strongly inhibited BLM hydrolase, suggesting that BLM hydrolases may have some similarities to other known peptidases. In agreement with our previous findings (Sebti & Lazo, 1987), however, the purified BLM hydrolase does not appear to act as an aminopeptidase.

Thus, rabbit pulmonary BLM hydrolase can now be purified to homogeneity. The described purification scheme for this biologically relevant enzyme should be useful for subsequent attempts to further characterize BLM hydrolase and to determine its amino acid sequence. The availability of pure BLM hydrolase will permit investigation of the potential endogenous substrates for this deamidating enzyme. In addition, substrate-specificity studies of the pure enzyme may result in the discovery of BLM analogues with better antitumor activity.

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8-Hydroxy-5-deazaflavin-Reducing Hydrogenase from *Methanobacterium thermoautotrophicum*: 1. Purification and Characterization†

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ABSTRACT: The 8-hydroxy-5-deazaflavin (coenzyme F_{420}) reducing hydrogenase from the obligate anaerobe *Methanobacterium thermoautotrophicum* ΔH has been purified 41-fold to apparent homogeneity. The major active enzyme form is a high molecular weight aggregate of M_r ca. 800 000, composed of three subunits, α (M_r 47K), β (M_r 31K), and γ (M_r 26K). The hydrogenase is purified aerobically in reversibly inhibited form, and conditions for anaerobic reductive activation with H_2, high salt, thiols, and electron acceptors have been defined. The minimal species transferring electrons from H_2 to coenzyme F_{420} appears to be an $\alpha\beta\delta$ (M_r 115K) complex. The tightly associated redox cofactors per 115K species are 0.6–0.7 nickel atom, 0.8–0.9 flavin adenine dinucleotide (FAD), and 13–14 iron atoms in iron–sulfur centers. The subunits have been separated by denaturing gel electrophoresis, which has permitted determination of amino acid composition, subunit N-terminal sequencing, and preparation of subunit-directed antibodies. There is iron associated with the α-subunit, but placement of the nickel and FAD has not been established.

The thermophilic methanogenic bacterium *Methanobacterium thermoautotrophicum* ΔH produces large fluxes of methane during growth on CO_2, H_2, and inorganic salts (including Ni^{2+} ions). The eight-electron reduction of CO_2 to CH_4 consumes four molecules of H_2, oxidized by hydrogenase action in the cell. As H_2 is the only source of reducing power,

$$4H_2 \rightarrow 8H^+ + 8e^-$$

$$CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2H_2O$$

$$\text{sum:}\quad 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

the hydrogenases play a critical role in biogenesis of methane. *M. thermoautotrophicum* ΔH contains two separable hydrogenase enzymes, distinguishable initially by assay with the methanogen 8-hydroxy-5-deazaflavin redox coenzyme, coenzyme F_{420}[1] (see Scheme I) (Jacobson et al., 1982; Walsh,

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[1] Abbreviations: F_{420}, 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphoryllactylglutamylglutamate; F_0, 8-hydroxy-5-deazariboflavin; MV, methylviologen; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; H_2ase, hydrogenase; DEAE, diethylaminoethyl; NAD, nicotinamide adenine dinucleotide; NADH, dihydronicotinamide adenine dinucleotide; QAE, quaternary aminoethyl; PMSF, phenylmethanesulfonyl fluoride; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; KP_i, potassium phosphate buffer; Me_2SO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EXAFS, extended X-ray absorption fine structure spectroscopy; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)-aminomethane; TBS, Tris-buffered saline; Bis-Tris propane, 1,3-bis-[[tris(hydroxymethyl)methyl]amino]propane; IgG, immunoglobulin G; DTT, dithiothreitol; TLC, thin-layer chromatography; EPR, electron paramagnetic resonance; GdmCl, guanidinium chloride; 2D, two dimensional; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; CAPS, 3-(cyclohexylamino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

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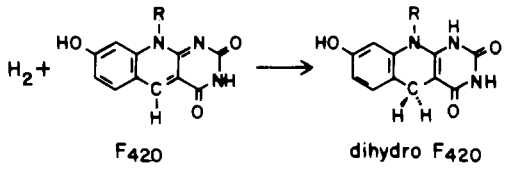
Scheme I



1986). Thus there is a coenzyme F_{420} reducing hydrogenase and an F_{420}-nonreactive hydrogenase; both species will reduce dyes such as methylviologen (Jacobson et al., 1982; Kojima et al., 1983; Fox, 1984; Jordan, 1985). This pattern of two hydrogenases also holds in other methanogenic bacteria, such as *Methanococcus vannielii* (Yamazaki, 1982) and *Methanobacterium formicicum* (Jin et al., 1983). An analogous set of two hydrogenases are found in other bacteria such as *Alcaligenes eutrophus* (Schink & Schlegel, 1980; Schneider & Schlegel, 1981; Schneider et al., 1984a) and *Nocardia opaca* (Schneider et al., 1984b), where one enzyme will pass electrons to NAD and the other only to methylviologen. We have noted elsewhere the similarities of deazaflavin and nicotinamide redox coenzymes (Jacobson & Walsh, 1984; Walsh, 1986).

The physiological roles for two hydrogenases in methanogenic and nonmethanogenic bacteria have yet to be fully delineated, although the transfer of electrons from H_2 to F_{420} or to NAD to yield $F_{420}H_2$ or NADH generates diffusible, low-potential reducing equivalents to drive cellular redox metabolism. The second, MV-reducing hydrogenase may be involved in the generation of membrane potentials and/or ATP synthesis. Despite the crucial role and relative abundance (~2% each) of the hydrogenases in the anaerobic methanogens, they had been resistant to purification. We have reported a preliminary investigation of the F_{420}-reducing hydrogenase (Jacobson et al., 1982), and in this and the following paper (Livingston et al., 1987) we fully detail the purification, properties, and catalytic behavior of this enzyme. Even though methanogens are fastidious anaerobes, it has proven more reliable to purify the F_{420}-reducing hydrogenase aerobically in an air-stable, reversibly inhibited form and then reductively activate it for assay. This hydrogenase is a three-subunit enzyme containing stoichiometrically bound nickel, FAD, and iron–sulfur centers as redox inventory. In this paper we report purification and activation conditions, subunit content and separation, and initial characterization and redox inventory. In the following paper we have investigated kinetic mechanism and deuterium kinetic and product isotope effects.

