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NECESSITY OF A MEMBRANE COMPONENT FOR NITROGENASE ACTIVITY IN *RHODOSPIRILLUM RUBRUM*

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SUMMARY

Acetylene reduction catalyzed by nitrogenase from *Rhodospirillum rubrum* has low activity and exhibits a lag phase. The activity can be increased by the addition of a chromatophore membrane component and the lag eliminated by preincubation with this component, which can be solubilized from chromatophores by treatment with NaCl. It is both trypsin- and oxygen-sensitive. Titration of the membrane component with nitrogenase and vice versa shows a saturation point. The membrane component interacts specifically with the Fe protein of nitrogenase, the interaction being ATP- and Mg^{2+}-dependent.

INTRODUCTION

Nitrogenase, which is a soluble enzyme complex, has been isolated from a number of organisms. The two components, the MoFe protein and the Fe protein, from *Clostridium pasteurianum*, *Klebsiella pneumoniae*, and *Azotobacter vinelandii* have been purified to homogeneity and studied extensively [1]. In all of these organisms the two nitrogenase components have been shown to be sufficient for full enzymatic activity.

A number of photosynthetic bacteria have been shown to synthesize nitrogenase under nitrogen-limiting conditions. The only nitrogenase component that has been purified to homogeneity, however, is the MoFe protein from *Chromatium* [2]. Investigations using cell-free extracts from *Rhodospirillum rubrum* [3–6] and *Chromatium* [7, 8] have also been reported and a crude preparation of the MoFe protein from *R. rubrum* [9] has been used in cross-reaction studies. We have previously reported that a membrane component is needed for full enzymatic activity in *R. rubrum* [6] and in this communication we report further studies on this component. Some fundamental properties of *R. rubrum* nitrogenase will be published elsewhere. Earlier reports on the occurrence of a third component in non-photosynthetic bacteria [10–12] have not been confirmed.

MATERIALS AND METHODS

R. rubrum, strain S1, was grown photoheterotrophically under an atmosphere of N_2/CO_2 (95 : 5, v/v) in the medium of Bose et al. [13] with omission of the ammonium sulphate. After harvesting and washing, the cells were suspended in 50 mM Tris · HCl, pH 7.7, containing 1 mM dithioerythritol and sodium dithionite (1 mg/ml). The cells were broken in a Ribi cell fractionator and cell debris was removed by centrifugation at 10 000 × *g* for 25 min. The supernatant was centrifuged at 114 00 × *g* for 120 min and the resulting supernatant, which will be referred to as crude extract, was used for producing a crude preparation of nitrogenase.

The membrane component was solubilized and partially purified from the chromatophores according to the scheme in Fig. 1. The buffer used throughout this procedure was 25 mM Tris · HCl, pH 7.7, containing 0.5 mg $Na_2S_2O_4$/ml. The procedure includes solubilization with salt, ammonium sulphate fractionation and gel filtration on Sephadex G-75. The membrane component was eluted from the Sephadex G-75 column and the fractions containing the membrane component were pooled and used in the assays. This pooled fraction will be referred to as MC.

A crude preparation of nitrogenase from the crude extract was produced by ion-exchange chromatography. The crude extract was mixed with an equal volume of DEAE-cellulose. The slurry was poured onto a column and washed with 0.1 M NaCl in buffer (25 mM Tris · HCl, pH 7.7, 1 mM dithioerythritol, 0.5 mg $Na_2S_2O_4$/ml).

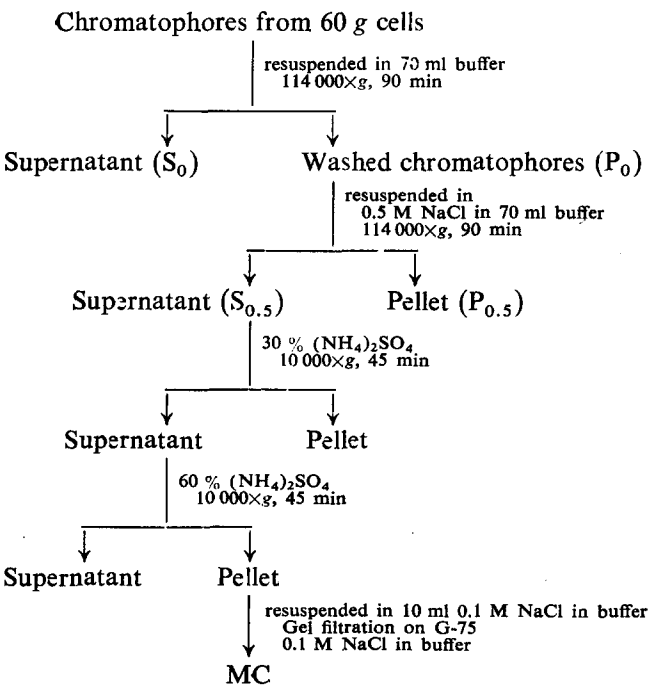

Fig. 1. Isolation of membrane component from chromatophores. Transfers of solutions were made under N_2.

