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FAD Requirement for the Reduction of Coenzyme F_{420} by Formate Dehydrogenase from *Methanobacterium formicicum*

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The partial purification of the formate dehydrogenase from cell-free extracts of *Methanobacterium formicicum* decreased the rate of coenzyme F_{420} reduction 175-fold relative to the rate of methyl viologen reduction. FAD, isolated from this organism, reactivated the coenzyme F_{420}-dependent activity of purified formate dehydrogenase and restored the activity ratio (coenzyme F_{420}/methyl viologen) to near that in cell-free extracts. Neither flavin mononucleotide nor $FADH_2$ replaced FAD. The reduced form of FAD inhibited the reactivation of coenzyme F_{420}-dependent formate dehydrogenase activity by the oxidized form. The results suggest that native formate dehydrogenase from *Methanobacterium formicicum* contains noncovalently bound FAD that is required for coenzyme F_{420}-dependent activity.

Formate dehydrogenase (FDH) is present in the aerobic and anaerobic eubacteria. Considerable diversity in electron acceptor specificities exists among FDHs from the anaerobic eubacteria. The highly purified FDHs from *Clostridium thermoaceticum* (17) and *Wolinella succinogenes* (*Vibrio succinogenes*) (9) utilize $NADP^+$ and quinones, respectively. Partially purified FDH from *Desulfovibrio vulgaris* reduces highly purified cytochrome *c*-553 (16). Of the eubacterial FDHs studied, only the NAD^+-dependent enzyme from the aerobe *Pseudomonas oxalaticus* is known to require flavin for activity (13).

Among the archaeobacteria, only the methanogenic bacteria are known to contain FDH. The FDHs of *Methanospirillum hungatei* (5) and *Methanobrevibacter ruminantium* (*Methanobacterium ruminantium*) (15) are linked to coenzyme F_{420} (F_{420}), a 8-hydroxy-5-deazaflavin derivative, which functions as an electron carrier in methanogenic bacteria (2). The F_{420} reduced by FDH in these organisms is linked to the reduction of $NADP^+$ by F_{420}:$NADP^+$ oxidoreductase. A 105,000-dalton FDH, a 5-deazaflavin, and a 5-deazaflavin:$NADP^+$ oxidoreductase recently were purified from *Methanococcus vannielii* and when recombined were shown to reduce $NADP^+$ with formate (7). The 5-deazaflavin is the physiological electron acceptor for the homogenous 105,000-dalton enzyme (8). In addition to FDH, several other F_{420}-dependent enzymes have been studied, but none is known to require flavins for activity (3, 6, 8, 18). The F_{420}-reducing hydrogenase from *Methanobacterium thermoautotrophicum*, purified to approximately 50% of homogeneity, contains tightly bound FAD, but a requirement for this flavin has not been shown (6). It is postulated that FAD could function to transfer electrons from one-electron centers to the two-electron acceptor F_{420} (6).

The purification of FDH from *Methanobacterium formicicum* decreases the F_{420} reduction rate relative to the rate of methyl viologen reduction (14). Here we describe the restoration of the F_{420}-dependent activity by FAD and present evidence that the native F_{420}-reducing FDH from this organism is a flavoprotein.

MATERIALS AND METHODS

Chemicals. The following were purchased from Sigma Chemical Co., St. Louis, Mo.: FAD grade III, flavin mononucleotide (FMN) grade I, AMP, ADP, ATP, methyl viologen (MV), 2-mercaptoethanol, and bovine serum albumin. Adenine was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Sephadex G-15 and Phenyl Sepharose CL-4B were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. DEAE-cellulose (DE-52) was purchased from Whatman, Inc., Clifton, N.J. Protein-dye reagent concentrate was obtained from Bio-Rad Laboratories, Richmond, Calif. All other chemicals were of reagent grade.

