Final Technical Report.

Enzymology of the Pathway for Acetate Conversion to Methane in *Methanosarcina thermophila*
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A. Regulation of enzyme synthesis.

Of all known methanogens, *Methanosarcina* species are the most catabolically diverse and therefore are most amenable to study the regulation of protein synthesis in response to the growth substrate. Our long term goal is to understand the molecular biology of this regulation.

1. Cloning, nucleotide sequence and transcription of the gene encoding a ferredoxin from *M. thermophila*.

Ferredoxin plays an important role in electron transport during the conversion of acetate to methane. Thus, the gene encoding this ferredoxin was cloned and sequenced to further characterize the protein and provide a starting point into the molecular genetics (Clements and Ferry, 1992). The results suggested the presence of a second ferredoxin that is likely to be involved in conversion of methanol to methane. The results also revealed conserved sequences in the promoter regions of the two genes that may be important for future studies on the regulated expression.

B. Activation of acetate to acetyl-CoA.

Prior to cleavage by the *Methanosarcina*, acetate is first activated to acetyl-CoA by acetate kinase and phosphotransacetylase. In addition to being an important intermediate in the metabolism of many anaerobic bacteria, acetyl-phosphate acts as a phosphoryl donor to transport proteins and to many response regulator proteins of two-component systems. More recently, it has been proposed that acetyl-phosphate functions as a global regulatory signal in *E. coli*. The sequence of any *pta* gene has not been reported, and the only gene encoding acetate kinase which has been cloned and sequenced is the *ackA* gene from *E. coli*. Our goal is to understand the biochemical mechanism of these enzymes and the molecular basis for regulated expression. Thus, we undertook the cloning, sequence analysis, and hyperexpression of the genes encoding phosphotransacetylase and acetate kinase from *M. thermophila* (Latimer and Ferry, 1993).

Both genes are present in only one copy per genome with the *pta* gene adjacent to, and upstream of, the *ack* gene. Consensus archaeal promoter sequences are found upstream of the *pta* coding region. The *pta* and *ack* genes encode predicted polypeptides with molecular masses of 35,198 and 44,482 daltons, respectively. A hydropathy plot of the deduced phosphotransacetylase sequence indicates that it is a hydrophobic polypeptide; however, no

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membrane-spanning domains are evident. Comparison of the amino acid sequences deduced from the *M. thermophila* and *E. coli* *ack* genes indicate similar subunit molecular weights and 44% identity (60% similarity). The comparison also revealed the presence of several conserved arginine, cysteine, and glutamic acid residues. Arginine, cysteine, and glutamic acid residues have previously been implicated at or near the active site of the *E. coli* acetate kinase. The *pta* and *ack* genes were hyperexpressed in *E. coli* and the hyperproduced enzymes were purified to homogeneity with specific activities higher than the enzymes previously purified from *M. thermophila*. The hyperproduced phosphotransacetylase and acetate kinase migrated at molecular masses of 37,000 and 42,000 daltons, respectively. The activity of the acetate kinase is optimal at 65°C and is protected from thermal inactivation by ATP. Diethylpyrocarbonate and phenylglyoxal inhibited acetate kinase activity in a manner consistent with the presence of histidine and arginine residues at or near the active site; however, the thiol-directed reagents 5,5'-dithiobis(2-nitrobenzoic acid) and *N*-ethylmaleimide were ineffective.

This is the first sequence reported for any *pta* gene, and only the second for a *ack* gene. The results provide a basis for continued studies on the regulated expression of these genes. The results also provide a foundation for continued biochemical studies on the mechanism of acetate kinase and phosphotransacetylase that will impact the broader field of anaerobic microbiology.

C. Biochemistry of acetyl-CoA cleavage.

Following activation of acetate to acetyl-CoA, the C-C and C-S bonds are cleaved. The objective of our research was to characterize the enzyme complex catalyzing this reaction with the goal of determining the biochemical mechanism of acetyl-CoA cleavage.

