Reconstitution of the Electron Transport System That Couples Formate Oxidation to Nitrogenase in *Methylosinus trichosporium* OB3b

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The electron transfer system that transports electrons from formate to nitrogenase was examined in extracts of the obligate methanotroph *Methylosinus trichosporium* OB3b. By supplementing a crude nitrogenase extract with NAD, formate dehydrogenase, NADH-ferredoxin reductase and FMN (an FDH cofactor), an electron transport system was established that coupled formate oxidation to nitrogenase-dependent acetylene reduction. The ferredoxin dependence of this reaction was demonstrated by its severe inhibition by antibodies to ferredoxin. The reaction sequence is as follows: formate → formate dehydrogenase → NADH → NADH-ferredoxin reductase → ferredoxin → nitrogenase.

INTRODUCTION

Methanotrophs can use methane and the intermediates of methane oxidation as energy sources for N₂ fixation (Dalton & Whittenbury, 1976; Toukdarian & Lidstrom, 1984), but it is not known how the oxidation of these one-carbon intermediates is coupled to the reduction of nitrogenase. With approximately 70% of the O₂-uptake used for the initial C₁ hydroxylation reaction (Anthony, 1982a), there appears to be relatively little reductant (NADH) available for the necessary biosynthetic reactions, much less for the reduction of N₂. Based on our current knowledge of these organisms, the demands for NADH, especially when the organism is fixing N₂, would seem to be greater than its supply. However, no alternative reductant seems to be available to the cells.

With NADH as its only known source of reducing power, the reduction of the nitrogenase (via a ferredoxin) would be difficult to achieve in *Methylosinus trichosporium* because most ferredoxins require a reductant with an oxidation–reduction potential of about –400 mV (Yoch & Carithers, 1979). Since all the electron transport processes that have been reported for this group of micro-organisms involve electron carriers whose redox potentials (E_0') are positive (Anthony, 1982b), it would suggest that N₂-fixing methanotrophs must have an additional redox system capable of transporting low potential electrons. By analogy with other N₂-fixing bacteria such a system would be expected to involve ferredoxins or flavodoxins that would either be reduced directly by a dehydrogenase, or by an NADH-linked oxidoreductase. While not excluding the first possibility, recent data from our laboratory have shown that the reduction of an endogenous ferredoxin from *M. trichosporium* OB3b could be coupled to formate oxidation by a reaction that requires an NAD-linked formate dehydrogenase and a newly discovered NADH-ferredoxin reductase (unpublished results).

In this communication we report the *in vitro* reconstitution of an electron transport system which couples formate oxidation to acetylene reduction by nitrogenase in *M. trichosporium* OB3b.

Abbreviations: FDH, formate dehydrogenase; NFR, NADH-ferredoxin reductase.

METHODS

Organism and growth. *Methylosinus trichosporium* OB3b (obtained from Dr Mary Lidstrom) was cultured in 500 ml media in 2 litre Erlenmeyer flasks shaken at 200 r.p.m. in a Lab-Line Orbit Shaker at 29 °C. The medium used for nitrogenase derepressed cultures was that of Dalton & Whittenbury (1976), modified by lowering the nitrate concentration to 2.5 mM. After 3 d, the culture turbidity was about 70 Klett units; at this point the nitrate was depleted and nitrogenase was derepressed. For the preparation of formate dehydrogenase and NADH-ferredoxin reductase, cells were grown on *nif*-repressing concentrations of nitrate (20 mM). The cultures were evacuated and regassed each day with an air/methane mixture (1:1, v/v).

Cell-free extracts. These were prepared from cells harvested anaerobically by resuspending the cell pellets (under a flow of Ar) in 2 vols Ar-saturated 300 mM-Tricine buffer, pH 7.8. The cell suspension was placed in a bottle fitted with a serum stopper, which was evacuated for 5 min then refilled with Ar depleted of O₂ by passing it through a column of hot, H₂-activated BASF catalyst R3-11. After the cells were cooled to 3 °C on ice, they were transferred to an anaerobic sonication vessel for disruption as described previously (Chen & Yoch, 1987). The extract was centrifuged at 27000 g for 30 min and the supernatant was removed anaerobically by syringe and placed in a degassed stoppered bottle. This crude nitrogenase extract was used in the experiments described below.

NADH-ferredoxin reductase (NFR). This was isolated from either nitrogenase repressed or derepressed *M. trichosporium* cultures by passing the crude extract (prepared as above) across a bed of DE-52 cellulose (2 × 10 cm) equilibrated with degassed 50 mM-Tris/HCl buffer, pH 7.0. After washing the column with 30 ml of this buffer, NFR was eluted with buffer containing 0.1 M-NaCl. Fractions with activity were pooled and chromatographed on a Sephadex G-100 column (0.9 × 50 cm) eluted with 50 mM-Tris/HCl buffer. The assay for NFR utilized spinach ferredoxin and consisted of measuring ferredoxin-dependent electron transport from NADH to horse heart cytochrome *c* (Yoch, 1973). Cytochrome *c*, which is reduced non-enzymically by reduced ferredoxin, was monitored by an increase in absorbance at 550 nm.

