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Factor 420-Dependent Pyridine Nucleotide-Linked Formate Metabolism of *Methanobacterium ruminantium*

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Methanobacterium ruminantium was shown to possess a formate dehydrogenase which is linked to factor 420 (F_{420}) as the first low-molecular-weight or anionic electron transfer coenzyme. Reduced F_{420} obtained from the formate dehydrogenase can be further linked to the formation of hydrogen via the previously described F_{420}-dependent hydrogenase reaction, thus constituting an apparently simple formate hydrogenlyase system, or to the reduction of nicotinamide adenine dinucleotide phosphate via F_{420}:nicotinamide adenine dinucleotide phosphate oxidoreductase. The results indicate that hydrogen and formate, the only known energy sources for *M. ruminantium* and many other methanogenic bacteria, should be essentially equivalent as sources of electrons in the metabolism of this organism.

Most known authentic species of methanogenic bacteria, exceptions being *Methanobacterium* strain MOH (1, 2), *Methanobacterium thermoautotrophicum* (22), *Methanosarcina barkeri* (17, 19), and strain F5 isolated by Prins et al. (13), use electrons generated from the oxidation of formate from growth and methane formation, yet very little is known concerning the electron transfer pathways of formate utilization. In studies on *Methanobacterium ruminantium*, Bryant et al. (1) were unable to prepare active cell-free extracts which produced methane from either H_2-CO_2 or formate. Subsequently, M. P. Sharma and Bryant (unpublished data) found that wet packed cells stored under hydrogen gas at −20 C for varying lengths of time and cell-free extracts prepared from fresh or stored cells failed to form methane; however, these crude preparations metabolized formate with formation of approximately stoichiometric amounts of hydrogen and carbon dioxide, showing that a formate hydrogenlyase system exists in *M. ruminantium*. In previous studies on the same organism, we (18) showed that factor 420 (F_{420}) (3), an electron transfer coenzyme, is involved in a nicotinamide adenine dinucleotide phosphate (NADP)-linked hydrogenase system.

The present investigation was initiated to determine the pathway for the transfer of electrons generated from the oxidation of formate and the pathway involved in the reduction of pyridine nucleotide by formate with cell-free extracts of *M. ruminantium*.

MATERIALS AND METHODS

Except as stated below, the materials and methods used were as indicated by Tzeng et al. (18).

Formate hydrogenlyase activity was assayed by measurement of hydrogen evolution with 0.2 ml of 6 M KOH in the center well of each Warburg vessel. The standard reaction mixture (total volume 3.0 ml) contained, in micromoles: potassium phosphate buffer (pH 7.0), 250; $MgCl_2$, 10; reduced glutathione, 10; sodium formate, 30; and 10 mg of protein from crude extract. Boiled crude extract, 2 μg of F_{420} from *M. ruminantium* or 0.5 μmol of methyl viologen, was added or sodium formate was deleted, as indicated in the results. All solutions were prepared with previously boiled and cooled water kept under argon gas. Vessels were gassed for 15 min with oxygen-free argon gas which was prepared by passing argon gas through a column of hot reduced copper wire and two 500-ml gas washing bottles containing photochemically reduced methyl viologen (21). After gassing, vessels were equilibrated for 10 min and the reaction was started by tipping in reduced glutathione plus crude extract from the side arm. Incubation was at 37 C.

The standard assay mixture (total volume 3.0 ml) for the spectrophotometric assay of F_{420}-linked formate dehydrogenase activity contained in each Thunberg cuvette 300 μmol of tris(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 8.0), 67 μg of F_{420} from *M. ruminantium*, and 1.5 mg of protein from extracts treated by passage through columns of diethylaminoethyl-cellulose and Sephadex G-25 (referred to as DEAE-G25-treated extract; 18); also, various amounts of sodium formate were added as indicated below. All solutions were prepared with previously boiled water and kept anaerobic under argon gas in serum bottles. Appropriate syringes were used to transfer reagents and enzyme from stock

solutions to Thunberg cuvettes. To obtain anaerobic conditions, each Thunberg cuvette was evacuated and flushed 10 times with oxygen-free argon gas prepared as described above. The reaction was started by tipping in DEAE-G25-treated extract from the side arm, and incubation was at room temperature. The enzymatic activity was assayed by estimation of the change of absorption of F_{420} at 420 nm with a Cary model 14 spectrophotometer. Reaction mixture minus formate was used as a control.