Purification of FDH. The growth of *M. formicicium* and the preparation of crude extract are described elsewhere (14). The following is a brief description of a modified procedure used to obtain partially purified FDH. The details of this procedure and general anaerobic methods appear elsewhere (14). Ammonium sulfate solutions were made with anaerobic buffer that contained 1 mM sodium dithionite, 2 mM 2-mercaptoethanol, and 10 mM sodium azide in 50 mM potassi-

um phosphate buffer (pH 7.5). Solid ammonium sulfate was added to crude extract (60 ml from 30 g [wet weight] of cells) to give 60% saturation (25°C) followed by centrifugation. The supernatant solution, which contained FDH, was applied to a DEAE-cellulose column (5 by 15 cm) equilibrated with buffer 60% saturated with ammonium sulfate (8.5 ml/min). The column was washed with 2 bed volumes of buffer 40% saturated with ammonium sulfate and then developed with a 1.2-liter linear gradient of ammonium sulfate (40 to 0% of saturation). The peak fraction of FDH eluted in buffer that was approximately 20% saturated with ammonium sulfate. The F_{420} which remained bound to the column was eluted with 100 ml of 1 M ammonium carbonate in deionized water and further purified as described below. The FDH-containing fractions (30 ml) from the DEAE-cellulose column were pooled (300 ml) and loaded onto a Phenyl Sepharose CL-4B column (2.5 by 10 cm) equilibrated with buffer 20% saturated with ammonium sulfate. The column was developed with a decreasing linear gradient (200 ml) of ammonium sulfate (20 to 0% of saturation) at 1.8 ml/min. The peak fraction of FDH eluted in buffer that was approximately 5% saturated with ammonium sulfate. Protein was determined by the Bradford assay with bovine serum albumin as the standard (1).

Low-molecular-weight compounds were removed from FDH preparations by pressure dialysis through a Diaflo YM-30 ultrafilter (Amicon Corp., Lexington, Mass.). The enzyme solution was first concentrated five-fold under N_2 followed by the addition of anaerobic buffer to the original volume. The enzyme was filtered in this manner seven times. The ultrafiltrates were pooled and concentrated to dryness by flash evaporation, and the residue was redissolved in deionized water before FAD analysis.

Purification of F_{420}. The F_{420} that eluted from the DEAE-cellulose column was concentrated and desalted by flash evaporation at 55°C. The residue that contained F_{420} was redissolved in a minimal amount of deionized water and applied to a second DEAE-cellulose column (2.5 by 10 cm) equilibrated with deionized water. The F_{420} was eluted with 0.5 M ammonium carbonate. The residue that remained after flash evaporation of the F_{420}-containing fractions was dissolved in 50 mM potassium phosphate buffer (pH 7.5) and applied to a Sephadex G-15 column (2.5 by 20 cm) equilibrated with phosphate buffer. The F_{420} was eluted with phosphate buffer and used in enzyme assays without further purification.

Purification of FAD. *M. formicicum* cell paste (25 g) was autoclaved (121°C, 15 min) in screw-cap polycarbonate centrifuge tubes (30 ml) and centrifuged at 41,000 × *g* for 20 min. The supernatant solution was applied to a DEAE-cellulose column (2.5 by 7 cm) equilibrated with 0.05 M ammonium carbonate (pH 8.8). The column was developed with a linear gradient (300 ml) of ammonium carbonate (0.05 to 0.5 M). The fractions were assayed for the ability to reconstitute F_{420}-dependent FDH activity. The active fractions were concentrated to dryness by flash evaporation at 55°C. The salt-free residue was redissolved in deionized water and applied to a Sephadex G-15 column (2.5 by 20 cm) equilibrated with 0.05 M ammonium carbonate (pH 8.8). The active fractions were concentrated as before and purified by reverse-phase high-performance liquid chomatography (HPLC) (11). The active fractions eluted from the HPLC column with a retention time identical to that of commercially available FAD. Spectra were obtained with a Perkin-Elmer 552 recording spectrophotometer. The isolated FAD was concentrated to dryness by flash evaporation, and the residue was redissolved in deionized water before use.

