Cloning, Sequence Determination, and Expression of the Genes Encoding the Subunits of the Nickel-Containing 8-Hydroxy-5-deazaflavin Reducing Hydrogenase from *Methanobacterium thermoautotrophicum* ΔH[†,‡]

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ABSTRACT: The genes *frh*A (1217 bp), *frh*B (845 bp), and *frh*G (710 bp) encoding the three known subunits, α, β, and γ, of the 8-hydroxy-5-deazaflavin (F_{420}) reducing hydrogenase (FRH) from the thermophilic methanogen *Methanobacterium thermoautotrophicum* ΔH have been cloned, sequenced, and shown to be tightly linked, indicative of a single transcriptional unit. The DNA sequence contains a fourth open reading frame, designated *frh*D (476 bp), encoding a polypeptide (δ) that does not copurify with the active enzyme. Expression of the *frh* gene cluster in *Escherichia coli* shows that four polypeptides are synthesized. When analyzed by SDS–PAGE, the proteins migrate with mobilities consistent with their calculated molecular weights. In order to understand the mechanism of H_2 oxidation by this enzyme, localization of redox cofactors (Ni, Fe/S, FAD) to specific subunits and information on their structure is needed. This has been hindered due to the refractory nature of the enzyme to denaturation methods needed in order to obtain individual subunits with cofactors intact. In this paper we discuss the possible localization of the redox cofactors as implicated from the DNA-derived protein sequences of the subunits. The amino acid sequences of the subunits of the FRH are compared with those of other Ni-containing hydrogenases, including the methyl viologen reducing hydrogenase (MVH) of *M. thermoautotrophicum* ΔH.

The thermophilic methanogenic archaebacterium *Methanobacterium thermoautotrophicum* ΔH can derive all of its energy by oxidizing H_2 gas concomitant with the reduction of CO_2 to produce CH_4. H_2 oxidation is mediated enzymatically by hydrogenase; therefore, this enzyme plays a key role in the metabolism of this species. Previous investigations in these laboratories have shown there are two hydrogenases in *M. thermoautotrophicum* ΔH: one that reduces the 8-hydroxy-5-deazaflavin cofactor F_{420},[1] known as the F_{420} reducing hydrogenase (FRH), and the second, which is nonreactive toward F_{420}, known as the methyl viologen reducing hydrogenase (MVH) (Jacobson et al., 1982; Walsh, 1986).

The FRH has been purified to homogeneity and shown to contain three subunits, α, β, and γ, with molecular weights of 47K, 31K, and 26K, respectively (Fox et al., 1987). These associate to form an $(\alpha_1\beta_1\gamma_1)_8$ complex with a molecular weight of approximately 800K (Wackett et al., 1988; Fox et al., 1987). The FRH contains Ni, Fe/S, and FAD cofactors, but because of the difficulties encountered in dissociating the FRH into its individual subunits, assignment of the cofactors to specific subunits has been unsuccessful (Fox et al., 1987). This has hindered our understanding of the function and mechanism of the FRH and its relationship to the MVH, which has also been shown to contain redox-active nickel (Jacobson et al., 1982; Kojima et al., 1983). The genes encoding the MVH have recently been cloned and sequenced (Reeve et al., 1989). Here we report the cloning and sequencing of the genes encoding the FRH. Homologies between the amino acid sequences of the two methanogen hydrogenases are shown and discussed in light of the known biochemistry of the Ni-containing hydrogenases.

Materials and Methods

All restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs. *Sac*I linkers were from New England Biolabs. T4 DNA ligase and the Cyclone kit were purchased from International Biotechnologies, Inc. Endoproteinase Lys-C and *Staphylococcus* V8 protease were from Boehringer Mannheim. Sequenase was from U.S. Biochemicals. *Escherichia coli* XL1-Blue was purchased from Stratagene and has the genotype *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 ($r_k^-m_k^+$), *sup*E44, *rel*A1, λ^-, *lac*, [F′, *pro*AB, *lac*I^{q}ZΔM15, Tn10 (tet^R)]. *E. coli* DH5α was purchased from Bethesda Research Laboratories and has the genotype F$^-$, φ80d*lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 ($r_k^-m_k^+$), *sup*E44, *thi*-1, λ^-, *gyr*A96, *rel*A1, Δ(*lac*ZYA–*arg*F), U169.

General. All molecular biological methods employed were standard techniques and were performed as described by Maniatis et al. (1986) or according to the manufacturers'

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[1] Abbreviations: aa, amino acid; dITP, deoxyinosine triphosphate; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; F_0, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; F_{420}, the natural product methanogen deazaflavin cofactor; FAD, flavin adenine dinucleotide; FDH, formate dehydrogenase; FRH, the F_{420} reducing hydrogenase; HPLC, high-pressure liquid chromatography; KP_i, potassium phosphate; MVH, the methyl viologen reducing hydrogenase; NAD, nicotinamide adenine dinucleotide; NBRF-PIR; National Biomedical Research Foundation Protein Identification Resource; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate.

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instructions unless otherwise noted. SDS–PAGE was performed as described by Laemmli (1970). Protein concentration was determined by the method of Lowry (1951).

Growth of Cells. M. thermoautotrophicum ΔH was grown anaerobically in a 16-L Chemap fermentor at 65 °C as previously described (Schonheit et al., 1979). The cells were harvested in late log phase by first cooling the culture to 20 °C while sparging with N_2, followed by pelleting with a Sharples continuous-flow centrifuge. Cell pellets were stored at −70 °C.

