CoA-TRANSFERASE AND 3-HYDROXYBUTYRYL-CoA DEHYDROGENASE: ACETOACETYL-CoA-REACTING ENZYMES FROM CLOSTRIDIUM BEIJERINCKII NRRL B593

by

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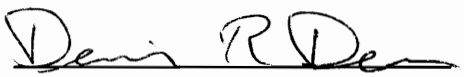

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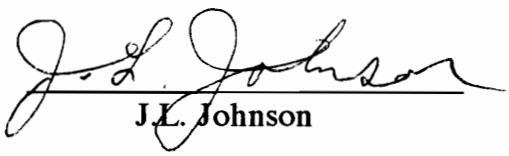

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Committee Chairman: J.-S. Chen
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(Abstract)

In acetone/butanol-producing clostridia, the metabolic intermediate acetoacetyl-CoA can be directed toward butyrate or butanol formation by the reaction catalyzed by 3-hydroxybutyryl-CoA dehydrogenase, or toward acetone formation by the reaction catalyed by acetoacetate:acetate/butyrate CoA-transferase. 3-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35 or 1.1.1.157) has been purified 45-fold to apparent homogeneity from the solvent-producing anaerobe Clostridium beijerinckii strain NRRL B593. The identities of 34 of the 35 N-terminal amino acid residues have been determined. The enzyme exhibited a native M_r of 213,000 and a subunit M_r of 30,800. It is specific for the (S)-enantiomer of 3-hydroxybutyryl-CoA. Michaelis constants for NADH and acetoacetyl-CoA were 8.6 and 14 μM, respectively. The maximum velocity of the enzyme was 540 μmol/(min·mg) for the reduction of acetoacetyl-CoA with NADH. The enzyme could use either NAD(H) or NADP(H) as cosubstrate; however, NAD(H) appeared to be the physiological substrate. In the presence of 9.5 μM NADH, the enzyme was inhibited by acetoacetyl-CoA at concentrations as

low as 20 μM, but the inhibition was relieved as the concentration of NADH was increased, suggesting a possible mechanism for modulating the energy efficiency during growth.

Acetoacetate:acetate/butyrate CoA-transferase (EC 2.8.3.9) has been purified 308-fold to apparent homogeneity from the same organism. The enzyme exhibited a native M_r of 89,100. The subunits of the enzyme were separated by preparative SDS-PAGE, and exhibited M_r values of 28,400 and 25,200. The identities of the 34 N-terminal amino acids of the large subunit and 38 of the 39 N-terminal amino acids of the small subunit were determined. The N-terminal region of the two subunits showed significant similarity with several other CoA-transferase enzymes. Michaelis constants for butyrate and acetoacetyl-CoA were 11.7 mM and 107 μM, respectively, while those for acetate and acetoacetyl-CoA were 424 mM and 118 μM, respectively. The value of k_{cat}/K_m was approximately 100 times higher with butyrate than with acetate.

Implications of the properties of these two enzymes for the acetone-butanol fermentation are discussed, and a model for the induction of the enzymes responsible for solvent production is suggested.

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3HBDH	3-Hydroxybutyryl-CoA Dehydrogenase
AcAc	Acetoacetate
AcAcCoA	Acetoacetyl-CoA
AcAcCoAR	Acetoacetyl-CoA Reductase
AcAcDC	Acetoacetate Decarboxylase
CoA	Coenzyme A
CoAT	Acetoacetate:Acetate/Butyrate CoA-Transferase
PHM	Pig Heart Mitochondrion
PTB	Phosphotransbutyrylase
UBA	Undissociated Butyric Acid

Purpose of Study

The course of research described in this dissertation was undertaken so that we might better understand the enzymology of the solvent-producing clostridia. The organism used in the study was Clostridium beijerinckii, strain NRRL B593 (VPI 13437), which produces acetone, butanol, isopropanol, and ethanol, in addition to acetate, butyrate, H_2, and CO_2. Much work has also been done in other laboratories using a related organism, C. acetobutylicum, which produces the same products, except isopropanol. A thorough knowledge of the properties of the enzymes leading to acid and solvent production in these organisms will serve not only as a basis for future investigations into the regulatory mechanisms of the organisms, but also as a starting point for future attempts at customizing the product patterns of these organisms.

In the solvent-producing clostridia, acetoacetyl-coenzyme A (AcAcCoA) is the branch-point intermediate of the two metabolic pathways leading to either the production of butyric acid or the production of the solvents acetone, isopropanol, and butanol. As such, the enzymes reacting with this compound are important determinants of the ratios and amounts of the acids and solvents produced. AcAcCoA is a substrate for both of the enzymes on which this study was focused, 3-hydroxybutyryl-coenzyme A dehydrogenase (3HBDH; EC 1.1.1.35 or 1.1.1.157) and acetoacetate-acetate/butyrate coenzyme A-transferase (CoAT; EC 2.8.3.9). During this study, both 3HBDH and CoAT were purified, and their physical and kinetic

properties were examined. In addition, the amino-terminal sequences of these two enzymes were determined, so that the genes encoding them may be identified.

Chapter I

Literature Review

Introduction

Louis Pasteur, in the second half of the nineteenth century, was the first to observe the production of butanol by a bacterial culture. In the first half of this century, fermentation was an important method for the industrial production of acetone and butanol. The development of petroleum refining technology in the second half of this century, however, led to cheaper ways of manufacturing these chemicals. As a result, the fermentative route of production is no longer used in most industrialized nations.

Interest in the acetone-butanol fermentation has been re-kindled in the past 15 years. This interest has been aroused for several reasons. The Arab oil embargo of 1972 and, more recently, the Persian Gulf conflicts of 1981-88 and 1991-92 reminded us of the strategic tenuousness of reliance on imported oil, and highlighted the importance of alternative sources of feedstock chemicals. Inevitably, the petroleum reserves of the world will be depleted. Also, as the more easily recovered deposits are consumed, the cost of petroleum production will necessarily increase, making the acetone-butanol fermentation economically favorable once more. The fermentation might also find practical application for the economic utilization of low-value carbohydrates (e.g. cellulose or ligno-cellulose; Mes-Hartree and Saddler, 1982)

or for the creation of specialized products (e.g., butanol for use in food preparation; Blaschek, 1986). Development of the fermentation will therefore yield both strategic benefit on a national scale and practical and economic benefits to individual users.

The most thoroughly-studied organism which carries out this fermentation, Clostridium acetobutylicum, is able to convert various carbohydrates to acetone, butanol, and ethanol. A similar organism, Clostridium beijerinckii, is able to produce isopropanol in addition to these products. Concurrent study of both organisms may allow insights to be gained by comparing the properties of the enzymes of the two organisms and the systems used by the two species to regulate the expression of these enzymes. By simultaneously studying and improving species with different product patterns, greater flexibility is offered to potential industrial users, since improvements made to one species may be applicable to another.

Reviews and discussions of the history, physiology, genetics, and process characteristics of the fermentation (Rogers, 1986; Jones and Woods, 1986 and 1989; Young et al., 1989; Cary et al., 1990a), the activities and properties of the enzymes relevant to it (Chen, 1993), and models of the industrial process (Srivastava and Volesky, 1990) and physiological mechanism (Grupe and Gottschalk, 1992) of the fermentation have been published previously.

Analysis of the fermentation necessarily began with observation and quantitation of the products of the fermentation. The solvent-producing clostridia exhibit a biphasic fermentation. Before solvent production is induced, acetate and

butyrate, in addition to CO_2 and H_2, are produced. As the concentrations of these acids increase, growth slows, the cells begin to re-assimilate the acids, and solvents are produced. Several factors have been correlated with the induction of solventogenesis. Several groups have observed that the addition of short-chain carboxylic acids to otherwise acidogenic fermentations of C. acetobutylicum resulted in solvent production (Bahl et al., 1982a; Martin et al., 1983; Holt et al., 1984; Fond et al., 1985). In addition, Ballongue et al. (1985) observed that addition of linear C_1-C_4 carboxylic acids induced acetoacetate decarboxylase (AcAcDC), one of the enzymes involved in solvent production. Although solvent production by either C. beijerinckii NRRL B592 (George and Chen, 1983) or C. acetobutylicum NCIB 8052 (Holt et al., 1984) can occur at neutral pH, a decrease in pH in conjunction with a high concentration carboxylic acids can shift an otherwise acidogenic fermentation to a solventogenic one (Bahl et al., 1982a; Monot et al., 1984; Holt et al., 1984). Some groups (Monot et al., 1984; Terracciano and Kashket, 1986; Hüsemann and Papoutsakis, 1988) have observed a close correlation between the onset of solventogenesis and the concentration of undissociated butyric acid (UBA), presumably a biochemically inert compound. Gottwald and Gottschalk (1985) suggest that the observed correlation between UBA and the onset of solventogenesis may be due to the intracellular accumulation of butyrate, butyryl-CoA, or butyryl-phosphate. It appears that the enzymes of the pathway leading to butanol production and of those leading to acetone/isopropanol production are separately

inducible. Grupe and Gottschalk (1992) noted that butanol can be induced by factors which inhibit H_2 formation, including carbon monoxide gassing, iron limitation, or addition of methyl viologen to the medium. They pointed out that lowering the pH, adding organic acids, or adding other uncoupling agents induce both acetone and butanol production. The physiological mechanism by which this metabolic switch is effected is not yet understood.

The next logical step toward understanding the fermentation was an analysis of the properties of the enzymes involved in acid and solvent formation. Improvements in protein purification technology facilitated the purification and characterization of these enzymes. Figure 1 (modified from Chen, 1993) illustrates the metabolic pathways used by the solvent-producing clostridia during the acid- and solventogenic phases. Most of these enzymes have been purified from C. acetobutylicum and/or C. beijerinckii (Chen, 1993). Andersch et al (1983) observed that the acid-forming enzymes phosphotransacetylase, phosphotransbutyrylase, acetate kinase, and butyrate kinase were present in C. acetobutylicum cells in acid-producing cultures at 2-10 times the levels present in cells in solvent-producing cultures. They also noted that CoA-transferase (CoAT) and AcAcDC activities were virtually undetectable in cells in acid-producing cultures but appeared shortly before the appearance of detectable levels of acetone and butanol in batch cultures. Yan et al. (1988) also noted a sharp increase in AcAcDC activity coincident with the onset of solvent formation in C. beijerinckii. They also observed a concurrent

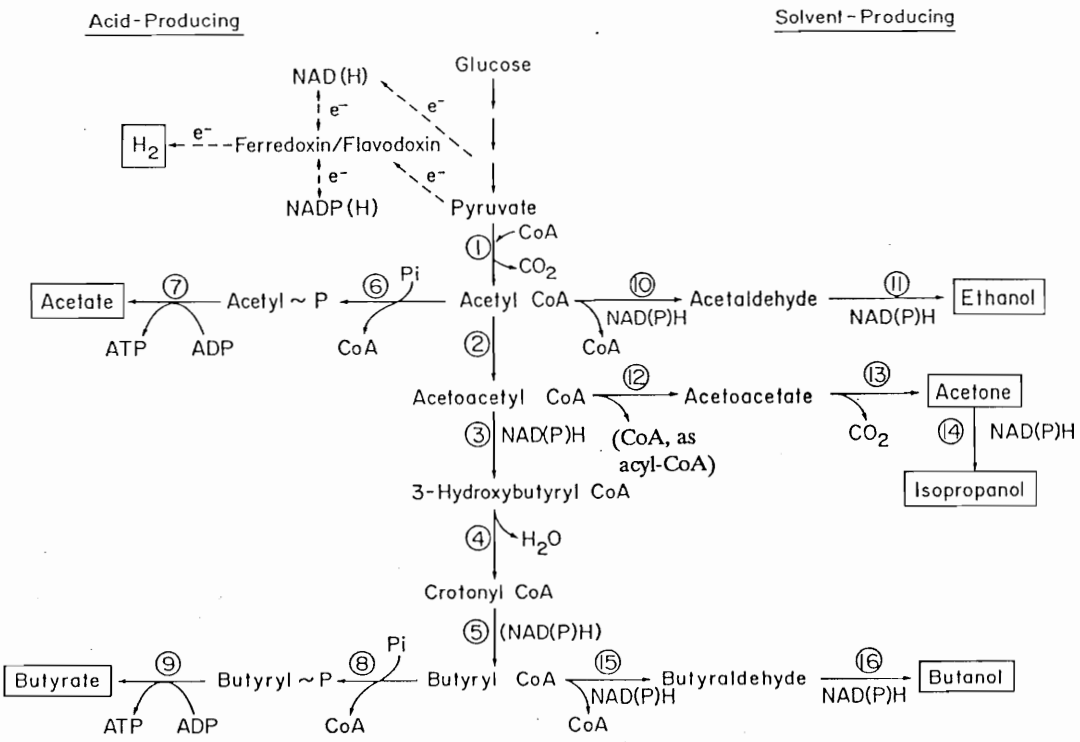

Figure 1. Metabolic pathways of acid and solvent production in clostridia (modified from Chen, 1993). Arrows represent physiological flow of metabolites. Numbered enzyme activities are:

1. Pyruvate:Ferredoxin Oxidoreductase
2. Acetoacetyl-CoA Thiolase
3. 3-Hydroxybutyryl-CoA Dehydrogenase
4. Crotonase
5. Butyryl-CoA Dehydrogenase
6. Phosphotransacetylase
7. Acetate Kinase
8. Phosphotransbutyrylase
9. Butyrate Kinase
10. Acetaldehyde Dehydrogenase
11. Ethanol Dehydrogenase
12. Acetoacetate:Acetate/Butyrate CoA-Transferase
13. Acetoacetate Decarboxylase
14. Isopropanol Dehydrogenase
15. Butyraldehyde Dehydrogenase
16. Butanol Dehydrogenase

increase in aldehyde dehydrogenase and butanol dehydrogenase (BDH) activities in a butanol-producing strain of C. beijerinckii and in both BDH and isopropanol dehydrogenase activities in a butanol- and isopropanol-producing strain.

The reactions whereby acetoacetyl-CoA (AcAcCoA) is converted to butyryl-CoA are common to both the acid- and solvent-producing pathways, and they appear to be coordinately regulated in C. acetobutylicum (Hartmanis and Gatenbeck, 1984). These researchers noted that the activity levels of these enzymes increased until they reached maximal activities after growth had ceased and solvent production had begun, and then declined.

Isolation of the enzymes involved in acid and solvent production has made the genes encoding these enzymes accessible. Studies of these genes and their expression may allow a better understanding of the control mechanism(s) of the fermentation. Genes for several of these enzymes have been cloned and expressed in Escherichia coli, including CoAT (Cary et al., 1990b), AcAcDC (Petersen and Bennett, 1990; Gerischer and Dürre, 1990; Mermelstein et al., 1992), three alcohol dehydrogenases (Youngleson et al., 1989a; Petersen et al., 1991; Walter et al., 1992), a putative alcohol dehydrogenase/aldehyde dehydrogenase bifunctional enzyme (Nair et al., 1993; Dürre, 1993), acetyl-CoA acetyltransferase (thiolase; Petersen and Bennett, 1991), 3-hydroxybutyrl-CoA dehydrogenase (3HBDH; Youngleson et al., 1989b), phosophotransbutyrylase (Cary et al., 1988), and butyrate kinase (Cary et al., 1988). In addition, an alcohol dehydrogenase gene from an isopropanol-producing strain of

C. beijerinckii has been cloned and sequenced (Rifaat and Chen, 1992). The ability to manipulate these genes (see, for example, Young et al., 1989 and Cary et al., 1990a) allows them to be included in experimental genetic systems wherein their control mechanisms may be elucidated.

A full understanding of the relevant physiology of the solvent-producing clostridia, including knowledge of the catalytic properties of the enzymes involved and the underlying mechanisms of genetic control of their expression may allow improvements to be made to the commercial process. By manipulating the expression of the genes encoding the enzymes responsible for acid and solvent production, it should be possible to improve the yield of the fermentation, or customize the ratio of products. Also, a thorough knowledge of the acid- and solvent-producing metabolic systems of the clostridia may enable them to be transferred in a modular fashion to an organism able to utilize more economical substrates. Such a system might be one of the first successful applications of the emerging discipline of metabolic engineering (Bailey, 1991).

Product Pattern

The product pattern of the fermentation may be manipulated without using the methods of genetic engineering. Each of the several species of solvent-producing clostridia has a characteristic pattern of solvent formation, which sometimes also varies among strains of the same species. The product pattern can also be altered

by varying the levels and types of nutrients in the growth medium, or by adjusting other culture conditions. The effects of non-nutrient additions has also been studied. Finally, mutant strains of solvent-producing organisms have been isolated, which produce altered solvent production patterns.

Strains of C. beijerinckii (Chen and Hiu, 1986), like those of C. acetobutylicum, are capable of producing butanol and acetone. Some strains of C. beijerinckii, including some previously identified as "C. butylicum" (George et al., 1983) and C. aurantibutyricum (George et al., 1983), are also capable of producing isopropanol. Other species of solvent-producing clostridia which have been studied include C. puniceum (Holt et al., 1988), and C. saccharoperbutylacetonicum (Soni et al., 1987), which produce acetone and butanol in various ratios, and C. tetanomorphum (Gottwald et al., 1984) and C. thermosaccharolyticum (Freier-Schröder et al., 1989), which produce butanol, but not acetone or isopropanol. C. acetobutylicum has been widely studied because it was the most commonly used organism in industrial fermentations and thus is well characterized. Several mutant strains of this organism, including some deficient in acetone production (e.g. Janati-Idrissi et al., 1987; Clark et al., 1989; Bertram et al., 1990), have been isolated which lack one or more of the enzymes necessary for solvent production. C. beijerinckii is studied in our lab so that an isopropanol-producing organism will be well-characterized and available for future improvements and industrial uses.