Experimental Procedures

Materials

MV, FMN, and FAD were purchased from Sigma Chemical Co. DEAE-Sephadex, Sepharose 6B, phenyl-Sepharose, and Mono Q were from Pharmacia. Bio-Rad protein assay solution was from Bio-Rad, as were all reagents used for polyacrylamide gel electrophoresis. Protein standards were either prepared from proteins obtained from Sigma or were purchased premixed from BRL. Native protein molecular weight standards were from Pharmacia. Oxygen scrubbers for argon and hydrogen gases were from VWR or from MG Scientific. Prepurified hydrogen and argon gases were purchased from Airco; mixtures of 80% H_2/20% CO_2 were from Matheson. Isoelectric focusing prepoured gels were from LKB. F_0 was the kind gift of W. Ashton of Merck and J. Honek of these laboratories.

Methods

Growth of Methanobacterium thermautotrophicum ΔH. Cells were grown according to the method of Schonheit et al. (1979). Starter cultures contained 0.2 mg/mL ampicillin to prevent contamination. Large-scale growth of methanogenic bacteria was carried out in 24- and 15-L New Brunswick fermentors or 14-L Chemap fermentors. Cells were cooled to 40 °C and then sparged with CO_2 for 1 h prior to concentration to a volume of 1 L with a Pelicon membrane filtration system from Millipore. The concentrate was centrifuged at 10000*g* for 20 min. The packed cells were frozen in liquid nitrogen and stored at −70 °C.

Purification of F_{420}-Reducing Hydrogenase. The following purification was modified from the procedure of Jacobson et al. (1982). The hydrogenase was prepared aerobically, typically from 60 g of frozen cells. All steps were performed at 4 °C unless otherwise indicated. The bacteria were thawed in 300 mL of 10 mM KP_i, pH 7.2/0.1 mM PMSF and ruptured by two passes through a French pressure cell at 19 000 psi. The cell extract was centrifuged at 6800*g* for 40 min to pellet cellular debris and then passed through a DEAE-Sephadex column (120 mL, 4 cm × 12 cm) equilibrated in 10 mM KP_i buffer, pH 7.2, at approximately 100 mL/h by gravity flow. The hydrogenase flowed through (cofactors and other proteins required added salt for elution) and was precipitated by slow addition of $(NH_4)_2SO_4$ to 80% saturation. After stirring for 30 min, the protein was sedimented by centrifugation at 9000*g* for 20 min. The protein (1.5 g total in ca. 50 mL) was dialyzed overnight with two 4-L changes of phosphate buffer and centrifuged at 9000*g* to sediment undissolved material. The dialysate was distributed over three Sepharose 6B columns (1.8 L, 90 cm × 5 cm) equilibrated in 10 mM KP_i buffer, pH 7.2. Hydrogenase was located by activity assays. Fractions reactive only toward MV were pooled, frozen at −70 °C, and used for subsequent purification of the separate F_{420}-nonreactive hydrogenase. Fractions reducing both F_0 and MV from the three columns were pooled, brought to 1 M in KCl, and adsorbed onto a phenyl-Sepharose column (40 mL, 2.5 × 8 cm) equilibrated in 1 M KCl/10 mM KP_i buffer, pH 7.2. The column was washed overnight with 600 mL of the same buffer. This was followed with 50 mL of 10 mM KP_i buffer, pH 7.2. When the hydrogenase band began to spread (the enzyme is brown-colored) over $^2/_3$ the column length, the buffer was changed to 25% Me_2SO/10 mM KP_i buffer, pH 7.2 at room temperature, to elute the enzyme in a defined, concentrated band. The hydrogenase was exhaustively dialyzed against 10 mM KP_i buffer, pH 7.2, to remove Me_2SO and either stored at this stage in 0.5-mL aliquots at −70 °C or purified to homogeneity by FPLC.

Fast Protein Liquid Chromatography of F_{420}-Reducing Hydrogenase. F_{420}-reducing hydrogenase (up to 20 mg per run) was applied to a 1-mL Mono Q (QAE monodisperse resin) column in 350 mM NaCl on an FPLC from Pharmacia. A gradient from 350 to 500 mM NaCl in 20 mM Bis-Tris propane buffer, pH 7.2, was applied at 2 mL/min. The hydrogenase eluted at 470 mM NaCl. Contaminants and inactive hydrogenase eluted in the initial 350 mM NaCl effluent from the column.

Protein Concentration. Protein concentrations were determined by either the Lowry (Lowry et al., 1951) or the Bradford/Bio-Rad (Bradford, 1976) protein assay using bovine serum albumin or carbonic anhydrase for standards, respectively.

SDS and Native Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed by using a Bio-Rad slab gel apparatus, Model 220, according to the method of Laemmli (1970). Native gradient PAGE (4–22% acrylamide) was performed by using a modification of the method of O'Farrell

(1975). Gels were stained for protein with Vesterberg's Coomassie Blue stain (Vesterberg, 1971) or with Amido Black 10B (Wilson, 1979). Molecular weights were estimated from a plot of log molecular weight of standard proteins vs. mobility. For MV and F_0 activity stains, the slab gel was sliced into lanes and placed in test tubes containing hydrogen gas saturated F_0 or MV activity assay solutions. The tubes were stoppered and made anaerobic under hydrogen. MV reduction appeared as dark blue bands after several minutes of incubation. Hydrogenase activity toward the deazaflavin was observed by using the oxidase reaction catalyzed by this enzyme. The gel was incubated in the F_0 activity assay solution until the F_0 chromophore was completely bleached. The gel was then removed from the tube and exposed to air. F_{420}-reducing hydrogenase was detectable by yellow bands of reoxidized F_0. R_f's of activity bands were noted and aligned with protein stained gels for molecular weight estimations. Mild SDS–PAGE was run with enzyme samples treated at room temperature with 2% SDS and electrophoresed at 4 °C according to Nishikimi et al. (1984).

Gels were stained for iron with a bathophenanthroline–thioglycolate stain (Ohmori et al., 1981). Gel slices were dissolved by heating for 8 h at 70 °C in 0.8 mL of 30% H_2O_2 and 0.6 mL of 70% $HClO_4$. A 0.5-mL of aliquot 8 M urea in 50 mM sodium phosphate, pH 7, was added to each sample prior to counting. Solutions were counted after overnight incubation in scintillation cocktail to quench chemiluminescence.

Subunit Molecular Weight and Stoichiometry. The size and stoichiometry of hydrogenase subunits were determined by SDS–PAGE. The molecular weights of hydrogenase subunits were extrapolated from a plot of log molecular weight vs. mobility. Subunit stoichiometry and enzyme purity were derived from scans of SDS–PAGE gels using a laser gel densitometer and integrator.

