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Enzymological Studies of One-Carbon Reactions in the Pathway of Acetate Utilization by Methanogenic Bacteria

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Several enzymes in the pathway of acetate conversion to methane and carbon dioxide have been purified from *Methanosarcina thermophila*. The mechanisms of these enzymes are under investigation utilizing biochemical, biophysical and molecular genetic approaches. Acetate kinase and phosphotransacetylase catalyzes the activation of acetate to acetyl-CoA. The primary structure of these enzymes will be determined through cloning and sequencing of the genes. Two protein components of the CO dehydrogenase complex are under investigation. The metal centers of each component have been characterized using EPR. Cloning and sequencing of the genes for the two subunits of each component is in progress. Results indicate that the Ni/Fe-S component cleaves the C-C and C-S bonds of acetyl-CoA followed by oxidation of the carbonyl group to carbon dioxide and transfer of the methyl group to the Co/Fe-S component. The enzymes and cofactors involved in transfer of the methyl group from the Co/Fe-S component to coenzyme M will be purified and characterized. Ferredoxin is an electron acceptor for the Ni/Fe-S component and also serves to reductively reactivate methylreductase which catalyzes the demethylation of methyl coenzyme M to methane. This ferredoxin is being characterized utilizing EPR and RR spectroscopic methods to determine the properties of the Fe-S centers. Genes encoding this and other ferredoxins have been cloned and sequenced to determine the primary structures. Carbonic anhydrase is being purified and characterized to determine the function of this enzyme in the pathway.

I. PROGRESS REPORT.

A. Biochemistry of the CO dehydrogenase complex from *M. thermophila*. *Methanosarcina thermophila* utilizes acetate to form CH_4 and CO_2. This pathway involves cleavage of acetate with transfer of the methyl group to 2-mercaptoethanesulfonic acid (CH_3-S-CoM); oxidation of the carbonyl group to CO_2 supplies the electron pair required for reductive demethylation of CH_3-S-CoM to CH_4. The enzyme mechanisms underlying these reactions are unresolved. It has been postulated that the CO dehydrogenase (CODH), abundant in acetate-grown methanogens, cleaves the C-C and C-S bonds of activated acetate (acetyl-CoA). Recently, acetyl-CoA synthesis and cleavage activity was reported for the CODH enzyme complex from *M. thermophila* as were the properties of the methyl coenzyme M methylreductase (see attached reprints).

The CODH enzyme complex from *M. thermophila* consists of five subunits of 89, 71, 60, 58, and 19 kDa. The complex contains nickel, iron, acid-labile sulfide, and cobalt in a corrinoid cofactor. Two components of the complex have been resolved by anion-exchange chromatography of the complex in the presence of dodecyltrimethylammonium bromide (DTAB) and Triton X-100: a 200 kDa Ni/Fe-S protein with the 89- and 19-kDa subunits and a 100 kDa corrinoid/Fe-S (Co/Fe-S) protein with the 60- and 58-kDa subunits (see attached reprint). The Ni/Fe-S component contains Ni, Zn, Fe, and acid-labile sulfide while the corrinoid/Fe-S component contains Co, factor III (Coα-[α-(5-hydroxybenzimidazolyl)-Coβ-cyanocobamide], Fe, and acid-labile sulfide. In collaboration with Dr. Steve Ragsdale and Dr. Wei-Ping Lu of the University of Nebraska-Lincoln, we have used electron paramagnetic resonance (EPR) spectroscopy and electrochemistry to identify the types of metal centers present in the CODH enzyme complex and the isolated components, and have measured their midpoint redox potentials.

We have studied the properties of the Fe-S cluster(s) in the Ni/Fe-S component and in the CODH enzyme complex in an effort to understand the composition and the structure of the metal centers. Three species of Fe/S clusters were detected in the isolated Ni/Fe-S component. The dominant species is the g_{av} = 1.94 species with apparent g values at 2.05, 1.94, and 1.89. A g_{av} = 1.86 species appears at very low redox potentials (<-530 mV) with apparent g values at 1.97 and 1.75. A g_{av} = 1.85 species has a high redox potential (> 0 mV) with g values at 2.04, 1.82, and 1.71. The latter species is only detected at temperatures below 16°K. Similar EPR spectra were observed for the CODH enzyme complex. Midpoint reduction potentials were deduced from Nernst plots. The oxidized CODH enzyme complex exhibits a narrow rhombic signal with a g value centered at 2.015, suggesting a [3Fe-4S] cluster. A similar signal was observed with the CODH from *Methanosarcina barkeri*.

