DOE/ER/13368--6

DE90 007660

ENZYMOLOGY OF ACETONE-BUTANOL-ISOPROPANOL FORMATION

Progress Report

February 1, 1989 - January 31, 1990

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DISCLAIMER

January 1990

PREPARED FOR THE U.S. DEPARTMENT OF ENERGY

UNDER GRANT NUMBER DE-FG05-85ER13368

I. Research Objectives.

The objectives of the project are to elucidate first the molecular properties of solvent-forming enzymes and then to apply the information gained from the enzymological study to elucidate the control mechanisms for the solvent-producing pathways and the expression of solvent-production genes.

Our research primarily involves two strains of Clostridium beijerinckii (Clostridium butylicum): C. beijerinckii NRRL B593 which produces isopropanol in addition to acetone, n-butanol, and ethanol, and C. beijerinckii NRRL B592 which produces acetone, n-butanol and ethanol, but not isopropanol. More recently, we started to include another solvent-producing organism, Bacillus macerans, in our study. B. macerans produces a high level of acetone and ethanol under anaerobic conditions. Because B. macerans does not produce butyric acid and butanol, it has a less complex metabolic pathway than that of C. acetobutylicum and C. beijerinckii. This simpler metabolic pathway serves as a useful system for comparison in our study of the enzymology and regulation of solvent formation.

Specific objectives for the present report period were

(1) Separation and characterization of acetoacetyl CoA-reacting enzymes from acetone-producing organisms,

(2) Purification and characterization of CoA-linked aldehyde dehydrogenase from C. beijerinckii,

(3) Purification and characterization of alcohol dehydrogenases from C. beijerinckii, and

(4) Cloning of the structural gene for the primary/secondary alcohol dehydrogenase of C. beijerinckii NRRL B593.

II. Summary of Scientific Progress.

(1) Separation and characterization of acetoacetyl CoA-reacting enzymes from acetone-producing organisms. Acetoacetyl CoA is a key metabolic intermediate in solvent-producing clostridia. It is the branch point for the pathway leading to the formation of butyric acid and butanol and the pathway leading to the formation of acetone and isopropanol. Characterization of enzymes involved in the conversion of acetoacetyl CoA to 3-hydroxybutyryl CoA (leading to butyric acid and butanol) and to acetoacetate (leading to acetone and isopropanol) is an important aspect of our research, as we aim to understand the mechanism that regulates the flow of acetoacetyl CoA into the two alternate pathways for solvent production. For this project, our emphasis has been on the enzyme(s) converting acetoacetyl CoA to acetoacetate.

We previously identified in C. beijerinckii a phosphate-dependent acetoacetyl CoA-utilizing activity which is independent of thiolase and phosphotransacetylase (PTA) activities. The reaction appears to be

Acetoacetyl CoA + Pi $\rightleftharpoons$ CoA + [acetoacetyl phosphate]

CoA has been identified as a product of the reaction, but the putative product acetoacetyl phosphate has not been reported previously and needs to be positively identified. During the report period, the enzyme responsible for this activity has been purified to homogeneity from C. beijerinckii NRRL B593 and characterized [see Appendix 1]. Results from a study of the enzyme's substrate specificity indicate that the enzyme may be designated as phosphotransbutyrylase (PTB). In summary, PTB of C. beijerinckii NRRL B593 has a native M_r of 205,000 and a subunit M_r of 33,000. Its activity is sensitive to pH changes within the physiological range of 6 to 8. It has a broad substrate specificity. At pH 7.5, the K_m values for butyryl CoA, acetoacetyl CoA, and acetyl CoA are 0.04, 1.10, and 3.33 mM, respectively. The V_{max} values with

butyryl CoA and acetoacetyl CoA are comparable, but the V_{max}/K_m is higher for butyryl CoA than for acetoacetyl CoA. If acetoacetyl phosphate is a significant metabolic intermediate, it would be energetically important because ATP may be formed from the compound through the action of a kinase.

In B. macerans, an active acetoacetyl CoA:acetate CoA-transferase has been measured, and it catalyzes a reaction analogous to that found in C. acetobutylicum. In C. beijerinckii, only a very low level of CoA transferase has been detected, but acetoacetyl CoA hydrolase and acetoacetyl CoA-reacting PTB have been identified. The property of the CoA transferase of C. beijerinckii and its physiological significance will remain a focus of our research.

