ENZYMOLGY OF ACETONE-BUTANOL-ISOPROPANOL FORMATION

Progress Report

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I. Research Objectives

The long-term goal of the project is to understand the fundamental properties of biological solvent production so that it would be possible to prevent degeneration of producing strains, to allow positive control of the solventogenic switch, and to regulate the product pattern according to market needs. Our approach is to elucidate first the molecular properties of solvent-producing enzymes and then to apply the information gained from the enzymological study to investigate control mechanisms for the solvent-producing pathways and the expression of solvent-production genes.

Our research primarily involves two strains of Clostridium beijerinckii: C. beijerinckii NRRL B593 which produces isopropanol in addition to acetone, butanol, and ethanol, and C. beijerinckii NRRL B592 which produces acetone, butanol and ethanol, but not isopropanol. In more recent studies, we also included another solvent-producing organism, Bacillus macerans, which is a facultative anaerobe. B. macerans produces a high level of acetone and ethanol under anaerobic conditions. Because B. macerans does not produce butyric acid, butanol, or isopropanol, it provides a simpler system for our study of the acetoacetyl-CoA-reacting enzymes.

The objectives for the reporting period were

- (1) To characterize the distinct types of alcohol dehydrogenases.
- (2) To purify and characterize acetoacetyl-CoA-reacting enzymes.
- (3) To clone and sequence the gene encoding the primary/secondary alcohol dehydrogenase of C. beijerinckii NRRL B593 and to search for the promoter region for solvent-production genes.

II. Summary of Scientific Progress.

- (1) Alcohol dehydrogenases (ADHs).

(a) Primary/secondary ADH. A primary/secondary ADH has been purified to homogeneity from two strains of Clostridium

beijerinckii (strains NRRL B593 and NESTE 255). The two ADHs exhibited similar structural and catalytic properties. The sequence of the first 21 amino acid residues is identical between the two ADHs, and both enzymes are oxygen-sensitive when in the crude extract but are stable in air after purification. However, their kinetic constants (k_{cat}/K_m) toward alternative substrates differ to some extent (see Table I), suggesting the presence of some structural differences between the two ADHs.

The structural gene for the ADH of C.

beijerinckii NRRL B593 has been cloned and sequenced (see section (3) below), and a comparison of the deduced amino acid sequence with those of other medium-chain ADHs revealed a unique feature. The primary/secondary ADHs from two obligate anaerobic bacteria, C. beijerinckii and Thermoanaerobium brockii, both lack a stretch of 18 amino acid residues that correspond exactly to the loop containing ligands for the structural zinc atom in horse liver ADH (see Rifaat and Chen, Appendix #1). The loop is also situated at the opening of the substrate-binding pocket. Because the substrate specificity and the thermal stability of the two primary/secondary ADHs differ significantly from the family of medium-chain ADHs, it should be interesting to find out whether the loop region or the structural zinc atom plays any role in the determination of the substrate specificity or thermal stability. Site-specific mutation of the cloned adh gene should provide a means for testing this aspect.

A comparison of the k_{cat}/K_m values with respect to aldehydes and ketones as substrates for the alcohol-forming reactions of the primary/secondary ADHs purified from C. beijerinckii suggests that the catalytic efficiency is a function of the size of the larger alkyl substituent on the carbonyl group (see Table I). A similar catalytic efficiency (2,000 to 3,000 for the two ADHs) was observed with 2-butanone and propionaldehyde, both of which contain an ethyl group. A comparable catalytic efficiency (between 100 and 200) was observed with 2-pentanone and butyraldehyde, which share a propyl group. In each of the two pairs of substrates, the other

ADH), whereas the other is active with both NADH and NADPH (the D/P-ADH). The two primary ADHs differ in thermal stability, and their activity responds differently to changes in the ionic strength. We focused on the D/P-ADH during this reporting period. The D/P-ADH has been purified 130-fold to give nearly constant specific activities. Further chromatographic treatments lowered the specific activity. On SDS-PAGE, the 130-fold purified ADH and the further chromatographed but less active preparations all gave two closely spaced bands ($M_r \approx 40,000$). However, the relative amount of the two bands varied, and it appears that the faster moving band correlates with the activity. At present, we postulate that the slower moving band on SDS-PAGE is derived from inactive ADH. We will investigate this problem during the coming project period.