Enzyme assays. All assays were performed at 35°C unless otherwise indicated. The assay for FDH was performed by following the formate-dependent reduction of F_{420} at 420 nm (ϵ_{420} = 42.5 mM^{-1} cm^{-1} at pH 7.5) (14) or the reduction of MV at 603 nm (ϵ_{603} = 11.3 mM^{-1} cm^{-1}) with a Perkin-Elmer model 552 spectrophotometer. The extinction coefficient for MV was experimentally determined. The reaction mixture (0.50 ml) contained (in micromoles): 2-mercaptoethanol, 10; potassium phosphate buffer (pH 7.5), 30; and either F_{420}, 0.024 or MV, 10. The reaction mixture was deoxygenated with N_2 and then transferred to a N_2-filled cuvette sealed with a serum stopper. Endogenous activity was recorded after the addition of enzyme. The reaction was initiated by the addition of 10 μmol of sodium formate. A unit of activity was the amount of enzyme that reduced 1 μmol of acceptor per min. Specific activity was reported as units per milligram of protein.

Reconstitutions of FDH with FAD were done in the assay cuvette. Deoxygenated solutions (5 to 40 μl) that contained FAD were added to the deoxygenated reaction mixture and preincubated 5 min with enzyme. After preincubation, endogenous activity was recorded, and the reaction was initiated with formate.

The $FADH_2$:F_{420} oxidoreductase assay was performed by following the $FADH_2$-dependent reduction of F_{420} at 420 nm. The reaction mixture (1 ml) contained (in micromoles): 2-mercaptoethanol, 20; potassium phosphate buffer (pH 7.5), 60; F_{420}, 0.024; $FADH_2$, 0.032. Deoxygenated reaction mixture was added to a N_2-filled cuvette, as in the FDH assay. The reaction was initiated by the addition of purified FDH (0.7 μg of protein per assay). Stock solutions of commercially available FAD were reduced with zinc dust.

RESULTS

The partial purification of FDH from *M. formicicum* resulted in a 175-fold decrease in the rate of F_{420} reduction relative to the rate of MV reduction (Table 1). In most experiments, the greatest loss in F_{420}-dependent activity occurred in the Phenyl Sepharose chromatography step of the purification. The dialysis of the purified

TABLE 1. Effect of purification and dialysis on relative rates of F_{420} and MV reduction by *M. formicicum* FDH

FDH prepn	Sp act (U/mg) assayed with:		Ratio of sp act (F_{420}/MV)
	F_{420}	MV	
Crude extract	2.7	6.5	0.42
Purified			
Before dialysis	0.067	28.4	0.0024
After dialysis	0.006	19.7	0.0003

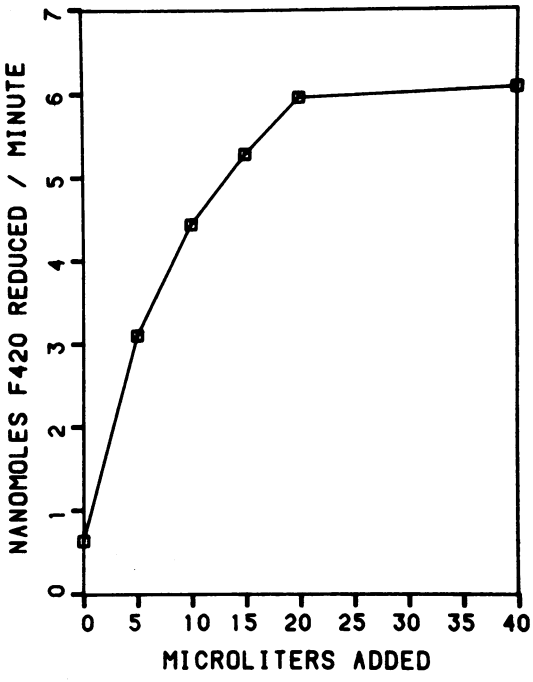

FIG. 1. Ability of boiled extract to stimulate the rate of F_{420} reduction by purified FDH from *M. formicicum*. The indicated volumes of extract were incubated with FDH before the assay (see the text). The reaction mixtures contained 10 μg of protein.

