STABILIZATION AND PARTIAL CHARACTERIZATION OF THE ACTIVATING ENZYME FOR DINITROGENASE REDUCTASE (Fe PROTEIN) FROM *RHODOSPIRILLUM RUBRUM*

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1. Introduction

Nitrogenase from *Rhodospirillum rubrum* grown on dinitrogen or glutamate as nitrogen source requires an activating factor in vitro. This factor can be solubilized from chromatophores of this organism by 0.5 M NaCl [1,2]. The activating factor acts on the Fe protein (i.e., dinitrogenase reductase) [1,2] by cleaving from it a covalently bound modifying group, thereby converting the inactive protein to its active form [3,4]. Preliminary evidence for the existence of this kind of activation of nitrogenase in other photosynthetic bacteria was provided in [5]. The activating factor has proven to be extremely labile and although a few suggestions were made for its purification [2,3] a reproducible method was missing.

We now have found that the activating factor can be stabilized in solutions containing 0.5 mM $MnCl_2$. Here, we provide evidence for its protein aceous nature and henceforth refer to it as the activating enzyme (AE). The enzyme is extremely oxygen-sensitive with a half-life in air of ~2 min. Its M_r as estimated by gel filtration was 20 500 ± 2000.

2. Materials and methods

The growth of *Rhodospirillum rubrum* S1, preparation of chromatophores, nitrogenase assay and the preparation of a crude nitrogenase, whose two components were not separated from each other, all followed published procedures [2]. When the activating enzyme was assayed for its capability to activate nitrogenase, the reaction mixture for acetylene reduction was made 0.5 mM in $MnCl_2$. The standard buffer for purifying AE was 25 mM Tris–acetate (pH 7.8) containing 1 mM dithioerythritol, 0.5 mM $MnCl_2$ and 0.5 mg $Na_2S_2O_4$/ml. Chromatophores were prepared as in [2] and resuspended in standard buffer with a Potter homogenizer. The suspension was centrifuged in the cold for 60 min at 204 000 × *g*. The washed chromatophores were extracted twice with 0.5 M NaCl in standard buffer. Each time they were spun for 90 min at 204 000 × *g* and were discarded after the last centrifugation. The supernatants of the two extraction steps were combined and desalted by ultrafiltration over a PM 10 membrane (Amicon) to ~10 mM residual NaCl. This material was pumped on top of a column of DEAE–Sepharose Cl-6B (2.4 × 3 cm), equilibrated with the Mn-containing standard buffer. The column was developed with a linear gradient of 10–150 mM NaCl in standard buffer (total vol. 160 ml). Fractions with AE started to elute at 50 mM Cl^-. They were concentrated by ultrafiltration to ≤1 ml and passed through a column of Sephacryl S-200 (1.4 × 40 cm), equilibrated with 0.15 M NaCl in standard buffer. Fractions containing AE were used in the experiments. Protein was determined by the method in [6].

3. Results and discussion

The key to successful handling of the activating enzyme was the finding of the stabilizing effect of Mn^{2+}. This was suggested by the requirement of a divalent metal for the activation process which is different from the metal requirement of nitrogenase

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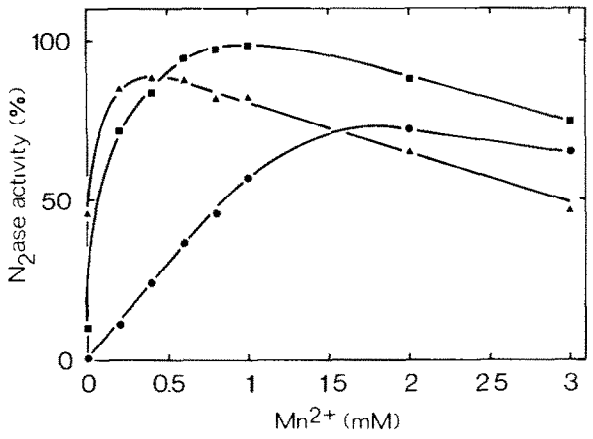

