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THE ELECTRON TRANSPORT SYSTEM IN NITROGEN FIXATION BY AZOTOBACTER

III. REQUIREMENTS FOR NADPH-SUPPORTED NITROGENASE ACTIVITY

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SUMMARY

Evidence has been obtained that NADPH may serve as a physiological source of reducing power for nitrogenase activity in *Azotobacter vinelandii*. NADH was ineffective. Electron transfer from NADPH to nitrogenase depended on four factors native to *A. vinelandii* cells: azotobacter ferredoxin, azotoflavin, a component replaceable by spinach ferredoxin-$NADP^+$ reductase and another soluble, heat-labile component not yet chemically characterized. The four factors probably constitute an electron transport chain between NADPH and nitrogenase.

INTRODUCTION

The capacity for biological nitrogen fixation is found in many, taxonomically different bacteria and blue-green algae. The nitrogenase enzyme complex involved in this process requires for its functioning both ATP and a strong reductant[1-3]. Dithionite (a nonphysiological reductant) is commonly used to measure the capacity of a given nitrogenase extract to reduce a substrate. In the obligate anaerobic bacteria represented by *Clostridium pasteurianum* the reductant for nitrogenase is reduced ferredoxin[1,2] that can be generated by an endogenous pyruvate clastic reaction[4,5].

Recently we reported the isolation from *Azotobacter vinelandii* cells of two electron carriers, azotoflavin and azotobacter ferredoxin, each able to mediate electron transfer from illuminated chloroplasts[6] to Azotobacter nitrogenase[7,8]. These experiments, while identifying two endogenous electron carriers linked to the Azotobacter nitrogenase system, left open the question how they are reduced prior to reacting with the nitrogenase system since Azotobacter[9], unlike *C. pasteurianum*, does not have a pyruvate clastic reaction that can generate reduced ferredoxin.

This paper presents evidence that the source of electrons transferred to Azotobacter nitrogenase by azotoflavin and azotobacter ferredoxin may be NADPH. Other components of an endogenous electron transport system for nitrogen fixation in Azotobacter are discussed.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid.

METHODS

A. vinelandii cells were grown and fractionated as previously described[7]. The cell extract preparation used in a number of experiments was the supernatant fluid obtained by centrifuging the disrupted cells for 30 min at 30000 × *g*. The cell extract was centrifuged at 105000 × *g* for 1 h, the pellet was discarded, and the supernatant fluid centrifuged at 145000 × *g* for 5 h. The sedimented pellet from the 5-h centrifugation (P_5) was the partly purified nitrogenase fraction. As discussed later, the corresponding supernatant fluid (S_5) contained a factor required for nitrogenase activity. In some experiments, the cell extract (15 ml) was passed through a column of DEAE-cellulose (2.5 cm × 4 cm) equilibrated with 0.02 M *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) to selectively remove the electron carriers, azotoflavin, and azotobacter ferredoxin.

Because the column became quickly saturated, only the more acidic proteins, such as azotobacter ferredoxin and azotoflavin, were bound to the DEAE-cellulose; more weakly acidic proteins, such as the Azotobacter nitrogenase complex, were not retained by the column and were recovered completely in the column effluent. Under different conditions (including a previous protamine sulfate treatment), other investigators have been able to bind Azotobacter nitrogenase to DEAE-cellulose[10,11]. However, this was not attempted in the present investigation.

The NADPH generator used in these experiments consisted of 0.01 mg glucose-6-phosphate dehydrogenase and the following in μmoles: glucose 6-phosphate, 20; $NADP^+$, 1.0; $MgCl_2$, 2; and HEPES buffer (pH 7.8), 20; in a volume of 0.1 ml. The ATP generator consisted of phosphocreatine kinase, 0.15 mg; creatine phosphate, 40 μmoles; and ATP, 4 μmoles.

Azotoflavin and azotobacter ferredoxin were prepared as previously described[7,8]. The coupling of the reducing power generated by illuminated spinach chloroplasts to Azotobacter nitrogenase activity was carried out as described by YOCH AND ARNON[6]. Nitrogenase activity, measured by the reduction of acetylene to ethylene, was determined by gas chromatography[12,13]. Spinach ferredoxin-$NADP^+$ reductase, a gift of Richard K. Chain, was prepared as described by SHIN *et al.*[14].