(2) Acetoacetyl-CoA-reacting enzymes.

During this reporting period, we purified to homogeneity 3-hydroxybutyryl-CoA dehydrogenase (catalyzing the first committed reaction toward butanol production) from C. beijerinckii NRRL B593 (see Colby and Chen, Appendix #4). We have characterized the kinetic property and the first 35 amino acid residues of the enzyme. Purification of the acetoacetyl-CoA:acetate/butyrate CoA-transferase is in progress. The CoA-transferase is stabilized by 1.5 M ammonium sulfate plus 20% (v/v) glycerol, which is a condition not required by all other solvent-forming enzymes. The physiological significance of this special requirement will be considered during the study of CoA-transferase.

(3) Cloning of alcohol dehydrogenase gene.

The structural gene for the primary/secondary ADH of C. beijerinckii NRRL B593 has been cloned and sequenced (see Rifaat and Chen, Appendix #1). The adh gene encodes a polypeptide of 351 amino acid residues. The first 30 amino acid residues deduced from the gene matched completely the sequence determined with the purified enzyme. A distinct feature of the polypeptide is its lack of a stretch of 18 amino acid residues which form a loop [see section (1) above and Fig. 4 in Appendix

#1] in horse liver ADH. The significance of the difference will be investigated.

Sequence analysis of the upstream region of the adh gene revealed an open-reading frame (ORF), which could encode a polypeptide of 447 amino acid residues (see Fig. 1 and Appendix #5). The ORF is separated by 264 base pairs from the adh gene. Multiple stem-and-loop structures may be formed in the intergenic region, suggesting that control elements may be present in this region. The nucleotide sequence of 2,493 base pairs has been submitted to GenBank and assigned the accession number of M84723.

A comparison of the ORF with protein sequences in the date bank indicated that the predicted protein product is related to the family of signal-transducing transcriptional regulator represented by NtrC. The central region (243 amino acid residues; the shaded region in Fig. 1) of the predicted protein corresponds to "domain D" of NtrC of Klebsiella pneumoniae; it has an identity between 40 and 51% with TyrR, XylR, NtrC, and NifA of three bacterial species. We have tentatively named the gene stc (for signal-transducing transcriptional control). This finding is one of the most exciting for us because a major goal of our enzymological study is to lead to the identification of the control elements for the expression of solvent-production genes. We are now investigating the role of the stc gene in relation to solvent production.

Figure 1. A map showing the locations of the adh (encoding the primary/secondary alcohol dehydrogenase) and the stc (for signal-transducing transcriptional control) genes of Clostridium beijerinckii NRRL B593. The shaded region has a 50% identity to NtrC of Klebsiella pneumoniae.

III. Publications.

Work reported in the following publications was supported by the Energy Biosciences Program.

A. Journal article:

1. Rifaat, M. and J.-S. Chen. 1992. Cloning and sequence analysis of a gene encoding a medium-chain zinc-containing alcohol dehydrogenase from the Gram-positive anaerobe Clostridium beijerinckii NRRL B593. (To be submitted to Gene)

B. Book Chapter:

1. Chen, J.-S. 1991. Properties of acid- and solvent-forming enzymes of clostridia. In D. R. Woods (ed.), The clostridia and biotechnology. Butterworth, Stoneham, Mass. (in press)

C. Abstracts:

1. Rifaat, M. and J.-S. Chen. 1991. Cloning of a primary/secondary alcohol dehydrogenase gene (adh) from Clostridium beijerinckii NRRL B593. Abstr. ASM General Meeting, O-48, p. 266.

2. Colby, G. D. and J.-S. Chen. 1992. Purification and characterization of 3-hydroxybutyryl-CoA dehydrogenase from Clostridium beijerinckii strain NRRL B593. Abstr. ASM General Meeting (submitted).

3. Rifaat, M. and J.-S. Chen. 1992. A transcriptional regulatory gene upstream from the adh gene of Clostridium beijerinckii NRRL B593: cloning and sequence analysis. Abstr. ASM General Meeting (submitted).

D. Thesis:

1. Yan, R.-T. 1991. Enzymology of butanol formation in Clostridium beijerinckii NRRL B592. Ph.D. Thesis. Virginia Polytechnic Institute and State University, Blacksburg.

IV. Appendix

Preprints of the listed article, book chapter, and abstracts are included as Appendices #1 to #5.

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