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PROPERTIES OF THE NITROGENASE SYSTEM FROM A PHOTOSYNTHETIC BACTERIUM, *RHODOSPIRILLUM RUBRUM*

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Summary

Soluble nitrogenase from *Rhodospirillum rubrum* has been isolated and separated into its two components, the MoFe protein and the Fe protein. The MoFe protein has been purified to near homogeneity and has a molecular weight of 215 000. It contains two Mo, 25—30 Fe and 19—22 acid-labile sulphide and consists of four subunits, M_w 56 000. The Fe protein has a molecular weight of 65 000. It contains approximately four Fe and four acid-labile sulphide and consists of two subunits, M_w 31 500. The highest specific activities for the purified components are 920 and 1260 nmol ethylene produced per min per mg protein, respectively. The purified components require the membrane component for activity (Nordlund, S., Eriksson, U. and Baltscheffsky, H. (1977) Biochim. Biophys. Acta 462, 187—195). Titration of the MoFe protein with the Fe protein shows saturation and excess MoFe protein over Fe protein is inhibitory. Addition of Fe^{2+} or Mn^{2+} to the reaction mixture increases the activity apparently through interaction with the membrane component.

Introduction

The soluble enzyme complex nitrogenase has been isolated and purified from a number of both non-photosynthetic and photosynthetic procaryotes. The most extensive studies have been carried out on nitrogenase from *Azotobacter vinelandii*, *Clostridium pasteurianum* and *Klebsiella pneumoniae*. From these three non-photosynthetic organisms the two components of the nitrogenase complex, the MoFe protein and the Fe protein, have been purified to homogeneity and extensively characterized [1].

Nitrogenase from photosynthetic organisms has been rather intensively studied, but reasonably active preparations have been obtained in only a few cases [2—7]. The only nitrogenase protein which has been highly purified is the MoFe protein from *Chromatium* [8].

The two nitrogenase components are both needed for activity in the ATP-dependent reduction of the substrate, molecular nitrogen to ammonia. In *Rhodospirillum rubrum*, however, there is also a requirement for a third protein, which is membrane associated as has been reported by us [5,9] and by Ludden and Burris [10].

In this paper we report the purification of the two soluble nitrogenase components from *R. rubrum* and give some of their characteristics. The fact that, in addition, a membrane component is necessary for high nitrogenase activity in *R. rubrum* made it important to investigate not only the nitrogenase system from this photosynthetic bacterium for its own sake, but also whether *R. rubrum* nitrogenase is different from nitrogenases from non-photosynthetic organisms in any other fundamental aspect. In particular, the possibility was realized that the membrane component may constitute a physiological link between the photosynthetic and the nitrogenase oxidation-reduction systems.

Materials and Methods

R. rubrum, strain S1, was grown heterotrophically under an atmosphere of N_2/CO_2 (95 : 5, v/v) in the synthetic medium of Bose et al. [11] with omission of the ammonium sulphate. The cells were harvested and broken, and a crude extract was produced as previously reported [9].

The buffer (buffer I) used throughout the purification of the two components was 25 mM Tris-HCl, pH 7.7, containing 1 mM dithioerythritol and sodium dithionite (0.75 mg/ml). Due to the great oxygen sensitivity of nitrogenase, all manipulations were performed under N_2 which had been passed over a heated BASF R3-11 catalyst. Hypodermic syringes which had been gassed with N_2 were used to transfer solutions containing the components. Fractions from the columns were collected in closed injection bottles which had been gassed with nitrogen. The columns were anaerobic as controlled by checking the content of sodium dithionite in the eluate.

The crude extract was applied onto a DEAE-Sepharose-CL-6B column (3.2 X 6 cm) and washed with 100 ml of 0.1 M NaCl and 200 ml of 0.15 M NaCl, which removed some red material. Nitrogenase was eluated as a dark brown band with 0.5 M NaCl using upward flow. This concentrated nitrogenase fraction was separated into the two components on a Sephacryl S-200 column (3.2 X 50 cm, flow rate 1 ml/min). Elution was carried out with 0.5 M NaCl. The MoFe protein eluted as the first brown band and was free from the Fe protein. The second brown band contained the Fe protein but also some MoFe protein. A third brown band contained ferredoxin.