Fig.1. Manganese and magnesium dependency of nitrogenase activity in the presence of the activating enzyme. Three series of standard assays for acetylene reduction contained: 5 mM (•); 10 mM (▪); 25 mM $MgCl_2$ (▲). Manganese was varied in each series from 0–3 mM. All assays contained the same amount of activating enzyme and were started by the addition of inactive nitrogenase. The reaction was run at 30°C and was stopped after 15 min by the addition of 0.5 ml 20% perchloric acid. A gas sample of 0.5 ml was analyzed for ethylene by gas chromatography. The experimental data were normalized for the activity obtained with 10 mM $MgCl_2$ at 1 mM $MnCl_2$. ATP in the assay was 5 mM.

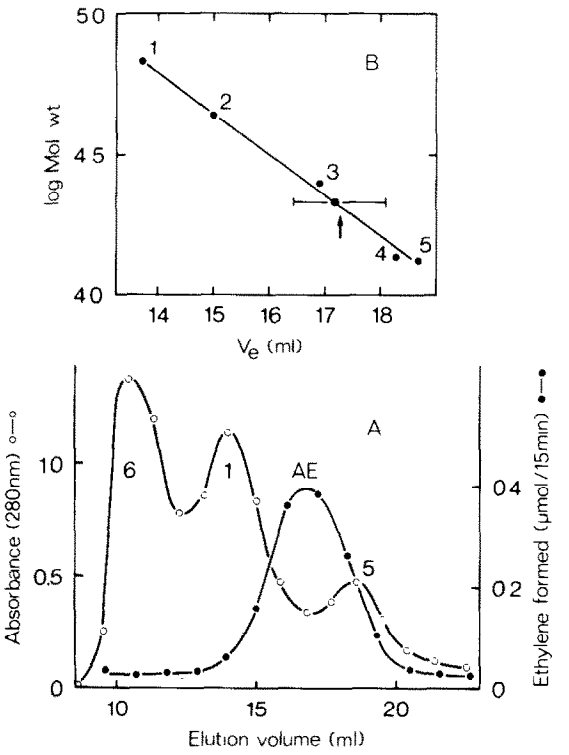

Fig.2. Elution profile of the activation enzyme from a Sephacryl S-200 column (1 × 26 cm) and M_r estimation. The column was equilibrated with 50 mM anaerobic standard buffer, containing 0.5 M NaCl and 0.5 mM $MnCl_2$. (A) The elution profile shows the positions of 2 mg blue dextran (6), 8 mg bovine serum albumin (1) and 3.5 mg cytochrome *c* (5), which were applied as a mixture in 0.7 ml total vol. The salt-solubilized and concentrated activating enzyme was applied in 0.8 ml in the same buffer as the standard proteins. Fractions of 0.8–1 ml (~5 min/fraction) were collected anaerobically and assayed for their capability to activate nitrogenase. AE denotes the position of the activating enzyme. (B) For M_r estimation the column was standardized with (1) bovine serum albumin (67 000); (2) ovalbumin (43 000); (3) chymotrypsinogen A (25 000); (4) ribonuclease A (13 700); (5) cytochrome *c* (11 700). The bar indicates the range of the elution volume for the activating enzyme for 6 determinations with the mean at 20 500 (→).

[1,7,8]. In [4] a model was proposed for the activation process and it was suggested that the metal might bind to the Fe protein or to AE, since it was required in excess of the [ATP] [1]. Fig.1 shows the magnesium and manganese requirement of nitrogenase from *Rhodospirillum rubrum* in the presence of AE. Under our conditions, we found maximal activation around 1 mM $MnCl_2$ in the presence of 5 mM ATP and 10 mM $MgCl_2$. The strongly skewed optimum-type curves indicate a rather complex interaction of both metals with nitrogenase and AE. The rate of activation as well as maximal activity were found to be functions of both metals. The stabilizing effect of Mn^{2+} supports the possibility of a metal-binding site on AE, whose occupation is necessary for its structural integrity. However, simple determination of the affinity constant for manganese [8] can be done only under experimental conditions were activation of the Fe protein is being controlled and can be studied separately from the nitrogenase reaction itself.

