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Thiolase, phosphotransbutyrylase, and CoA transferase and their role in product formation in *Clostridium acetobutylicum* ATCC 824

Wiesenborn, Dennis Paul, Ph.D.
Rice University, 1989
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THIOLASE, PHOSPHOTRANSIBUTYRYLASE, AND COA TRANSFERASE
AND THEIR ROLE IN PRODUCT FORMATION
IN CLOSTRIDIUM ACETOBUTYLICUM ATCC 824

by

DENNIS PAUL WIESENBERG

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ABSTRACT

Thiolase, Phosphotransbutyrylase, and CoA Transferase and their Role in Product Formation in Clostridium acetobutylicum ATCC 824

Dennis Paul Wiesenborn

Three key enzymes of the Clostridium acetobutylicum ATCC 824 metabolism, namely thiolase, phosphotransbutyrylase (PTB), and CoA transferase, were purified to homogeneity. Each of these enzymes catalyzes the first reaction after a branch-point in the fermentative pathway, and may therefore be an important point in the regulation of the fermentation. A number of physical and kinetic properties, namely native and subunit molecular weight, $K_m$ values, kinetic binding mechanism, optimum pH, substrate specificity, and effect of cofactors and metabolites on enzyme activity were determined with the objective of identifying important regulatory factors for each enzyme.

Thiolase is inhibited by micromolar levels of CoASH in the condensation direction, and this may be the most important factor in modulating the net rate of condensation of acetyl CoA. The enzyme is relatively insensitive to change in pH within the physiologic range. Studies of thiolase specific activity under various types of continuous fermentations show that regulation of this enzyme at both the genetic and enzyme level is important.

PTB is sensitive to pH change in the butyryl phosphate-forming direction, within the physiologic range of pH
5.5 to 7; relative activity decreases with decreasing pH to about 5% at pH 6. This indicates that change in internal pH may be one important factor in the regulation of the enzyme. The enzyme can use a number of substrates in addition to butyryl CoA, but has the highest relative activity with butyryl CoA, isovaleryl CoA and valeryl CoA.

CoA transferase is very unstable *in vitro* unless high concentrations of both glycerol (20%) and ammonium sulfate (0.5 M) are present; virtually all activity is lost within an hour without either stabilizer. The $K_m$ values for butyrate and acetate of 660 mM and 1200 mM, respectively, are high relative to the intracellular concentrations of these species; consequently, *in vivo* enzyme activity is expected to be sensitive to changes in those concentrations. In addition to the carboxylic acids listed above, this CoA transferase is able to activate propionate, valerate, isobutyrate, and crotonate. The enzyme activity is relatively independent of pH, but is inhibited by physiologic levels of acetone and butanol, suggesting a feedback inhibition mechanism.
To Diane, Paul and Jesse,
and to my mother and father.
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<thead>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK</td>
<td>butyrate kinase</td>
</tr>
<tr>
<td>Ches</td>
<td>2-(N-cyclohexylamino)-ethanesulfonic acid</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CoASH</td>
<td>Coenzyme A, reduced form</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5′-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HOADH</td>
<td>β-hydroxyacyl CoA dehydrogenase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
</tr>
<tr>
<td>$K_A$</td>
<td>$K_m$ for substrate A (see Section 3.4)</td>
</tr>
<tr>
<td>$K'_A$</td>
<td>inhibition constant in general bisubstrate rate equation (see Section 3.4)</td>
</tr>
<tr>
<td>$K_B$</td>
<td>$K_m$ for substrate B (see Section 3.4)</td>
</tr>
<tr>
<td>$K_i$</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>$K_{iA}$</td>
<td>inhibition constant for substrate A in the case of substrate inhibition (see Equation B-5)</td>
</tr>
<tr>
<td>$K_{iB}$</td>
<td>inhibition constant for substrate B in the case of substrate inhibition (see Equation B-5)</td>
</tr>
<tr>
<td>$K_{iP}$</td>
<td>product inhibition constant based on a change in the $y$-intercept on a double-reciprocal plot (see Equation B-4)</td>
</tr>
<tr>
<td>$K_{iS}$</td>
<td>product inhibition constant based on a change in the slope on a double-reciprocal plot (see Equation B-4)</td>
</tr>
</tbody>
</table>
\( K_m \) Michaelis constant

Mes 4-morpholineethanesulfonic acid

Mops 4-morpholinepropanesulfonic acid

MW molecular weight

PEG polyethylene glycol-12,000

PHB poly-\( \beta \)-hydroxybutyrate

PTA phosphotransacetylase

PTB phosphotransbutyrylase

SPR specific product rate, defined in Section 4.2.3

\( v \) reaction velocity

\( V_{\text{max}} \) maximum reaction velocity

[ ] brackets denote concentration; for example, [A] denotes concentration of substrate A
CHAPTER 1

INTRODUCTION

1.1 Historical Background

The synthesis of butanol by bacteria was first reported by Louis Pasteur in 1861 (38). A shortage of natural rubber in England in the early 1900's spurred the search for microbes that can synthesize butadiene for production of synthetic rubber. This led Chaim Weizman in 1912 to isolate a clostridium which produced acetone and butanol and which was named Clostridium acetobutylicum. A large demand for acetone arose during World War I, because acetone was used to produce the explosive cordite. Weizman's acetone-butanol fermentation was successfully employed to meet this demand, and this event marks the beginning of industrial microbiology (30,38,53).