The product pattern of the fermentation is affected by the levels of nutrients in the growth medium. Monot et al. (1982) observed that in batch cultures in a defined medium, C. acetobutylicum ATCC 824 produced more butanol relative to acetone, and more solvents overall, as the initial concentration of glucose was increased. While the amount of ammonium ion present must be non-limiting to allow solvent production by C. acetobutylicum NCIB 8052 (Gottschal and Morris, 1981; McNeil and Kristiansen, 1987) in continuous cultures, the ratio of butanol to acetone produced is not significantly affected by the ammonium concentration (Monot et al., 1982). Solvent production under phosphate-limitation has also been demonstrated (Bahl et al., 1982b). Monot et al. (1982) observed that the weight ratio of butanol to acetone (g butanol/g acetone; B/A weight ratio) produced increased with increasing concentration of Mg^{++} ions. However, in a Mg-limited chemostat, McNeil and Kristiansen (1987) observed a high yield of solvents (35.5% of glucose, by weight, converted to solvents) and high B/A weight ratio (9.8).

Several groups (Monot et al., 1982; Bahl et al., 1986; Junelles, 1988) have observed the effect of Fe-limitation on the fermentation. Monot et al. (1982) determined that the B/A weight ratio was twice as large when a batch culture was grown in medium to which no Fe had been added than when the medium was supplemented with $FeSO_4 \cdot 7\ H_2O$ at concentrations between 1 and 50 mg/liter. Using the same organism and a similar medium, Junelles et al. (1988) found that the B/A weight ratio was increased by more than 3-fold when the amount of $FeSO_4 \cdot 7$

H_2O was decreased from 10 mg/l to 0.2 mg/l. Furthermore, this group found that the specific activities of two Fe-containing enzymes, hydrogenase and AcAcDC, were 40% and 25%, respectively lower in the iron-limited (0.2 mg/l) culture. One explanation for the increase in the B/A ratio caused by iron limitation of the medium is that the reducing equivalents that would otherwise be dissipated by the action of hydrogenase must be dissipated by re-directing carbon flow through the pathway leading to butanol production, in order to recycle electron carriers. The reduction in AcAcDC activity may also contribute to the increase in the B/A ratio by limiting the carbon flow through the acetone-producing branch of the fermentation. Bahl et al. (1986) determined that the limited iron content of whey significantly contributed to the high B/A molar ratio (mol. butanol/mol. acetone = 100) observed for the fermentation of whey by strains DSM 792, 1731, and 1732 of C. acetobutylicum. Fermentation of pentoses by C. beijerinckii strains NRRL B592 and NRRL B593 and C. acetobutylicum strains NRRL B3179 and NRRL 527 resulted in lower values for the conversion of substrate to solvents and lower solvent production rates than those obtained from analogous hexose fermentations (Volesky and Szczesny, 1983). A complete understanding of the effects of nutrient levels on the fermentation will allow optimization of the industrial process by intelligent supplementation of the available substrates.

Non-nutritive medium supplements can be used to influence the ratio of solvents produced by the fermentation. Martin et al. (1983) and Fond et al. (1985)

observed that when acetate and/or butyrate, the normal products of the acidogenic phase of the fermentation, were added to batch or fed-batch cultures of C. acetobutylicum ATCC 824, increased amounts of acetone and butanol were produced. Hüsemann and Papoutsakis (1990) demonstrated that the addition of either acetate or propionate to batch cultures of this organism increased the levels of solvents produced. These authors suggested that the buffering capacity imparted to the medium by the acid additions was the cause of the increase in solvent production. Bryant and Blaschek (1988) had previously shown that increasing the buffering capacity of the growth medium resulted in an increase in butanol production. Treatment of C. acetobutylicum cultures with CO has been shown to increase the specificity of the fermentation for butanol production (Kim et al., 1984; Meyer et al., 1986; Hüsemann and Papoutsakis, 1989a). The physiological reasons behind this effect appear to be two-fold. Hüsemann and Papoutsakis (1989a) found that AcAcDC was completely inactivated in a chemostat culture (strain ATCC 824) sparged with CO. Meyer et al. (1986) observed that CO-sparging of chemostats (strain ATCC 824) inhibited H_2-formation by up to 100%. Hydrogenase is inhibited by CO (Adams et al., 1981). Kim et al. (1984) vented the headspaces above both stirred and non-stirred batch cultures (strain ATCC 4259) and found that H_2-production was decreased and the B/A ratio was increased. Inhibition of hydrogenase by CO forces the cells to dispose of reducing equivalents by other means. C. acetobutylicum may recycle its electron carriers by shifting carbon flow

to the butanol production pathway, or (in some strains, including ATCC 824) by reducing pyruvate to lactate (e.g., Meyer et al., 1986). Junelles et al. (1987) observed that addition of pyruvate to cultures of C. acetobutylicum ATCC 824 actively producing solvents resulted in a sharp decline in the specific rates of butyrate uptake and butanol production, with concomitant consumption of pyruvate. Partial or total inhibition of AcAcDC will reduce or eliminate acetone production.

Other methods of altering the electron flow of C. acetobutylicum will also influence the product pattern of the fermentation. Rao and Mutharasan (1988) found that growth of the organism (strain ATCC 824) in the presence of reducing agents caused a shift in favor of the production of H_2 and butanol, and ascribed this shift to the need of the organism to dispose of more reducing equivalents due to the reduced nature of the environment. The same authors (1987) added viologen dyes to a continuous culture and observed that H_2 and acid formation rates were depressed, while butanol and ethanol rates simultaneously increased. Apparently, the electron flow which would otherwise have been directed through hydrogenase was routed instead through the butanol- and ethanol-producing pathways, consuming the carbon flow which would otherwise have contributed to acetone production. Kim and Kim (1988) used an electrochemical method of reducing $NAD(P)^+$ (via methyl viologen) to shift the product pattern of a batch culture of a lactate non-producing C. acetobutylicum strain (ATCC 4259) toward butanol production.

Induction of Solvent Production

The physiological mechanism by which a clostridial culture is switched from acid to solvent production is not well understood. Huesemann and Papoutsakis (1986) noted that acetoacetate (AcAc) was able to induce solvent formation in otherwise acidogenic batch cultures of C. acetobutylicum ATCC 824, either with or without pH control. These authors demonstrated that while the addition of either acetate or propionate to batch cultures of this organism increased the levels of solvents produced, the onset of solvent production did not occur immediately following addition of the acid. Ballongue et al. (1985) had previously noted that C_1-C_4 straight-chain carboxylic acids and AcAc are able to induce AcAcDC activity in C. acetobutylicum ATCC 824. Among the acids investigated, addition of AcAc caused induction of the highest levels of AcAcDC activity at all acid concentrations studied. Several groups (Monot et al., 1984; Terracciano and Kashket, 1986; Bryant and Blaschek, 1988; Hüsemann and Papoutsakis, 1988 and 1990) have observed an apparently close correlation between the onset of solvent production and an extracellular concentration of UBA in the range 9-20 mM. This apparent relationship does not explain why solvent production by C. acetobutylicum NCIB 8052 was induced by 9 mM butyrate in a batch culture maintained at pH 5.0 ([UBA] = 5.5 mM), but 100 mM butyrate and acetate was required to induce solvent production in a batch culture maintained at pH 7.0 ([UBA] = 0.5 mM; Holt et al., 1984). In C. beijerinckii NRRL B592, the levels of UBA present at the onset of

solventogenesis were not the equal for batch cultures maintained at different pH values (George and Chen, 1983). These authors also observed that a batch culture maintained at pH 6.8 had to be supplemented with both acetate and butyrate in order to accelerate the induction of solvent production. Gottwald and Gottschalk (1985) suggested the biochemical inertness of UBA and proposed that elevated intracellular concentrations of butyryl-CoA and/or butyryl-phosphate are responsible for inducing the shift to solventogenesis. However, Grupe and Gottschalk (1992) demonstrated that the intracellular concentrations of both acetyl- and butyryl-CoA decreased during the period when a continuous culture of C. acetobutylicum DSM 1731 shifted from acid to solvent production, following a cessation of pH control. They have proposed that the induction of solvent-producing enzymes depends on two separate signals, one responsible for inducing the enzymes leading to acetone production, the other responsible for inducing the enzymes leading to butanol production.

3-Hydroxybutyryl-CoA Dehydrogenase and Acetoacetate:Acetate/Butyrate CoA-Transferase

The goal of the work described in this dissertation is to add to the knowledge of the physiology of acid and solvent production by analyzing the properties of two enzymes, 3HBDH and CoAT, from an isopropanol-producing strain of C. beijerinckii. During the acidogenic phase of the fermentation, essentially all of the

net AcAcCoA produced from acetyl-CoA by thiolase is converted to butyrate. 3HBDH catalyzes the first committed step toward butyrate production. Thompson and Chen (1990) noted that phosphotransbutyrylase from this organism was also capable of reacting with AcAcCoA, although the Michaelis constant (K_m) of this enzyme for AcAcCoA was much higher than the K_m values for AcAcCoA of either 3HBDH (Colby and Chen, 1992) or CoAT (this study). Little or no CoAT activity is observed during the acidogenic phase of the fermentation (Andersch et al., 1983; Hüsemann and Papoutsakis, 1989b).

During the solventogenic phase of the fermentation, both 3HBDH (Hartmanis and Gatenbeck, 1984) and CoAT (Andersch et al., 1983; Hartmanis et al., 1984; Hüsemann and Papoutsakis, 1989b) activities are present. Since both enzymes utilize AcAcCoA as a substrate, they are in competition. AcAcCoA that reacts with 3HBDH forms either butyrate or butanol, while that reacting with CoAT is used for formation of acetone and/or isopropanol and also results in the re-assimilation of previously-formed acetate and butyrate. The ratio of AcAcCoA consumption by the two pathways affects the reduction state of the cell, the product pattern of the fermentation, and the rate of toxicity increase in the growth medium. Production of butyrate from AcAcCoA via 3HBDH consumes four reducing equivalents and contributes to the toxicity caused by butyrate in the medium (Kell et al., 1981; Bowles and Ellefson, 1985). Production of butanol from AcAcCoA via 3HBDH consumes eight reducing equivalents. Production of butanol by uptake of butyrate

via CoAT consumes four reducing equivalents and de-toxifies the medium by lowering the butyrate concentration. In addition, another two reducing equivalents may be consumed by conversion of acetone (formed by decarboxylation of AcAc by AcAcDC) to isopropanol. The implications of acetate uptake via CoAT are more complicated. It is nonetheless clear that the metabolic pathway or combination of pathways used by solvent-producing cells will have broad implications for the energy charge and reduction state of the cells and the transient toxicity of the medium. An understanding of the kinetic properties of 3HBDH and CoAT may allow more accurate prediction of the influence of the two enzymes on the product pattern of the fermentation.

The gene encoding 3HBDH from C. acetobutylicum P262 has been cloned and expressed in E. coli by Youngleson et al. (1989b). They also noted that the deduced amino acid sequence of the enzyme showed significant similarity to several mammalian enzymes. A model of the three-dimensional structure of one of these enzymes, that from pig heart mitochondria, has been proposed (Birktoft et al., 1987). This model proposes that the coenzyme A moiety of AcAcCoA does not interact with the enzyme, but rather that the binding occurs via the acetoacetyl portion of the molecule. The clostridial enzyme had not been extensively studied before this report.

CoAT from C. acetobutylicum ATCC 824 has been purified and characterized previously (Wiesenborn et al., 1989). Exceptionally high values are observed for the Michaelis constants for acetate and butyrate - 1.2 M and 0.66 M, respectively. The

enzyme also has unusual stability requirements *in vitro*, and was stable only in the presence of 20% (vol/vol) glycerol and 0.5 M ammonium sulfate. However, the enzyme appears to be stable for at least 2-3 hours *in vivo* (Welch et al., 1992). The genes encoding the enzyme have been cloned and expressed in E. coli (Cary et al., 1990b), although the transcription appeared to originate from a promoter in the vector. The sequences of the large (Gerischer and Dürre, 1990; Petersen et al., 1993) and small (Petersen et al., 1993) subunits of CoAT from C. acetobutylicum have been published. The genes are located adjacent to the gene encoding AcAcDC, and are transcribed independently. The genes encoding CoAT are separated from that encoding AcAcDC by a single apparent Rho-independent terminator. Nair et al. (1993) and Dürre (1993) have identified a gene encoding a putative aldehyde dehydrogenase/alcohol dehydrogenase bifunctional enzyme adjacent to the CoAT genes, which appears to be co-transcribed with them. CoAT and AcAcDC activities appear concomitantly *in vivo* and increase proportionally (Hüsemann and Papoutsakis, 1989b). Furthermore, sharp increases in the mRNA species encoding AcAcDC and CoAT are observed simultaneously in C. acetobutylicum cells undergoing the switch from acid- to solvent-production (Gerischer and Dürre, 1992). It appears that the two enzymes share a common regulatory mechanism.

CoAT enzymes have been isolated from several sources, including from pig heart mitochondria (Hersh and Jencks, 1967a). Jencks and his colleagues have thoroughly characterized this enzyme and described the reaction mechanism (Jencks,

1973). The reaction exhibits ping-pong kinetics (Hersh and Jencks, 1967b) and proceeds through the formation of a stable covalent enzyme-CoA complex. The site of CoA esterification has been identified as a glutamate residue (Solmon and Jencks, 1969). The rate acceleration of the reaction by the enzyme is primarily effected by the application of energy gained from specific binding interactions between the enzyme and the 3'-phospho-ADP moiety of CoA to the enzyme-CoA complex destabilized by the pantotheine moiety (Moore and Jencks, 1982; Fierke and Jencks, 1986). A cDNA with the gene encoding this enzyme has been sequenced and described (Lin and Bridger, 1992).

CoAT from E. coli has been purified and characterized (Sramek and Frerman, 1975a; Sramek and Frerman, 1976b). In addition, the genes encoding it have been located. They lie adjacent to the gene encoding thiolase II, in the same orientation (Jenkins and Nunn, 1987a). The transcription of these genes is induced by a positive regulatory element which is activated by the presence of AcAc in the medium (Pauli and Overath, 1972; Jenkins and Nunn, 1987b). The regulatory properties of the activator are similar to those of MalT protein in E. coli, the activator of the three operons containing genes encoding the enzymes responsible for the uptake and activation of maltose and maltodextrins (Schwartz, 1987).

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Chapter II

Purification and Properties of 3-Hydroxybutyryl-CoA Dehydrogenase from Clostridium beijerinckii (syn. C. butylicum) Strain NRRL B593

INTRODUCTION

3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and acetoacetyl-CoA reductase (AcAcCoAR, EC 1.1.1.36) in both eukaryotic and prokaryotic systems have been studied. These enzymes catalyze analogous reactions and are common to several metabolic pathways including ß-oxidation of fatty acids (Wakil et al., 1954; Stern, 1957; Hashimoto, 1982), short-chain fatty acid elongation (Madan et al., 1973; Hillmer and Gottschalk, 1985), ketone body formation (Prasad et al., 1984), and polyhydroxybutyrate synthesis (Ritchie et al., 1971; Shuto et al., 1981; Packter and Flatman, 1983; Fukui et al., 1987; Haywood et al., 1988). Since polyhydroxybutyrate is a polymer of (R)-3-hydroxybutyrate, the enzymes catalyzing the interconversions of polyhydroxybutyrate and acetoacetyl-CoA (AcAcCoA) are specific for that enantiomer. The enzymes of the other pathways may be specific for one enantiomer or the other (Wakil et al., 1954; Madan et al., 1973; Noyes and Bradshaw, 1973; Osumi and Hashimoto, 1980; Shuto et al., 1981; Prasad et al., 1984; Haywood et al., 1988) or may lack stereospecificity (Haywood et al., 1988).

3-Hydroxybutyryl-CoA Dehydrogenase (3HBDH, EC 1.1.1.35 or 1.1.1.157) in clostridia (Madan et al., 1973; Hillmer and Gottschalk, 1974; Sliwkowski and Hartmanis, 1984; Youngleson et al., 1989; this work) catalyzes the reduction of AcAcCoA by NAD(P)H. This reaction is the first committed step toward production of butyrate and butanol. Although there is a high degree of similarity in the amino acid sequences of many of the 3-hydroxyacyl-CoA dehydrogenases (Youngleson et al., 1989), there is less similarity in kinetic properties and less still in quaternary structure (Madan et al., 1973; Noyes and Bradshaw, 1973a; Noyes and Bradshaw, 1973b; Osumi and Hashimoto, 1980; Shuto et al., 1981; Haywood et al., 1988; Yang et al., 1991).