RESULTS AND DISCUSSION

Since NADPH and NADH are carriers of reducing power liberated from various substrates by cellular dehydrogenases, one or both of these dinucleotides might mediate in Azotobacter cells the transfer of reducing power to the nitrogenase system. Such a transfer would involve the reduction of ferredoxin by NADH or NADPH—a reaction that is thermodynamically unfavorable since the oxidation–reduction potential of the nicotinamide adenine dinucleotides is about 100 mV more positive than that of ferredoxins[15,16]. (The oxidation–reduction potential of azotobacter ferredoxin[8] has not yet been determined, but it seems likely to be similar to that of other bacterial ferredoxins.) Nevertheless, the reduction of spinach ferredoxin by NADPH has been shown to proceed[15,17], particularly when linked to an NADPH-generating system such as isocitrate dehydrogenase which maintains a high NADPH/$NADP^+$ ratio[17]. Further evidence for an effective electron flow from these dinucleotides to clostridial ferredoxin was recently reported by JUNGERMANN *et al.*[18] and THAUER

et al.[19] who obtained, in a cell-free system of *Clostridium kluyveri*, a ferredoxin-dependent formation of molecular hydrogen using NADPH or NADH as electron donors. There is thus little reason to doubt, on thermodynamic grounds, that metabolically generated NADPH or NADH could serve (*via* ferredoxin) as an electron donor for nitrogenase activity in cells.

NADPH can serve not only as an electron donor for ferredoxin but also as a reductant for the FMN prosthetic group of azotoflavin (Fig. 1). This reaction was mediated by spinach ferredoxin–$NADP^+$ reductase. The increase in absorbance at 580 nm indicates the reduction of the oxidized flavoprotein to the blue semiquinone[20,21]. There was no subsequent decrease of absorbance at 580 nm, indicating that the reduction of azotoflavin did not go beyond the semiquinone state. The lag period in the reduction was minimal under anaerobic conditions but appreciable under aerobic conditions.

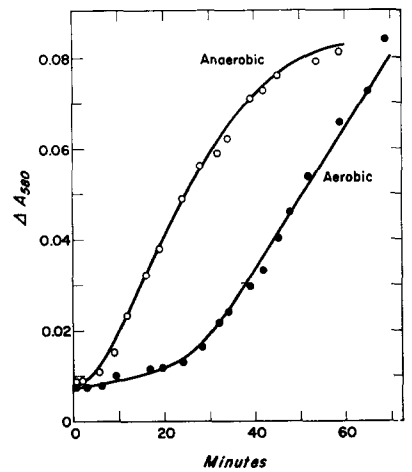

Fig. 1. Reduction of azotoflavin to the semiquinone by an NADPH generating system. The complete reaction mixture (final volume 1.0 ml) contained an NADPH generator, 0.032 μmole azotoflavin, and 0.015 mg spinach ferredoxin–$NADP^+$ reductase. For anaerobic conditions the stoppered cuvette was flushed with argon through a syringe needle for 5 min.

Several investigators have used NADH as an electron donor for nitrogenase[2,22] but we have found no similar systematic investigations with NADPH. YATES AND DANIEL[23] have recently described experiments with cell-free preparations of *Azotobacter chroococcum* in which either substrate amounts of NADH or catalytic amounts of NAD^+ in the presence of carbon substrates served as the electron donor for nitrogenase. No NADPH (or catalytic amounts of $NADP^+$) were tried although the substrate that was most effective in their experiments, glucose 6-phosphate, undergoes dehydrogenation by an $NADP^+$-linked dehydrogenase (see below). The activity of the Azotobacter nitrogenase in these experiments was low[23], possibly because of a lack of $NADP^+$.

The ability of NADPH (continuously generated) to act as an electron donor was tested in a model system comprised of partly purified *A. vinelandii* nitrogenase, clostridial ferredoxin, and spinach ferredoxin–$NADP^+$ reductase, an enzyme that

reversibly mediates electron transfer between NADPH and ferredoxin. As seen in Table I, NADPH-supported nitrogenase activity was appreciable and amounted to about 40 % of that obtained when ferredoxin was photochemically reduced by spinach chloroplasts. (The activity of the chloroplast-nitrogenase system was comparable to that of the dithionite-nitrogenase system—approx. 6000 nmoles ethylene formed per 30 min with the same amount of Azotobacter nitrogenase.) The significance of this experiment is that an NADPH-generating system can support (in a model system) ferredoxin-mediated nitrogenase activity at an appreciable rate.