During measurements of the specific activity of the reduction of F_{420}, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) by formate with the formate dehydrogenase system, the standard reaction mixture and experimental procedures used were the same as described above, except that 20 μmol of sodium formate as an electron donor and 79 μg of F_{420} isolated from *M. ruminantium* or 0.2 μmol of FAD or FMN were used, and that 10 μmol of dithiothreitol (DTT) plus various amounts of DEAE-G25-treated extract, as indicated below, was added to the side arm and reduced glutathione was omitted. Reaction mixture minus formate served as controls. The reaction rate of the reduction of FAD or FMN was measured by following the change of absorption at 450 nm.

During the spectrophotometric assay of the pyridine nucleotide reduction linked to the formate dehydrogenase system, all procedures were the same as described above, except that the standard reaction mixture (total volume 3.0 ml) in each Thunberg cuvette contained 90 μmol of sodium formate, 250 μmol of potassium phosphate buffer (pH 8.0), 3 μmol of NADP or nicotinamide adenine dinucleotide (NAD), 1.0 μg of F_{420} from *Methanobacterium* strain MOH, and 8.4 mg of protein from DEAE-G25-treated extract. This assay was measured by following the change of absorption of pyridine nucleotide at 340 nm. Reaction mixture minus formate was used as the control.

RESULTS

Formate hydrogenlyase system. Results in Fig. 1 show that extracts of *M. ruminantium*, passed through a column of Sephadex G-25 to remove low-molecular-weight materials and through a column of DEAE-cellulose to remove strongly anionic materials, required only F_{420} for hydrogen evolution from formate. Other experiments showed that, even with crude extract (10 mg), addition of formate was necessary for hydrogen evolution and that F_{420} (2 μg) or boiled crude extract (13 mg of protein) more than doubled the reaction rate. The data (Fig. 1) also show that neither the flavins nor crude ferredoxin, proven to be active in the *Clostridium pasteurianum* pyruvate-ferredoxin oxidoreductase reaction (18), replaced F_{420} in the reaction; however, FAD stimulated the system when included with F_{420}. The failure of the

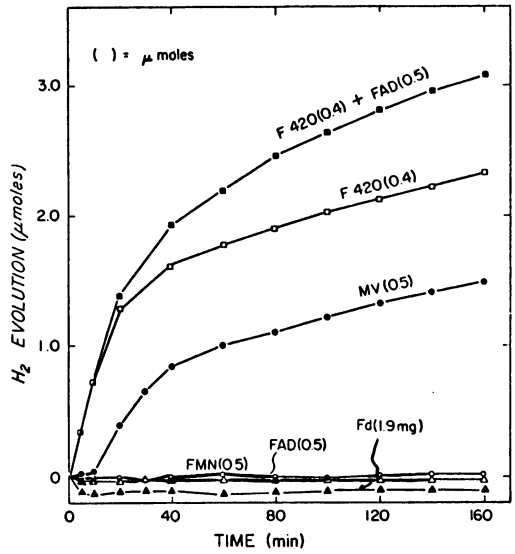

FIG. 1. *Effect of F_{420}, flavins, and ferredoxin in the formate hydrogenlyase system in DEAE-G25-treated extracts of M. ruminantium. The reactions were carried out as described in the text except that 18 mg of protein from DEAE-G25-treated extract, 10 μmol of DTT in place of reduced glutathione, 40 μmol of sodium formate, and FAD, FMN, methyl viologen (MV), ferredoxin (Fd) from C. pasteurianum, and F_{420} from M. ruminantium were added as indicated. All data shown were corrected for the control which contained all reagents described above except flavins, MV, Fd, and F_{420}.*

system to produce hydrogen from formate when a flavin replaced F_{420} is of interest since both the hydrogenase (18) and formate dehydrogenase allow flavin reduction. Apparently the reaction of the flavin-linked hydrogenase can effectively proceed in only one direction, toward flavin reduction.