Butanol was initially an unwanted byproduct; however, in the late 1910's, butanol was found to make an excellent quick-drying solvent for nitrocellulose lacquers used in the automobile industry (38). This rekindled commercial interest in the acetone-butanol fermentation. Development of new strains with improved yields and the ability to use a variety of inexpensive substrates such as corn and molasses contributed to a high interest in this fermentation throughout the world up to the end of World War II. Throughout much of the early part of this century, this was the second most important fermentation, next to the ethanol fermentation (67).
By the 1950's, processes were available for synthesizing acetone and butanol from petroleum. The acetone-butanol fermentation was perceived as not being an economically viable process, and by the 1960's the fermentation had been discontinued in virtually all countries with free-market economies. The most noteworthy exception was in South Africa, where the fermentation was continued until 1982 (38,67).

The acetone-butanol fermentation had several principal disadvantages that contributed to its downfall (38,52). The cost of the substrate tended to be very high and unpredictable. Also butanol is toxic to the bacteria, and therefore the concentration of solvents in the broth could not exceed about 2%. This in turn resulted in high distillation and equipment costs. Several detailed accounts of the history of this fermentation are available (30,38,53,67).

1.2 Future Prospects for the Acetone-Butanol Fermentation

Butanol is valued as a solvent and feedstock for the chemical industry, and there is also interest in butanol as a high-octane fuel extender (38,57). Although the acetone-butanol fermentation is not currently used commercially in the U.S.A. or Europe, there continues to be much interest in the process. The large number of papers and several major review articles concerning a variety of aspects of this fermentation testify to that interest (38,52,57). This continued interest results from recognition of an inevitable world-wide shortage of petroleum plus realization that technological advancements, particularly
in biology, will likely enable the acetone-butanol fermentation to again become economically attractive.

There are several areas of research that promise to improve the economics of this fermentation (38,52). In the past, the fermentation was carried out using batch or semi-continuous reactors, whereas various continuous fermentation strategies are now being shown to be both feasible and substantially more productive (18,45,47,75,79). New methods are being developed that should significantly improve the cost of product recovery (38,52). At the same time, efforts are under way to increase the resistance of the bacteria to butanol, thereby making it possible to obtain higher solvent concentrations in the broth (38,52,57,75).

The traditional substrates have been corn mash, molasses, or other sources of carbohydrate, which are now relatively expensive. Currently, however, much research has been aimed at the use of low-cost substrates, such as cheese whey, Jerusalem artichokes, wastes from the pulp and paper industry, hydrolyzed lignocellulose, and even unhydrolyzed lignocellulose (10,38,78,79).

Perhaps the most exciting prospects are those which seek to apply mutagenesis and recombinant DNA techniques for strain improvement. The ultimate goal of this approach is to engineer the metabolism to improve the selectivity and yield for the most valuable product(s), and to endow the bacteria with enzymes that yield new products such as polymers or allow the rapid
conversion of low-cost substrates. Formerly, efforts at strain improvement were limited to isolating new strains from the wild (38,52,57).

1.3 Biochemistry of Product Formation

Several species of clostridia, most notably C. acetobutylicum and C. beijerinckii, can be used to carry out the acetone-butanol fermentation. These are rod-shaped, saccharolytic, spore-forming obligate anaerobes. Typically, a batch acetone-butanol fermentation has two distinct stages. Initially, growth is rapid and the main products are hydrogen, carbon dioxide, acetate, and butyrate. The carboxylic acids lower the external pH and also break down the pH gradient across the cell membrane such that the internal pH decreases from about 7.0 to as low as 5.5 (34). In the second stage, growth stops and the carboxylic acids are taken up from the media, causing the external pH to rise. The final products are butanol, acetone, and ethanol, and in C. acetobutylicum are typically present in a 6:3:1 ratio (38,67). Other butanol-forming clostridia have different characteristic product distributions, and C. acetobutylicum fermentations can be manipulated to give a range of product distributions (38,47,79). Also, acetoin and lactate are sometimes produced.

Much research has been directed towards understanding what triggers the initiation of solventogenesis. It has been found that a variety of conditions can induce solventogenesis, such as
glucose-rich media, low intracellular pH, high concentrations of acetate and/or butyrate, inhibition of hydrogenase activity, and cell-recycle. These conditions have been reviewed recently (38,52; C. L. Meyer, Ph.D. thesis, Rice University).

1.3.1 Metabolic Pathway

Figure 1-1 shows the branched metabolic pathway of *C. acetobutylicum* leading from the key intermediate acetyl CoA to the major products. An additional step (not shown) in some strains of *C. beijerinckii* converts acetone to isopropanol. Acetyl CoA is produced from pyruvate by pyruvate ferredoxin oxidoreductase. Pyruvate is synthesized from hexose sugars via the Embden-Meyerhof pathway. Pentose sugars can also be metabolized via the pentose phosphate pathway into intermediates of glycolysis (15,24).

