Mechanistic Studies of the Coenzyme F_{420} Reducing Formate Dehydrogenase from *Methanobacterium formicicum*†

Neil L. Schauer‡ and James G. Ferry*

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

John F. Honek,§ William H. Orme-Johnson, and Christopher Walsh

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT: Mechanistic studies have been undertaken on the coenzyme F_{420} dependent formate dehydrogenase from *Methanobacterium formicicum*. The enzyme was specific for the *si* face hydride transfer to C_5 of F_{420} and joins three other F_{420}-recognizing methanogen enzymes in this stereospecificity, consistent perhaps with a common type of binding site for this 8-hydroxy-5-deazariboflavin. While catalysis probably occurs by hydride transfer from formate to the enzyme to generate an EH_2 species and then by hydride transfer back out to F_{420}, the formate-derived hydrogen exchanged with solvent protons before transfer back out to F_{420}. The kinetics of hydride transfer from formate revealed that this step is not rate determining, which suggests that the rate-determining step is an internal electron transfer. The deflavo formate dehydrogenase was amenable to reconstitution with flavin analogues. The enzyme was sensitive to alterations in FAD structure in the 6-, 7-, and 8-loci of the benzenoid moiety in the isoalloxazine ring.

The strictly anaerobic methanogenic bacteria obtain energy for growth by the reduction of carbon dioxide with electrons from the oxidation of formate or H_2 catalyzed by FDH[1] or hydrogenase. About 50% of the methanogenic bacteria contain FDH, and in these organisms the oxidation of formate drives reduction of the 8-hydroxy-5-deazaflavin, coenzyme F_{420} (Eirich et al., 1978) (Figure 1). F_{420} is a low-potential ($E^{\circ\prime}$ = −360 mV) electron carrier present in high concentrations (up to 100 mg/kg of cells). The dihydro form, $F_{420}H_2$, drives NADPH formation via an NADP:F_{420} oxidoreductase (Jones & Stadtman, 1980). The F_{420}-reducing FDH from *Methanococcus vannielii* (Jones & Stadtman, 1980) and *Methanobacterium formicicum* (Schauer & Ferry, 1982, 1983, 1986; Barber et al., 1983; May et al., 1986) has been characterized. The *M. formicicum* enzyme contains zinc, molybdenum, molybdopterin, iron–sulfur clusters, and FAD and in these latter four cofactors resembles the oxidation–reduction inventory of the well-studied milk xanthine oxidase (Hille et al., 1981). The F_{420}-reducing hydrogenase of *Methanobacterium thermoautotrophicum* and *M. formicicum* is also a multicomponent oxidation–reduction inventory catalyst containing nickel, iron–sulfur centers, and bound FAD (Kojima et al., 1983; Nelson et al., 1984). The FAD component of these F_{420}-reducing enzymes apparently functions as an oxidation–reduction switch to collect electrons from one-electron centers and donate an electron pair to the obligate two-electron F_{420} acceptor (Jacobson et al., 1982; Schauer & Ferry, 1983; Nelson et al., 1984).

The FAD of the *M. formicicum* FDH dissociates when the enzyme is reduced, which yields the deflavoenzyme that is incompetent toward F_{420} reduction (Schauer & Ferry, 1986). We report here initial studies on reconstitution of the deflavoenzyme with FAD analogues to probe the oxidation–reduction mechanism in this enzyme. We also report studies with isotopically labeled formate, F_o (a hydrolytic derivative of F_{420}), and solvent to analyze the mode of hydrogen transfer and the stereochemical outcome for this enzyme.

Experimental Procedures

Flavins and Flavin Analogues. Coenzyme F_{420} was prepared as previously described (Schauer & Ferry, 1983). F_o, a hydrolytic derivative of F_{420}, was prepared by the method of Ashton et al. (1978). 10-*N*-Dealkyl-F_o was prepared by the method of Yamazaki et al. (1982). The 9-aza-, 8-chloro-, and 8-hydroxyriboflavin analogues of F_{420} were generous gifts of Merck and Co.

The [5-^{3}H]F_o was generated chemically by borotritiide reduction followed by reoxidation as previously described (Yamazaki et al., 1980). The k_H/k_T during reoxidation allowed preparation of oxidized 5-tritio-F_o of high specific radioactivity useful for stereochemical studies. Radioactivity was counted on a Beckman LS1800 scintillation counter with ACS (Amersham) as scintillation fluid.

Riboflavin-level analogues were a generous gift from Dr. W. T. Ashton, Merck Sharp & Dohme, West Point, PA. The 6-hydroxyriboflavin utilized in this study was a gift from Dr. Vincent Massey. Enzymatic synthesis of the FAD levels of the various flavin analogues was performed by the use of

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* Address correspondence to this author.

‡ Present address: Genetics Institute, Cambridge, MA, 02140.

§ Present address: Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1.