The acid- and solvent-producing pathways of the clostridia are shown in Figure 1 of Chapter I. As evidenced by its position at a branch point in the fermentation, AcAcCoA is an important intermediate in the metabolism of these organisms. The metabolic fate of this compound directly affects the product pattern of the fermentation. Thiolase (Wiesenborn et al., 1988) and CoA transferase (Wiesenborn et al., 1989), which react with AcAcCoA, have been isolated from a solvent-producing organism, Clostridium acetobutylicum ATCC 824. Thompson and Chen (1990) have purified and characterized phosphotransbutyrylase, which also reacts with AcAcCoA, from C. beijerinckii NRRL B593. Characterization of the other enzymes reacting with this intermediate should yield insight into the metabolic control mechanisms for the

fermentation. A thorough knowledge of the control mechanisms will make it possible to customize the product pattern of the fermentation for various industrial uses.

Youngleson et al. (1989) have cloned and sequenced the *hbd* gene encoding 3HBDH from C. acetobutylicum P262 and expressed it in Escherichia coli. The *hbd* gene is located upstream from the *adh-1* gene (encoding an alcohol dehydrogenase) and was identified as the structural gene for 3HBDH because its encoded amino acid sequence has a high similarity to that of 3-hydroxyacyl-CoA dehydrogenases. Although 3HBDH activity was observed at a high level in E. coli harboring a plasmid containing the *hbd* gene, the enzyme has not been purified or extensively studied. In this chapter the purification and characterization of 3HBDH from C. beijerinckii NRRL B593 is described.

MATERIALS AND METHODS

Source of chemicals. $NAD(P)^+$, NAD(P)H, coenzyme A, 3-hydroxybutyryl-CoA, glycine, chloride determination kit, 2-(N-morpholino)ethanesulfonic acid (MES), Sephacryl S-300 (Pharmacia), lysozyme (chicken egg white), DNase I, ferritin (horse spleen), alcohol dehydrogenase (baker's yeast), conalbumin (chicken egg white), thyroglobulin (bovine), bovine serum albumin, ovalbumin (chicken), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), trypsin inhibitor (soybean), carbonic anhydrase (bovine erythrocyte), and ß-lactoglobulin (bovine milk) were obtained from Sigma Chemical Co.; α-toluenesulfonyl fluoride (PMSF), ammonium peroxydisulfate, acrylamide, and N,N'-methylenebisacrylamide were obtained from Eastman Kodak Co.; diketene was obtained from Aldrich Chemical Co.; tryptone and yeast extract were obtained from Difco Laboratories; Matrex Gel Red A, ultrafiltration membranes, and Centricon 30 ultrafiltration units were obtained from Amicon Corp.; Q-Sepharose (fast flow) was obtained from Pharmacia, Inc.; and dye-binding protein assay kit, N,N,N',N'-tetramethylene-diamine, and sodium dodecyl sulfate were obtained from Bio-Rad Laboratories.

Organism and growth conditions. Spores of C. beijerinckii NRRL B593 (VPI 13437), produced in potato extract-glucose medium (George et al., 1983), were stored in the same medium at -70°C. Heat-shock and growth conditions

were as previously described (Thompson and Chen, 1990), except that glucose was used in place of sucrose in 600-ml and 8-liter cultures incubated at 34°C. The cells were allowed to grow until the A_{550} of the culture, measured with a Hitachi model 100-40 spectrophotometer, increased by less than 0.5 over a two-hour period (typically about 10 hours after inoculation of the 8-l culture). Cells were harvested by centrifugation at 13,000 x g for 30 min at 4°C. The packed cells were resuspended in about 1 liter of 50 mM potassium phosphate buffer (pH 7.0) and centrifuged again for 45 min to remove remaining growth medium and fermentation products. Crude extracts (see below) were made from freshly-prepared cell paste. However, crude extracts could also be made from cell paste stored at -70°C with no apparent adverse effects on the activity of the enzyme.

Synthesis of acetoacetyl-CoA. AcAcCoA was synthesized by a modification of the method of Senior and Dawes (1973). The reaction mixture was maintained at 0°C, and diketene was added in 2-μl aliquots 10 min apart. After acidification (by addition of 1 N HCl to pH 4), extraction with diethyl ether to remove excess diketene, and gentle sparging with nitrogen to remove residual ether, the AcAcCoA preparation was stored at -20°C in 1-ml aliquots. Under these storage conditions, the preparation was stable for at least 2 months. The final concentration of a typical AcAcCoA preparation was about 10 mM.

Enzyme Assays. The activity of 3HBDH was routinely assayed in air at room temperature (about 23°C) in the physiological (3-hydroxybutyryl-CoA-

forming) direction by monitoring the decrease in A_{340} of NADH. The standard assay mixture contained 50 mM sodium-MES (pH 6.0), 200 μM NADH, 200 μM AcAcCoA, and as much as 0.2 U of 3HBDH in a total volume of 1 ml. The reaction was initiated by addition of AcAcCoA. Activities were corrected for oxygen-linked oxidation of NAD(P)H when crude preparations were assayed. One unit (U) of activity is defined as the amount of enzyme which results in the oxidation of 1 μmol of NAD(P)H per minute. The extinction coefficient used (340 nm) for NAD(P)H was 6.22 mM^{-1} cm^{-1}.

To determine whether the inhibition observed with AcAcCoA was caused by free CoA, a possible contaminant of the AcAcCoA preparation, 3HBDH was assayed in the presence and absence of CoA. The reaction mixture contained 50 mM sodium-MES (pH 5.5), 60 μM AcAcCoA, 100 μM NADH, 0.2 U of 3HBDH, and either 0 or 200 μM CoA in a total volume of 1 ml. The suitability of the CoA preparation for enzyme assay was confirmed by assaying for the previously-observed (Yan et al., 1988) CoA-dependent AcAcCoA thiolase activity in a crude extract prepared from C. beijerinckii NRRL B592. The reaction mixture contained 100 mM Tris-Cl (pH 7.5), 100 μM AcAcCoA, 20 mM $MgCl_2$, 60 μM CoA, and 2 μl of crude extract (about 40 μg of protein) in a final volume of 1 ml. The reaction was initiated by the addition of CoA. The activity was determined by monitoring the decrease in A_{310} of the Mg-AcAcCoA chelate; the extinction coefficient used was 8.0 mM^{-1} cm^{-1} (Stern, 1956).

Protein Assays. Protein concentrations were determined by the dye-binding assay (Bradford, 1976), with bovine gamma globulin as the standard.

Other Assays. The concentration of AcAcCoA in the stock preparation was determined by measuring the A_{310} of a mixture composed of 100 mM Tris-Cl (pH 7.5), 20 mM $MgCl_2$, and 20 µl of the AcAcCoA stock solution in a total volume of 1 ml.

Chloride concentration was determined with the Sigma chloride determination kit.

Purification of 3HBDH. All purification steps were performed at room temperature (about 23°C) under aerobic conditions. Between steps, enzyme samples were stored at 4°C. Except where noted, the buffer used was 50 mM potassium phosphate buffer (pH 7.0, hereafter referred to as phosphate buffer).

(i) **Preparation of crude extract.** Cell paste (about 20 g) was suspended in phosphate buffer (2 ml per g cell paste). Lysozyme (2.25 mg/ml of buffer) and DNase I (1.2 mg/ml of buffer) were added to the suspension. A solution of the protease inhibitor PMSF (50 mg/ml) in 95 % (vol/vol) ethanol was added to the suspension in aliquots (0.25 mg PMSF/ml cell suspension) at the beginning of the procedure and every 30 min thereafter, since PMSF decomposes in aqueous solution. The procedure was continued for a total of 90 minutes with constant mixing. The resulting lysate was centrifuged for 30 min at 37,000 x g at 4°C to remove cell debris from the crude extract (the supernatant).

(ii) Q-Sepharose column. The crude extract (about 1.2 g of protein) was applied directly to a Q-Sepharose column (2.5 by 17 cm) which had been equilibrated with phosphate buffer. The column was washed with 500 ml of phosphate buffer, and activity was eluted with a linear gradient of KCl (0 to 0.4 M in phosphate buffer, total volume, 2000 ml). The flow rate was maintained at 300 ml/hr, and 50 ml fractions were collected.

(iii) Matrex Gel Red A column. Fractions (about 300 ml) from the Q-Sepharose column containing high 3HBDH activity were pooled. To reduce the concentration of KCl, pooled fractions were twice concentrated by ultrafiltration through a YM30 membrane in a stirred cell to about 20 ml and diluted 10-fold with phosphate buffer and finally concentrated to about 20 ml. This preparation was applied to a Matrex Gel Red A column (1.5 by 6.0 cm) which had been equilibrated with phosphate buffer. The column was washed with 100 ml of phosphate buffer, and activity was eluted by a linear gradient of KCl (0 to 0.75 M in phosphate buffer; total volume 400 ml). The flow rate was maintained at 50 ml/hr throughout the step, and 5.0 ml fractions were collected.

(iv) Sephacryl S-300 column. Fractions (about 35 ml) from the Matrex Gel Red A column containing high 3HBDH activity were pooled and concentrated by ultrafiltration through a YM30 membrane in a stirred cell to about 4 ml. The concentrated sample was applied to a Sephacryl S-300 (fast flow) column (2.5 by 52 cm) which had been equilibrated with phosphate buffer.

The flow rate was maintained at 50 ml/hr throughout the step, and 2.0 ml fractions were collected.

Determination of native and subunit molecular weights. The native molecular weight of the enzyme was determined by measurement of its elution volume on a calibrated Sephacryl S-300 (fast flow) column (2.5 by 52 cm) which had been equilibrated with phosphate buffer. A 2.0 ml sample was applied, and 2.5 ml fractions were collected at 50 ml/hr. The routine assay was used to assay the fractions for 3HBDH. Molecular weight standards used were (M_r in parentheses) thyroglobulin (669,000), ferritin (440,000), yeast alcohol dehydrogenase (150,000), and conalbumin (78,000).

The purity and subunit molecular weight of the enzyme were determined by polyacrylamide gel electrophoresis on a 12.5 % gel in the presence of SDS (Laemmli, 1970). Before being applied to the gel, purified 3HBDH and protein standards were equilibrated with SDS-PAGE stacking gel buffer (0.5 M Tris-Cl [pH 6.8]) through extensive washing in Centricon 3 ultrafiltration units and then heated at 100°C for 2 min in the presence of SDS and 2-mercaptoethanol. Protein was stained with Coomassie brilliant blue R-250.

Kinetic studies. Michaelis constants of 3HBDH for the physiological (AcAcCoA-consuming) reaction were determined by using 50 mM sodium MES buffer (pH 5.5) at 26°C, and the reaction was initiated by the addition of AcAcCoA. All measurements were made at least in duplicate.

The substrates and concentrations used during the determination of Michaelis constants for the reaction in the physiological direction, with NADH as the co-substrate were NADH (9.5 to 70 μM) and AcAcCoA (10 to 100 μM). The apparent K_m and V_{max} values for NADPH was found at 100 μM AcAcCoA and 35 to 1000 μM NADPH. The apparent K_m value for AcAcCoA with NADPH as co-substrate was measured at 400 μM NADPH and 9 to 100 μM AcAcCoA.

Apparent Michaelis constants for the non-physiological (AcAcCoA-forming) reaction were determined by using 50 mM Tris-Cl (pH 8.0) at 26°C, and the reaction was initiated by the addition of 3-hydroxybutyryl-CoA. Apparent K_m values for 3-hydroxybutyryl-CoA were determined at 100 μM NAD^+ or 10 mM $NADP^+$ and 17 to 100 μM 3-hydroxybutyryl-CoA. Apparent K_m values for NAD^+ and $NADP^+$ were found at 200 μM 3-hydroxybutyryl-CoA and either 7.5 to 75 μM NAD^+ or 1 to 10 mM $NADP^+$.

Effect of pH on 3HBDH activities. During the study of the effect of pH on the activity of 3HBDH in the physiological direction, the substrates and concentrations used were AcAcCoA (200 μM) and either NADH (200 μM) or NADPH (200 μM). For the nonphysiological direction, the substrates and concentrations used were 3-hydroxybutyryl-CoA (100 μM) and NAD^+ (100 μM) or 3-hydroxybutyryl-CoA (200μM) and $NADP^+$ (200 μM). The buffers (50 mM) used for the assays were Na-MES (pH 5.0 to 6.5), sodium phosphate (pH 6.5 to 8.0), and Tris-Cl (pH 8.0 to 9.0).

Determination of the stereospecificity of 3HBDH. The stereospecificity for 3-hydroxybutyryl-CoA of 3HBDH from C. beijerinckii was determined by comparison with the pig heart enzyme, which is specific for the (S)-enantiomer (Noyes and Bradshaw, 1973). In one set of assays, a reaction mixture (1.5 ml) containing 50 mM Na-MES (pH 5.5), 200 μM AcAcCoA, and 200 μM NADH was allowed to react in air in the presence of either the pig heart enzyme (5 U) or the enzyme from C. beijerinckii (5 U) until no further change in A_{340} was observed (about 2 min; about 75% of AcAcCoA was reduced). After the initially present enzyme had been removed by passing the solution through a Centricon 30 ultrafiltration unit, the other enzyme was added to the filtrate along with 3 mM NAD^+. The oxidation of 3-hydroxybutyryl-CoA was monitored by observing the increase in A_{340} as a result of production of NADH. In a second set of assays, a reaction mixture (1.5 ml) containing 200 mM Tris-Cl (pH 8.0), 100 μM (RS)-3-hydroxybutyryl-CoA, 3 mM NAD^+, and 8 U of the pig heart enzyme was allowed to react in air until no further change in A_{340} was observed (about 3 min; about 50% of 3-hydroxybutyryl-CoA was oxidized). The reaction mixture was passed through a Centricon 30 ultrafiltration unit to remove the pig heart enzyme. 3HBDH from C. beijerinckii (5 U) was added to the filtrate, and the A_{340} of the reaction mixture was monitored.

Determination of N-terminal amino acid sequence. The N-terminal amino acid sequence of purified 3HBDH was determined with an Applied Biosystems

Model 477A sequencer (35 cycles by Dr. C.L. Rutherford and R. Peery of the Protein Sequencing Facility, Virginia Polytechnic Institute and State University, Blacksburg). The enzyme sample was washed extensively with distilled water in a Centricon 30 ultrafiltration unit before analysis. Approximately 50 μg of protein was used for the analysis.

RESULTS

Purification of 3HBDH. The 3HBDH was purified 45-fold to apparent homogeneity, as indicated by the presence of a single band after SDS-PAGE when as much as 24 μg of protein was loaded (Figure 1). In addition, N-terminal amino acid sequencing revealed no additional residues at each cycle. A summary of a typical purification of 3HBDH is presented in Table 1. The procedures for each step are described in the Materials and Methods section. No loss of activity was detected in a sample of purified 3HBDH (0.8 mg/ml) either when stored in 50 mM potassium phosphate, pH 7.0 at 4°C for as long as 60 days or when frozen as droplets in the same buffer containing 20% (vol/vol) glycerol and stored immersed in liquid nitrogen.

Determination of Native and Subunit Molecular Weight. 3HBDH had a native molecular weight of 213,000 ± 6,000 (mean ± standard deviation) by gel filtration. SDS-PAGE gave a subunit molecular weight of 30,800 ± 500 (Figure 1).

When crude extracts were fractionated by successive gel filtration steps on a Sephacryl S-300 column, 3HBDH activity was recovered in fractions with elution volumes corresponding to molecular weights of 360,000, 235,000, and 120,000, suggesting that 3HBDH may exist as different multimers (data not shown). However, purified 3HBDH did not show any change in its molecular weight

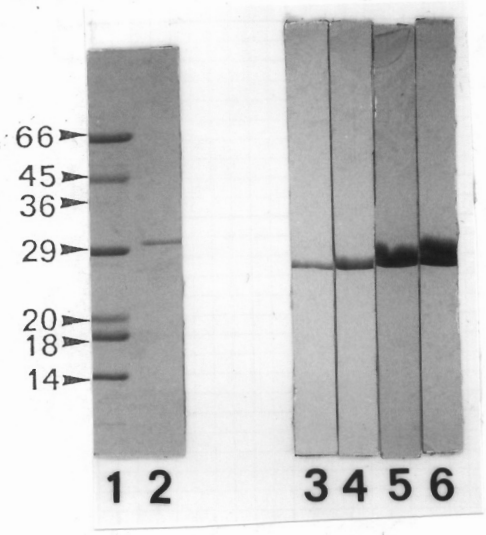

Figure 1. Examination of purity and estimation of subunit molecular weight of 3HBDH purified from C. beijerinckii NRRL B593 by SDS-PAGE. Lanes 1 and 2 contain protein standards and purified 3HBDH, respectively. Lanes 3, 4, 5, and 6 contain 4, 8, 16, and 24 μg, respectively, of purified 3HBDH. Protein was visualized with Coomassie brilliant blue R-250 staining. The molecular weight (in thousands) of each of the standards is indicated beside the corresponding band. Molecular weight standards used were bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), trypsin inhibitor (M_r 20,100), ß-lactoglobulin (M_r 18,400), and lysozyme (M_r 14,300). Glyceraldehyde-3-phosphate dehydrogenase gave a faint band.

TABLE 1. Purification of 3HBDH from C. beijerinckii NRRL B593

Step	Protein (mg)	Total Activity[a] (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Crude Extract	1,180	9,050	7.65	1	100
Q-Sepharose	156	6,780	43.5	5.7	75
Matrex Gel Red A	18.7	4,560	244	32	50
Sephacryl S-300	6.4	2,060	323[b]	42	23

[a]One unit of activity is the amount of enzyme that oxidizes 1 μmole of NADH per minute in the presence of acetoacetyl-CoA.

