen when a time course study was performed. ATP'ase activity of 50 nmole of Pi released per min per mg of MoFe protein was obtained but no dinitrogen reduction was detected even after prolonged incubation.

DISCUSSION

Early research (5) (6) indicated that cross reactions of nitrogenase components of various organisms was higher with phylogenetically related organisms than with non-related ones. Of concern in this paper is the cross reaction of nitrogenase components from two phylogenetically unrelated organisms. The nitrogenase complex formed between the Fe protein of C. pasteurianum and MoFe protein of A. cylindrica gives 50% of the activity observed with the homologous nitrogenase of A. cylindrica. This finding suggests that the evolutionary variability of the Fe proteins from the photosynthetic alga and the strictly anaerobic bacterium is not high. A time course study of the above heterologous system shows no lag period in H^+, acetylene or N_2 reduction. This result is different from a previously reported cross reaction between the Fe protein of C. pasteurianum and the MoFe protein of K. pneumoniae (9) where an even longer lag of over 30 min for N_2 reduction was observed. The latter studies suggested that different forms of the enzyme complex were generated at different times after combination of the components and complexes that reduce one of the substrates do not necessarily reduce the others. Our findings indicate that one of the heterologous enzyme complexes between Anabaena and Clostridium is similar to that of the homologous systems involving only a single form of enzyme complex which is capable of reducing H^+, acetylene, and N_2, although substrate may not bind with the same affinity (18). Complexes similar to those formed by the Clostridial-Klebsiella

system may be formed too rapidly in the Clostridial-Anabaena system to result in a lag in any of the activities. Since the N_2 'ase complex of Clostridium-Anabaena that reduces N_2 forms rapidly, one would not expect lag periods with any of the other substrates.

The cross reaction between the Fe protein of A. cylindrica and the MoFe protein of C. pasteurianum gives only 1.5% of the acetylene reduction activity of the homologous system from A. cylindrica and no N_2 reduction was observed. This indicates that the two proteins do complex, but that the enzyme complex formed is unable to effectively reduce acetylene or dinitrogen. Apparently the algal MoFe protein has evolved with less specificity than the clostridial MoFe protein since it can complex fairly well with both Fe proteins whereas clostridial MoFe protein complexes effectively only with its own Fe protein. A similar finding was seen in the hybridization between A. cylindrica and Chloropseudomonas ethylica (3), although in the latter case nitrogenase actually comes from Chlorobium (19). The algal MoFe protein forms an active complex with the bacterial Fe protein, but the reciprocal cross was inactive. MoFe protein of C. pasteurianum was reported to give only one active non-homologous cross, namely that with the Fe protein of Bacillus polymyxa (6) and the former does not complex effectively with Fe proteins from either K. pneumoniae (9) or Azobacter vinelandii (6). The inability of the MoFe protein of C. pasteurianum to complex with other Fe proteins might be related to its low tryptophan content compared with K. pneumoniae or A. vinelandii (20). An explanation of the extremely low efficiency of the cross between algal Fe protein and bacterial MoFe protein will require more structural knowledge of the proteins concerned.

The $Pi/2e^-$ ratio of the heterologous nitrogenase is always higher than the homologous nitrogenase. A higher $Pi/2e^-$ ratio has also been reported in other cases of cross reactions between bacteria (7) (9). ATP hydrolysis seems to be partially uncoupled from substrate reduction in the heterologous system and such systems have a lower efficiency (higher ratio of ATP hydrolyzed: electron used for reduction). This probably means that the complex of the two proteins is less tightly coupled.

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