FRH Purification, Isolation of Subunits, and Determination of N-Terminal Sequences. FRH was purified by the method of Fox et al. (1987) as modified by Bastian (1987) except that fractions from the Sepharose CL-4B column were brought to 1 M KCl and loaded onto the phenyl-Sepharose column at room temperature. This column was washed with 500 mL of 10 mM KP_i/1 M KCl buffer (pH 7.2) at 30 mL/h, FRH was eluted with a buffer change to 10 mM KP_i, pH 7.2. Fractions active for F_0 reduction were pooled and stored frozen at −70 °C. The individual subunits of the FRH were isolated as described by Fox et al. (1987). For determination of the N-terminal amino acid sequences, the subunits were separated by SDS–PAGE (10%) and blotted to PVDF membranes for sequencing at the Harvard Microchemistry Facility.

Proteolysis of the α Subunit. To 1 mg of the α subunit in 1 mL of 25 mM Tris/0.2 M glycine buffer (pH 8.4) was added EDTA (2 μL of 0.5 M, pH 8.0). The solution was heated to 90 °C for 3 min and cooled on ice. Dithiothreitol was added (10 μL of 200 mM) and the solution was purged with argon for several minutes followed by incubation for 60 min at 37 °C. Under an argon atmosphere, iodoacetamide was added (100 μL of 50 mM) and the solution incubated for 1 h at 37 °C in the dark. The reaction was stopped by the addition of β-mercaptoethanol (1% v/v). The buffer was changed to 0.1 M NH_4HCO_3/0.1% SDS (pH 9.0) with the use of a Centricon-30. The reduced, alkylated α subunit (500 μg) was resuspended into 0.1 M NH_4HCO_3/0.1% SDS (300 μL, pH 9.0), heated to 90 °C for 3 min, and cooled on ice. This solution was treated with endoproteinase Lys-C (5 μg) while being shaken at 37 °C for 24 h. A second aliquot (10 μg) of protease was added and incubation continued for 72 h. The reaction was stopped by the addition of trifluoroacetic acid (0.1% v/v) and frozen at −20 °C. The products were separated on a 10% SDS–polyacrylamide gel. Following electrophoretic separation, one lane was stained by Coomassie blue to visualize the peptide fragments and the remainder was blotted to a PVDF membrane for amino acid sequencing at the Harvard Microchemistry Facility.

Proteolysis of the β Subunit. The β subunit (400 μg), in 1 mL of 25 mM Tris/0.2 M glycine buffer (pH 8.4), was reduced and alkylated as described above for the α subunit and resuspended in 0.1 M NH_4HCO_3/0.1% SDS buffer (pH 9.0, 400 μL). This solution was treated with *Staphylococcus* V8 protease (8 μg) and incubated for 24 h at 37 °C. A second aliquot (8 μg) of protease was added and incubation continued for 12 h. The reaction mixture was acidified with trifluoroacetic acid and frozen at −20 °C. The proteolytic fragments were then purified by reverse-phase HPLC on a Vydac C_4 analytical column with an increasing linear gradient (10–90%) of 95% acetonitrile/0.1% trifluoroacetic acid at a flow rate of 1 mL/min. Identical peaks from several runs were pooled, concentrated in vacuo, and sequenced at the Harvard Microchemistry Facility.

Synthetic Oligonucleotides. Oligonucleotides were synthesized by using solid-phase phosphoramidite chemistry on a Pharmacia Gene Assembler and purified according to the manufacturer's recommendation. The oligonucleotides were synthesized on the basis of the N-terminal protein sequences previously obtained for the FRH (Fox et al., 1987), except in the case of the γ subunit, which differed at residue 13 when resequenced (see Results). The oligonucleotide α-I (ATGGAIGTIGAIGAIGAIGGIATIGTIACIAA) was based on residues 4–14 of the α subunit, the β-I probe (ACIGAIIGIGAIATICAIAAIITIGCICAIGAIGGIGG-IATIGTIACIGG) was based on residues 14–30 of the β subunit, and γ-I (GCIGAIGAIAAIGCIAAICCIIGIAT-IGGITAIATICAICT) was based on residues 1–14 of the γ subunit. These probes were end-labeled with γ-[^{32}P]ATP (Maniatis et al., 1986). Additional oligonucleotides used as sequencing primers were P-2, CCCTCATCATCGACTTC-CATG; P-4, GAAGAGTACTTCAAGGATAG; P-5, AGAAATTAGCCCAGGATGGA; P-6, GGTCTGGCA-GGGTATTGCAA; P-7, ATTTCCACATTGGGAGCTGA; ALEX-7, GAAGATGATAGTTGTGGATG; and ALEX-8, TGCGCAACACACGTGATGGTTG.

Isolation of Genomic DNA from M. thermoautotrophicum ΔH. Genomic DNA from *M. thermoautotrophicum ΔH* was isolated as described by Kiener et al. (1987).

Southern Blotting of Genomic DNA with α-I, β-I, and γ-I Oligonucleotide Probes. M. thermoautotrophicum ΔH genomic DNA (5 μg) was digested with a series of restriction enzymes for 2–3 h in the appropriate buffer (20 μL). DNA fragments from each digestion were separated by agarose gel electrophoresis and transferred to nitrocellulose by standard procedures (Smith & Summers, 1980; Southern, 1979) except the acid depurination step was omitted. Hybridization conditions were as follows: 40 °C for the α-I probe, 42 °C for the β-I and γ-I probes, and 54 °C for the *Sph*I/*Bam*HI 700-bp fragment of pB6-7. All filters were hybridized in a solution containing 5× SSCP, 5× Denhardt's solution, 2.5 mM EDTA (pH 8.0), 0.1% SDS, and 100 μg/mL sonicated salmon sperm DNA (500-bp lengths). Washing of the filters was done in 6× SSC/0.1% SDS solution at the hybridization temperature. (1× SSC is 150 mM NaCl and 15 mM $Na_2C_6H_6O_7$; 1× SSCP is 120 mM NaCl, 15 mM $Na_2C_6H_6O_7$, 10 mM Na_2HPO_4, and 10 mM NaH_2PO_4.)