The cobamide in the Co/Fe-S component is a methyl carrier. The methyl group is transferred to HS-CoM probably with the assistance of a methyltransferase. Since only the Co^{1+} form of cobamide can accept the methyl group, the midpoint potential for the $Co^{2+/1+}$ couple of the protein is of importance in the reaction. Furthermore, the conformation of the cobamide in the protein, such as base-on or -off, may have implications in the chemical mechanism of the methyl transfer reaction. The spectrum of the isolated corrinoid/Fe-S component indicates a low spin Co^{2+}. Eight vertical lines in the spectrum arise from a hyperfine splitting due to the interaction with the cobalt nucleus (I = 7/2). The hyperfine splitting constant ($A_{z,Co}$) is 140 gauss (measured as the interval between each pair of lines), and there is no superhyperfine splitting from the nitrogen nucleus (I = 1/2) on the 5-hydroxybenzimadazole base. These observations demonstrate that a weak ligand, such as H_2O, replaces the nitrogen of the base at the axial fifth (low) position of the cobamide. This observation is consistent with the results from the UV-VIS spectrum. A similar EPR spectrum was observed for the CODH enzyme complex, suggesting that the coordination state of the cobamide is not changed after isolation of Co/Fe-S component from the complex. Redox titration of the $Co^{2+/1+}$ couple was performed with the CODH enzyme complex. The Nernst plot of the data suggests an E_m of -525 mV. The value is close to that reported for the Co/Fe-S protein from *C. thermoaceticum* (-505 mV). The Co/Fe-S component from *M. thermophila* also contains an Fe-S cluster. The spectrum for the Fe-S cluster is very similar to that of the clostridial protein suggesting that the iron cluster in the *M. thermophila* corrinoid/Fe-S component is a [4Fe-4S] cluster.

The NiFeC signal, generated from the interaction of CO with a metal cluster containing Ni,Fe and S in the CODH enzyme complex, is probably indicative of an intermediate in such reactions as the isotopic CO/acetyl-CoA exchange, CO oxidation, CO_2 reduction, and acetyl-CoA synthesis or hydrolysis. Study of the signal will provide information on the structure of the metal cluster(s) and its environment and catalytic role in the protein. After treatment with CO, the CODH enzyme complex exhibits the NiFeC signal at liquid nitrogen temperature (100°K) as reported previously. However, there are some differences between the spectra presented here and that reported before. A near axial signal with apparent g values of 2.05 and 2.03 is generated upon addition of CO. Computer simulation of the spectrum yielded g values of 2.059, 2.051, and 2.029. This is in contrast to the previous observation where a rhombic signal with apparent g values of 2.074, 2.05, and 2.03 was noted upon treatment with CO and converted to the near axial signal (signal II) after further addition of acetyl-CoA. The discrepancy is apparently not due to the presence or absence of ethylene glycol in the sample buffer. The signal was strong with 0.6 spin per mol of the CODH enzyme complex.

A similar axial NiFeC signal was observed with the isolated Ni/Fe-S component,indicating that the isolated Ni/Fe-S component itself is sufficient to generate the NiFeC signal and the signal is not affected by the presence of other components, such as the Co/Fe-S protein. Addition of acetyl-CoA increased the signal intensity about two-fold. The spin quantitation of the spectrum was low (about 0.09 spin/mol of protein) compared with that for the complex. This is in agreement with the observation of low Ni content (approx. 0.2 Ni/dimer) in the isolated protein.

B. Cloning and analysis of genes from *M. thermophila* encoding ferredoxin. A mixed 17-mer oligonucleotide corresponding to a sequence in the N-terminus of a ferredoxin from Methanosarcina thermophila was used to probe a lambda gtll library prepared from M. thermophila genomic DNA. Positive clones contained either a 5.7-kilobasepair or 2.1-kilobasepair EcoRI insert; these inserts were approximately the same size as fragments identified in Southern blot analyses of genomic DNA digested to completion with EcoRI. An open reading frame (fdxA) located within the 5.7-kilobasepair insert had a deduced amino acid sequence that was identical to the first 26 N-terminal residues reported for a ferredoxin isolated from M. thermophila, with the exception of the initiator methionine. FdxA had the coding capacity for a 6,230-Da protein which contained two domains of cysteine clusters typical of proteins which coordinate [3Fe-XS] or [4Fe-4S] clusters. An open reading frame (ORF-1) located within the 2.1-kbp EcoRI fragment had the potential to encode a small protein (5,850 Da) which contained eight cysteines spaced in two domains and could coordinate either [3Fe-XS] or [4Fe-4S] clusters. The similarities between the predicted ORF-1 protein and 2[4Fe-4S] ferredoxins suggested that ORF-1, if expressed, may encode a second ferredoxin in M. thermophila. FdxA and ORF-1 were present as single copies in the genome and the two sequences were separated by at least 7.0 kilobasepairs in M. thermophila.

