Substrate and Accessory Protein Requirements and Thermodynamics of Acetyl-CoA Synthesis and Cleavage in *Methanosarcina barkeri*†

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ABSTRACT: Enzymological studies on the multienzyme acetyl-CoA decarbonylase synthase (ACDS) complex from *Methanosarcina barkeri* have been conducted in order to identify and characterize physiologically relevant substrates and reactions in acetyl-CoA synthesis and decomposition in methanogens. Whereas previous investigations employed carbon monoxide as substrate and reducing agent for acetyl-CoA synthesis, we discovered that bicarbonate (or CO_2) acts as a highly efficient carbonyl group precursor substrate in the presence of either hydrogen or Ti^{3+}•EDTA as reducing agent. In reactions with Ti^{3+}•EDTA, synthesis of acetyl-CoA was strongly dependent on ferredoxin, and in reactions with H_2, dependence on ferredoxin was absolute. Two major hydrogenases were resolved from the enzyme complex preparation by HPLC gel filtration. One of these hydrogenases was shown to be active in reconstitution of acetyl-CoA synthesis in CO_2-containing reactions with H_2 as reducing agent. The hydrogenase active in reconstitution was capable of reducing ferredoxin, but was unreactive toward the 8-hydroxy-5-deazaflavin derivative coenzyme F_{420}. In contrast, the hydrogenase that did not reconstitute acetyl-CoA synthesis was reactive with F_{420} but was unable to reduce ferredoxin. Further experiments were performed in which the value of the equilibrium constant (K_{eq}) was determined for the reaction: $H_2 + CO_2 + CH_3\text{-}H_4SPt +$ CoASH ⇌ acetyl-CoA + $H_4SPt + H_2O$. where $CH_3\text{-}H_4SPt$ and H_4SPt stand for *N^5-methyl*-tetrahydrosarcinapterin and tetrahydrosarcinapterin, respectively. K_{eq} for this reaction was found to be $2.09 \times 10^6\ M^{-1}ATM_{H_2}{}^{-1}$ at 37 °C. Calculations of thermodynamic values for additional, related reactions were made and are discussed. The findings indicate that the hydrogen partial pressure is critical in determining whether net synthesis or cleavage of acetyl-CoA is favored. As partial pressures of H_2 drop below approximately 10^{-3} atm, acetyl-CoA synthesis becomes more and more unfavorable. The results support the theory that redox potential inside the cell or hydrogen availability may regulate carbon flow through the ACDS complex in methanogens.

Methanogens derive energy from the anaerobic oxidation of various substrates by different metabolic pathways that share in common the final production of methane as the mechanism for disposal of reducing equivalents. Although acetate is a major source of methane formed in nature, relatively few methanogens are capable of exclusive utilization of acetate as a source of both carbon and energy. However, most species of methanogens are capable of growth on a mixture of hydrogen and carbon dioxide. *Methanosarcina barkeri* can grow on a number of substrates including CO_2 plus H_2, acetate, methanol, and mono-, di-, and trimethylamine. The overall reactions for methane production from CO_2 plus H_2 and from acetate are shown in eqs 1 and 2.

$$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O \quad (1)$$

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \quad (2)$$

In the process of growth on CO_2 plus H_2, oxidation of hydrogen provides the electrons required for reduction of CO_2 to methane. In the conversion of acetate to methane, oxidation of the carbonyl group of acetate (after conversion to acetyl-CoA) provides the electrons needed for reduction of the methyl group to methane.

In methanogens, reversible cleavage and synthesis of acetyl-CoA are catalyzed by a multienzyme complex composed of five different subunits (Grahame, 1991, 1993; Ferry, 1992). Enzymatic activities of the complex include CO_2/CO oxidoreductase (sometimes termed CO dehydrogenase or CODH[1]), Co-β-methylcobamide/tetrahydropteridine methyltransferase, and acetyl-CoA synthase (Grahame, 1993). At this time, designation of a complete systematic name for the complex is not possible because all of the reactants and products of the individual partial reactions have not been identified. The enzyme complex has previously been designated as the CODH−corrinoid complex. Although use of this nomenclature has the benefit of denoting the separate corrinoid and CO_2/CO oxidoreductase subcomponents within

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[1] Abbreviations: CODH, carbon monoxide dehydrogenase; ACDS, acetyl-CoA decarbonylase synthase; H_4SPt, tetrahydrosarcinapterin; $CH_3\text{-}H_4SPt$, *N^5*-methyltetrahydrosarcinapterin; Fd, ferredoxin; HPLC, high-pressure liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; tricine, *N*-tris(hydroxymethyl)methylglycine; H_2ase-Fd, ferredoxin specific hydrogenase; F_{420}, 8-hydroxy-5-deazaflavin derivative (coenzyme F_{420}); H_2ase-F_{420}, coenzyme F_{420} specific hydrogenase.

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the complex, a drawback of its usage is that the redox reactions catalyzed do not result in substrate dehydrogenation. In addition, carbon monoxide is a substrate that may be physiologically irrelevant. For these reasons, the enzyme complex will here be referred to as the acetyl-CoA decarbonylase synthase complex (or ACDS complex). The name acetyl-CoA decarbonylase synthase is descriptive of the characteristic ability of the complex to bring about decomposition or synthesis of acetyl-CoA by insertion or excision of the acetyl-CoA carbonyl group.

The ACDS complex appears to be universal in methanogens, as it is found not only in acetate-utilizing organisms, *Methanosarcina* (Grahame, 1991, 1993; Ferry, 1992) and *Methanothrix* (S. H. Zinder and D. A. Grahame, unpublished results), but also in a methanogen that grows on single carbon units, *Methanococcus vannielii* (D. A. Grahame & E. DeMoll, unpublished results). Presumably, the ACDS complex is used for synthesis of acetyl-CoA by methanogens growing on one-carbon compounds. However, in methanogens growing with acetate as sole carbon and energy source, the enzyme complex is used to produce C_1 units from acetyl-CoA for introduction into the methane biosynthetic pathway. Methanogens growing on acetate have increased levels (approximately 5% of total protein) of the enzyme complex over methanogens growing on C_1 units (Krzycki et al., 1982; Krzycki & Zeikus, 1984). Apparently, this high level of the enzyme is needed in order to generate a flux of C_1 units sufficient to insure an adequate level of methane synthesis.