1. Synthesis of acetyl-CoA by the carbon monoxide dehydrogenase complex from acetate-grown *M. thermophila*.

A CO dehydrogenase complex is present at high levels when *M. thermophila* is grown on acetate, and it had been proposed that the enzyme complex cleaves acetyl-CoA. The first biochemical evidence supporting this proposed function was obtained when it was demonstrated that the purified complex synthesizes acetyl-CoA (Abbanat and Ferry, 1990). In addition to supporting the proposed function of the complex and further characterization, the results suggested different sites on the enzyme complex for acetyl-CoA synthesis and CO oxidation which we further documented in our more recent studies (Lu et al., 1994).

2. Demonstration of carbon-carbon bond cleavage of acetyl-CoA using isotopic exchange catalyzed by the CO dehydrogenase complex from acetate-grown *M. thermophila*.

Although the synthesis of acetyl-CoA supported the proposed cleavage function for the complex, this had yet to be demonstrated and characterized. Thus, the acetyl-CoA C-C and C-S bond cleavage activity was examined by assaying the enzyme for the ability to exchange the carbonyl group of acetyl-CoA with CO as well as the exchange of CoA with acetyl-CoA (Raybuck et al., 1991).

The kinetic parameters for the carbonyl exchange were: K_m (acetyl-CoA) = 200 μM , V_{max} = 15 min^{-1} . CoA was a strong inhibitor of the exchange (K_i = 25 μM) and is formed under the assay conditions because of a slow but detectable acetyl-CoA hydrolase activity. Comparison of the kinetic parameters with those for the clostridial enzyme suggests differences that reflect the different physiological functions in acetyl-CoA synthesis and cleavage.

In addition to supplying further evidence for the proposed function of the CO dehydrogenase enzyme complex, the results indicated fundamental differences from the clostridial enzyme.

3. Resolution of component proteins in an enzyme complex from *M. thermophila* catalyzing the synthesis or cleavage of acetyl-CoA.

In order to better understand the function of the five subunits in the complex, it was resolved into distinct enzyme components that were initially characterized (Abbanat and Ferry, 1991). The properties of the component enzymes support a mechanism in which the nickel/iron-sulfur component cleaves acetyl-CoA, oxidizes the carbonyl group to CO_2 and then transfers the methyl group to the corrinoid/iron-sulfur component.

4. Characterization of the metal centers of the nickel/iron-sulfur component of the CO dehydrogenase enzyme complex from *M. thermophila*.

The nickel/iron-sulfur component of the enzyme complex was further characterized in collaboration with Dr. Steve Ragsdale to obtain a more detailed understanding of the proposed mechanism (Lu et al., 1994).

Three metal centers in the nickel/iron-sulfur component were characterized by electron paramagnetic resonance (EPR) spectroscopy and spectroelectrochemistry and pre-steady state kinetics. Center A contains nickel and iron and forms an EPR active adduct with CO, which is called the NiFeC species. The EPR spectrum of the NiFeC species has g values of 2.059, 2.051, and 2.029 and is observable at temperatures as high as 150 K. This signal had previously been observed only in the carbon-monoxide dehydrogenase complex of *M. thermophila* and the acetyl-CoA synthase from acetate-producing bacteria. Incubation of the CO-reduced Ni/Fe-S component with acetyl-CoA resulted in an increase in intensity of the NiFeC signal, which supports a role

for the component in the cleavage of acetyl-CoA. Generation of the NiFeC EPR signal occurs with a rate constant of 0.4 s^{-1} , a result that demonstrates the kinetic competence of this species in the acetyl-CoA cleavage reaction but rules it out as the site of oxidation of CO to CO_2 . Center B is likely to be a $[\text{4Fe-4S}]^{2+/1+}$ center with g values of 2.04, 1.93, and 1.89 ($g_{\text{av}}=1.95$) and a standard reduction potential (E'_0) of -444 mV. At potentials less than -500 mV, another EPR signal develops that appears to originate from another state of Center B. Center C is a fast relaxing center with g values of 2.02, 1.88, and 1.71 ($g_{\text{av}}=1.87$) and an E_m of -154 mV.

The results firmly support the proposed role for the nickel/iron-sulfur component in cleavage of the C-C and C-S bonds of acetyl-CoA, and offers additional evidence for separate sites involved in acetyl-CoA cleavage and carbonyl group oxidation.

