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Isolation, Characterization, and Biological Activity of Ferredoxin-NAD$^+$ Reductase from the Methane Oxidizer *Methylosinus trichosporium* OB3b

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A ferredoxin-NAD$^+$ oxidoreductase (EC 1.18.1.3) has been isolated from extracts of the obligate methanotroph *Methylosinus trichosporium* OB3b. This enzyme was shown to couple electron flow from formate dehydrogenase (NAD$^+$ requiring) to ferredoxin. Ferredoxin-NAD$^+$ reductase was purified to homogeneity by conventional chromatography techniques and was shown to be a flavoprotein with a molecular weight of 36,000 ± 1,000. This ferredoxin reductase was specific for NADH (K_m, 125 μM) and coupled electron flow to the native ferredoxin and to ferredoxins from spinach, *Clostridium pasteurianum*, and *Rhodospirillum rubrum* (ferredoxin II). *M. trichosporium* ferredoxin saturated the ferredoxin-NAD$^+$ reductase at a concentration 2 orders of magnitude lower (3 nM) than did spinach ferredoxin (0.4 μM). Ferredoxin-NAD$^+$ reductase also had transhydrogenase activity which transferred electrons and protons from NADH to thionicotinamide adenine dinucleotide phosphate (K_m, 9 μM) and from NADPH to 3-acetylpyridine adenine dinucleotide (K_m, 16 μM). Reconstitution of a soluble electron transport pathway that coupled formate oxidation to ferredoxin reduction required formate dehydrogenase, NAD$^+$, and ferredoxin-NAD$^+$ reductase.

In aerobic methylotrophs and obligate methanotrophs, the oxidation of methane ($CH_4 \rightarrow CH_2OH \rightarrow CHO \rightarrow HCOOH \rightarrow CO_2$) provides the energy for growth. The only known source of reductant for biosynthesis is NADH generated by formate dehydrogenase (FDH), although some species also have an NAD-linked formaldehyde dehydrogenase (5). Therefore, in studying the electron transport system that couples to biosynthetic reactions such as those involving nitrogenase in the methanotrophs, NADH and enzymes that oxidize it are the only known sources of reductant available for ferredoxin (Fd) reduction. The generation of a reductant for nitrogenase in all aerobic and microaerophilic bacteria is a matter of long-standing interest; however, the published work on the subject has not produced an explanation of it (for reviews, see references 7 and 24). While Fds that couple to nitrogenase have been found in all N_2-fixing aerobes, including the obligate methanotroph *Methylosinus trichosporium* (4), the enzymes responsible for Fd reduction have not been found. Even though the oxidation-reduction potential of the NAD(P)H-NAD(P)$^+$ couple is approximately 100 mV more positive than is the average Fd_{red}-Fd_{ox} redox couple, it has been clearly demonstrated in several clostridial species that electrons from reduced pyridine nucleotides could be coupled to Fd reduction by endogenous Fd-NAD(P)$^+$ oxidoreductases and could support either nitrogenase (10) or hydrogenase (11, 21) activities at high rates.

In this paper, we report the isolation and characterization of an Fd-NAD$^+$ reductase (FNR) from *M. trichosporium* OB3b. This enzyme (a flavoprotein) has been purified and shown to be a critical component in coupling formate oxidation (via NAD$^+$) to Fd reduction. Although this enzyme reduces Fd in aerobes, it functions in reverse to reduce pyridine nucleotides in anaerobes and phototrophs, thus earning the name Fd-NAD(P)$^+$ oxidoreductase (EC 1.18.1.3) (9). In a previous paper (4), we called this enzyme NADH-Fd reductase because of its role in reducing Fd in *M. trichosporium*, but here we have conformed to the Enzyme Commission-recommended FNR.

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MATERIALS AND METHODS

M. trichosporium OB3b was kindly supplied by Mary Lidstrom, California Institute of Technology. It was cultured in 9-liter carboys filled with 8 liters of mineral salts medium (6) containing 10 mM sodium nitrate as a nitrogen source and gassed with air- methane (5:1). This mixture was dispersed in the culture by passing it through stone diffusers and stirring with a magnetic stir bar. Alternatively, 800-ml cultures were grown in 2-liter Erlenmeyer flasks containing air-methane (1:1), with daily regassing, and were incubated at 30°C on a platform shaker operated at 200 rpm (3).