Previous studies showed that both the isolated methanogen CO_2/CO oxidoreductase component and the intact complex catalyze the transfer of electrons from carbon monoxide to ferredoxin (Grahame & Stadtman, 1987; DeMoll et al., 1987; Terlesky & Ferry, 1988). Fischer and Thauer (1990) demonstrated with soluble, crude extracts of *M. barkeri* that ferredoxin is involved in the transfer of electrons in acetate decomposition to methane. Somewhat later it was confirmed that electrons derived during cleavage of acetyl-CoA by the isolated ACDS complex enter into reduction of iron−sulfur centers in both added ferredoxin and the complex itself (Grahame, 1991). In the overall synthesis of acetyl-CoA, the methyl group of acetyl-CoA is derived from *N*^{5}*-methyl*-tetrahydrosarcinapterin (CH_3-H_4SPt) (Grahame, 1993). Carbon monoxide is capable of serving as a precursor for the carbonyl moiety of acetyl-CoA; however, CO is a substrate that may be neither physiologically nor environmentally relevant to acetate synthesis and decomposition by methanogens. Pezacka and Wood (1984) showed that acetyl-CoA synthesis from CO_2 and H_2 could occur in a system derived from *Clostridium thermoaceticum*. However, the protein fraction employed was unresolved, and it was unexplained why ATP was required by this system.

Herein the reversible synthesis of acetyl-CoA is demonstrated from physiologically important substrates, CO_2, H_2, CH_3-H_4SPt, CoA, and ferredoxin (Fd). All of the substrates and accessory proteins required for both synthesis and cleavage of acetyl-CoA in *M. barkeri* were isolated and are identified. Under *in vivo* conditions, the overall acetyl-CoA synthesis/cleavage reaction (eq 3) is indicated to be composed of two physiologically relevant partial reactions—one catalyzed by a ferredoxin specific hydrogenase (eq 4) and the other brought about by the ACDS complex (eq 5).

$$H_2 + CO_2 + CH_3\text{-}H_4SPt + CoASH \rightleftharpoons \text{acetyl-CoA} + H_4SPt + H_2O \quad (3)$$

$$H_2 + 2\ Fd_{ox}(Fe^{3+}) \rightleftharpoons 2\ Fd_{red}(Fe^{2+}) + 2\ H^+ \quad (4)$$

$$CO_2 + 2\ Fd_{red} + CH_3\text{-}H_4SPt + CoASH + 2\ H^+ \rightleftharpoons \text{acetyl-CoA} + H_4SPt + 2\ Fd_{ox} + H_2O \quad (5)$$

Reaction 5 in itself comprises several interesting partial reactions including CH_3-H_4SPt:Co(I)cobamide transmethylation, CO_2:carbonyl oxidation/reduction, and acetyl-CoA carbonylation/decarbonylation. Herein we also present the results of thermodynamic studies on the overall process of acetyl-CoA synthesis (eq 3). Implications of the thermodynamic parameters are discussed in relation to variability in carbon and energy utilization by methanogens with different lifestyles for carbon and energy metabolism.

MATERIALS AND METHODS

Reagents. Coenzyme A, disodium salt (>96%, HPLC), and acetyl-CoA, trilithium salt (>95%, HPLC), were purchased from Fluka Chemical Corp. *N*^{5}*-Methyl*-tetrahydrosarcinapterin (CH_3-H_4SPt) was prepared from tetrahydrosarcinapterin (H_4SPt) as described (Grahame, 1991). Ferredoxin was purified from *M. barkeri* by gel filtration and anion exchange chromatography according to the protocol given previously (Grahame, 1991). Unless otherwise stated, all other chemicals were commercial products of analytical reagent grade. All gases were humidified and further purified by washing with an aqueous solution of reduced methylviologen as described previously (Grahame, 1993).

Anaerobic stock 0.50 M bicarbonate was prepared by addition of sodium bicarbonate powder to argon-sparged water inside a Coy anaerobic chamber. Sparging was discontinued after addition of the powder. The carbon dioxide concentration produced by addition of bicarbonate to reaction mixtures of specified pH was calculated by use of the Henderson−Hasselbalch equation as follows:

$$\text{pH(reaction mixture)} = 6.1 + \log \frac{[HCO_3^-]}{[CO_2]}$$

with the requirement that the total bicarbonate added must equal the sum of the final concentrations of CO_2 and HCO_3^- produced by dissociation. Thus, reaction mixtures buffered at pH 8.0 to which 10 mM $NaHCO_3$ was added are calculated to contain 124 μM CO_2. Spectrophotometric measurements were made with a Hewlett-Packard 8452A diode array spectrophotometer installed inside a Coy anaerobic chamber. Unless otherwise noted, all reactions and final preparations of reagents were completed inside the anaerobic chamber.

Acetyl-CoA Decarbonylase Synthase Complex. The ACDS enzyme complex was isolated from acetate-grown cells of *M. barkeri* by anaerobic gel filtration on Sepharose 6B-CL, as previously described (Grahame, 1991). Further purification was carried out by stepwise elution from phenyl-Sepharose 4B. Details of the methodology and results of phenyl-Sepharose purification were as described in Grahame, (1993). Preparations were stored frozen in a liquid N_2

storage dewar. Protein was assayed by the method of Bradford (1976), by employing the dye reagent supplied by Bio-Rad Laboratories, Inc., with bovine γ-globulin as standard.

Analytical Superose 6 Gel Filtration. Analytical gel filtration was carried out at approximately 23 °C under strictly anaerobic conditions inside a Coy anaerobic chamber by use of a column of Superose 6 HR 10/30 (Pharmacia). The column (10 mm × approximately 300 mm) was equilibrated with a solution saturated with 100% H_2, containing 50 mM Na_2SO_4 and 50 mM sodium 3-(*N*-morpholino)-propanesulfonate (MOPS), pH 7.2, "equilibration solution". A sample of the enzyme complex was removed from storage, transferred into the anaerobic chamber, and thawed therein under hydrogen. An aliquot of the enzyme complex (120 μL) was mixed with an equal volume of H_2-saturated equilibration solution, and the entire mixture (containing approximately 6.4 nmol of cobamide) was injected onto the column. Elution was carried out at a flow rate of approximately 0.48 mL/min. Spectrophotometric monitoring of the effluent was accomplished by use of an 80 μL, flow-through, quartz spectrophotometer cell attached directly to the column outlet tubing. Fractions were collected at intervals (1 or 2 min) beginning 10 min after injection.

Hydrogenase. Assay of benzylviologen-reducing hydrogenase was carried out at 24 °C by addition of an aliquot of the enzyme sample to be measured (5−40 μL) to 1.00 mL of a 100% H_2-saturated solution containing 50 mM MOPS and 1.0 mM benzylviologen, pH 7.2. The increase in absorbance at 578 nm was converted to units of activity by use of the molar absorptivity of reduced benzylviologen, 1.1 × 10^4 M^{-1} cm^{-1} (Axley et al., 1990). One unit (1 U) of activity is defined as the amount of enzyme required for reduction of 1 μmol of benzylviologen/min under the conditions of assay.