TABLE I

A COMPARISON OF AZOTOBACTER NITROGENASE ACTIVITY COUPLED TO PHOTOCHEMICALLY GENERATED REDUCING POWER OR TO NADPH

The complete reaction mixture (final volume 1.5 ml) contained 10 mg Azotobacter nitrogenase (P_5); 100 μg clostridial ferredoxin; 50 μmoles HEPES buffer (pH 7.4); 5 μmoles Mg^{2+}; and a ATP generator (see METHODS). In addition the vessels contained either spinach chloroplasts (300 μg chlorophyll) or a NADPH generating system (see METHODS) and spinach ferredoxin–$NADP^+$ reductase, 0.15 mg. Gas phase, 73% argon and 27% acetylene. The reaction was carried out at 30°. Illumination, 10000 ft-candles.

Source of reducing power	*Ethylene formed/30 min (nmoles)*
Illuminated chloroplasts	5060
NADPH generator	1950

In subsequent experiments, the effectiveness of NADPH and a number of organic substrates as electron donors for nitrogenase was investigated using freshly prepared *A. vinelandii* cell extracts supplemented with azotoflavin. Azotobacter ferredoxin appeared to be sufficient in the crude extracts used. As shown in Table II, the substrates best able to support nitrogenase activity were malate, glucose 6-phosphate, and isocitrate. Appreciable increases over the control were also obtained with α-ketoglutarate, citrate, and β-hydroxybutyrate. Coenzyme A and lipoic acid, added in the presence of pyruvate and α-ketoglutarate, had no effect.

NADPH (added either in substrate quantities or as an NADPH generating system) was as good a hydrogen donor for nitrogenase as any of the substrates tested (Table II). NADH had no activity in these experiments.

Since the most effective substrates for nitrogenase (malate, glucose 6-phosphate, and isocitrate, in Table II) have $NADP^+$-linked dehydrogenases, it seemed possible that their effectiveness (without added $NADP^+$) was due to endogenous $NADP^+$ (and the respective dehydrogenases) in the crude extract. To test this possibility, the effects of glucose 6-phosphate and isocitrate on nitrogenase activity were tested in a dialyzed extract. Both substrates showed a marked stimulation of nitrogenase activity (0.25 and 0.14 nmoles ethylene/min per mg protein, respectively) caused by the addition of $NADP^+$ to dialyzed cell extract. The addition of NAD^+ gave zero activity; nor did substrates that are known to have NAD^+-linked dehydrogenases (β-hydroxybutyrate and malate) show any activity with this cofactor.

The cell extract was dialyzed anaerobically (0.025 M HEPES buffer, pH 7.4) for only 4 h since longer dialysis (14–18 h), while not damaging dithionite-supported

TABLE II

EFFECT OF ORGANIC SUBSTRATES ON ACETYLENE REDUCTION BY AN AZOTOBACTER EXTRACT

The complete reaction mixture (final volume 1.5 ml) contained crude extract, 14 mg protein, and the following in μmoles: azotoflavin, 0.036; HEPES buffer (pH 7.4), 50; Mg^{2+}, 5; and an ATP generator. In addition, each vessel contained one of the following: 40 μmoles of a substrate, 10 μmoles NADPH or NADH, or a NADPH generator. Gas phase, 73% argon and 27% acetylene. The reaction was carried out at 30°.

Substrate	*Ethylene formed/30 min (nmoles)*
None	30
NADPH generator	195
NADPH	188
NADH	31
Malate	220
Glucose 6-phosphate	180
Isocitrate	150
α-Ketoglutarate	103
Citrate	98
β-Hydroxybutyrate	67
Glyceraldehyde 3-phosphate	32
Pyruvate	33
Formate	31

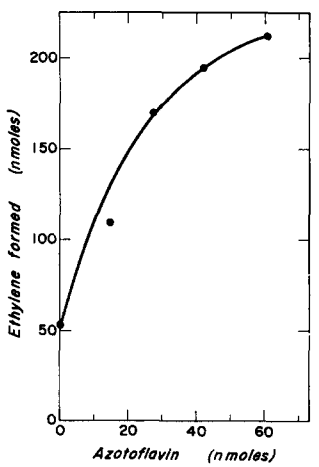

Fig. 2. Effect of azotoflavin on NADPH-supported nitrogenase activity. The complete reaction mixture (final volume 1.5 ml) contained an ATP and an NADPH generator, 12.1 mg Azotobacter extract (S_1), and the following in μmoles: azotobacter ferredoxin, 0.021; HEPES buffer (pH 7.4), 50; Mg^{2+}, 5; and azotoflavin as indicated. Other experimental conditions were as described for Table I.