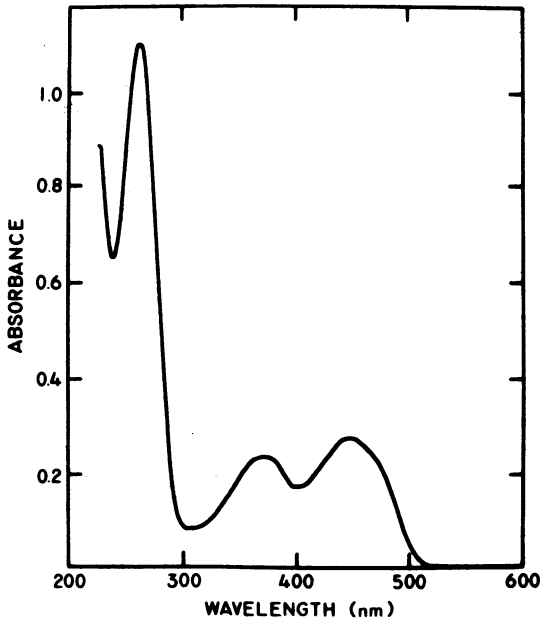

FIG. 2. UV-visible absorption spectrum of the activating component isolated from *M. formicicum*. The sample and reference buffers were 20% methanol and 5 mM ammonium acetate (pH 6.0).

FDH produced an additional eight-fold decrease in the ratio of F_{420} to MV activity (Table 1). An air-oxidized and boiled extract from *M. formicicum* stimulated 10-fold the rate of F_{420} reduction by purified FDH (Fig. 1). The boiled extract did not affect the rate of MV reduction (74 nmol of MV reduced min^{-1}/10 μg of protein). These results suggested that purification or dialysis removed a heat-stable, low-molecular-weight component(s) from FDH specifically required for the reduction of F_{420}. The component isolated from the extract eluted from the HPLC column with a retention time identical to that of commercially available FAD. The UV-visible absorption spectrum of the component had maxima at 263, 375, and 450 nm (Fig. 2). The fluorescence maxima (pH 7.0) of the component and FAD were identical, and the fluorescence emission intensity (526 nm) of the component increased when the pH was lowered to 1.0. These results suggest that the isolated component is similar in structure to FAD. The isolated

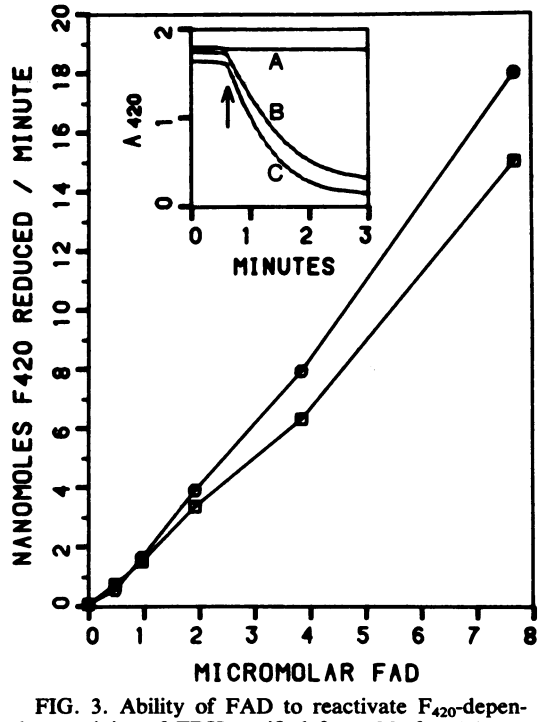

FIG. 3. Ability of FAD to reactivate F_{420}-dependent activity of FDH purified from *M. formicicum*. Residual FAD was removed from the purified FDH by dialysis as described in the text. The reaction mixtures contained 9.6 μg of protein. Symbols: □, FAD purified from *M. formicicum*; ○, commercially available FAD. Inset: time courses of F_{420} reduction by purified FDH preincubated with (A) no FAD, (B) 7.7 μM FAD purified from *M. formicicum*, (C) 7.7 μM commercially available FAD. The reaction was initiated with formate at the arrow.

component and commercially available FAD were equivalent in the reactivation of F_{420}-dependent FDH activity (Fig. 3). The rate of F_{420} reduction by purified FDH was increased approximately 200-fold when 7.6 μM FAD from either source was preincubated with the FDH (Fig. 3).

