

The Nitrogenase System of *Spirillum lipoferum*

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The nitrogenase system of *Spirillum lipoferum* was separated into three components, the normal Mo-Fe and Fe proteins as well as an activating factor for the Fe protein. The rate of activation is increased by Mn²⁺ or by an excess of Mg²⁺, and the process requires ATP.

Nitrogen fixation by *Spirillum lipoferum* has been studied intensively since its rediscovery as a nitrogen-fixing organism by Döbereiner & Day (1976). Associations with a variety of non-leguminous plants have been described (Von Bülow & Döbereiner, 1975), and the physiology of the organism has been investigated (Okon *et al.*, 1976a,b). Okon *et al.* (1977) described crude extracts from *S. lipoferum* that had nitrogenase activity; their results indicated that *S. lipoferum* has a three-component nitrogenase system, Mo-Fe protein, Fe protein and an activating factor for the Fe protein similar to that found in *Rhodospirillum rubrum* (Ludden & Burris, 1976). In the present paper, we report the separation of *S. lipoferum* nitrogenase into three components, none of which has any activity by itself, and describe some of the requirements for activation of the Fe protein.

Materials and Methods

S. lipoferum cells were grown under micro-aerophilic conditions (Okon *et al.*, 1977). The cells were collected anaerobically by sparging centrifuge bottles with N₂ while siphoning the cells into them. The centrifuge bottles were sealed with rubber O-ring tops (DuPont Instruments-Sorvall, Newton, CT, U.S.A.) during centrifugation, and the cells were scooped out of the tubes and into liquid N₂ immediately after collection. Crude extracts were prepared with a French press (Okon *et al.*, 1977) from about 100g of cell paste at a time. All operations were performed anaerobically in the presence of Na₂S₂O₄.

The buffer used in preparing extracts was rather concentrated (300mM-Tris acetate, pH 8.5), so the extracts were diluted 3-fold into 50mM-Tris/acetate (tris(hydroxymethyl)aminomethane adjusted to pH 7.7 with acetic acid) before applying them to an anaerobic DEAE-cellulose column (2.5cm × 5cm). After washing this first column with 50mM-NaCl (50ml in 50mM-Tris acetate, pH 7.7), the Mo-Fe protein was eluted with 200mM-NaCl (30ml in Tris acetate, pH 7.7). This Mo-Fe protein was diluted with 1.5 vol. of 50mM-Tris acetate, pH 7.7, and was applied

to a second DEAE-cellulose column (2.5cm × 5cm); it was eluted with a gradient from 50 to 300mM-NaCl in 50mM-Tris acetate, pH 7.7 (total gradient volume equalled 5 column bed volumes). The Mo-Fe protein at this stage was free from background activity of either Fe protein or activating factor and had a specific activity of about 120nmol of acetylene reduced/min per mg of protein.

Fe protein was eluted from the first DEAE-cellulose column with 450mM-NaCl in 50mM-Tris acetate, after the Mo-Fe protein had been removed, and then the Fe protein fraction was diluted and applied to another DEAE-cellulose column (1cm × 5cm) to concentrate the protein. The protein was washed off immediately with 450mM-NaCl in 50mM-Tris acetate, pH 7.7. The concentrated Fe protein was desalted (50mM-Tris/acetate buffer, pH 7.7) on a Sephadex G-25 column before being purified by preparative polyacrylamide-gel electrophoresis on a 3cm diameter polyacrylamide gel with an 8.3% separating gel (4cm deep) and a 4% acrylamide stacking gel (2cm deep). The gel buffer and reservoir buffer was 100mM-Tris/borate, pH 8.6, and the eluting buffer was 100mM-Tris acetate, pH 8.0. The gel was pre-run for 3h at 60V in the presence of 1mM-Na₂S₂O₄ before loading the protein under the upper reservoir buffer. The protein was electrophoresed into the gel overnight at 50V (150 pulses/s) with an Ortec pulsed power supply, and it was then run into the eluting buffer the next morning at 150V (150 pulses/s). Fe protein obtained in this way appears to be over 95% pure on sodium dodecyl sulphate/polyacrylamide gels and has a specific activity of 510nmol of acetylene reduced/min per mg of protein.