All of the pathways shown in Figure 1-1 have actually been demonstrated in *C. acetobutylicum*. It has been speculated that additional pathways identified in other bacteria might be present in *C. acetobutylicum*, namely an ATP-dependent butyryl CoA synthetase, and butyryl CoA:acetate CoA transferase (1,28). Efforts to detect these activities have not been successful; however, some of the other enzymes in Figure 1-1 have unusual stability requirements that have only recently been identified (50; this work, Chapter 6; R. W. Welch and F. B. Rudolph, submitted). Thus, there may be as yet unidentified enzymes relevant to Figure 1-1. Acetoin is sometimes also an important
Figure 1-1. Metabolic pathways of product formation in *C. acetobutylicum*. Enzymes involved are (1) thiolase -- note that 2 acetyl CoA molecules are condensed to form 1 acetoacetyl CoA molecule, (2) β-hydroxybutyryl-CoA dehydrogenase, (3) crotonase, (4) butyryl-CoA dehydrogenase, (5) phosphotransacetylase, (6) acetate kinase, (7) acetaldehyde dehydrogenase, (8) ethanol dehydrogenase, (9) phosphotransbutyrylase, (10) butyrate kinase, (11) acetoacetyl CoA:acetate (butyrate) CoA transferase, (12) acetoacetate decarboxylase, (13) butyraldehyde dehydrogenase, and (14) butanol dehydrogenase. Acetyl CoA is formed from glucose via glycolysis and pyruvate ferredoxin oxidoreductase (broken line).
product, but the exact pathway for its formation in this organism has not yet been identified (15, 24, 51).

Formation of acetate yields twice as much ATP per mole of acetyl CoA than formation of butyrate. Formation of butanol and ethanol from acetyl CoA does not yield any ATP but does allow the cell to regenerate more NAD (or NADP) from NADH (NADPH) than formation of acids.

1.3.2 Variation of Enzyme Activity with Fermentation Conditions

The change in specific activity during batch fermentations and various types of continuous fermentations has been studied for the enzymes in Figure 1-1. Autor, in 1970, documented the increase in acetoacetate decarboxylase in a batch fermentation by at least 40-fold. The increase began late in the fermentation at about the time that external pH reached a minimum (A. P. Autor, Ph.D. thesis, Duke University, 1970).

Andersch and coworkers measured the specific activities of several enzymes in both batch and continuous fermentations (1). They reported that the specific activities of the two kinases and two phosphotransacylases during solventogenesis were only 10 to 50% of their activities during acidogenesis. Also, they showed that, in addition to acetoacetate decarboxylase, the activities of CoA transferase, butyraldehyde dehydrogenase, and butanol dehydrogenase increased at the onset of solventogenesis; however, subsequent research by others has revealed some serious technical deficiencies in their work with these latter three
enzymes (17,50; this work, Chapter 6; R. W. Welch and F. B. Rudolph, submitted).

Hartmanis and coworkers measured specific activities of several intermediary enzymes during batch fermentations, and obtained some results that were very different from those just described (27). Those workers found at the onset of solventogenesis that butyrate kinase activity increased 2.5 to 10 fold, while PTB activity rapidly dropped to below the detection level. The consecutive enzymes thiolase, β-hydroxybutyryl-CoA dehydrogenase, and crotonase appeared to be coordinately expressed and reached peak activity at the onset of solventogenesis.

Recently, Yan and coworkers presented specific activities from several enzymes in batch fermentations using C. beijerinckii strains NRRL B592 and NRRL B593 (83). They reported significant increases in the levels of butyraldehyde dehydrogenase, butanol dehydrogenase, and acetoacetate decarboxylase that began about one hour before initiation of solventogenesis. Meanwhile the enzymes glucose-6-phosphate isomerase and thiolase, which are needed for both acidogenesis and solventogenesis, showed only a comparatively slight change in activity. Significant differences between the two strains were noted in the degree of increase for several enzymes, and butyraldehyde dehydrogenase was not detected in strain NRRL B593.

Palosaari and Rogers assayed butyraldehyde dehydrogenase activity in a batch fermentation of C. acetobutylicum and found over a 200-fold increase in activity corresponding to the onset
of solventogenesis (50). This increase in activity could be blocked with rifampin, showing that the increase is dependent on protein synthesis. Also, antibodies were used to show that the enzyme was not present in an inactive form prior to the increase. Activity decreased sharply towards the end of the fermentation.

Huesemann and Papoutsakis recently studied the levels of several enzymes in both batch and solvent fermentations of C. acetobutylicum (manuscript submitted). In continuous fermentations, they found no correlation between in vitro and in vivo activities of the two kinases, the two phosphotransacylases, acetoacetate decarboxylase, and butanol dehydrogenase. In batch fermentations, the increase in alcohol dehydrogenase activity preceded that of the decarboxylase by about an hour, and the activities of the kinases and phosphotransacylases showed little change. Aldehyde dehydrogenase and CoA transferase activities were not reported, but subsequent results show that butyraldehyde dehydrogenase activity seems to correlate with the specific rate of solvent formation in at least some continuous fermentations and that CoA transferase activity increases sharply in coordination with the decarboxylase activity at the onset of solventogenesis in batch cultures (M. Huesemann and E. T. Papoutsakis, personal communication).

In summary, some conflicting results have been reported which may be partly attributed to differences in species or strains. Nevertheless, at least in batch fermentations, it
appears that the levels of enzymes of the solvent pathways increase in activity at about the time of initiation of solventogenesis, but that the enzymes are not always coordinately expressed. The enzymes of the acid pathways may or may not decrease during solventogenesis, but there is probably almost always significant activity such that the cessation of acid formation does not result from the absence of the necessary enzymes.

1.3.3 Enzymes of the Acetone-Butanol Fermentation Already Purified and Characterized

Enzymes shown in Figure 1-1 which have previously been purified to homogeneity and characterized to varying degrees are acetoacetate decarboxylase (81,84), crotonase (80), butyrate kinase (26), butanol dehydrogenase (R. W. Welch and F. B. Rudolph, submitted), and butyraldehyde dehydrogenase (50) in C. acetobutylicum and two alcohol dehydrogenases in C. beijerinckii (32). With the exception of the decarboxylase and crotonase, the purification of these enzymes have been announced only since 1987, which illustrates the widespread interest in gaining a better understanding of this fermentation. Unfortunately, in the case of the decarboxylase, crotonase, and kinase in particular, few if any characteristics of physiologic interest were reported.