[1] Abbreviations: FDH, formate dehydrogenase; F_{420}, coenzyme F_{420}; $F_{420}H_2$, dihydrocoenzyme F_{420}; F_o, 7,8-didemethyl-8-hydroxy-5-deazariboflavin, riboflavin level of coenzyme F_{420}; F_oH_2, dihydro-F_o; FAD, flavin adenine dinucleotide; $FADH_2$, fully reduced FAD; FMN, flavin mononucleotide; HPLC, high-performance liquid chromatography; MV, methyl viologen; NMR, nuclear magnetic resonance. The flavin analogues at the riboflavin level are as follows: 7-chloro-8-demethyl, 7-chloro-7,8-didemethylriboflavin; 8-chloro, 8-chloro-8-demethylriboflavin; 6-methyl, 6-methylriboflavin; 6-hydroxy, 6-hydroxyriboflavin; 1-deaza, 1-deoxy-1-[2,3-dihydro-7,8-didemethyl-1,3-dioxopyrido[3,4-*b*]-quinoxalin-5(1*H*)-yl]-D-ribitol; 5-deaza, 1-deoxy-1-[3,4-dihydro-7,8-dimethyl-2,4-dioxopyrimido[4,5-*b*]quinolin-10(2*H*)-yl]-D-ribitol.

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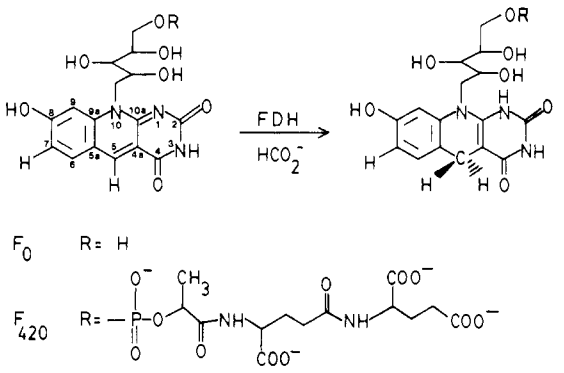

FIGURE 1: Reduction of coenzyme F_{420} or F_o catalyzed by FDH from *M. formicicum*.

flavokinase and FAD synthetase partially purified from *Brevibacterium ammoniagenes* as previously reported (Spencer et al., 1976; Hausinger et al., 1986). FAD (grade III), FMN (grade I), and riboflavin were purchased from Sigma.

Preparation of Holo- and Deflavo-FDH. The purification of FDH from *M. formicicum* and preparation of the deflavoenzyme was as previously described (Schauer & Ferry, 1986). The purified enzyme was repeatedly ultrafiltered and resuspended in buffer (50 mM potassium phosphate, pH 7.5) to remove unbound FAD from the preparation. This form of FDH was utilized in all kinetic experiments that required holoenzyme. The deflavoenzyme was prepared by removing bound FAD by anoxic pressure dialysis of formate-reduced holoenzyme.

Enzyme Assays and Incubations. The general anaerobic procedures for the preparation of solutions, enzyme assays, and incubations were as previously described (Schauer & Ferry, 1982). The standard assay for FDH was performed by following the formate-dependent reduction of electron acceptor with a Perkin-Elmer Model 552 spectrophotometer. The standard reaction mixture (0.75 mL) contained the following (μmol): 2-mercaptoethanol (15), potassium phosphate buffer (pH 7.5) (45), and MV (15), F_{420} (0.025), FMN (0.025), or F_o (0.025). Endogenous activity was recorded after the addition of enzyme, and the reaction was initiated by the addition of 10 μmol of sodium formate. The following extinction coefficients were used: MV, 11.3 mM^{-1} cm^{-1} at 603 nm; F_{420}, 42.5 mM^{-1} cm^{-1} at 420 nm (pH 7.5); F_o, 39.4 mM^{-1} cm^{-1} at 420 nm; FMN, 12.2 mM^{-1} cm^{-1} at 450 nm. A unit was the amount of enzyme that reduced 1 μmol of acceptor/min.

Deuterioformate Studies. The effect of deuterioformate vs. protioformate on the rate of reduction of F_{420} was determined by following the kinetics of reduction at 420 nm. A 10-μL solution of holoenzyme (0.232 mg/mL) was added to standard assay mixtures that contained various concentrations of F_{420} (5, 10, 20, 30, and 35 μM) followed by the addition of 10 μL of an anoxic solution (pH 7.5) of either 1 M sodium protioformate or 1 M sodium deuterioformate to initiate the reaction. A 1.0 M solution of deuterioformate was prepared by neutralizing 1.93 mL of [1-^{2}H]formic acid (99 atom% deuterium, Merck) with 1 N NaOH to pH 7.71.