After washing, nitrogenase was eluted with 0.5 M NaCl in buffer and collected in closed injection bottles which had been gassed with nitrogen. This crude nitrogenase preparation will be called N_{cr}.

A partial purification and separation of the two nitrogenase components was achieved on a second DEAE-cellulose column. N_{cr} was diluted four times with buffer before pumping onto the column. After washing with 0.1 M NaCl in buffer, the two components were eluted with a linear gradient of 0.1–0.35 M NaCl in buffer and collected as above. Due to the extreme oxygen sensitivity of the enzymes all manipulations of nitrogenase-containing solutions were made with syringes and under nitrogen.

Nitrogenase activity was measured as the rate of reduction of acetylene [14]. Protein concentrations were determined by the method of Lowry et al. [15].

Iron was determined by the bathophenanthroline method of Van De Bogart and Beinert [16]. Molybdenum was determined by the toluene dithiol method [17] with the modification that excess H_2O_2 was decomposed by titration with 4 % potassium permanganate and extraction of the complex was carried out with chloroform instead of iso-amyl acetate.

RESULTS

As was briefly reported earlier [6], nitrogenase activity was found in the crude extract but was strongly enhanced when chromatophores were added. The component needed for full activity is membrane associated and can be solubilized by washing with salt (Table I). The optimal concentration was found to be 0.5 M NaCl (Fig. 2).

The membrane component was partially purified by ammonium sulphate precipitation and gel filtration. The active membrane component, MC, was eluted after the major protein peak. The activity of MC was lost upon treatment with trypsin. The active protein in MC is apparently oxygen-sensitive, since activity was lost upon

TABLE I

ACTIVITY OF CRUDE EXTRACT WITH ADDITION OF DIFFERENT MEMBRANE COMPONENT FRACTIONS

0.1 ml of crude extract (protein concentration, 16.0 mg/ml) and 0.1 ml of the other fractions (see Fig. 1) were used. The reaction was carried out in 12.5-ml injection bottles. The reaction mixture contained 4 mM ATP, 20 mM $MgCl_2$, 10 mM creatine phosphate, 1.5 units creatine phosphokinase/ml in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5. After gassing with argon, 0.1 ml of 1.2 mg $Na_2S_2O_4$/ml was added immediately prior to addition of enzyme fractions and 1 ml of acetylene. The total volume was 1.7 ml. The assay was run at 30 °C and the reaction stopped by addition of 0.5 ml 20 % $HClO_4$. 0.5 ml of the gas phase was analyzed for ethylene by gas chromatography.

	nmol ethylene formed after 15 min	
	No addition	+Crude extract
Crude extract	220	–
Chromatophores	50	670
S_0	40	360
P_0	0	640
$S_{0.5}$	0	1050
$P_{0.5}$	0	490

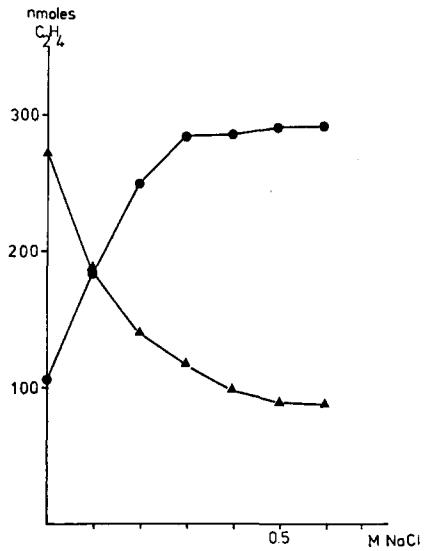

Fig. 2. The effect of different salt concentrations on solubilization of the membrane component from washed chromatophores, P_0. P_0 was resuspended in buffer containing different salt concentrations and centrifuged. The activity of the resulting supernatant (● — ●) and pellet (▲ — ▲) were measured with 0.1 ml of nitrogenase fraction N_{cr}. The assay was performed as described in the legend of Table I.