Acetoacetate decarboxylase has been extensively studied since its detection in 1942, but the research was directed
mainly towards gaining a detailed understanding of the kinetic mechanism, or studying inactivators or inhibitors with little apparent physiologic significance. Some interesting characteristics have been reported, however, such as an almost doubling of activity obtained by heating to 70°C for 60 minutes. The enzyme is specific for acetoacetate, with a $K_m$ of 8 mM (A. P. Autor, Ph.D. thesis, Duke University, 1970). An optimum pH has apparently not been reported for \textit{C. acetobutylicum} enzyme, although the \textit{Clostridium madisonii} enzyme has an optimum $V_{\text{max}}$ at pH 6.1 in water and 5.6 in 30% ethanol (82).

In the case of crotonase and butyrate kinase, the few kinetic experiments performed to obtain values of $K_m$ and optimum pH were not in the usual physiologic direction (26, 80). Butyrate kinase shows broad substrate specificity with respect to carboxylic acid in the acid-phosphorylation direction. Its pH optimum with butyrate is 7.5 (26).

Hiu and coworkers isolated two different alcohol dehydrogenases in \textit{C. beijerinckii}, one from strain NRRL B592 and the other from NRRL B593 (32). Each alcohol dehydrogenase is NAD(P)H-linked, and appears to be the dominant one in the respective strains, although low levels of NAD(H)-linked alcohol dehydrogenase were detected in crude extracts. The two isolated dehydrogenases show striking differences in substrates used, values of $K_m$, and optimum pH. In the physiologic direction, the NRRL B592 enzyme reduces both acetaldehyde and butyraldehyde, with a $K_m$ of 6 μM for butyraldehyde and optimum activity at
about pH 9. The NRRL B593 enzyme reduces the same aldehydes plus acetone, with a $K_m$ of 9.5 mM for butyraldehyde and optimum activity at about pH 6.5.

Welch and Rudolph isolated a butanol dehydrogenase from \textit{C. acetobutylicum} ATCC 824 (manuscript submitted). Unlike the \textit{C. beijerinckii} alcohol dehydrogenases, this enzyme requires a metal ion such as Zn, Fe, Mn, or Co, and is NAD(H)-linked. An NADP(H)-linked enzyme is also present in \textit{C. acetobutylicum} ATCC 824, but it is not clear which, if any, is dominant. The optimum pH of the NAD(H)-linked enzyme is near 5.5, whereas that of the NADP(H)-linked enzyme appears to be much higher. It is not uncommon for dehydrogenases performing the same function in different species or strains to differ in their affinity for NAD(H) and NADP(H) (32).

An NAD(H)-linked aldehyde dehydrogenase activity was isolated from \textit{C. acetobutylicum} by Palosaari and Rogers (50). In the physiologic direction, the optimum pH is near 7.0. The $K_m$ for butyryl CoA, NADH, and NADPH are 0.045 mM, 0.003 mM, and 0.04 mM, respectively. Hence, activity with NADPH is expected to be low.

The preceding review of the enzymology of the acid and solvent metabolism shows a considerable amount of progress in recent years. Yet there is still much to be learned about the properties of the enzyme catalysts, such as the effects of metabolites and cofactors on activity. An understanding of the fundamental mechanisms governing product formation still eludes
us, and such an understanding is needed to ensure the effectiveness of engineering the metabolism to improve the fermentation.
CHAPTER 2

APPROACH AND RATIONALE

2.1 Levels of Metabolic Regulation

Organisms have evolved manifold ways of regulating their metabolism; only a very brief outline of some features of regulation particularly relevant to bacteria is given here. A metabolic reaction can be regulated either by regulating the amount of active enzyme, which means controlling the rate at which it is synthesized and degraded, or by modulating the in vivo activity of the enzyme through small molecules that may or may not be a product or substrate of the reaction.

The amount of enzyme present is regulated mainly at the genetic level, that is, at the level of transcription and/or translation. Given an accurate enzyme assay, it is straightforward to determine the extent to which the amount of native enzyme changes, by measuring the specific enzyme activity under various growth conditions. This information indicates how tightly the enzyme is regulated at the genetic level, and might suggest, for example, whether a particular substrate or product acts as a repressor or inducer, or whether the amount of enzyme is regulated in coordination with the amounts of other enzymes on the same pathway. The changes in in vivo activity of the enzyme can also be calculated from the rate of accumulation of products and biomass, and compared to the specific activity assayed in vitro. If the in vivo and in vitro activities
associated with an enzyme correlate well, then the enzyme likely catalyzes a step that limits the rate of its branch of metabolism. This type of analysis has already been applied to several enzymes of the *C. acetobutylicum* metabolism, as mentioned in Chapter 1. Although useful information is obtained by this approach, a comprehensive understanding of metabolic regulation at the level of transcription and translation is contingent upon the identification and characterization of the relevant genetic components through recombinant DNA techniques.