[b]The highest specific activity observed was 349 U/mg, which corresponds to a purification of 45-fold.

(213,000 ± 6,000) when samples at concentrations between 3 and 0.05 mg/ml were applied to a Sephacryl S-300 column. It is not clear why 3HBDH, prior to its purification, would undergo apparent changes in its molecular weight.

pH Dependence of 3-HBDH Activity. NADH-linked 3HBDH activity in the physiological (3-hydroxybutyryl-CoA-forming) direction decreased steadily over the pH range studied (5.0 to 8.0). The activity at pH 8.0 was about 60% of that at pH 5.0. NADPH-linked 3HBDH activity changed little between pH 5.0 and 5.5, but decreased steadily as the pH was increased from 5.5 to 8.0. The activity of 3HBDH at pH 8.0 was approximately 10% of that at pH 5.0. At pH 5.0, NADH-linked activity was about 5 times higher than NADPH-linked activity.

In the nonphysiological (AcAcCoA-forming) direction, NAD^+-linked activity varied little between pH 8.0 and 9.0 but increased steadily over the pH range of 6.0 to 8.0, with the activity at pH 6 being about 10% of that at pH 8. $NADP^+$-linked activity exhibited a maximal value at pH 8.0 and decreased 70% when the pH was either increased to 9.0 or decreased to 6.5. At pH 6.5 and 8.0, NAD^+-linked activities were about 1,600 and 1,300 times higher, respectively, than $NADP^+$-linked activities.

Stereospecificity of 3HBDH. (S)-3-Hydroxybutyryl-CoA, produced by the pig heart 3-hydroxyacyl-CoA dehydrogenase, was oxidized by the C. beijerinckii enzyme (data not shown). 3-Hydroxybutyryl-CoA produced by 3HBDH from C. beijerinckii was also oxidized by the pig heart enzyme. Furthermore, when

3HBDH from C. beijerinckii was added to an assay solution that originally had contained a mixture of the (R)- and (S)-enantiomers (equivalent to the D[+] and L[-] enantiomers, respectively) of 3-hydroxybutyryl-CoA but had reacted with the (S)-specific pig heart enzyme [leaving primarily (R)-3-hydroxybutyryl-CoA], no further reaction was observed. The 3HBDH from C. beijerinckii was therefore specific for the (S)-enantiomer of 3-hydroxybutyryl-CoA.

Kinetic Studies. Values of the kinetic constants for the physiological reaction involving NADH were obtained using replots of slopes and intercepts of double-reciprocal plots (Figure 2). The maximal velocity of the enzyme was 540 U/mg, with a k_{cat} value of 115,000 min^{-1}. The Km for NADH was 8.6 μM, and that for AcAcCoA was 14 μM.

A pattern typical of substrate inhibition was observed on the double-reciprocal plot of a family of lines with NADH as the changing fixed substrate (Figure 2), indicating that substrate inhibition by AcAcCoA was occurring at concentrations as low as 20 μM when NADH was present at 9.5 μM. The concentration of AcAcCoA required for inhibition increased as the concentration of NADH increased, suggesting that NADH relieved the inhibition caused by AcAcCoA. At 70 μM NADH, no inhibition could be observed at AcAcCoA concentrations as high as 100 μM.

The presence of 200 μM CoA in the assay mixture caused less than 10% inhibition of 3HBDH activity when the concentrations of NADH and AcAcCoA

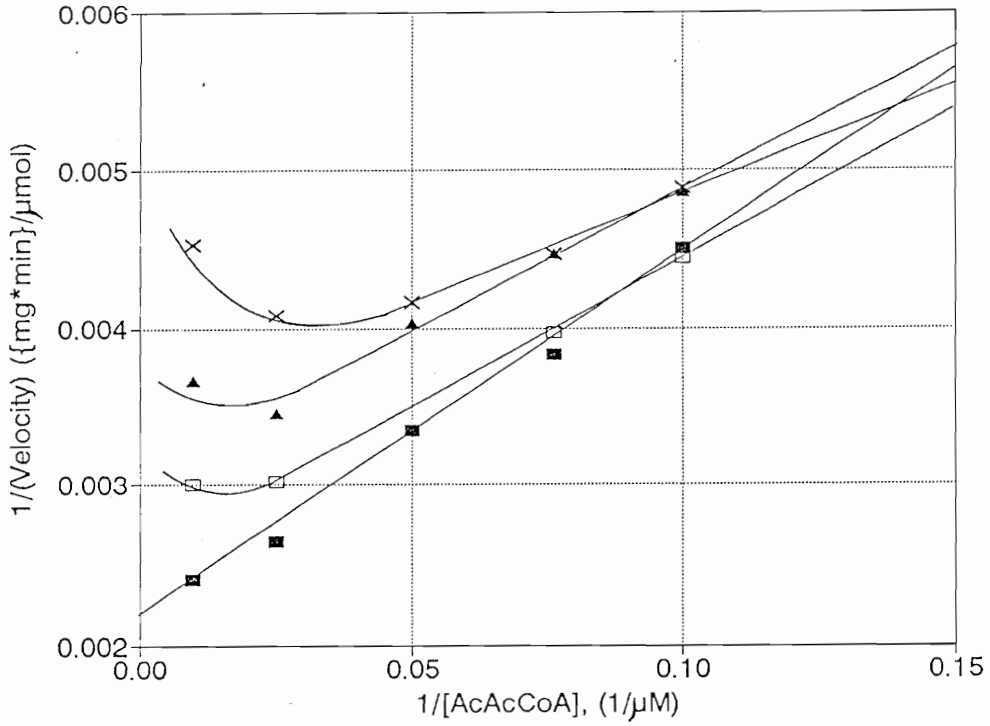

Figure 2. Double-reciprocal plot of initial velocity data in the physiological direction with AcAcCoA as the variable substrate and NADH as the changing fixed substrate. Details are described in Materials and Methods. NADH concentrations were 70 μM (■), 28 μM (□), 14 μM (▲), and 9.5 μM (x). AcAcCoA concentrations were 100, 40.1, 20.0, 13.1, and 10.0 μM. The assay mixture contained 0.053 units of purified 3HBDH.

were 100 and 60 μM, respectively. Therefore, the inhibition of 3HBDH by AcAcCoA was not caused by the possible contamination of the AcAcCoA preparation with CoA.

The apparent Km for NADPH was 150 μM (at 100 μM AcAcCoA), whereas the apparent Km value for AcAcCoA was 8 μM (at 400 μM NADPH). The apparent V_{max} for the NADPH-linked reaction was 153 U/mg (at 100 μM AcAcCoA and 0.040 to 1.0 mM NADPH), with an apparent k_{cat} value of 32,600 min^{-1}. In the non-physiological direction, the apparent Km values for NAD^+ and $NADP^+$ were, respectively, 19 μM and 5 mM (at 200 μM 3-hydroxybutyryl-CoA). The apparent Km values for 3-hydroxybutyryl-CoA were 24 μM (at 100 μM NAD^+) and 500 μM (at 10 mM $NADP^+$).

N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence of 3HBDH from C. beijerinckii NRRL B593 was Met-Lys-Lys-Ile-Phe-Val-Leu-Gly-Ala-Gly-Thr-Met-Gly-Ala-Gly-Ile-Val-Gln-Ala-Phe-Ala-Gln-Lys-Gly-(xxx)-Glu-Val-Ile-Val-Arg-Asp-Ile-Lys-Glu-Glu-. The identity of residue 25 has not been determined. Of 34 residues identified in the C. beijerinckii enzyme, 32 are identical to the N-terminal amino acid sequence deduced from the gene encoding 3HBDH in C. acetobutylicum P262 (Youngleson et al., 1989). In the C. beijerinckii enzyme, residues 2 and 34 were lysine and glutamate, respectively, whereas residues 2 and 34 of the C. acetobutylicum enzyme are glutamate and aspartate, respectively. According to the rules of Chou and Fasman (1978), the

residues which differ (residues 3 and 34) should not disrupt the predicted ß-α-ß secondary structure for coenzyme binding (Youngleson et al., 1989).

Discussion

Purification of C. beijerinckii 3HBDH to homogeneity resulted in a 45-fold increase in specific activity over that of the crude extract. The NADP-specific 3HBDH was previously purified from C. kluyveri by two research groups (Madan et al., 1973; Sliwkowski and Hartmanis, 1984), and they achieved 14- and 18-fold purification. These results suggest that 3HBDH is present at high levels, which may be a characteristic of the butyrate-producing clostridia.

3HBDH from C. beijerinckii shares a nearly identical N-terminal region with the enzyme from C. acetobutylicum, including a putative dinucleotide-binding site (Youngleson et al., 1989). The subunit size of the C. beijerinckii enzyme (M_r, 30,800) is comparable to the deduced size of the C. acetobutylicum enzyme (M_r, 31,435). Youngleson et al. (1989) noted that significant sequence similarities are present among the C. acetobutylicum 3HBDH, the pig heart muscle mitochondrial 3-hydroxyacyl-CoA dehydrogenase, and the 3-hydroxyacyl-CoA dehydrogenase portion of the rat peroxisomal bifunctional enzyme. The subunit size and kinetic properties (Noyes and Bradshaw, 1973a and 1973b) of the pig heart muscle mitochondrial 3-hydroxyacyl-CoA dehydrogenase are similar to those of the C. beijerinckii enzyme, and both are inhibited by AcAcCoA at concentrations near their Km value. This is further evidence of a possible common evolutionary origin of the two enzymes.

Birktoft et al. (1987) have determined the three-dimensional structure of the pig heart mitochondrial enzyme and proposed a model of acyl-CoA binding to the enzyme in which the CoA moiety is not specifically bound by the enzyme. Our observations that the enzyme is inhibited by AcAcCoA, but not by CoA, lend support to this model. The kinetic and physical properties of the rat peroxisomal enzyme are not as similar (Osumi and Hashimoto, 1980) to those of 3HBDH from C. beijerinckii as are those of the pig enzyme.

Although 3HBDH from C. beijerinckii 3HBDH is very similar in subunit size and kinetic properties to the NADH-linked AcAcCoAR of Alcaligenes eutrophus (Haywood et al., 1988), 3HBDH was specific for (S)-3-hydroxybutyryl-CoA, whereas the AcAcCoAR lacks stereospecificity. The kinetic constants and subunit sizes of the C. beijerinckii 3HBDH and several other AcAcCoARs (Ritchie et al., 1981; Shuto et al., 1981; Packter and Flatman, 1983; Prasad et al., 1984; Fukui et al., 1987; Haywood et al., 1988) did not show any significant similarity.

The catalytic efficiency [k_{cat}/K_m with respect to NAD(P)H] of the NADH-linked reaction was 1.3×10^7 min^{-1} mM^{-1}, whereas that of the NADPH-linked reaction was 2.2×10^5 $min^{-1}mM^{-1}$. Since NADH-linked activity varied less with pH and had a greater catalytic efficiency than the NADPH-linked activity, NADH appeared to be the physiological cosubstrate of 3HBDH from C. beijerinckii.

The broad pH profile of the enzyme activity suggests that it remains active throughout the internal pH range (6.7 to 5.5) reported for the C. acetobutylicum cell (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985; Huang et al., 1985; Terracciano and Kashket, 1986). Although *in vivo* levels of AcAcCoA have not been determined for C. beijerinckii or reported for any other solvent-producing clostridia, the fact that inhibition by AcAcCoA was observed at concentrations at or above the Michaelis constant when NADH concentration was near the Michaelis constant suggests that the metabolic intermediate plays some role in the regulation of 3HBDH activity *in vivo*. It is possible that the inhibition of 3HBDH by AcAcCoA when there is not a great excess of NADH allows the cell to generate more ATP from acetyl-CoA and to maintain a redox balance. It is known that 1 mol of ADP may be phosphorylated per mole of acetyl-CoA if the phosphate is derived from acetylphosphate, whereas only 0.5 mol of ADP may be phosphorylated per mole of acetyl-CoA if the phosphate is derived from butyrylphosphate. Inhibition of 3HBDH by AcAcCoA may therefore serve to increase the energetic efficiency. Under conditions where the NADH concentration rises, the inhibition by AcAcCoA is relieved so that more 3-hydroxybutyryl-CoA may be formed to lead to the production of butyrate or butanol, thereby allowing the oxidation of more NADH. A more complete understanding of the role of AcAcCoA in the regulation of metabolism in C.

beijerinckii awaits the characterization of the other AcAcCoA-reacting enzymes from this organism.

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Chapter III

Purification and Properties of Acetoacetate:Acetate/Butyrate Coenzyme A-Transferase from Clostridium beijerinckii (syn. C. butylicum) Strain NRRL B593

Introduction

Production of acetone, isopropanol, and butanol by anaerobic fermentations of plant biomass represents a possible alternative to the petrochemical-based syntheses. The ability of several species within the genus Clostridium to perform such fermentations has been extensively investigated. The widely-studied organism Clostridium acetobutylicum is able to produce acetone, butanol, and ethanol. Another of the solvent-producing clostridia, C. beijerinckii is characterized by the production of isopropanol in addition to these solvents (George et al., 1983). Solvent formation occurs both by the conversion of hexose and by the uptake and conversion of acids from the medium. Figure 1 of Chapter I (Chen, 1993) shows the metabolic pathways used during each mode of fermentation in these clostridia and identifies the enzymes which catalyze each reaction.

Assimilation of acetate and butyrate from the medium serves to detoxify the medium and to provide these organisms with an alternative terminal electron acceptor. Meyer et al. (1986) demonstrated that butyrate uptake could occur by a

reversal of the phosphotransbutyrylase (PTB)/butyrate kinase (BK) system in C. acetobutylicum when the culture was continually gassed with carbon monoxide, which inhibits acetoacetate decarboxylase (AcAcDC) and hydrogenase. However, the results of Hartmanis et al. (1984) strongly suggest that the PTB/BK system is not used for butyrate uptake, but rather that butyrate and acetate are re-assimilated by transfer of the coenzyme A (CoA) moiety from AcAcCoA - the reaction catalyzed by acetoacetate: acetate/butyrate CoA-transferase (CoAT).

CoAT from C. acetobutylicum ATCC 824 has been purified and characterized (Wiesenborn, 1989). The genes encoding both of the CoAT subunits from this organism have been cloned and expressed in Escherichia coli (Cary et al., 1990b) and their sequences have been published (Petersen et al., 1993). The values of the Michaelis constants observed for C. acetobutylicum CoAT are large - 1,200 mM, 1,000 mM, and 660 mM for acetate, propionate, and butyrate, respectively. Also, although CoAT appears to be stable in vivo for at least several hours (Welch et al., 1992), extracts of C. acetobutylicum have to be supplemented with 20 % (vol/vol) glycerol and 0.5 M ammonium sulfate to maintain the enzyme's activity (Wiesenborn et al., 1989).

Characterization of the CoAT from C. beijerinckii is an important step toward understanding this organism's fermentation. Since CoAT catalyzes the first committed step toward acetone and isopropanol production and is responsible for butyrate and acetate uptake under most conditions, its kinetic

properties will directly affect the product pattern of the fermentation. Knowledge of the N-terminal amino acid sequences of the subunits of CoAT will allow identification of the structural genes and measurement of their transcription. Also, a better understanding of the structure and function of CoAT may allow future manipulation of its substrate specificity, and thereby customization of the product pattern of the fermentation.

MATERIALS AND METHODS

Source of chemicals

CoA, glycine, N(2-hydroxyethyl)piperazine-N'-(2 ethanesulfonic acid) (HEPES), Tris, 1,3-bis(tris [hydroxymethyl]methylamino)-propane (Bis-Tris-Propane), 3-(N-morpholino)propanesulfonic acid (MOPS), glycerol, Cibacron blue 3GA-agarose (Type 3000-CL), hexyl-agarose, Sephacryl S-300 (high resolution; Pharmacia), butyric acid, ferritin (horse spleen), alcohol dehydrogenase (baker's yeast), conalbumin (chicken egg white), α-chymotrypsinogen A (bovine pancreas), ribonuclease A (bovine pancreas), ovalbumin (chicken), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), carbonic anhydrase (bovine erythrocyte), trypsinogen (bovine pancreas), trypsin inhibitor (soybean), ß-lactoglobulin (bovine), lysozyme (chicken egg white), α-lactalbumin (bovine), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), acrylamide, N,N'-methylenebisacrylamide, ammonium peroxydisulfate, N,N,N',N'-tetramethylenediamine, and antifoam C were obtained from Sigma Chemical Co.; ethylenediaminetetraacetic acid (EDTA) and ammonium sulfate were obtained from Fisher Scientific; butyl-Sepharose 4B was obtained from Pharmacia, Inc.; ultrafiltration membranes and Centricon 10 ultrafiltration units were obtained from Amicon Corp.; sodium dodecyl sulfate (SDS), Silver Stain Plus kit, and the dye-binding protein assay kit were obtained from Bio-Rad Laboratories; diketene was obtained from Aldrich Chemical Co; d-biotin was obtained from United States Biochemical Corp; para-

aminobenzoic acid was obtained from Matheson Coleman & Bell Manufacturing Chemists.

Organism and growth conditions

One liter of minimal medium contained the following: 1.0 g ammonium sulfate, 3.5 g dibasic potassium phosphate, 10 mg d-biotin, 10 mg para-aminobenzoic acid, 60 g glucose, and 1 ml of a mineral salts solution (George et al., 1983). The pH of the medium was adjusted to 6.8 before autoclaving, and glucose was autoclaved separately to prevent caramelization. After autoclaving, the medium was cooled to 34°C while nitrogen flowed through the headspace over it.