Source of Hydride in Reduced F_{420}. The source of the hydrogen donated to the C-5 position of F_{420} was determined by performing the reduction in the presence of either deuterioformate and H_2O or protioformate and D_2O catalyzed by FDH. A 1 M anoxic solution of sodium deuterioformate (100 μL) and 50 μL of holoenzyme (0.232 mg/mL) were added to a 5-mL anoxic solution of 630 μM F_{420} in 50 mM potassium phosphate buffer (pH 7.5). The incubation was under argon for 2 h at 23 °C after which an additional 150 μL of FDH and 100 μL of deuterioformate solutions were added. Progress of the reaction was monitored at 450 nm and was complete after a total of 3 h. In the alternate protioformate/D_2O incubation, F_{420} was enzymically reduced under identical conditions to those described above for the deuterioformate/H_2O incubation except that the F_{420} solution was lyophilized and dissolved in D_2O for two cycles and finally dissolved in D_2O before incubation. In this experiment, a 1 M anoxic solution of sodium formate dissolved in D_2O was the reductant. After completion, the reaction mixtures in both experiments were heated under argon at 100 °C for 10 min to inactivate FDH and then quickly frozen. Oxidized and reduced F_{420} were separated by HPLC utilizing isocratic conditions [10% methanol/90% 20 mM potassium phosphate (pH 7.5)] on a C-18 reverse-phase Bondpak semianalytical column. HPLC was performed on a Du Pont 850 chromatography system. The HPLC fractions containing reduced F_{420} were analyzed for deuterium incorporation at the C_5 position by proton NMR spectrometry. Samples for spectrometry were prepared by dissolving the lyophilized material in anoxic D_2O under argon. The spectrometry was performed in deuterium oxide (99 atom% ^{2}H, Aldrich) with (trimethylsilyl)propanesulfonate (Aldrich) as internal standard, and the spectrum was obtained on a Bruker 250-MHz Fourier-transform spectrometer. The position of the protons in oxidized and reduced F_{420} were previously assigned (Eirich et al., 1978). On reduction, the C_5–H signal at 8.3 ppm in oxidized F_{420} shifts to 3.35 ppm for the C_5 CH_2 group of $F_{420}H_2$. The ratio of C_5 protons (at 3.35 ppm) to aromatic protons gave the ratio of deuterium incorporated at C_5. The NMR spectra of reduced F_{420} produced during incubation with deuterioformate/H_2O or protioformate/D_2O were in complete agreement with published spectra (Fox et al., 1986). The incubation conditions used with the number of protons at the C_5 position of F_{420} determined by integration were as follows: protioformate/H_2O, 2 protons; deuterioformate/H_2O, 2.2 protons; protioformate/D_2O, 1.3 protons.

Stereochemistry of 5-Deazaflavin Reduction. The stereochemistry of the reduction of F_o by FDH from *M. formicicum* was determined in the following manner. The solution of [5-^{3}H]F_o (9.5 Ci/mol) was made up in 2 mL of anoxic buffer [100 mM potassium phosphate (pH 7.5) containing 10 mM 2-mercaptoethanol]; the total radioactivity was 7×10^5 dpm. The absorbance at 400 nm was 0.526 (ϵ_{400} = 25.5 mM^{-1} cm^{-1}). The solution was made anoxic under argon and brought to 37 °C followed by the addition of 30 μL of FDH (0.23 mg/mL) and 20 μL of 1 M sodium formate to initiate the reaction. The reaction mixture was incubated for 15 min, which resulted in the complete disappearance of yellow color and 400-nm absorbance. The incubation was continued for a total of 90 min after which an additional 5 μL of FDH and 10 μL of formate solutions were added. The mixture was cooled to 10 °C, and an Amicon Centricon-10 (Amicon Corp., Lexington, MA) fitted with a YM10 membrane was used to anoxically separate reduced 5-deazaflavin from the FDH. The transfer of the anoxic solution to the Amicon chamber was completed under argon and then centrifuged at 3000 rpm for 20 min to complete the anoxic filtration. An additional 525 μL of anoxic buffer was added to the Centricon-10 chamber, and after centrifugation the filtrates were pooled. The reduced F_o was then oxidized with pure F_{420}-dependent hydrogenase prepared from *M. thermoautotrophicum* as previously described (Fox et al., 1986). The hydrogenase (90 μL of a 1.9 μg/mL anoxic solution) and 50 μL of 4 M potassium chloride were added to

60 μL of anoxic buffer to activate the enzyme. A 25-μL aliquot of the activated hydrogenase solution was added to the pooled anoxic filtrates (2.5 mL) and incubated at 50 °C under argon. The progress of the reoxidation was monitored spectrophotometrically at 400 nm and reached completion after 15 min. The incubation was continued for an additional 75 min, after which the increase in absorbance was 0.412, representing a 96–100% reoxidation of reduced F_o. The hydrogenase was removed from solution by Centricon-10 ultrafiltration, and the membrane was washed with 400–500 μL of buffer as described above. The total volume of the pooled filtrates was brought to 3 mL, which contained 6.5×10^5 dpm, representing 93–95% recovery of radioactivity. The tritium washout was analyzed by distillation of the 3-mL sample in a miniature all-glass apparatus. The first fraction (1.94 mL) contained 5.5×10^4 dpm. An additional 3 mL of water was added, and a further 3.9 mL of distillate was collected, which contained a total count of 3.2×10^4 dpm. The yellow residue of F_o was dissolved in 2.4 mL of water, which contained a total count of 4.9×10^5 dpm. The total recovery of radioactivity from all fractions was 88–93%.