Construction of the BamHI and KpnI Size-Selected Libraries. M. thermoautotrophicum ΔH DNA fragments (4–6 kb), produced from *Bam*HI digestion of genomic DNA, were isolated by electroelution from an agarose gel with an IBI Model UEA electroeluter. These fragments were then ligated into the *Bam*HI site of pUC18 (Yanisch-Perron et al., 1985) and transformed into *E. coli* DH5α. Recombinants were selected, restreaked onto plates, and lifted onto nitrocellulose, and the DNA was screened with end-labeled β-I probe under the hybridization conditions described above. Plasmids from clones showing a positive hybridization signal were isolated and digested with *Bam*HI, and the resulting fragments were separated by agarose gel electrophoresis. These fragments were transferred to nitrocellulose and screened for hybridization to both the β-I and γ-I probes. Clone pB6-7 was chosen for all further studies.

The construction of the *Kpn*I size-selected library used the same methodology as described above for the *Bam*HI size-selected library except that the electroeluted DNA fragments isolated (3.6–8.0 kb) were produced from the *Kpn*I digestion of genomic DNA. Clone pK6-1-4 was chosen for all further studies.

DNA Sequencing. DNA sequencing was by the dideoxy method (Sanger et al., 1977) using Sequenase. Problems

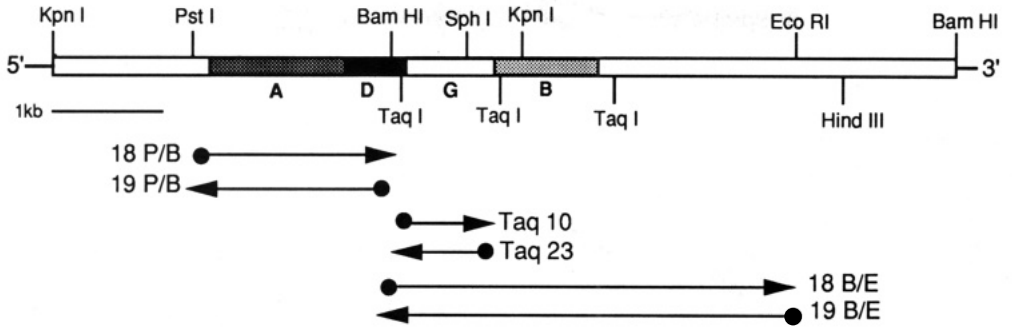

FIGURE 1: Restriction map of the total DNA cloned from *M. thermoautotrophicum* ΔH. The M13 clones constructed and sequenced are indicated below the map as solid dots with arrowed lines to indicate direction of sequencing.

related to secondary structure were circumvented by using dITP in place of dGTP (Mills & Kramer, 1979). Overlapping M13 clones were constructed by generating sets of nested deletions (Dale et al., 1985) with a Cyclone kit. The M13 clones constructed for sequencing are shown in Figure 1. *E. coli* XL1-Blue was used as the host of all M13 work. Gaps in the sequence and the region flanking the *Bam*HI site that is common to pK6-1-4 and pB6-7 were determined by using the sequencing primers described above.

Expression of the frhA, frhB, frhD, and frhG Genes. *Sac*I linkers were ligated onto the 1.4-kb *Dra*I–*Bam*HI fragment from pK6-1-4, which contained the *frh*A gene and part of *frh*D. The fragment was cloned into pT7-5 digested with *Sac*I and *Bam*HI to yield pT75LA12. A 3.6-kb *Bam*HI-*Hin*dIII fragment from pB6-7 was then cloned into *Bam*HI/*Hin*dIII-digested pT75LA-12 to give pT75LA12-15. Plasmids pT75LA12 and pT75LA12-15 were transformed into *E. coli* XL1-Blue carrying pGP1-4. Cells carrying both plasmids were grown, and the polypeptide products produced were labeled with [^{35}S]methionine as described by Tabor and Richardson (1985). Labeled polypeptides were detected by autoradiography after electrophoresis (12.5% SDS–polyacrylamide gel).

Results

Peptide Sequences. The N-terminal amino acid sequences of the α, β, and γ subunits had previously been determined (Fox et al., 1987) and were redetermined in this work to facilitate design of the DNA probes described under Materials and Methods. The following changes were indicated: cysteine to glycine at residue 3 and valine to arginine at residue 28 of the β subunit, and glycine to histidine at residue 13 of the γ subunit. Also, 16 additional residues, SGXTGDAMSLTEXTDI, were obtained for the γ subunit.

The following peptide sequences were obtained from endoproteinase Lys-C digestion of the α FRH subunit: α-LysC-2, RACSTIPLYDGRNVEVGPRAXMVEF, and α-LysC-4, APETAPVIVQRIXGVXPIPHTLA. The sequence β-3, HGYEQAGCKIEKDYVAE, was obtained from *Staphylococcus* V8 protease digestion of the β FRH subunit (X indicates an undetermined residue).

Cloning of the frhB, frhG, and frhA Genes Encoding the α, β, and γ Subunits of the F_{420} Reducing Hydrogenase from M. thermoautotrophicum ΔH. Oligonucleotide probes were designed on the basis of the N-terminal amino acid sequences of the α, β, and γ subunits of the FRH (this work; Fox et al., 1987). Inosine was used in places of degeneracy in the codons. It has been shown by Ohtsuka et al. (1985) that substitution of inosine in a degenerate position of a codon in oligonucleotide probes does not bias duplex formation. Therefore, this enables one to use longer regions of amino acid sequence to design oligonucleotide probes. This was important, as the N-terminal amino acid sequences for the three FRH subunits contain many amino acids that have highly degenerate codons.