Regulation at the enzyme level means the modulation of the rate of a reaction per amount of native enzyme. When *in vivo* activity is compared with *in vitro* activity for enzymes of the acidogenic and solventogenic pathways of *C. acetobutylicum*, there may be no close correlation, indicating that regulation at the enzyme level is definitely important. Also, the *in vivo* activity is typically in the range of a few percent down to several thousandths of a percent of the *in vitro* activity (27). The mechanisms which potentially regulate metabolism at the enzyme level vary in type and complexity, and include the dependence of enzyme activity on the concentration of substrate(s), product(s), or cofactor(s) of the particular reaction, and the inhibition or activation by other intermediates, end products, and cofactors not directly involved in the reaction.
2.2 Approaches to Understanding Metabolic Regulation

A fuller understanding of the mechanisms for regulating the branched fermentation pathway of \textit{C. acetobutylicum} is desirable. One approach is to try to identify one or a few key regulatory enzymes that essentially control a whole metabolic pathway. In a sense, though, all enzymes are regulatory enzymes (54). Also, some metabolites and cofactors may have a key regulatory role, by virtue of their effect on enzyme activity. This is seen in the butanol fermentation, where under some fermentation conditions, ATP appears to regulate solvent formation by \textit{C. acetobutylicum} (C. L. Meyer, Ph.D. thesis, Rice University, 1987), and it has been suggested that NADH also sometimes has a regulatory role (14,38,52). Consequently, we are faced with the problem of weighing the relative impact of a number of enzymes, metabolites, and cofactors on the overall regulation of the branched fermentation pathway, and that relative impact likely changes depending on the fermentation conditions.

Despite this daunting complexity, experience shows that particular enzyme reactions can control a whole pathway and therefore should be characterized first. Reactions that are far from equilibrium tend to be rate-limiting and are often among the most important points of regulation (54,71). Equilibrium constants have been determined for many of the reactions of the branched fermentation; however, there is little or no data available on the intracellular concentrations of intermediates which are needed to gauge the closeness to equilibrium.
Reactions that are essentially irreversible are, of course, usually far from an equilibrium state. But of the enzymes of the branched fermentation, only the acetoacetate decarboxylase reaction could be considered irreversible. On the other hand, as Krebs has pointed out, reactions that are very close to equilibrium may also be important regulatory enzymes by maintaining equilibrium between two or more key intermediates that are in turn substrates or products of other reactions (54).

Branch-point enzymes are also particularly likely to be regulatory enzymes (71). By controlling a branch of a metabolic pathway at the first step of that branch, the accumulation of excessive amounts of an intermediate can be avoided. On the pathways from acetyl CoA to the acid and solvent products (see Figure 1-1), there are at least six branch-point enzymes in _C. acetobutylicum_, since the acetaldehyde and butyraldehyde dehydrogenase may be the same enzyme. Because branch-point enzymes often have interesting regulatory properties, three of these branch-point enzymes, namely thiolase (acetyl-CoA acetyl transferase, E.C. 2.3.1.9), phosphotransbutyrylase (PTB; phosphate butyryltransferase, E.C. 2.3.1.19), and CoA transferase (acetoacetyl CoA:acetate(butyrate) CoA-transferase, E.C. 2.8.3.-), are the focus of this study.
2.3 Outline and Significance of Approach

Regulation of branches of the fermentative pathway by thiolase, PTB, and CoA transferase was studied by purifying these enzymes to homogeneity from cell-free extracts of C. acetobutylicum ATCC 824, and then characterizing relevant physical and kinetic properties of these enzymes. The study of these enzymes in vitro should help explain variations in their in vivo activity under different fermentation conditions and give insight into the very low in vivo activities of these enzymes relative to their in vitro activities. Such information should ultimately help us predict how changes in the fermentation conditions or the levels of enzymes by metabolic engineering will affect in vivo activity.

Purification to homogeneity of enzyme activity offers several advantages over studying the characteristics of the enzymes in crude or partially pure preparations. Purification removes other enzymes that might compete for the substrate added to carry out kinetic experiments. It is also desirable to add other metabolites and cofactors to determine their effect on the reaction; however, these also may be acted on by other enzymes. The subunit molecular weight, and thus the number of subunits cannot be determined without a homogeneous preparation. With respect to regulation, this is of interest because enzymes consisting of subunits are more likely to be subject to complex regulation (54). With pure preparations of the enzyme it is possible to determine part of the amino acid sequence, which can
then be used to develop probes to locate the gene for that enzyme. This in turn facilitates the cloning of the gene and should ultimately help elucidate the mechanisms of regulating enzyme activity on the genetic level. Finally, homogeneous enzyme preparations can be used to raise polyclonal antibodies to the enzyme (antigen) which are useful in detecting the presence of inactive enzyme in crude extracts and as an additional tool for detecting the expression of the cloned gene.

Kinetic properties which were investigated are values of $K_m$, kinetic binding mechanism, pH dependence, substrate specificity, and effect of products and other metabolites on the reaction. The knowledge obtained from these in vitro studies must be combined with an understanding of the intracellular pH and concentrations of metabolites and cofactors in an effort to predict which factors are most important in vivo in determining the enzyme activity. Unfortunately, little is known about the intracellular concentrations of most intermediates and cofactors in C. acetobutylicum, but methods for assaying most of these are available (5).