The fractions containing MoFe protein were pooled, diluted three times with buffer I and applied onto a DEAE-Sepharose CL-6B column (2.4 X 10 cm) equilibrated with 0.15 M NaCl. After washing with 100 ml 0.15 M NaCl and 100 ml 0.2 M NaCl, the MoFe protein was eluted with a linear gradient 0.2—0.3 M NaCl. The fractions containing MoFe protein were pooled, diluted two times with buffer I and applied onto a DEAE-Sepharose CL-6B column (2.4 X 2.4 cm) from which the concentrated MoFe protein was eluted with 0.5 M NaCl. A second Sephacryl S-200 column (3.2 X 50 cm, flow rate 0.6 ml/min) in 0.3 M NaCl was used as the last purification step.

The Fe protein fraction from the first Sephacryl S-200 column was further purified according to the same steps as the MoFe protein with the following exceptions: the linear gradient was 0.2—0.35 M NaCl and the second Sephacryl S-200 column was 2.4 X 50 cm, flow rate 0.4 ml/min.

The membrane component was solubilized from chromatophores as described [9]. The ionic strength of the solution containing the membrane component was decreased by repeated dilution with buffer II (25 mM Tris-HCl, pH 8.5 and 0.5 mg $Na_2S_2O_4$ per ml) and concentration on an Amicon Diaflo cell with a PM-10 membrane. The concentrated solution was then pumped on a column of DEAE-Sepharose CL-6B (2.4 X 2 cm) equilibrated with 0.15 M NaCl in buffer II. The membrane component activity passed through the column and was collected as described above. The fractions were concentrated in an Amicon Diaflo cell and this solution, which will be referred to as membrane component, was used in the experiments.

Analytical gel electrophoresis was performed essentially as described by Davis [12]. All solutions were gassed with nitrogen, the top electrode buffer contained 0.2 mg $Na_2S_2O_4$/ml and was gassed with nitrogen during the electrophoresis. Coomassie Brilliant Blue G-250 was used to stain the gels. Sodium dodecyl sulphate (SDS) electrophoresis was performed to determine the molecular weight of the subunits according to Alvares and Siekevitz [13] with the buffer system of Neville [14]. The gels were stained with Coomassie Brilliant Blue R-250. The following proteins were used as molecular weight standards: phosphorylase *a* (100 000), bovine serum albumine (68 000), catalase (58 000), ovalbumine (43 000), carbonic anhydrase (29 000), trypsin inhibitor (17 000) and cytochrome *c* (11 700). Molecular weights were determined from a log M_w vs. relative migration diagram.

Molecular weights of the nitrogenase components were estimated from gel filtration on a column of Sephadex G-200 (1.4 X 93 cm). The flow rate was 4.0 ml/h and 1.3-ml fractions were collected. The following proteins were used as molecular weight standards: catalase (240 000), fumarase (194 000), lactate dehydrogenase (140 000), malate dehydrogenase (70 000), carbonic anhydrase (29 000) and cytochrome *c* (11 700). Molecular weights were determined from a log M_w vs. eluation volume diagram.

Nitrogenase activity was measured as the rate of reduction of acetylene [15]. Protein concentrations were determined by the methods of Lowry et al. [16], Bradford [17] and Goa [18]. Bovine serum albumine was used as standard.

Iron was determined by the bathophenanthroline method of van de Bogart and Beinert [19]. Molybdenum was determined by the toluene dithiol method [20] modified as described previously [9]. Dithionite and dithioerythritol were removed by gel filtration on Sephadex G-25 before determination of acid-labile sulphide by the method of King and Morris [21].

Specific activities for the separated components were determined with one of the components and the membrane component in excess. The protein concentration used in the calculations was that of the limiting component. Specific activities are expressed as nmol ethylene produced per min per mg protein.

Results

Purification of the nitrogenase components

The standard purification procedure developed by us for *R. rubrum* nitrogenase is described above. After this procedure the MoFe protein appeared as a single band both on analytical gel electrophoresis and on SDS gel electrophoresis. No other band could be detected. The Fe protein produced a multiple band pattern on analytical gel electrophoresis even when anaerobic precautions were taken but showed one major band on SDS gel electrophoresis, being slightly contaminated with other proteins. No band that ran as the MoFe protein could be detected. Although not pure, this fraction of the Fe protein was used in the following experiments since the contamination was small and did not contain the MoFe protein.

Characteristics of the separated components

The results of molecular weight determinations and of the analysis of Mo, Fe and acid-labile sulphide are summarized in Table 1. From these data it can be calculated that the MoFe protein consists of four subunits of identical molecular weight and that the Fe protein consists of two subunits with a molecular weight of 31 500.