Synthesis of Acetyl-CoA from CoASH, CH_3-H_4SPt, and CO_2. Protein samples were analyzed for catalytic activity in acetyl-CoA synthesis in reaction mixtures containing substrates CO_2 (from bicarbonate), CH_3-H_4SPt, and coenzyme A. The reactions were carried out in a stoppered semimicrocuvette at 37 °C, pH 8.0, in a mixture (600 μL) saturated with hydrogen containing 200 μM coenzyme A, 250 μM CO_2 (from 20 mM $NaHCO_3$ added), 75 μM CH_3-H_4SPt, 6.2 μM ferredoxin, 0.1 M tricine·HCl, and a suitable amount of the enzyme complex for analysis. All components except for the enzyme complex and sodium bicarbonate were added to the cuvette and bubbled with 100% H_2 for 5−8 min by use of a 25 gauge syringe needle. At this point the enzyme was added to the cuvette, which was then stoppered (by use of the Hungate technique) and placed in the cell compartment of the spectrophotometer maintained at 36−37 °C. After temperature equilibration for 5 min, the reaction was initiated by addition of the stock sodium bicarbonate. Acetyl-CoA synthesis was monitored based on measurement of the concomitant demethylation of CH_3-H_4SPt. Reaction mixtures containing Ti^{3+}·EDTA at a final concentration of 1.0 mM were prepared by addition of a stock solution composed of 64 mM $TiCl_3$, 129 mM EDTA, and 0.32 M Tris·HCl, pH 8.0. The demethylation of CH_3-H_4SPt to form H_4SPt was measured by the increase in absorbance at 312 nm, with the value of 3600 M^{-1} cm^{-1} used as the difference in molar absorptivity (Grahame, 1991).

Equilibrium Measurements of Acetyl-CoA Synthesis/Cleavage. Four separate reactions (A−D) were carried out, each at 37 °C in a stoppered semimicrocuvette containing 164 μM CoASH, approximately 120 μM CH_3-H_4SPt, 10 mM $NaHCO_3$, H_2 (0.933 atm), hydrogenase, 6.2 μM ferredoxin, ACDS complex (1.7 μM cobamide), and 0.1 M tricine, pH 8.0. Acetyl-CoA was added in initial concentrations (μM) as follows: A, 0; B, 154; C, 617; D, 2467. Reactions were carried out as described above for acetyl-CoA synthesis. The partial pressure of H_2 was calculated to be 0.933 atm. This value takes into account the contributions from the vapor pressure of H_2O (0.062 atm) and CO_2 (0.005 atm). Reactions were allowed to proceed until equilibrium was attained, as judged by the lack of further change in A_{312}. That equilibrium had indeed been reached was also supported by the final results, in that all four measurements, although requiring different lengths of time to arrive at equilibrium, nevertheless yielded approximately the same value for the K_{eq}. At equilibrium aliquots of the reaction mixtures were frozen in liquid N_2. The aliquots were subsequently thawed and quantitatively analyzed for CoASH, acetyl-CoA, H_4SPt, and CH_3-H_4SPt as described under HPLC Analysis of Components of Acetyl-CoA Synthesis and Cleavage. All manipulations were carried out anaerobically.

HPLC Analysis of Components of Acetyl-CoA Synthesis and Cleavage. Quantitative reversed phase HPLC analysis was performed under anaerobic conditions as described previously (Grahame, 1991) with the following modifications. Aliquots from reaction mixtures were thawed under nitrogen by addition of an equal volume of an anaerobic solution containing 0.5 M sodium citrate and 20 mM 2-mercaptoethanesulfonate, pH 4.0. Heating and filtration steps were omitted, and sample injection was performed immediately upon thawing. No adjustment of pH was made on aqueous KH_2PO_4 used in HPLC solvents. Dual wavelength recording at 300 and 260 nm was employed to enable quantification of all four reaction products/substrates (CoASH, acetyl-CoA, H_4SPt, and CH_3-H_4SPt) in each chromatographic run, as described earlier [Figure 6 in Grahame (1991)].

Midpoint Potential of the CO_2/CO Couple. The value of −499 mV was used as the midpoint potential for the CO_2/CO couple at pH 6.0 and 25 °C, as determined previously (Grahame, 1993).[2] This is equivalent to a potential of −558 mV at pH 7.0 (standard biological conditions) and −617 mV at pH 8.0. The half-reaction 6 is

$$CO_2 + 2\,H^+ + 2\,e^- \rightleftharpoons CO + H_2O \qquad (6)$$

The value for E_o of −558 mV determined experimentally may be compared with the value of −524 mV calculated by Thauer (1990).

RESULTS

It was found previously that carbon monoxide would function as precursor of the carbonyl moiety of acetyl-CoA in a ferredoxin-independent reaction catalyzed by the enzyme

[2] By similar methods as used previously (Grahame, 1993), redox potential measurements on the CO_2/CO couple were also made at pH 7.2 with both platinum and graphite indicator electrodes (Grahame, unpublished results). The midpoint reduction potential at pH 7.2 was found to be approximately −560 to −570 mV (graphite).

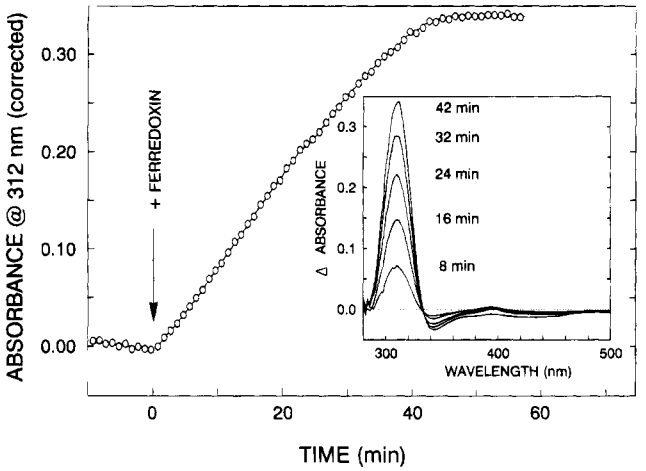


FIGURE 1: Ferredoxin requirement for acetyl-CoA synthesis with CO_2 as carbonyl group precursor. The acetyl-CoA synthesis reaction was catalyzed by the *M. barkeri* acetyl-CoA decarbonylase complex. Reaction mixtures contained the enzyme complex (1.6 μM cobamide), a trace of H_2ase-Fd in the ACDS preparation, 100 μM CH_3-H_4SPt as methyl group donor, 164 μM coenzyme A, and 124 μM CO_2 (from 10 mM sodium bicarbonate). The reaction mixture, 600 μL, was buffered at pH 8.0 with 50 mM tricine and saturated with hydrogen at 37 °C. Demethylation of CH_3-H_4SPt was monitored by absorbance change at 312 nm. At the time indicated by the arrow, purified *M. barkeri* ferredoxin (6.2 μM) was added. Plotted data have been corrected for dilution and background absorbance due to ferredoxin. The inset shows the difference spectra that resulted at times indicated following the addition of ferredoxin.

