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The acetyl-CoA decarbonylase synthase (ACDS) complex has been detected in a variety of methanogens including species of *Methanosarcina*, *Methanothrix* (i.e., *Methanosaeta*), and *Methanococcus*. The multienzyme complex from *Methanosarcina barkeri* is composed of five different subunits, possibly arranged in an $\alpha_6\beta_6\gamma_6\delta_6\varepsilon_6$ structure with the individual subunits of molecular masses (kDa) of 89, 60, 50, 48, and 20, respectively (Grahame, 1991). This progress report summarizes our work from the past 21 months on studies directed toward understanding how the ACDS complex functions in the physiology of acetate-cleaving, and acetate-synthesizing methanogens. Significant findings from these, and other studies on methanogen biochemistry and physiology have been included in four (4) papers, published during this time, listed as follows:

P1. Grahame, D.A., and DeMoll, E. (1995) *Substrate and Accessory Protein Requirements and Thermodynamics of Acetyl-CoA Synthesis and Cleavage in Methanosarcina barkeri.* ***Biochemistry*** **34,** 4617-4624.

P2. Grahame, D.A., Khangulov, S., and DeMoll, E. (1996) *Reactivity of a Paramagnetic Enzyme-CO Adduct in Acetyl-CoA Synthesis and Cleavage.* ***Biochemistry*** **35,** 593-600.

P3. Grahame, D.A., and DeMoll, E. (1996) *Partial Reaction Catalyzed by Protein Components of the Acetyl-CoA Decarbonylase/Synthase Enzyme Complex from Methanosarcina barkeri.* ***J. Biol. Chem.*** **271,** 8352-8358.

P4. LeClerc, G.M., and Grahame, D.A. (1996) *Methylcobamide:Coenzyme M Methyltransferase Isozymes from Methanosarcina barkeri: Physicochemical characterization, cloning, sequence analysis and heterologous gene expression.* ***J. Biol. Chem.*** **271,** in press.

Protein and cofactor requirements for acetyl-CoA synthesis or cleavage by the ACDS complex from *Methanosarcina barkeri*.

In **P1** we elucidated the exact requirements for acetyl-CoA synthesis and cleavage by the ACDS complex (equation [1]) and determined the K_{eq} of equation [3] -- the overall net reaction (sum of Eq. [1] and [2]). By use of a resolved system we provided the first direct demonstration that the route of electron transfer to and from the ACDS complex *during the acetyl-CoA synthesis and cleavage reactions* occurs via ferredoxin (designated Fd) according to equations [1] and [2].

[1] $CO_2 + 2\ Fd_{red}\ (Fe^{2+}) + CH_3\text{-}H_4SPt + CoASH + 2\ H^+ \rightleftharpoons$ acetyl-CoA + H_4SPt + $2\ Fd_{ox}\ (Fe^{3+})$ + H_2O

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

[3] $H_2 + CO_2 + CH_3\text{-}H_4SPt + CoASH \rightleftharpoons$ acetyl-CoA + $H_4SPt + H_2O$

In the above reactions H_4SPt and $CH_3\text{-}H_4SPt$ stand for tetrahydrosarcinapterin and *N^5-methyl*-tetrahydrosarcinapterin, respectively (these are functional equivalents of tetrahydrofolic acid and *N^5-methyl*-tetrahydrofolate in methanogens).