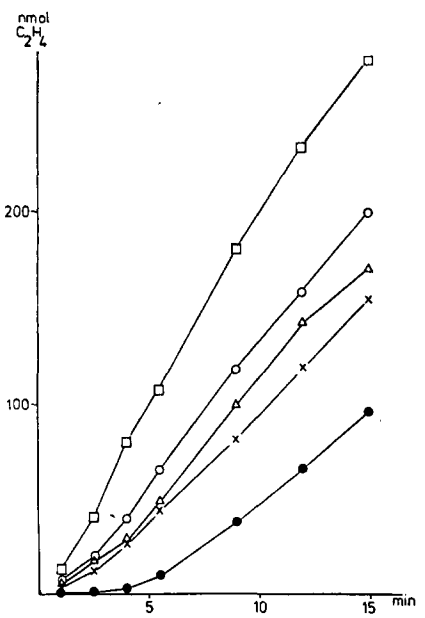

Fig. 3. Preincubation of membrane component, MC, with nitrogenase fraction, N_{cr}. The assay was performed as described (Table I) with the following exceptions: total volume, 3.5 ml; 2 ml acetylene, 0.2 ml $Na_2S_2O_4$ in 22.5-ml injection bottles. Samples, 0.5 ml, were removed at different time intervals and analyzed for ethylene. ● — ●, no preincubation; × — ×, 3 min; △ — △, 5 min; ○ — ○, 7 min; and □ — □, 10 min preincubation.

shaking in air. We have not been able to detect molybdenum or iron in MC by the methods used. Membrane component activity was also found in chromatophores from cells grown with ammonium sulphate though the activity was lower on a bacteriochlorophyll basis.

The crude nitrogenase preparation, N_{cr} was obtained by using ion-exchange chromatography; nitrogenase was eluted with 0.5 M NaCl and the specific activity of these preparations varied between 50 and 100 nmol ethylene/min per mg protein. This preparation, N_{cr} was used in studies with the membrane component, MC. N_{cr} was preincubated with MC before the reaction was started by adding acetylene. Preincubation was carried out in the reaction mixture (ATP, Mg^{2+}, creatine phosphate and creatine phosphokinase) contained in the injection bottle in which the assay was to be run. Without preincubation there was a lag of 5–6 min before the rate became linear. Increasing the preincubation time decreased the lag and 10 min preincubation eliminated it completely (Fig. 3).

The crude nitrogenase preparation, N_{cr}, was titrated with MC and vice versa (Figs. 4A and 4B). A preincubation of 4 min was used and the rate between 5 and 15 min was calculated. It can be seen from the inserted diagrams (Figs. 4A and 4B) that N_{cr} can be saturated with MC and vice versa.