Table I: Correlation of Experimental Protein Data from the DNA Sequence

gene	size (bp)	poly-peptide	predicted MW from DNA sequence	observed MW from SDS gel
*frh*A	1217	α	44.7	47
*frh*B	845	β	30.7	31
*frh*G	710	γ	25.7	26
*frh*D	476	δ	17.6	NA

Screening of *M. thermoautotrophicum* ΔH genomic DNA with the β-I and γ-I oligonucleotide probes led to the identification and subsequent cloning of the genes encoding the β and γ subunits, *frh*B and *frh*G, respectively, on a 4.8-kb *Bam*HI fragment. The gene encoding the α subunit, *frh*A, was located on an overlapping 4.2-kb *Kpn*I fragment by hybridization of a *Sph*I/*Bam*HI restriction fragment, containing *frh*G, and the α-I oligonucleotide probe. The region encoding the total cloned DNA is shown in Figure 1.

Correlation of Genes and Gene Products and Identification of frhD within the FRH Gene Cluster. The DNA sequence from the *Pst*I site to the 3′-most *Taq*I site in Figure 1 is shown in Figure 2. Analysis of the DNA sequence revealed three ORFs (*frh*A, *frh*B, and *frh*G) that encoded the known subunits (α, β, and γ) of the FRH, an assignment based on identification of experimentally determined protein sequences with the amino acid sequence predicted from translation of the DNA sequence (Figure 2). As shown in Table I, the molecular weights of the α, β, and γ subunits of the purified FRH, as determined by SDS–PAGE (Fox et al., 1987), correlate well with those predicted from translation of the DNA sequence. In addition, the amino acid composition is in agreement with earlier work by Fox et al. (1987). The DNA sequence showed the existence of a fourth ORF, *frh*D, that encoded a polypeptide that has not been found to copurify with the active FRH.

Features of the DNA Sequence. The four *frh* genes are preceded by sequences that conform to the consensus sequence for methanogen ribosome binding sites (Brown et al., 1989). Most methanogen protein-encoding genes begin with an ATG codon; however, there are examples of GTG initiation codons (Bokranz & Klein, 1987; Inatomi et al., 1989). Both *frh*D and *frh*B begin with ATG, while *frh*G uses GTG. The *frh*A gene apparently uses TTG as an initiation codon. The codon TTG is very rarely used for initiation in *E. coli* (Gren, 1984) and has not been previously reported as an initiation codon for a methanogen gene.

The region upstream of *frh*A is AT rich and contains two sequences, CTTATAAC beginning at nucleotide 230 and GTTATAAT beginning at nucleotide 249, that conform to the consensus methanogen promoter element TTTAWATA

