8-Hydroxy-5-deazaflavin-Reducing Hydrogenase from *Methanobacterium thermoautotrophicum*: 2. Kinetic and Hydrogen-Transfer Studies†

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Appendix: Derivation of a Steady-State Rate Equation for Deazaflavin-Reducing Hydrogenase

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ABSTRACT: Steady-state kinetic parameters have been obtained for the pure 8-hydroxy-5-deazaflavin-reducing hydrogenase. With H_2 and 8-hydroxy-5-deazariboflavin (F_0) as substrates, $K_m(H_2)$ = 12 μM, $K_m(F_0)$ = 26 μM, and k_{cat} = 225 s^{-1}. In the back-direction, F_0H_2 is reoxidized (anaerobically) at 225 s^{-1}. Initial velocity patterns, product inhibition patterns, dead-end inhibition by carbon monoxide, and transhydrogenation to Procion Red HE-3B suggest a two-site hybrid ping-pong mechanism. A kinetic derivation for the rate equation is provided in the Appendix. Studies with D_2 and with D_2O reveal that no steps involving D transfer are substantially rate determining. Further, D_2 yields F_0H_2 with no deuterium at C_5 while in D_2O a 5-monodeuterio F_0H_2 product is formed, indicating complete exchange of hydrogens from H_2 with solvent before final transfer of a hydride ion out from reduced enzyme to C_5 of F_0.

The 8-hydroxy-5-deazaflavin coenzyme, F_{420} (Eirich et al., 1978, Ashton et al., 1982), is present in high concentrations in methanogenic bacteria at greater than 100 mg/kg in *Methanobacterium thermoautotrophicum* (*Mb. thermoautotrophicum*) ΔH (Van Beelen et al., 1983). F_{420} is also present in lower concentrations in *Streptomyces* (McCormick & Morton, 1982), in *Mycobacteria* (Naraoka et al., 1984), and in the blue green alga *Anacystis nidulans* (Eker & Fichtinger-Schepman, 1975). The 8-OH-5-deazaflavin coenzyme is structurally a hybrid between the tricyclic flavin and the monocyclic nicotinamide coenzymes and has a lower redox potential (−360 to −370 mV) than either flavins (−210 mV) or nicotinamides (−320 mV). As such, F_{420} can function as a general low-potential reducing currency in anaerobic methanogen metabolism, provided $F_{420}H_2$ can be generated (Walsh, 1986).

Methanogens can grow on H_2 and CO_2 or on formate as energy sources. Both the $H_2/2H^+ + 2e^-$ and the $HCOO^-/CO_2 + H^+$ two-electron redox couplex, at −420 mV, can provide the driving force for the reduction of F_{420}:

$$HCOO^- + F_{420} \rightarrow CO_2 + F_{420}(red)$$

$$H_2 + F_{420} \rightarrow F_{420}H_2$$

The corresponding enzyme, F_{420}-reducing formate dehydrogenase, has been purified from *Methanococcus vannielii* (*Mc. vannielii*) (Jones & Stadtman, 1980) and *Methanobacterium formicicum* (Schauer & Ferry, 1983), while F_{420}-reducing hydrogenases have been purified from *Mb. thermoautotrophicum* (Jacobson et al., 1982), *Mc. vannielli* (Yamazaki, 1982), and *Mb. formicicum* (Nelson et al., 1984). Essentially no mechanistic studies of these enzymes have been reported. Hydrogenase activities are the key energy-providing catalysts for *Mb. thermoautotrophicum*, which grows on H_2 and CO_2 chemolithoautotrophically, and this enzyme constitutes 2–3% of the cell protein. We have detailed the aerobic purification, characterization, and optimization for anaerobic reactivation of the F_{420}-reducing hydrogenase in the preceding paper (Fox et al., 1987).

In this paper, we report studies on the steady-state kinetic mechanism of this three-subunit, three-redox cofactor hydrogenase and note evidence for separate sites for electron output to one-electron or two-electron (F_{420}) acceptors, as well as a distinct site for initial H_2 oxidation. We also report kinetic and product hydrogen-transfer studies and show that despite the fact that F_{420} is a hydride-transfer coenzyme (Jones & Stadtman, 1980; Yamazaki et al., 1980; Walsh, 1986), hydrogen atoms from H_2 do not end up in $F_{420}H_2$ but rather are released to the solvent.

This is consistent with our earlier observations (Daniels et al., 1980) that $8e^-$ oxidation of $4D_2$ and reduction of CO_2 to methane generates CH_4 and essentially no deuterium-containing methane species.

Experimental Procedures

Materials. Procion Red HE-3B was purchased from Sigma. Prepurified hydrogen and argon gases were from Airco or Matheson. H_2/Ar and H_2/CO analyzed gas mixtures were obtained from Matheson (highest grade), as was deuterium gas (99.5% minimum). Deuterium oxide, 99.96% and 99.8% minimum, was from Aldrich. Solvents for high-performance liquid chromatography (HPLC) were from either Omnisolv or Mallinkrodt. Reverse-phase C-18, preparative and analytical, was from either Du Pont, Waters, or Alltech. Norganic trace organic removal cartridges were purchaed from Millipore. F_0 (8-OH-5-deazariboflavin) was the kind gift of W. Ashton of Merck and J. Honek of these laboratories. F_{420} was isolated from *Mb. thermoautotrophicum* in this laboratory or was a gift from Professor R. Thauer (Marburg).

Equipment. Spectrophotometric kinetic assays were performed on Perkin-Elmer λ3 and λ5 spectrophotometers in-

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terfaced to a Model 3600 data station for data acquisition and calculation of initial velocity rates. Fluorescence measurements were performed on a Perkin-Elmer L3 spectrofluorometer.

Enzyme Purification. F_{420}-reducing hydrogenase was purified as described in the preceding paper (Fox et al., 1987). The homogeneity of the enzyme was determined by densitometry of a Coomassie Blue stained sodium dodecyl sulfate (SDS)–polyacrylamide gel or estimated by the x-fold purification number derived from comparison of activity in crude extract vs. that in purified enzyme. The purity of the enzyme sample was reestimated for each experiment by comparison of the activity observed with a standard 40 μM F_0 assay tube with the activity observed when the enzyme was first isolated. This estimate was used to correct V_{max} values.

Enzyme Assays and Activation. Analysis of Kinetic Data. Initial velocity data was analyzed by a weighted least-squares analysis according to Wilkinson (1961). Michaelis constants and V_{max} values and relative errors were derived from data for variable substrate at single fixed-substrate concentrations; i.e., no assumption about the kinetic model was made during analysis of data in the primary replot. Calculation of K_m for H_2, F_0, or F_0H_2 was performed by secondary replot of slope of $1/V_{max,app}$ vs. substrate concentration (Segel, 1975). The secondary replots took the same form in the steady-state equation derived in the Appendix as those derived for an ordered bi-uni reaction (Segel, 1975).

Hydrogen Gas Oxidation Reactions. F_{420}-reducing hydrogenase was assayed for hydrogenase activity by using F_0, methylviologen, or F_{420} as cosubstrate with H_2 according to the method for hydrogenase activation and assay described in the preceding paper (Fox et al., 1987). The concentrations of F_0 and F_{420} assay mixtures were measured by absorbance at 400 (ϵ_{mM} = 25.5) and 420 nm (ϵ_{mM} = 40), respectively. Assays were usually performed under 100% H_2 atmosphere. When lower concentrations of H_2 or D_2 were required, assay tubes were first made anaerobic under argon and then flushed with the appropriate gas. Gas mixtures were either prepared by using a mercury manometer or purchased from Matheson Gas Co.

Anaerobic Oxidation of F_0H_2 by Hydrogenase. Assay tubes for the anaerobic oxidation contained 2 mL of 0.5 M KCl/10 mM 2-mercaptoethanol/50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.2, buffer. These assay tubes were degassed and left under an Ar atmosphere. F_0 was chemically reduced with $NaBH_4$ (Jacobson & Walsh, 1984) and then dissolved in assay buffer, filtered through an HPLC prefilter, and degassed under Ar. The concentration of F_0H_2 was determined spectrophotometrically at 320 nm (ϵ = 10.5 mM^{-1}). Prior to assay, F_0H_2 was injected into the assay tubes to achieve the desired concentration of substrate; F_{420}-reducing hydrogenase was first activated under H_2 and then placed under an Ar atmosphere (Fox et al., 1987; Kojima et al., 1983). F_0H_2 oxidation was initiated by the addition of enzyme and monitored at 400 nm by following the appearance of the F_0 chromophore. The assay tubes were exposed to air to allow complete reoxidation of F_0H_2. The absorbance of each assay solution was measured at 400 nm to determine the exact concentration of substrate. Reaction rates were adjusted to compensate for variations in assay volume caused by injection of different quantities of F_0H_2 solutions.