Flavin Content. Flavin was extracted from hydrogenase according to a modification of the procedure of Mayhew and Massey (1969). A 95-μL volume of 100% TCA was added. The sample was vortexed and incubated on ice for 10 min. Protein was pelleted by centrifugation, washed with 50 μL of 5% TCA, and recentrifuged. The supernatants were pooled and brought to pH 6.0 by the addition of 30 μL of 2 M KH_2PO_4. Standards of 5 μM FMN and 5 μM FAD were prepared in 0.3 M KH_2PO_4/5% TCA and separated by HPLC on a 10-μm C-18 reverse-phase analytical column. An elution program with a linear gradient of 5–20% acetonitrile/50 mM ammonium formate was used. Effluent was monitored at 436 nm. The HPLC (from Du Pont) was interfaced to an integrator (Hewlett-Packard Model HP3390A). Flavin standards were used to define a standard curve of integration units vs. picomoles of flavin. Aliquots of hydrogenase extract were injected onto the HPLC, and the flavins present were identified by elution time and coinjection with standard and quantitated by integration of peak area.

Atomic Absorption Spectrometry. Nickel and iron content was determined by atomic absorption spectrometry using a Perkin-Elmer Model 2380 atomic absorption spectrometer with an HGA-400 graphite furnace and programmer.

Activation and Assay of F_{420}-Reducing Hydrogenase. Hydrogenase assays for MV and F_0-reducing activities were performed as described by Jacobson (1981). Hydrogenase was typically activated in 50 μM F_0, 1 M KCl, and 10 mM 2-mercaptoethanol in 50 mM Tris buffer, pH 7.5. Enzyme concentrations in these preincubations were 0.14–0.3 mg/mL. Hydrogenase was first made anaerobic with four cycles of evacuation and flushing with argon on an all glass/metal/Teflon gas train. Hydrogen gas was then introduced, and samples were cycled twice more. After 5 min at room temperature, six more cycles under hydrogen gas were carried out, followed by incubation at 45 °C for 1 h. The activation tubes were placed on ice and then cycled 6 more times under hydrogen. Enzyme was kept on ice or at room temperature prior to assay.

Standard F_0 assay tubes contained 40 μM F_0, 10 mM 2-mercaptoethanol, and 0.5 M KCl in 50 mM Tris buffer, pH 7.5 at room temperature. Standard MV assay tubes contained 2 mM MV and 2 mM 2-mercaptoethanol in 50 mM Tris buffer, pH 7.5. Assays were performed at 32 °C. Assay tubes were made anaerobic under hydrogen gas by six cycles on the gas train. Prior to assay, six cycles under hydrogen gas were repeated. All assays were performed under 1.2 atm of hydrogen gas in 2 mL of solution. Reduction was initiated by enzyme addition anaerobically via a reduced MV scrubbed Hamilton syringe. Both activities were monitored at pH isosbestic points. F_0 reduction was monitored at 400 nm (ϵ = 25 mM^{-1} cm^{-1}) (Jacobson, 1981; Jacobson & Walsh, 1984) and MV reduction at 560 nm (ϵ = 8 mM^{-1} cm^{-1}) by using a Perkin-Elmer λ3 spectrophotometer interfaced to a Perkin-Elmer 3600 data station. Variations in activation and assay procedure for specific experiments are noted in figure legends.

Separation of α-, β-, and γ-Subunits. Enzyme solution at >2 mg/mL in 30 mM Bis-Tris propane (pH 7.2) was denatured by addition of SDS to 2% final concentration and heating to 90 °C for 3 min. Preparative SDS–PAGE electrophoresis in 10 × 15 cm × 0.3 cm gels were run with 5–10 mg of protein per gel (Guellen et al., 1984). Gels were run at 40-V constant voltage for 12–16 h. Protein was visualized by soaking in ice-cold 3 M KCl for 30 min (Nelles & Bamburg, 1976) or by rapid Coomassie Blue staining of a strip of the gel. Protein bands were then cut out of the gel and treated by one of two methods. For preparation of acrylamide suspensions, the gel selections were fixed in 10% acetic acid/25% isopropanol for 1 h and soaked in 0.9 M NaCl at 4 °C overnight and the sections homogenized with a Brinkmann Polytron with an equal volume of cold 0.9 M NaCl. Homogenates were stored at −20 °C. For electroelution of subunit polypeptides, gel strips were soaked in Tris/glycine running gel buffers for 1 h and electroeluted on an ISCO electroelution apparatus at 4 °C for 2–5 h.

α-Subunit was also prepared from enzyme by SDS denaturation followed by hydroxyapatite chromatography. One-millilter hydroxyapatite columns (Bio-Rad HPHT) were run at 2.5 mL/h in 10 mM sodium phosphate, pH 6.4, with 0.1 SDS. Enzyme was treated with SDS as described above and applied to the column. The column was eluted with 0.2–0.5 M sodium phosphate gradient in a total volume of 12 mL. Fractions (0.5 mL) were collected and analyzed by modified micro-Lowry protein determination and by SDS–PAGE.

Antibody Preparation. Male New Zealand White rabbits were injected with 0.5–1 mg either of holoenzyme or separated subunits emulsified 1:1 with Freund's complete adjuvant. Rabbits were boosted twice at 2-week intervals with 50–100 μg of protein. Anti-subunit sera were prepared with initial injections and first boosts of polyacrylamide homogenates and second boosts of soluble subunit polypeptides prepared by electroelution. Rabbits were bled from ear arteries and ca. 30-mL samples collected per bleed. All blood samples were allowed to clot overnight at 4 °C and centrifuged for 10 min in a clinical centrifuge, and the sera were purified by affinity chromatography on protein A–Sepharose (Ey et al., 1978).

The IgG fractions were dialyzed vs. PBS buffer, pH 7.2, at 4 °C overnight and stored in multiple aliquots at −70 °C.

Immunoprecipitation. Ten micrograms of enzyme was incubated with 10 μL of IgG in PBS for 30 min at 4 °C. Samples were centrifuged in a microfuge for 2 min. The supernatant fractions were then assayed for F_0-reducing activity.

Immunoinhibition. Assay tubes containing 1.8 mL of TBS (pH 7.5), 100 mM DTT, 1–10 μL of IgG, and 10 μL of a 1 mM solution of $Fe(NH_4)SO_4$ were stoppered and cycled 6 times under vacuum and H_2. Tubes were incubated at 37 °C. This procedure effects complete anaerobiosis (Knappe & Blaschkowski, 1975), as confirmed by comparison with our standard assay procedures for F_0 or MV reduction, where stoppered tubes were allowed to sit overnight after cycling under vacuum and N_2 and cycled again immediately before use. Activated H_2ase was added to the tubes, followed by room temperature incubation for 20 min. Aliquots of anaerobic F_0 or MV were then injected into the tubes to begin the reaction.

Amino Acid Composition. Holoenzyme and subunits prepared by electroelution were dialyzed into 50 mM $NaHCO_3$, pH 7.0 buffer. A total of 0.5–1.0 nmol of each polypeptide was used per analysis. Samples were acid-hydrolyzed at 100 °C in sealed tubes overnight. Cysteine was determined as cysteic acid by performic acid oxidation. Analyses were performed on a Beckmann amino acid analyzer.