C. Purification and characterization of carbonic anhydrase from *M. thermophila*. Carbonic anhydrase activity is expressed when cells are shifted to growth on acetate suggesting a role for this enzyme in acetate conversion to methane. Thus, the enzyme was purified and characterized to learn more of its properties.

About 80% of carbonic anhydrase activity was recovered in the soluble fraction after ultracentrifugation of cell extract for 2 hours at 130,000 x g. The enzyme was purified over 10,000 fold from cell extract to apparent homogeneity as indicated by the presence of a single protein band on a denaturing gel. The specific activity of the purified enzyme (5175 ± 443 units/mg) was comparable to that of the human carbonic anhydrase II which was 7870 ± 574 units/mg in the assay system used. Gel filtration chromatography of the native carbonic anhydrase gave an estimated M_r = 84,000. SDS gel electrophoresis revealed one protein band with a M_r = 40,000 suggesting that the native enzyme was purified

as a α_2 homodimer. Also, N-terminal analysis only detected one N-terminal sequence in the first 26 amino acids analyzed. The N-terminus showed some identity (27-31% and 39% overall) to a centrally located peptide common to all 4 human carbonic anhydrase isozymes sequenced so far. The carbonic anhydrase from *M. thermophila* lost little activity when incubated for 15 minutes at 65°C. After preincubation at the same temperature HCA II had already lost almost all of its initial activity. The inhibitor constants of various compounds known to inhibit carbonic anhydrase activity noncompetively in other organisms were determined for the enzyme and compared to those obtained for HCA II. Inhibitors of the sulfonamide type were 10^3 - 10^4 times less effective inhibitors for the enzyme from *M. thermophila* than for HCA II. Monovalent ions were also less effective, however to a lesser extent (in average only ~ 10-fold). The enzyme was especially insensitive to iodide.

D. Formate dehydrogenase from *M. formicicum*. The overlapping fdhA and fdhB genes of Methanobacterium formicicum, which encode the α and β subunits of formate dehydrogenase, were cotranscribed as part of an approximately 4.5-kb transcript. An additional gene (fdhC) upstream of fdhA was cotranscribed with fdhA and fdhB. The deduced amino acid sequence suggested fdhC has the potential to encode an hydrophobic polypeptide with a calculated molecular weight of 29,417. An hydropathy plot of the hypothetical polypeptide indicated several potential membrane-spanning regions. The putative fdhC gene product had 28% identity with the deduced amino acid sequence of the nirC gene from Salmonella typhimurium. Northern (RNA) blot analyses, primer extension assays, and DNA sequence analysis located a transcription start site 268 bp upstream of the initiation codon of fdhC. A sequence identical to the consensus promoter sequence for methanogenic organisms was situated between -35 and -25 bp from the proposed transcription start site. In addition to the 4.5-kb transcript, Northern blot analyses detected a 1.1-kb transcript using a fdhC-specific probe and a 3.4-kb transcript using either fdhA- or fdhB-specific probes. The levels of all three transcripts were significantly greater in cells grown in media supplemented with molybdate.

The pterin cofactor in formate dehydrogenase was identified as molybdopterin guanine dinucleotide (see reprint). The pterin, stabilized as the alkylated, dicarboxyamidomethyl derivative, was shown to have absorption and chromatographic properties identical to those of the previously characterized authentic compound. Treatment with nucleotide pyrophosphatase produced the expected degradation products GMP and carboxamidomethyl molybdopterin. The molybdopterin guanine dinucleotide released from the enzyme by treatment with 95% dimethyl sulfoxide was shown to be functional in the *in vitro* reconstitution of the cofactor-deficient nitrate reductase in extracts of the *Neurospora crassa* nit-1 mutant.

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