Formate dehydrogenase (FDH). This was eluted from the DE-52 cellulose column described above when the NaCl concentration in the buffer was increased to 0.2 M. Fractions (1 ml) were collected anaerobically in Eppendorf tubes which were then stoppered, evacuated and refilled with Ar. All samples, whether further purified or not, were stored on ice until used. Activity was stable for at least 2 weeks under these conditions. FDH was further purified by passage over an anaerobic Sephadex G-100 column (0.9 × 50 cm) eluted with the Tris buffer. Formate dehydrogenase was assayed in a reaction mixture containing 40 mM-Tris/HCl (or potassium phosphate) buffer, pH 7.0, 10 mM-sodium formate, 2 mM-NAD and 2.5 µM-FMN. A cuvette with the reaction mixture was closed with a serum stopper; it was evacuated and regassed three or four times with Ar through a syringe needle. The reaction was started by adding FDH anaerobically by syringe. The reduction of NAD was monitored by its increase in absorbance at 340 nm.

Nitrogenase activity. This was measured in cell-free extracts in 5 ml Fernbach flasks. The complete reaction mixture contained, in a final volume of 1.5 ml: 50 mM HEPES/NaOH buffer, pH 7.4, 24 mM-phosphocreatine, 20 µg creatine phosphokinase, 15 mM-MgCl₂ and 2.9 mM-ATP. The reductant was either 33 mM-sodium dithionite (the control) or 10 mM-sodium formate. Acetylene was added to each flask to a final concentration of 5% (v/v). Additional details of the formate-driven nitrogenase reaction are given in the legend to Table 1. Nitrogenase activity was measured by the reduction of acetylene to ethylene over a 15 min period. These two gases were separated on an alumina column eluted with N₂ at 195 °C, and detected by gas chromatography with a flame ionization detector. Peaks were integrated on an HP 3390 (Hewlett-Packard) integrator.

Ferredoxin antiserum. This was obtained by vaccinating a male New Zealand white rabbit three times over a 6 week period with approximately 100 µg pure *Rhodospirillum rubrum* ferredoxin II (in the native state) plus incomplete adjuvant. The antibody (IgG) fraction was isolated as the 0 to 50% ammonium sulfate precipitate. This precipitation was repeated on the resolubilized immunoglobulin fraction which was then dialysed extensively against 0.9% NaCl, and frozen as individual samples until needed.

RESULTS

When *M. trichosporium* is grown on methane under nitrogen-limited conditions, nitrogenase is derepressed. Such cells oxidize formate by a constitutive FDH for the generation of both the ATP and the reductant needed for nitrogenase (acetylene reduction) activity. The concentration of formate which supported maximal rates of activity was between 10 and 15 mM, with severe inhibition beyond this concentration (Fig. 1). We have previously shown that under these same culture conditions a constitutive hydrogenase will also support nitrogenase activity (Chen & Yoch, 1987).

Cell-free extracts prepared from nitrogen-limited cells had nitrogenase activity when dithionite was used as a direct reductant of the Fe protein and an ATP-regenerating system was

Fig. 1. Formate-dependent acetylene reduction activity by *M. trichosporium* OB3b. Cells for this experiment were grown on methane and a limiting concentration of nitrate to derepress nitrogenase.

Table 1. *In vitro* reconstitution of formate-dependent nitrogenase activity

The complete reaction mixture contained 1.65 mg crude extract protein, 2 mM-NAD, 10 mM-formate, 6 μ M-FMN, 9 μ g NFR and 15 μ g FDH. The ATP-regenerating system and the general techniques for the nitrogenase assay are described in Methods.

Reaction mixture	Acetylene reduction activity [nmol ethylene produced min ⁻¹ (mg protein) ⁻¹]
Plus dithionite (control)*	6.14
Complete	6.97
Minus NAD	0.07
Minus formate	0.06
Minus FMN	1.48
Minus NFR	0.96
Minus FDH	1.33

* The control was assayed in the absence of the reconstitution system.

provided (Table 1). When formate and NAD were substituted for dithionite, however, no activity was observed. It was only when the nitrogenase extracts were also supplemented with formate dehydrogenase and NADH-ferredoxin reductase (NFR) that any activity was observed with formate. NAD reduction by formate dehydrogenase was stimulated by FMN (data not shown), and since electron transport to nitrogenase appeared to proceed through NADH, we added 6 μ M-FMN to the reaction mixture. The addition of FMN stimulated this reconstituted system 5-fold. The acetylene reduction activity of the complete formate-driven reconstituted system was equivalent to that supported by dithionite (Table 1).

The response of formate-driven acetylene reduction activity to increased concentrations of NFR and FDH is seen in Fig. 2(a) and Fig. 2(b), respectively. These proteins were used directly from the initial DE-52 cellulose column, so the protein concentrations are quite high. While NFR was not saturating in this experiment, maximal acetylene reduction was observed between 60 and 75 μ g NFR protein in another experiment.