In **P1** we also demonstrated that a specific hydrogenase was required in order to catalyze Eq. 2. Further characterization of the *M. barkeri* hydrogenases in **P1**, confirmed that

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a separate hydrogenase (designated H_2ase-F_{420}) catalyzes the oxidation and reduction of the 8-hydroxy-5-deazaflavin redox cofactor F_{420}. We then went on to show for the first time that this hydrogenase is incapable of catalyzing the oxidation and reduction of ferredoxin, and that H_2ase-F_{420} was unable to reconstitute acetyl-CoA synthesis with electrons supplied from H_2. We discovered instead, that a second, membrane-associated hydrogenase activity (at present designated H_2ase-Fd), [probably via one or more additional electron transfer steps (one apparently being via cytochrome b)], catalyzes the oxidation and reduction of ferredoxin, but that this hydrogenase does not catalyze the oxidation and reduction of F_{420}. This result, coupled with recent work of Kemner and Zeikus (1993; 1994) identified the role of that previously ambiguous hydrogenase (Weiss & Thauer, 1993). Kemner and Zeikus showed that a membrane associated hydrogenase is linked to cytochrome b reduction, but that the purified hydrogenase could not catalyze *direct* reduction of ferredoxin (implying a role for other, as yet unidentified electron transport components). This membrane-associated hydrogenase appears to be the same enzyme (Fd-H_2ase) that we demonstrated to be associated with ferredoxin reduction. However, the number and identity of other protein(s) involved in electron transfer to and from H_2ase-Fd has not been established. It is still unclear as to which membrane protein(s) couple to ferredoxin in the transfer of electrons to and from the H_2ase-Fd. This membrane-bound H_2ase also may be associated with electron transfer to the heterodisulfide reductase complex involved in methane biosynthesis (Heiden, *et al.* 1993). It is still unknown how electron transfer is *partitioned* between methane synthesis and acetyl-CoA synthesis in methanogens growing in the absence of acetate as carbon source.

In **P1** we presented overwhelming evidence in support of the premise that ferredoxin is the physiologically relevant electron carrier to and from the ACDS complex. We showed that with hydrogen as the source of reducing eqiuvalents, the dependence of acetyl-CoA synthesis on ferredoxin was absolute. No detectable acetyl-CoA synthesis occurred in otherwise complete reaction mixtures that were lacking in ferredoxin (H_2-saturated mixtures at pH 8.0 containing ACDS complex, Fd-H_2ase, CH_3-H_4SPt, CoA, and CO_2/HCO_3^-). Addition of ferredoxin initiated immediate acetyl-CoA synthesis. Ferredoxin transfers electrons to and from one or more Fe/S centers, and either directly or indirectly to and from the Co^{2+}/Co^{1+} corrinoid center and substrates being transformed by the ACDS complex. Ultimately, we require specific knowledge of the identity and number of sites for electron transfer between ferredoxin and the ACDS complex.

It was only until after we developed in **P1** a means for analysis of acetyl-CoA cleavage reactions in which *all* of the substrates were the physiological ones, that we were able to then measure the ovrall equilibrium constant for the physiologically relevant process (Eqn 3.). The measurement of the K_{eq} of this reaction was very important because the results have profound significance to our understanding of the physiology of methanogenesis from acetate, and of acetate synthesis in methanogens. The results explain *quantitatively* why synthesis of acetate is favorable in cultures grown with hydrogen, or when hydrogen is present in the environment, but that cleavage is dictated at low levels of hydrogen, such as in cultures of acetate-cleaving *M. barkeri*, and in fresh-water environments in which availability of hydrogen is also very low. These findings are summarized in **P1**, and also shown here in Table 1. Furthermore, it is now possible to use these results for semi-quantitative assessment of electron flow to competing pathways of CO_2 assimilation versus methanogenesis, and we are now able to predict how energy is partitioned between these pathways under different

environmental conditions.

An EPR active center and its involvement in acetyl-CoA synthesis and cleavage by the methanogen ACDS complex.

EPR studies have been used extensively to study the reaction of carbon monoxide with the acetyl-CoA synthesizing ACS system from *Clostridium thermoaceticum* -- primary by S.W. Ragsdale and P.A. Lindahl and their associates. Much of that work has been interpreted to support the idea that the enzyme-carbonyl species that generates the so-called NiFeC EPR signal is a physiologically relevant one in the mechanism of acetyl-CoA synthesis from CO_2. The ACDS complex from *Methanosarcina thermophila* has also been the subject of studies that employed EPR spectroscopy (Terlesky *et al.*, 1986; 1987; Lu, *et al.*, 1994). Although a number of similarities were observed between the clostridial and the methanogen systems, certain other aspects were in conflict with each other, and conclusions that one could make, especially about the methanogen ACDS complex, were unclear. Consequently, we undertook several experiments with the hope of clarifying some of the abiguities.