N-Terminal Sequencing. One to three nanomoles of each subunit prepared by electroelution was reelectrophoresed by SDS–PAGE and electrophoretically transferred to nitrocellulose paper. Microsequencing was performed on an Applied Biosystems 470A gas-phase protein sequencer. PTH-amino acids were identified by reverse-phase HPLC employing an IBM cyanopropyl column and a Waters HPLC.

Nickel Removal. A total of 220 μCi of ^{63}Ni-containing H_2ase in Tris buffer was treated with a 7–8-fold molar excess of methyl methanethiosulfonate (Jaffe et al., 1984). Several incubations were performed at varying temperatures for 1 h. The samples were then applied to a 1 cm × 11 cm P6DG column (Bio-Rad) to separate protein from free nickel. Fractions (0.5 mL) were collected, and 50-μL aliquots were counted in 5-mL Liquiscint cocktail on a Beckman LS2300 scinitillation counter in the tritium window. Aliquots (15 mL) were analyzed by a modified micro-Lowry protein assay (Bensadoun & Weinstein, 1976).

Perchloric acid denaturation experiments were done with 50 μL of ^{63}Ni enzyme. Cold 0.1 N $HClO_4$ (100 μL) was added to enzyme solution, incubated on ice for 5 min, and centrifuged for 2 min. Acid-denatured protein was separated from cofactor by gel filtration chromatography on a 0.7 cm × 48 cm calibrated Sephadex G-15 column run at 6.8 mL/h in 1 mM HCl. Aliquots of fractions were neutralized with NaOH, counted, and used for protein determinations.

Flavin Removal. FAD was removed from holoenzyme by $CaCl_2$ treatment (Komai et al., 1969). A solution containing 100 μL of enzyme (0.4 mg), 100 μL of 12 M urea, 200 μL of 0.1 M Tris buffer (pH 8.0), and 600 μL of 3.4 M $CaCl_2$ was incubated at 35 °C for 90 min. FAD removal was monitored by fluorescence increase (λ_{ex} = 450 nm, λ_{em} = 530 nm) as free FAD is hydrolyzed to FMN. Control reactions contained KCl replacing F_0- and MV-reducing activity with and without added FAD in the activation tube. Apoenzyme was incubated with FAD for 15 min prior to activation.

[^{14}C]FAD was synthesized from [^{14}C]riboflavin and ATP enzymatically (Spencer et al., 1976). The product was purified by filtration through a Centricon (Amicon) followed by FPLC reverse-phase chromatography on a PepRC column. FAD was separated from riboflavin and FMN by a 5 mM NH_4CH_3COO–MeOH gradient (Light et al., 1980). The product was checked for purity by TLC on cellulose and lyophilized for storage at −20 °C. Final specific activity was 32 μCi/μmol. Apoflavoenzyme was reconstituted with [^{14}C]FAD in 3:1 molar ratio at 30 °C for 1 h. Excess FAD was removed by gel filtration on P6DG.

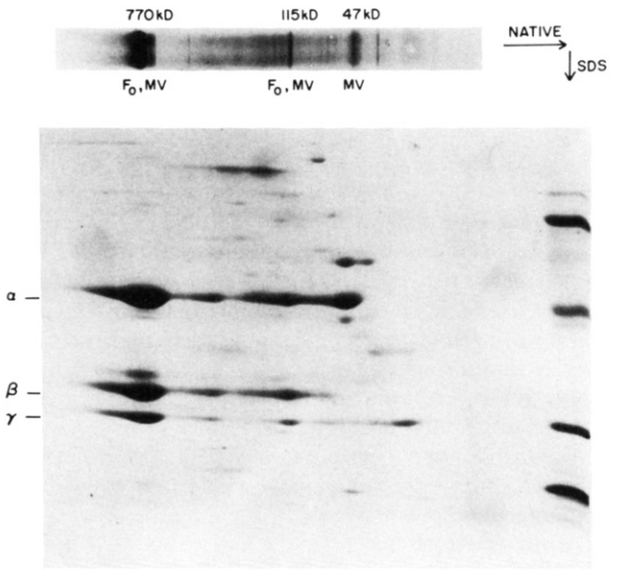


FIGURE 1: Two-dimensional native SDS–PAGE of F_{420}-reducing hydrogenase. Native PAGE (150 μg of protein) was performed as described in the text. The gel was then incubated in Laemmli sample buffer (Laemmli, 1970) for 1 h at room temperature and then for 15 min at 100 °C. The gel was annealed to the top of an SDS–PAGE gel with agarose, electrophoresed, and strained with Coomassie Blue (Vesterberg, 1971). Hydrogenase samples were run in parallel and stained for activity toward F_0 and MV as described above.

RESULTS

Purification and Properties of the Coenzyme F_{420} Reducing Hydrogenase. There are two detectable H_2-oxidizing enzymes in *M. thermoautotrophicum* ΔH, and as we have observed previously (Jacobson et al., 1982), they can be separated by column chromatography on Sepharose 6B and distinguished by assay with methylviologen (both reactive) and the 8-hydroxy-5-deazaflavin coenzymes F_0 and F_{420} (only one H_2ase reactive). We have further purified the F_{420}-reducing hydrogenase by phenyl-Sepharose and FPLC anion-exchange on a Mono Q column to yield a protein fraction that on native gradient gel electrophoresis reproducibly gives a slowly migrating major band and more rapidly migrating minor band, both of which display both F_0- and MV-reducing activity. Each of these hydrogenase bands, when examined by SDS gel electrophoresis in the second dimension, contains three subunits, α, β, and γ, as shown in Figure 1. The three-subunit composition of F_{420}-reducing hydrogenase is reproducible in more than 30 preparations conducted over the past 5 years.

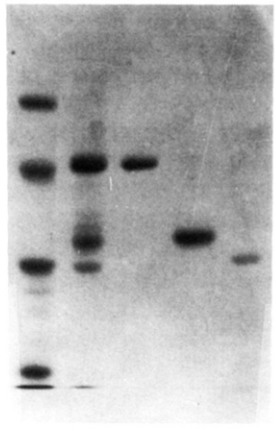
The four-step procedure summarized in Table I permits isolation of large quantities of purified enzyme (45–60 mg) in ~50% overall recovery. The 41-fold purification suggests the enzyme is 2–2.5% of the soluble cell protein in this methanogenic thermophilic bacterium.