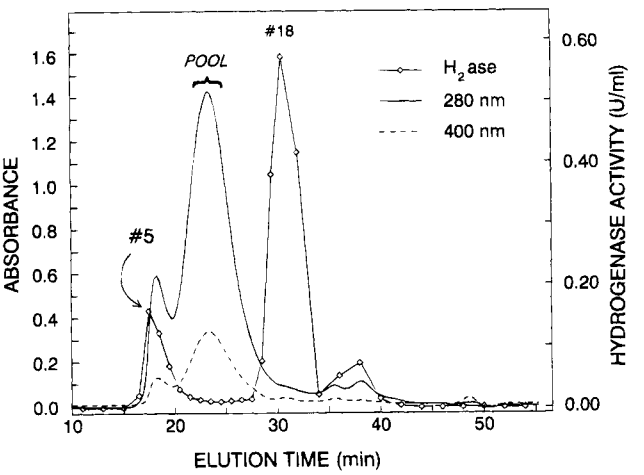


FIGURE 2: Resolution of hydrogenase activity from the acetyl-CoA decarbonylase complex by analytical HPLC gel filtration. Analytical HPLC gel filtration was carried out under anaerobic conditions on a column of Superose 6 HR 10/30 (Pharmacia) by use of a hydrogen-saturated eluant as described under Materials and Methods. Assay of hydrogenase (benzylviologen-reducing) and spectral monitoring of the effluent were carried out as described under Materials and Methods. Hydrogenase activities measured on the fractions are plotted at elution time points corresponding to the average time of collection. Four fractions were subsequently combined in the region designated "*POOL*", for later analysis of acetyl-CoA synthetic activity.

complex (Grahame, 1993). However, since CO is not an important compound in the environment or metabolism of methanogens, methods were developed to replace CO with substrates bearing physiological significance. A reaction mixture was constructed under an atmosphere of H_2 containing a sample of the enzyme complex and all of the components needed for acetyl-CoA synthesis, except that 10 mM sodium bicarbonate (equivalent to 124 μM CO_2) was added instead of carbon monoxide. As shown in Figure 1, efficient synthesis of acetyl-CoA was not observed in this mixture by itself. However, acetyl-CoA synthesis commenced promptly upon addition of ferredoxin (6.2 μM). Acetyl-CoA synthesis proceeded at a constant rate of 1.46 nmol/min for about 30 min. In contrast to the abrupt cessation previously observed in reaction with carbon monoxide (Grahame, 1993), the reaction rate then decreased gradually. After approximately 45 min the reaction appeared to have ceased, since no further demethylation of CH_3-H_4-SPt could be detected.

In an otherwise identical reaction carried out under an atmosphere of 100% argon, synthesis of acetyl-CoA could not be detected. This finding suggested that hydrogen acts to reduce ferredoxin via hydrogenase activity present in the ACDS complex preparation.[3] Reduced ferredoxin would then serve as an electron donor to the enzyme complex in order to bring about reduction of CO_2 to the redox level of the carbonyl group in acetyl-CoA. To test this hypothesis, further purification of the enzyme complex by HPLC gel filtration was undertaken to attempt complete removal of hydrogenase activity.

Analytical HPLC gel filtration of the ACDS complex on Superose 6 resulted in elution of the enzyme as the major peak of absorbance at both 280 and 400 nm (shown in Figure 2). Residual hydrogenase activity of the enzyme complex preparation was resolved into two well-defined peaks. The two peaks of hydrogenase eluted at positions that were also distinct from that of the enzyme complex. Several fractions that were very high in concentration of the enzyme complex were also essentially devoid of hydrogenase activity. Four of these fractions, in the region marked "*POOL*" in Figure 2, were combined for subsequent analyses of acetyl-CoA synthesis and dependence of the reaction on hydrogenase. The results of these analyses are shown in Figure 3.

In the absence of added hydrogenase, acetyl-CoA synthesis by the pooled fractions containing the enzyme complex was extremely slow. The product versus time profile of the reaction without added hydrogenase (not shown) was virtually identical to the curve obtained when hydrogenase from the second peak from HPLC gel filtration was added (Figure 3, "*POOL* + #18"). In contrast, addition of hydrogenase from the first eluted peak resulted in active synthesis of acetyl-CoA, as shown in Figure 3, "*POOL* + #5". In the presence of higher levels of hydrogenase, a further increase in the rate of acetyl-CoA synthesis was observed (Figure 3, "*POOL* + BULK H_2ase"). The data indicated that the process of hydrogen- and ferredoxin-dependent synthesis of acetyl-CoA with CO_2 as carbonyl group precursor was also dependent on hydrogenase. The results also indicated that specificity exists for hydrogenase(s) capable of efficient reduction of ferredoxin. To test this hypothesis, separate reaction mixtures were constructed to assay for the ability

[3] Hydrogenase activity (capable of reduction of both benzylviologen and ferredoxin) has been previously detected in the ACDS complex preparation (Grahame, 1993). As isolated by gel filtration on Sepharose 6B (Grahame, 1991), the ACDS complex preparation contained approximately 0.7% of the total hydrogenase activity (benzylviologen-reducing) recovered from the column. No peak of hydrogenase could be discerned at the elution position of the enzyme complex. Instead the hydrogenase activity profile reached a minimum in this region (data not shown). Further purification of the enzyme complex on phenyl-Sepharose, as described in Grahame (1993), removed a substantial amount but did not completely eliminate residual hydrogenase activity.