Activating factor was prepared from cells that were grown at a low O₂ concentration (about 0.5%) on 0.5g of NH₄Cl/litre and were then collected anaerobically. NH₃-grown cells make activating factor, but no Mo-Fe or Fe protein, so extracts have no nitrogenase background activity. Cells were broken with a French press and were centrifuged at 45000g for 30min at 5°C. Activating factor was precipitated from the supernatant with 30% (w/w)

poly(ethylene glycol) (PEG 4000; Union Carbide Corp., New York, NY, U.S.A.), and the precipitate was resuspended in 50mM-Tris acetate buffer, pH 7.7. Activating factor from *R. rubrum* can also be prepared from anaerobically collected NH_3 -grown cells; it sediments when centrifuged at 45000g for 120min and can be solubilized from the pellet with 0.5M-NaCl. We also precipitated it with 30% poly(ethylene glycol) before use.

Nitrogenase activity was assayed by the acetylene-reduction technique (Burriss, 1972). ATP and creatine kinase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and phosphocreatine was obtained from Pierce Chemical Co., Rockford, IL, U.S.A.

Results and Discussion

S. lipoferum nitrogenase, like nitrogenase from *R. rubrum*, follows a non-linear time course for acetylene reduction, and its activity is enhanced by Mn^{2+} (Fig. 1). In contrast, nitrogenases such as those from *Clostridium pasteurianum* or *Azotobacter vinelandii* give linear responses and are influenced very little by Mn^{2+} . The role of Mn^{2+} presumably is in the activation process and not in the catalysis of acetylene reduction. Approximately 0.5mM- Mn^{2+} is optimal for both *S. lipoferum* and *R. rubrum* nitrogenases.

Table 1 shows that no two components of *S. lipoferum* nitrogenase are active when incubated with ATP, $\text{Na}_2\text{S}_2\text{O}_4$ and metal cations. All three components are required to support acetylene reduction. Table 1 also shows that *R. rubrum* activating factor will substitute for *S. lipoferum* activating factor.

Because the Mo-Fe protein from *S. lipoferum* is active as isolated when crossed with the Fe protein from *K. pneumoniae*, and shows no unusual metal cation requirements (results not shown), it appears that the Mo-Fe protein from it does not require activation. Fig. 2 shows that there was linear reduction of acetylene when the Mo-Fe protein was added to a reaction mixture whose components (activating factor, reductant, ATP, Fe protein and metal cations)

had been preincubated together for 20min. Thus Mo-Fe protein is not the activated species nor is it required for activation. If activating factor was withheld during the incubation, there was a lag in acetylene reduction on addition of activating factor and Mo-Fe protein (Fig. 2). ATP is also required for activation, because there is a lag when it is withheld during incubation (Fig. 2). *R. rubrum* activating factor was used in the present experiment,

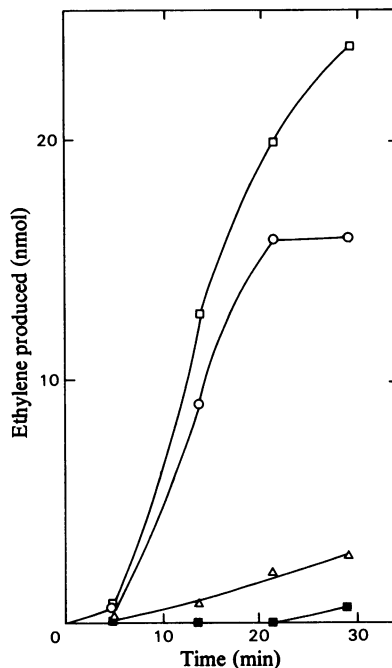


Fig. 1. Time-course of acetylene reduction by nitrogenase from *S. lipoferum* when components of the reaction mixture were not preincubated together

□, 20mM- Mg^{2+} , 0.5mM- Mn^{2+} ; ○, 10mM- Mg^{2+} , 0.5mM- Mn^{2+} ; △, 20mM- Mg^{2+} , no added Mn^{2+} ; ■, 10mM- Mg^{2+} , no added Mn^{2+} .