Native Molecular Weight, Subunit Separation, and Subunit Composition. The native molecular weight of F_{420}-reducing hydrogenase was estimated by gel filtration on a calibrated Sephacryl S-300 column and by 4–22% native gradient PAGE (O'Farrell, 1975; Figure 1, first dimension). With both

Table I: Purification of F_{420}-Reducing Hydrogenase

step	act. (μmol min^{-1} mg^{-1})[a]	total protein (g)	purification (x-fold)	yield (%)[b]
crude extract	1.2	2.9		
DEAE-Sephadex, $(NH_4)_2SO_4$ precipitation	2.1	1.5	1.75	90
Sepharose 6B, phenyl-Sepharose	30.7	0.070	26	57
FPLC on Mono Q	49.0	*c*	41	*c*

[a] Activity was measured with 40 μM F_0 assay tubes as described under Methods. [b] Yield refers to recovery of activity units. [c] F_{420}-reducing hydrogenase was purified by FPLC on Mono Q when homogeneous protein was required. Recovery of F_{420}-reducing hydrogenase was 62%.



1 2 3 4 5

FIGURE 2: Hydrogenase subunits after separation by preparative SDS–PAGE: lane 1, molecular weight standards (M_r 13.7 26K, 43K, and 65K); lane 2, 15 μg of holoenzyme; lane 3, 3 μg of α-subunit; lane 4, 3 μg of β-subunit; lane 5, 3 μg of γ-subunit.

techniques the majority of F_{420}-reducing activity was associated with a high molecular weight aggregate of M_r 775 000–800 000. These data are for enzyme purified under aerobic conditions, as detailed under Methods, requiring reductive anaerobic activation before catalytic activity regain. On anaerobic, reducing conditions in native gradient PAGE, the active enzyme major forms appears slightly larger at M_r 900 000–950 000, suggesting aggregation per se is not an artifact of aerobic isolation. Electrophoresis of SDS-treated enzyme under mild conditions (Ohmori et al., 1984) revealed that >90% of the protein remained in high molecular weight aggregates. We have recently determined this hydrogenase shows discrete structure by electron microscopy, and image reconstruction confirms a molecular weight of ca. 850 000 for the enzyme particles (Wackett et al., 1987).

As noted above, activity staining of native gels with F_0 shows protein bands of lower molecular weight with deazaflavin-reducing activity of which the smallest band is M_r 115 000. Both the M_r 800 000 and M_r 115 000 bands also stain for H_2-oxidizing methylviologen-reducing activity. A band at 47K in the native gradient gel is also active at MV reduction but not F_0 reduction. The molecular weights of the three subunits (Figures 1 and 2) are estimated from SDS gel electrophoresis to be 47 000 (α), 31 000 (β), and 26 000 (γ). An $\alpha\beta\gamma$ trimer would have a minimal unitary molecular weight of 105 000 for a species active in F_0 and F_{420} reduction. An octamer of trimers would account for a higher molecular weight aggregate at M_r 840 000 (Wackett et al., 1986). Attempts to determine $\alpha\beta\gamma$ subunit stoichiometry using densitometry of SDS–PAGE-separated subunits stained with Coomassie Blue (Vesterberg, 1971) have been inconclusive, with 2:2:1 ratios of α:β:γ a typical result. Repetition of the experiment with Amido Black 10B staining (Wilson, 1979) suggested instead a 2:1:1 ratio. We provisionally assign a 1:1:1 ratio based on the 115K band and the 800–840K major active aggregate but realize this question requires further investigation and the ambiguity in subunit stoichiometry also constrains redox cofactor stoichiometry and placement, as discussed below. The isoelectric point of the F_{420}-reducing hydrogenase is 5.7.

Table II: Composition and Cofactor Content of F_{420}-Reducing Hydrogenase

subunits	F_{420}-reducing active forms	redox cofactor content per M_r 115 000 unit
α (47K)	M_r 115 000, minor form ($\alpha\beta\gamma$:?)	13–14 Fe
β (31K)	M_r 800 000, major form ($\alpha_8\beta_8\gamma_8$:?)	0.6–0.7 Ni
γ (26K)		0.8–0.9 FAD

Cofactor Content of F_{420}-Reducing Hydrogenase. F_{420}-reducing hydrogenase contains iron–sulfur centers, as do all known hydrogenases (Adams et al., 1981). Preliminary analysis by iron EXAFS has indicated Fe_4S_4 clusters are present (Lindahl et al., 1984) but does not rule out the possibility of other centers. By atomic absorption analysis (several preparations) there are 13–14 mol of Fe per M_r 115 000 $\alpha\beta\gamma$ trimer. As we noted briefly in the initial communication (Jacobson et al., 1982) and have studied subsequently by ^{61}Ni EPR (Kojima et al., 1983), by nickel EXAFS (Lindahl et al., 1984), and by growth of cells on radioactive ^{63}Ni (data not shown), the F_{420}-reducing hydrogenase is a nickel enzyme. On average there is 0.6–0.7 mol of nickel per M_r 115 000 unit. The third redox cofactor is tightly associated FAD, and it is present at 0.8–0.9 molecules per M_r 115 000 unit, as summarized in Table II.

Subunit Separation and Characterization. A variety of methods were attempted to resolve the hydrogenase into component subunits with preservation of active structures and/or coenzyme contents, but this proved quite difficult. Two molar urea or guanidinium chloride (GdmCl) has negligible effects on enzyme activity, and we were unable to separate subunits by prolonged incubation in 6 M GdmCl, 8 M urea, or 0.1% Triton X-100 (with or without thiols present) followed by ion-exchange or hydroxyapatite chromatography. Subunits also remained complexed after several freeze–thaw cycles with the potent chaotrope sodium trifluoroacetate (3 N). Even reverse-phase chromatography in 0.1% TFA gave a single peak, still containing all three subunits by gel analysis. These data, consistent with the EM analysis (Wackett et al., 1987), suggest remarkably stable subunit interactions, which may be requisite for proper functioning in its physiological intracellular milieu of 0.8 M KCl at 65 °C (Daniels et al., 1984). Ultimately, incubation in SDS revealed that, at 0.1% detergent, the F_0-reducing activity fell to 15% while the MV-reduction activity actually increased ~25%; dilution of the SDS restores F_0-reducing activity. Complete inhibition of F_0 reduction occurs at 0.25% SDS. Hydroxyapatite chromatography of enzyme boiled in 2% SDS and run in buffers containing 0.1% SDS (Moss & Rosenblum, 1972) allowed resolution of α-subunit (selectively retained) from a mixture of β- and γ-subunits on reasonable scale, but we finally used preparative SDS–PAGE to get all three subunits apart (Figure 2). From 15 mg of holoenzyme, we could recover 2 mg of α-subunit, 1 mg of β-subunit, and 0.8 mg of γ-subunit.