The in vitro conditions under which kinetic properties are determined are inherently very different from the intracellular environment of the enzyme. For example, the intracellular environment of the enzyme has several orders of magnitude greater protein concentration, plus there may be weak associations between different enzymes on the same pathway. The roles of internal concentration gradients and diffusion limitations are
other unknowns in this type of analysis. These and other limitations to extrapolating \textit{in vitro} results to \textit{in vivo} conditions are discussed in detail by Price and Stevens (54).
CHAPTER 3

METHODS

3.1 Organism and Growth Conditions

*C. acetobutylicum* ATCC 824 was grown from heat-shocked spores in a 14-liter fermentor (Microferm, New Brunswick Scientific, Edison, NJ). Maintenance of the organism, including storage, transfer, and heat-shocking, has been described previously (46). The medium contained, per liter distilled water: 
KH$_2$PO$_4$, 0.75 g; K$_2$HPO$_4$, 0.75 g; MgSO$_4$•H$_2$O, 0.4 g; MnSO$_4$•H$_2$O, 0.01 g; FeSO$_4$•7H$_2$O, 0.01 g; NaCl, 1.0 g; asparagine, 2.0 g; yeast extract, 5.0 g; (NH$_4$)$_2$SO$_4$, 2.0 g; antifoam C, 0.2 mL; and glucose, 50 g. The broth was sparged with prepurified nitrogen to maintain anaerobicity at least until growth reached mid-exponential phase. The broth was constantly agitated and the temperature was controlled at 37°C. The pH was controlled at a fixed setpoint by the addition of 3 M NaOH. The setpoint varied depending upon the enzyme to be purified.

3.2 Analytical Methods

Protein Assay: For the purpose of determining thiolase specific activity, protein was assayed by the method of Lowry (44) with lysozyme the standard. Otherwise protein was assayed by the dye-binding method of Bradford (7) with bovine serum albumin the standard.
**CoASH Assay:** The concentration of CoASH in stock solutions of CoASH and acyl CoA solutions used in kinetic and substrate specificity studies was determined by adding DTNB, then measuring the initial absorbance change at 412 nm. An extinction coefficient of 13.6 mM\(^{-1}\) cm\(^{-1}\) was used (19). Each assay contained 70 mM potassium phosphate, pH 8.0, 0.08 mM DTNB and CoASH diluted with distilled water to give an absorbance change of less than 0.8.

**Acyl CoA Assay:** The concentration of acyl CoA, such as acetyl CoA and acetoacetyl CoA, in stock solutions used in kinetic and substrate specificity studies was determined by incubating the acyl CoA compound with neutral hydroxylamine. Neutral hydroxylamine hydrolyzes the acyl CoA bond, thereby releasing CoASH which can then be assayed as described above (23). The neutral hydroxylamine was prepared just prior to use by combining equal volumes of 2 M KOH and 2 M hydroxylamine hydrochloride. To calculate the acyl CoA concentration, the concentration of free CoA as determined prior to hydrolysis with neutral hydroxylamine must be subtracted away. The amount of CoASH present in these stock solutions was less than 1% of the acyl CoA concentration, with the exception of solutions of acetoacetyl CoA purchased from Sigma, which contained up to 6% CoASH.

**Butyryl Phosphate Assay:** Butyryl phosphate used in kinetic studies was assayed by the method of Lipmann and Tuttle (43) as modified by Avison (3). Butyryl phosphate samples were
incubated with 0.67 M neutral hydroxylamine in a total of 3 mL for 10 minutes to form butyrohydroxamic acid. 3 mL of 0.063 M FeCl₃ in 1 M HCl was then added to the incubated solution and the absorbance at 540 nm measured. The standard was acetohydroxamic acid which has approximately the same extinction coefficient as butyrohydroxamic acid under these conditions (3). Solutions of butyryl phosphate were assayed before and after kinetic studies and showed no more than a 2% loss.

**Enzyme Assays:** Enzyme assays are described under the relevant chapter. All enzyme assays were performed spectrophotometrically (Model 250, Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with a chart recorder (Servogor 210, Brinkmann Instruments, Westbury, NY).

3.3 Enzyme Purification Procedures

Enzyme purification procedures are described in detail under the relevant chapter.

In the case of thiolase, cells were lysed by sonic disruption (Model W-225R, Heat Systems-Ultrasonics, Inc., Plainview, NY). In the case of PTB and CoA transferase, cells were lysed by passage through a French pressure cell (Model FA-073, SLM Instruments, Inc., Urbana, IL). With the French pressure cell, large volumes of cell suspension were processed more rapidly and easily and with the same or higher enzyme activity in the cell-free extract, as compared to sonic disruption; however,
the French pressure cell became available only after work with thiolase was completed.

A variety of chromatography techniques employing ion-exchange, gel filtration, hydrophobic-interaction, and dye-ligand affinity media were tested in order to determine an efficient method of purifying each enzyme. Also both low pressure columns using flow adaptors and high-pressure liquid chromatography (HPLC) columns were used. The particular chromatographies used to purify each enzyme were selected on the basis of yield, degree of purification, and ease of use. Each chromatography method separates proteins on the basis of different protein characteristics, and consequently it is good strategy to employ a variety of these chromatographies, as opposed to using variations of the same type of chromatography. Ion-exchange chromatography separates proteins by electrostatic charge, gel filtration by size, hydrophobic interaction by hydrophobic amino acids on the protein surface, and dye-ligand affinity by interaction with a large dye molecule that is structurally similar to certain cofactors involved in some enzyme reactions (73).

A detailed comparison of the advantages and disadvantages of each type of chromatography are beyond the scope of this discussion; however, hydrophobic and dye-ligand affinity chromatography exhibit interesting properties worth noting. Hydrophobic interaction chromatography is a relatively new approach, which binds enzymes at high ionic strength and elutes them at low ionic strength. Unlike ion-exchange and dye-ligand
chromatographies, enzyme partially purified by ammonium sulfate precipitation can be applied without dialysis. Also, the running buffer can contain relatively large amounts of stabilizing salts and glycerol which prevent enzymes from binding to ion-exchange and dye-ligand media (see Chapter 6).