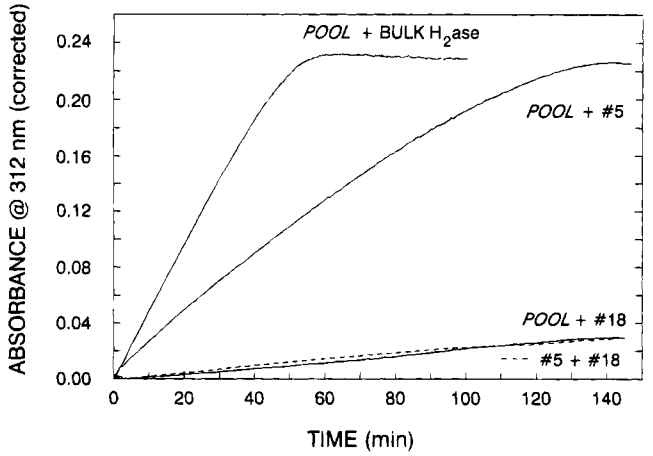


FIGURE 3: Hydrogenase requirement for acetyl-CoA synthesis with CO_2 as carbonyl group precursor. Analysis of acetyl-CoA synthesis was conducted as described under Materials and Methods. Samples of the enzyme complex (90 μg of protein) taken from the pooled, major peak fractions from HPLC gel filtration (in the region marked "*POOL*" in Figure 2) were used in test reactions of acetyl-CoA synthetic activity. The reaction mixtures were supplemented with samples of the indicated hydrogenase fractions obtained from HPLC gel filtration (Figure 2). The amounts of hydrogenase added to reaction mixtures from fractions 5 and 18 were 0.016 and 0.029 unit, respectively. The hydrogenase addition indicated as "BULK H_2ase" consisted of 28.3 units from a major, well-defined peak that contained the bulk of the soluble benzylviologen-reducing hydrogenase recovered during initial fractionation of a crude *M. barkeri* extract by low-pressure gel filtration as described in Grahame (1991) (hydrogenase assay data not shown). The time trace marked "#5 + #18" was produced by a reaction mixture that contained both hydrogenase fractions 5 and 18 (0.016 and 0.029 unit, respectively) but none of the pooled ACDS complex.

of hydrogenase samples to catalyze direct reduction of ferredoxin. Spectrophotometric measurements on reactions carried out in the presence of hydrogen confirmed that hydrogenase activity in the first eluted peak (fraction no. 5) did catalyze the reduction of ferredoxin. In contrast, the second hydrogenase peak (fraction no. 18), although significantly more active than the first in catalyzing the reduction of benzylviologen, was almost completely inactive in catalyzing the reduction of ferredoxin. Furthermore, when tested for reduction of the 8-hydroxy-5-deazaflavin derivative F_{420} from *M. vannielii*, the second hydrogenase peak was the only fraction found to be active. Thus, synthesis of acetyl-CoA was dependent on a non-F_{420}-reactive hydrogenase specific for reduction of ferredoxin.

In order to characterize further the dependence of acetyl-CoA synthesis on reduced ferredoxin, chemical reduction of ferredoxin with Ti^{3+}•EDTA was tested as a means for providing reducing equivalents needed for carbonyl group incorporation from CO_2. Direct reduction of enzyme Fe-S and corrinoid centers by Ti^{3+}•EDTA (1 mM Ti^{3+} and 1.7 μM enzyme cobamide) occurred over a time course of tens of minutes. In contrast, reduction of 6 μM ferredoxin by Ti^{3+}•EDTA (1 mM Ti^{3+}) occurred within seconds (data not shown). Since the enzyme complex and ferredoxin are widely separated during the initial steps of purification by gel filtration, it is unlikely that the slow reduction of the Fe-S and corrinoid centers in the enzyme complex by Ti^{3+} is mediated by residual ferredoxin in the enzyme preparation.

As shown in Table 1, with CO_2 as precursor of the carbonyl group and Ti^{3+}•EDTA as reductant, addition of 6 μM ferredoxin increased the rate of acetyl-CoA synthesis

Table 1: Rate of Acetyl-CoA Synthesis with Various Substrates and Reducing Agents[a]

carbonyl group precursor substrate[b]	reducing agent[c]	turnover[d] $(min)^{-1}$
CO	CO	33.8
CO_2	H_2	0.0
CO_2	H_2 + ferredoxin (6 μM)	4.9
CO_2	Ti^{3+}	3.1
CO_2	Ti^{3+} + ferredoxin (6 μM)	60.2
CO_2	Ti^{3+} + ferredoxin (12 μM)	70.4

[a] The ACDS preparation contained a trace amount of H_2ase-Fd. [b] CO_2 was added as bicarbonate, and CO was introduced into reactions as CO-saturated water (Grahame, 1991). [c] Saturating H_2 in all but the reaction with CO. [d] Acetyl-CoA formed, mol/mol of enzyme cobamide (reaction mixtures contained 0.24 nmol of enzyme cobamide).

Table 2: Product/Reactant Ratios in Acetyl-CoA Synthesis Reactions at Equilibrium

	[product]/[reactant] ratio[b]		equilibruim expression ratio
reaction[a]	$\frac{[\text{acetyl-CoA}]}{[\text{CoASH}]}$	$\frac{[CH_3\text{-}H_4SPt]}{[H_4SPt]}$	$\frac{[\text{CoASH}][CH_3\text{-}H_4SPt]}{[\text{acetyl-CoA}][H_4SPt]}$
A	4.86	50.9	247
B	6.24	37.6	235
C	7.64	32.9	251
D	12.0	19.6	236

[a] Acetyl-CoA synthesis reactions A−D were performed starting with different initial levels of acetyl-CoA as described under Materials and Methods. The ACDS preparation contained a trace amount of H_2ase-Fd. [b] Product/reactant ratios were calculated at equlibrium as described under Materials and Methods.

by about 20 times. Addition of twice this level of ferredoxin raised the turnover rate about 17% further. In the presence of 1 mM Ti^{3+}, these concentrations of ferredoxin are essentially completely reduced.[4] Therefore, the results indicated that levels of reduced ferredoxin approach saturation at the ratio of around 50 mol of reduced ferredoxin/mol of enzyme cobamide. Turnover rates under these conditions were about 2 times higher than observed with carbon monoxide alone. These data indicated that reduced ferredoxin functioned very efficiently as a substrate to provide immediate electron donation to the ACDS complex for carbon dioxide incorporation into the carbonyl group of acetyl-CoA. The results contribute further evidence that ferredoxin serves *in vivo* as the proximal electron carrier for synthesis and cleavage of acetyl-CoA.

Determination of the K_{eq} for Acetyl-CoA Synthesis and Cleavage (Eq 3). Reactions were established and allowed to proceed until no further change in CH_3-H_4SPt concentration (A_{312}) was observed. The micromolar quantities of CoASH, acetyl-CoA, H_4SPt, and CH_3-H_4SPt were then measured directly by HPLC analysis as described under Materials and Methods. Product/reactant ratios [acetyl-CoA]/[CoASH] and [H_4SPt]/[CH_3-H_4SPt] were obtained and are presented in Table 2. Under conditions in which the initial concentration of acetyl-CoA was varied from 0 to 2467 μM, the resulting final ratios of [acetyl-CoA]/[CoASH] and [H_4SPt]/

[4] On the basis of the values of the redox midpoint potentials of $\text{ferredoxin}_{ox/red}$ and $Ti^{4+/3+}$•EDTA couples, we calculate that addition of 1 mM Ti^{3+}•EDTA to 6 μM ferredoxin causes 99.94% reduction of ferredoxin, whereas under reaction conditions with saturating H_2 at pH 8.0 ferredoxin is about 88.8% reduced.