Some reactions with the separated components

We have obtained MoFe protein and Fe protein with specific activities of 920 and 1260, respectively, and the preparations routinely used had specific activities between 600 and 900 nmol ethylene produced/min per mg protein for both of the components. The time curve of the purified components (Fig. 1) shows a lag which can be eliminated by preincubation with the membrane component as previously reported [9]. The nitrogenase reaction (in presence of membrane component) shows a pH optium between 7.0 and 7.5.

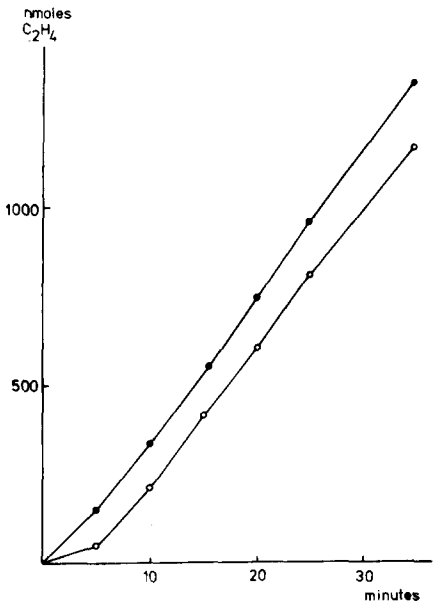
Saturating amounts of membrane component which was determined by titration [9] was used in all experiments. Titration of the MoFe protein with the Fe protein and vice versa is depicted in Fig. 2. The results show that excess of the MoFe protein over the Fe protein is inhibitory. On the other hand, titration of the MoFe protein with the Fe protein shows saturation. These results are in good principal agreement with the results from studies with nitrogenase systems from non-photosynthetic bacteria lacking a membrane component [1].

TABLE I

CHARACTERISTICS OF THE MoFe PROTEIN AND THE Fe PROTEIN FROM *R. RUBRUM*

Analyses were performed as described in Materials and Methods. The numbers for Mo, Fe and acid labile sulphide were based on the determined molecular weights. S^{2-} denotes acid-labile sulphide.

	MoFe protein	Fe protein
Molecular weight	215 000	65 000
Mo	2	—
Fe	25—30	3.8—4.5
S^{2-}	19—22	2.8—5.0
Molecular weight of subunits	56 000	31 500



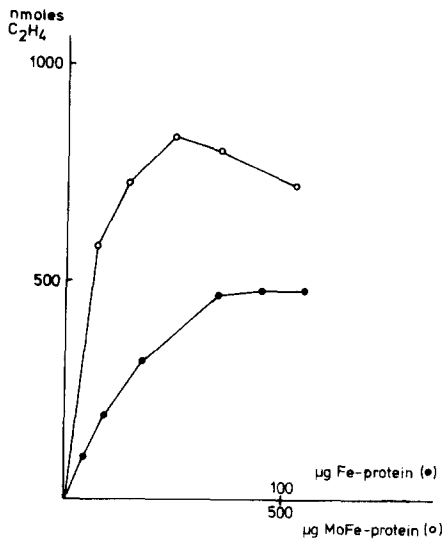


Fig. 1. Preincubation of the purified MoFe protein and Fe protein with membrane component. The reaction was carried out in 22.5-ml injection bottles. The reaction mixture contained 4 mM ATP, 20 mM $MgCl_2$, 10 mM creatine phosphate, 1.5 units of creatine phosphokinase/ml in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), pH 7.4. After gassing with argon, 0.2 ml of 1 mg $Na_2S_2O_4$/ml was added immediately prior to addition of enzyme fractions and 2 ml of acetylene. The total volume was 3.5 ml. The assay was run at 30° C. 0.5-ml gas samples were removed at different time intervals and analyzed for ethylene by gas chromatography. 245 µg MoFe protein, 45 µg Fe protein and 420 µg membrane component were used. o———o, no preincubation; •———•, 6 min preincubation.

Fig. 2. Titration of MoFe protein with Fe protein and vice-versa. The assays were carried out in 12.5-ml injection bottles. The reaction mixture was the same as described in Fig. 1, but 0.1 ml instead of 0.2 ml $Na_2S_2O_4$ was added; total volume 1.7 ml. The reaction was stopped by addition of 0.5 ml 20% $HClO_4$. 0.5-ml gas samples were analyzed for ethylene. o———o, 45 µg of Fe protein, MoFe protein varied; •———•, 35 µg of MoFe protein, Fe protein varied. In both cases 420 µg membrane component was added.

