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Enzymology of Acetoclastic Methanogenesis August 1994-July 1997

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Specific Aims

The overall aims of the previous proposal were to identify intermediates in the process of methane formation from acetate. The specific aims were:

- (i) to characterize the mechanism of acetyl-CoA conversion to $\text{CH}_3\text{-H}_4\text{MPT}$, CO, and CoA and elucidate the mechanism of CO oxidation by the *Methanosarcina thermophila* CO dehydrogenase/acetyl-CoA synthase (CODH/ACS), and
- (ii) to study the mechanism of methyl-CoM reduction to methane by developing an activation protocol for methyl-CoM reductase (MCR) and identifying intermediates in the MCR mechanism.

We also made significant progress toward accomplishing two other specific aim that were not included in the original proposal. These were:

- (iii) to characterize the *M. thermophila* heterodisulfide reductase (HDR), and
- (iv) to characterize the methyltetrahydromethanopterin:Coenzyme M methyltransferase from *Methanosarcina mazei* strain Gö1 in collaboration with Gerhard Gottschalk's group.

1. Characterizing the mechanism of acetyl-CoA conversion to $\text{CH}_3\text{-H}_4\text{MPT}$, CO, and CoA and elucidating the CO oxidation mechanism

1.1. Characterization of CODH/ACS from *M. thermophila*.

In collaboration with Professor Greg Ferry at Penn State, we characterized the CODH/ACS (12) from *M. thermophila*. The properties of the CODH/ACS component of the multienzyme complex from *M. thermophila* were found to be highly similar to those of other CODHs. The properties of the metal centers of the corrinoid iron-sulfur proteins (CFeSPs) from *M. thermophila* and *Clostridium thermoaceticum* were also found to be highly similar in work published before the grant was funded (6). The similarity between the spectroscopic properties of metal centers of the acetogenic and the methanoarchaea proteins suggests that the methylnickel and iron-carbonyl (8, 13) intermediates that were characterized for the *C. thermoaceticum* CODH/ACS are likely to be present as catalytic intermediates in the acetoclastic reactions. In addition, it indicates that the metal centers in the acetogenic and methanogenic proteins share similar structures. It also supports the concept that the initial steps in methane synthesis from acetic acid occurs by a direct reversal of the final steps in acetogenesis. Finally, it provides biochemical evidence that the Wood-Ljungdahl pathway of autotrophic growth and the pathway of acetoclastic methanogenesis may have evolved from a progenitor of *archaea* and *bacteria* that predated the evolutionary branching of *archaea* and *bacteria*. Wächtershäuser recently described experiments that support the concept that the Wood-Ljungdahl pathway may have been the first metabolic cycle (18).

1.2. Characterization of ferredoxin from *M. thermophila*.

In collaboration with Ferry's group, we also characterized the ferredoxin (Fd) that acts as the electron acceptor from CODH/ACS (4). This was found to be a rather typical Fd containing two $[\text{Fe}_4\text{S}_4]$ units that interact strongly through a dipolar mechanism when both clusters are reduced.

1.3. Characterization of the iron-sulfur flavoprotein from *M. thermophila*

In collaboration with Ferry's group, we characterized an iron-sulfur flavoprotein (Isf) from *M. thermophila* that had been recently suggested to be involved in electron transfer reactions involving CO metabolism (10). Isf was found to contain one FMN and one $[\text{4Fe-4S}]^{2+/1+}$ cluster per monomeric unit. The midpoint potential (E_m) value for the FMN/FMNH₂ couple was found to be -0.277 V. Neither the red or blue semiquinone was observed during potentiometric titrations. The reduced $[\text{4Fe-4S}]$ cluster had g values of 2.06, 1.94 and an unusual split signal at its lowest g value at 1.86 and 1.82. The E_m value for the 2+/1+ redox couple of the cluster was found to be -0.394 V. Although the existence of a FMN semiquinone radical is thermodynamically unfavorable,

low amounts of the radical were observed in freeze quench EPR studies when Isf was reacted with CO and the CO dehydrogenase/acetyl-CoA synthase complex from *M. thermophila*. A paper was been submitted describing these results (3).

1.5. Characterization of the mechanism of the CODH from *M. thermophila*

We grow *M. thermophila* under pH controlled conditions that yield high specific activities of the enzymes we are studying, CODH/ACS, HDR, and MCR. Eisuke Murakami, a graduate student who is working on CODH/ACS, routinely isolates the CODH/ACS complex with CODH and ACS

Fig. 2, CODH Mechanism

activities of ~ 500 and 0.3 U/mg, respectively; these are both $\sim$ six-fold higher than the values reported earlier (14, 17). The ACS activity is ~ 10 -fold higher than the value measured for the *M. soehngenii* enzyme (7). The spin intensity of the NiFeC EPR signal is routinely observed to be as high as ~ 0.7 spins per mol of enzyme, which is even higher than that for the *C. thermoaceticum* enzyme.