Substrate Flavin Analogues. Riboflavin-level analogues of F_{420} were studied for the effect of oxidation–reduction potential on their rate of reduction by FDH. The standard reaction mixture was used. Final concentrations of analogues were in the range 30–240 μM. The riboflavin analogue reductions were monitored spectrophotometrically as previously reported (Walsh et al., 1978). The 10-*N*-dealkyl-F_o was assayed at 400 nm (ϵ_{400} = 25.5 mM^{-1} cm^{-1}).

Reconstitution of Deflavo-FDH with FAD Analogues. A 5-μL aliquot of a 0.43 mM anoxic solution of normal FAD or FAD analogue or an equal aliquot of buffer was added to 25 μL of deflavo-FDH (2 μg) and incubated at 35 °C for 5 min. A 5-μL aliquot of the reconstituted enzyme solution was assayed in the standard assay. Kinetic constants were obtained with the following acceptor concentrations in the assay cuvette: F_o, 15, 20, 24, 29, 34, 39, and 49 μM; F_{420}, 10, 15, 21, 26, 36, 41, and 51 μM.

In the FAD challenge experiments, 10 μL of 0.5 mM FAD was added to the assay cuvette after a sustained rate was obtained; increases in the rate of reduction of the electron acceptor were recorded after a short lag. The results were reported as the percent of increase expected when deflavo-FDH was challenged with FAD.

Results and Discussion

Studies with Deuterioformate and D_2O. FDH from *M. formicicum* contains molybdenum and iron–sulfur oxidation–reduction centers that are likely to participate in the intramolecular transfer of reducing equivalents from formate to the FAD prosthetic group (Barber et al., 1983). Obligate two-electron reduction of the cosubstrate F_{420} occurs from reduced FAD (Schauer & Ferry, 1986). In analogy with the prototypic molybdenum, iron–sulfur, flavoprotein xanthine oxidase, where catalysis is initiated by oxidation of substrate via a hydride transfer to the Mo^{VI} cofactor of the enzyme, it is unlikely that *M. formicicum* FDH oxidizes formate to CO_2 with corresponding H^- transfer to the Mo^{VI} prosthetic group (Barber et al., 1983). To probe whether this H^- transfer step or any subsequent transfers of this substrate-derived hydrogen occur in any kinetically significant transition states, we determined the steady-state kinetics of pure FDH with deuterioformate and protioformate. The results with protioformate (K_m = 12 ± 3 μM; V_{max} = 15 ± 2 units/mg) and deuterioformate (K_m = 9 ± 3 μM; V_{max} = 15 ± 2 units/mg) showed no significant V_H/V_D or $(V/K)_D$ isotope effects, ruling out

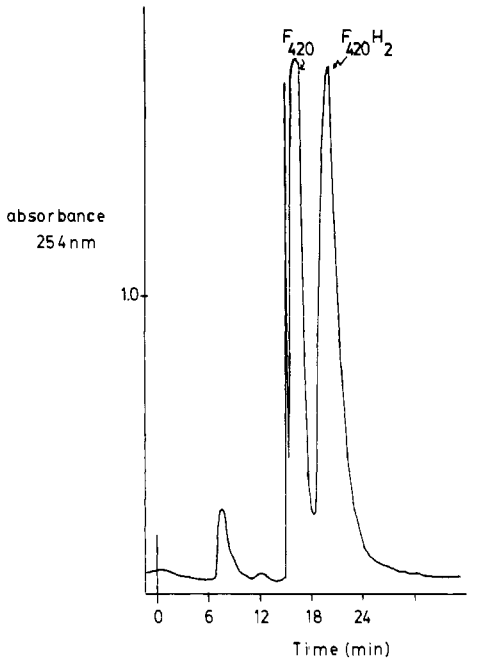

FIGURE 2: HPLC separation of oxidized and enzymically reduced F_{420}.

rate-limiting transfers of the substrate-derived deuterium in catalysis. Given the anticipated formation of an enzymic Mo^{IV}–D species, the subsequent fate of that deuterium was analyzed. The F_{420} undergoes net two-electron reduction to generate a methylene group at C_5. It was previously shown that this is a nonexchangeable carbon site and that F_{420} is nicotinamide-like in undergoing obligate two-electron transfers via hydride (Fisher & Walsh, 1974). Thus, it was possible that the D^- equivalent transferred into the enzyme would be handed along and passed back out as a D^- equivalent to yield $[5\text{-}D_1]F_{420}H_2$.