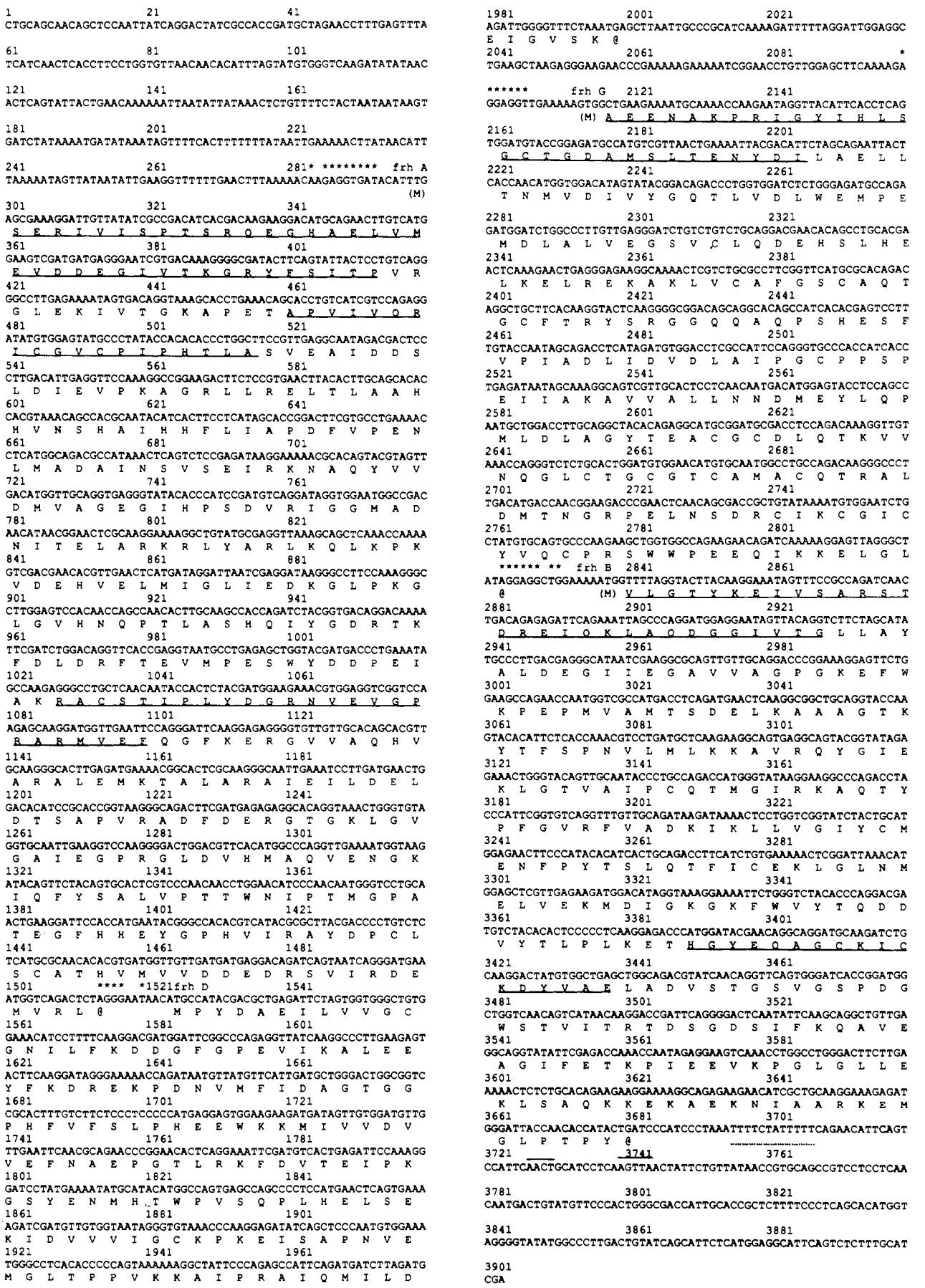

FIGURE 2: The sequence of the *frh* genes and gene products. Ribosome binding sites are indicated by asterisks. Experimentally determined protein sequences are underlined. The DNA sequence shown is 3903 bases long, comprising the DNA from the *Pst*I site to the 3′-most *Taq*I site in Figure 1. The potential transcription terminator is shown by the dotted line downstream of *frh*B.

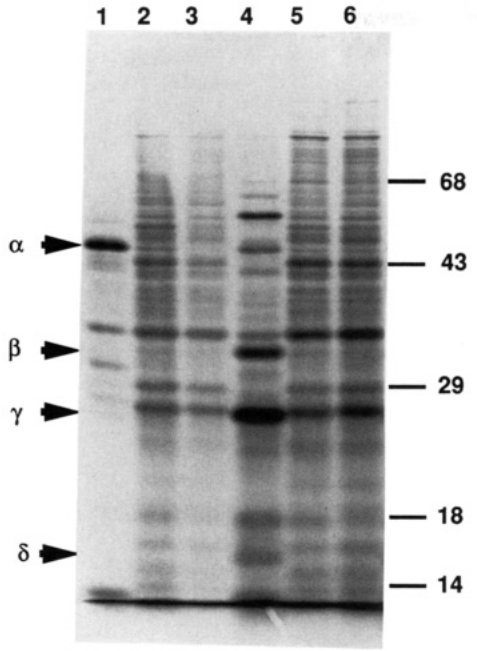

FIGURE 3: Autoradiogram (overnight exposure) of ^{35}S-labeled proteins produced from pT75LA12 (lanes 1–3) and pT75LA12-15 (lanes 4–6). Cells that are uninduced are shown in lanes 3 and 6. Cells that have been induced (heat-shocked) are shown in lanes 2 and 5. Cells that have been induced with rifampicin added are shown in lanes 1 and 4. The location of the labeled polypeptides produced from the *frh* genes are indicated by the arrows on the left of the figure. Molecular weight standards are indicated $\times 10^{-3}$.

(W = A or T) (Brown et al., 1989).[2] The sequence TTTTCTATTTTT located directly downstream of *frh*B (Figure 2) is similar to sequences implicated in transcription termination in methanogens (Brown et al., 1989).

Expression of the frh Genes in E. coli. Plasmids pT75LA12 and pT75LA12-15 were constructed to provide *frh*A and the entire operon, respectively, behind the T7 promoter. When these plasmids were transformed into *E. coli* XL1-Blue carrying the plasmid pGP1-4, which directs the synthesis of T7 RNA polymerase (S. Tabor, personal communication), polypeptide synthesis was detected by incorporation of [^{35}S]-methionine. By adding rifampicin to induced cells, the incorporation of the radiolabel could preferentially be directed into polypeptides whose genes are under transcriptional control of the T7 promoter (Tabor & Richardson, 1985). Proteins migrating with approximately the same electrophoretic mobility as the α, β, and γ subunits of purified FRH were observed (Figure 3, lane 4). In addition, a peptide was expressed at low levels that migrated with an electrophoretic mobility expected for the *frh*D gene product, although this does not prove it is derived from *frh*D. The *frh*A gene was not expressed well in this system and may have resulted from the utilization of the rare TTG initiation codon in *E. coli* and the poor ribosomal binding site preceding the gene.

Discussion

The genes encoding the F_{420} reducing hydrogenase are adjacent and, although as yet unproven, appear to form a single transcriptional unit organized as *frh*ADGB. The existence of *frh*D was a surprise, as protein purification studies have previously indicated the FRH from *M. thermoautotrophicum* ΔH to be a three-subunit enzyme (Fox et al., 1987). The product of *frh*D does not appear to be needed in vitro to produce an active hydrogenase, since the three-subunit FRH $(\alpha_1\beta_1\gamma_1)_8$ has a k_{cat}/K_m of 10^7 M^{-1} s^{-1} (Livingston et al., 1987). The *frh*D gene product has no obvious sequence similarities with the proteins in the NBRF-PIR database (version 20) or with the sequences of other hydrogenase subunits. The role of the *frh*D gene product at this point is not understood. However, it should be noted that the FRH isolated from another methanogen, *Methanococcus voltae*, is a four-subunit Ni-containing hydrogenase (Muth et al., 1987). Although no sequence data for the *M. voltae* FRH have been reported, perhaps the *frh*D gene product has an analogous function to one of the subunits of that protein and is easily dissociated upon purification of the *M. thermoautotrophicum* ΔH FRH.