Amino acid compositions (Table III), at a single 24-h time point (with that attendant unreliability), establish each poly-

Table III: Amino Acid Composition of Subunits of F_{420}-Reducing Hydrogenase

	residues/mol		
	α	β	γ
Asp + Asn	40.0	17.3	20.9
Thr	20.2	16.0	10.1
Ser	16.3	10.4	10.1
Glu + Gln	45.2	30.5	28.7
Pro	23.9	10.8	12.6
Gly	39.9	34.5	21.2
Ala	30.9	20.2	18.7
Val	31.5	17.5	11.5
Met	8.4	7.3	6.4
Ile	27.5	14.6	10.3
Leu	30.9	20.6	24.9
Tyr	8.7	8.9	5.8
Phe	11.2	8.0	3.5
His	14.0	1.6	3.5
Lys	18.5	29.8	9.7
Arg	23.2	6.6	7.3
Cys	5.3	3.9	11.5
Trp	1.1	ND[a]	ND[a]

[a] ND, not determined.

peptide is unique, with the β-subunit high in lysine and the small γ-subunit rich in cysteine (11–12 residues). N-Terminal sequencing of each isolated subunit produced the sequence information of Table IV, with identical residues for α and β at positions 24 and 26–29.

Because of the tenacity of association of subunits and the resultant strong denaturing conditions needed for separation, it has been difficult as yet to localize redox cofactors to specific subunits. Iron was detected in isolated α-subunit by three approaches. A mild SDS–PAGE procedure, followed by bathophenanthroline staining, showed non-heme iron present in both high molecular weight aggregates and the 47K α-subunit. In 2D gels (e.g., Figure 1) both 47K α-subunit and a 70K $\alpha\gamma$ complex stained for non-heme iron. Lastly, on electroelution of α-subunit after preparative SDS gel electrophoresis, iron was detected by visible spectroscopy at 400 nm. By inference from the high cysteine content of γ-subunit (11–12 Cys, compared to 5–6 Cys for α and ca. 4 Cys for β) and the iron content (13–14 Fe per α,β,γ trimer) and the indication of Fe_4S_4 clusters by EXAFS (Lindahl et al., 1984; Bastian, Walsh, and Orme-Johnson, unpublished data), it is likely that γ-subunit will also contain Fe/S clusters. Activity staining of 2D gels did indicate the α-subunit alone can reduce methylviologen from H_2 and may be consistent with it as a core H_2-oxidizing subunit. Bound FAD is essential for F_0 or F_{420} reduction by the cognate hydrogenase from *M. formicicum* (Nelson et al., 1984). Similarly, $CaCl_2$ treatment of holoenzyme removes the bulk of FAD from this hydrogenase and drops the F_0/MV reduction ratio 5-fold, from 0.20 in control to 0.04 in treated enzyme. Incubation of this apoflavoenzyme with stoichiometric amounts of FAD restored the activity ratio to 0.19. Attempts at reconstitution and localization with [^{14}C]FAD give only 0.1 radiolabeled FAD per 115K species after gel filtration and may reflect instability of reconstituted holoenzyme. The only clue to date on FAD localization is again from the 2D gel electrophoretic studies which showed the $\alpha\beta\gamma$ 115K species reduces F_0 but the 70K $\alpha\gamma$ complex does not, suggesting one might focus on β-subunit in the future as a possible FAD binding site.

Studies with ^{63}Ni-containing purified enzyme revealed that perchloric acid and TCA precipitation of protein quantitatively solubilize the radioactive nickel which then chromatographs on Sephadex G-15 at a position equivalent to $^{63}NiCl_2$ standards, consistent with the absence of a discrete nickel cofactor structure, e.g., such as the methanogen F_{430} nickel corphin (Pfaltz et al., 1982). ^{63}Ni was removed efficiently at 55 °C from native enzyme by methyl methanethiosulfonate (MMTS), a reagent known to disrupt Zn–S bonds in porphobilinogen synthase (Jaffe et al., 1984). Analysis of ^{63}Ni content in enzyme on native gradient gels confirmed radiolabel in the 800K aggregate. After mild SDS–PAGE gel electrophoresis there was a small amount of radioactivity detectable with α-subunit, but localization of nickel to α-subunit on this basis is tentative.

Stability of F_{420}-Reducing Hydrogenase and Procedures for Anaerobic Reductive Activation of the Isolated Enzyme. Although *M. thermoautotrophicum* ΔH is an obligate anaerobe and it was known that hydrogenase activity was air-sensitive, our successful large-scale purifications were done aerobically, from cells depleted of reducing power, yielding a stable protein that can readily be reactivated to hydrogenase with high k_{cat}/K_m values (see following paper in this issue). In fact, attempts at anaerobic purification have not been fully satisfactory because the enzyme may lose redox cofactors on chromatographic steps (e.g., $FADH_2$ may be less tightly bound than FAD). Enzyme isolated in its aerobic, inactivation form can be stored for more than a year at −70 °C with gradual loss of ca. 30% of its activity when subsequently assayed by reductive activation.

Once the enzyme is reductively activated, however, if it is reexposed to air, irreversible inactivation occurs almost at once. Thus enzyme that has its redox inventory oxidized before exposure to air is reversibly inhibited and can regain full activity on anaerobic reduction, but enzyme with a reduced redox inventory (Fe/S, Ni, FAD) on exposure to air is irreversibly inhibited. This irreversible oxidation may arise from O_2^- or H_2O_2 generated when O_2 reacts with reduced centers on the enzyme. For example, in manipulation of the hydrogenase for catalytic or spectroscopic assays after reductive activation with H_2, it is possible to displace H_2 with argon on the gas line and then expose the enzyme to air without damage. This enzyme when reduced and assayed anaerobically has full activity. The removal of H_2 gas draws electrons out of the enzyme at low partial pressures of H_2 and reoxidizes Fe/S, nickel, and FAD centers (Kojima et al., 1983), rendering the exposure to O_2 harmless. Thus, aerobic purification of enzyme

Table IV: N-Terminal Sequences of Hydrogenase Subunits

subunit	sequence
α	1 Ser - Glu - Arg - Ile - Val - Ile - Ser - Pro - Thr - Ser - 11 Arg - Gln - Glu - Gly - His - Ala - Glu - Leu - 21 Val - Met - Glu - Val - Asp - Asp - Glu - Gly - Ile - Val - Thr - Lys
β	1 Val - Leu - Cys - Thr - Tyr - Lys - Glu - Ile - Val - Ser - 11 Ala - Arg - Ser - Thr - Asp - Arg - Glu - Ile- 21 Gln - Lys - Leu - Ala - Gln - Asp - Gly - Gly - Ile - Val - Thr - Gly - 31 Leu
γ	1 Ala - Glu - Glu - Asn - Ala - Lys - Pro - Arg - Ile - Gly - 11 Tyr - Ile - Gly - Leu