To test for net hydride transfer from formate to deazaflavin, we used F_{420} as cosubstrate and conducted an experiment with deuterioformate in H_2O on a scale large enough to isolate sufficient $F_{420}H_2$ for NMR analysis (see Experimental Procedures). The HPLC separation of oxidized and reduced F_{420} is shown in Figure 2. The analysis showed two protons at the C_5 CH_2 group of $F_{420}H_2$ when integrated against the internal C_6, C_7, and C_8 proton signals. Thus, the deuteride equivalent from deuterioformate was washed out to solvent before transfer to the deazaflavin cosubstrate. The cognate experiment, using protioformate in deuterated buffer, revealed one integratable proton at C_5 of $F_{420}H_2$, confirming formation of $[5\text{-}D_1]F_{420}H_2$. Thus, there was an exchange with one solvent hydrogen at an intermediate stage in catalysis, and one likely site is N_5 of the enzyme-bound $FADH_2$ from which deuteride transfer to F_{420} would then generate the observed $[5\text{-}D_1]F_{420}H_2$. This interpretation is consistent with the result that deflavo-FDH cannot reduce F_{420} (or F_o) and that the enzyme-bound FAD is the required oxidation–reduction switch interfacing between one-electron centers and the obligate two-electron acceptor F_{420}. While enzyme–FAD is an obvious site of solvent wash prior to hydride transfer to F_{420}, the site of wash-out of deuterium from deuterioformate could be earlier, possibly as a proton dissociation from Mo^{IV}–H, and remains to be analyzed.

Stereochemical Analysis of 5-Deazaflavin Reduction. Carbon 5 of $F_{420}H_2$ is structurally analogous to C_4 of NADH (i.e., a dihydronicotinamide that bears prochiral diasteriotopic methylene hydrogens at a nonexchangeable carbon locus in

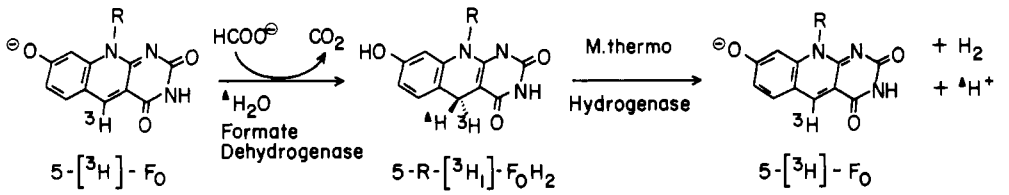

FIGURE 3: Source and chirality of hydride equivalent in enzymically reduced F_{420} by FDH from *M. formicicum* and comparison to the F_{420} hydrogenase from *M. thermoautotrophicum*.

contrast to the single exchangeable hydrogen at nitrogen 5 of dihydroflavins). As an aspect of FDH mechanism, we analyzed the stereochemical outcome of the hydride transfer out from the reduced enzyme to the deazaflavin cosubstrate. The [5-^{3}H]F_o was quantitatively reduced with FDH and formate in the presence of H_2O. The [5-^{3}H]F_oH_2 product was then reoxidized anaerobically by pure F_{420}-dependent hydrogenase from *M. thermoautotrophicum* (Figure 3). This hydrogenase has good activity (k_{cat} = 250 s^{-1}; Fox et al., 1986) toward $F_{420}H_2$ or F_oH_2 reoxidation, and the tritium removed from the C_5 of F_oH_2 exchanges with solvent before any significant HT formation. After the hydrogenase-catalyzed reoxidation of the [5-^{3}H]F_oH_2, 85 ± 5% of the tritium remained in the F_o and 10–15% was volatilized as HTO. In control studies, approximately 10% nonenzymic reoxidation of [5-^{3}H]F_oH_2 samples occurred under the experimental conditions; on the basis of this assumption, the 85 ± 5% could have approached quantitative retention of tritium in the hydrogenase-reoxidized F_o molecules (Jacobson, 1981). Alternatively, stereochemical randomization by comproportionation could have accounted for the 10–15% volatilized as HTO. The [5-^{3}H]F_oH_2 generated by reduction of [5-^{3}H]F_o by this same hydrogenase transfers tritium quantitatively on reoxidation by *re* face specific *Beneckea harveyii* NADH oxidoreductase (Jacobsen & Walsh, 1984); thus, the retention of ^{3}H in the above experiment was not complicated by nonreleasable tritium.