The *frh*A-encoded polypeptide is clearly related to the large subunits of several other Ni-containing hydrogenases. An alignment of the *frh*A-encoded protein and the large subunit of the MV hydrogenase (Reeve et al., 1989), encoded by *mvh*A in *M. thermoautotrophicum* ΔH, is shown in Figure 4. It was expected that these two polypeptides would contain conserved sequences, since anti-α-FRH antibodies (Fox et al., 1987) have been observed to cross-react with the *mvh*A-encoded protein (Reeve et al., 1989). Such similar epitopes suggest common structural elements between the two large subunits of the Ni hdyrogenases in this organism. The amino acid sequences conserved between the *frh*A and *mvh*A gene products are also found in large subunits of Ni-containing hydrogenaes from several eubacteria [see Reeve et al. (1989) for discussion]. Blocks of conserved amino acid residues exist at both the amino and carboxy termini of all these eubacterial and archaebacterial polypeptides, which each contain a pair of cysteine residues (Cys_{62} and Cys_{65} at the N-terminus and Cys_{379} and Cys_{382} at the C-terminus of *frh*A) and several invariant histidine residues as well. These four conserved cysteine residues are prime candidates for the Ni active site. Data from Ni EXAFS on the FRH and MVH suggest that there are three to four thiol ligands to the Ni atom (Lindahl et al., 1984). Evidence for cysteine ligands to nickel have also been reported for the Ni–Fe hydrogenase of *Desulfovibrio gigas* (Scott et al., 1984), the Ni–Fe–Se hydrogenase of *Desulfovibrio baculatus* (Eidsness et al., 1989), and the Ni hydrogenase of *Chromatium vinosum* (Albracht et al., 1986). It should be noted that one of the cysteine residues in the "conserved" C-terminal pair is replaced by selenocysteine in the *D. baculatus* hydrogenase, and Eidsness et al. (1989) have reported EXAFS evidence for direct Se coordination to the Ni atom in this hydrogenase. Additionally, previous investigations from this group showed, by spin-echo EPR spectroscopy, evidence for a nitrogen atom 3.5 Å from the Ni atom of the F_{420} hydrogenase (Tan et al., 1984). This distance would be consistent with a distal N atom in an imidazole coordinated to Ni and could implicate one or more of the conserved histidine residues as being near the active site.

There are 12–13 Fe atoms/$\alpha_1\beta_1\gamma_1$ unit of the FRH (Fox et al., 1987) and both EPR and EXAFS data suggest the presence of 4Fe/4S clusters (Bastian et al., 1987). The *frh*G gene encodes the γ subunit of the FRH, which contains 16 cysteine residues. The C-terminal eight cysteine residues are arranged in a ferrodoxinlike sequence, suggestive of two 4Fe/4S clusters. The best overall similarity is with the ferrodoxin of *Clostridium thermosaccharolyticum* (Tanaka et al., 1971, 1973). However, as shown in Figure 5, there also exists a similarity with the ferrodoxin domains of *mvh*B, the gene encoding the polyferredoxin of the MVH (Reeve et al.,

[2] It has been noted in review that there are additional nucleotide sequences at 143, 195, and 210 that could potentially serve as promoter sequences.

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FRH A     SERIVISPTSRQEGHAELVMEVDDEGIVTKGRYFSITPVRGLEKIVTGKAPETAPVIVQRICGVCPIPHTLA72
            ** * P *R EGHA * * *DD G V   R * *   RG*EK** G** E AP IV RICG*C * H LA
MVH A     MVKLTMEPVTRIEGHAKITVRLDDAGNVEDTR-LHVMEFRGFEKFLQGRPIEEAPRIVPRICGICDVQHHLA71

FRH A     SVEAIDD--SLDIE--VPKAGRLLRELTLA-AHHVNSHAIH-HFLIAPDFVP--E----N---LMADAINSV129
          *  A*D   *** E  *P A ** RE* *  *  **SH**H  FL APDF**  E    N   ** DA ***
MVH A     AAKAVDACFGFEPEDVLPAAYKM-REI-MNWGSYMHSHGLHFYFLAAPDFIAGKDRKTRNVFQIIKDAPDAL141

FRH A     --SEIRKNAQYVVDMVAGEGIHPSDVRIGGMADNITELARK----------------------RLY-ARLKQL-170
             E*RKNA  *V   *G  IHP*    GG** ** * **K                        **   * *L
MVH A     QAIELRKNALELVRATGGRPIHPTSSTPGGISTELDDETQKDLLKKAQRNVELAEATLELAVPIFEENIIDLV214

FRH A     KPKVDEHVE-LMIGLIEDKGL--P-KGLGVH-NQPTLASHQIYGD-R-TKFDLDRFTE-VMP-ESW----Y--236
          ** ** **E    GL* D G*     G  V* **    ** ** *   * *  D **E V P  SW    Y
MVH A     NS-LG-NIETYHTGLVKD-GVWDVYDGI-VRIKD-K-EGN-MFREF-PADYA-DTIAEHVTPY-SWLKFPYIK278

FRH A     D-D-PE-IAKRACSTIPLYDGR-NV-E-V---GPRARMVE-FQGFKER-GVVAQ-----HVARALEMKTALA291
          D * P* *  R  S  PL  *R NV * *   *P*A*  E F* F*E  G  AQ*  * H AR L E*  LA
MVH A     DLGYPDGVY-RV-S--PL--SRLNVADKMPDAAPKAQ--EHFKEFRENFGY-AQQTLLYHWAR-LIEL--LA338

FRH A     -RAIEI-LDELDTSAPVRAD-F-D--ERGRGKLGVGAIEGPRG-LDVHMA-QVENGKI-QFYSALVPTT-WNI354
            A E   D L* *  * ** F D  ER *G  GVG *E*PRG L  H * * ENG I *  * *V*T   N
MVH A     CA-E-ECAADALE-GD-LSGEKFPDSLERQAGD-GVGIVEAPRGTLTHHYTCD-ENGLITK-ANIVVATIQ-NN403