Eisuke Murakami has nearly completed the elucidation of the CO oxidation reaction by steady-state and presteady-state kinetics. His results are in overall agreement with those recently determined for the *C. thermoaceticum* enzyme (Fig. 2) (15). This work will be submitted for publication in the next few months when the kinetic simulations are completed.

2. Activating and characterizing methyl-CoM reductase

Don Becker in my laboratory recently developed a reliable and simple procedure to activate the MCRs from *M. thermophila* and *M. thermoautotrophicum*. He found that adding sodium sulfide to the culture before harvesting quantitatively converted the enzyme to a state (previously labeled MCR_{ox1}) that is inactive, but can be reduced to the active form (labeled MCR_{red1}). The MCR_{ox1} state is relatively stable and MCR in this state can be purified to homogeneity and MCR can be activated at any stage during the purification procedure. In addition, the enzyme can be stored in the MCR_{ox1} form at liquid nitrogen temperatures, thawed, and activated. The specific activities of the activated *M. thermophila* and *M. thermoautotrophicum* MCR, 7 U mg^{-1} and 40 U mg^{-1} , respectively, are higher than those required to explain the *in vivo* rate of methane formation! Don has found that these activities represent between 40-70% active sites. He has characterized the activation process using EPR and UV-visible spectroscopic methods. A manuscript describing this work has been submitted (3).

3. Purification and properties of the heme containing heterodisulfide reductase from *Methanosarcina thermophila*

Mihaela Simianu, a postdoctor in my laboratory, has purified the heterodisulfide reductase (HDR) from acetate-grown *M. thermophila* to homogeneity. The purified protein contained two subunits with apparent molecular masses of 52.5 and 28 kDa. The protein contained two types of heme b associated with the small subunit and two $[Fe_4S_4]$ clusters that apparently are located in the large subunit per α, β dimer. The *M. thermophila* HDR has an extremely high specific activity of ~ 200 U mg^{-1} . The highest specific activity previously reported for any HDR was 60 U mg^{-1} for the *M. barkeri* enzyme (9). Based on gel exclusion chromatography behavior, the enzyme had an apparent molecular mass of 155 ± 20 kDa; however, sedimentation equilibrium experiments revealed the presence of a significant amount of lipid associated with the purified enzyme resulting

in a high partial specific volume ($0.9 \text{ cm}^3/\text{g}$). The two b-hemes had distinguishable electrochemical and R. Raman spectroscopic properties; the midpoint potential (E_m) of the $\text{Fe}^{3+/2+}$ couple of one was -20 mV and that of the other was -200 mV . The two clusters in HDR was also distinguishable by electrochemistry, with E_m values of -100 and -440 mV . The two clusters apparently are located in close proximity resulting in EPR spectra of the fully reduced protein that reveal strong dipolar coupling similar to those observed for 8 Fe ferredoxins. CO and H_2 could reduce the enzyme in the presence of ferredoxin and CODH or hydrogenase, respectively, and could also reduce the heterodisulfide substrate to free Coenzyme M and mercaptoheptanoylthreonine phosphate. This activity was stimulated by an electron carrier protein(s) associated with the membrane. A manuscript describing this work has been submitted (16).

4. Characterizing the N^5 -methyltetrahydromethanopterin(H_4MPT): coenzyme M methyltransferase.

In collaboration with Gerhard Gottschalk's group, we purified and characterized a membrane-bound cobalamin-containing methyltransferase (MeTr) of *Methanosarcina mazei* strain G61 (11) that couples the methylation of CoM-SH by methyltetrahydrosarcinopterin ($\text{CH}_3\text{-H}_4\text{SPT}$) to the translocation of Na^+ across the cell membrane (2). The enzyme was found to share similarities with the analogous enzyme from *M. thermoautotrophicum* strain Marburg (5). The enzyme requires ATP and a reductant, such as Ti(III) for activity (1, 2, 5).

It was shown to contain a base-on cobamide with an E_m for the $\text{Co}^{2+/1+}$ couple of -426 mV . Besides the cobamide, MeTr was shown to contain at least one iron-sulfur cluster that is essential for activity. The Fe-S cluster appeared to be a $[\text{4Fe-4S}]^{2+/1+}$ type with an E_m value of -215 mV . When the methyltransferase was performed at various controlled redox potentials, it was shown that the enzyme is activated by a one-electron reduction with half-maximum activity occurring at -235 mV in the presence of ATP and -450 mV in its absence. No activation was observed when ATP was replaced by other nucleoside triphosphates or nonhydrolyzable ATP analogs.

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Simianu, M., Brewer, J.M., & Ragsdale, S.W. Purification and properties of the heme and iron-sulfur containing heterodisulfide reductase from *Methanosarcina thermophila*, submitted.

Abstracts to meetings during funding period:

Ragsdale, S.W. (1997) Discovery Of A Bio-Organometallic Reaction Sequence Involving Vitamin B₁₂ And Nickel/Iron-Sulfur Clusters, Fourth European Symposium on Vitamin B₁₂ and B₁₂-proteins, Sept. 2-6.

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