[CH_3-H_4SPt] were found to vary by factors of about 2.5 and $^1/_{2.5}$ respectively. However, as shown in Table 2, the equilibrium product ratio

$$\frac{[\text{acetyl-CoA}][H_4\text{SPt}]}{[CH_3\text{-}H_4\text{SPt}][\text{CoASH}]}$$

value was independent of initial product concentration. The average value of this ratio was 242 with a relative error of 2.9%. As indicated under Materials and Methods, the partial pressure of H_2 was maintained at 0.933 atm and the concentration of CO_2 was determined to be 124 μM. Substituting these values into the equilibrium eq 7:

$$K_{eq} = \frac{[\text{acetyl-CoA}][H_4\text{SPt}]}{[H_2][CO_2][CH_3\text{-}H_4\text{SPt}][\text{CoASH}]} \quad (7)$$

yields a K_{eq} of 2.09×10^6 M^{-1} $ATM_{H_2}^{-1}$ with [CO_2] in molar units and [H_2] in atm, at 37 °C.

Midpoint Potential of the Oxidizing Half-Reaction in the Synthesis of Acetyl-CoA. The standard free energy change in the synthesis of acetyl-CoA (reaction 3) is calculated from the value of K_{eq} according to eq 8.

$$\Delta G^\circ = -RT \ln K_{eq} \quad (8)$$

Therefore, ΔG° is −8.96 kcal/mol (−37.5 kJ/mol). Knowledge of the standard free energy change allows us to calculate ΔE_o according to eq 9.

$$\Delta G^\circ = -n\mathcal{F}\Delta E_o \quad (9)$$

Consequently, the value of ΔE_o for reaction 3 is 194 mV. Since the midpoint potential of the reducing half-reaction 10 in the synthesis of acetyl-CoA is −474 mV at pH 8.0

$$2\,H^+ + 2\,e^- \rightleftharpoons H_2 \qquad E_o = -474 \text{ mV at pH } 8.0 \quad (10)$$

E_o for the oxidizing half-reaction 11 is given by ΔE_o of the complete reaction (194 mV, reaction 3) minus E_o of the reducing half-reaction. Thus, the E_o value obtained for the oxidizing half-reaction 11 is −280 mV.

$$2\,H^+ + 2\,e^- + CO_2 + CH_3\text{-}H_4\text{SPt} + \text{CoASH} \rightleftharpoons \text{acetyl-CoA} + H_4\text{SPt} + H_2O \quad (11)$$

At pH 7.0, where E_o' for reaction 10 is −414 mV, E_o' for reaction 11 becomes −220 mV. When our calculation is done with CO_2 expressed in atm units, we obtain a value for E_o' of approximately −270 mV, which may be compared with the value (−200 mV) calculated by a method that employed other standard free energy data (Thauer, 1990).

Determination of K_{eq} for the Synthesis of Acetyl-CoA with CO as Carbonyl and Electron Donor. Acetyl-CoA synthesis with CO as the carbonyl group donor (reaction 12) is composed of the oxidizing half-reaction 11, $E_o = -280$ mV (pH 8.0), and the reducing half-reaction 6 with $E_o = -617$ mV (pH 8.0). Thus, the overall ΔE_o of eq 12 is found to be 337 mV. (Although E_o values of the individual half-reactions are dependent on pH, ΔE_o of eq 12 is pH independent.) From eq 9 a value of −15.5 kcal/mol (−65.0 kJ/mol) for ΔG° is obtained. This yields a K_{eq} of 9.09×10^{10} M^{-1} for the net synthesis reaction 12 at 37 °C.

$$CO + CH_3\text{-}H_4\text{SPt} + \text{CoASH} \rightleftharpoons \text{acetyl-CoA} + H_4\text{SPt} + H_2O \quad (12)$$

The large magnitude of K_{eq} indicates that when acetyl-CoA synthesis is carried out with CO as carbonyl source and reducing agent, the reaction is essentially irreversible.

DISCUSSION

Acetyl-CoA Synthesis from Physiologically Relevant Substrates. The acetyl-CoA decarbonylase synthase enzyme complex from *M. barkeri* was shown to utilize carbon dioxide as an efficient carbonyl group precursor for acetyl-CoA synthesis. In reactions with CO_2 as substrate, reduced ferredoxin was required as the proximal source of reducing equivalents for reduction of CO_2 to the redox level of the carbonyl group in acetyl-CoA. Although it had been previously demonstrated that the enzyme complex was competent to incorporate carbon monoxide in ferredoxin-independent synthesis of acetyl-CoA, the results presented here demonstrate acetyl-CoA synthesis from substrates that all have physiological significance.

As a reducing agent, reduced ferredoxin from *M. barkeri* [$E_o(Fd_{ox}/Fd_{red}) = -420$ mV (Grahame, 1991)] is substantially less powerful than carbon monoxide [$E_o(CO_2/CO) = -558$ mV, pH 7.0 (Grahame, 1993)]. Nevertheless, under optimal conditions CO-containing reactions yielded a rate of acetyl-CoA synthesis that was only approximately one-half of that obtained when CO_2 plus reduced ferredoxin was employed, even though the equilibrium constant for acetyl-CoA synthesis from CO is about 43 500 times larger than from CO_2. The lower rate of synthesis observed with CO as compared with CO_2 may be related to the artificial characteristics of CO as substrate.

Accessory Protein Requirements for Acetyl-CoA Synthesis from H_2 and CO_2. The ACDS-catalyzed synthesis and cleavage of acetyl-CoA requires two additional proteins to transfer electrons from H_2. Original preparations of the enzyme complex contained low levels of hydrogenase activity; however, the complex is obtained virtually free from hydrogenase by analytical HPLC gel filtration chromatography. A specific hydrogenase resolved by this procedure catalyzes the reduction of ferredoxin. In turn, reduced ferredoxin transfers electrons to the ACDS complex. Previously it was indicated that electron transfer proceeds via iron−sulfur centers in the enzyme complex (Grahame, 1993). The reduced iron−sulfur centers provide the low-potential electrons required for CO_2 reduction and in addition maintain the enzyme complex-bound corrinoid moiety in the active Co^{1+} state (Grahame, 1993).