Spores of C. beijerinckii NRRL B593 (VPI 13437), produced in potato-extract-glucose medium (George et al., 1983), were stored in the same medium at -70°C. The spores were heat-shocked and transferred to 50 ml of chopped-meat carbohydrate (CMC) medium as previously described (Thompson and Chen, 1990). The CMC culture was incubated for 2 h at 34°C before shaking (150 RPM in an Orbit Environ-Shaker, Lab-Line Instruments, Inc., Melrose Park, Ill.) was begun. When the optical density at 550 nm (OD_{550}) of the culture, as measured with a Hitachi model 100-40 spectrophotometer, was about 2 (4-6 h after shaking began), the entire CMC culture was used to inoculate 600 ml of minimal medium in a 1-liter Erlenmeyer flask fitted with gassing and sampling ports. The nitrogen flow through the headspace of the 600 ml culture was stopped shortly following

inoculation. The gases produced by the culture were allowed to vent, and the pressure above the culture was maintained at 4 inches of water above atmospheric pressure. When the OD_{550} of this culture reached 1.5-2 (usually 4 h after inoculation), the entire culture was transferred to 7.4 liters of minimal medium in a 9-liter carboy fitted with gassing and sampling ports, to which had been added 8 ml of sterilized 50% (vol/vol) antifoam C. The nitrogen flow to the headspace of this culture was stopped, and the culture was vented as before. Cells were allowed to grow until the OD_{550} of the culture no longer increased significantly over a 4 h span (typically 24-36 h after inoculation of the 8-liter culture). The cells were harvested by centrifugation at 13,000 x g for 30 min at 4°C. The packed cells were resuspended in 1 liter of degassed buffer containing 50 mM Na-MOPS, pH 7.0 ,20% (vol/vol) glycerol, and 1.5 M ammonium sulfate and centrifuged for 2 h. The cell paste (typically 25-30 g) was immediately frozen in liquid nitrogen and was stored at -70°C until it was used to prepare crude extract.

Synthesis of AcAcCoA

AcAcCoA was synthesized by a previously described (Colby and Chen, 1992) modification of the method of Senior and Dawes (Senior and Dawes, 1973). AcAcCoA was stored at -20°C, and was stable for at least 2 months.

Extinction coefficient of AcAcCoA

The concentration of AcAcCoA in the stock preparation was determined by measuring the A_{310} of a mixture composed of 100 mM Tris-Cl (pH 7.5), 20 mM

$MgCl_2$, and 20 µl of the AcAcCoA stock solution in a total volume of 1 ml. The extinction coefficient used was 8.0 mM^{-1} cm^{-1} (Stern, 1956).

The extinction coefficient of AcAcCoA was affected by the concentration of monovalent cations, the buffer system used, and the temperature of the assay mixture. Because of this variation, the extinction coefficient to be used was determined by measuring the A_{310} of a known concentration of AcAcCoA in the specific assay system before each experiment.

Enzyme assays

The activity of CoAT was routinely assayed in air at room temperature (about 23°C) in the physiological (AcAcCoA-consuming) direction by monitoring the decrease in A_{310} of the Mg:AcAcCoA chelate. The standard assay mixture contained 100 mM K-HEPES (pH 7.0), 5% (vol/vol) glycerol, 20 mM $MgCl_2$, 20 mM Na-butyrate (pH 7.0), 300 µM AcAcCoA, and as much as 0.02 U of CoAT in a total volume of 1 ml. One unit of activity is defined as the amount of enzyme which results in the consumption of 1 µmol of AcAcCoA per min in the presence of acetate or butyrate.

Protein assays

Protein concentrations were estimated by measuring A_{230} during the purification, and by the dye-binding assay (Bradford, 1976), with bovine gamma globulin as the standard, for estimation of relative amounts of protein.

Purification of CoAT

All purification steps except for crude extract preparation were performed at room temperature (about 23°C) under aerobic conditions. Between steps, enzyme samples were stored at 4°C. The buffer (hereafter referred to as MOPS buffer) used throughout the purification scheme was 50 mM Na-MOPS (pH 7.0) containing 20% (vol/vol) glycerol and ammonium sulfate in the amount specified in each step.

(i) **Preparation of crude extract.** Crude extract was prepared anaerobically. Cell paste (144 g) was suspended in 288 ml of anaerobic MOPS buffer containing 1.5 M ammonium sulfate. After the suspension was homogenized by mixing, 40 ml of it was transferred to a French pressure cell (Aminco model J4-3398A) which was constantly flushed with argon. The suspension was passed through the French pressure cell at 15,000 psi and was collected in a degassed centrifuge tube. The remainder of the suspension was processed in this manner. The lysate was centrifuged for 45 min at 37,000 x g at 4°C to remove cell debris and precipitated protein. The supernatant was combined with anaerobic MOPS buffer containing 1.5 M ammonium sulfate (0.5 ml buffer/ml supernatant) in a degassed flask and stored at 4°C with gentle stirring. After 8 h, the precipitated protein was removed by centrifugation at 37,000 x g for 2 h. The resulting supernatant was the crude extract. Its protein content was about 38,000 ml*A_{230}.

(ii) Butyl-Sepharose columns. The crude extract was divided into three portions. One portion was applied to a butyl-Sepharose column (2.5 by 5.3 cm) which had been equilibrated with MOPS buffer containing 1.5 M ammonium sulfate. Activity was eluted by applying the same buffer at 100 ml/h, and 10 ml fractions were collected. The column was washed with distilled water and 6 M urea and re-equilibrated with buffer before the next portion of crude extract was loaded.

To improve the yield of the step, those fractions (180 ml) which were collected before the bulk of CoAT activity and had specific activities in the range of 3-15 U/(ml*A_{230}) were pooled and concentrated by ultrafiltration through a YM30 membrane in a stirred cell to 93 ml. This sample was then applied to a butyl-Sepharose column as described above.

(iii) Sephacryl-S300 HR column. Fractions (1030 ml) from the four preceding butyl-Sepharose columns containing specific activities of CoAT above 15 U/(ml*A_{230}) were pooled and concentrated by ultrafiltration through a YM30 membrane to 8.2 ml. This preparation was applied to a Sephacryl-S300 HR column (2.5 by 53.0 cm) which had been equilibrated with MOPS buffer containing 1.5 M ammonium sulfate. The flow rate was maintained at 25 ml/h, and 5-ml fractions were collected.

(iv) Cibacron blue 3GA-agarose columns. Fractions (30 ml) from the Sephacryl S-300 HR column containing high CoAT activity were pooled and

concentrated by ultrafiltration through a YM30 membrane to 5.0 ml and twice diluted 10-fold with MOPS buffer containing 0.5 M ammonium sulfate, and finally concentrated to 6.5 ml. This sample was applied to a Cibacron blue 3GA-agarose column (1.5 by 21.2 cm) which had been equilibrated with MOPS buffer containing 0.5 M ammonium sulfate. Activity was eluted by applying the same buffer at 40 ml/h, and 5 ml fractions were collected.

The fold of purification effected by this step was increased by repeating it. Fractions (25 ml) containing CoAT activity were pooled and concentrated by ultrafiltration through a YM30 membrane to 5.3 ml. The Cibacron blue 3GA-agarose column was washed with 6 M urea and distilled water and re-equilibrated with buffer. The concentrated enzyme sample was applied and eluted as before.

(v) Hexyl-agarose column. Fractions (50 ml) from the second Cibacron blue 3GA-agarose column were pooled and concentrated by ultrafiltration through a YM30 membrane to 5.0 ml and twice diluted 10-fold with MOPS buffer containing 1.5 M ammonium sulfate, and finally concentrated to about 5 ml. This sample was applied to an hexyl-agarose column (2.5 by 8.4 cm) which had been equilibrated with MOPS buffer containing 1.5 M ammonium sulfate. The column was washed with 50 ml of the same buffer, and activity was eluted by using a linear gradient of ammonium sulfate (1.5 to 1.0 M in MOPS buffer; total volume, 200 ml). The flow rate was maintained at 50 ml/h, and 5 ml fractions were collected.

Determination of native and subunit molecular weights

The native molecular weight of CoAT was determined by measurement of its elution volume on a calibrated Sephacryl-S300 HR column (2.5 by 52.9 cm) which had been equilibrated with MOPS buffer containing 0.5 M ammonium sulfate. A 2.5-ml sample was applied, and 2.5-ml fractions were collected at 50 ml/h. The routine assay was used to determine CoAT activity in the fractions. Molecular weight standards and M_r values used were ferritin (440,000), yeast alcohol dehydrogenase (150,000), conalbumin (78,000), and α-chymotrypsinogen A (25,000).

The purity and the subunit molecular weight of CoAT were determined by polyacrylamide gel electrophoresis (PAGE) on a 12.5% gel in the presence of SDS (Laemmli, 1970). Before being applied to the gel, purified CoAT and protein standards were equilibrated with SDS-PAGE stacking gel buffer (0.5 M Tris-Cl [pH 6.8]) by dialysis against stacking gel buffer and then incubated for 3 min in a boiling water bath in the presence of SDS and 2-mercaptoethanol. Protein was stained with the Silver Stain Plus kit (Bio-Rad).

Kinetic studies

The extinction coefficient of AcAcCoA varied among the various assay conditions used. The pH of buffer and carboxylic acid substrate solutions was adjusted by addition of Bis-Tris-Propane in an attempt to minimize this variation. The assay system used for the determination of Michaelis constants of AcAcCoA

and butyrate in the physiological direction contained 100 mM (Bis-Tris-Propane)-HEPES (pH 7.0), 20 mM $MgCl_2$, 77.8, 97.9, 130, 194, or 482 μM AcAcCoA, and 8.00, 10.0, 13.5, 20.0, or 40.0 mM (Bis-Tris-Propane)-butyrate (pH 7.0) and was initiated by addition of 150 ng of purified CoAT. The extinction coefficient of AcAcCoA in this assay system varied between 1.9 and 2.3 $mM^{-1}*cm^{-1}$.

The assay system used for the determination of Michaelis constants of AcAcCoA and acetate in the physiological direction contained 100 mM (Bis-Tris-Propane)-HEPES (pH 7.0), 20 mM $MgCl_2$, 26.8, 33.5, 49.6, 101, or 255 μM AcAcCoA, and 67.0, 80.0, 100, 133, 200, or 400 mM (Bis-Tris-Propane)-acetate (pH 7.0) and was initiated by addition of 150 ng of purified CoAT. The extinction coefficient of AcAcCoA in this assay system varied between 2.4 and 3.2 $mM^{-1}*cm^{-1}$.

The assay system used for the determination of Michaelis constants of AcAc and Butyryl-CoA in the non-physiological direction contained 100 mM (Bis-Tris-Propane)-HEPES (pH 7.0), 20 mM $MgCl_2$, 0.5, 0.65, 1, 2, or 5 mM Li•AcAc (pH adjusted to 7.0 with HCl), and 50, 65, 100, 200, or 500 μM Butyryl-CoA, and was initiated by addition of 3 μg of purified CoAT. The extinction coefficient of AcAcCoA in this assay system varied between 2.3 and 2.7 $mM^{-1}*cm^{-1}$.

The assay system used for the determination of Michaelis constants of AcAc and Acetyl-CoA in the non-physiological direction contained 100 mM (Bis-Tris-Propane)-HEPES (pH 7.0), 20 mM $MgCl_2$, 0.5, 0.67, 1, 2, or 5 mM Li•AcAc

(pH adjusted to 7.0 with HCl), and 200, 235, 285, 385, or 500 μM Acetyl-CoA, and was initiated by addition of 3 μg of purified CoAT. The extinction coefficient of AcAcCoA in this assay system varied between 1.6 and 2.4 $mM^{-1}*cm^{-1}$.

Separation of the subunits of CoAT

The two subunits of CoAT were separated using a Bio-Rad Model 491 continuous elution preparative electrophoresis cell. Purified CoAT was dialyzed against stacking gel buffer (125 mM Tris-Cl [pH 6.8]) and incubated for 3 min in a boiling water bath in the presence of SDS and mercaptoethanol. A 10 % (wt/vol) polyacrylamide gel (5.0 x 2.8 cm) was used, and 800 μg of purified CoAT was loaded in a total volume of 1 ml. The separated subunits were collected in a buffer (25 mM Tris, 192 mM glycine, 0.1% [wt/vol] SDS), which was pumped through the elution chamber at 60 ml/h; 3 ml fractions were collected.

Determination of the N-terminal amino acid sequence

Fractions containing about 300 μg of either of the separated subunits were combined and concentrated in Centricon-10 units, and washed extensively with distilled water before sequencing. Approximately 500 pmol of each subunit, estimated by the dye-binding method, was submitted for sequencing. The N-terminal amino acid sequences of each of the subunits of CoAT were determined with an Applied Biosystems Model 477A sequencer (by C.L. Rutherford and S. Peters-Weigel of the Protein Sequencing Facility, Virginia Polytechnic Institute and State University, Blacksburg).

RESULTS

Purification of CoAT

Before CoAT from C. beijerinckii could be purified and characterized, it was necessary to formulate a buffer system in which the enzyme was stable. Wiesenborn et al (1989) found that a buffer composed of 25 mM Na-MOPS (pH 7.0), 20 % (vol/vol) glycerol, and 0.5 M ammonium sulfate was sufficient to maintain the activity of CoAT from C. acetobutylicum. This buffer system also significantly increased the stability of CoAT from C. beijerinckii relative to the same buffer lacking glycerol, ammonium sulfate, or both. However, the stability of CoAT from C. beijerinckii was increased further by increasing the concentration of ammonium sulfate to 1.5 M (data not shown).

CoAT was purified 308-fold to apparent homogeneity, as indicated by the presence of two bands following SDS-PAGE and silver staining (Figure 1). In addition, N-terminal amino acid sequencing revealed little additional residues at each cycle for either of the two subunits. A summary of the purification of CoAT is presented in Table 1. The procedures for each step are described in Materials and Methods. Glycerol (20% [vol/vol]) and at least 0.5 M ammonium sulfate was required to maintain the stability of CoAT. CoAT activity was 40% lower in aerobically-prepared crude extract than in anaerobically-prepared crude extract after one month of storage at 4°C. After the first purification step, the enzyme was no longer oxygen sensitive. CoAT could be stored either at 4°C in MOPS

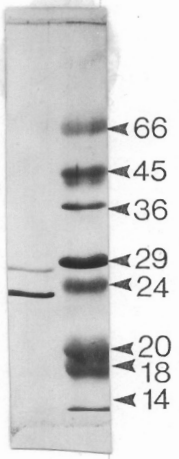


Figure 1. Examination of purity and estimation of subunit molecular weight by SDS-PAGE of CoAT purified from C. beijerinckii NRRL B593. Lane 1 contains approximately 2.5 μg purified CoAT. Lane 2 contains protein standards. Experimental details are described in the Materials and Methods section. MW values listed are in thousands.

TABLE 1. Purification of CoAT from C. beijerinckii NRRL B593

Step	CoA Transferase Activity[a] (U)	Total Protein (A_{230}*ml)	Specific Activity (U/{ml*A_{230}})	Purification (Fold)	Yield (%)
Crude Extract	74,900	38,200	1.96	1.00	100
Butyl-Sepharose	43,500	1,870	23.2	11.8	58
Sephacryl S-300 HR	35,100	852	41.2	21.0	47
Cibacron Blue 3GA	21,300	119	179	91.3	28
Hexyl-Agarose	13,300	23.2	572[b]	292	18

[a] One unit of activity is the amount of enzyme that consumes 1 µmol of AcAcCoA per min in the presence of butyrate.

[b] The highest specific activity observed was 604 U/(ml*A_{230}), which corresponds to a purification of 308-fold.

buffer containing 20% (vol/vol) glycerol and 1.5 M ammonium sulfate for one month or at -20°C in the same buffer containing 1.0 M ammonium sulfate for four months without noticeable loss of activity.

Despite the fact that the CoAT enzyme from C. beijerinckii showed a high degree of N-terminal sequence identity to that from C. acetobutylicum, it could not be purified using the same procedure. CoAT from C. acetobutylicum was recovered from octyl- and phenyl-Sepharose columns with yields of about 45% and 70%, respectively (Wiesenborn et al., 1989). However, when CoAT from C. beijerinckii was applied to these columns under identical conditions, no activity could be recovered.

Determination of native and subunit molecular weight

CoAT had a native molecular weight of 89,100 ± 4,600 (mean ± standard deviation) by gel filtration. SDS-PAGE gave subunit molecular weights of 28,400 ± 500 and 25,200 ± 400. Coomassie brilliant blue protein determination of the separated subunits of CoAT suggested that the two were present in roughly equimolar amounts.

Kinetic studies

Another difficulty which had to be overcome before CoAT could be characterized was that of developing a reliable assay for the enzyme. The physiological reaction of CoAT (transfer of CoA from AcAcCoA to acetate or butyrate) can be followed directly by measuring the A_{310} of the Mg-chelate of the

enolate form of AcAcCoA (Stern, 1956). If CoAT is able to react with only one form (keto or enol) of AcAcCoA, then the effective concentration of the true substrate will vary as the extinction coefficient varies. Assays performed in the presence and absence of $MgCl_2$ showed no difference in CoAT activity (data not shown), so AcAcCoA concentrations reported herein represent total concentrations (keto + enol forms). However, the value of the extinction coefficient of the chelate varies with the concentrations of monovalent cations and acid substrates in the assay mixture, as well as with temperature and pH. For this reason, it was necessary to measure the extinction coefficient at each assay condition used.