Aerobic Oxidation of F_0H_2 by Hydrogenase. A solution of F_0H_2 was prepared as described above, except the solution was not made anaerobic. Assays were performed aerobically in 1-mL quartz cuvettes by diluting the stock solution of F_0H_2 to the appropriate concentration with 0.5 M KCl/10 mM 2-mercaptoethanol/50 mM Tris, pH 7.2, buffer. F_{420}-reducing hydrogenase was placed in the buffer usually used for reductive activation but was not activated by incubation with hydrogen gas. Enzyme in this state cannot catalyze hydrogen gas oxidation (Fox et al., 1987). F_0H_2 oxidation was initiated by enzyme addition and followed at 400 nm.

Product Inhibition Studies. Two-milliliter assay tubes at various concentrations of F_0 or hydrogen gas were prepared. An anaerobic solution of chemically reduced F_0H_2 was made as described above. F_{420}-reducing hydrogenase was activated, and the hydrogen gas atmosphere was replaced by argon. F_0H_2 was anaerobically transferred into the assay tubes, and reduction was initiated by the addition of enzyme and monitored at 400 nm.

Carbon Monoxide Inhibition Studies. For inhibition vs. H_2, three-way gas mixtures of H_2/Ar/CO were prepared by manometric mixing of gases. For inhibition vs. F_0, analyzed mixtures of H_2/CO from Matheson Gas were used. Assay tubes of appropriate F_0 concentration were first equilibrated with Ar, followed by equilibration with the appropriate gas mixture. Enzyme assay was as described above.

Fluorescent Measurement of F_0 Binding to Enzyme. The fluorescence of F_0 is quenched upon binding to enzyme. The excitation wavelength used was 280 nm. Fluorescence emission at 470 nm was monitored for an aerobic solution of 3 mg/mL unactivated enzyme in 0.1 M Tris buffer, pH 7.5, after additions of 5–10-μL aliquots of a stock solution of F_0 in the same buffer. Measurements were performed on control solutions containing only enzyme or F_0 at equivalent concentrations to those of the composite solution. Differences in fluorescence between the composite and control solutions were calculated. The fractional saturation of the enzyme at a given [F_0] was then calculated from the ratio of the fluorescence difference at that concentration divided by the difference at saturating [F_0].

Kinetic and Solvent Isotope Effects for F_0 Reduction. H_2 or D_2 gas mixtures were prepared manometrically and introduced into anaerobic (under Ar) 60 μM F_0 assay tubes. F_0 assays were performed at pH 7.2 and at 32 °C.

For determination of solvent isotope effects, assay tubes were prepared in 99.8% D_2O from stock solutions of F_0, Tris, 2-mercaptoethanol, and KCl that had been made up in D_2O. The pDs of the buffers were adjusted with a pH meter according to the equation pD = pH − 0.4.

Hydrogen Transfer into C_5 of F_0. The origin of the hydrogen incorporated into F_0 upon enzymatic reduction was determined by 250-MHz ^1H NMR. A method of recovering reduced F_0 from the reaction mixture was devised to both concentrate and separate the product from unreduced F_0 and hydrogenase for NMR analysis. The reaction mixture was injected on a 10-μm reverse-phase C-18 analytical column for HPLC. Protein was retained by the guard column; reduced F_0 and oxidized F_0 were separated and eluted from the C-18 column. All aqueous buffers used for HPLC were filtered through a Norganic trace organic removal cartridge to remove contaminants. The recovered F_0H_2 was lyophilized, dissolved in 99.8% D_2O, lyophilized, and finally dissolved in 0.4–6 mL of 99.6% D_2O by alkalinization with the addition of small amounts of 30% NaOD or Na_2CO_3, for analysis on a Bruker WM 250-MHz NMR.

Three reductions were carried out by using the same general procedure, differing only in whether the reactions were done with deuteriated (D_2) or regular (H_2) gas or solvent. The three combinations of gas and solvent used were H_2/H_2O, D_2/D_2O, and H_2/D_2O. One milligram of F_0 was dissolved in 2 mL of

Table I: Kinetic Parameters for Reactions Catalyzed by F_{420}-Reducing Hydrogenase[a]

	K_m	$K_m(H_2)$ (μM)	k' (s^{-1})	k'/K_m (M^{-1} s^{-1})	$k'/K_m(H_2)$ (M^{-1} s^{-1})	K_i (μM)
F_0 reduction	26 ± 5 μM	12 ± 2	225 ± 15	4.5×10^6	1.9×10^7	5[b]
F_{420} reduction	36 μM		205	5.7×10^6		
MV reduction	1.5 mM	7	225	1.5×10^5	3.2×10^7	
F_0H_2 oxidation (anaerobic)	100 μM		225	1.6×10^5		
F_0H_2 oxidation (aerobic)	140 μM		27	3.3×10^6		

[a] All measurements were at 22 °C, except F_0H_2 aerobic oxidation was at 32 °C. [b] Measured by fluorescence quenching of F_0.

0.1 M NaCl/5 mM sodium phosphate, pH 7.2 (or pD 7.6). The pH was adjusted to 7.2 with appropriate base (NaOH or NaOD). The solution was filtered through an HPLC prefilter into a standard assay tube, stoppered, wrapped in foil, and made anaerobic under Ar or H_2. The tube was incubated at 45 °C for 1 h and flushed again with the appropriate gas (deuterium or hydrogen gas). Hydrogenase was activated in 1 M KCl (2.1 nmol of hydrogenase with 75 μL of 4 M KCl to give a volume of 275 μL). Two hundred microliters (1.5 nmol) of activated hydrogenase was transferred anaerobically to the F_0 assay tubes via H_2- or D_2-scrubbed Hamilton syringes. Reduction was allowed to go to 90% completion (by absorbance at 400 nm). The reaction mixture was removed from the assay tube with a H_2- or D_2-scrubbed 2-mL glass syringe fitted with a 22-gauge disposable needle. The needle was replaced with a blunt one prior to injection onto C-18 10-μm reverse-phase HPLC column through a prefilter. With this method of transfer, more than 80% of the deazaflavin product remained reduced. The oxidized and reduced F_0 were separated isocratically with 15% MeOH and 5 mM potassium phosphate buffer, pH 7.2, as the aqueous component. Buffer was made up in D_2O for the recovery of reduced F_0 from the incubation in this solvent. The eluant was monitored at 214 and 436 nm or at 254 nm.

A fourth incubation was done in 40% D_2O/60% H_2O with 4 mg of F_0 reduced in 8 mL. Four consecutive injections were made onto a 10-μm C-18 preparative column. Reduced F_0 fractions were pooled and lyophilized as for the other reductions.

Transhydrogenation between Reduced F_0 and Procion Red HE-3B. Schneider et al. (1984) have reported purification of several hydrogenases by chromatography on Red–agarose, which is Procion Red HE-3B dye attached covalently to agarose. The free dye behaves as an inhibitor for hydrogenase from *Alcaligenes eutrophus*. Our earlier attempts to purify methanogen hydrogenases had included trials with several dye–agarose absorbants. F_{420}-reducing hydrogenase did bind to both blue and red dye columns. Free Procion Blue HB and Procion Red HE-3B were screened as potential inhibitors of this hydrogenase. Both dyes emerged as excellent substrates for the enzyme (data not shown). Procion Red HE-3B was used to demonstrate transhydrogenation between reduced F_0 and oxidized dye. Transhydrogenation was observed at two concentrations of Procion Red HE-3B, 5 and 20 μM, in the presence of 40 μM F_0H_2 in 0.5 M KCl/50 mM Tris, pH 7.2, under an argon atmosphere. Dye solutions were made up by weight from a 1 M stock solution. An approximate extinction coefficient at 585 nm of 7.8 mM^{-1} was used to calculate reaction rates. Nonenzymatic rates of transhydrogenation were negligible. Transhydrogenation was monitored by following the reduction of red dye by decrease in absorbance at 585 nm. Appreciable rates of red dye reduction were seen only after anaerobic addition of fully activated enzyme that had been placed under argon after activation.