Table 1: Influence of H_2 Levels on the Direction of Acetyl-CoA Synthesis and Cleavage

$[H_2]$ atm	environment	$\frac{[\text{acetyl-CoA}]^a}{[\text{CoASH}]}$
2.4×10^{0}	standard culture[b]	3.8×10^{3}
2.0×10^{-3}	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).

Three important new discoveries were reported **P2**. These are as follows:

1. We found that neither CoA nor acetyl-CoA has any significant effect on the primary EPR signal (signal 1) generated by the *M. barkeri* ACDS complex in the presence of saturating carbon monoxide. [This signal has been observed by others both in the clostridial system and in the ACDS complex from *M. thermophila*. However, our results differed significantly from those in clostridia (Ragsdale *et al.*, 1985), in which CoA, but not acetyl-CoA had an influence on the spectrum, and also differed from those in *M. thermophila* in which effects from acetyl-CoA, but not CoA were found (Terlesky *et al.*, 1987.)] In addition, we showed that a second signal (signal 2), that had been observed by others at widely divergent levels in various clostridial and methanogen preparations, was produced as an artifact of storage. Although this signal has been a prominent feature in some recent studies from other laboratories, our results suggest that it is difficult to justify further studies on this form of the enzyme.
2. We demonstrated in **P2** that the natural methyl donor CH_3-H_4SPt was the only physiological substrate that had any influence on the EPR spectrum. Addition of relatively low concentrations of CH_3-H_4SPt to the EPR-active enzyme-carbon monoxide adduct resulted in nearly complete loss of the EPR signal. This indicated that under these conditions an EPR-silent enzyme-acetyl intermediate in acetyl-CoA synthesis may be formed. These results are highly encouraging because they indicate a high probability that further studies will succeed in revealing much more information about this critical (but somewhat elusive) acetyl-enzyme species. Detailed knowledge of the characteristics of this enzyme-acetyl is highly important to our understanding of the overall mechanism of acetate cleavage and physiological functions of the ACDS complex in methanogens.

3. We discovered that the EPR-silent form of the ACDS complex (generated in the reaction with both CO and CH_3-H_4SPt) is highly reactive in acetyl-CoA synthesis. As shown in **P2**, this form of the enzyme is more reactive by a factor of 44 than any other species of the ACDS complex yet reported. This finding is very important because our ultimate understanding of the mechanism of the overall reaction must involve knowledge of the individual step(s) that are rate-limiting. Such steps can be identified and studied only when the reaction conditions are established such that maximal rates of synthesis and cleavage, i.e., comparable to those observed *in vivo*, are produced.

Assignment of partial reactions catalyzed by the ACDS complex to individual protein subcomponents.

As a result of our work in this past year it is now possible to assign unique enzymatic activities to all three protein subcomponents of the ACDS complex. A necessary prerequisite to these studies was to establish conditions whereby the ACDS complex could be quantitatively dissociated without destroying catalytic activity in the various partial reactions. A problem with previous attempts to do this (Abbanat & Ferry, 1991) was that in those studies the ACDS complex was not quantitatively dissociated and all of the individual components were not fully resolved. We worked out a procedure of limited proteolysis which allowed us to efficiently dissociate the enzyme complex, and to obtain purified preparations of all of the major subunits and/or subcomponent proteins. The results of these studies are summarized next, and are fully described in **P3**.