Fig.2 shows the elution profile of AE from a small Sephacryl S-200 column which was equilibrated with 0.5 mM $MnCl_2$. AE activity eluted between bovine serum albumin and cytochrome *c*. When manganese was omitted from the chromatography buffer, no or substantially less activity was recovered. This observation provided the clue for using manganese as a stabilizing effector for AE. Standardization of the same column with marker proteins gave a M_r estimate of 20 500 ± 2000 (SD, $n = 6$) (fig.2).

Evidence for the protein nature of the activating component is shown in fig.3. Incubation of AE with trypsin abolished all activating capability after 20 min,

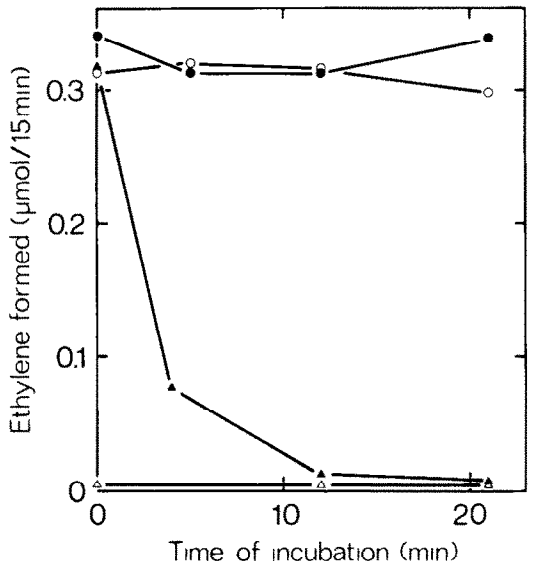

Fig.3. Trypsin sensitivity of the activating enzyme. Three incubation mixtures with the activating enzyme contained the following components: (A) (•) Activating enzyme in 0.5 ml 25 mM Tris–acetate buffer (pH 7.8), 0.15 mM in NaCl, 1 mM in dithioerythritol, 2 mM in $Na_2S_2O_4$ and 0.5 mM in $MnCl_2$. (B) (▲) The same mixture as (A), to which 1 mg trypsin dissolved in 0.1 ml 50 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.3) was added. To (A) 0.1 ml Hepes buffer was added instead of trypsin. (C) (○) The same components as (B) containing in addition 1.9 mg soybean trypsin inhibitor. At the time intervals indicated in the graph, 50 μl samples were withdrawn from series (A–C) and injected into complete reaction mixtures for acetylene reduction. Immediately afterwards 0.9 mg inactive nitrogenase was added and the reaction run under standard conditions. In the control experiment (△) nitrogenase was assayed without the addition of the activating enzyme. To account for a possible non-specific effect of the trypsin inhibitor on nitrogenase and to stop proteolysis in series (B) all nitrogenase reaction mixtures contained 0.5 mg trypsin inhibitor.