Inhibition experiments with Procion Blue HB were carried out with enzyme activated at 0.48 mg/mL as described above. Following incubation for 1 h at 45 °C, the activated enzyme was assayed for reduction of 70 μM F_0 or 2 mM methylviologen (MV). An aliquot of an anaerobic dye solution was added to the activation mixture. A total of 2.4 μg of enzyme was used per assay with varying dye concentrations in the assay.

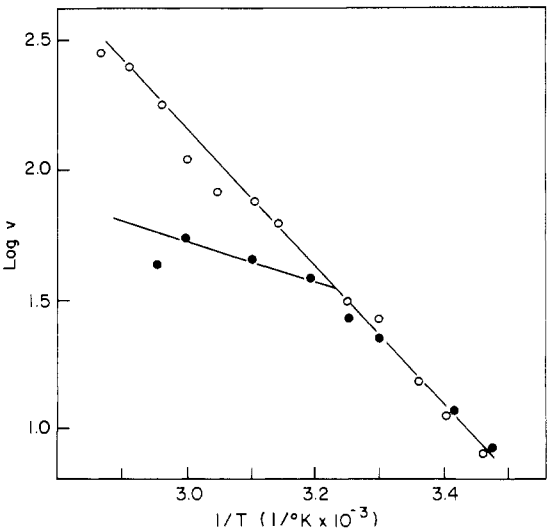


FIGURE 1: Arrhenius replots of temperature profiles of F_0 (●) and MV (○) reduction by enzyme. F_0 and MV assays were performed as described in text. The buffer system was 50 mM potassium phosphate with 0.5 M KCl, 10 mM 2-mercaptoethanol, and either 40 μM F_0 or 4 mM MV at pH 7.2.

Results and Discussion

The kinetic constants for the F_{420}-reducing hydrogenase catalyzed reduction of F_0, F_{420}, and MV as well as for the oxidation of F_0H_2 were determined and are summarized in Table I.

Under 100% H_2 atmosphere, F_{420}-reducing hydrogenase (H_2ase) reduces F_0, F_{420}, and MV with respective K_m values of 26 μM, 36 μM, and 1.5 mM (Table I). Substrate inhibition occurs with F_0 as acceptor at concentrations above 50 μM (data not shown). The pseudo-second-order bimolecular rate constants k'/K_m are shown in Table I for each reaction, where k' is the turnover number. F_{420}-reducing hydrogenase, activated according to the method described in the preceding paper (Fox et al., 1987), is an efficient catalyst for both F_{420} and F_0 reduction. The k'/K_m for these two reductions, 5.7×10^6 M^{-1} s^{-1} and 9×10^6 M^{-1} s^{-1}, respectively, show that the hydrogenase may function at close to diffusion-limited efficiency at 32 °C. *Mb. thermoautotrophicum* grows optimally at 62 °C; at this temperature the F_{420}-reducing hydrogenase should be approximately 8-fold faster, operating near the diffusional limits of catalysis. This prediction is borne out by the temperature profile for hydrogenase-catalyzed F_0 reduction shown in Figure 1 in Arrhenius form. Below 43 °C, an activation energy of 12 kcal/mol is derived from the slope of the graph. Above 42 °C there is a break in the plot for F_0 reduction (but not MV), reflecting a change in activation energy to only 3.5 kcal/mol, consistent with catalysis limited by a step (or steps) with very low barriers, e.g., diffusion. This con-

Table II: Initial Velocity Reciprocal Plot Patterns for F_{420}-Reducing Hydrogenase

	exptl	predicted			
		random	ordered (H_2 first)	ordered (F_0 first)	hybrid ping-pong
F_0 as variable substrate	intersecting	intersecting	intersecting	intersecting	intersecting
H_2 as variable substrate	intersecting	intersecting	intersecting	intersecting	intersecting
F_0H_2 vs. H_2	noncompetitive	competitive	noncompetitive[a]	competitive	noncompetitive
F_0H_2 vs. F_0	noncompetitive	competitive	competitive	noncompetitive[b]	competitive
CO vs. H_2	competitive	competitive	competitive	competitive	competitive
CO vs. F_0	uncompetitive	competitive	competitive	uncompetitive	uncompetitive

[a] Saturating F_0 abolishes inhibition by F_0H_2. [b] Saturating H_2 abolishes inhibition by F_0H_2.

clusion may be tested in future experiments by measurement of maximal rates in media of varying viscosity.

Reduction of the artificial electron acceptor, MV, is also extremely rapid. The k' for this substrate is the same as for deazaflavins, 225 s^{-1}, although the K_m for this substrate (1.5 mM) is 2 orders of magnitude higher than for F_0 (26 μM) and F_{420} (36 μM). The $E°$ of methylviologen is −460 mV (Stombaugh et al., 1976), 40 mV lower than that of hydrogen gas (−420 mV) and a full 100 mV lower than that of F_0 (−360 mV) (Jacobson & Walsh, 1984). These data suggest that electron transfer out to reducible substrates is not rate-limiting in hydrogenase catalysis (Light & Walsh, 1980).

The K_m for H_2 with all three substrates is extremely low, 7–12 μM, and reflects the methanogen's ability to scavenge hydrogen gas under the low partial pressures of gas present in its environment (Wolfe, 1971). k'/K_m for H_2 with any of the three reducible cosubstrates (Table I) shows that, with respect to H_2, F_{420}-reducing hydrogenase also acts with bimolecular rate constants within the range of diffusion-limited rates of catalysis.

Initial Velocity Patterns. For a classical bireactant enzyme, there are two possible classes of kinetic mechanism. One is obligate formation of a ternary complex by sequential addition (random or ordered) of substrates. Alternatively, one substrate may add, followed by release of product with formation of a modified enzyme species. Normally, such a "ping-pong" mechanism is detected by parallel lines in initial velocity reciprocal plots. However, in the present case, the immediate product of H_2 oxidation is a proton. Since protonated enzyme species are in equilibrium with solvent protons under the reaction conditions, a reversible connection is established between substrate additions. Intersecting patterns may result. Therefore, ping-pong and sequential mechanisms may be considered degenerate in qualitative interpretation of the initial velocity patterns. The plots shown in panels A and B of Figure 2 are intersecting for both F_0 and H_2 as variable substrate.

The scatter in Figure 2 reflects imprecision in data which must be accumulated in a range of subinhibitory concentrations of F_0 (due to substrate inhibition) and may be responsible for the lack of common intersection points.

Binding of 8-OH-5-deazaflavin. The binding of F_0 was analyzed by fluorescence. The fluorescence of 8-OH-5-deazaflavin (F_0) is quenched upon binding to enzyme. A saturation curve can then be measured and converted to Scatchard form by plotting $\Delta f/\Delta f_\infty$ vs. concentration of total deazaflavin. This analysis enabled us to calculate a K_d and the number of sites for F_0 binding to unactivated enzyme. These values are 50 μM and 1.4, respectively, per minimum molecular weight of 115K. These data, taken together with enzyme activation studies, imply that enzyme can bind deazaflavin or H_2 randomly, as the enzyme can be reductively activated by H_2 in the absence of F_0. Further, it appears that more than one molecule of F_0 can bind per molecule of enzyme FAD site.

