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PHYSIOLOGICAL ELECTRON DONOR SYSTEMS TO THE NITROGENASE OF THE BLUE-GREEN ALGA *ANABAENA CYLINDRICA*

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SUMMARY

Reduced ferredoxin was found to function as electron donor to nitrogenase present in extracts prepared from the blue-green alga *Anabaena cylindrica*. Two physiological mechanisms of reducing ferredoxin were identified. The major physiological mechanism of reducing ferredoxin was a process linked to the photosynthetic electron transport chain which was dependent on light. An alternative source of reducing potential was $NADP^+$ reduced in dark reaction by substrates such as isocitrate or pyruvate. Transfer of electrons from NADPH to nitrogenase was dependent on ferredoxin–$NADP^+$ reductase and ferredoxin.

INTRODUCTION

The ability to fix atmospheric nitrogen is found in some species of blue-green algae in aerobic and anaerobic bacteria and in bacterioids from leguminous root nodules. In studies with cell-free extracts of these organisms it has been found that requirements for nitrogenase activity are ATP and a reductant[1]. The artificial reductant commonly used experimentally is sodium dithionite. The physiological reductant for nitrogenase in the anaerobic bacterium *Clostridium pasteurianum* has been identified as ferredoxin[2]. The ferredoxin is reduced by pyruvate in the phosphoroclastic reaction. Pyruvate has also been shown to support nitrogenase activity in cell-free extracts of *Bacillus polymyxa*[3] and *Chloropseudomonas ethylicum*[4]. By employing illuminated chloroplasts as a source of reducing potential two possible electron carriers to Azotobacter nitrogenase, azotoflavin and Azotobacter ferredoxin were demonstrated[5,6]. YOCH *et al.*[7] showed that a protein similar to Azotobacter ferredoxin was also a possible electron carrier for the nitrogenase system of Rhizobium bacteriods. These organisms lack the phosphoroclastic reaction and the mechanism for reducing all these electron carriers is unknown.

There have only been limited studies on nitrogen fixation in photosynthetic microorganisms. YOCH AND ARNON[8] showed that photoreduced ferredoxin could act as electron donor to Chromatium, nitrogenase. However, they employed spinach chloroplasts to reduce the ferredoxin rather than a physiological system from the

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; HEPES, *N*-2-hydroxyethylpiperizine-*N'*-2-ethanesulfonate.

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organism. EVANS AND SMITH[4] demonstrated that light-dependent ferredoxin reduction by chlorophyll-containing particles prepared from the green photosynthetic bacterium *Chl. ethylicum* could be coupled to nitrogenase from this organism.

The present communication reports evidence that ferredoxin is the physiological electron donor to the nitrogenase of the blue-green alga *Anabaena cylindrica*. It seems likely that the main source of reducing potential for nitrogen reduction in this organism is the photosynthetic electron transport chain. We have found that ferredoxin reduced by lamellar particles can function as electron donor to the nitrogenase. An alternative source of reducing potential is $NADP^+$ reduced in dark reactions by substrates such as isocitrate or pyruvate. The transfer of electrons from reduced $NADP^+$ to nitrogenase requires ferredoxin.

MATERIALS AND METHODS

A. cylindrica was grown as described previously[9,10]. Cell-free extracts were prepared by sonication as described previously[9,10]. $MgCl_2$ was added to the suspension before sonication to give a concentration of 0.01 M $MgCl_2$. The extract was centrifuged for 30 min at 78000 × *g* and the supernatant used for subsequent experiments without further treatment. In experiments employing NADPH as electron donor the supernatant was passed through a column of Sephadex G-75 to remove ferredoxin and other low molecular weight compounds.

Preparation of particles

The cell paste was suspended 1:4 (w/v) in 0.02 M potassium *N*-2-hydroxyethylpiperizine-*N'*-2-ethanesulfonate (HEPES) buffer (pH 7.5) containing 0.4 M sucrose and 0.01 M NaCl. After briefly homogenizing the suspension it was sonicated for 5 min and the crude extract centrifuged for 15 min at 40000 × *g* to remove unbroken cells and large particles. $MgCl_2$ was then added to give a concentration of 0.01 M $MgCl_2$ and the supernatant was centrifuged for 1 h at 40000 × *g*. The pellet was resuspended in extracting medium diluted 1:10 to give a concentration of 1.0 mg chlorophyll per ml. Before use in experiments the particles were heated for 5 min at 55° to destroy their ability to evolve oxygen. The particles could be conveniently stored in liquid nitrogen without loss of activity.