Although the existence of at least two separate hydrogenase activities in methanogens has been recognized, only the role of the enzyme that catalyzes the reduction of F_{420} was known (Weiss & Thauer, 1993). We have now demonstrated that a role of the second hydrogenase, which cannot catalyze the reduction of F_{420}, is to catalyze the reduction of ferredoxin. The results from both preparative gel filtration on Sepharose 6B (data not shown) and analytical HPLC gel filtration (Figure 2) show that *M. barkeri* expresses multiple hydrogenases when grown with acetate as sole carbon and energy source. Two major hydrogenase peaks were detected in ACDS complex preparations. One of these hydrogenases (H_2ase-Fd) catalyzes the transfer of electrons between

Table 3: Influence of H_2 Levels on the Direction of Acetyl-CoA Synthesis and Cleavage

[H_2] atm	environment	[acetyl-CoA][a] / [CoASH]
2.4×10^{0}	standard culture[b]	3.8×10^{3}
2.0×10^{-2}	rumen postfeeding	1.3×10^{1}
1.9×10^{-3}	rumen basal	1.2×10^{0}
2.7×10^{-4}	sewage sludge	1.7×10^{-1}
3.7×10^{-5}	fresh water sediments	2.3×10^{-2}

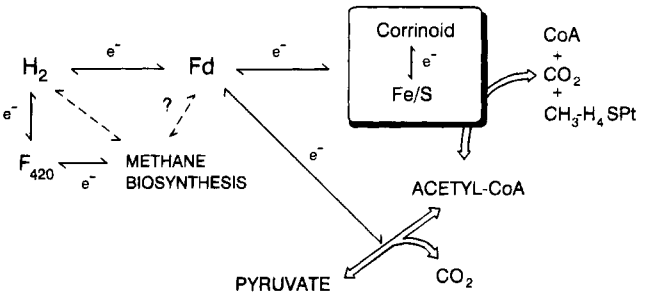
[a] Ratio of [acetyl-CoA]/[CoASH] must be less than this value for acetyl-CoA synthesis to be favorable. Calculations are based on the value of the equilibrium constant for eq 3 of 2.09×10^{6} M^{-1} $ATM_{H_2}^{-1}$ assuming 0.003 M CO_2 (CO_2 gas phase of ≈10%) and a constant ratio of H_4SPt/CH_3-H_4SPt of 10/1. [b] Calculated with CO_2 concentration of 0.018 M (80/20 H_2/CO_2 gas mixture at ≈3 atm).

hydrogen and ferredoxin and supports H_2-dependent synthesis of acetyl-CoA. The other hydrogenase is an F_{420}-reducing hydrogenase (H_2ase-F_{420}) that catalyzes the transfer of electrons between H_2 and the deazaflavin derivative coenzyme F_{420}. The H_2ase-F_{420} is unable to catalyze the transfer of electrons between H_2 and ferredoxin and therefore does not support acetyl-CoA synthesis (Figure 3). The role for the H_2ase-Fd in cells that use H_2 as an energy source would be to allow formation of reduced ferredoxin required as a substrate of the ACDS complex for efficient biosynthesis of acetyl-CoA. In different methanogens, or in *M. barkeri* grown on substrates other than acetate, the presence of a second hydrogenase may provide a means for apportioning electron flow to either methane synthesis and energy production via the H_2ase-F_{420} or acetyl-CoA synthesis, and thus to additional biosynthetic pathways via the H_2ase-Fd.

Implications of the K_{eq} of Eq 3 for Utilization of Acetate or H_2 by Methanogens. The results presented herein, along with previous measurements of the ACDS complex corrinoid cofactor redox midpoint potential (Grahame, 1993), suggest that for cells growing on CO_2 plus H_2 both the rate and extent of acetyl-CoA synthesis are controlled by the level of hydrogen in the environment. Under environmentally relevant levels of hydrogen, approximately 5×10^{-5} atm (Thauer, 1990), the redox potential available to the ACDS complex inside the cell would be approximately −286 mV (pH 7.0, 25 °C). At this potential less than 0.2% of the enzyme complex would exist in the active Co^{1+} form [E_o of the enzyme cobamide Co^{2+}/Co^{1+} couple = −426 mV (Grahame, 1993)]. This would restrict the available H_2 almost exclusively to methane synthesis. Substantial acetyl-CoA synthesis would occur only when additional reducing equivalents became available.

As measured, the K_{eq} for reaction 3, the biosynthesis of acetyl-CoA (2.09×10^{6} M^{-1} $ATM_{H_2}^{-1}$), appears to be strongly favored. However, the direction in which the reaction will proceed depends also on the actual concentrations of all substrates and products. The level of H_2 in particular, because of its great variability, would play a major role in determining whether net synthesis or cleavage of acetyl-CoA is favored. Based on the value of K_{eq} of eq 3, [acetyl-CoA]/[CoASH] ratios that would exist at equilibrium were obtained as a function of environmental H_2 concentration, as shown in Table 3. The results indicate that acetyl-CoA synthesis is decidedly favored in the presence of high concentrations of hydrogen, such as would be employed in cell culture at 3 atm of a standard gas mixture (80% H_2 and 20% CO_2). However, the reverse reaction of acetyl-CoA cleavage becomes spontaneous in environments with low levels of hydrogen, such as in sewage digestors and sediments of fresh bodies of water. In the rumen the relatively high levels of acetate obviate the need for methanogen acetyl-CoA synthesis via the ACDS complex. Moreover, the high levels of H_2 present under postfed conditions would inhibit breakdown of acetate as a source of carbon for methane biosynthesis.

Scheme 1: Electron Flow to Competing Pathways of CO_2 Assimilation and Methanogenesis[a]



[a] Single lines represent electron flow. Double lines show carbon flow. Dashed lines are other possible routes of electron flow. The ACDS complex containing iron−sulfur and corrinoid centers is represented by the box.

Electron Flow to Competing Pathways of CO_2 Assimilation and Methanogenesis. The presence of the H_2ase-Fd in acetate-grown cells suggests that electrons derived from the carbonyl group of acetyl-CoA and transferred via the ACDS complex to ferredoxin may eventually be used in the synthesis of H_2. It is likely that this accounts for the significant level of H_2 detected during growth of *M. barkeri* on acetate (Krzycki et al., 1987). Hydrogen production via the H_2ase-Fd would appear to be a waste of energy that could otherwise provide reducing equivalents for the final reductive step in methane biosynthesis. The complete pathway of electron transfer from the carbonyl group of acetyl-CoA to the final step in methane biosynthesis is unknown, but there appears to be two avenues for the fate of H_2. Hydrogen could be a required intermediate, with electron flow to methane proceeding via capture of formed H_2 by H_2ase-F_{420} or another hydrogenase. Electrons might also be transferred to the final step of methanogenesis via a pathway that bypasses free H_2 (Scheme 1).