Product Inhibition and Dead-End Inhibition by Carbon Monoxide. In considering the inhibition data, three orders

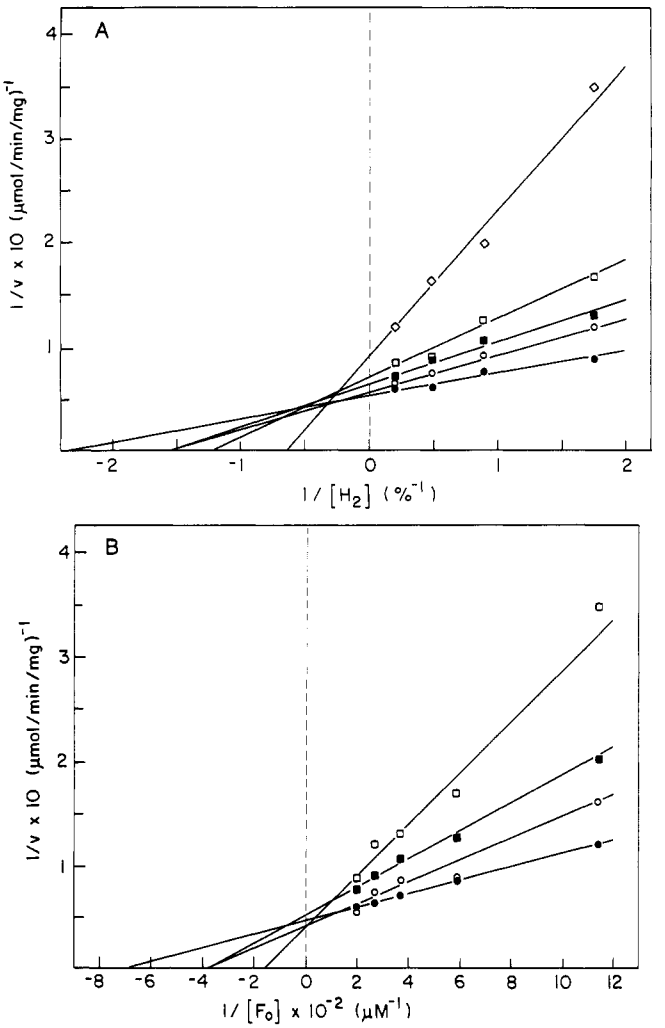


FIGURE 2: Initial velocity patterns for F_0 reduction by enzyme. F_0 reduction was observed with five concentrations of deazaflavin and four concentrations of H_2. (A) Lineweaver–Burk plot with H_2 as variable substrate at fixed concentrations of F_0: 50.4 (●), 36.4 (○), 27.2 (■), 17 (□), and 8.8 μM (◇). (B) Lineweaver–Burk plots with F_0 as variable substrate at fixed H_2 concentrations: 5% (●), 2.05% (○), 1.12% (■), and 0.57% (□).

of substrate addition to a bi-uni *single-site* enzyme are possible, random and ordered with H_2 or F_0 adding first to enzyme.

Table II summarizes the inhibition patterns we observed for dead-end inhibition by CO and end-product inhibition by F_0H_2. F_0H_2 acts noncompetitively vs. either substrate. Further, CO is a competitive inhibitor for H_2 but is uncompetitive vs. F_0.

Either uncompetitive or noncompetitive CO inhibition eliminates from consideration the random addition of both substrates to a single site and ordered addition with H_2 adding first. Since CO-inhibition experiments with F_0 as variable substrate were carried out under 100% H_2 atmosphere (saturating H_2), ordered addition with F_0 adding first must also be eliminated from consideration. An alternate mechanism

is then required to explain these data.

Two-Site Hybrid Ping-Pong Mechanism. F_{420}-reducing hydrogenase is a complex enzyme with multiple redox centers (Ni, Fe/S, FAD) (Fox et al., 1987). It is reasonable to assume that redox reactions of H_2 and 8-OH-5-deazaflavin substrates in fact occur at *distinct sites* and that the component reactions occur in nonsequential fashion, a so-called "hybrid ping-pong" mechanism. Such a mechanism was first considered for the biotin carboxylases (Northrop, 1969), and more recently for several multisite redox enzymes: xanthine dehydrogenase (Coughlan & Rajagopalan, 1980), glutamate synthase (Rendina & Orme-Johnson, 1978), and nitrate reductase (Renosto et al., 1981). This mechanism has been suggested for soybean nodule bacteroid hydrogenase, but an explicit rate equation to account for the kinetic data was not derived (Arp & Burris, 1981).

We have now derived a steady-state rate equation for two-site hydrogenase with H_2 oxidation and 8-OH-5-deazaflavin reduction occurring at distal sites mediated by intramolecular electron transfer (two-site hybrid ping-pong). The rate equation is derived on the basis of the following assumptions: (1) F_0 and F_0H_2 bind in mutually exclusive fashion at one productive binding site; (2) H^+ release is at equilibrium; (3) binding of H_2 and that of deazaflavin do not influence each other; and (4) binding constants are the same in any redox oxidation state. We derive the rate equation according to the method of Cha (1968) in the Appendix. The resultant equation takes similar form to that of a classical bi-uni ordered enzyme (Segel, 1975) and predicts intersecting initial velocity patterns, as observed. Further, uncompetitive inhibition of F_0 reduction by CO is a clear consequence of the derivation. This is observed and provides strong evidence for distinct binding sites. The equation predicts noncompetitive product inhibition vs. H_2 (which we observe) and competitive inhibition vs. F_0. This last prediction is the sole inconsistency, in that the data in Table II show F_0H_2 is noncompetitive vs. F_0. This may be related to the substrate inhibition at high F_0 levels and the greater than stoichiometric amount of 8-OH-5-deazaflavin bound. There is evidence, therefore, that 8-OH-5-deazaflavin might bind at a second inhibitory site. In the case of F_0H_2 product inhibition, this may lead to the mixed-type inhibition observed. The derived equation (Appendix) could include a term for an additional deazaflavin site; however, we refrain from developing this form, since at present we could assign this site to the H_2 binding site or a third distal site.

We can check the derived rate equation against our experimental results by comparing the values of $V_{max,f}/K_m(H_2)$ and $V_{max,r}/K_i(H_2)$ (see Table I). Both of these expressions reduce to k_1/K_4 (see Appendix) and are equal to 5.6 and 4.5 μM^{-1} s^{-1}, respectively (see Table I). These values agree within experimental error.

A useful calculation can be made from the expression for $K_i(F_0) = k_{-1}k_{-2}K_5/k_2k_3$. Since K_i and K_5 ($=K_D$ for F_0) are 87 and 50 μM, respectively, the ratio of $k_{-1}K_{-2}/k_2k_3 = 1.8$. If k_2 and k_{-2} are equal (a reasonable assumption for electron transfer between centers of similar redox potential), then the ratio of $k_{-1}/k_3 = 1.8$. This result predicts hydride transfer out to F_0 by enzyme–$FADH_2$ to be slower than evolution of H_2 from a metallocenter in the reverse reaction. Further, since $V_{max,f}/K_{m,F_0} = k_2k_3/(k_2 + k_{-2})K_5$ (see Appendix), $k_2k_3/(k_2 + k_{-2}) = 450$ s^{-1}. If $k_2 = k_{-2}$, then $k_3 = 900$ s^{-1}, which exceeds the measured $V_{max,f}$ of 225 s^{-1}. If this value is correct, then $k_{-1} = 1620$ s^{-1}, which exceeds the $V_{max,r}$. This analysis shows that the rate-limiting step in the forward direction is not hydride transfer from flavin to 8-OH-5-deazaflavin substrate. The reverse reaction is likewise not limited by H_2 evolution.

Table III: Effect of Procion Blue HB on MV and F_0 Reduction by F_{420}-Reducing Hydrogenase

[Procion Blue] (μM)	F_0 reduction (μM H_2 min^{-1} mg^{-1})	MV reduction (OD/min)
0	43.7	1.42
62.5	21.5	
187	8.7	
250		3.07

Partial Reactions. We have sought and found additional evidence to support a hybrid ping-pong mechanism. Common to enzymes with independent substrate binding sites is the ability to catalyze exchange and partial reactions in the absence of cosubstrate. Several of these reactions were investigated:

(*1*) *Transhydrogenation.* Procion Red H-E 3B, a triazine dye, was used to investigate hydride or electron transfer from reduced deazaflavin to acceptor in the absence of H_2 cosubstrate. F_{420}-reducing hydrogenase catalyzed this transhydrogenation at rate of 1.7 nmol/(min·mg) under argon atmosphere. Because the protons of the reduced dye are exchange-labile, isotope exchange experiments to examine direct proton transfer from $[^3H]F_0H_2$ to dye are not possible.