At this juncture it was possible to establish an absolute configurational assignment to the C_5 center in F_oH_2 generated by *M. formicicum* FDH activity on the basis of recent knowledge that the *M. thermoautotrophicum* hydrogenase is *si* face specific at C_5 of F_o. Yamazaki et al. (1985) and Teshima et al. (1985) studied the F_{420}-reducing hydrogenase from *M. vannielii*. They employed [5-^{2}H]F_o and after hydrogenase reduction degraded the [5-^{2}H$_1$]F_oH_2 to (*R*)-[4-^{2}H]-3,4-dihydro-7-hydroxy-1-(hydroxyethyl)quinoline. This corresponds to an (*R*)-[5-^{2}H$_1$]F_o configurational assignment; thus, the H^- equivalent added by the *M. vannielii* hydrogenase is introduced on the *si* face. With the absolute configuration known, they could relate previous studies (Yamazaki et al., 1980) to establish that the *M. vannielii* F_{420}-NADP reductase and the *M. thermoautotrophicum* hydrogenase are also *si* face specific at C_5 of F_o and F_{420}. In turn, the data of this paper show that the *M. formicicum* FDH is likewise *si* face specific. All four F_{420}-processing methanogen enzymes thus far examined are *si* face specific while the non-methanogen bioluminescent *B. harveyii* is *re* face specific with respect to NADH (Fisher & Walsh, 1974).

Reconstitution of Deflavo-FDH with FAD Analogues. Pressure dialysis in the presence of sodium formate as previously described (Schauer & Ferry, 1986) produced deflavoenzyme with less than 10% residual F_{420}-dependent activity. The effect of FAD concentration on the reconstitution of F_o-dependent activity is shown in Figure 4. Reconstitution of deflavoenzyme with normal FAD resulted in complete restoration of both the F_o-dependent and the F_{420}-dependent specific activity of the holoenzyme (Table I), reflecting the

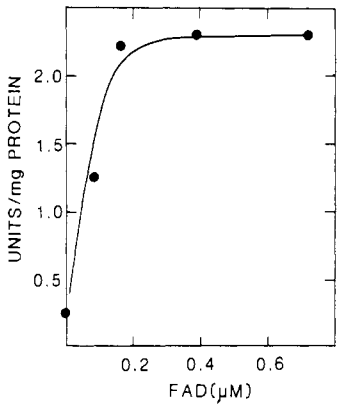

FIGURE 4: Reconstitution of deflavo-FDH from *M. formicicum*: effect of FAD concentration. Deflavoenzyme was reconstituted with FAD and assayed in the standard assay for F_o as described under Experimental Procedures. The various FAD concentrations shown were those in the assay cuvette; the FAD concentrations in the reconstitution mixtures were 100-fold greater.

Table I: Reconstitution of Deflavo-FDH from *M. formicicum* with Normal FAD and FAD Analogues

FAD analogue	$E^{\circ\prime}$[a]	V_{max} (μmol min^{-1} mg^{-1})[b]		K_m (μM)[b]	
		F_{420}	F_o	F_{420}	F_o
5-deaza	−311	NA[c]	NA	NA	NA
1-deaza	−280	6.9	7.3	14	160
6-hydroxy	−265	7.7	7.1	14	160
6-methyl	−219	NA	NA	NA	NA
normal FAD	−208	10.5	6.7	13	150
8-chloro	−152	11.1	1.5	45	135
7-chloro-8-desmethyl	−128	NA	NA	NA	NA

[a] Two-electron midpoint potential at the riboflavin level. [b] The initial rate of reduction (at least two determinations) with each acceptor concentration was linear, and double-reciprocal plots of initial velocity vs. acceptor concentration were also linear with correlation coefficients of 0.98 or greater (see Experimental Procedures). The residual activity of the apoenzyme was subtracted when calculating the kinetic constants. [c] No activity.

stability of deflavoenzyme during preparation and reconstitution. The MV-dependent activity (66 units/mg) was essentially unaffected, clearly proving MV accepted electrons from other oxidation–reduction centers in the FDH, most likely from iron–sulfur clusters by one-electron transfer. A third electron acceptor for holoenzyme, FMN, can in principle accept electrons from both one-electron and two-electron oxidation–reduction centers. The FMN was reduced by deflavoenzyme at 57% of the rate of holoenzyme (data not shown), suggesting that it apparently accepted electrons from the molybdenum and/or iron–sulfur oxidation–reduction centers.

Table I shows the midpoint potentials of normal FAD and the FAD analogues and kinetic constants for F_o and F_{420} reduction after the deflavoenzyme was reconstituted with these flavins. The FDH apparently was very sensitive to alterations in FAD structure in the 6-, 7-, and 8-loci of the benzenoid position of the isoalloxazine ring, the 6-methyl-FAD and 7-

Table II: Kinetic Isotope Effects on Coenzyme F_{420} Reduction Catalyzed by FDH from *M. formicicum* Reconstituted with Normal FAD and FAD Analogues

FAD analogue	$E^{\circ\prime}$ (mV)	activity[a] (μmol min^{-1} mg^{-1}) in H_2O	activity[a] (μmol min^{-1} mg^{-1}) in D_2O
1-deaza	−280	3.41 ± 0.31	2.87 ± 0.03
normal FAD	−208	4.87 ± 0.13	4.27 ± 0.06
8-chloro	−153	3.54 ± 0.10	2.01 ± 0.01

[a] Mean of three determinations in the standard assay with 30 μM F_{420}.

chloro-8-desmethyl-FAD enzymes were inactive, and 8-chloro-FAD increased the K_m for F_{420}.