Dye-ligand chromatography can potentially be very selective, resulting in high purification in a single step; however, recovery is frequently low and it may be necessary to screen a variety of dye-ligand media to find one that works well (73).

3.4 Kinetic Studies

Procedures for kinetic studies are described in detail under the relevant chapter. All studies were performed at least in duplicate at 30°C. Reagents were incubated at 30°C for 2 minutes prior to initiation of a reaction. Often several enzyme assays were used, depending upon whether the purpose was the study of optimum pH, substrate specificity, effect of metabolites and cofactors, or determination of kinetic constants and kinetic binding mechanism.

Kinetic data for determining binding mechanism and the $K_m$ values were obtained by measuring the initial reaction rate over a range of concentrations of both substrates. The range of concentrations of each substrate were in the vicinity of the $K_m$ value for the respective substrate, and were selected by a preliminary experiment that gave an approximate value for each $K_m$. 
Straight line fits were determined by linear regression with a Hewlett-Packard 11C calculator.

The three enzymes studied here carry out bisubstrate reactions. A general equation that applies to bisubstrate enzyme reactions is (20,54):

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} \left[ 1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K'_A \cdot K_B}{[A][B]} \right]$$

where $v$ = reaction velocity, $V_{\text{max}}$ = maximum reaction velocity, $K_A$ and $K_B = K_m$ value for substrates A and B respectively, $[A]$ and $[B]$ = concentrations of substrates A and B respectively, and $K'_A =$ inhibition constant. The $K_m$ value for a substrate is the concentration at which $v = V_{\text{max}}/2$ when the enzyme is fully saturated with the other substrate (that is, the concentration of the other substrate is extrapolated to infinity). It can be seen from the preceding equation that, when the concentration of substrate B is fixed, a plot of $1/v$ versus $1/[A]$ should be linear, with:

slope $= \frac{1}{V_{\text{max}}} \left[ K_A + \frac{K'_A \cdot K_B}{[B]} \right]$  

intercept $= \frac{1}{V_{\text{max}}} \left[ 1 + \frac{K_B}{[B]} \right]$  

When slopes and intercepts have been determined for a range of
[B], the slopes and intercepts can be plotted versus 1/[B], and those plots should also be linear. Such replots can be used to determine the value of $V_{\text{max}}$, $K_A$, $K_B$ and $K'_A$. Analogous equations for slope and intercept are obtained when the concentration of substrate A is fixed and 1/v is plotted versus 1/[B]. In this case, replots of intercepts and slopes can be used to generate second estimates of $V_{\text{max}}$, $K_A$, $K_B$ and $K'_A$ (20).

The double-reciprocal plots described above can also be used to determine the kinetic binding mechanism. If the mechanism is Ping Pong Bi Bi, in which the first product dissociates from the enzyme before the second substrate binds, a plot of 1/v versus 1/[A] will yield a family of parallel lines corresponding to different fixed concentrations of B (20). Plots of 1/v versus 1/[B] show a similar pattern. Other possible bisubstrate binding mechanisms are Random Bi Bi, Ordered Bi Bi, and Equilibrium Ordered. Random Bi Bi and Ordered Bi Bi cannot actually be distinguished from each other using only double-reciprocal plots; in this case, product inhibition data are also needed (59).

In practice, kinetic data on double-reciprocal plots cannot always be fitted with straight lines. Enzymes which exhibit Ping Pong kinetics are particularly likely to be inhibited by high concentrations of one or both substrates, and kinetic data are hyperbolic on double-reciprocal plots. References are available which discuss this more complex kinetic behavior, (9,20,61).
3.5 Determination of Native and Subunit Molecular Weight

The native molecular weight was determined by measuring elution volume on a calibrated gel filtration column (2). The column (90 x 1.5 cm) was packed with Sephacryl S-300 (Pharmacia). The composition of the running buffer varied depending on the enzyme as follows: thiolase, 100 mM potassium phosphate, pH 7.1; PTB, 50 mM potassium phosphate, pH 7.0 with 100 mM (NH₄)₂SO₄, 5% (v/v) glycerol and 2 mM dithiothreitol; CoA transferase, 25 mM Mops, pH 7.0 with 0.5 M (NH₄)₂SO₄ and 20% (v/v) glycerol. Samples were applied in 2.0 mL and run at 15 to 24 mL/h with 1.25 to 1.60 mL fractions. The following standards were used to calibrate the column (molecular weights in parentheses): carbonic anhydrase (29,000), serum albumin (66,000), alcohol dehydrogenase (150,000), β-amylase (200,000), apoferritin (443,000), and thyroglobulin (669,000). The column was calibrated for each enzyme using the running buffer for the respective enzyme.

Subunit molecular weight was determined by denaturing gel electrophoresis on a 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (42). Protein bands were stained with Coomassie blue. The following standards were used (subunit molecular weight in parentheses): α-lactalbumin (14,200), trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000) and serum albumin (66,000). In the case of thiolase, a different set of standards was used:
phosphorylase B (95,500), glutamate dehydrogenase (55,000), lactate dehydrogenase (36,000), carbonic anhydrase (29,000), lactoglobulin (18,400) and cytochrome C (12,400).

3.6 Amino Acid Analyses

Amino acid composition was determined for thiolase and PTB. Purified, dialyzed enzyme was hydrolyzed in 6 N HCl for 48 hours at 110°C in vacuo. Analysis was carried out on a Beckman 121 MB amino acid analyzer at the Protein Sequencing Center, University of Texas in Austin, TX.