(*2*) *F_0 Oxidase Activity.* The values of k'/K_m for anaerobic and aerobic oxidation of F_0H_2 are both high (10^5–10^6 M^{-1} s^{-1}; see Table I). The latter is an oxidase activity generating H_2O_2 (Jacobson, 1981). This reaction occurs in the absence of H_2 with either activated or unactivated aerobic enzyme. The latter is not competent for H_2 evolution but clearly can bind oxidized F_0 (as determined by fluorescence quenching) or reduced F_0H_2, as required for oxidase activity.

(*3*) *H_2–D_2 Exchange.* This is a reaction catalyzed by all hydrogenases examined for the reaction (Krasna & Rittenberg, 1954; Arp & Burris, 1981). We have not examined gas-phase exchange reactions for this enzyme. We have investigated the related incorporation of hydrogen isotopes into reduced 8-OH-5-deazaflavin, which is not exchange-labile at the C_5 position.

Hydride Incorporation into 8-OH-5-deazaflavin. The hydrogen atom incorporated into C_5 of F_0 during reaction by the F_{420}-reducing hydrogenase might originate from solvent or from hydrogen gas. We reduced the substrate F_0 in four different combinations of H_2/H_2O, D_2/H_2O, H_2/D_2O, and H_2(60% D_2O, 40% H_2O). F_0H_2 was isolated from the reaction mixture by HPLC and then analyzed by 250-MHz NMR for hydrogen content at the C_5 position by integration relative to the protons seen at C_7, C_8, and C_9. The results are in Table III. The two C_5 hydrogen atoms of F_0H_2 do not exchange with solvent during isolation. ^1H NMR spectra of enzymically reduced F_0 appear in Figure 3. When F_0 is reduced in D_2O, only one proton resonance is found at C_5 of the substrate. Conversely, the incubation with deuterium gas in H_2O results in the incorporation of a hydrogen at C_5; two proton resonances are seen in the NMR spectrum at this position. The hydrogen incorporated into F_0 through F_{420}-reducing hydrogenase mediated reduction originates from solvent and not from hydrogen gas. Exchange of both H_2 hydrogens with solvent at one of three enzymic redox centers (Ni, Fe/S, FAD) must occur faster than substrate turnover, or less likely, F_0 is reduced by two electrons to the C_5 dihydro-F_0 anion and subsequently protonated.

Selective Inhibition of Deazaflavin Reduction. Additional evidence for multiple substrate binding sites came from competitive inhibition experiments with Procion Blue HB. This

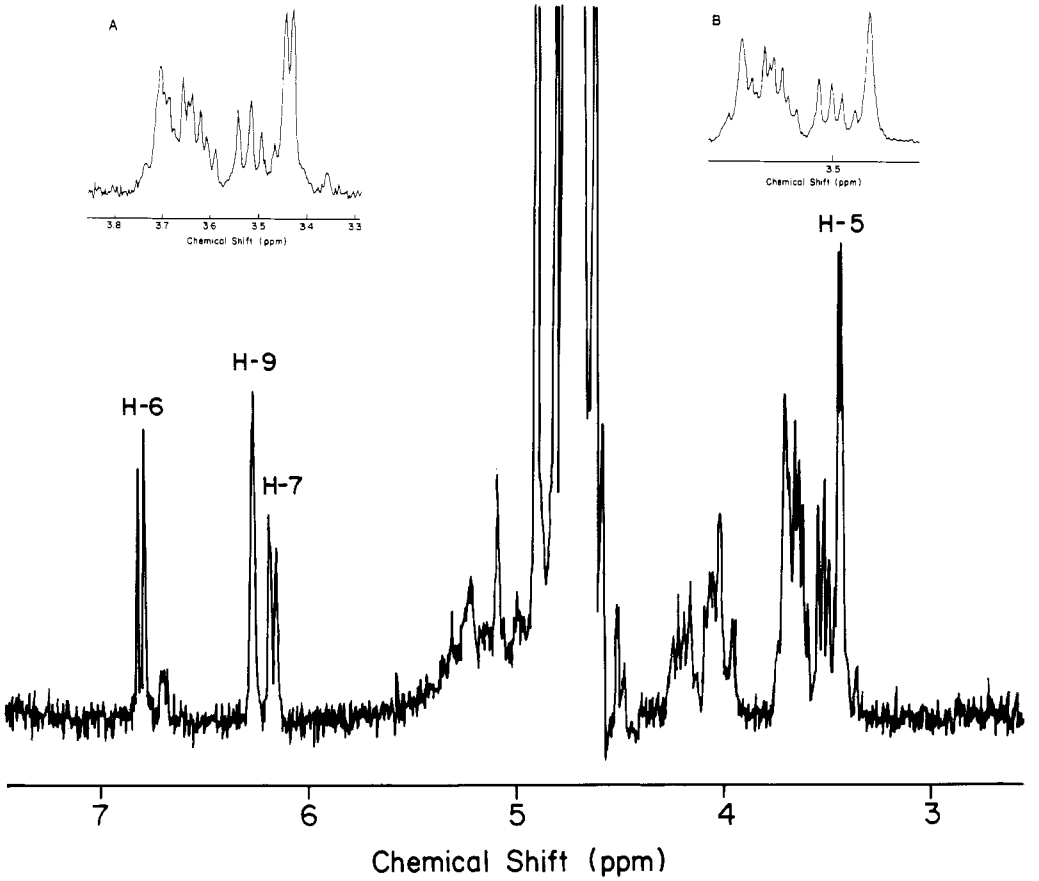
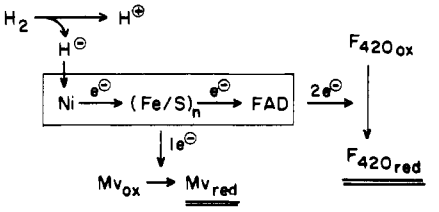


FIGURE 3: Hydrogen transfer to C_5 of F_0 by enzyme: ^1H NMR spectrum of reduced F_0 under H_2 atmosphere in H_2O. Insets are expanded region of C_5 proton of reduced F_0 in H_2O (A) and D_2O (B). F_0 was reduced enzymatically and isolated by reverse-phase HPLC as described in the text.

compound is an inhibitor of F_0 reduction but actually stimulates reduction of methylviologen (Table III). This experiment suggests that the site of the action of the dye is distal to the H_2 binding site and possibly to the site of viologen dye reduction. Attempts to determine whether MV was a noncompetitive inhibitor of F_0 reduction were unsuccessful due to overlapping optical spectra of the acceptor substrates. However, distal MV and F_0 sites would be consistent with the activity-stained native gels and FAD-reconstitution experiments described in the preceding paper (Fox et al., 1987). Binding of Procion Blue at the $F_0(F_{420})$ site and blockage of electron transfer there might permit greater flux of electron transfer out to the one-electron acceptor MV at an upstream redox site.

Substrate and Solvent Kinetic Isotope Effects for F_0 Reduction. The net reduction of F_0 and F_{420} by F_{420}-reducing hydrogenase probably involves heterolytic cleavage of a hydrogen–hydrogen bond in the H_2 substrate, by precedent with other hydrogenases (Yagi et al., 1973; Tamiya & Miller, 1963; Krasna & Rittenberg, 1954; Arp & Burris, 1981), followed by hydride transfer out from the last enzymic redox center ($FADH_2$) to the 8-OH-5-deazaflavin. The effects of heavy isotopes of hydrogen on the kinetic parameters, V_{max} and K_m, of deazaflavin reduction were examined to see if substrate and/or solvent kinetic isotope effects would be observed for these hydrogen-transfer steps. The kinetic parameters for F_0 reduction were determined with D_2 in H_2O, H_2 in D_2O, and D_2 in D_2O and compared with those obtained for the analogous reductions with H_2 and H_2O. Both substrate and solvent kinetic isotope effects were ≤ 1.1. For hydrogen vs. deuterium gas, these data are consistent with the possibility of diffusion-limited rates of catalysis by F_{420}-reducing hydrogenase

Scheme I: Distinct Sites for Methylviologen and F_{420} Reduction by F_{420}-Linked Hydrogenase



(Table I), which is discussed below. The minimal solvent isotope effect suggests hydride transfer from enzyme $FADH_2$ (where N_5 H is rapidly exchanged) to the nonexchangeable C_5 locus of 8-OH-5-deazaflavin is not rate-determining.