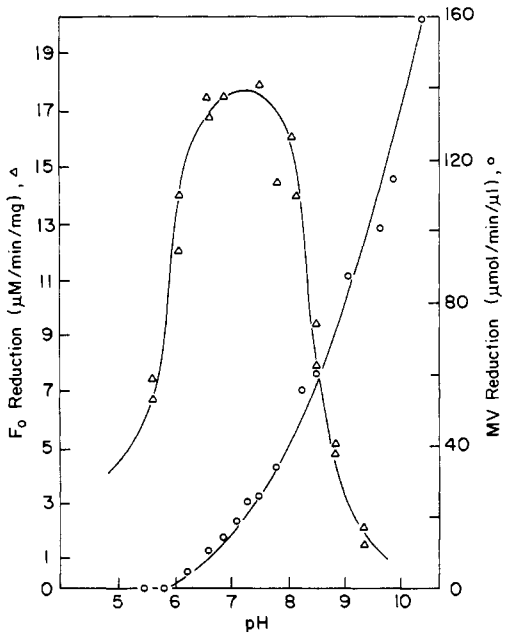


FIGURE 3: pH profiles of F_0 and MV reduction by F_{420}-reducing hydrogenase. F_0 assays (Δ) were performed at 25.5 °C with 40 μM F_0 in 50 mM CAPS/MES/TAPS/0.5 M KCl buffer adjusted to pH ranging from 5.6 to 10.0. MV reduction (○) was followed in the same buffer without salt and with 2 mM MV and 2 mM 2-mercaptoethanol.

from cells, depleted of reducing equivalents before cell breakage and aerobiosis, is the preferred method for reliable isolation of an enzyme form that can be reproducibly converted to active enzyme.

Anaerobic activation of the isolated, purified enzyme has been systematically studied and optimized, both in the anaerobic activation process and in the subsequent anaerobic assay. The final obtainable activity was stimulated 10-fold by inclusion of salt at 1 M during activation and then at at least 10^{-4} M in assay buffer. There was no significant preference for K^+ vs. Na^+ despite an estimated intracellular concentration of 0.78 M K^+ in *M. thermoautotrophicum* ΔH (Daniels et al., 1984), nor was activation by halide ions correlatable with chaotropicity, as had been suggested for *Megasphera elsdernii* hydrogenase (van Dijk et al., 1980).

For optimal activation a minimal concentration of 0.12 mg/mL enzyme is needed in the activation buffer (BSA cannot substitute), and concentrations in the 4 mg/mL range obviated the need for electron acceptor (Jacobson, 1981; Fox, 1984). After activation, the enzyme can be assayed in the microgram per milliliter range without problem. Thiol is needed during activation (Jacobson, 1981), and inclusion of mercaptoethanol routinely yielded enzyme stable to multiple manipulations of gas phase (e.g., H_2 to argon to H_2) on the gas line. Lastly, inclusion of electron acceptors, 50 μM F_0 or 50 μM MV, in reactivation buffers led to maximally active enzyme (Fox, 1984). Reactive activation was routinely carried out at 45 °C for the thermophilic enzyme, leading to a full regain of activity by 30–60 min when subsequently assayed with either F_0 or MV as electron acceptors. The enzyme thus activated is stable to multiple assay (>65 per day) and maintains >80% specific activity after 24 h at room temperature.

The pH profile of F_0-reducing activity and MV-reducing activity differ as shown in Figure 3, whereas deazaflavin-reducing activity is optimal at pH 6.5–7.5 but the MV reduction rate increases up to pH 11, beyond which the enzyme is not stable. The increase of the potential of the MV^{2+}/MV^+ couple and the decrease of the $H_2/2H^+$ potential, with increasing pH, may contribute to the observed pH vs. V_{max} profile.

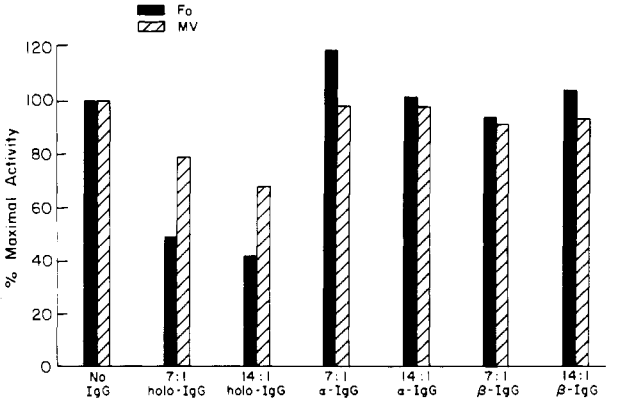


FIGURE 4: Immunoinhibition of F_0- and MV-reducing activity by IgG against holoenzyme and separated subunits. Ratios indicate micrograms of IgG to micrograms of hydrogenase.

Antibodies to Holoenzyme and Subunits. Antibodies were raised against holoenzyme and subunits isolated by SDS gel electrophoresis. Antisera to holoenzyme gave detectable immunoprecipitation with holoenzyme on Ouchterlony double-diffusion plates but antibodies to subunits were not precipitated in this assay. They were monospecific for subunit antigens when used as probes for Western blots of *M. thermoautotrophicum* ΔH crude cell extracts (P. Hamilton, personal communication), but the titer with anti-γ antibody was low. Dot-blotting and ELISA detection assays indicated that anti-holoenzyme IgG cross-reacted with holoenzyme and β- and γ-subunits. Anti-α IgG was specific for α-subunit. Anti-β IgG recognized holoenzyme and β- and γ-subunits. The anti-holoenzyme antiserum (10 μL) was inhibitory to both F_0- and MV-reducing activity of pure hydrogenase (10 μL of enzyme) while anti-subunit IgG in equivalent amounts was noninhibitory under the same conditions. To look for selective inhibition of MV reduction vs. F_0 reduction activity of enzyme, we required a different protocol from the normal activation conditions (1 M KCl, 45 °C, 60 min under H_2) since antigen–antibody complexes are not stable under these conditions. A rapid alternate O_2 removal, H_2 equilibration, was accomplished by adaptations of a nitrogenase assay (Knappe & Blaschkowski, 1975), with IgG in sealed assay tubes under H_2 in a solution containing TBS, pH 7.5, and a catalytic reducing system of 5 mM DTT and 5 μM $Fe(NH_4)SO_4$ which goes completely anaerobic in 30 min at 37 °C. As shown in Figure 4, the anti-holoenzyme IgG exhibits preferential inhibition of F_0-reduction activity, consistent with our proposal of spatially distinct F_0 and MV reduction sites in the enzyme in the following paper (Livingston et al., 1987).