Values of the kinetic constants for the physiological reactions (AcAcCoA consumption) involving acetate and butyrate were obtained by using replots of the vertical intercepts of double-reciprocal plots. They are listed in Table 2. The correlation coefficients of the lines fitted to the data in Figures 2 and 3 (least-squares method) were greater than 0.98 for the reactions in the physiological direction. It appeared from the nearly parallel lines fitted to the data that the reaction proceeded by a ping-pong mechanism. In addition, double reciprocal plots of assays in which the ratio of substrates was fixed yielded straight lines, again indicating a ping-pong mechanism. It was also apparent from the double-reciprocal plots (Figures 2 and 3) that substrate inhibition was occurring, both by butyrate at concentrations as low as 40 mM when the AcAcCoA concentration

Table 2. Kinetic Constants of Acetate/Butyrate:Acetoacetate CoA-Transferase from Clostridium beijerinckii NRRL B593.

Physiological Reaction - Acid + AcAcCoA → Acyl-CoA + AcAc

Substrate	K_M (mM)	V_{MAX} (μmol/[min*mg])	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}*mM^{-1}$)
Butyrate	11.7	2890	2.60×10^5	2.22×10^5
AcAcCoA	0.107			2.43×10^6
Acetate	424	1120	1.01×10^5	238
AcAcCoA	0.118			8.54×10^5

Reverse (Non-Physiological) Reaction - Acyl-CoA + AcAc → Acid + AcAcCoA

Substrate	K_M (mM)	V_{MAX} (μmol/[min*mg])	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}*mM^{-1}$)
Butyryl-CoA	0.107	160	1.44×10^4	1.35×10^5
AcAc	0.339			4.25×10^4
Acetyl-CoA	0.141	19.4	1.75×10^3	1.24×10^4
AcAc	0.161			1.08×10^4

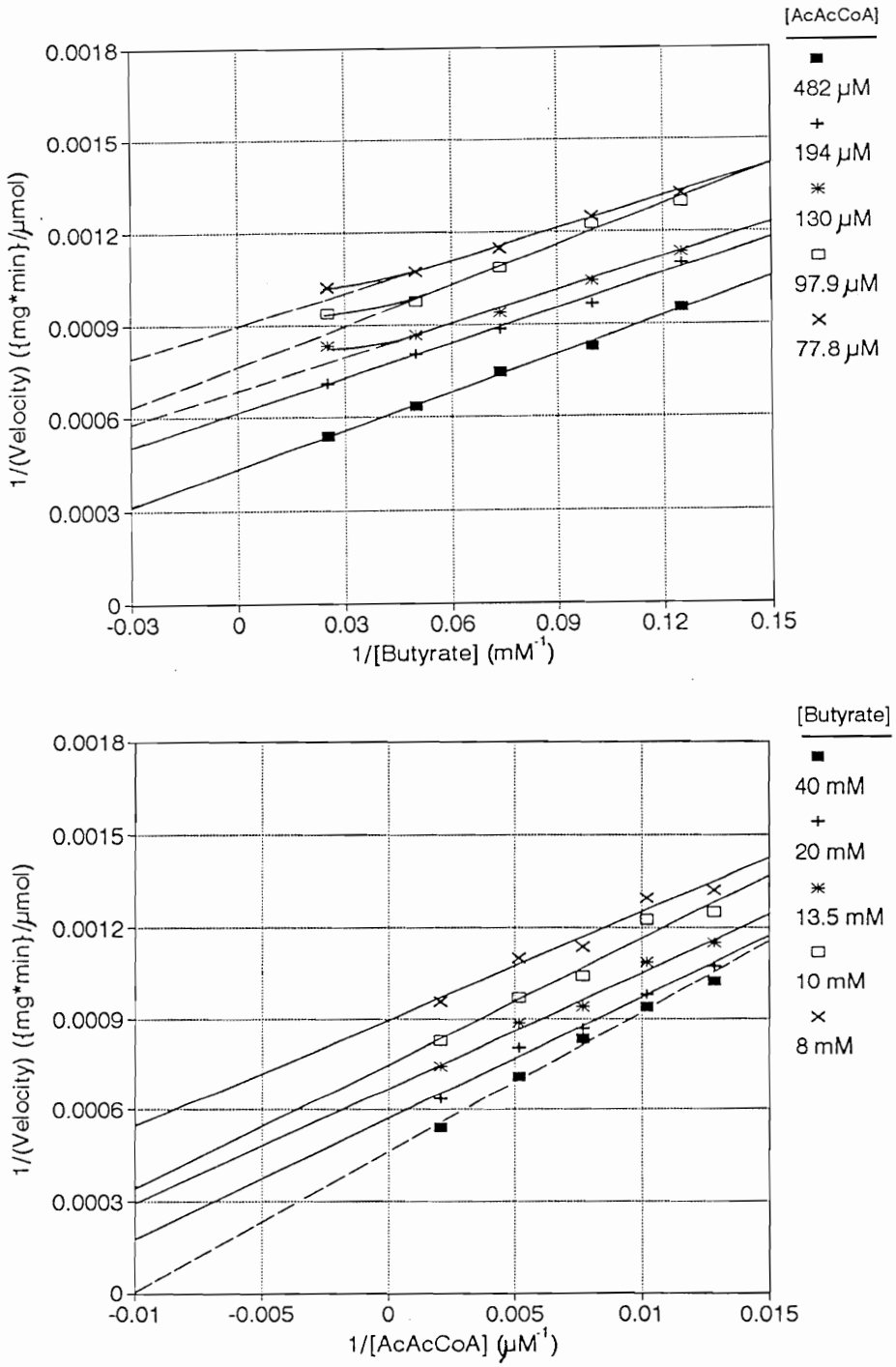
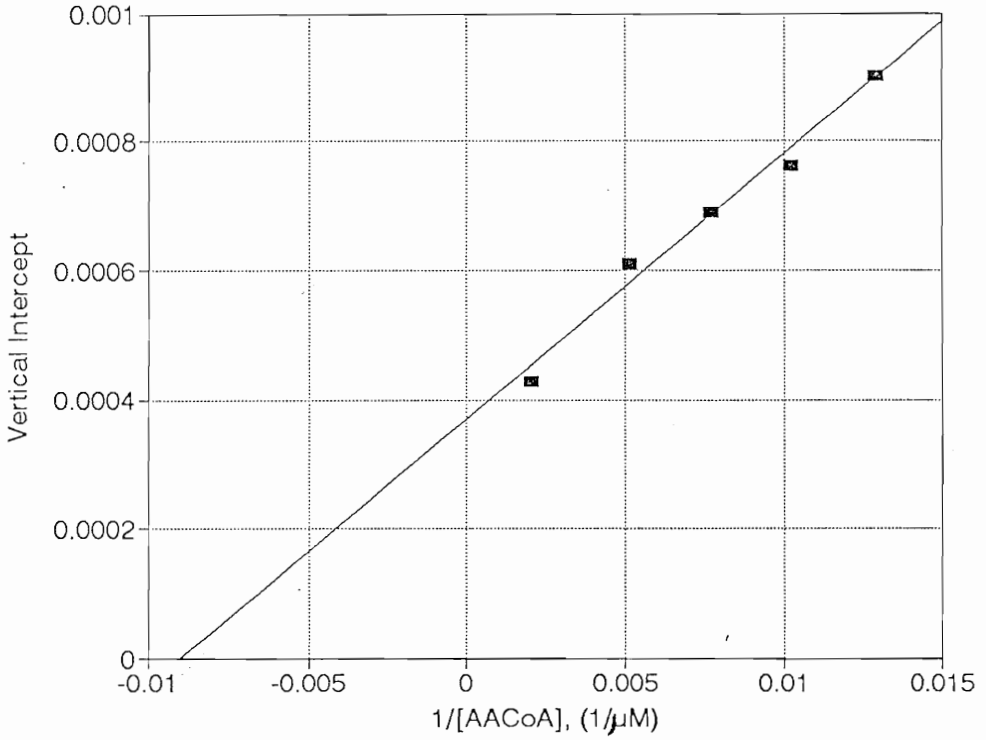


Figure 2a. Double-reciprocal plots of kinetic data for the reaction between butyrate and AcAcCoA in the physiological (AcAc-forming) direction. Assay conditions and substrate concentrations are listed in the Materials and Methods section. 0.2 Units of CoAT were used in each assay. Dashed lines indicate assay conditions under which substrate inhibition was observed. These data were not used for the determination of kinetic constants.



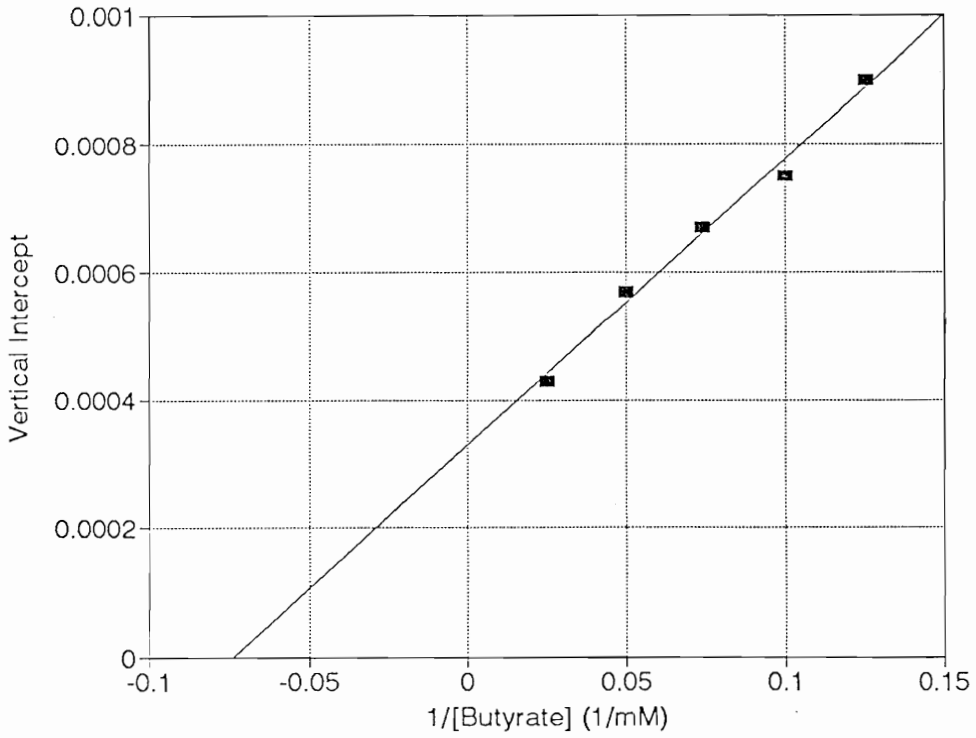


Figure 2b. Replots of the vertical intercepts of the lines fitted to the data in the plots of Figure 2a. Values for the kinetic constants for CoAT were deduced from the replots.

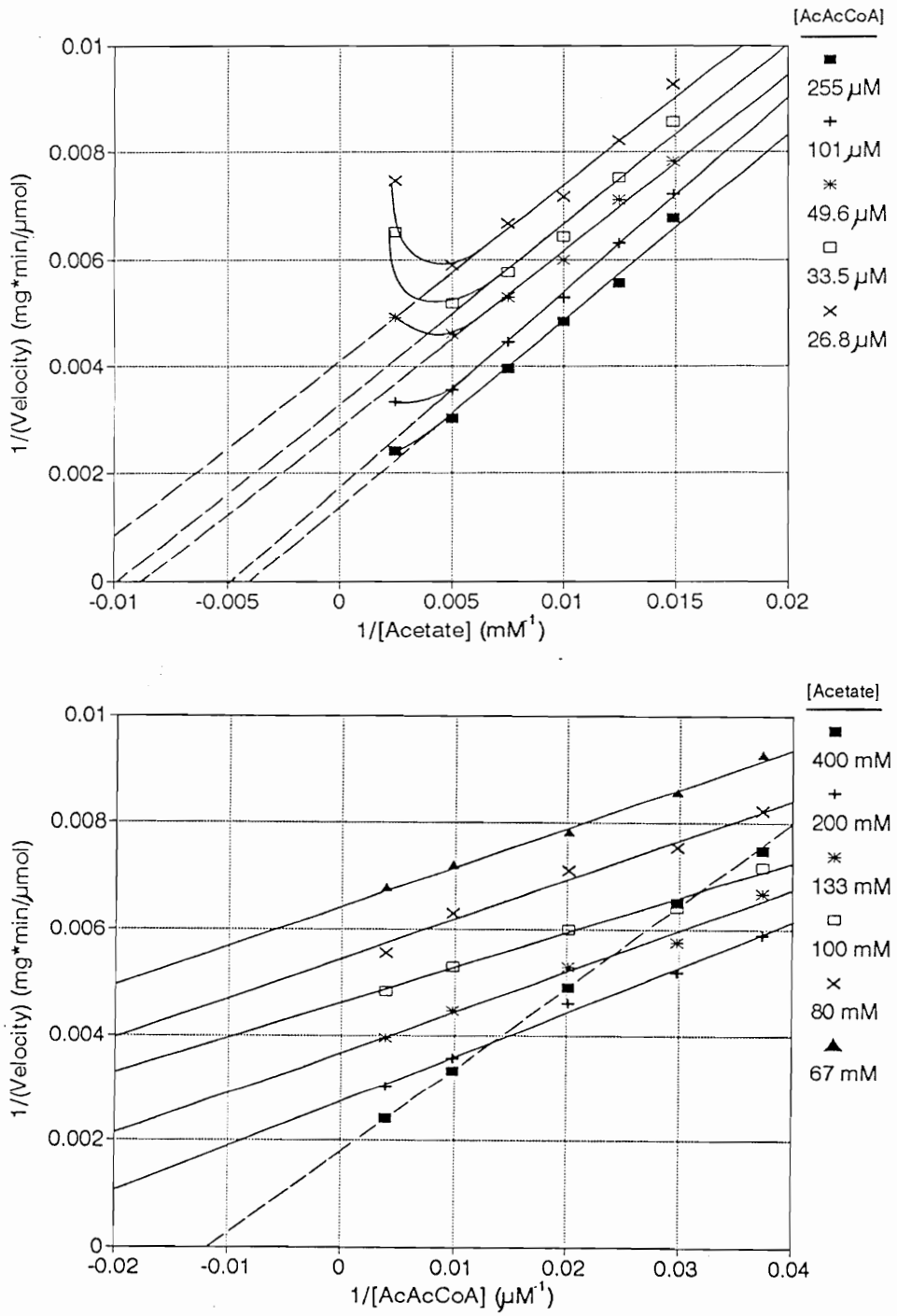
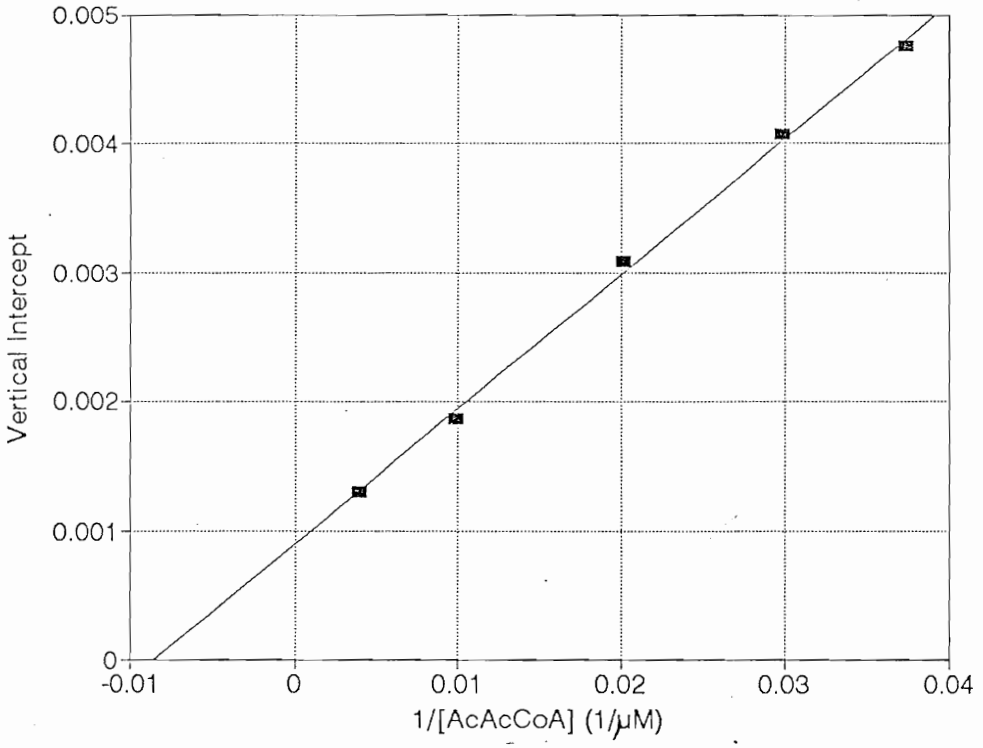


Figure 3a. Double-reciprocal plots of kinetic data for the reaction between acetate and AcAcCoA in the physiological (AcAc-forming) direction. Assay conditions and substrate concentrations are listed in the Materials and Methods section. 0.2 Units of CoAT were used in each assay. Dashed lines indicate assay conditions under which substrate inhibition was observed. These data were not used for the determination of kinetic constants.