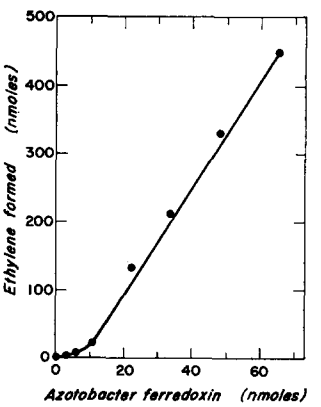

Fig. 3. Effect of azotobacter ferredoxin on NADPH-supported nitrogenase activity of a DEAE-cellulose treated cell extract. The complete reaction mixture (final volume 1.5 ml) contained an ATP and an NADPH generator, DEAE-cellulose treated Azotobacter extract, 12 mg, and the following in μmoles: spinach ferredoxin–$NADP^+$ reductase, 0.004; azotoflavin, 0.028; HEPES buffer (pH 7.4), 50; Mg^{2+}, 5; and azotobacter ferredoxin as indicated. Other experimental conditions were as described for Table I.

nitrogenase activity (17 nmoles ethylene formed/min per mg protein), completely abolished the NADPH-supported nitrogenase activity even when the reaction mixture was supplemented with azotoflavin and azotobacter ferredoxin. It is possible that longer dialysis inactivated one or both of the other endogenous factors needed for NADPH-supported nitrogenase activity (see below).

When NADPH (or substrates having $NADP^+$-linked dehydrogenases) served as source of reducing power, the nitrogenase activity of the cell extract was limited by the endogenous concentration of azotoflavin. As shown in Fig. 2, adding a nearly saturating amount of azotoflavin to the cell extract increased nitrogenase activity about 400 %. By contrast, the level of azotobacter ferredoxin normally present in the cell extract was sufficient for nitrogenase activity. Adding increasing amounts of azotobacter ferredoxin to the cell extract gave only small increases in nitrogenase activity. The activity of this extract was still limited, however, by the amount of endogenous ferredoxin–$NADP^+$ reductase present.

A requirement of azotobacter ferredoxin for NADPH-supported nitrogenase activity was demonstrated by passing the cell extract through a DEAE-cellulose column to remove the endogenous ferredoxin (Fig. 3). Control experiments showed that this treatment removed all of the endogenous ferredoxin (assayed using illuminated chloroplasts and an electron donor[6]) but not the nitrogenase (see dithionite control, Table III). However, the DEAE-cellulose treated cell extract gave no nitrogenase activity with NADPH as the reductant unless azotobacter ferredoxin was added (in the presence of azotoflavin and spinach ferredoxin–$NADP^+$ reductase discussed below). Fig. 3 shows that NADPH-supported nitrogenase activity was proportional to added azotobacter ferredoxin up to a concentration of at least 60 nmoles per 1.5 ml.

Clostridial ferredoxin was consistently more effective than the azotoflavin-azotobacter ferredoxin system in supporting NADPH-dependent nitrogenase activity. (Compare Table I and Fig. 3.) The system with clostridial ferredoxin and azotoflavin–azotobacter ferredoxin gave activities of 1950 and 500 nmoles ethylene formed/30 min,

TABLE III

REQUIREMENTS FOR NADPH-SUPPORTED NITROGENASE ACTIVITY BY A DEAE-CELLULOSE TREATED AZOTOBACTER CELL EXTRACT

The complete reaction mixture (final volume 1.5 ml) contained 12 mg DEAE-cellulose treated extract; NADPH generator; ATP generator; and the following in μmoles: HEPES buffer (pH 7.2), 50; Mg^{2+}, 5; azotoflavin, 0.036; azotobacter ferredoxin, 0.028; and spinach ferredoxin–$NADP^+$ reductase, 0.004. The control contained dithionite (20 μmoles) in place of the NADPH generator (no electron carriers added). Gas phase, 73% argon and 27% acetylene. The incubation was at 30°.