Redox Cofactor and Hydrogen Exchange Sites. A speculative view of the H_2 oxidation and 8-OH-5-deazaflavin reduction sites in F_{420}-reducing hydrogenase catalysis is suggested in Scheme I. It is quite likely that H_2 oxidation is a net hydride transfer into a redox site on the enzyme and F_0 and F_{420} reduction is a net hydride transfer out, the last via the enzyme–FAD site utilizing the FAD as one-electron/two-electron redox switch. There is no precedent for FAD acting as a hydrogenation catalyst, so H_2 oxidation and F_{420} reduction must occur at separate sites chemically in accordance with the two-site kinetic proposal. The one-electron reduction of methylviologen occurs at yet a third site and by analogy with other Fe/S proteins is likely to occur at one of the (three or four) $(Fe/S)_4$ cluster sites in hydrogenase, upstream from the F_{420} site. While Scheme I depicts linear alignment of redox centers, no information about linear vs. branching arrays is available.

The provisional assignment of the enzyme-bound nickel as site of the H_2 oxidation is speculative and is based on recent

observations by Slater and colleagues on the nickel-containing hydrogenase from *Chromatium* (Van der Zwaan et al., 1985). It should be remembered, however, that all hydrogenases are iron–sulfur proteins, and only some of them additionally contain Ni.

Our hydrogen-transfer experiments analyze the fate of hydrogens from H_2 during deazaflavin reduction by determining the origin of the hydrogen incorporated into reduced F_0. Data from these studies showed that hydrogen introduced into F_0 upon reduction is derived from solvent and not H_2. The absence of solvent isotope effect suggests that hydrogens from H_2 must exchange with solvent from an enzyme-bound redox center at a rate 10–100-fold faster than turnover (225 s^{-1} at 32 °C).

F_{420}-reducing hydrogenase contains several redox-active cofactors that are possible sites of exchange—nickel (Kojima et al., 1983; Van der Zwann et al., 1985), Fe/S centers (Lindahl et al., 1984), and FAD (Jacobson et al., 1982; Fox et al., 1987). The role of nickel in the hydrogenase is as yet unclear, although it is redox-active (Kojima et al., 1983). If this center is the site of H_2 activation, then hydrogen exchange, via dissociation of H^+ from a low-valent Ni–H species, may occur at this locus. Slater and co-workers recently presented electron paramagnetic resonance (EPR) evidence of nickel being an exchange site in *Chromatium* hydrogenase (Van der Zwaan et al., 1985). Photolysis of that reduced enzyme under argon in D_2O induced a measurable broadening of the Ni^1 resonance.

Hydride ion and the proton resulting from heterolytic cleavage of hydrogen gas at the Fe/S centers of several hydrogenases exchange with solvent (H_2–D_2–HD). The rate of hydrogen exchange for the hydride equivalent is slower than for the proton (Krasna & Rittenberg, 1954; Tamiya & Miller, 1963; Yagi et al., 1973; Arp & Burris, 1981; Averill & Orme-Johnson, 1979; Egerer et al., 1982), consistent with a finite lifetime of an enzyme–hydride species.

Flavin is also a reasonable exchange site for hydrogens with solvent from the N_5 position of reduced flavin (Walsh, 1979). For example, complete washout of tritium label is observed by using tritiated NADH to reduce the flavin center of dehydroorotate dehydrogenase. The turnover number for this enzyme is 11 s^{-1}, 30-fold slower than the methanogen F_{420}-reducing hydrogenase (Walsh, 1979). Exchange rates from a secondary amine range from 10^3 to 10^7 s^{-1} and are p*K*-dependent, but solvent exchange may be mediated by an interposed basic group at the active site of an enzyme (Walsh, 1979; Averill & Orme-Johnson, 1978).

We note that the absence of substrate (D_2) and solvent (D_2O) kinetic isotope effects in the catalytic action of 8-hydroxy-5-deazaflavin-reducing hydrogenase rules out either the initial H^- step into the enzyme or the final H^- step out of the enzyme from occurring in a kinetically significant transition state. The lack of effect of alteration of $E^{\circ\prime}$ of an acceptor over a 100-mV range on V_{max} similarly shows a dramatic insensitivity of k_{cat} to electron-transfer potential at either the F_{420} site or the separate methylviologen site, suggesting inter redox center electron-transfer steps are not slow either. When these observations are meshed with a very low E_a for catalysis above 42 °C and k'/K_m values close to 10^7 M^{-1} s^{-1}, it may be that physical binding and dissociation steps are rate-limiting for this hydrogenase. Although methanogenic bacteria are of the archaebacterial kingdom and in that sense are presumed to be ancient and primordial, there has been a corresponding long period for this contemporary enzyme to evolve toward efficient catalysis.

Comparison with Other Hydrogenases. Kinetic investigation of three hydrogenases from diverse sources have been reported. The enzymes from *Clostridium pasteurianum* (Erbes & Burris, 1978) and soybean root nodule bacteroids (Arp & Burris, 1981) were studied with methylene blue and methylviologen electron acceptors under H_2 atmosphere. Studies with physiological electron acceptors were not performed. The NAD-reducing uptake hydrogenase from *A. eutrophus* has been studied with several acceptors including NAD, the physiological acceptor (Egerer et al., 1982; Egerer & Simon, 1982). We believe the latter enzyme to bear the most mechanistic similiarity to the F_{420}-reducing hydrogenase. Both hydrogenases reduce obligate two-electron acceptors (F_{420} or NAD) and have bound flavin cofactors. However, none of these published reports have addressed all of the criteria listed above in determining the kinetic mechanism. We now review the data presented in this study and compare the behavior of the methanogen enzyme to the other hydrogenases examined. Initial velocity patterns for H_2 and deazaflavin are intersecting for F_{420} hydrogenase and for all other hydrogenases examined. Intersecting initial velocity patterns have been reported in preliminary studies by Fuchs et al. (1979) on a partially characterized hydrogenase from *Mb. thermoautotrophicum*, Marburg strain. It is likely that this preparation was predominantly the hydrogenase competent to reduce MV, but not deazaflavin. Intersecting initial velocity patterns have also been reported for *C. pasteurianum* and bacteroid hydrogenases (Erbes & Burris, 1978; Arp & Burris, 1981).

Hydrogenases differ from the observed behavior of other multisite redox enzymes which show the expected parallel patterns for ping-pong enzymes. Intersecting patterns are predicted qualitatively if one of the products is saturating. The likely candidate species in the hydrogenase reaction are protons produced from H_2 oxidation. Further, our derivation of a rate equation for F_{420} hydrogenase (see Appendix) explicitly predicts intersecting patterns, as the form of the denominator is similar to that of the steady-state equation for a bi-uni enzyme (Siegel, 1975).

Substrate inhibition by deazaflavin in the methanogen enzyme is precedented: substrate inhibition by oxidized methylene blue (competitive vs. H_2) was found in studies of bacteroid hydrogenase (Arp & Burris, 1981).

The most obvious explanation for substrate inhibition is binding of more than one acceptor molecule to enzyme. Our fluorescence quenching experiment indicates that greater than stoichiometric amounts of oxidized deazaflavin may bind to the oxidized methanogen enzyme. At this stage, we cannot distinguish between competition for the H_2 binding site and a distal site distinct from the productive site for deazaflavin reduction.

It is perhaps not surprising that polycyclic dye molecules may adhere to enzymes in adventitious inhibitory modes. End-product inhibition patterns for the methanogen hydrogenase are potentially difficult to obtain, since the product (F_0H_2) must be held at sufficiently low concentration to prevent reverse reaction. Reverse reaction is observed at H_2 partial pressures below 0.5%. However, at the higher H_2 tensions, F_0H_2 as a noncompetitive inhibitor of H_2 stands revealed. This may be interpreted as product and H_2 binding to different sites or different forms of the enzyme. We favor the former interpretation, since both H_2 and F_0H_2 can certainly bind to activated enzyme held under Ar. These data are also compatible with reduced deazaflavin binding at two sites, with one site being the same as the H_2 binding site.