Table 1. Activation of the Fe protein from *S. lipoferum*

Assay conditions were: 5mM-ATP, 20mM-phosphocreatine, 0.025 mg of creatine kinase, 20mM- Mg^{2+} , 0.5mM- Mn^{2+} , 10mM- $\text{Na}_2\text{S}_2\text{O}_4$, in a total volume of 0.5 ml at 30°C. The Mo-Fe protein concentration was 2 mg/ml and the Fe protein concentration was 3 mg/ml. Abbreviations: AF, activating factor from *S. lipoferum*; RAF, activating factor from *R. rubrum*.

Mo-Fe protein (μl)	Fe protein (μl)	AF (μl)	RAF (μl)	Acetylene reduced (nmol)		
				5 min	15 min	25 min
20	0	50	0	0	0	
0	10	50	0	0	0	
20	10	0	0	0	0	
20	10	50	0	1.2	12	
20	10	0	50	8	48	
						19
						72

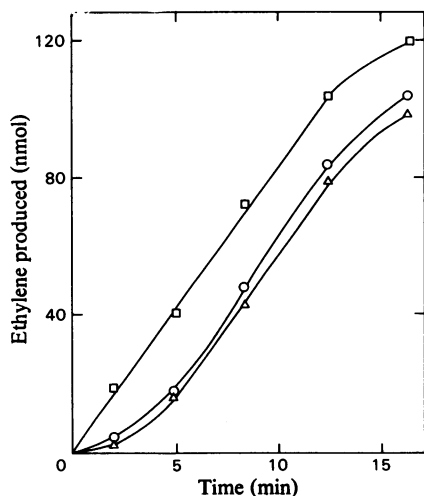


Fig. 2. Time course of acetylene reduction by the nitrogenase from *S. lipoferum*.

Symbols: □, 30 μ l of Fe protein from *S. lipoferum* was incubated for 20 min at 30°C with 20 mM-Mg²⁺, 0.5 mM-Mn²⁺, 10 mM-Na₂S₂O₄, 50 μ l of *R. rubrum* activating factor, 5 mM-ATP and an ATP-generating system; ○, Fe protein incubated as in (□) except that activating factor was added with the Mo-Fe protein after the 20 min incubation; △, Fe protein incubated as in (□) except that ATP was added with the Mo-Fe protein after the 20 min incubation.

because we were able to prepare it with higher activity.

Although *R. rubrum* is a photosynthetic and normally anaerobic bacterium and *S. lipoferum* is non-photosynthetic and aerobic, their metabolism is similar. Both depend primarily on tricarboxylic acid-cycle enzymes for cellular energy, and neither grows well on carbohydrates (Okon *et al.*, 1976b; Kondrat'eva, 1965). Whether the three-component system is restricted to N₂-fixing bacteria with such metabolism remains to be established. Nitrogenase from *Mycobacterium flavum*, a micro-aerophilic N₂-fixer that grows poorly on carbohydrates, has been

partially purified by Biggins *et al.* (1971). Their results give no indication of an *R. rubrum*-type activating factor, but the authors do not discuss linearity of substrate reduction with time.

We have only found two N₂-fixing bacteria that require an activating factor for their Fe protein. This third protein may well be involved in the regulation of their nitrogenases. Although all nitrogenases studied require both Fe and Mo-Fe proteins to function, it is probable that a variety of non-genetic controls are used by different micro-organisms for the regulation of their nitrogenase activity (Shah *et al.*, 1972; Seto & Mortenson, 1973; Kleiner, 1975).

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