FDH assayed with either MV or F_{420} eluted from the DEAE-cellulose column in coincident profiles with an activity ratio (F_{420}/MV) of 0.28 in the peak fraction (Fig. 4A). This ratio was 67% of the activity ratio (F_{420}/MV) in cell-free extract (Table 1), which suggested that little F_{420}-dependent FDH activity was lost in this step. The apparent FAD in the DEAE-cellulose column fractions was estimated by the ability of boiled samples to reactivate F_{420}-dependent FDH activity (Fig. 4B). A comparison of Fig. 4A and B shows coincident profiles of FDH and FAD. HPLC analysis of boiled samples from the peak fraction of the FDH profile confirmed the presence of FAD. HPLC analysis also confirmed the presence of FAD in the ultrafiltrates of purified FDH. These results suggested that FAD copurified with FDH.

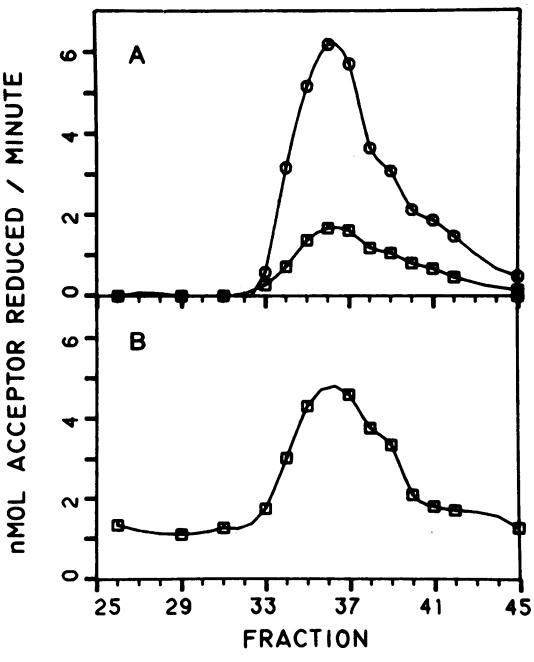

FIG. 4. Elution of FDH and FAD from a DEAE-cellulose column. (A) FDH assayed with F_{420} (□) and MV (○). Samples of 10 μl from each fraction were assayed. (B) FAD as estimated by reactivation of F_{420}-dependent FDH activity. The source of FDH was the FDH peak fraction from which residual FAD was removed by dialysis. Boiled samples (40 μl) of column fractions were incubated with the FDH (10 μg of protein) before the assay (see the text).

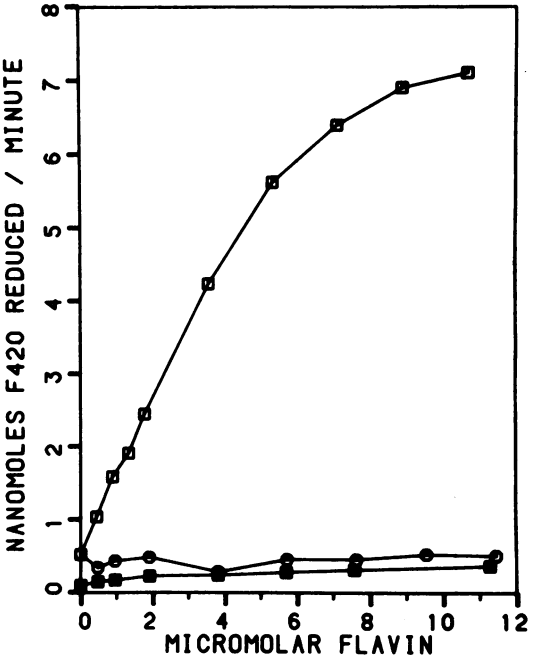

FIG. 5. Effect of the concentration of flavins on reactivation of F_{420}-dependent activity of purified FDH from *M. formicicum*. The assays contained 0.7 μg of protein. Symbols: □, commercially available FAD; ■, $FADH_2$; ○, FMN. The FAD was reduced with zinc dust.