In cells growing with hydrogen and CO_2 as sole carbon and energy source, the extent of electron flux from hydrogen in any one direction is determined by ratios of substrates and products of the individual reactions, as indicated in Scheme 1. Electron flow into the methane biosynthetic pathway is coupled to at least two F_{420}-dependent reactions, reduction of *N^5,N^{10}-methenyl*-H_4SPt to *N^5,N^{10}-methylene*-H_4-SPt and reduction of the latter compound to *N^5-methyl*-H_4-SPt. In *Methanobacterium* spp. the first of these reactions is catalyzed by an enzyme that does not use F_{420} but instead uses hydrogen directly (Zirngibl et al., 1990). Our results, and the results of Thauer (1990), show that significant differences exist between the standard free energies of reactions in which H_2 is utilized for acetyl-CoA synthesis versus those in which H_2 is employed for methane synthesis, as shown by eqs 3, 13, and 14. Introduction of H_2 into the pathways represented by eqs 13 and 14 is mediated by F_{420}, but the F_{420} term cancels when the overall equations are written.

$$H_2 + CO_2 + CH_3\text{-}H_4SPt + CoASH \rightleftharpoons \text{acetyl-CoA} + H_4SPt + H_2O \quad \Delta G^\circ = -8.96 \text{ kcal/mol } (-37.5 \text{ kJ/mol}) \quad (3)$$

$$3\,H_2 + N^5,N^{10}\text{-methenyl-}H_4SPt \rightleftharpoons CH_4 + H_4SPt + H^+ \quad \Delta G^\circ = -32.8 \text{ kcal/mol } (-137 \text{ kJ/mol}) \quad (13)$$

$$2\,H_2 + N^5,N^{10}\text{-methylene-}H_4SPt \rightleftharpoons CH_4 + H_4SPt \quad \Delta G^\circ = -31.5 \text{ kcal/mol } (-132 \text{ kJ/mol}) \quad (14)$$

Not only does the acetyl-CoA synthetic reaction have to compete with methanogenesis for CO_2 and H_2, but it must also compete for a third substrate, CH_3-H_4SPt, which is an intermediate in the methane biosynthetic pathway.

Additionally, since it has been shown that the level of ACDS complex in the active Co^{1+} form is a function of the redox potential imposed by the available H_2 (Grahame, 1993), electron flux into acetyl-CoA synthesis is restricted further. Since the usual environmental levels of hydrogen [approximately 5×10^{-5} atm (Thauer, 1990)] would allow less than 0.2% of the ACDS complex corrinoid cofactor to exist in the active, reduced form, high levels of acetyl-CoA synthesis (and therefore growth) theoretically would occur only when higher than usual levels of hydrogen become available. Electron flow through membrane components, such as might be needed for reverse electron flow to reduce CO_2-methanofuran to the formyl level (Thauer, 1990), is another important reaction (not specifically addressed in Scheme 1) that competes with acetyl-CoA synthesis for available reducing equivalents.

In cells growing with acetate as sole carbon and energy source, electron flux through the ACDS complex during acetyl-CoA cleavage likely maintains a significant fraction of the corrinoid moiety of the enzyme complex in the active Co^{1+} state (Grahame, 1991). The ACDS complex transfers electrons to ferredoxin. Electrons from ferredoxin are then passed into hydrogen via H_2ase-Fd. It is not known whether other possible mechanisms exist for redox coupling of ferredoxin to the methane biosynthetic pathway. Under conditions of acetate depletion, growth would be restricted not only by carbon availability but also because levels of reduced ferredoxin would be low, thus restricting pyruvate synthesis via pyruvate-ferredoxin oxidoreductase (Scheme1), thereby limiting growth. Under conditions of abundant acetate, a higher level of reduced ferredoxin would be present, and therefore pyruvate synthesis and growth would become less restricted. This scheme may also account for the observation that pyruvate provides a rapid and efficient source of reducing equivalents for methanogens (T. C. Stadtman, personal communication; Bock et al., 1994).

We have provided substantial evidence in support of ferredoxin as the physiologically important component required for efficient transfer of electrons from H_2 to the acetyl-CoA decarbonylase synthase complex. In an *in vitro* acetyl-CoA-synthesizing system (Figure 1), the extent of acetyl-CoA formation is negligible or extremely low until ferredoxin is added. Lowering the redox potential of the reaction mixture by addition of 1 mM Ti^{3+}•EDTA generates electron transfer to the ACDS complex; however the rate of transfer in the absence of ferredoxin is still very low, and thus, the rate of acetyl-CoA formation is limited (Table 1). In previous studies, slow direct reduction of the enzyme complex was also noted when the potential of the ACDS enzyme solution was poised by adjusting the H^+/H_2 ratio in the absence of ferredoxin (Grahame, 1993). In reactions with Ti^{3+} as the electron source, the rate of acetyl-CoA synthesis increases 20-fold by addition of ferredoxin (Table 1). Therefore it may be concluded that while a favorable redox potential is necessary to provide electrons for acetyl-CoA synthesis, this alone is insufficient to allow for efficient electron transfer. In order for optimum acetyl-CoA synthesis to occur, an efficient coupling agent, in this case ferredoxin, must also be present.

Finally, our discovery of a second hydrogenase as an enzyme capable of catalyzing the reduction of ferredoxin not only identifies the function of that previously ambiguous hydrogenase (Weiss & Thauer, 1993) but also supports the role of ferredoxin as the mediator of electron flow from hydrogen to acetyl-CoA synthesis. It should be emphasized that ferredoxin acts as a coupling agent in both directions. When the redox potential inside the cell becomes less negative due to a scarcity of available reducing equivalents such as H_2, electrons are transferred out of the ACDS complex, thus causing the Co^{2+}/Co^{1+} equilibrium of the corrinoid moiety to shift more to the oxidized, catalytically inactive form (Grahame, 1993).

In contrast to the high amounts of both ferredoxin and the ACDS complex present in *M. barkeri* cells that are actively converting large quantities of acetate to methane, it is interesting to note that comparatively low quantities of these two proteins exist in formate-grown cells of *M. vannielii* which require only low levels of acetyl-CoA synthesis to be sufficient for production of cell components. This observation is understandable in view of the proposed action of ferredoxin as the factor that couples electron transfer between H_2 and the ACDS complex *in vivo*.

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