After challenge of the reconstituted enzymes with FAD, a large increase in F_{420}-dependent activity occurred only with the 6-methyl-FAD enzyme (85% and 91% of expected increase with F_o and F_{420} as acceptors). Three assumptions were made in the interpretation of the FAD challenge results: (i) increases in the rate of F_{420}-dependent reduction, which occurred after challenge with FAD, resulted from the subsequent reconstitution of only free deflavoenzyme with FAD; (ii) the free deflavoenzyme in the assay cuvette resulted from the failure of the original FAD analogue to bind to the FAD site on the enzyme; (iii) once an analogue became bound to the FAD site, it did not dissociate from that site during the course of the experiment. The FAD challenge results imply that 6-methyl-FAD may not have been incorporated into the FAD site in the enzyme. Failure of the 6-methyl-FAD to associate with the FAD site could be explained by steric hindrance from the 6-methyl group. Furthermore, the 6-hydroxy-FAD reactivated the deflavoenzyme, which indicated that the size of the group at position 6 may have influenced binding to the FDH. On the other hand, the third assumption made in the challenge experiments (above) may not have applied to the 6-methyl-FAD; therefore, it cannot be ruled out that this FAD analogue was incorporated but was inactive.

Although the FAD challenge experiments suggested that 5-deaza-FAD was incorporated into deflavoenzyme, no significant activity was detected. The inactivity of the 5-deaza-FAD enzyme could have resulted from the fact that, like F_{420}, the parent 5-deazaflavin system in 5-deaza-FAD is also restricted to two-electron transfer steps and thus was incompetent to accept electrons from obligate one-electron donating centers.

The semilog plot of the data in Table I for flavin two-electron potential and the V_{max} for F_{420} reduction is shown in Figure 5. In interpreting these data, it was assumed that the nonreconstitutable enzyme molecules did not interfere in the assays. The 1-deaza-FAD and 6-hydroxy-FAD with potentials of 72 and 57 mV more negative than that of FAD yielded enzymes with a V_{max} for F_{420} reduction of 66 and 73% of that of the FAD enzyme. However, the 8-chloro-FAD with a potential 56 mV less negative than that of FAD produced an enzyme with a V_{max} less than expected based on the relative potentials (Figure 5). The rate of F_{420}-dependent activity for the 8-chloro-FAD enzyme was decreased by 43% when assayed in D_2O whereas the rate was decreased by 15 and 12% for the 1-deaza-FAD and normal FAD enzymes (Table II). These kinetic isotope effects suggest that electon transfer out from 8-chloro-FAD to F_{420} was more difficult than those of the 1-deaza-FAD or normal FAD, which may have resulted from the large difference between the potentials of 8-chloro-FAD ($E^{\circ\prime}$ = −152 mV) and F_{420} ($E^{\circ\prime}$ = −369 mV). On the other hand, the K_m for F_{420} of the 8-chloro-FAD enzyme was 3-fold greater than the K_m for F_{420} of the normal FAD enzyme (Table I), suggesting that substituents in the 8-loci of the benzenoid ring of enzyme-bound FAD affected the interaction with F_{420}, an 8-phenoxide species. The 8-chloro-FAD and normal FAD showed the same K_m for F_o (Table I), suggesting that the effect of 8-chloro-FAD on the interaction of F_{420} with enzyme was focused on the side chain of F_{420}. Hence, reduction of 8-chloro-FAD should have been easier compared to FAD, on the basis of relative oxidation–reduction potentials, but reoxidation by transfer of reducing equivalents to F_{420} apparently was more complex with the 8-chloro-FAD enzyme.

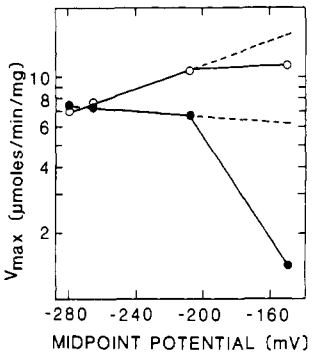

FIGURE 5: Variation in V_{max} of F_{420} and F_o reduction with the midpoint potential of the FAD analogue used to reconstitute the deflavo-FDH from *M. formicicum*. The analogues and midpoint potentials at the riboflavin level are as follows (left to right): 1-deaza-FAD, −280 mV; 6-hydroxy-FAD, −265 mV; normal FAD, −208 mV; 8-chloro-FAD, −152 mV. Symbols: (○) F_{420}; (●) F_o.