The ferredoxin requirement for formate-dependent acetylene reduction is not seen in Table 1 because its concentration in crude extracts was sufficient for maximal nitrogenase activity. An indirect means was used to demonstrate the involvement of an endogenous ferredoxin in the

Fig. 2. Effect of NFR (a) and FDH (b) concentration on formate-driven nitrogenase activity in *M. trichosporium*. The reaction components are described in the legend to Table 1, with NFR and FDH added as indicated.

Fig. 3. Immunoblots showing cross-reactivity of *M. trichosporium* ferredoxin with antiserum against *R. rubrum* ferredoxin II. Lane A, *M. trichosporium* ferredoxin fraction; blots 1-4, 6.6, 3.3, 1.3 and 0.3 μg protein, respectively. Lane B, *R. rubrum* ferredoxin II; blots 1-4, 8.0, 4.0, 1.6 and 0.4 μg protein, respectively. Lane C, *R. rubrum* ferredoxin I; blots 1-4, 7.5, 3.25, 1.3 and 0.325 μg protein, respectively. All proteins were applied in the native state.

formate-driven nitrogenase reaction. After demonstrating that antibody to *R. rubrum* ferredoxin II shows measurable specificity toward the *M. trichosporium* Fd (Fig. 3, lane A) compared to a positive control (lane B) and a negative control (lane C), the antibody was added in increasing concentrations to the complete formate-driven nitrogenase reaction mixture. The results show

Fig. 4. Inhibitory effect of ferredoxin antibody on formate-dependent nitrogenase activity in extracts of *M. trichosporium*. Antibody prepared against *R. rubrum* ferredoxin II was added at increasing concentrations to extracts in which dithionite was the electron donor (●) and to 'complete' reaction mixtures, as described in Table 1, in which formate was the electron donor (○). The crude extract in the complete reaction mixture contained 0.66 mg protein; there was 180 μg (protein) FDH and 60 μg NFR per vessel.

Fig. 5. Reaction sequence coupling electron transport from formate to nitrogenase by *M. trichosporium*.

that acetylene reduction activity was progressively inhibited as the antibody level was increased which suggests a role for ferredoxin in this reaction (Fig. 4). The control shows that when the antibody to ferredoxin was added to the dithionite-dependent reaction mixture, there was no effect since dithionite reduces the nitrogenase Fe protein directly. This indirect evidence for ferredoxin involvement in the formate-driven reaction is reinforced by the fact that the only way NFR could contribute to this activity (Table 1) would be if a ferredoxin was involved. The reaction scheme showing the coupling of formate oxidation to nitrogenase acetylene reduction is seen in Fig. 5.

DISCUSSION

The limited amount of reductant that *M. trichosporium* seems to have available for biosynthesis may manifest itself in its low *in vivo* rates of N₂-fixation. Formate-dependent acetylene reduction activity ranges from 5 to 10 nmol C₂H₄ formed min⁻¹ (mg cell protein)⁻¹ (Dalton & Whittenbury, 1976; Dalton, 1980; Toukdarian & Lidstrom, 1984), compared to values of approximately 100 nmol min⁻¹ (mg cell protein)⁻¹ commonly seen in *Klebsiella pneumoniae* (Shanmugam *et al.*, 1975). *In vitro*, where reductant and ATP could be provided in excess, the specific activity of *Methylosinus* nitrogenase was still lower than that of *Klebsiella*, but

our finding that NADH-supported nitrogenase activity was equivalent to that supported by dithionite was surprising in light of the low rates achieved with NADH (compared to those supported by dithionite) in cell extracts of other aerobic N_2 -fixers (Klucas & Evans, 1968; Yates & Daniel, 1970; Benemann *et al.*, 1971).

The problem of reducing nitrogenase with NADH is really one of reducing ferredoxin. The reduction of a ferredoxin with an E'_0 of -420 mV from NADH is thermodynamically unfavourable ($\Delta G'^0 = +18$ kJ mol $^{-1}$). This endothermic reaction becomes feasible only if the ferredoxin remains primarily in an oxidized state by being rapidly re-oxidized after it is reduced. This situation exists in cells containing nitrogenase and an excess of ATP. *In vitro* this reaction also requires, in addition to ATP, an enzyme that facilitates the efficient transfer of electrons from NADH to ferredoxin. With the discovery and purification of NFR in *M. trichosporium* (unpublished results) this enzyme could be added to the crude extract to enhance ferredoxin reduction. Without an exogenous source of this enzyme, NADH-supported nitrogenase activity in *Azotobacter vinelandii* (Yates & Daniel, 1970; Benemann *et al.*, 1971) and *Rhizobium bacteroids* (Klucas & Evans, 1968) is extremely low. In *Clostridium pasteurianum*, which has a stable, functional NFR, nitrogenase activity supported by NADH is about 25% of that supported by dithionite (Jungermann *et al.*, 1974).

In summary an electron transport pathway has been reconstituted that supports significant levels of acetylene reduction from FDH, NFR and an endogenous ferredoxin.

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