Treatment	*Ethylene formed/30 min (nmoles)*
Complete	244
minus azotoflavin	91
minus azotobacter ferredoxin	0
minus ferredoxin–$NADP^+$ reductase	0
Dithionite control	6860 *

* Corresponds to 105% of the activity of the untreated cell extract.

respectively, with comparable levels of protein. This shows a significant difference in the biological activity of the azotobacter and clostridial ferredoxins which cannot, at this time, be related to their chemical and physical differences[8]. Comparable rates might be expected when all the components (see below) of the azotoflavin–azotobacter ferredoxin system are purified and added back at their optimal concentrations.

In addition to ferredoxin, the DEAE-cellulose treatment of Azotobacter cell extract removed two other factors that were required for NADPH-supported nitrogenase activity: azotoflavin and an endogenous factor (not yet characterized) that was replaceable by ferredoxin–$NADP^+$ reductase from spinach chloroplasts. As shown in Table III, without added azotoflavin, the DEAE-cellulose treated cell extract still retained some nitrogenase activity which was, however, markedly increased by the addition of azotoflavin. Furthermore, the DEAE-cellulose treatment has not only completely removed azotobacter ferredoxin but it has also removed (or inactivated) the Azotobacter factor corresponding to ferredoxin–$NADP^+$ reductase, bringing nitrogenase activity (without added spinach ferredoxin–$NADP^+$ reductase) to a complete halt. Thus, the DEAE-cellulose treatment was useful for establishing that the NADPH-supported Azotobacter nitrogenase activity requires three endogenous factors: azotobacter ferredoxin, azotoflavin, and a factor replaceable by spinach ferredoxin–$NADP^+$ reductase.

Experiments in which the DEAE-cellulose treated extract was replaced by partly purified nitrogenase (P_5) showed that, aside from these three components, NADPH-supported Azotobacter nitrogenase activity required a fourth factor: a soluble component present in the supernatant fluid (S_5) corresponding to the P_5 fraction (see METHODS). Approx. 85 % of the nitrogenase activity was lost when the S_5 fraction was omitted from the reaction mixture. (The "complete" system had a specific activity of 0.35 nmole ethylene/min per mg protein.) Even the remaining 15 % activity was lost when the nitrogenase preparation (P_5) was washed by sedimentation and resuspension in fresh buffer.

Further evidence for the fourth soluble factor required for NADPH-supported nitrogenase activity was obtained by mild heating (55° for 10 min) of the cell extract —a treatment which did not impair the dithionite-supported nitrogenase activity. The heat treatment resulted in a complete loss of the NADPH-supported nitrogenase activity which could not be restored by the addition of azotobacter ferredoxin, azotoflavin, and spinach reductase. Adding the S_5 supernatant fraction partly restored nitrogenase activity to the heated extract. The nature of this soluble, heat-labile component remains to be determined.

CONCLUDING REMARKS

The well-known role of the pyruvate phosphoroclastic reaction in generating reductant (reduced ferredoxin) for the nitrogenase[24,4] of *C. pasteurianum* has, in the past, somewhat obscured the physiological importance of alternate electron donor systems in nitrogen fixation, particularly in aerobic and symbiotic nitrogen fixers in which no "clastic" reaction is known to exist.

The dehydrogenases of the substrates which most effectively supported nitrogen fixation by *A. vinelandii* extracts are $NADP^+$-linked. Among those supporting the highest activity were malate, glucose 6-phosphate, α-ketoglutarate and isocitrate

(Table II). Isocitrate dehydrogenase ($NADP^+$-linked) has been found to make up about 1% of the total soluble cell protein of *A. vinelandii*[25]. It seems likely, therefore, that NADPH rather than NADH[22,23] is the electron donor for Azotobacter nitrogenase.

The joint requirement of both azotoflavin and azotobacter ferredoxin for the NADPH-supported nitrogenase reaction (Figs. 2 and 3) indicates that they and the two other endogenous factors required for the nitrogenase system act as an electron transport chain between NADPH and nitrogenase. There is a definite possibility that the $NADP^+$-linked electron transport system in the Azotobacter nitrogenase system may serve as a model for other aerobic and symbiotic nitrogen fixers.

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