(2) Purification and characterization of CoA-linked aldehyde dehydrogenase from C. beijerinckii. An aldehyde dehydrogenase is required for the conversion of acyl CoA to aldehyde in the production of butanol and ethanol. We have purified the CoA-linked aldehyde dehydrogenase from C. beijerinckii NRRL B592 to homogeneity and characterized its catalytic properties [see Appendix 2]. The aldehyde dehydrogenase has a native M_r of 100,000 and a subunit M_r of 55,000. Kinetic data indicate that the enzyme is responsible for the formation of both acetaldehyde and butyraldehyde and a ping pong mechanism is involved. The enzyme is active with either NAD(H) or NADP(H) as a coenzyme, but the K_m for NAD(H) is much lower than that for NADP(H). The enzyme is O_2-sensitive, but it could be protected against O_2 inactivation by dithiothreitol. The O_2 inactivated enzyme can be reactivated by incubating the enzyme with CoA in the presence or absence of dithiothreitol prior to assay.

(3) Purification and characterization of alcohol dehydrogenases from C. beijerinckii. The two strains of C. beijerinckii that we use in our study have very different alcohol dehydrogenases (ADHs). The isopropanol-producing strain (B593) has an NADP(H)-specific primary/secondary ADH, whereas the isopropanol-non-producing strain (B592) has two primary ADHs, one active with NADP(H) only and the other active with both NAD(H) and NADP(H).

We have characterized further the kinetic properties of the purified primary/secondary ADH from strain B593. True K_m values for physiological substrates have been determined, and kinetic data are consistent with a sequential mechanism. Inhibitor constants with alternate substrates have also been measured. A manuscript is being prepared to report the findings.

The NADH/NADPH-reactive ADH of strain B592 has been extensively purified. We expect to purify this ADH during the next project period and to make a comprehensive comparison of the enzymic properties between this primary ADH and the primary/secondary ADH of strain B593. The ADHs of strain B592 are O_2 sensitive and are inhibited by the chelating agents o-phenanthroline and 2,2'-dipyridyl. Recently, we observed a 5- to 10-fold increase in specific activities of ADH in cell-free extracts prepared from cells grown with glucose, instead of sucrose, as the carbon source. This change in growth medium also eliminated the large amount of capsular material which was always produced in the sucrose-based medium. We will measure other solvent-forming enzyme activities in extracts prepared from glucose-grown cells to see if there is a similar increase in enzyme activities.

(4) Cloning of the structural gene for the primary/secondary alcohol dehydrogenase of C. beijerinckii NRRL B593. Cloning of the alcohol dehydrogenase gene is in progress. From the N-terminal amino acid sequence we

obtained from the protein, two sets of oligonucleotide probes have been synthesized. One set of 17-mers is in the sense orientation (corresponding to amino acid residues 4 to 9), and the other set of 20-mers is in the antisense orientation (corresponding to amino acid residues 14 to 20). The fully degenerated probes are 192- and 256-fold in complexities, but we also used probes with 24- and 32-fold degeneracies for the two regions. These probes allowed us to identify putative DNA restriction fragments containing the adh gene sequence, and we have obtained clones presumed to contain the adh fragment in pUC18 plasmid. DNA sequencing will be performed to confirm the isolation of the adh gene. More recently, we started to use the oligonucleotide probes (primers) and the polymerase chain reaction (PCR) to generate DNA sequences containing the adh gene. The PCR products have been used as more sensitive and specific probes for the screening of recombinant clones. Once the adh gene is sequenced, we will focus on the upstream region to search for and study the control region for the solvent-production genes.

III. Publications.

All publications listed below are attributable to DOE funding.

A. Journal articles:

1. Thompson, D. K. and J.-S. Chen. 1990. Purification and properties of an acetoacetyl coenzyme A-reacting phosphotransbutyrylase from Clostridium beijerinckii ("Clostridium butylicum") NRRL B593. Appl. Environ. Microbiol. 56 (March issue)

2. Yan, R.-T. and J.-S. Chen. 1990. Coenzyme A-linked aldehyde dehydrogenase of Clostridium beijerinckii NRRL B592: purification and properties. (To be submitted)

B. Abstract:

1. Thompson, D. K., R.-T. Yan, M. B. Walker, and J.-S. Chen. 1989. Phosphotransbutyrylase, phosphotransacetylase and thiolase from Clostridium beijerinckii. Abstract Annual Meeting of American Society for Microbiology O-77, p. 317.

C. Thesis:

!. Thompson, D. K. 1989. Acetoacetyl coenzyme A-reacting enzymes in solvent-producing Clostridium beijerinckii NRRL B593. M.S. Thesis. Virginia Polytechnic Institute and State University, Blacksburg.

IV. Appendix

Articles #1 and #2 are included as Appendices #1 and #2.

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