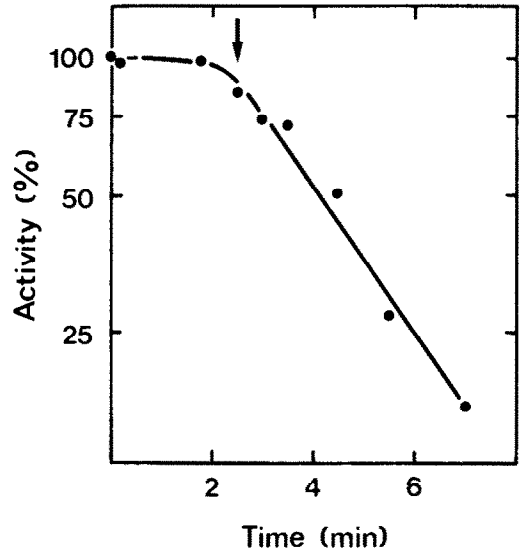

Fig.4. Oxygen sensitivity of the activating enzyme. Activating enzyme (~1 ml) dissolved in standard buffer with 0.15 mM NaCl and 0.5 mM $MnCl_2$, was exposed to air simultaneously with vigorous magnetic stirring. To indicate the complete oxidation of dithionite (→), a trace of methyl viologen was added. At the time intervals indicated in the graph, 50 μl samples were withdrawn and injected into complete dithionite-containing assay mixtures for acetylene reduction from which only nitrogenase was omitted. All samples were started at the same time by injecting 0.9 mg inactive nitrogenase into the assay mixture. The reaction was run under standard conditions.

however, activity was fully preserved when AE was incubated simultaneously with trypsin and trypsin inhibitor from soybean. The lability of AE was due not only to its requirement for Mn^{2+}, but also because of its sensitivity towards oxygen. On exposure to air, we found a half-life for AE of 2 min (fig.4). All handling of and the entire purification procedure for AE thus has to be done under anaerobic conditions, facilitated by the use of dithionite in all solutions as protective reductant. A study of the processes that inactivate AE either by metal depletion or oxygen has to await the availability of highly purified samples.

Ion-exchange chromatography on Sepharose Cl-6B and gel filtration on Sephacryl S-200 provided the first means of a partial purification of AE after its successful stabilization. We define as one unit of activity that amount of AE that activates 1 nmol Fe protein/min at 30°C. Since the conditions for a controlled activation process, physically separated from the nitrogenase reaction, are not yet established, we used the non-linear time course of ethylene production to follow the progress of purification. Samples of AE which were to compare were adjusted to a concentration that gave the same pattern of activation. Once activation of the Fe protein had occurred constant rates of ethylene production which depended on the amount of AE present, were observed (fig.5). An increase in specific activity over the salt-solubilized material of ~10-fold was obtained with the present purification method. From 60 g cell paste ~1 mg partially purified protein was isolated wherein AE contributed <10% to total protein, as estimated from

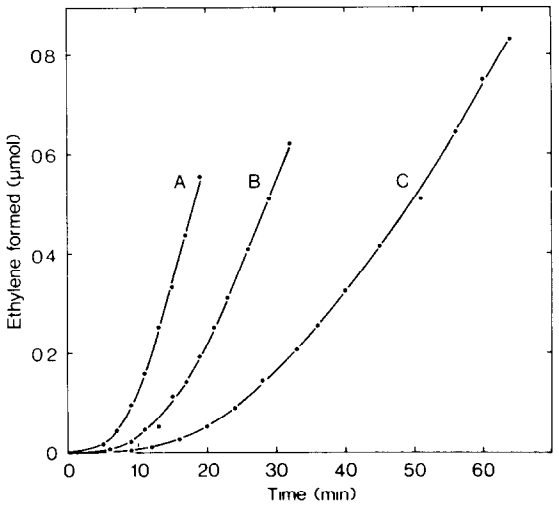

Fig.5. Kinetics of ethylene formation by nitrogenase under the simultaneous action of the activating enzyme. Three standard assays for acetylene reduction contained 200 µl (A), 100 µl (B), or 50 µl (C) of the activating enzyme. Reactions were started by the addition of 1.6 mg inactive nitrogenase at the times indicated in the graph. Samples of 25 µl from the head space of the reaction mixture were analyzed for ethylene. The activity of the activating enzyme alone was nil; the specific activity of nitrogenase prior to activation was 0.05 nmol. (min . mg protein)$^{-1}$.

electrophoretic analysis. Technical limitations in preparing anaerobically large quantities of chromatophores will have to be resolved, before further purification can be achieved. The minute amounts of AE, however, are consistent with its proposed role as a regulatory enzyme.

Acknowledgements

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