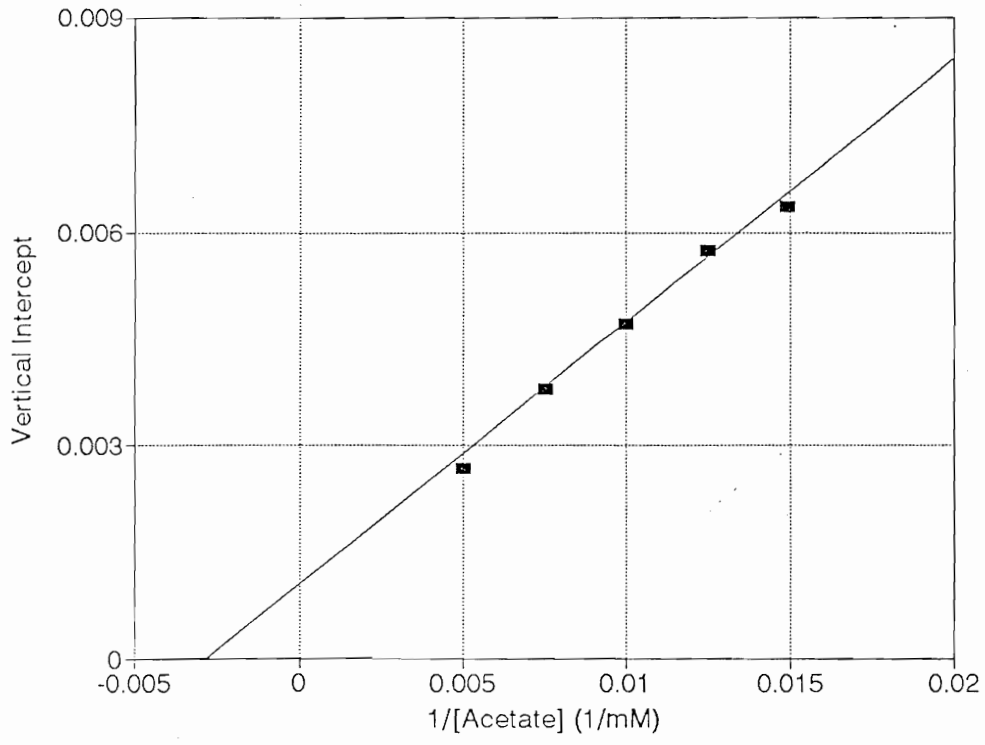


Figure 3b. Replots of the vertical intercepts of the lines fitted to the data in the plots of Figure 3a. Values for the kinetic constants for CoAT were deduced from the replots.

was 130 μM and by acetate at a concentration of 400 mM at all AcAcCoA concentrations studied.

Table 2 also lists the kinetic constants for the non-physiological reactions (AcAc-CoA formation) involving acetyl-CoA and butyryl-CoA, as deduced from replots of the vertical intercept values obtained from double-reciprocal plots (Figures 4 and 5). The correlation coefficients of the lines fitted to the data from the non-physiological reactions varied between 0.92 and 0.99. The decreasing slope of the lines with increasing substrate concentration is indicative of substrate inhibition by the substrates of the non-physiological direction. This pattern is not unusual, since substrate inhibition is usually more pronounced in the non-physiological direction than in the physiological.

N-terminal amino acid sequences

The N-terminal amino acid sequence of the large subunit of CoAT from C. beijerinckii NRRL B593 was Met-Ile-Val-Asp-Lys-Val-Leu-Ala-Lys-Glu-Ile-Ile-Ala-Lys-Arg-Val-Ala-Lys-Glu-Leu-Lys-Lys-Gly-Gln-Leu-Val-Asn-Leu-Gly-Ile-Gly-Leu-Pro-Thr-Leu-Val-Ala-Asn-Tyr-Val-Pro-Lys-Glu-Tyr-Met-. Of 45 amino acid residues identified in the C. beijerinckii enzyme, 34 (76%) are identical to the N-terminal amino acid sequence deduced from the gene encoding the large subunit of CoAT in C. acetobutylicum ATCC 824 (Gerischer and Dürre, 1990; Petersen et al., 1993). Of the 11 non-identical residues, 4 are conservative replacements.

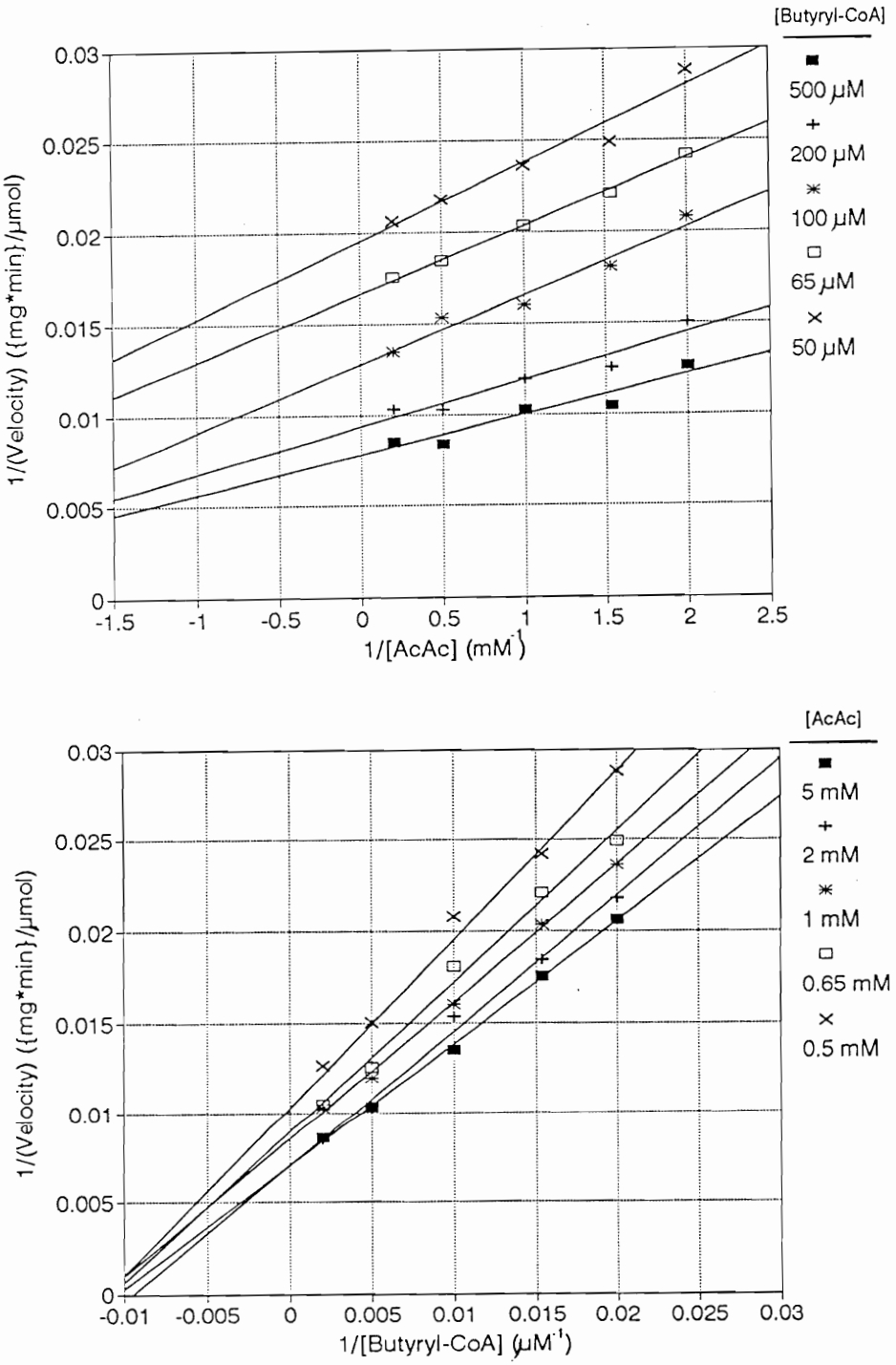


Figure 4. Double-reciprocal plots of kinetic data for the reaction between butyryl-CoA and AcAc in the non-physiological (AcAc-consuming) direction. Assay conditions and substrate concentrations are listed in the Materials and Methods section.

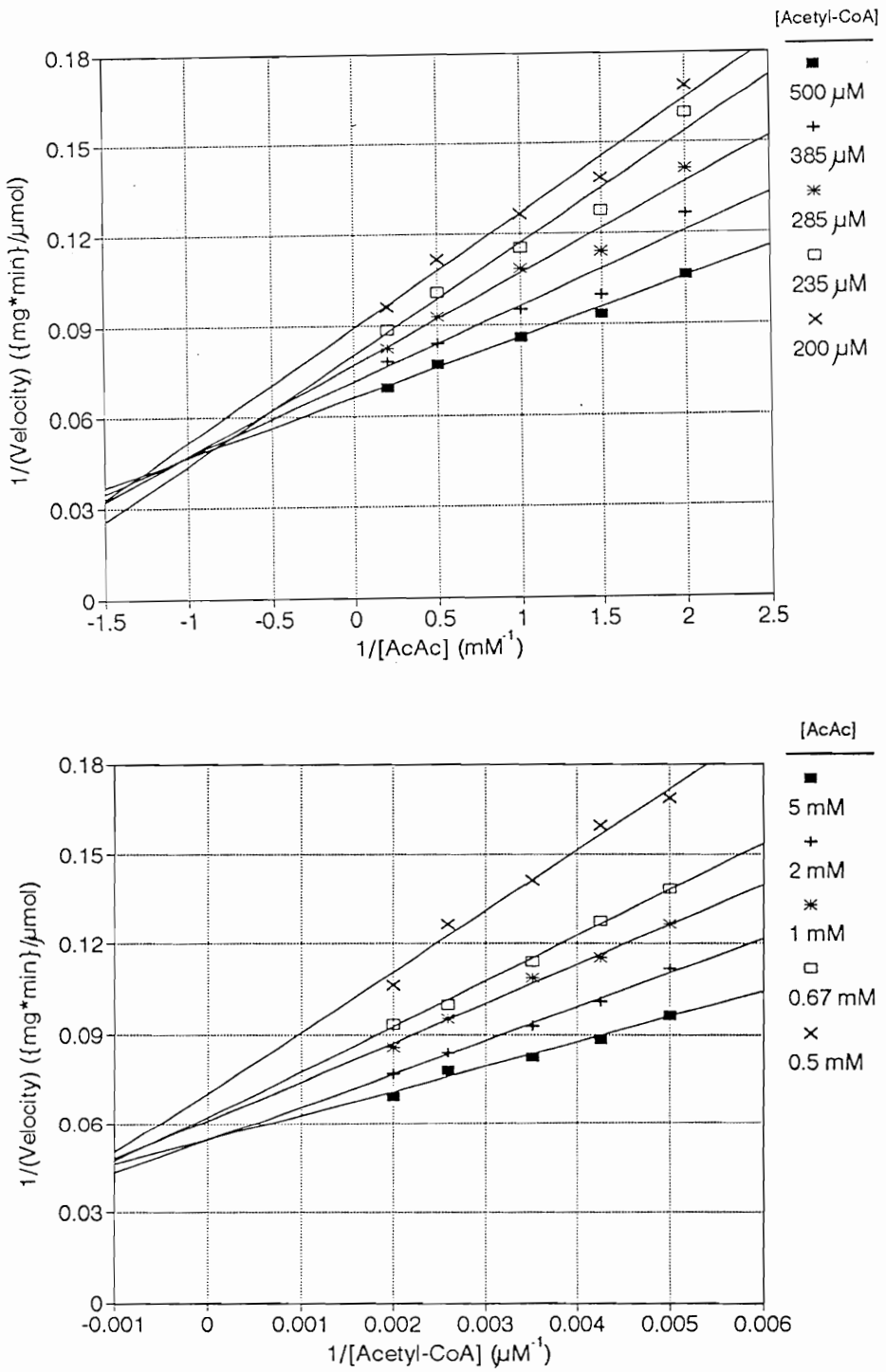


Figure 5. Double-reciprocal plots of kinetic data for the reaction between acetyl-CoA and AcAc in the non-physiological (AcAc-consuming) direction. Assay conditions and substrate concentrations are listed in the Materials and Methods section.

The N-terminal amino acid sequence of the small subunit of the C. beijerinckii CoAT was Met-Asn-Lys-Leu-Val-Lys-Leu-Thr-Asp-Leu-Lys-Arg-Ile-Phe-Lys-Asp-Gly-Met-Thr-Ile-Met-Val-Gly-Gly-Phe-Leu-Asp-(XXX)-Gly-Thr-Pro-Glu-Asn-Ile-Ile-Asp-Met-Leu-Val-Asp-. Of 39 amino acid residues identified in the small subunit of the C. beijerinckii enzyme, 22 (56%) are identical to the N-terminal amino acid sequence deduced from the gene encoding the small subunit of CoAT in C. acetobutylicum ATCC 824 (Petersen et al., 1993). Of the 17 non-identical residues, 4 are conservative replacements. In addition, significant similarity was observed between the N-terminal sequences of the subunits of CoAT from C. beijerinckii and CoAT enzymes from Pseudomonas putida (Parales and Harwood, 1992), Acinetobacter calcoaceticus (Parales and Harwood, 1992), and pig heart mitochondria (Lin and Bridger, 1992), as shown in Figure 6.

DISCUSSION

Stability of CoAT

The CoAT enzymes from C. beijerinckii NRRL B593 (this study) and C. acetobutylicum ATCC 824 (Wiesenborn, 1989) require the presence of glycerol and ammonium sulfate in the solution to remain active. No similar requirements were observed among the other CoAT enzymes which have been studied (Allen, et al. 1964; Hersh and Jencks, 1967a; Sramek and Frerman, 1975a; Tung and Wood, 1975; Barker et al., 1978; Buckel et al., 1981; Eikmanns and Buckel, 1990; Scherf and Buckel, 1991). The stabilizing conditions for the CoAT enzymes from the two solvent-producing clostridia must reflect the intracellular conditions during solvent production, since the C. acetobutylicum enzyme has been shown to be stable *in vivo* (Welch et al., 1992).

Glycerol is capable of contributing to the conformational stabilization of proteins by creating a zone of preferential hydration at the protein-solvent interface (Timasheff and Arakawa, 1989). Inclusion of otherwise shielded hydrophobic regions of the protein in this hydration zone will force more water molecules to assume an ordered configuration, resulting in an increase in the free energy of the system. Hydration of hydrophilic regions of the protein does not require well-ordered water structure, and results in a smaller increase in free energy. Since natural systems tend toward situations of minimum free energy, hydrophobic regions of the protein will be preferentially excluded from the

protein-solvent interface, resulting in a stabilized conformation. Increasing the hydrophilic nature of the zone of hydration, by adding glycerol, for instance, will increase the free energy difference between protein conformations with various degrees of hydrophobic region exposure. The situation is complicated by the fact that glycerol can also stabilize proteins by interacting with polar residues on their surfaces. Ammonium sulfate exerts its stabilizing effects by increasing the surface tension of water, thereby creating a similar zone of preferential hydration about the protein (Timasheff and Arakawa, 1989). These effects apply similarly to interactions between different regions of a single peptide and between regions of different peptides. Therefore, the stable quaternary structure of proteins is also affected by these phenomena.

It is unclear what cellular component of C. beijerinckii or C. acetobutylicum might be able to substitute for ammonium sulfate and glycerol in vivo. It has been reported (Reysenbach, et al., 1986) that granulose is accumulated intracellularly, which might stabilize proteins in the same manner as glycerol - by causing preferential hydration due to exclusion of the polymer from the zone around proteins. It is possible that CoAT forms a complex with another protein, or that one of the heat-shock proteins induced in C. acetobutylicum during the shift to solventogenesis (Pich et al., 1990; Terracciano et al., 1988) is able to either maintain the native conformation of CoAT or aid denatured CoAT in re-folding to its proper configuration. Stabilization and catalysis of refolding of

malate dehydrogenase (Hartman et al., 1993) and citrate synthase (Buchner et al., 1991) by the mammalian heat-shock protein GroE has been demonstrated. Further study is required to test these hypotheses.

Similarities to other CoAT enzymes

The kinetic constants calculated from replots of double reciprocal plots for CoAT from C. beijerinckii are presented in Table 2. It is apparent from these data that butyrate is preferred to acetate as a substrate, since the turnover number, k_{cat} (min^{-1} calculated using an estimated native molecular weight of 90,000), is roughly 100 times higher for butyrate than for acetate. In contrast, the C. acetobutylicum enzyme was more active with acetate than butyrate (relative k_{cat} values of 4.2 and 1, respectively; Wiesenborn et al., 1989). Also, the Michaelis constants of CoAT from C. beijerinckii for acetate and butyrate (424 and 11.7 mM, respectively) are far lower than those reported for CoAT from C. acetobutylicum (1,200 and 660 mM, respectively; Wiesenborn et al., 1989). The Michaelis constant for butyrate of CoAT from C. beijerinckii is approximately an order of magnitude or more below the range of intracellular butyrate concentrations (80-700 mM) reported for C. acetobutylicum at the point when the organism switches from the acid-producing to the solvent-producing phase of growth (Terracciano and Kashket, 1986; Wiesenborn et al., 1989; Grupe and Gottschalk, 1992). This preference for butyrate exhibited by the C. beijerinckii CoAT is a possible explanation for the greater reassimilation of butyrate, relative

to acetate, observed in several studies of solvent-producing clostridia (Bahl et al., 1982; Andersch et al., 1983; George and Chen, 1983; Grupe and Gottschalk, 1992). Hartmanis and coworkers (1984) presented strong evidence that essentially all reactivation of acids by C. acetobutylicum was catalyzed by CoAT.