Discussion

Coenzyme F_{420} reducing hydrogenase is present in substantial quantity in *M. thermoautotrophicum* and doubtless in other methanogens as well. The 5-deazaflavin coenzyme F_{420} is a marker molecule for methanogenic bacteria and is present at up to 100 mg/kg of cells. This hydrogenase probably provides reducing equivalents for methane production (Nagle & Wolfe, 1983). For example, anti-holoenzyme antibody blocks H_2-dependent synthesis of CH_4 in crude extracts of these cells (D. Livingston and L. Wackett, unpublished observations). The $F_{420}H_2$ generated by this hydrogenase during growth of cells on H_2 subsequently generates NADPH, by action of the F_{420}–NADPH transhydrogenase, necessary for reductive biosynthetic metabolism (Yamazaki et al., 1980; Jacobson & Walsh, 1984). The redox potentials at pH 7 for the H_2/H^+ couple, $F_{420}/F_{420}H_2$ couple, and NADP/NADPH

couple are −420, −360, and −320 mV, respectively (Jacobson & Walsh, 1984; Walsh, 1986). Consistent with its central role in methanogen metabolism, the F_{420}-reducing hydrogenase constitutes some 2% of the soluble cell protein. The ability to purify, stabilize, and activate this enzyme is essential to further understanding of the biological production of methane as well as for insight into the mechanism of enzymatic activation of molecular hydrogen.

Isolation of hydrogenases that are catalytically active only under anaerobic, reducing conditions by deliberate aerobic isolation in reversibly inactive form has now succeeded in several cases, including the soluble, uptake hydrogenases from *A. eutrophus* H16 and 21 (Schneider & Schlegel, 1976; Egerer & Simon, 1982; Egerer et al., 1982; Popov et al., 1983; van Dijk et al., 1979, 1980) and *Desulfovibrio gigas* (Lissolo et al., 1984). It is likely that success in aerobic purification in general requires the redox centers (e.g., iron–sulfur clusters) to be oxidized before exposure to air. If they are reduced, then O_2 reduction to superoxide, peroxide, or hydroxyl radical can damage the redox centers and irreversibly inactivate the hydrogenases. Therefore, we gas cells with CO_2 but not H_2 before exposure to air and cell breakage. Activation of hydrogenases back to a catalytically active species requires both oxygen removal and reductive conditions. The process may involve autocatalytic and collisional propagation of reduction among active hydrogenase molecules (Schneider & Schlegel, 1976) and/or conformational state alteration (Popov et al., 1982). We see no obvious change in aggregation state, but we have observed reduction of nickel, iron, and flavin centers during reactivation (Kojima et al., 1983) and have carefully defined experimental conditions for optimization of anaerobic reductive activation. We have elsewhere noted the EPR-detectable nickel(III) form of enzyme is a signal for aerobic, inactive enzyme and is altered on hydrogenase activation (Kojima et al., 1983).

Striking features of the F_{420}-reducing hydrogenase are the large size of the major form of the enzyme and the hydrophobic interaction of the subunits. The latter property is reflected, for example, in the need for 25% dimethyl sulfoxide for elution from phenyl-Sepharose columns and in the remarkable resistance of subunits to separation in denaturants and upon heating. The enzyme may be peripherially associated with cellular membranes but is certainly not an intrinsic membrane enzyme. The aggregate displaying a molecular weight around 800 000 is of likely physiological significance and represents a discrete structure detected by electron microscopy both in crude extracts (aerobic and anaerobic) and in pure enzyme (Wackett et al., 1987). While determination of the stoichiometry of heterooligomeric proteins is at best difficult and is an object of continuing study, it seems likely that an $\alpha\beta\gamma$ species may be the minimal form active in deazaflavin reduction and correspondingly an $\alpha_8\beta_8\gamma_8$ form may be the major species characterized by electron microscopy.

The cofactor inventory associated with this hydrogenase is complex. Nickel has now been detected in at least a dozen other bacterial hydrogenases functioning in the H_2 oxidation direction [see Teixera et al. (1985) and Bastian et al. (1987)]. Nickel is redox active and is apparently in ligation to cysteine thiolates (Lindahl et al., 1984; Scott et al., 1984). The iron is a ubiquitious constituent of hydrogenases as iron–sulfur clusters, and EPR and EXAFS data suggest the anticipated four iron–four sulfur clusters (Bastian et al., 1987). Flavin, as FAD, is not nearly so common a constituent of hydrogenases but is found in the nicotinamide-reducing hydrogenases from *A. eutrophus* (Schneider & Schlegel, 1978; Friederich et al., 1982; Schneider et al., 1979, 1984a) and *N. opaca* (Schneider et al., 1984a,b). In studies on redox chemistry of the F_0 and F_{420} coenzyme (Jacobson & Walsh, 1984; Walsh, 1986) we have noted that, in analogy to NAD, they are hydride-transfer coenzymes in the ground state. As obligate two-electron redox acceptors they need a $1e^-/2e^-$ redox intermediary to accept electrons from the one-electron Fe/S clusters. Thus the bound FAD can serve this one-electron/two-electron redox switch (Walsh, 1980). Indeed, during anaerobic isolation of the F_{420}-reducing hydrogenase by Ferry and colleagues (Nelson et al., 1984), the $FADH_2$ dissociated from enzyme during column chromatography to yield a flavin-free hydrogenase, inactive at F_{420} reduction until exogenous FAD was added back.

In addition to evaluation of the precise redox roles of nickel, iron–sulfur centers, and flavin, we would like to know placement of the redox centers within the $\alpha\beta\gamma$ subunit array. The resistance of subunits to separation except under fully denaturing conditions has made this a difficult venture to date, but as noted, there is clearly iron in the α-subunit, and the high cysteine content of the γ-subunit makes it a strong candidate for a ferredoxin-like role. The 47K α-subunit will oxidize H_2 and reduce MV and so may be a core hydrogenase. This could be the site of nickel binding or nickel could span subunits in thiol ligation (e.g., $\alpha + \gamma$). The fact that $\alpha\gamma$ complex cannot reduce F_{420} but $\alpha\beta\gamma$ species can suggests that β-subunit may be a site for FAD binding. There is the prospect that use of antibody and N-terminal sequence data will permit subunit cloning and sequencing (an effort in progress with J. Reeve, Ohio State University) and that sequence information may help in cofactor placement (e.g., FAD binding sites are recognizable in primary sequence data). In this context, the DNA sequence for the gene encoding the eubacterial *Desulfovibrio vulgaris* uptake hydrogenase has been reported (Voordouw & Brenner, 1985), and the sites of some of the iron–sulfur centers are obvious.

The following paper describes kinetic characterization of the F_{420}-reducing hydrogenase and evidence for a multisite hybrid ping-pong mechanism as well as deuterium studies to probe rate-determining steps and regio- and stereochemistry of hydrogenase catalysis (Livingston et al., 1987).

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Registry No. 8-Hydroxy-5-deazariboflavin, 71415-45-7; coenzyme F_{420} hydrogenase, 65099-08-3.

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