The overall reaction of acetyl-CoA synthesis or cleavage may be divided into several possible partial reactions. One of these is CO:acceptor oxidoreductase (CO dehydrogenase). This reaction may be written as follows,

[4] $CO_2 + 2H^+ + 2e^- \rightleftharpoons CO + H_2O$

and, it has been known for over ten years that this reaction is catalyzed by an $\alpha_2\varepsilon_2$ subcomponent of the ACDS complex (Krzycki & Zeikus, 1984). We have now been able to assign two additional reactions to two other protein subcomponents: (a) CH_3-H_4pteridine:cob(I)amide-protein methyltransferase (Reaction [5]), catalyzed by a protein component that contained the intact γ subunit and fragments of the δ subunit, and (b) acetyltransferase (Reaction [6]), catalyzed by a truncated form of the β subunit. Furthermore, we showed that the ability of the ACDS complex to catalyze reaction 1 was directly proportional to level of intact 60 kDa β subunit that remains during proteolytic digestion. The results demonstrated for the first time that the β subunit contains the site(s) for binding of CoA and acetyl-CoA, and further indicated that acetyl-enzyme formation occurs on the β subunit.

[5] cob(I)amide-protein + CH_3-H_4SPt + H^+ $\rightleftharpoons$ CH_3-cob(III)amide-protein + H_4SPt

[6] acetyl-CoA + 3'-dephospho-CoA $\rightleftharpoons$ *S*-acetyl-3'-dephospho-CoA + CoA

In **P3** we also carried out equilibrium measurements on the reaction catalyzed by the γδ sub-

component. We found that methyl group transfer from the ACDS corrinoid component to the cellular pool of H_4SPt (physiological concentrations) was both rapid and thermodynamically favorable. We thereby demonstrated the significance of the value of K_{eq} for equation [5] to the *in vivo* process of methyl group transfer.

As revealed by SDS PAGE, low levels of chymotrypsin act selectively on the 60 kDa β subunit to produce a truncated subunit (β*), of about 50 kDa, that possesses a high specific activity of acetyltransferase. Similar results were also found with other proteases such as bromelain and trypsin. Thus, the presence is suggested of a structural region of the β polypeptide that is highly susceptible to general proteolytic attack. In experiments in which the extent of digestion was varied, overall acetyl-CoA synthetic activity is lost in direct proportion to the loss of the intact β subunit. However, more extensive digestion was required to bring about dissociation of the complex. Loss of the intact 48 kDa δ subunit occurred as the level of digestion was further increased, and correlated with the ability to obtain high resolution of all three protein components (and with the absence of residual undissociated enzyme). Therefore, the results allow us to formulate the hypothesis that integrity of the δ subunit may be essential for maintaining the quaternary structure of the enzyme complex. Furthermore, since loss of acetyl-CoA synthetic activity occurs without overall dissociation of the enzyme complex, the involvement of the β subunit apparently extends beyond that of a structural role or that of the ability to carry out acetyl transfer. Further investigations are needed to explain the precise mechanism for the decline in overall ACDS activity coinciding with the loss of the intact β subunit.

Kinetics of acetyl transfer and the nature of the acetyl-enzyme intermediate

In as yet unpublished work we have initiated studies on the acetyltransferase reaction (Eq. [6]) in the intact ACDS complex and with the isolated β subunit in order to establish the mechanism of acetyl group transfer. The rationale for such studies on individual partial reactions is ultimately to establish an overall picture of the far more complex kinetic mechanism of the complete reaction of acetyl-CoA synthesis and cleavage. Furthermore, highly useful information will be derived from these studies that will ultimately tell us about the chemical nature of the critical (but somewhat elusive) acetyl-enzyme adduct that must be formed in the reaction on the β subunit.