Preparation of ferredoxin

Extracts for ferredoxin isolation were prepared by suspending the cell paste in 0.02 M potassium phosphate buffer (pH 7.5), containing 0.01 M $MgCl_2$ (1:4, w/v). The suspension was passed through an A.P.V. Manton-Gaulin Laboratory homogenizer at a pressure of 5000 lb/inch^2 and then centrifuged at 22000 × *g* for 30 min. The green pellet was discarded. The dark blue supernatant was then passed through a 10 cm × 4 cm column containing DEAE-cellulose DE-23 equilibrated with buffer containing 0.2 M NaCl. Ferredoxin was eluted with 0.8 M NaCl in buffer. Subsequent purification procedures were the same as those described by EVANS *et al.*[11].

Experimental procedures

The assay procedures for nitrogenase activity were those described previously[9,10] employing the reduction of acetylene to ethylene[12]. A Varian Aerograph gas chroma-

togram with a column (6 ft × 0.25 inch) of Poropak R was used to estimate ethylene. Chlorophyll was estimated by the method of HAGER AND MEYER-BERTENRATH[13]. Protein was estimated by the phenol method, as modified by RABINOWITZ AND PRICER[14]. Spinach ferredoxin-$NADP^+$ reductase was prepared as described by SHIN AND ARNON[15]. The level of the enzyme present in extracts was estimated by the method of DAVENPORT AND HILL[16]. Creatine phosphate and DL-isocitrate was obtained from Sigma Chemical Co. London, Great Britain. Isocitrate dehydrogenase, creatine kinase, ATP and $NADP^+$ were obtained from Boehringer Corp., London. HEPES buffer was supplied by Calbiochem. Other compounds were analytical reagent grade.

RESULTS

The addition of $MgCl_2$ to the crude extract resulted in the removal of mucilaginous material and chlorophyll-containing particles which were present in the extracts[9,10]. Table I shows the requirements for nitrogenase activity employing *A. cylindrica* ferredoxin photoreduced by particles prepared from *A. cylindrica*. This nitrogenase system shows requirements for ATP, the ATP generating system and ferredoxin and also for light and for *A. cylindrica* particles. Because of the oxygen sensitivity of the *A. cylindrica* nitrogenase[10] reduced 2,6-dichlorophenolindophenol (DCIP) was employed as electron donor to *A. cylindrica* particles instead of the oxygen-evolving water system. The optimum *A. cylindrica* ferredoxin concentration for this system was 6.0 mM. Increased levels of ferredoxin were inhibitory. The reaction proceeded linearly for 20 min at 30°.

Although good rates of acetylene reduction were obtained with this assay procedure there was a possibility that ferredoxin was not donating electrons directly to the nitrogenase. Since $NADP^+$ is the electron carrier between the photosynthetic electron transport chain and biosynthetic enzyme systems[17] it seemed possible that $NADP^+$ might act as an electron carrier between ferredoxin and nitrogenase.

TABLE I

REQUIREMENTS FOR ACETYLENE REDUCTION BY EXTRACTS OF *A. cylindrica* WITH FERREDOXIN AS ELECTRON DONOR

Complete reaction mixture contained in a final volume of 1.5 ml: potassium HEPES buffer (pH 7.3), 100 μmoles; ATP, 4.0 μmoles; creatine phosphate, 10 μmoles; creatine kinase, 50 μg; $MgCl_2$, 10 μmoles; sodium ascorbate, 20 μmoles; DCIP, 0.2 μmole; *A. cylindrica* ferredoxin, 100 μg; nitrogenase extract containing 5.0 mg protein; *A. cylindrica* particles containing 0.2 mg of chlorophyll *a*. The gas phase was 90% argon and 10% acetylene. The incubation was for 10 min at 30° at a light intensity of 70000 lux.

Conditions	C_2H_4 *formed* (*nmoles*)
Complete light	232
Complete dark (ferredoxin not reduced)	0
ATP omitted	0
Creatine phosphate and creatine kinase omitted	0
Ferredoxin omitted	27
Particles omitted	0
Extract omitted	0
$MgCl_2$ omitted	18
Ascorbate and DCIP omitted	0

The experiments described above were repeated in the presence of a saturating level of ferredoxin–$NADP^+$ reductase and a range of concentrations of $NADP^+$ from 0.015 to 1.5 mM. No increase in the rate of acetylene reduction was observed, and at concentrations of $NADP^+$ above 0.75 mM acetylene reduction was inhibited completely. However, these experiments did not exclude the possibility that the light-dependent cell-free nitrogenase activity required low levels of $NADP^+$ and ferredoxin–$NADP^+$ reductase present in the crude extract. An alternative assay procedure not involving light and *A. cylindrica* particles was therefore devised in which NADPH was produced by a regenerating system dependent on isocitrate and isocitrate dehydrogenase in a dark reaction. After optimisation of the concentrations of reactants for this procedure rates of acetylene reduction obtained were the same as in the light-dependent system. However, the possibility still remained that NADPH was not donating electrons directly to nitrogenase but that ferredoxin–$NADP^+$ reductase and ferredoxin present in the extract were required. After gel filtration of the extract to remove ferredoxin it was possible to show a ferredoxin requirement for the NADPH system.