CoAT enzymes have been isolated from several organisms besides C. beijerinckii and C. acetobutylicum. Although the physiological roles of these enzymes vary significantly, the CoAT enzymes from C. beijerinckii and C. acetobutylicum show various degrees of similarity to many of them. The native and subunit molecular weights of the enzymes from the solvent-producing clostridia are similar to those of the CoAT from E. coli (Sramek and Frerman, 1975a). Also, the true Michaelis constants for acetyl-CoA and AcAcCoA reported for the E. coli (Sramek and Frerman, 1975b) enzyme are within an order of magnitude of those found for C. beijerinckii.

The Michaelis constants and turnover numbers of the pig heart mitochondrial (PHM) CoAT using succinyl-CoA and AcAc (its physiological substrates) in both the forward and reverse directions (Hersh and Jencks, 1967b; Moore and Jencks, 1982) are within an order of magnitude of the Michaelis constants and turnover numbers of the C. beijerinckii enzyme using butyryl-CoA and AcAc. However, even though the native molecular weights of the PHM CoAT (M_r= 92,000; White and Jencks, 1976b) is similar to that of C. beijerinckii, the single subunit of the PHM CoAT is approximately equal in size to the two C.

beijerinckii enzyme subunits combined. Furthermore, as illustrated in Figure 6, the primary amino acid sequences of the C. beijerinckii enzyme (and those deduced from the genes encoding CoAT in C. acetobutylicum [Petersen et al., 1992]) can be aligned with similar regions of the amino acid sequence deduced from a cDNA encoding the PHM CoAT (Lin and Bridger, 1992). The amino acid sequences deduced from the genes encoding the subunits of the less thoroughly-characterized CoAT enzymes from Pseudomonas putida and Acinetobacter calcoaceticus (Parales and Harwood, 1992) also show significant similarity to the PHM CoAT and the enzymes from C. beijerinckii and C. acetobutylicum. It appears from the comparison of the primary sequences of the proteins listed in Figure 6 that the PHM CoAT represents a fusion of the protein domains present as separate subunits in the other enzymes. It is interesting that enzymes from such different physiological systems, including acid re-utilization, ketone body catabolism, and ß-ketoadipate metabolism, exhibit so much sequence similarity. Lin and Bridger (1992) noted that the region linking the two regions of the PHM CoAT similar to the subunits of the other enzymes is significantly more hydrophilic than either of the conserved regions. In fact, it has been observed (Moore and Jencks 1982) that this enzyme may be proteolytically cleaved in this hydrophilic region (Lin and Bridger 1992) without loss of enzymatic activity. The CoAT enzyme which has been extensively purified from Clostridium SB4 (Barker et al., 1978) also had native and subunit molecular

```
     1                                                  50
CBS  .......... .......... .......... .........m nklvkLtDLk RiFkdGMTIM VGGFLdxGtP ENIIdMLV..
CAS  .......... .......... .......... ........mn skiirFenLr sfFkdGMTIM IGGFLnCGtP tkLIdFLVnl
PPL  .......... .......... .......... .......lin KtYesIAsAV efItdGsTIM VGGFGtaGMP seLIdGLIaT
ACS  .......... .......... .......... .......mid KsaatLtEAL sqIhdGATIL IGGFGtaGqP aeLIdGLIel
PHM  MAALTLLSSR LRLCASAYRS GGAWSQGCAG YFSTSTRRHT KFYTDAVEAV KDIPNGATVL VGGFGLCGIP ENLIGALLKT
CBL  .......... .......... .......... .......... .......... .......... .......... ..........
CAL  .......... .......... .......... .......... .......... .......... .......... ..........
PPS  .......... .......... .......... .......... .......... .......... .......... ..........
ACL  .......... .......... .......... .......... .......... .......... .......... ..........

                    100                                                            150
CBS  .......... .......... .......... .......... .......... .......... .......... ..........
CAS  nIKNLTIISN dtcypNtGIG kLisnnQVKK LIASYI..GS NPDtgKklfn nELEVELSPQ GTLVERIRAG GsGLGGVlTk
PPL  GArDLTIISN NAGngeiGLA sLLmagsVRK VVCSFPrqsD syvFDelYrA GkIELEVvPQ GnLAERIaAA GsGIGAFFSp
ACS  GrKnLTIVSN NAGngdyGLA kLLkTgaVKK IICSFPrqAD syvFDelYrA GkIELEIvPQ GnLAcRIqAA GMGLGPIYTp
PHM  GVKELTAVSN NAGVDNFGLG LLLQSKQIKR NISSYV..GE NAEFERQYLA GELEVELTPQ GTLAERIRAG GAGVPAFYTS
CBL  .......... .......... .......... .......... .......... .......... .......... ..........
CAL  .......... .......... .......... .......... .......... .......... .......... ..........
PPS  .......... .......... .......... .......... .......... .......... .......... ..........
ACL  .......... .......... .......... .......... .......... .......... .......... ..........

                                        200
CBS  .......... .......... .......... .......... .......... .......... .......... ..........
CAS  TGlGTLIekG .......... ........Kk kisiNGteYL LElPLtADiA LIKGsiVDeA GNtfYKgTtK NFNPyNamAA
PPL  TGYGTLLaEG .......... ........KE tREidGrmYV LEmPLhADFA LIKAHKGDrw GNLTYRKaAR NFgPINamAA
ACS  TGFGTLLaEG .......... ........Kp tlnFdGkdYV LEnPIKADFA LIKAYKGDrw GNLvYRKSAR NFgPINamAA
PHM  TGYGTLVQEG GSPIKYNKDG SIAIASKPRE VREFNGQHFI LEEAIRGDFA LVKAWKADQA GNVTFRKSAR NFNLPMCKAA
CBL  .......... .......... .......... .......... .......... .......... .......... ..........
CAL  .......... .......... .......... .......... .......... .......... .......... ..........
PPS  .......... .......... .......... .......... .......... .......... .......... ..........
ACL  .......... .......... .......... .......... .......... .......... .......... ..........

          250                                                            300
CBS  .......... .......... .......... .......... .......... .......... .......... ..........
CAS  kTvIVEAEnL VsceklekEk AmtPefl... .......... .......... .......... .......... ..........
PPL  kTaIAqVDqV VELGeldPEh IitPgIFVQR VVaVsgaaas siakaI.... .......... .......... ..........
ACS  nvTIAqVsEV VaLGeldPEn VvtPgIFVQh VVpVqstpas Aap....... .......... .......... ..........
PHM  ETTVVEVEEI VDIGSFAPED IHIPKIYVHR LVKGEKYEKR IERLSVRKEE DVKTRSGKLG D.NVRERII. KRAALEFEDG
CBL  .......... .......... .......... .......... .......... .......mIV DkvlakeIIa KRVAkElkkG
CAL  .......... .......... .......... .......... .......... .......mIn DkNlakeIIa KRVArElknG
PPS  .......... .......... .......... .......... .......... ......mtIt kklsRteMAq .RVAAdiqEG
ACL  .......... .......... .......... .......... .......... .......msy hkltRDqIAq .RVAqdipEG

                                   350
CBS  .......... .......... .......... .......... .......... .......... .......... ..........
CAS  .......... .......... .......... .......... .......... .......... .......... ..........
PPL  .......... .......... .......... .......... .......... .......... .......... ..........
ACS  .......... .......... .......... .......... .......... .......... .......... ..........
PHM  MYANLGIGIP LLASNFISPN MTVHLQSENG ILGLGPYPLQ NEVDADLINA GKETVTVLPG ASYF.SSDES FAMIRGGHVN
CBL  qlVNLGIGLP tLVaNYVpke ym..... .......... .......... .......... .......... ..........
CAL  qlVNLGVGLP tmVadYIpkN fkItfQSENG IVGMGaSPki NEADkDVVNA GgDytTVLPd gTFFdSSV.S FsLIRGGHVd
PPS  AYVNLGIGAP tLVaNYL.gd keVFLhSENG LLGMGPsPAp gEeDdDLINA GKqhVTLLtG gaFFhhaD.S FsMMRGGHLd
ACL  sYVNLGIGLP tkIasYLpAd kdVFLhSENG LLAfGPpPAa gEeDPELINA GKEyVTMLeG gCFFhhgD.S FAMMRGGHLd

     400                                                  450
CBS  .......... .......... .......... .......... .......... .......... .......... ..........
CAS  .......... .......... .......... .......... .......... .......... .......... ..........
PPL  .......... .......... .......... .......... .......... .......... .......... ..........
ACS  .......... .......... .......... .......... .......... .......... .......... ..........
PHM  LTMLGAMQVS KYGDLAMNMI .PGKLVKGMG GAMDLVSSAK TKVVVTMEHS AKGNAHKIME KCTLPLTGKQ CVNRIITEKA
CBL  .......... .......... .......... .......... .......... .......... .......... ..........
CAL  VTVLGALQVD ekGnIAMV.I vPGKMLsGMG GAMDLVngAK .KVIIsMrHT nKG.qpKILk KCTLPLTAKs qANlIVTElG
PPS  IAVLGAfQVS vkGDLAMVht gAegsIpAVG GAMDLAtgAr .qVfVmMDHl tKtgesKLVp eCTyPLTGia CVsRIyTDlA
ACL  IcVLGAfQIA anGDLAMVht gAPdAIpsVG GAMDLAvgAK .KVfVTtDHv tKkgepKIVa elTyPATGqk CVdRIyTDlc
```

```
                                  500                 520
CBS  .......... .......... .......... .......... ......
CAS  .......... .......... .......... .......... ......
PPL  .......... .......... .......... .......... ......
ACS  .......... .......... .......... .......... ......
PHM  VFDVDRKKGL TLIELWEGLT VDDIKKSTGC DFAVSPKLIP MQQVTT
CBL  .......... .......... .......... .......... ......
CAL  VlEVind.GL lLtEInkntT IDEIRslTAa DlLISneLrP Mav...
PPS  VlEVtpe.GL kVVEIcadid FDELqKlsGv plIk...... ......
ACL  IIDVvpe.GL kVIEkvEGLS FEELqRlTGa tlIdatqG.. ......
```

Figure 6. Amino acid sequences of various CoAT enzymes. Single-letter amino acid codes are used. For convenience, all enzymes are compared to the PHM sequence. Bold-face letters represent amino acid residues identical to the corresponding PHM residue. Capital letters indicate amino acid residues similar or identical to the corresponding PHM residue.

Protein abbreviations: CBS, Clostridium beijerinckii NRRL B593 CoAT, small subunit (this work); CAS, C. acetobutylicum ATCC 824 CoAT, small subunit (Petersen et al., 1993); PPL, Pseudomonas putida ß-Ketoadipate:Succinate CoAT, large subunit (Parales and Harwood, 1992); ACS, Acinetobacter calcoaceticus ß-Ketoadipate:Succinate CoAT, small subunit (Parales and Harwood, 1992); PHM, Pig Heart Mitochondrial Acetoacetae:Succinate CoAT (Lin and Bridger, 1992); CBL, C. beijerinckii NRRL B593 CoAT, large subunit (this work); CAL, C. acetobutylicum ATCC 824 CoAT, large subunit (Petersen et al., 1993); PPS, P. putida CoAT, small subunit (Parales and Harwood, 1992); ACL, A. calcoaceticus CoAT, large subunit (Parales and Harwood, 1992). Numbers correspond to PHM CoAT sequence.

weights similar to those of the CoAT enzymes from C. beijerinckii and C. acetobutylicum. Its apparent Michaelis constants for acetyl-CoA and butyryl-CoA are within an order of magnitude of the true Michaelis constants determined for the C. beijerinckii enzyme. CoAT from Acidaminococcus fermentans (Buckel et al., 1981) also contains approximately equal amounts of two subunits, and was able to react with acetate, propionate, and butyrate, in addition to its physiological substrate, glutaconate. As with the PHM enzyme, an enzyme-CoA intermediate has been demonstrated with CoAT from Peptostreptococcus elsdenii (Tung and Wood, 1975). This enzyme and that from Clostridium aminobutyricum (Scherf and Buckel, 1991) contain two copies of a single subunit, again like the PHM CoAT. The similarities among these various CoAT enzymes suggest that they form a single family, and are perhaps evolutionarily related.

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Chapter IV

Concluding Remarks

Induction of Solvent-Forming Enzymes in Clostridia

By an as yet uncharacterized mechanism, the enzymes responsible for acetone/isopropanol production (acetoacetate:acetate/butyrate CoA-transferase [CoAT], acetoacetate decarboxylase [AcAcDC], and isopropanol dehydrogenase [if applicable]) and the enzymes responsible for butanol production (butyraldehyde dehydrogenase and butanol dehydrogenase) are induced. Grupe and Gottschalk (1992) discussed the fact that the enzymes responsible for butanol production can be induced independently of those responsible for acetone production in C. acetobutylicum DSM 1731.

Attempts to customize the product pattern of the fermentation will be aided by an understanding of the control mechanisms of the fermentation. Whether ultimately proven or disproven, a model of the regulatory mechanisms for the induction of solvent production which is consistent with currently available data can be considered as a starting point for future investigations. A model might be analogous to the regulation of the *ato* operon in E. coli.

Expression of the genes of the *ato* operon in E. coli is necessary for AcAc catabolism, as well as for the catabolism of butyrate and valerate. Both CoAT and a thiolase are strongly induced by addition of AcAc to the growth medium (Pauli and Overath, 1972). The *atoC* gene encodes a positive regulatory element

capable of inducing the transcription of the *atoDAB* genes when AcAc is present in the medium. The superficial similarity between the CoAT enzymes from C. beijerinckii and C. acetobutylicum and the CoAT from E. coli, and the similar adjacency of the induction of CoAT with the induction of another enzyme in its physiological pathway (AcAcDC in the clostridia, thiolase II in E. coli) suggests that a comparison of the conditions causing induction in the clostridia and in E. coli may be instructive. A model for the regulation of the genes encoding the enzymes leading to acetone production may be postulated, based on an understanding of the properties of 3HBDH and CoAT and by analogy with the regulation of the E. coli *ato* operon.

The behavior of batch cultures of C. beijerinckii or C. acetobutylicum might be thus explained: Prior to the onset of solvent production in C. acetobutylicum, low levels of CoAT activity (Andersch et al., 1983) and of mRNA which hybridized to a fragment of one of the genes encoding the subunits of CoAT (Gerischer and Dürre, 1992) have been observed. CoAT and 3HBDH compete for the available AcAcCoA. Since the acid substrates of CoAT will be at low concentrations initially, and since the Michaelis constant of 3HBDH for AcAcCoA, as determined for the enzyme purified from C. beijerinckii (Colby and Chen, 1992), is ten-fold lower than that of CoAT, essentially all AcAcCoA will be directed to the production of butyrate.

Before batch cultures enter the solventogenic phase, the intracellular concentration of acids, particularly butyrate, increases (Terracciano and Kashket, 1986; Wiesenborn et al., 1989a; Grupe and Gottschalk, 1992) and the intracellular pH decreases (Bowles and Ellefson, 1985; Gottwald and Gottschalk, 1985; Huang, et al., 1985; Terraciano and Kashket, 1986). The activity of phosphotransbutyrylase (PTB) from both C. beijerinckii NRRL B593 (Thompson and Chen, 1990) and C. acetobutylicum ATCC 824 (Wiesenborn et al., 1989b) has been shown to decrease sharply with declining pH in the range 7-5.5. Hartmanis and Gatenbeck (1984) demonstrated that the specific activity of PTB decreased sharply following the onset of solventogenesis in batch cultures of C. acetobutylicum ATCC 824. The increasing intracellular butyrate concentration may lead to a further decrease in the metabolic flux through the pathway leading to butyrate formation. These combined effects and the substrate inhibition of 3HBDH by AcAcCoA previously observed (Colby and Chen, 1992) may result in an accumulation of some of the intermediates of the butyrate-producing pathway, including AcAcCoA.

As butyrate and AcAcCoA concentrations increase, the ability of CoAT to compete with 3HBDH for AcAcCoA will be enhanced, and AcAc production will occur. In the absence of high levels of AcAcDC activity, AcAc will accumulate, and could cause induction or de-repression of the enzymes responsible for solvent production. As proposed by Grupe and Gottschalk (1992), production of the

enzymes leading to butanol, but not acetone/isopropanol, formation could be induced by another signal, perhaps related to the ability of the cell to dispense with its reducing power.

Future Work

A thorough understanding of all of the enzymes of the acid and solvent production pathways of the clostridia will allow the effects of changes made to the system to be intelligently predicted. Furthermore, purification and N-terminal sequencing of the enzymes will allow the genes to be identified, cloned, sequenced, and inserted into experimental systems. These genetic manipulations may allow elucidation of the control mechanisms of the fermentation. This knowledge will allow attempts at optimization and customization of the industrial process to be made.

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