FRH A     PTMGPATEGFHHEY---GPH----------VIRAYDPCLSCATHVMVVDDEDR--SV-I--RD-EMV-RL404
          P*M   *G   **Y   G *           VIRAYDPCLSCATH  * D * R  ** *   D **V R*
MVH A     PAMEMGIGKVAQDYIKPGVEVDDKIFNLMEMVIRAYDPCLSCATHT-I-DSQMRLATLEVYDSEGDLVKRI472
```

FIGURE 4: Alignment of the protein sequences encoded by *frh*A and *mvh*A. Conserved cysteine and histidine residues are shown in boldface type. An extended region of amino acid sequence conservation in the C-terminal region is also shown in boldface type. Asterisks show conservation of functional amino acids.

```
C. thermosaccharolyticum  AHIITDE-CISCGACAAECPVEA--IHEGTGKYEVDADTCIDCGACEAVCPTGAVKAE
R. rubrum                 AYKIEET-CISCGACAAECPVNA--IEQGDTIFVVNADTCIDCGNCANVCPVGAPVAE
FRH G                     TKVVNQGLCTGCGTCAMACQTRA--LDMTNGRPELNSDRCIKCGICYVQCPRSWWLEE
MVH B DOMAIN 3            GPIFIAD-CVGCGMCVPECPVDAITLSKVGGVIEIDEDTCIKCGVCAQTCPWNAVYIS
M. barkeri                ATVNADE-CSGCGTCVDECPNDAITLKEEKGIAVVDNDECVECGACEEACPNQAIKVE
```

FIGURE 5: Alignment of the C-terminal protein sequence of *frh*G, encoding the γ subunit, with the sequences of 4Fe/4S-containing ferredoxins from *C. thermosaccharolyticum* (Tanaka et al., 1971, 1973), *R. rubrum* (Matsubara et al., 1983), *mvh*B, the "polyferredoxin" (the *mvh*B gene encodes a protein with six repeating ferredoxin domains) of *M. thermoautotrophicum* ΔH (Reeve et al., 1989) and a 3Fe/3S ferredoxin of *M. barkeri* (Hausinger et al., 1982).

```
FRH B     VLGTYKEIVSARSTDREIQKLAQDGGIVTGLLAYALDEGIIEGAVVAGPGKEFWKPEPMVAMTSDELKAAA
          ** T ***  A*S*D EI   ** GG VT LL * L EGI*** *  *** **    P**   ****  AA
FDH B     MINT-NDMFYAKSSDAEIAEAGEYGGAVTTLLKFLLKEGIVDAVLAVDSSADLYDVVPILIEDPEDVVKAA

FRH B     GTKYTFSPNVLMLKKAVRQY--GIEKLG-TVAI-PCQTMGIRKAQTYPFGVRFVADKIKLLVGIYCMENFP
          G*   F*   L L K V *Y  G * *  *V** PC**M *          *  AD**. **VG* C    P
FDH B     GS-LHFG--TLNLAKVVTRYLDGAQDMKIAVTVKPCDAMTMVELMK-R-E-KVNADNV-IMVGLNCGGTMP

FRH B     YTSLQTFICEKL-GLNMELVEKMDIGKGKFWVYTQDDV-YTLPLKE--THGYE-QAGCKICKDYVAELADV
              *  * E *  ** * V K *I*KGK* V T*D*    *P* E   *G*  ** C* C   ** *AD*
FDH B     PVKGRQMM-EEFYEVDPDSVVKEEIAKGKLIVETEDGTEKEIPEDELEDEGFGRRTNCRRCEVNIPRMADL

FRH B     STG--SVGSP-DGWSTVITR-TDSGDSIFKQAVEAGIFE-TKPI---EEVKPGL-GLLEKLSAQKKEKAE-
          * G  *V *P  G *T I   *  G ** *A EAG***   PI    E**  * G * KL A*K *  *
FDH B     ACGNWGVIGPLAGKATFIEVCSPKGAEVLEKAKEAGVIDLEDPIPKGIEIREKIDGAMVKL-ADKWQGNDW