TABLE II

REQUIREMENTS FOR NADPH-SUPPORTED ACETYLENE REDUCTION BY A SEPHADEX G-75 TREATED *A. cylindrica* EXTRACT

Complete reaction mixture contained in a final volume of 1.5 ml: potassium HEPES buffer (pH 7.3), 100 μmoles; ATP, 4.0 μmoles; creatine phosphate, 10 μmoles; creatine kinase, 50 μg; $NADP^+$, 0.4 μmole; isocitrate, 10 μmoles; isocitrate dehydrogenase, 50 μg; ferredoxin–$NADP^+$ reductase, 65 μg; *A. cylindrica* ferredoxin, 100 μg; Sephadex G-75 treated nitrogenase extract containing 2.4 mg protein. The gas phase was 90% argon, 10% acetylene. The incubation was for 10 min at 30°.

Conditions	C_2H_4 *formed* (*nmoles*)
Complete	211
ATP omitted	9
Creatine phosphate and creatine kinase omitted	13
Ferredoxin omitted	40
Ferredoxin–$NADP^+$ reductase omitted	211
Isocitrate dehydrogenase omitted	249
Isocitrate omitted	6
$NADP^+$ omitted	4
$MgCl_2$ omitted	0

The requirements for NADPH-supported acetylene reduction are shown in Table II. The system shows requirements for ATP and an ATP generating system, which are also requirements for the light-dependent particle system. There are also requirements for $NADP^+$, isocitrate and $MgCl_2$. There is no requirement for added ferredoxin–$NADP^+$ reductase or isocitrate dehydrogenase because of the presence of these enzymes in the Sephadex G-75 treated extract. Their activities separately determined were as follows: isocitrate dehydrogenase, 0.07 μmole $NADP^+$ reduced per mg protein per min; ferredoxin–$NADP^+$ reductase, 1.1 μmoles cytochrome *c* reduced per mg protein per min.

In previous studies we have failed to obtain nitrogenase activity in the presence

of pyruvate[10]. However, in the present experiments we found that the addition of pyruvate instead of isocitrate in the presence of $NADP^+$ resulted in good rates of acetylene reduction. Presumably the reductant for *A. cylindrica* nitrogenase in this system is NADPH, which is reduced by pyruvate oxidase. Electrons are then transferred to nitrogenase *via* the intermediate electron carriers ferredoxin–$NADP^+$ reductase and ferredoxin as in the system with isocitrate as electron donor.

DISCUSSION

These experiments show ferredoxin is the electron donor to nitrogenase in *A. cylindrica*. The electron transport requirements for nitrogen fixation in *A. cylindrica*, are summarised in Fig. 1. Both the light-dependent particles system and the NADPH system show ferredoxin dependency. The ferredoxin from *A. cylindrica* used in these experiments is a normal plant-type ferredoxin. It is a one electron transfer protein, unlike the ferredoxin from other nitrogen fixing organisms, which transfer 2 electrons per mole. It seems probably that the major physiological mechanism of nitrogen reduction by blue-green algae is a light-dependent process linked by ferredoxin to the photosynthetic electron transport chain. There is no evidence to suggest that any intermediate electron carriers between ferredoxin and nitrogenase are required.

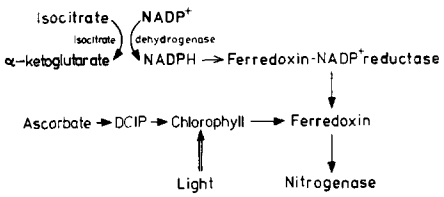

Fig. 1. Electron transport requirements for nitrogenase activity by *A. cylindrica* extracts, employing ferredoxin as electron donor.

The reduction of ferredoxin by NADPH linked to a system which regenerates reduced $NADP^+$ has been shown by SHIN AND ARNON[15]. However, the NADPH-linked mechanism of nitrogen fixation in *A. cylindrica* is probably of limited physiological importance since only about 5 % of the light saturated rate of acetylene reduction is observed in cells incubated in the dark[13]. This type of system may be of physiological importance in organisms such as Azotobacter and the Rhizobium bacteriods which lack light dependent or the phosphoroclastic mechanism of ferredoxin reduction. It seems possible that the nitrogenase activity observed by COX AND FAY[18] in extracts of *A. cylindrica* in the presence of pyruvate may be explained by the presence of $NADP^+$ in their extracts. Similarly, the low rates of acetylene reduction recently reported by YATES AND DANIEL[19] in cell-free extracts of *Azotobacter chroococcum* with added NAD^+ may depend on the presence of $NADP^+$ and ferredoxin in their extracts.

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