Methyl viologen replaced F_{420} in the system but, as was the case with the NADP-linked hydrogenase system (18), the rate of the reaction was considerably slower. Other experiments indicated that F_{420} isolated from *Methanobacterium* strain MOH was as effective as that from *M. ruminantium* in the formate hydrogenlyase system.

Passage of the crude extract through DEAE-cellulose and Sephadex G-25 columns caused a lag of 30 to 40 min to develop in the formate hydrogenlyase system. The lag was not seen when the reaction was carried out with crude extracts. The lag was due to the formate dehydrogenase and the length of the lag was dependent upon the concentration of formate added (see below). DTT at an optimal concentration of

about 2.5 mM largely eliminated this lag, whereas 6.7 mM Na_2S, glutathione, or cysteine plus vitamin B_{12} (0.2 mM) did not. DTT did not contribute to hydrogen evolution in the absence of formate.

Formate dehydrogenase. Since the formate hydrogenlyase system of *M. ruminantium* is composed of at least two enzymes, i.e., formate dehydrogenase and hydrogenase, and the activity of the DEAE-G25-treated extract requires addition of only one co-factor, F_{420}, it was evident that F_{420} is a low-molecular-weight and anionic electron acceptor for the formate dehydrogenase. As illustrated in Fig. 2, with DEAE-G25-treated extracts, F_{420} is reduced by formate. Higher concentrations of formate shortened the lag period in the reduction of F_{420} by formate seen when DTT was deleted. This indicates that the lag seen in the formate hydrogenlyase system was due to the dehydrogenase rather than the hydrogenase.

The apparent K_m of formate for the F_{420}-linked formate dehydrogenase is 8.3×10^{-4} M at pH 8.0 (Fig. 3).

Results indicated in Fig. 4 show that FAD and FMN are also linked to the formic dehydrogenase and that the reaction rate with either the flavins or F_{420} as acceptor is proportional to the amount of extract included. The specific activity for F_{420} is 0.11 μmol per min per mg of protein or about three and five times the activity for FAD and FMN, respectively. Other experiments showed that both methyl and benzyl viologen link to the formate dehydrogenase in DEAE-G25-treated extracts.

Pyridine nucleotide-linked formate dehydrogenase system. As expected from the above results and the previous study (18) showing that DEAE-G25-treated extracts of *M. ruminantium* contain F_{420}-dependent NADP-linked hydrogenase activity, results (Fig. 5) show that NADP but not NAD is reduced by formate and the reaction is dependent upon F_{420}. Other experiments indicated that crude ferredoxin from *C. pasteurianum*, FAD, and FMN were

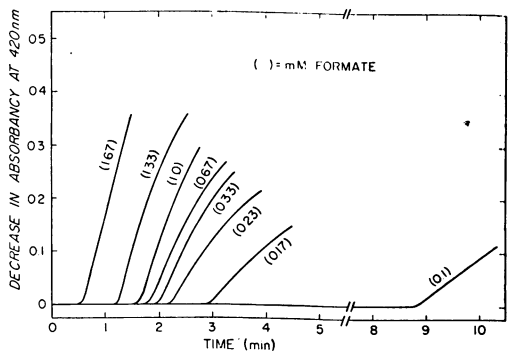

FIG. 2. *Reduction of F_{420} by various amounts of formate with DEAE-G25-treated extracts of M. ruminantium. Conditions were as described.*

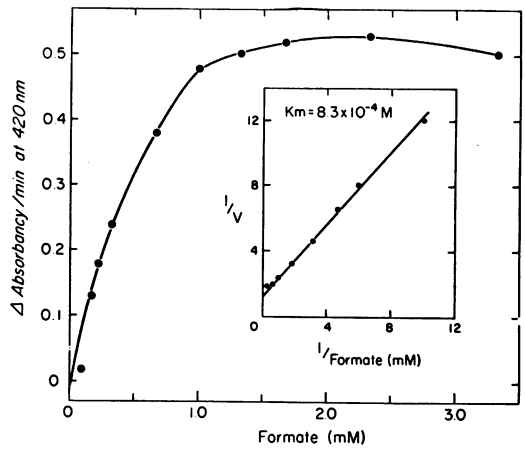

FIG. 3. *The effect of formate concentration on the rate of F_{420} reduction by the formate dehydrogenase of M. ruminantium and a Lineweaver-Burk plot used to determine the K_m for formate. The experiment was that described in Fig. 2. The rate of change in absorbancy was estimated from the slope of a straight line drawn as a tangent through the estimated points of maximal slope of the recorded change in absorbancy with time (Fig. 2).*