5. Characterization of the metal centers of the corrinoid/iron-sulfur component of the CO dehydrogenase enzyme complex from *M. thermophila* by EPR spectroscopy and spectroelectrochemistry.

The corrinoid/iron-sulfur component of the enzyme complex was further characterized in collaboration with Dr. Steve Ragsdale to obtain a more detailed understanding of the proposed mechanism (Jablonski et al., 1993). The results further support a methyl transfer function for this component and provides a better understanding of the mechanism for reductive activation of the corrinoid.

D. Electron transport.

Acetotrophic methanogens obtain energy for growth by an electron transport phosphorylation. A goal of our research was to identify electron carriers and begin to reconstruct the electron transport chain with purified and partially purified components *in vitro*.

1. Purification and properties of methyl coenzyme M methylreductase from acetate-grown *M. thermophila*.

The terminal steps in the electron transport chain involve reduction of methyl coenzyme M ($\text{CH}_3\text{-S-CoM}$) with HS-HTP resulting in the heterodisulfide CoM-S-S-HTP which is reduced back to the sulfhydryl forms of the cofactors with electrons derived from oxidation of the carbonyl group of acetate by the CO dehydrogenase complex. The methylreductase was purified for the purpose of characterizing this enzyme from an acetate-grown *Methanosarcina* species for comparison with similar enzymes from the acetotroph *Methanotherix* and CO_2 -reducing methanogens (Jablonski and Ferry, 1991).

The enzyme was purified 16-fold from cell extract to apparent homogeneity as determined by native polyacrylamide gel

electrophoresis. 94% of methylreductase activity was recovered in the soluble fraction of cell extracts. The estimated native molecular weight of the enzyme was between $132,000 \pm 1200$ and $141,000 \pm 1200$ daltons. Denaturing polyacrylamide gel electrophoresis revealed three protein bands corresponding to molecular weights of $69,000 \pm 1200$, $42,000 \pm 1200$, and $33,000 \pm 1200$ daltons and indicated a subunit configuration of $\alpha_1\beta_1\gamma_1$. The as-isolated enzyme was inactive but could be reductively reactivated with titanium (III) citrate or reduced ferredoxin. ATP stimulated enzyme reactivation and was postulated to be involved in a conformational change of the inactive enzyme from an "unready" to a "ready" state which could be reductively reactivated. The temperature and pH optima for enzyme activity were 60°C and between 6.5-7.0 respectively. The active enzyme contained one mole of coenzyme F_{430} per mole of enzyme ($M_r = 144,000$). The K_m for $\text{CH}_3\text{-S-CoM}$ and HS-HTP were 3.3 mM and 59 μM respectively.

The results revealed a novel mechanism for reductive reactivation of the enzyme involving ferredoxin as the electron donor and identified a second function for this electron carrier in acetotrophic metabolism.

2. Structural characterization and physiological function of component B from *M. thermophila*.

HS-HTP (component B), or a derivative thereof, is the electron donor for the methylreductase (Jablonski and Ferry, 1991). The literature presented some controversy over the structure of HS-HTP in CO_2 -reducing methanogens. This fact, and a need to determine the structure in acetotrophic *Methanosarcina*, prompted us to determine the structure in *M. thermophila* (Clements et al., 1993).

HS-HTP was isolated as the 7-methyl derivative and characterized. Gas chromatography-mass spectrometry and ^1H NMR analyses identified this derivative as 7-methylthioheptanoylthreonine phosphate ($\text{CH}_3\text{-S-HTP}$), indicating that the original component B had the same structure (HS-HTP) as previously determined for component B from *Methanobacterium thermoautotrophicum*. The heterodisulfide of HS-HTP and coenzyme M (HS-CoM, 2-mercaptoethanesulfonate) was enzymatically reduced in cell extracts using electrons supplied by either H_2 or CO , confirming that HS-HTP was a functional molecule in *M. thermophila*.

The results showed that the structure of component B in *Methanosarcina* was the same as HS-HTP. The ability of H_2 to donate electrons to the heterodisulfide reductase is an interesting result that continues the debate on the potential role of H_2 in electron transport during growth on acetate.