Enzyme assays. FNR activity in *M. trichosporium* was assayed by its ability to catalyze the Fd-dependent reduction of horse heart cytochrome *c* from NADH, as shown in the following reaction: NADH + $Fd_{ox} \rightarrow NAD^+ + Fd_{red} \rightarrow$ cytochrome *c*.

The transhydrogenase activity of the reductase was measured by using analogs of NAD (3-acetylpyridine adenine dinucleotide [APAD$^+$]) and NADP$^+$ (thionicotinamide adenine dinucleotide phosphate [thioNADP$^+$]) as electron acceptors with NADPH and NADH, respectively. Reduction of thioNADP$^+$ was monitored at 395 nm (ε_{395}, 11.7 mM^{-1} cm^{-1}), and APAD$^+$ was monitored at 375 nm (ε_{375}, 5.1 mM^{-1} cm^{-1}) (18). Since these analogs absorb light at wavelengths other than 340 nm, their reduction could be monitored in the same reaction mixture in which NAD(P)H oxidation was occurring.

Fd reduction was monitored indirectly by its coupling of electrons (nonenzymatically) to cytochrome *c* (17), whose reduction was measured at 550 nm in a Perkin-Elmer Lambda 3 spectrophotometer. The reaction mixture contained 40 mM phosphate buffer (pH 7.0), 0.25 mM NADH, Fd (for routine assays, 0.4 μM spinach Fd was used), and 50 μM horse heart cytochrome *c*. Although the reductase is not sensitive to O_2, Fd is auto-oxidizable; therefore, anoxic

TABLE 1. Purification of FNR from *M. trichosporium* OB3b

Treatment	Vol (ml)	Protein		Activity[a]			Sp act (U/mg)	Purification (fold)
		Concn (mg/ml)	Total (mg)	Concn (U/ml)	Total (U)	Yield (%)		
Crude extract	270	7.0	1,890	131	35,370	100	19	1
DE-52 cellulose	18	13.2	240	1,480	26,640	75	111	6
DEAE–Sephadex A-50	18	1.3	2.40	755	13,590	38	566	30
Sephacryl S-300	9	0.34	3.9	300	2,700	8	750	40

[a] One unit of FNR activity is defined (in Materials and Methods) by using a saturating level of spinach Fd as the electron carrier.

conditions are required for this assay. The reaction mixture was placed in a modified cuvette which was closed with a serum bottle stopper, evacuated, and refilled three times with argon before the reductase was added with a gastight syringe. The extinction coefficient (ε_{550}, 27 mM^{-1} cm^{-1}) was used to calculate cytochrome *c* reduction. One unit of FNR activity was calculated to be the amount of enzyme necessary to reduce 1 nmol of cytochrome *c* per min with a saturating concentration of Fd present.

M. trichosporium Fd activity was assayed by substituting this protein for spinach Fd in the standard FNR assay (described above). Fd activity was also measured by its ability to catalyze the photoreduction of NADP via the bound plant FNR (17, 23). In this assay, spinach chloroplasts were disrupted by osmotic shock, which releases the native Fd. After the chloroplast fragments were washed and suspended, they were heated at 55°C for 5 min to kill their O_2-evolving capacities. Because the heated chloroplasts could not use water as a source of electrons for photochemistry, they had to be supplied with an artificial electron donor such as ascorbate-reduced 2,6-dichlorophenol-indophenol.

FDH was assayed in a reaction mixture containing 10 mM sodium formate, 40 mM phosphate buffer (pH 7.0), 0.2 mM NAD^+, and 2.5 μM flavin mononucleotide. Under certain conditions of isolation, flavin mononucleotide (but not flavin adenine dinucleotide) is required for FDH activity (unpublished data). FDH was added to the cuvette to start the reaction, which was monitored by measuring the reduction of NAD at 340 nm.