In order to study acetyltransferase catalysis by the ACDS complex, it was first necessary to develop a routine and reliable method for analysis of this activity. The new assay was based on the analogous reaction of [3′-^{32}P]CoA/acetyl-CoA exchange. The method we developed uses an HPLC separation step, however, radioactively labeled coenzyme A, which is unavailable commercially, is not required. Instead the coenzyme A analog 3′-dephospho-CoA is used. Coenzyme A, acetyl-CoA, dephospho-CoA, and acetyl-dephospho-CoA are all readily distinguishable and quantifiable by our HPLC analysis method. We have characterized the kinetic mechanism of acetyltransferase by measuring the initial rate of formation of 3′-dephospho-acetyl-CoA and CoA as a function of acetyl-CoA concentration ([A]) at two levels of dephospho-CoA ([B]). The results indicated that the reaction proceeds via a modified ping pong mechanism. Figure 1 in the main body of the proposal shows the kinetic mechanism and the equation that was found to fit the initial velocity data. The kinetic constants, K_m^A and K_m^B were experimentally determined to be 28.5 (± 12.1) μM and 41.7 (± 7.3) μM, respectively. The V_{max}^f was calculated to be 72.8 (± 10.3) μmol-min^{-1}-mg enzyme^{-1}.

Two major findings from these studies are that 1. the V_{max} corresponds a significantly

higher specific activity for acetyl transfer than had been reported in any of the isotope exchange experiments so far published. And, 2. that a *cooperative* influence of acetyl-CoA was firmly established that has never before been demonstrated in any previous study. This finding is likely to have major significance to our eventual understanding of the mechanism of the overall reaction. It means that there exists an apparent requirement for a second molecule of acetyl-CoA for the acetyl transfer reaction to occur. This would be consistent with one of a limited number of enzymological possibilities that we plan to distinguish in future experiments [e.g., an *allosteric* site for binding of a second molecule of acetyl-CoA, the requirement for a second acetyl-transfer step (in this case the initially cleaved acetyl-CoA molecule might or might not subsequently dissociate from the enzyme), or the presence of two active sites or the binding of two molecules of acetyl-CoA at a single active center.]

And finally, we have also made significant progress toward solution of the Haldane relationships (see below) that will ultimately provide a value for the *thermodynamic stability* of the acetyl-enzyme intermediate. Further studies on the reverse reaction are still needed before this work will be completed.

$$\frac{V^f_{max}{}^2 K_m^P K_m^Q}{V^r_{max}{}^2 K_m^A K_m^B} = K_{eq} \text{ (overall reaction,equation[6])}$$

$$\frac{V^f_{max} K_m^P}{V^r_{max} K_m^A} = K_{eq} \quad (\textit{E-acetyl}) \qquad \frac{V^r_{max} K_m^B}{V^f_{max} K_m^Q} = K_{eq} \quad (\textit{E'-acetyl})$$

The above equations describe the K_{eq} for the enzyme-acetyl formation in equations [7] and [8]:

[7] dephospho-acetyl-CoA + Enzyme ⇌ dephospho-CoA + Enzyme-acetyl

[8] acetyl-CoA + Enzyme ⇌ CoA + Enzyme-acetyl

Since it is the yet uncharacterized acetyl-enzyme intermediate that is the actual species that undergoes C-C bond cleavage, the importance of determining the identity and properties of this proximal intermediate is unmistakable.

Purification and characterization of the acetyl-CoA decarbonylase/synthase complex from *Archaeoglobus fulgidus*.

These experiments are in the process of being compiled into a manuscript. They topic is are slightly outside of our original research plan, however we chose to study this, because if *A. fulgidus* did possess the ACDS complex, it would be the first instance in which that enzyme complex would have been found in a non methanogen. We had all of the necessary equipment and biochemical techniques with which to do the study, and we were able to obtain the cells from Dr. P. Hartzell, so the rewards for doing the work were greater than the costs.

We found that *A. fulgidus* does indeed have an ACDS complex, and that it resembles closely the methanogen ACDS complex both in structure and in catalytic properties. Moreover, antibodies made to subunits of the enzyme complex from *M. barkeri* crossreact with subunits of