In the above experiments preincubation was carried out in the reaction mix-

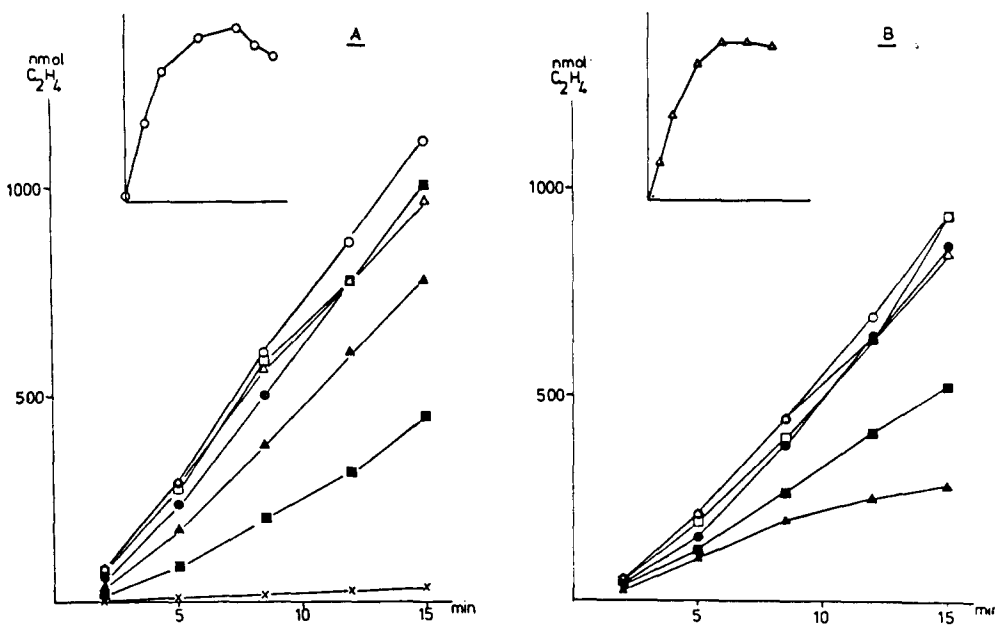

Fig. 4. Titration of MC with N_{cr} and vice versa. The assay was performed as described in Fig. 3. The reaction was started after 4 min preincubation, by the addition of acetylene. (A) Titration of 3.0 mg N_{cr} with different amounts of MC (1.2 mg protein/ml). (B) Titration of 2.7 mg MC with different amounts of N_{cr} (30 mg protein/ml). The inserted diagrams show the rate of the reaction (nmol ethylene/min) between 5 and 15 min as a function of added MC and N_{cr}, respectively. (A) × — ×, 0 ml MC; ■—■, 0.05 ml MC; ▲— ▲, 0.1 ml MC; ●—●, 0.2 ml MC; ○—○, 0.3 ml MC; □—□, 0.35 ml MC; △— △, 0.4 ml MC; (B) ▲— ▲, 25 μl N_{cr}; ■—■, 50 μl N_{cr}; △— △, 100 μl N_{cr} ○—○, 150 μl N_{cr}; □—□, 200 μl N_{cr}; ●—●, 250 μl N_{cr}.

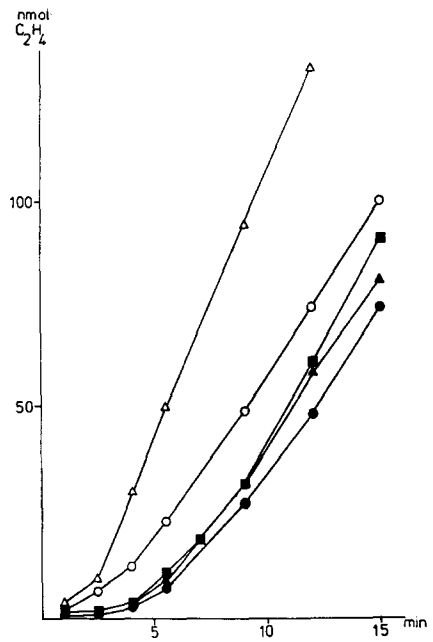

Fig. 5. The effect of preincubation with different preincubation mixtures. The preincubation (6 min) was carried out in the same injection bottle as the assay. Before adding acetylene to start the assay, additions were made to make the reaction mixture complete. In all cases preincubation was run in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5, with 0.1 ml of $Na_2S_2O_4$ added. Total volume, 3.0 ml. ● — ●, no preincubation; ▲ — ▲, 4 mM ATP; ■ — ■, 19 mM $MgCl_2$; ○ — ○, 4 mM ATP + 19 M $MgCl_2$; △ — △, 4 mM ATP + 19 mM $MgCl_2$ + 10.5 mM creatine phosphate + 1.5 units creatine phosphokinase/ml in the preincubation mixture. 3.0 mg N_{cr} and 0.24 mg MC were used.

ture. In Fig. 5 are depicted the results of studies with different preincubation mixtures. It is clear that use of the complete reaction mixture, i.e. ATP, Mg^{2+}, creatine phosphate and creatine phosphokinase, gave the shortest lag and the highest rate of acetylene reduction. Preincubation with ATP or Mg^{2+} alone gave the same lag and rate as with no preincubation in the complete reaction mixture.

The two nitrogenase components were partially separated by ion-exchange chromatography of N_{cr}. The MoFe protein was eluted with 0.2–0.25 M NaCl and was identified on the basis of the Mo/Fe ratio between 15 and 20 which is in good agreement with values reported for MoFe proteins in other organisms. The Fe protein, which was eluted with 0.25–0.35 M NaCl, was not completely separated from the MoFe protein or from the membrane component, but addition of MoFe protein in the presence of MC led to a 4-fold increase in activity (Table II). These partially separated components were used in experiments designed to define which of the components was needed to eliminate the lag when preincubated with MC. The results of preincubation experiments (Fig. 6) clearly demonstrate that incubation of the Fe protein with MC gave the same effect as when both components are preincubated with MC. There is, on the other hand, no effect when the MoFe protein is preincubated with MC or with the Fe protein.