It has been reported [10] that Mn^{2+} stimulates the *R. rubrum* nitrogenase. The results of addition of Mn^{2+} and/or Fe^{2+} to the reaction mixture are shown in Table 2. If no membrane component was added, the metal ions alone did not

TABLE II

EFFECT OF Fe^{2+} AND Mn^{2+}

The assay was carried out as described in Fig. 5. MoFe protein, Fe protein and membrane component were added in optimal concentrations.

Metal ion	Relative activity
None	100
0.3 mM Fe^{2+}	210
0.3 mM Mn^{2+}	205
0.15 mM Fe^{2+} + 0.15 mM Mn^{2+}	205
0.3 mM Fe^{2+} + 0.3 mM Mn^{2+}	210

stimulate nitrogenase. CO^{2+}, Zn^{2+}, Ca^{2+} were also tested but had no effect. In preliminary experiments the membrane component was treated with EDTA. After removal of EDTA, the membrane components had lost 93% of this activity. Reactivation by addition of Mn^{2+} has been partially successful. We have regained approx. 20% of full activity.

Like other nitrogenase systems, *R. rubrum* nitrogenase cannot use nucleotide triphosphates other than ATP as energy source. Inorganic pyrophosphate was also tried, since it has been shown to be produced in *R. rubrum* chromatophores [22] and used as energy donor [23], but it had no effect. We have been unable to find any physiological reductant. NADH, NADPH, malate, succinate and pyruvate were tested using crude nitrogenase preparations with chromatophores added, both in presence and absence of light, but none of them could function as reductant.

Discussion

The results reported in this paper show that, in spite of the absolute requirement of the membrane component for activity, *R. rubrum* nitrogenase has properties very similar to those of other nitrogenase systems. The MoFe protein which has been purified to a very high degree has a molecular weight (215 000) which falls in the range of those reported for other MoFe proteins [24—26]. The MoFe protein seems to consist of four identical subunits with M_w 56 000 which is the same as reported for the MoFe protein from *Rhizobium* and *A. vinelandii* [26—28]. The Mo, Fe and S^{2-} content of the MoFe protein is in good agreement with that of other MoFe proteins [1]. The Fe protein has not yet been purified to quite the same high degree as the MoFe protein, but the data obtained are very similar to those of other Fe proteins [1].

The nitrogenase reaction exhibits a lag when studied with crude preparations of nitrogenase as we have reported earlier [9]. We have shown that this is also the case with totally separated components. The lag can be eliminated by preincubation with the membrane component. Titration of the MoFe protein and Fe protein with each other shows the same pattern as other nitrogenase systems [24,27,29,30], i.e., excess MoFe protein over Fe protein is inhibitory and titration of the MoFe protein with the Fe protein shows saturation. We do not draw any conclusions from these data regarding the obtained ratio of the components since the Fe protein preparations were not sufficiently pure to allow this.

The stimulatory effect of Mn^{2+} in crude extract has previously been reported by Ludden and Burris [10] and it has now been confirmed by us with purified components. In addition we have shown that Fe^{2+} stimulates the *R. rubrum* nitrogenase to the same degree as Mn^{2+}. From the data it can also be concluded that Fe^{2+} and Mn^{2+} probably function in the same way, and that the metal ion effect can apparently be ascribed to interaction with the membrane component.

It is clear from the data reported in this paper that the *R. rubrum* nitrogenase differs in only one major aspect from other nitrogenase systems and that is the requirement of the membrane component. Further studies on this component are needed to understand its function in the nitrogenase system. The fact that it seems to require Fe^{2+} and/or Mn^{2+} for activity could indicate that it

plays some role in the electron transport from a hydrogen donor to nitrogenase. Both the requirement of a membrane protein component for the function of nitrogenase in *R. rubrum* and the effect exerted upon its action by Fe^{2+} and Mn^{2+} are in line with our working hypothesis that a metabolic link may exist in photosynthetic bacteria between their photosynthetic and nitrogenase systems, not only by means of the ATP produced in photophosphorylation. Thus there might exist a direct coupling between light induced electron transport and the nitrogenase system, which requires reducing equivalents at a high energy level in order to catalyze the reduction of molecular nitrogen to ammonia.

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