Although a clear relationship between flavin potential and V_{max} for either F_{420} or F_o was precluded by the limited amount of data (Figure 5), the different results obtained with F_{420} and F_o implied that hydride transfer from FAD to F_o was more difficult than transfer from FAD to F_{420}. This could possibly have resulted from the 12-fold greater K_m for F_o compared to F_{420} (Table I) (Schauer & Ferry, 1983). As was the case for F_{420}, the V_{max} for F_o reduction by the 8-chloro-FAD enzyme was lower than expected (Figure 5); again, these results may have reflected the sensitivity of the enzyme to FAD substituted in position 8 of the benzenoid ring.

Finally, in an analysis of other flavins (at the riboflavin level) to replace F_{420} as electron-accepting cosubstrates, we analyzed 9-azariboflavin ($E^{\circ\prime}$ = −135 mV), 8-chlororiboflavin ($E^{\circ\prime}$ = −152 mV), riboflavin ($E^{\circ\prime}$ = −208 mV), 8-hydroxyriboflavin ($E^{\circ\prime}$ = −340 mV), 8-hydroxy-5-deazariboflavin (F_o) ($E^{\circ\prime}$ = −360 mV), and the 8-hydroxy-5-deaza-N^1-lumiflavin ($E^{\circ\prime}$ = −350 mV) to function in turnover. The 9-azariboflavin (V_{max} = 7 units/mg, K_m = 9 μM), 8-chlororiboflavin (V_{max} = 24 units/mg, K_m = 22 μM), riboflavin (V_{max} = 11 units/mg, K_m = 7 μM), and 8-hydroxy-5-deazariboflavin (V_{max} = 198 units/mg, K_m = 63 μM) were substrates; the others were not. Why 8-hydroxy-5-deazariboflavin but not 8-hydroxyriboflavin was a substrate for FDH is unclear. The 10-*N*-dealkyl-F_o analogue, lacking the ribityl side chain for binding, was not reduced by FDH; however, *M. thermoautotrophicum* hydrogenase reduced this flavin.

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Registry No. F_{420}, 64885-97-8; F_o, 37333-48-5; FDH, 87110-40-5; FAD, 146-14-5; 5-deaza-FAD, 57818-88-9; 1-deaza-FAD, 64183-67-1; 6-hydroxy-FAD, 52301-43-6; 6-methyl-FAD, 76524-29-3; 8-chloro-

FAD, 68385-36-4; 7-chloro-8-demethyl-FAD, 76510-45-7; D_2, 7782-39-0.

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Comparison of Aspartate Transcarbamoylases from Wheat Germ and *Escherichia coli*: Functionally Identical Histidines in Nonhomologous Local Sequences†

Stephen C. J. Cole and Robert J. Yon*

School of Biological Sciences and Environmental Health, Thames Polytechnic, London SE18 6PF, U.K.

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ABSTRACT: Aspartate transcarbamoylase (ATCase) from wheat germ and the catalytic subunit of the enzyme from *Escherichia coli* are trimers of similar size. The former is a regulatory enzyme in its trimeric state, while the latter is a component of a complex regulatory dodecamer. In a comparison of the two enzymes, reaction with diethyl pyrocarbonate revealed a highly active, essential histidine residue in each case. The two histidines (i.e., one in each enzyme) behaved nearly identically with respect to the following functional properties: (1) kinetics of acylation (ethoxyformylation) and concomitant inactivation; (2) kinetics of deacylation by hydroxylamine and concomitant reactivation; (3) hyperbolic dependence of the apparent first-order rate constant (k_{app}) on diethyl pyrocarbonate concentration; (4) pH dependence of k_{app}; (5) failure of active-center ligands to protect the residue against diethyl pyrocarbonate, producing instead near-identical increases in the inactivation rate. These similarities point to an essential, highly conserved histidine in each enzyme, in a functional microenvironment that has changed relatively little since the divergence of plants and bacteria. Ethoxyformylated peptides were isolated from tryptic digests of the two inactivated enzymes. Sequencing of the major labeled peptide in each case showed the wheat and *E. coli* histidines embedded in nonhomologous primary segments, suggesting that, contrary to expectation, these segments are not part of the conserved microenvironment. In the case of the *E. coli* enzyme, the essential residue was identified as His-134 in the known sequence, which has a potential catalytic role on crystallographic evidence [Krause, K. L., Volz, K. W., & Lipscomb, W. N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1643–1647]. A second, much less reactive histidine was identified as His-64. Since the full primary and tertiary structures of the wheat-germ enzyme are not known, it is not possible at present to compare the environments of the essential histidine in the two enzymes.

Aspartate transcarbamoylase (EC 2.1.3.2) catalyzes an early step in a major growth pathway, the de novo synthesis of pyrimidines. It exhibits great phylogenetic diversity in structural and regulatory properties. Structural types vary from relatively small (ca. 100 kDa) enzymes with few (3–4) chains in some bacteria and plants, through the larger (ca. 300 kDa) and more complex dodecameric structure of *Escherichia coli* and other enterobacteria, to very large complexes with

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* Author to whom correspondence should be addressed.

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