Figure 5 shows the effect of flavin concentration on the reactivation of F_{420}-dependent FDH activity. The MV-dependent FDH activity (42 μmol min^{-1}/mg of protein) was not affected by the concentrations of the flavins tested. Above 10 μM FAD, the reconstituted activity ratio (F_{420}/MV) was 0.23. This ratio is 60% of that found in the cell-free extract (Table 1). Neither FMN or $FADH_2$ substituted for FAD at the concentrations tested (Fig. 5). Air-oxidized $FADH_2$ was equivalent to FAD in the reactivation of F_{420}-dependent FDH activity (data not shown).

A plot of the FAD concentration versus the reactivation of F_{420}-dependent FDH activity was nonlinear when $FADH_2$ was present (Fig. 6). This result was unlike that obtained in the absence of $FADH_2$ (Fig. 5), which indicates that $FADH_2$ competes with FAD for a specific site on the enzyme but is inactive. These results could be a consequence of the pronounced structural differences between FAD and $FADH_2$. Compounds that did not reactivate F_{420}-dependent FDH activity when present in the reaction mixture at 10μM included AMP, ADP, ATP, $NADP^+$, NAD^+, and adenine.

Partially purified FDH from *M. formicicum* reduces both F_{420} and FAD (14), which present-

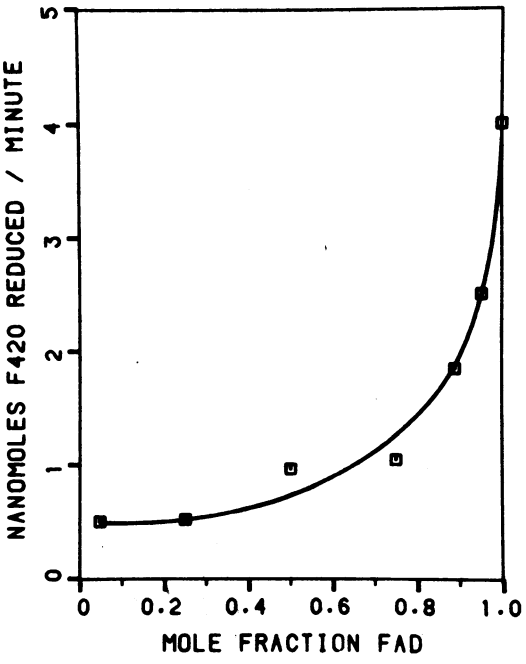

FIG. 6. Effect of $FADH_2$ on FAD reactivation of F_{420}-dependent activity in purified FDH from *M. formicicum*. The mole fraction FAD was FAD/(FAD + $FADH_2$), and the total flavin concentration was 12 μM. Commercially available FAD was used. The FAD was reduced with zinc dust. Each assay contained 0.7 μg of protein.

ed the possibility that the FDH preparations contained a $FADH_2$:F_{420} oxidoreductase which mediated the electron flow from FDH to F_{420}. Under the assay conditions used, no $FADH_2$:F_{420} oxidoreductase activity was detected.

The reactivation of F_{420}-dependent FDH activity by FAD was time dependent (Fig. 7). The rate of reactivation was also dependent on the temperature. Maximum reactivation required 5 min at 35°C with 10 μM FAD. These results were consistent with the incorporation of FAD into apoprotein.

DISCUSSION

FAD is present in the membranes of *Methanobacterium bryantii* (10) and is required for a NADH diaphorase isolated from *M. hungatei* (12). The partially purified hydrogenase from *M. thermoautotrophicum* contains tightly bound FAD (6). Both FAD and FMN are reduced by hydrogenase and FDH (6, 8, 14), but the present evidence does not support an electron carrier function for free FAD or FMN in the methanogenic bacteria. The results presented here suggest that free FAD did not function as an electron carrier between FDH and F_{420} in the *M. formicicum* FDH preparations. This suggestion was supported by the time-dependent reactivation of F_{420}-dependent FDH activity upon incubation with FAD. Furthermore, no $FADH_2$:F_{420} oxidoreductase activity was detected in the FDH preparations. The results indicate that the FDH from *M. formicicum* is a F_{420}-reducing flavoprotein and that purification by Phenyl Sepharose column chromatography produced a MV-reducing deflavo species of FDH unable to reduce F_{420}. A function ascribed to flavins is the intramolecular transfer of electrons from one-electron centers to two-electron acceptors, such as NAD^+ or $NADP^+$. Perhaps FAD performs a similar function in the two-electron reduction of F_{420} by FDH from *M. formicicum*. The purified FDH from this organism contains molybdenum, possibly in a molybdenum cofactor, and iron-sulfur clusters (14; unpublished data) which are candidates for the one-electron centers. The removal of FAD from the FDH or reconstitution with FAD had no effect on the formate-depen-