Purification of FNR. To purify *M. trichosporium* FNR, 100 g of cell paste was suspended in 3 volumes of 50 mM phosphate buffer (pH 7.0) and sonicated in 50-ml batches. The extract was clarified by centrifugation at 35,000 × *g* for 15 min. The extract was initially passed over a DE-52 cellulose collecting column equilibrated with phosphate buffer (as described above). The column was washed with buffer containing (in steps of 50 ml) 0, 0.1, 0.2, and 0.4 M NaCl. The fractions with FNR activity were then applied to a DEAE-Sephadex A-50 column (0.9 by 50 cm) which was eluted with 100 ml of a 50 to 300 mM NaCl gradient in 50 mM phosphate buffer. The four fractions with the highest activities were concentrated to 0.5 ml in a dialysis bag placed on a bed of polyethylene glycol 8000. After being treated with 100 μg of DNase, the sample was applied to a Sephacryl S-300 gel filtration column (1.7 by 81 cm) that was equilibrated with 50 mM phosphate buffer. Several of these fractions showed the enzyme to be homogeneous (see below). The purification of FNR is summarized in Table 1.

Other methods. The weight of native FNR was estimated by the gel filtration method of Andrews (1). Gel electrophoresis methods were as described previously (8).

M. trichosporium Fd was purified after being eluted from the first DE-52 cellulose column with 0.4 M NaCl (Fig. 1). This protein was pure after being eluted from a DE-52 cellulose column (1 by 25 cm) with a 0 to 500 mM NaCl gradient and chromatographed on a Sephadex G-100S column (1.5 by 90 cm).

RESULTS

Purification and molecular weight of FNR. Figure 1 shows an elution profile of a crude extract that was applied to a DE-52 cellulose column and eluted with a sodium chloride step gradient. A typical elution profile showed that FNR activity eluted with buffer containing 0.1 M NaCl, FDH eluted at 0.2 M NaCl, and Fd eluted from the column with 0.4 M NaCl. The purification of FNR proved to be not very difficult because it was essentially pure after only two additional steps of purification (starting with 100 g of cell paste [Table 1]).

When FNR was chromatographed on a Sephadex G-100 column which had been calibrated with protein standards, it eluted in a volume indicating an M_r of approximately 36,000 ± 1,000 when interpolated from a plot of elution volume versus log M_r for molecular weight standards (Fig. 2). The Fig. 2 inset shows the purity of two of the peak fractions from Sephacryl S-300 which were subjected to M_r analysis on the calibrated gel filtration column.

The spectrum of purified FNR is that of a flavoprotein

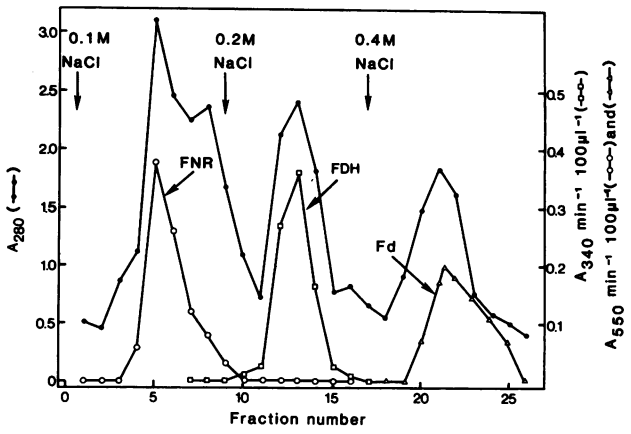

FIG. 1. Chromatography elution profile of *M. trichosporium* FNR, FDH, and a ferredoxin fraction. Crude extract (170 mg of protein) was applied anaerobically to a DE-52 cellulose column (1.7 by 17 cm) equilibrated with 50 mM of degassed phosphate buffer (pH 7.0). Protein was eluted from the column by washing with 30 ml of buffer containing no salt and then washing with buffer containing 0.1, 0.2, and 0.4 M NaCl, which was added at the points indicated by arrows. Fractions (2 ml) were collected at a rate of 20 ml/h. FNR, FDH, and Fd activities were assayed as described in Materials and Methods. All enzymes were collected and assayed anaerobically.

