Work supported under DE-FG02-00ER15108 resulted in much new insight into unusual mechanisms of metalloenzymes involved in anaerobic metabolism in methanogens, other archaea, and bacteria, and was published in a series of high quality publications supported by funding which spanned from 2000 to around 2009. The extensive results from these efforts are briefly summarized for each of the major publications as follows.

1. Gencic, S., LeClerc, G. M., Gorlatova, N., Peariso, K., Penner-Hahn, J. E., and Grahame, D. A. (2001) Zinc-thiolate intermediate in catalysis of methyl group transfer in *Methanosarcina barkeri*. *Biochemistry* **40**, 13068-13078.

Both zinc and cobalt were found to be functional at the active site of methylcorrinoid:CoM methyltransferase. Direct stoichiometric binding of substrate CoM thiolate to the metal site was established by UV-visible and X-ray spectroscopic studies. The pH dependence of metal-thiolate formation indicated that substrate binding takes in a separate step from proton release, which occurs with a pKa of around 6.4. Thermodynamics of substrate binding indicated that metal-thiolate bond formation is a major driving force that contributes to the overall highly favorable process of methyl group transfer to CoM in a step that is a major contributor to the overall bioenergetics of methanogenesis.

2. Gencic, S., and Grahame, D. A. (2003) Nickel in subunit beta of the acetyl-CoA decarbonylase/synthase multienzyme complex in methanogens. Catalytic properties and evidence for a binuclear Ni-Ni site. *J. Biol. Chem.* **278,** 6101-6110.

Cleavage of the acetyl-CoA C-C bond in methanogens metabolizing large quantities of acetate was shown to require nickel at the active site of the acetyl-CoA synthase (ACS) beta subunit in the multienzyme complex acetyl-CoA decarbonylase/synthase (ACDS). Nickel reconstitution experiments performed with the recombinant beta subunit expressed in *E. coli* under anaerobic conditions demonstrated that an intact [4Fe-4S] cluster was present in the apo enzyme and the stoichiometric incorporation of 2 mol of Ni per mole of [4Fe-4S] cluster to form the mature A cluster. Cysteine residues involved in Fe/S cluster assembly versus Ni coordination were distinguished by site-directed mutagenesis. Characterization of the recombinant system in these studies set the stage for further advances to study acetyl-CoA synthase in the strict absence of CO dehydrogenase (CODH) which otherwise complicates redox control and levels of the CO as a common substrate for both enzymes.

3. Gu, W., Gencic, S., Cramer, S. P., and Grahame, D. A. (2003) The A-cluster in subunit beta of the acetyl-CoA decarbonylase/synthase complex from *Methanosarcina thermophila*: Ni and Fe K-edge XANES and EXAFS analyses. *J. Am. Chem. Soc.* **125**, 15343-15351.

In a collaborative effort, data from X-ray absorption spectroscopic analysis of the Ni reconstituted ACDS beta subunit were satisfactorily simulated using both a computational approach and by using spectra of model compounds with known geometries, which indicated that 40 to 50% of the Ni in the enzyme is present in tetrahedral geometry with the remaining likely in a square planar arrangement. A working model was developed with the proximal Ni atom as a tetrahedral site and distal Ni in square planar geometry. EXAFS studies indicated coordination geometries and coordination numbers consistent with this model. Neither copper

nor zinc was present in the wild-type or recombinant methanogen enzymes, which differed from the two original crystallographic reports by others on bacterial CODH/ACS. Those metals were later concluded to be contaminants in the preparations used for those studies, however, our spectroscopic data and other studies remain uncomplicated by the presence of adventitious Zn or Cu.

4. Funk, T., Gu, W., Friedrich, S., Wang, H., Gencic, S., Grahame, D. A., and Cramer, S. P. (2004) Chemically distinct Ni sites in the A-cluster in subunit beta of the acetyl-CoA decarbonylase/synthase complex from *Methanosarcina thermophila*: Ni L-edge absorption and X-ray magnetic circular dichroism analyses. *J. Am. Chem. Soc.* **126**, 88-95.