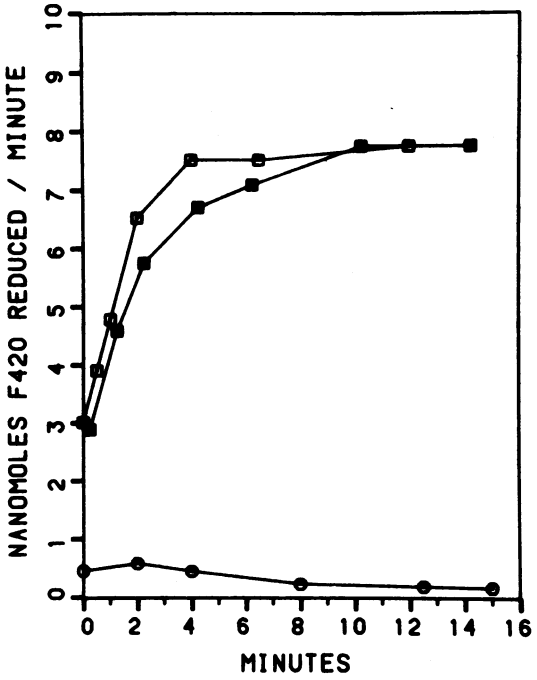

FIG. 7. Time course of reactivation of F_{420}-dependent activity of FDH purified from *M. formicicum*. Purified FDH was anaerobically preincubated in the assay cuvette for the indicated times at various temperatures and assayed at 35°C. Reaction mixtures contained 0.7 μg of protein. Symbols: □, preincubation at 35°C with 10 μM commercially available FAD; ■, preincubation at 25°C with 10 μM commercially available FAD; ○, preincubation at 35°C without FAD.

dent reduction of MV, which shows that FAD is not required for the initial oxidation of formate by FDH. Further research is required to determine the precise function of FAD in the F_{420}-dependent FDH activity of *M. formicicum*.

The removal of FAD by purification or dialysis showed that it is not covalently bound to the FDH from *M. formicicum*. The FDH from this organism appears similar to the *P. oxalaticus* FDH, from which FMN can be reversibly removed and whose deflavo species is unable to reduce the two-electron acceptor NAD^+ (13). However, unlike the methanogen FDH, the deflavo species is unable to reduce dyes. The *M. formicicum* and *P. oxalaticus* enzymes are similar in that both are poorly reactivated by the reduced form of the appropriate flavin.

The iron-sulfur, NAD^+-dependent FDH from *P. oxalaticus* is the only bacterial FDH quantitatively studied that is known to require flavin. Both FMN and FAD stimulate NAD^+ reduction by FDH in extracts of *Rhodopseudomonas palustris* (19). Flavins are not detectable in the *Escherichia coli* or *W. succinogenes* FDHs (4, 9). Both of these enzymes contain molybdenum but are distinguished from other bacterial FDHs by their association with cytochromes and the ability to reduce quinones but not NAD^+ or $NADP^+$. Flavins were not reported in the homogeneous tungsten-selenium-iron FDH of *C. thermoaceticum* that reduces $NADP^+$ (17). The homogeneous FDH from *M. vannielii* is an iron-sulfur molybdoenzyme that reduces F_{420} (8). Both FAD and FMN are also reduced by the *M. vannielii* FDH, but it was not determined whether flavins are present or required for the reduction of F_{420} (8). The considerable diversity in prosthetic groups and specificity of electron acceptors among bacterial FDHs suggests different intramolecular electron transport mechanisms. Further research on the function of FAD in *M. formicicum* FDH should provide information on the mechanisms of F_{420} reduction.

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