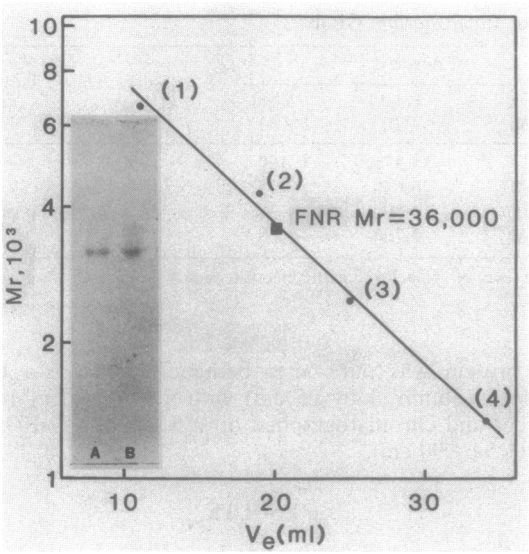

FIG. 2. M_r estimation of *M. trichosporium* NADH-FNR. The protein markers were as follows: 1, bovine serum albumin, 67,000; 2, ovalbumin, 43,000; 3, chymotrypsinogen A, 25,000; and 4, RNase, 13,700. The standards (2 mg each) were applied one at a time to a Sephadex G-100 column (0.9 by 51 cm) equilibrated and eluted with 50 mM phosphate buffer (pH 7.0). The elution volume (V_e) of NADH-Fd reductase was determined by assaying the fractions in the standard Fd-cytochrome *c*-coupled reaction (see Materials and Methods). A 15% native polyacrylamide gel stained with Coomassie blue shows FNR fractions with the highest activities from the Sephacryl S-300 column (inset, A and B). Both lanes were loaded with approximately 5 μg of protein.

(Fig. 3) with absorbance peaks at 275, 368, and 458 nm. The spectrum is similar to that of FNR purified from spinach chloroplasts (17) and *Bacillus polymyxa* (23), except that the plant enzyme has peaks at 275, 385, and 456 nm and the extinction of the UV-absorbing component (275 nm) is 30%

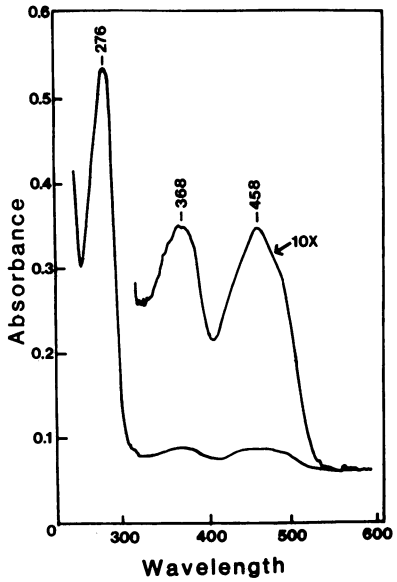

FIG. 3. Absorbance spectrum of NADH-Fd reductase. The flavoprotein was in 50 mM Tris hydrochloride buffer (pH 7.2) at a concentration of 400 μg of protein per ml.

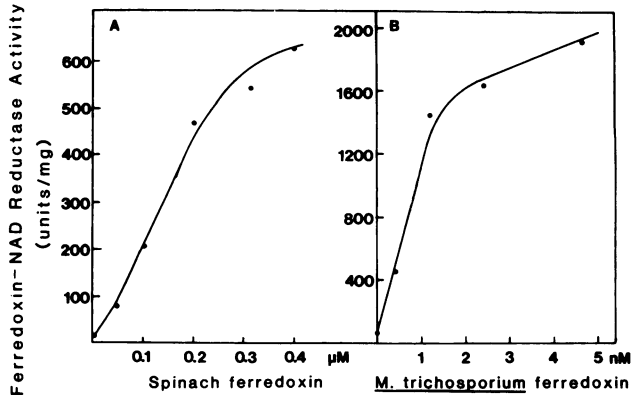

FIG. 4. Effectiveness of spinach (A) and *M. trichosporium* (B) Fds coupling to FNR. The reaction (Fd reduction) was measured by coupling electrons from Fd to cytochrome *c*. The FNR concentration was 8 μg of protein per ml of reaction mixture. The concentrations of spinach (micromolar) and *M. trichosporium* Fds (nanomolar) were calculated from millimolar extinction coefficients of 9.68 at A_{410} and 27.1 at A_{400}, respectively.

greater than that of FNR from *M. trichosporium*. Although the flavin cofactor of the plant reductase has been identified as flavin adenine dinucleotide, its identity in the *M. trichosporium* enzyme has not yet been determined.