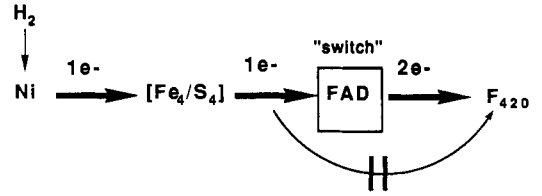
FRH B     KNIAARKEMGLPTPY280
           * A*R   ** T Y
FDH B     EDKAGREIFSVLTEYMDDFSRC296
```

FIGURE 6: Homology shared between residues 1–280 of the β subunit of F_{420} hydrogenase (FRH B) and residues 1–296 of the β subunit of formate dehydrogenase (FDH B). Conserved cysteine residues are in boldface type, and conserved functional amino acids are indicated by asterisks. These regions show 25.8% identity and 54% homology including conservative changes.

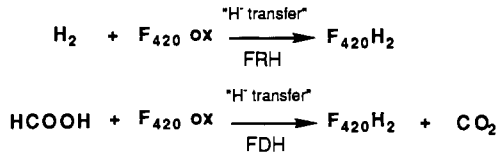
1989), a ferredoxin from *Rhodospirillum rubrum* (Matsubara et al., 1983), and a 3Fe/3S ferredoxin of *Methanosarcina barkeri* (Hausinger et al., 1982). The N-terminal part of the γ subunit of FRH also has regions conserved in the γ subunit of MVH and the small subunits of other eubacterial Ni-containing hydrogenases, including four conserved cysteine moieties (residues 17, 92, 112, and 142 in γ).

All three subunits of the FRH are necessary for F_{420} reduction; however, $\alpha\gamma$ complexes can reduce one-electron acceptor dyes such as methyl viologen (Livingston et al., 1987; Fox, 1984). This implicates the β subunit, or an intersubunit contact region involving β, as the site of F_{420} reduction, and

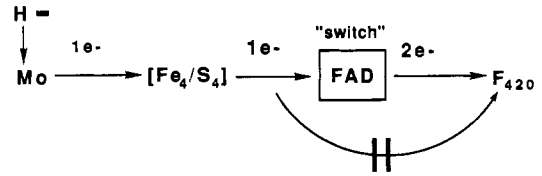
Scheme I



Scheme II



Scheme III



it is, therefore, expected to have a binding site for the F_{420} cosubstrate. In ground-state biochemistry, F_{420} appears to function as a two-electron redox coenzyme in strong analogy to NAD (Jacobson & Walsh, 1984; Walsh, 1986) and may accept a hydride equivalent from $FADH_2$ at the electron output side of the FRH. The subunit that recognizes F_{420} may, therefore, also contain FAD, although the β subunit does not contain a GXGXXG sequence found in many FAD-binding proteins (Wierenga & Hol, 1983).

A plausible route for electron transfer in the F_{420} hydrogenase would involve H_2 oxidation at the Ni site followed by single-electron transfer steps via Fe/S clusters to FAD as shown in Scheme I.

As FAD can mediate both one- and two-electron transfer processes, it could function as a redox "switch" that collects electrons singly from the metal centers of the FRH and passes them off as hydride equivalents to reduce the F_{420} cosubstrate. Intriguingly, the amino acid sequence of the β subunit of FRH shows 26% identity with the sequence of the β subunit of formate dehydrogenase (FDH) of *Methanobacterium formicicum*. If conservative substitutions are considered, the sequences show 54% identity (Figure 6). FDH oxidizes formate to CO_2 with concomitant reduction of a F_{420} cosubstrate by a net hydride transfer (Schauer & Ferry, 1982). The overall stoichiometry of FRH and FDH are similar (Scheme II). FDH is composed of two subunits; *fdh*A encodes the α subunit of 85K, and *fdh*B encodes the β subunit of 53K (Shuber et al., 1986). FDH contains a bound molybdopterin, FAD, 2 Zn atoms, and 21–24 Fe atoms as cofactors (Schauer & Ferry, 1986). There is also no consensus FAD-binding motif found in FDH. It could be envisioned that this protein transfers redox equivalents among the molybdenum, Fe/S, and FAD cofactors with molecular logic similar to that of the F_{420} hydrogenase (Scheme III). As both FRH and FDH reduce F_{420}, most likely mediated by bound $FADH_2$, this ability is probably reflected in the conserved amino acid sequences between the β subunits of these two proteins (Figure 6). Obtaining the sequences of several more FAD-, NAD-, or F_{420}-containing methanogenic proteins, and ultimately a crystal structure, may allow us to define a distinct, perhaps primordial nucleotide-binding site for FAD and F_{420} for these archaebacterial proteins.

The genes for each of the two multisubunit hydrogenases that enable the thermophilic archaebacterium *M. thermoautotrophicum* ΔH to live chemolithoautotrophically on H_2 as the sole oxidizable energy source have now been cloned and sequenced (this work; Reeve et al., 1989). Structural elements and candidates for ligands for the redox-active nickel and iron centers are identifiable by comparison between the two methanogen hydrogenases and to the significant number of other bacterial hydrogenase genes reported over the past two years. With heterologous expression systems for one or more of the α, β, γ, and δ subunits, reconstitution of one or more of the redox sites may be possible to confirm metal or organic coenzyme location within the multienzyme complex.

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Spontaneous Incorporation of Bacteriorhodopsin into Large Preformed Vesicles†

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ABSTRACT: Bacteriorhodopsin, either as purple membrane sheets or as detergent-solublized protein, was found to incorporate spontaneously into both large unilamellar vesicles (LUVs) and sized multilamellar vesicles (MLVs) under either gel-phase or liquid-phase conditions. These results were obtained with LUVs of various lipid compositions, including dimyristoylphosphatidylcholine (DMPC), DMPC and cholesterol, dioleoylphosphatidylcholine (DOPC), and DOPC and cholesterol. The lipid to protein (L/P) ratio of all proteoliposomes arising from these preformed vesicles continually increased in the presence of protein-free vesicles. Under fluid-phase conditions, the mixing of LUVs of DMPC with proteoliposomes showed vesicle growth independent of lipid concentration, suggesting that growth was due to lipid transfer. However, under gel-phase conditions a more rapid, concentration-dependent increase in the L/P ratio of the proteoliposome was observed, suggesting that growth occurred by a mechanism other than lipid transfer from vesicles to proteoliposomes. The use of the proteoliposome as an artificial lipid–protein membrane model is discussed.

Integral membrane proteins spontaneously insert into preformed small unilamellar vesicles (SUVs)[1] to form a variety of protein–lipid bilayer vesicles, designated proteoliposomes (Scotto & Zakim, 1985, 1986, 1988; Scotto et al., 1987). Several different integral membrane proteins have been incorporated by this procedure: these include bacteriorhodopsin, as crystalline arrays or detergent-solubilized monomers, UDP-glucuronosyltransferase, cytochrome oxidase (Scotto & Zakim, 1985, 1988), and the thrombospondin receptor (Berk et al.,

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; MLVs, multilamellar lipid vesicles; LUVs, large unilamellar vesicles; SUVs, small unilamellar lipid vesicles.

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