Bacteroid hydrogenase shows end-product inhibition by H_2 when H_2 evolution is measured (Arp & Burris, 1981). This noncompetitive inhibition vs. reduced methylene blue can also be explained by distinct sites for H_2 and acceptor. We find CO to be a competitive inhibitor of H_2 and uncompetitive to F_0. CO is found to completely inhibit H_2 in hydrogenase from clostridia and bacteroids (Erbes & Burris, 1978; Arp & Burris, 1981). CO is found to be noncompetitive vs. methylene blue for both these hydrogenase. O_2 can act as a reversible inhibitor of bacteroid hydrogenase. It is competitive vs. methylene blue and noncompetitive vs. H_2. These patterns are difficult to reconcile with a single-site bi-uni ping-pong enzyme, and they are, in fact, similar to inhibition patterns of several dead-end inhibitors of glutamate synthase described by Rendina and Orme-Johnson (1978). In combination with the end-product inhibition we report, the kinetic patterns for the F_{420} hydrogenase strongly suggest separable binding sites for H_2 and acceptor.

Partial reactions in the absence of cosubstrate are common to hydrogenases. *A. eutrophus* hydrogenase catalyzes transhydrogenation between NADH and acetylpyridine adenine dinucleotide (APAD) under Ar atmosphere (Egerer & Simon, 1982). Bacteroid hydrogenase catalyzes an active D_2–H_2O exchange which is in fact inhibited by electron acceptor (Arp & Burris, 1981). We observe two partial reactions catalyzed by F_{420} hydrogenase in the absence of H_2. F_0H_2–triazine transhydrogenation under Ar occurs at appreciable rates. F_0H_2 oxidase activity occurs even with unactivated enzyme under O_2 atmosphere.

Studies of the NAD^+-reducing hydrogenase, also a Ni, Fe/S flavoenzyme, from *A. eutrophus* with [^{3}H]NADH show a washout of tritium label during NADH oxidation; transhydrogenation between [^{3}H]NADH and APAD results in unlabeled, reduced APAD (Egerer & Simon, 1982). Consistent with our observations for F_{420}-reducing hydrogenase, Egerer and Simon (1982) saw essentially no substrate kinetic isotope effect for hydrogen gas oxidation by NAD^+-reducing hydrogenase from *A. eutrophus* (V_H/V_D = 1.0–1.2). However, in contrast to the methanogen enzyme, solvent isotope effects of 2.95, 2.0, and 1.25, respectively, were seen for NAD^+ reduction, MV reduction, and transhydrogenation between NADH and APAD. So these hydrogen-transfer rates may begin to be partially rate limiting in that enzyme. Low deuterium and kinetic isotope effects are perhaps to be expected in hydrogenases. Hydrogenations mediated by several transition metals used in homogeneous catalysis (Cu, Rh, Ru) display kinetic and solvent deuterium isotope effects ranging from 0.9 to 1.5 and from 0.9 to 1.0, respectively (Zhou et al., 1985). Overall, then, the design and behavior of the *A. eutrophus* and *Mb. thermoautotrophicum* hydrogenases are very similar—as anticipated for catalysts that apparently do a two-electron oxidative step in H_2 oxidation and one-electron transfer via Fe/S clusters, followed by collection of two electrons by bound flavin for the hydride output step to NAD, or to that "nicotinamide in flavin clothing," the 5-deazaflavin, F_0 or F_{420}.

ACKNOWLEDGMENTS

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Registry No. F_0, 71415-45-7; F_0H_2, 108395-20-6; F_{420}, 64885-97-8; MV, 1910-42-5; CO, 630-08-0; hydrogen, 1333-74-0; coenzyme F_{420} hydrogenase, 65099-08-3.

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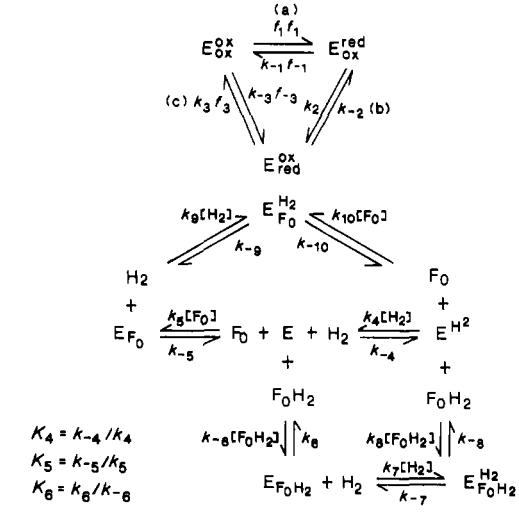
Appendix: Derivation of a Steady-State Rate Equation for Deazaflavin-Reducing Hydrogenase

The methanogen hydrogenase enzyme catalyzes a bi-uni reaction involving redox reactions of substrates and intramolecular electron transfer between cofactors. The derivation of a steady-state rate equation for a multisite redox enzyme is possible by using methods presented by Cha (1968). For hydrogenase, enzyme species of specified redox state are interconverted by reversible steady-state reactions. The steady-state steps (a, b, and c, below) are respectively H_2-dependent reduction of enzyme, intramolecular electron transfer, and FAD-mediated reduction of F_{420}. These interconverted species are, in fact, collections of a number of enzyme–substrate–product ligation states. These aggregate species (demoted E^{ox}_{ox}, E^{ox}_{red}, etc.) are expanded in rapid-equilibrium segments.



The velocity equation for the steady-state diagram is derived by the King-Altman method:

$$v/[E]_t = (k_1k_2k_3f_1f_3 - k_{-1}k_{-2}k_{-3}f_{-1}f_{-3})/[k_1(k_2 + k_{-2})f_1 + k_2k_3f_3 + k_1k_3f_1f_3 + k_{-3}(k_2 + k_{-2})f_{-3} + k_{-1}k_{-2}f_{-1} + k_{-1}k_3f_{-1}f_3 + k_{-1}k_{-3}f_{-1}f_{-3}]$$

The fractional occupancy factors, which represent the ratio of productive enzyme complex to total complex, both in the specified oxidation state, are

$$f_1 = \frac{\sum[E^{ox}_{ox}H_2]}{\sum[E^{ox}_{ox}]_t} = \frac{[H_2]/K_4}{1 + [H_2]K_4} = \frac{[H_2]}{K_4 + [H_2]} = \frac{[H_2]}{d_1}$$

$$f_{-1} = \frac{\sum[E^{ox}_{red}]}{\sum[E^{ox}_{red}]_t} = \frac{1}{1 + [H_2]/K_4} = \frac{K_4}{K_4 + [H_2]} = \frac{K_4}{d_1}$$

$$f_3 = \frac{\sum[E^{ox}_{red}F_0]}{\sum[E^{ox}_{red}]_t} = \frac{[F_0]/K_5}{1 + [F_0]/K_5 + [F_0H_2]/K_6} = \frac{K_6[F_0]}{K_5K_6 + K_6[F_0] + K_5[F_0H_2]} = \frac{K_6[F_0]}{d_2}$$

$$f_{-3} = \frac{\sum[E^{ox}_{red}F_0H_2]}{\sum[E^{ox}_{red}]_t} = \frac{[F_0H_2]/K_6}{1 + [F_0]/K_5 + [F_0H_2]/K_6} = \frac{K_5[F_0H_2]}{K_5K_6 + K_6[F_0] + K_5[F_0H_2]} = \frac{K_5[F_0H_2]}{d_2}$$

where

$$d_1 = K_4 + [H_2]$$

$$d_2 = K_5K_6 + K_6[F_0] + K_5[F_0H_2]$$

Inserting fractional occupancy factors and multiplying numerator and denominator by d_1 and d_2:

$$v/[E]_t = (k_1k_2k_3K_6[H_2][F_0] - k_{-1}k_{-2}k_{-3}K_4K_5[F_0H_2])/[k_1(k_2 + k_{-2})[H_2]d_2 + k_2k_3K_6[F_0]d_1 + k_{-1}k_{-2}K_4d_2 + k_{-3}(k_2 + k_{-2})K_5[F_0H_2]d_1 + k_1k_3K_6[H_2][F_0] + k_{-1}k_3K_4K_6[F_0] + k_{-1}k_{-3}K_4K_5[F_0H_2]]$$