Substrate specificities and kinetic constants. The specificity of *M. trichosporium* FDH for NAD^+ (19) suggests that a pyridine nucleotide-linked reductase coupling electron flow to Fd in this organism is specific for NADH. This was confirmed when it was observed that the reduction of cytochrome *c* in an Fd-dependent reaction was five times faster with NADH than with NADPH (data not shown). The K_m for NADH was 0.125 mM when NADH concentrations were varied from 0.05 to 0.5 mM.

The specificity of the reductase for Fd is shown in Fig. 4. Spinach Fd was used in the routine assays for this enzyme because it was available before the native Fd had been purified. The native Fd is a brown bacterial-type Fd whose spectrum resembles that of *Azotobacter vinelandii* FdI. The *M. trichosporium* Fd was more effective in terms of V_{max} than was spinach Fd (cf. Fig. 4A and B), and it saturated the reaction at less than 1/100 the concentration that spinach Fd did (2 to 3 versus 400 nM). The specific activities of FNR with saturating concentrations of Fds from spinach, *Clostridium pasteurianum*, and *Rhodospirillum rubrum* (FdII) were 750, 739 and 1,055 U/mg, respectively. When pure *M. trichosporium* Fd was used in the assay, the specific activity of FNR was 1,502 U/mg.

Transhydrogenase activity. FNR from *M. trichosporium*, like that from plants (17, 25), also has transhydrogenase activity. The rates were measured in both directions by using analogs of NAD^+ ($APAD^+$) and NADP ($thioNADP^+$) as electron acceptors with NADPH and NADH, respectively, as the electron donors. The transhydrogenase activity from NADPH to $APAD^+$ was used to calculate a K_m of 16 μM for $APAD^+$. The K_m of $thioNADP^+$ in the transfer of electrons from NADH to $thioNADP^+$ was 9 μM. These results suggest that the transhydrogenase activity (NADH→NADP) of the reductase can easily provide *M. trichosporium* with all the NADPH that might be required for the biosynthetic needs of the cell.

Formate-dependent Fd reduction. Protein components in crude extracts of *M. trichosporium* which separate on DE-52

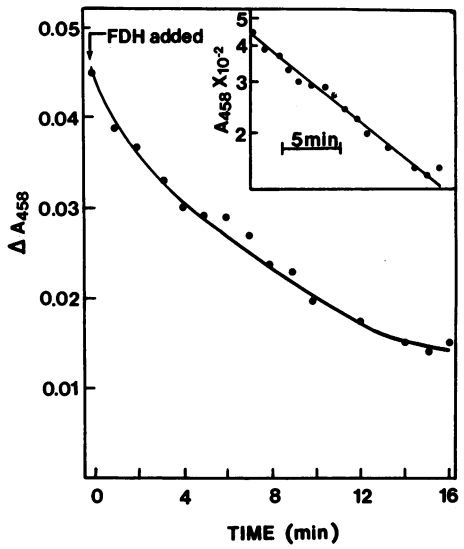

FIG. 5. Reduction of the flavin moiety of FNR by FDH plus NAD^+. The reaction mixture contained FNR, 600 μg of protein, FDH, 40 μg of protein, 0.5 mM NAD, 25 mM formate, 2.5 μM flavin mononucleotide and 50 mM phosphate buffer. The reaction mixture was made anaerobic before the FDH was added. A first-order rate plot of these data is also shown (inset).

cellulose columns can be purified and reconstituted into a system (along with NAD^+) capable of coupling electron flow from formate to Fd. To first demonstrate the coupling of electron flow from FDH to the reductase, the reduction (bleaching) of the flavin was monitored at 458 nm following the addition of FDH (Fig. 5). The concentration of the FDH in the reaction mixture was kept low in order to control the rate of reduction. If this low concentration of FDH resulted in NADH levels below the K_m of the reductase, the pseudo-first-order rate constant calculated from these data (Fig. 5, inset) would have little meaning.