TABLE II

ACETYLENE REDUCTION BY DIFFERENT COMBINATIONS OF THE MoFe PROTEIN, Fe PROTEIN AND MC PREPARATIONS

The assay was performed as in Table I. 0.25 mg MoFe protein, 0.25 mg Fe protein and 0.1 mg MC were used.

	nmol ethylene formed after 15 min
Complete	140
– MoFe protein	35
– Fe protein	0
– Membrane component	20
Only MoFe protein	0
Only Fe protein	5

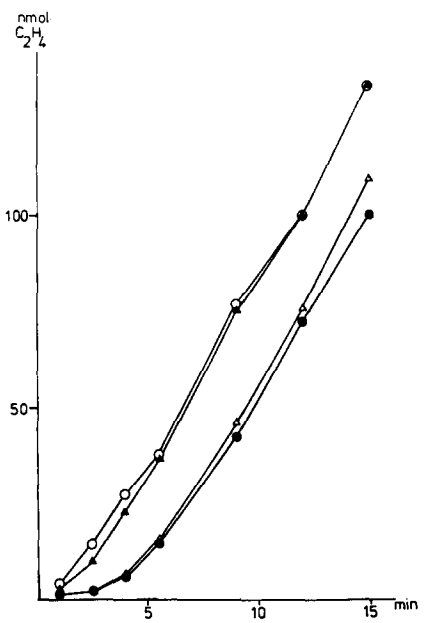

Fig. 6. Effect of preincubation of MC with the MoFe protein and/or the Fe protein. The different combinations were preincubated in the reaction mixture in the same injection bottle as the assay was run. Preincubation, 6 min. ○—○, MoFe protein+Fe protein +MC; ▲—▲, Fe protein +MC. MoFe protein added after preincubation. △—△, MoFe protein +MC. Fe protein added after preincubation. ●—●, MoFe protein +Fe protein +MC, no preincubation (the same curve as with preincubation of only MoFe protein +Fe protein or Fe protein alone). In each assay 0.4 mg MoFe protein, 0.4 mg Fe protein and 0.2 mg MC was used. The assay was performed as described in Fig. 3.

DISCUSSION

The results reported in this communication provide strong evidence in support of our earlier claim [6] that nitrogenase from *R. rubrum* is dependent on a membrane-associated component. This component, which is trypsin and oxygen sensitive, can be solubilized from the membrane by NaCl. The nitrogenase reaction exhibits a lag period indicating the existence of a time-dependent interaction between nitrogenase and the membrane component. This is consistent with the fact that the lag can be eliminated by preincubation of the membrane component with nitrogenase. The most effective preincubation was achieved with ATP, Mg^{2+} and an ATP-generating system present. It is, however, not possible to conclude that ATP is consumed during the interaction of the membrane component and nitrogenase since the preincubation is carried out in the presence of dithionite. Since nitrogenase also can reduce protons to hydrogen in an ATP- and electron-dependent process, this could also explain the ATP consumption.

Titrations of the membrane component and nitrogenase with each other showed that nitrogenase can be saturated with the membrane component and vice versa. This would indicate that there is formation of a complex between the membrane component and nitrogenase which is active during the nitrogenase reaction. The alternative, which would not be favoured by our data, would involve interaction of the membrane component with nitrogenase, before the nitrogenase reaction, and result in transformation of nitrogenase to an "active" state.

Nitrogenase in bacteria in general is known to consist of two components, the MoFe protein and the Fe protein. By using partially separated components we have shown that the interaction of the membrane component is with the Fe protein. There is no indication that there is any interaction with the MoFe protein, at least not prior to the interaction with the Fe protein.

Our interpretation of the results on the interaction of the membrane component with nitrogenase, i.e. the Fe protein, is that nitrogenase is associated with the membranes via the membrane component when the nitrogenase functions physiologically. Since the reduction of nitrogen catalyzed by nitrogenase has a high ATP requirement, the localization of nitrogenase on the photosynthetic apparatus indeed would appear to be favourable to the cell.

Some of the data reported in this communication are similar to those given in a report by Ludden and Burris [18] published during the completion of this work. Further investigations are clearly needed to give a detailed picture of the functioning of the membrane component and its molecular properties as well as of the entire nitrogenase system. It would also be of great interest to find out whether such membrane components occur in nitrogen fixing photosynthetic bacteria in general.

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