3. Characterization of the Iron-Sulfur Clusters in Ferredoxin from Acetate-Grown *M. thermophila*.

Ferredoxin is the electron acceptor for the CO dehydrogenase complex and plays a central role in electron transport during acetotrophic methanogenesis. Previously, it was suggested that a three-iron center may exist in a similar ferredoxin from *Methanosarcina*; however, the deduced amino acid analysis suggested the protein is more likely to contain two four-iron centers (Clements and Ferry, 1992). Thus, biochemical properties of this protein from *M. thermophila* were determined in collaboration with Drs. Ragsdale and Spiro (Clements et al., 1994). In addition to further characterization, the results showed that this is a $2 \times [4\text{Fe}-4\text{S}]$ ferredoxin and is unlikely to contain a three-iron center that is physiologically relevant.

4. Characterization of a CO:heterodisulfide oxidoreductase system from acetate-grown *M. thermophila*.

Although it is known that ferredoxin is the electron acceptor for the CO dehydrogenase complex and that electrons are passed to the heterodisulfide, the involvement of membranes and other electron carriers is unknown. Thus, reconstruction of an electron transport chain was attempted with purified and partially purified components (Peer and Ferry, unpublished).

A CO:CoM-S-S-CoB oxidoreductase system from acetate-grown *M. thermophila* was reconstituted with purified CO dehydrogenase enzyme complex, ferredoxin, membranes, and heterodisulfide reductase partially purified from soluble proteins. Coenzyme F_{420} was not required and no significant CO: F_{420} oxidoreductase activity was detected in cell extracts. The membranes contained a *b*-type cytochrome that was reduced with CO and oxidized with CoM-S-S-CoB. The cytochrome was purified from solubilized membrane proteins in a complex with six other polypeptides.

The results suggest that a novel CoM-S-S-CoB reducing system operates during acetate conversion to CH_4 and CO_2 . In this system, ferredoxin transfers electrons from the CO dehydrogenase complex to membrane-bound electron carriers, including cytochrome *b*, which are required for electron transfer to the heterodisulfide reductase.

5. Cloning, sequence analysis, and hyperexpression of a novel iron-sulfur flavoprotein from *M. thermophila*.

In the course of cloning the genes encoding the acetate activating enzymes, an open reading frame (ORF) was discovered upstream of the *pta* gene. The deduced amino acid sequence suggested it may function in electron transport; thus, the gene was hyperexpressed and the hyperproduced protein initially characterized (Latimer et al., unpublished).

Biochemical characterization of the hyperproduced protein showed it was a novel iron-sulfur flavoprotein. Western blotting shows the protein is present in acetate- and methanol-grown cells, and it is proposed that this novel electron carrier may

function as a one-electron/two-electron switch. These preliminary results provide a basis for determining the function and further biochemical and molecular genetic characterization (please see text of research proposal).

E. Other enzymes implicated in the pathway of acetate conversion to methane.

Levels of carbonic anhydrase (CA) are elevated in acetate-grown *Methanosarcina* and several other acetotrophic anaerobes indicating a broad role for this enzyme in acetate metabolism by anaerobic microbes. Our goal was to purify CA from *M. thermophila* with the purpose to help define the physiological function (Alber and Ferry, 1994).

The CA was purified over 10,000-fold (22% recovery) to apparent homogeneity and a specific activity of 4872 units/mg. The estimated native molecular mass of the enzyme is 84,000 Da based on gel filtration chromatography. SDS/PAGE revealed one protein band having an apparent molecular weight of 40,000. The *M. thermophila* CA is less sensitive than the human isozyme II towards inhibition by sulfonamides and monovalent ions. The gene encoding this CA was cloned into pUC18 and sequenced. *Escherichia coli* harboring the recombinant plasmid expresses CA activity (2.3 units/mg cell extract protein). Comparison of the deduced amino acid sequence with the N-terminal sequence of the purified protein shows that the gene encodes an additional 34 N-terminal residues with properties characteristic of signal peptides in secretory proteins. The calculated molecular mass (22.9 kDa) and pI (4.0) suggests SDS/PAGE overestimates the subunit size and further suggests a tetrameric structure for the native enzyme. The deduced amino acid sequence has no significant identity to any known CAs, but has 35% sequence identity to the first 197 deduced N-terminal amino acids of a proposed CO₂-concentrating-mechanism protein from *Synechococcus* PCC7942, and 28% sequence identity to the deduced ferripyochelin binding protein from *Pseudomonas aeruginosa*.