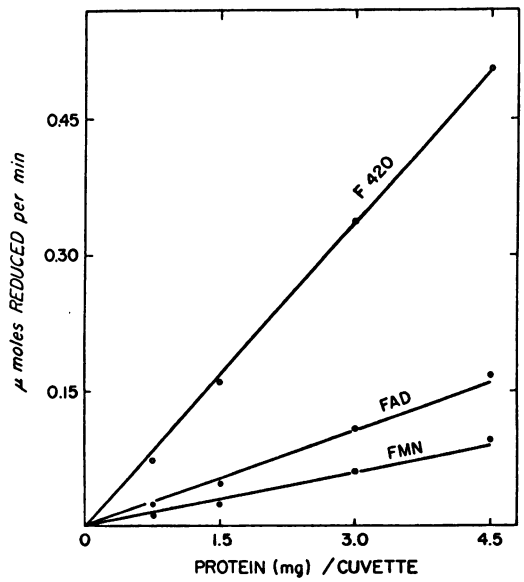

FIG. 4. *Relationship between the rate of F_{420}, FAD, and FMN reductions and the amount of DEAE-G25-treated extract. The reactions were carried out as described. The reaction rates were estimated as indicated in Fig. 3.*

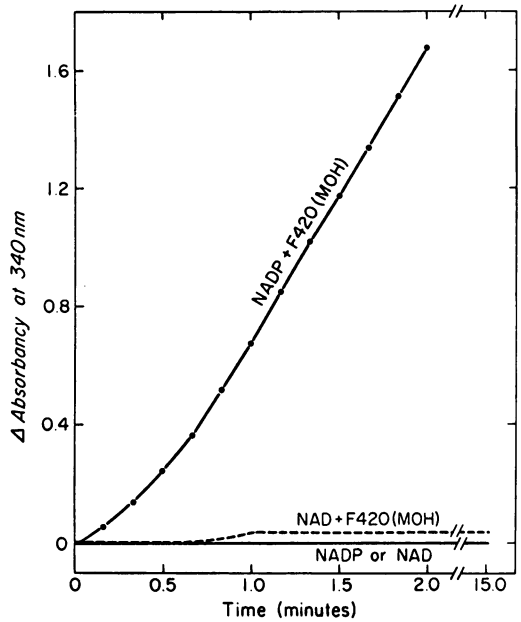

FIG. 5. *The reduction of NADP by formate via F_{420} with DEAE-G25-treated extract of M. ruminantium. Conditions were as described.*

inactive in the system. The inactivity of flavins in this system confirms the earlier results in which H_2 was the electron donor showing that no flavin:NADP transhydrogenase activity is present in *M. ruminantium*. Whether flavin reduction by reduced NADP could be catalyzed by the extracts was not studied.

DISCUSSION

The present results together with those previously presented (18) indicate that transport of electrons between formate, hydrogen, and NADP in *M. ruminantium* involve pathways which require F_{420} as a necessary intermediate electron carrier, i.e., NADP-linked, F_{420}-dependent hydrogenase and NADP-linked, F_{420}-dependent formate dehydrogenase activities. Both the hydrogenase and the formate dehydrogenase linked to F_{420} as the low-molecular-weight carrier. Whether hydrogen (18) or formate is the source of electrons, the electron carriers utilized by the NADP reducing system remain the same. The features of the hydrogenase activity seem to be essentially the same when studied via hydrogen evolution with the formate hydrogenylase reaction, as done here or with the previously used hydrogen uptake systems (18).

Since both the hydrogenase and the formate dehydrogenase are linked to F_{420}, it is evident that hydrogen and formate, the only known energy sources for *M. ruminantium*, should be essentially equivalent as sources of electrons in the metabolism of this organism.