In collaboration with S. Cramer and T. Funk, we obtained L-edge spectroscopic data on Ni in the active site of the ACDS beta subunit, and further characterized the A cluster by magnetic circular dichroism at the nickel L edge. The methods served to confirm the presence of two chemically distinct Ni atoms at the active site and probe changes in their reactivity in different states of oxidation and upon reaction of the enzyme with CO.

5. Grahame, D. A., Gencic, S., and DeMoll, E. (2005) A single operon-encoded form of the acetyl-CoA decarbonylase/synthase multienzyme complex responsible for synthesis and cleavage of acetyl-CoA in *Methanosarcina thermophila*. *Arch. Microbial.* **184**, 32-40.

In this work we established that a single ACDS complex, encoded by a one operon, serves both catabolic (acetyl C-C fragmentation) and anabolic (acetyl group synthesis) functions in a methanogen. Previously it was speculated that separate ACDS complexes might be involved, and even separately regulated, in anabolic versus catabolic pathways. These ideas were based largely on genomic identification of duplicated operons in certain species of methanogens, and erroneous interpretation of two forms of CO dehydrogenase observed by ion exchange chromatography. Our evidence from genomic DNA analyses on *M. thermophila* TM-1 showed that only a single operon exists in this organism. MALDI MS-MS revealed identical sets of ACDS peptides in cells grown either on methanol or on acetate. Thus, we found that the single operon-encoded ACDS complex is the sole form of the complex used both for acetyl-CoA synthesis in cells grown on methanol as well as for acetyl-CoA cleavage in cells grown on acetate. The role of the second operon found in other species of methanogens still remains poorly understood, however, from our studies it is clear that this is not due to expression of "anabolic versus catabolic" forms of the complex.

6. Gencic, S., and Grahame, D. A. (2008) Two separate one-electron steps in the reductive activation of the A cluster in subunit beta of the ACDS complex in *Methanosarcina thermophila*. *Biochemistry* **47**, 5544-5555.

To understand the mechanism of catalysis at the A cluster, knowledge is required of the redox properties of the cluster as well as changes in the redox state that take place during turnover. In these studies we established that reduction of the A cluster, needed to generate the fully active state of the enzyme, requires the stoichiometric addition of 2 low-potential electron equivalents. For this work, we developed a reliable method for routine measurement of high activity acetyl-CoA synthesis catalyzed by the recombinant beta subunit, by employing the "base-off"

compound methylcobinamide, rather than base-on methylcobalamin, to afford a better mimic of the reactivity of the natural base-off methylcorrinoid cofactor of the corrinoid-Fe/S protein. This allowed the first kinetic and redox characterization experiments of acetyl-CoA synthesis to be performed on an A cluster protein in a system without interference from CO dehydrogenase. The results showed that 2 electrons are required to fully activate the enzyme, and that they appear to be added one at a time in two separate one-electron steps. The kinetics provided the first evidence for burst formation of acetyl-CoA, which would be expected if an intermediate acetyl-enzyme species was formed initially by reaction of the enzyme with methylcobinamide and CO, and then subsequently rapidly and stoichiometrically transferred upon addition of CoA to form acetyl-CoA. Indeed, direct analysis of the burst stoichiometry over a range of protein concentrations indicated that almost exactly 1 mol acetyl group per enzyme had formed on the enzyme that was rapidly transferred to CoA in the burst. Furthermore, we showed that the acetyl enzyme could be isolated chromatographically, thus establishing a direct means to study the acetyl enzyme intermediate, a species that previously had been postulated only on the basis of kinetic exchange data. Since nearly 100% formation of the acetyl-enzyme was achieved, this indicated substantial thermodynamic stability, and further analyses showed that two low potential electrons were taken up and stabilized by being sequestered in the acetyl-enzyme intermediate. The results explain how the difficult reductive activation of the A cluster is driven by generation of stable methyl-Ni or acetyl-Ni forms in which low potential electrons become trapped. Thus, under physiological conditions, the mechanism of redox activation of the enzyme likely relies on the process of attendant electron transfer, as found here during alkylation or acylation of Ni in the A cluster.