The reconstitution of the entire pathway linking formate oxidation to Fd reduction is shown in Table 2. Fd reduction from formate was coupled to the nonenzymatic reduction of cytochrome *c* in a reaction that required FDH to reduce NAD^+ and FNR to couple electrons from NADH to Fd. Controls in which either Fd or FNR was deleted showed no reduction of cytochrome *c*, which meant that there was no reduction of the Fd. The sequence of the reconstituted reaction was as follows: formate → FDH → NAD^+ → FNR → Fd → Cytochrome *c*.

TABLE 2. Fd reduction coupled to formate oxidation[a]

Source of Fd	Fd concn (μM)	Fd reduction (nmol of cytochrome *c* reduced/min)
M. trichosporium	0.005	4.0
C. pasteurianum	0.50	1.9
R. rubrum (FdII)	0.30	2.1
Spinach	0.30	1.5
None		0.5
None (FNR deleted)		0.1

[a] The reaction mixture (2 ml) contained in an anaerobic cuvette was 10 mM sodium formate, 50 mM phosphate buffer (pH 7.0), 200 μM NAD, 0.75 μM horse heart cytochrome *c*, 2.5 μM flavin mononucleotide, 8 μg of FDH, 11.4 μg of FNR, and Fds as indicated above.

DISCUSSION

The mechanism of Fd reduction in aerobic bacteria has been a matter of speculation for many years (7, 24). A key step in elucidating the pathway of electron transport to the nitrogenase in various anoxic species has been finding an Fd-linked dehydrogenase. In *C. pasteurianum* and *Klebsiella pneumoniae*, this problem was solved when it was found that pyruvate dehydrogenases coupled pyruvate oxidation to the reduction of an Fd (15) and a flavodoxin (2), respectively. In other anaerobes, such as *Clostridium kluyveri* (10, 11), *B. polymyxa* (23), and S organism (16), Fd-NAD(P) reductases which coupled Fd reduction to the oxidation of NAD(P)H were found. In the N_2-fixing aerobes *Azotobacter* and *Rhizobium* spp., Fds and flavodoxins have been isolated (7, 24), but there are no reports of dehydrogenases or NAD(P)H-linked reductases that couple to these low-redox electron carriers. There is one report, however, that the aerobe *Halobacterium halobium* can reduce its native 2Fe-2S Fd by a coenzyme A-dependent pyruvate dehydrogenase (13).

This is the first report of the purification of an FNR from a methanotroph, although comparable flavin enzymes have been isolated from various pseudomonads (12, 20). FNR from the methanotroph is also very similar to plant FNR (25) and adrenodoxin reductase found in adrenal-cortex mitochondria (14). All are flavoproteins of comparable molecular weights, i.e., 34,000 to 37,000. *M. trichosporium* FNR appears to be distinct from component C of the methane monooxygenase system of this organism (5) in that the latter is 8 to 10 kilodaltons smaller and has relatively poor NADH-cytochrome *c* reductase activity in the absence of Fd. The importance of finding FNR in an aerobe as it relates to NADH-supported nitrogenase activity is more than hypothetical, as we have been able to use it to reconstitute an NAD- and Fd-dependent electron transport system in *M. trichosporium* which couples the oxidation of formate, a key intermediate in methane utilization, to the reduction of nitrogenase (4). In other methanotrophs which have active NAD-linked aldehyde dehydrogenases, these enzymes can presumably act either in concert with FDH or alone to drive electron transport to nitrogenase via FNR and a native Fd. It is important to point out, however, that these observations do not preclude the possibilities that methanotrophs may have an Fd-linked formate or pyruvate dehydrogenase.

In summary, the purification of a flavoprotein with FNR activity from an aerobic obligate methanotroph made it possible to demonstrate the following mechanism of Fd reduction in vitro: formate → FDH → NADH → FNR → Fd.

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