In the F_{420} linkage, the formate dehydrogenase of *M. ruminantium* seems to differ from other formate dehydrogenases. The formate dehydrogenases isolated from a particulate preparation of *Enterobacter* (*Aerobacter*) *aerogenes* (10) or *Escherichia coli* (4, 12) or a soluble fraction of *Rhodopseudomonas palustris* (14) were not linked to the reduction of NADP or NAD, but they catalyzed the reduction of artificial dyes such as methylene blue and viologens. A soluble cytochrome b_1-linked formate dehydrogenase has been obtained when the particulate preparation of *E. coli* grown aerobically or anaerobically was treated with deoxycholate and snake venoms (5) or deoxycholate and ammonium sulfate (16, 20). In some other anaerobic bacteria, the formate dehydrogenases differ from that of *M. ruminantium* in that they link directly to NAD or NADP; that isolated from *Clostridium thermoaceticum* (9) is linked to NADP whereas those of *Clostridium formicoaceticum* (11) and S organism are linked to NAD (19). That isolated from *Clostridium acidiurici* (8) is linked to NAD via ferredoxin. Jacobs and Wolin (6) could not demonstrate that the formate dehydrogenase from *Vibrio succinogenes* is linked to pyridine nucleotides; however, small amounts of H_2O_2 are formed during the oxidation of formate by oxygen and both cytochrome *b* and *c* are reduced by formate. In aerobic bacteria, Johnson and Quayle (7) demonstrated that direct reduction of NAD by formate was catalyzed by soluble formate dehydrogenases from two different species of *Pseudomonas* grown on methanol.

The lag period in the reaction of the formate dehydrogenase is probably due to oxidation of the enzyme(s) during passage of extracts through DEAE-cellulose and Sephadex G-25 columns. That the reducing agent DTT or higher levels of formate diminishes the lag period suggests that sulfhydryl groups or other moiety activated by reduction may be necessary for enzyme activity. A similar lag period has been observed in formate dehydrogenase reactions of a number of organisms. For example, the lag in the NAD-linked reaction carried out by DEAE-cellulose-treated extracts of S organism (15) could be greatly shortened by adding ferredoxin, which presumably allowed reduction of an essential moiety via a ferredoxin-linked reaction. The NADP-specific enzyme of *C. thermoaceticum* was inhibited by the introduction of oxygen into the system and it required a sulfhydryl compound for activity

(9). The inhibition due to oxygen of the formate dehydrogenase from *R. palustris* could not be reversed by the addition of catalase. This enzyme was also inhibited by a very low concentration of *p*-chloromercuribenzoate, and the inhibition could be completely reversed by the addition of an excess of cysteine (14). The formate dehydrogenase from *C. acidiurici* was sensitive to oxygen and light, and it also required a reducing agent to enhance the rate of electron transfer (8).

The formate hydrogenlyase system in *M. ruminantium* which may consist of only two components, the F_{420}-linked formate dehydrogenase and hydrogenase activities, seems to contain fewer components than that of other organisms so far studied. The formate hydrogenlyase system of S organism consists of an NAD-dependent formate dehydrogenase, an NAD:ferredoxin oxidoreductase, and ferredoxin-dependent hydrogenase activity (15). The formate hydrogenlyase system of *R. palustris* (14) is different from that of *M. ruminantium* but has many properties in common with that of coliform organisms (4, 12). The system from *E. coli* appears to consist, at least in part, of a soluble formate dehydrogenase and a particulate hydrogenase, and evidence suggests that one or more unidentified intermediate electron carriers is involved in this system. The participation of cytochrome c_3 as electron carrier in the formate hydrogenlyase of *Desulfovibrio desulfuricans* has been reported by Williams et al. (Bacteriol. Proc., p. 110, 1964).

Whether or not formate can be synthesized from CO_2 by reversal of the formate hydrogenlyase system in *M. ruminantium* was not determined. Stadtman (17) showed that formate was synthesized from carbon dioxide and hydrogen by cell suspensions and cell-free extracts of *Methanococcus vannielli*.

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