7. Gencic, S., Duin, E. C., and Grahame, D. A. (2010) Tight coupling of partial reactions in the acetyl-CoA decarbonylase/synthase (ACDS) multienzyme complex from *Methanosarcina thermophila*: Acetyl C-C bond fragmentation at the a cluster promoted by protein conformational changes. *J. Biol. Chem.* **285**, 15450-15463.

This work set out to resolve a long-standing question of why methanogen ACDS and bacterial CODH/ACS differ in their abilities to catalyze CO exchange with the carbonyl group of 1-14[C] acetyl-CoA. In answering this question we also gained substantial insight into the role of protein conformational changes in controlling the reactivity of the A cluster. We measured the kinetics of overall acetyl-CoA synthesis and of separate CO and acetyl exchange partial reactions, and characterized the enzyme Ni(I)-CO EPR spectroscopic properties in parallel with three different forms of acetyl-CoA synthase, archaeal ACDS beta subunit, bacterial ACS, which contains a 317-amino acid N-terminal domain not found in the archaea, and an N-terminally truncated bacterial ACS enzyme constructed to resemble the N-terminal domain-lacking native beta subunit from ACDS. All proteins were highly active in catalyzing the acetyltransferase partial reaction and in overall acetyl-CoA synthesis, however, only the full length bacterial enzyme was capable of exchange of the carbonyl group of acetyl-CoA with CO. The results revealed an unanticipated direct role of the N-terminal domain in promoting acetyl C-C bond fragmentation. In the intact ACDS complex, CO exchange was also weakly active, however, the reaction with CO2 was up to 350 times faster than with CO, providing evidence that acetyl fragmentation at the A cluster is tightly coupled to CO oxidation at the C cluster. Protein conformational changes, related to “open/closed” states were indicated to have direct effects on the coordination geometry

and stability of the A cluster Ni-acetyl intermediate, controlling Ni-acetyl fragmentation and Ni-(CO)(CH3) condensation. Two different forms of the Ni(I)-CO EPR signal observed previously were recognized here to correspond to open versus closed conformational states of the protein. To exert control over fragmentation of the acetyl C-C bond at the A cluster, it was concluded that coupling of A and C cluster function involves ACS interdomain interactions in bacteria versus CODH/ACS subunit-subunit interactions in archaea. The data support a mechanism for the methanogen ACDS complex which ensures that loss of CO does not occur, because the A cluster-acetyl species remains stable and unfragmented in the absence of a productive intersubunit contact with CODH. In contrast to the methanogen system, acetyl fragmentation in bacteria is promoted by interdomain interactions involving only the ACS subunit, separate from interaction with CODH. Hence CO exchange is a characteristic of the full-length bacterial enzyme but not seen in the N-terminally truncated form or the isolated ACDS beta subunit. The upshot in methanogens is that CO is released only under conditions in which appropriate beta (ACS)-to-alpha/epsilon (CODH) intersubunit contacts exist. Once these interactions are in place, then CO can be released and efficiently transferred to the C cluster for rapid oxidation to CO2. Because the resultant transfer in ACDS is highly efficient, the carbonyl exchange activity is observed predominantly with CO2 rather than CO. In ACDS, the escape of CO would be harmful to cells carrying out methanogenesis by disproportionation of acetate, due to the loss of low potential electrons needed to drive methyl group reduction to methane, coupled with energy conservation. Thus, the structural arrangement in ACDS appears to be optimized to prevent wasteful loss of CO by ensuring that transfer of CO to the C cluster coincides with fragmentation of the acetyl group and release of CO at cluster A.