Replacing d_1 and d_2, grouping terms, and simplifying by assuming that $k_{-1} < k_2$, k_{-2}:

$$v/[E]_t = (k_1k_2k_3K_6[H_2][F_0] - k_{-1}k_{-2}k_{-3}K_4K_5[F_0H_2])/[k_{-1}k_{-2}K_4K_5K_6 + k_1(k_2 + k_{-2})K_5K_6[H_2] + k_2k_3K_4K_6[F_0] + k_{-3}(k_2 + k_{-2})K_4K_5[F_0H_2] + K_6[k_1(k_2 + k_{-2} + k_3) + k_2k_3][H_2][F_0] + K_5(k_2 + k_{-2})(k_1 + k_{-3})[H_2][F_0H_2]]$$

Replacing rate constants with kinetic constants:

$$v = V_{max}([H_2][F_0] - [F_0H_2]/K_{eq})/[K_{i,F_0}K_{m,H_2} + K_{m,F_0}[H_2] + K_{m,H_2}[F_0] + [H_2][F_0] + (K_{i,F_0}K_{m,H_2}/K_{m,F_0H_2})[F_0H_2] + (K_{m,F_0}/K_{i,F_0H_2})[H_2][F_0H_2]]$$

where

$$K_{eq} = \frac{num_1}{num_2} = \frac{k_1k_2k_3K_6}{k_{-1}k_{-2}k_{-3}K_4K_5}$$

$$V_{max,f} = \frac{num_1}{coeff_{[H_2][F_0]}} = \frac{k_1k_2k_3}{k_1(k_2 + k_{-2} + k_3) + k_2k_3}$$

$$V_{max,r} = \frac{num_2}{coeff_{[F_0H_2]}} = \frac{k_{-1}k_{-2}}{k_2 + k_{-2}}$$

$$K_{m,H_2} = \frac{coeff_{[F_0]}}{coeff_{[H_2][F_0]}} = \frac{k_2k_3K_4}{k_1(k_2 + k_{-2} + k_3) + k_2k_3}$$

$$K_{m,F_0} = \frac{coeff_{[H_2]}}{coeff_{[H_2][F_0]}} = \frac{k_1(k_2 + k_{-2})K_5}{k_1(k_2 + k_{-2} + k_3) + k_2k_3}$$

$$K_{m,F_0H_2} = \frac{const}{coeff_{[F_0H_2]}} = \frac{k_{-1}k_{-2}K_6}{k_{-3}(k_2 + k_{-2})}$$

$$K_{i,H_2} = \frac{const}{coeff_{[H_2]}} = \frac{k_{-1}k_{-2}K_4}{k_1(k_2 + k_{-2})}$$

$$K_{i,F_0} = \frac{const}{coeff_{[F_0]}} = \frac{k_{-1}k_{-2}K_5}{k_2k_3}$$

$$K_{i,F_0H_2} = \frac{coeff_{[H_2]}}{coeff_{[H_2][F_0H_2]}} = \frac{k_1K_6}{k_1 + k_{-3}}$$

Useful relationships from these definitions are

$$\frac{V_{max,f}}{K_{m,H_2}} = \frac{V_{max,r}}{K_{i,H_2}} = \frac{k_1}{k_4}$$

and

$$K_{m,F_0}K_{i,H_2} = K_{m,H_2}K_{i,F_0}$$

The velocity equation can be written for the forward direction as a function of $[H_2]$ or $[F_0]$:

$$1/v = \frac{1}{[H_2]}\frac{K_{m,H_2}}{V_{max}}\left(1 + \frac{K_{i,F_0}}{[F_0]} + \frac{K_{i,F_0}[F_0H_2]}{[F_0]}\right) + \frac{1}{V_{max}}\left(1 + \frac{K_{m,F_0}}{[F_0]} + \frac{K_{m,F_0}}{K_{i,F_0}}\frac{[F_0H_2]}{[F_0]}\right)$$

$$1/v = \frac{1}{[F_0]}\frac{K_{m,F_0}}{V_{max}}\left(1 + \frac{[F_0H_2]}{K_{i,F_0}} + \frac{K_{i,H_2}}{[H_2]} + \frac{K_{m,F_0H_2}K_{i,H_2}}{K_{i,F_0H_2}[H_2]}\right) + \frac{1}{V_{max}}\left(1 + \frac{K_{m,H_2}}{[H_2]}\right)$$

In the absence of product (F_0H_2), the preceding equations predict intersecting initial velocity patterns. The predicted product inhibition patterns are noncompetitive vs. $[H_2]$ and competitive vs. $[F_0]$.

The fractional occupancy factors in the presence of a competitive inhibitor (I) for H_2 (e.g., CO) are modified:

$$f_1' = \frac{[H_2]/K_4}{1 + [H_2]/K_4 + [I]/K_I} = \frac{K_I[H_2]}{K_IK_4 + [H_2]K_I + K_4K_I} = \frac{K_I[H_2]}{d_1'}$$

$$f_{-1}' = \frac{1}{1 + [H_2]/K_4 + [I]/K_I} = \frac{K_I}{K_IK_4 + [H_2]K_I + K_4[I]} = \frac{K_I}{d_1'}$$

where

$$d_1' = K_IK_4 + [H_2]K_I + K_4K_I$$

The velocity equation is derived with f_1' and f_{-1}. The reciprocal forms are as follows:

$$1/v = \frac{1}{[H_2]}\frac{K_{m,H_2}}{V_{max}}\left(1 + \frac{K_{i,F_0}}{[F_0]} + \frac{[I]}{K_i}\right) + \frac{1}{V_{max}}\left(1 + \frac{K_{m,F_0}}{[F_0]}\right)$$

$$1/v = \frac{1}{[F_0]}\frac{K_{m,H_2}}{V_{max}}\left(1 + \frac{K_{m,F_0H_2}K_{i,H_2}}{K_{i,F_0H_2}[H_2]}\right) + \frac{1}{V_{max}}\left(1 + \frac{K_{m,H_2}}{[H_2]} + \frac{K_{m,H_2}}{[H_2]}\frac{[I]}{K_i}\right)$$

A competitive inhibitor of $[H_2]$ is predicted to be uncompetitive vs. F_0 (in the presence of a constant amount of $[H_2]$).

Evidence for an Essential Histidine in Neutral Endopeptidase 24.11[†]

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ABSTRACT: Rat kidney neutral endopeptidase 24.11, "enkephalinase", was rapidly inactivated by diethyl pyrocarbonate under mildly acidic conditions. The pH dependence of inactivation revealed the modification of an essential residue with a pK_a of 6.1. The reaction of the unprotonated group with diethyl pyrocarbonate exhibited a second-order rate constant of 11.6 M^{-1} s^{-1} and was accompanied by an increase in absorbance at 240 nm. Treatment of the inactivated enzyme with 50 mM hydroxylamine completely restored enzyme activity. These findings indicate histidine modification by diethyl pyrocarbonate. Comparison of the rate of inactivation with the increase in absorbance at 240 nm revealed a single histidine residue essential for catalysis. The presence of this histidine at the active site was indicated by (a) the protection of enzyme from inactivation provided by substrate and (b) the protection by the specific inhibitor phosphoramidon of one histidine residue from modification as determined spectrally. The dependence of the kinetic parameter V_{max}/K_m upon pH revealed two essential residues with pK_a values of 5.9 and 7.3. It is proposed that the residue having a kinetic pK_a of 5.9 is the histidine modified by diethyl pyrocarbonate and that this residue participates in general acid/base catalysis during substrate hydrolysis by neutral endopeptidase 24.11.

Neutral endopeptidase 24.11 (NEP, "enkephalinase", EC 3.4.24.11) is a mammalian membrane-bound zinc metalloendopeptidase that was first described by Kerr and Kenny (1974a,b). The enzyme has been purified to homogeneity from a number of sources (Kerr & Kenny, 1974a; Orlowski & Wilk, 1981; Gafford et al., 1983; Almenoff & Orlowski, 1983) and its substrate specificity studied in several laboratories (Turner et al., 1985; Pozsgay et al., 1986; Hersh & Morihara, 1986). NEP resembles the bacterial neutral metalloproteases in that it cleaves peptide bonds on the amino side of hydrophobic residues. However, the enzyme also displays a preference (but not absolute requirement) for a substrate containing a free COOH-terminal carboxylate, cleaving these substrates preferentially near their COOH terminus (Hersh & Morihara, 1986).

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