The results indicate that this archaeal enzyme represents a novel class of CAs and provide a basis to determine the physiological roles for CA in acetotrophic anaerobes.

F. Books, review articles, and methods papers.

A book (Ferry [ed.], 1993), five review articles (Ferry, 1992a; Ferry, 1992b; Alber et al., 1993; Ferry, 1993a; Ferry 1993), and three methods papers (Latimer and Ferry, 1994; Clements and Ferry, 1994; Rasche et al., 1994) were published or are in press.

III. PUBLICATIONS ATTRIBUTABLE TO DOE FUNDING.

A. Original research articles.

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Raybuck, S.A., S.E. Ramer, *D.R. Abbanat, *J.W. Peters, W.H. Orme-Johnson, J.G. Ferry and C.T. Walsh. 1991. Demonstration of carbon-carbon bond cleavage of acetyl-CoA using isotopic exchange catalyzed by the CO dehydrogenase complex from acetate-grown *Methanosarcina thermophila*. Journal of Bacteriology 173:929-932.

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*Jablonski, P. E., W.-P. Lu, S. W. Ragsdale and J. G. Ferry. 1993. Characterization of the metal centers of the corrinoid/iron-sulfur component of the CO dehydrogenase enzyme complex from *Methanosarcina thermophila* by EPR spectroscopy and spectroelectrochemistry. Journal of Biological Chemistry 268:325-329.

*Clements, A. P., R. H. White and J. G. Ferry. 1993. Structural characterization and physiological function of component B from *Methanosarcina thermophila*. Archives of Microbiology 159:296-300.

*Latimer, M. T. and J. G. Ferry. 1993. Cloning, sequence analysis, and hyperexpression of the genes encoding phosphotransacetylase and acetate kinase from *Methanosarcina thermophila*. Journal of Bacteriology 175:6822-6829.

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Methanosarcina thermophila. Journal of Biological Chemistry 269:9736-9742.

*Alber, B. E. and J. G. Ferry. A novel carbonic anhydrase from the archaeon *Methanosarcina thermophila*. Proceedings of the National Academy of Sciences of the United States of America (in press).

*Clements, A.P., L. Kilpatrick, W.-P. Lu, S. W. Ragsdale, and J. G. Ferry. Characterization of the Iron-Sulfur Clusters in Ferredoxin from Acetate-Grown *Methanosarcina thermophila*. Journal of Bacteriology 176:2689-2693.

B. Review articles.

Ferry, J. G. 1992. Methane from acetate. Journal of Bacteriology 174:5489-5495.

Ferry, J. G. 1992. Biochemistry of Methanogenesis. Critical Reviews in Biochemistry and Molecular Biology 27:473-503.

*Alber, B. E., *Clements, A. P., *Jablonski P. E., *Latimer, M. T. and Ferry, J. G. 1993. Enzymology of the methanogenic fermentation of acetate by *Methanosarcina thermophila*. In (C. Murrell and D. Kelly, Eds.) Microbial Growth on C₁ Compounds. Intercept, Ltd., UK pp 163-170.

Ferry, J. G. 1993. Fermentation of Acetate. In Methanogenesis (J. G. Ferry, Ed.). Chapman and Hall, New York pp. 304-334.

Ferry, J. G. 1993. CO Dehydrogenase of Methanogens In Acetogenesis (H. L. Drake, Ed.). Chapman and Hall, New York (in press).

C. Books edited.

Ferry J.G. (ed.) *Methanogenesis* 1993. Chapman and Hall, London, 536 pp.

D. Methods articles.

*Latimer, M. T. and J. G. Ferry. 1994. Purification of overproduced *Methanosarcina thermophila* acetate kinase and phosphotransacetylase from *Escherichia coli*. In Protocols for Archaeal Research (F. T. Robb et al. Eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (in press).

*Clements, A. P. and J. G. Ferry. 1994. Purification of the carbon monoxide dehydrogenase-linked ferredoxin from *Methanosarcina thermophila*. In Protocols for Archaeal Research (F. T. Robb et al. Eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (in press).

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