3.7 Source of Chemicals

Acetoacetyl CoA used in kinetic studies of thiolase was a gift from Dr. H. F. Gilbert of the Baylor College of Medicine, Houston, TX. Butyryl phosphate was synthesized by the method of Avison (3). The dye-binding protein assay kit was purchased from Bio-Rad Laboratories, Richmond, CA. Low pressure chromatography media used to purify enzymes, namely DEAE-Sephacel, Sephacryl S-300, Phenyl Sepharose, Octyl Sepharose, and Blue Sepharose CL-6B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Molecular weight standards for the thiolase denaturing gel were purchased from Diversified Biotech, Newton Centre, MA. All other chemicals were purchased from Sigma Chemical Company, St. Louis, MO.
CHAPTER 4

THIOLASE AND ITS ROLE IN DETERMINING

THE RATIO OF PRODUCTS

4.1 Background

Thiolase condenses two molecules of acetyl CoA to acetoacetyl CoA:

\[ 2 \text{acetyl CoA} \rightarrow \text{acetoacetyl CoA + CoASH} \]

The equilibrium constant is between $1.5 \times 10^{-5}$ and $8.7 \times 10^{-5}$ within the pH range of 6 to 9 (22). The reverse reaction is referred to here as the thiolysis reaction as opposed to the forward condensation reaction. Thiolase has been most fully characterized in Zooglea ramigera, in which case the amino acid sequence of the protein and the nucleotide sequence of the gene has been determined (13,48), and in other bacteria that synthesize poly-β-hydroxybutyrate (PHB) (49,62,74). The enzyme is thought to play a key role in the regulation of the synthesis of PHB.

Thiolase has not previously been purified or characterized from clostridia that are of interest for butanol formation. Although thiolase is found in high amounts in clostridia, it has only been isolated in two species of this genus, Clostridium pasteurianum (6) and Clostridium kluveri (29,64,65). These two species of clostridia are significantly different from C. acetobutylicum. For example, C. pasteurianum shows little DNA homology with C. acetobutylicum (12) and produces mostly
acetate and butyrate (57, 66). C. kluysteri produces mostly butyrate or caproate, or produces succinate by CO₂ fixation (15). Among all clostridia, the kinetic properties of thiolase have previously been characterized only for thiolase from C. pasteurianum. Like thiolase in PHB-synthesizing bacteria, the enzyme of C. pasteurianum has a Ping Pong mechanism and is inhibited by CoASH in the condensation direction (6). C. pasteurianum thiolase has optimal activity at pH 8.0, and its relative activity falls sharply with decreasing pH to about 65% at about pH 7.3. The relative activity was not reported for lower pH values, but it may be that in C. pasteurianum, internal pH is an important factor in the regulation of the enzyme.

Hartmanis and Gatenbeck studied the change in the level of thiolase in a batch C. acetobutylicum fermentation (27). They found significant levels of thiolase activity throughout the fermentation, with peak activity occurring shortly after growth ceased. Thiolase appeared to be coordinately expressed with at least two of the three other enzymes that, together with thiolase, convert acetyl CoA to butyryl CoA. Those workers speculated that thiolase in C. acetobutylicum is very sensitive to changes in internal pH, in the same way that pH affects thiolase in C. pasteurianum. Changes in external and internal pH are important in regulating product yields and selectivities in the acetone-butanol fermentation.
4.2 Methods

4.2.1 Purification of Thiolase

Cells were grown in a 14-liter batch fermentor at pH 6.0 as described in Section 3.1. The cells were harvested by centrifugation when the optical density at 600 nm reached 7.5, then stored at -20°C. All subsequent operations were carried out at 0-4°C with one exception: separation using HPLC was done at room temperature. Buffers were degassed and sparged with nitrogen, then 1 mM dithiothreitol was added just prior to use.

Step 1: Preparation of Crude Extract. Frozen cells (40-50 g wet weight) were thawed in two volumes of 50 mM 4-morpholinepropanesulfonic acid (Mops), pH 7.0. The cells in suspension were lysed by sonic disruption (Heat Systems-Ultrasonics, Inc. Plainview, NY, Model W-225R) in a salt-ice-water bath for 10 minutes with 50% pulse and full power. The lysed suspension was then centrifuged at 25,000 x g for 60 minutes. The supernatant is here referred to as crude extract.

Step 2: Ammonium Sulfate Precipitation. Recrystallized ammonium sulfate was added to 41% saturation. After 30 minutes of stirring, this was centrifuged at 25,000 x g for 15 minutes. The pellet was discarded and ammonium sulfate then added to the supernatant to 67% saturation followed by a repeat of the stirring and centrifugation. The pellet obtained by this second precipitation was resuspended in 40 mL of 50 mM Tris-HCl, pH
7.7, and dialyzed overnight against 500 mL of the same buffer.

**Step 3: DEAE-Sephadex Chromatography.** The dialyzed enzyme preparation was applied to a DEAE-Sephadex (Pharmacia) column (2.5 x 50 cm) equilibrated with 50 mM Tris-HCl, pH 7.7. The column was washed with 150 mL of equilibration buffer, then thiolase activity was eluted with a linear gradient from 0 to 80 mM (NH₄)₂SO₄ in a total of 700 mL 50 mM Tris-HCl, pH 7.7. Fractions of 7 mL were collected, and those with high activity were combined and concentrated essentially to dryness by dialysis overnight against 500 mL 10% (w/v) polyethylene glycol-12,000 (PEG) in 20 mM Tris-HCl, pH 7.5, then resuspended in 5 mL of fresh dialysis buffer without PEG.

**Step 4: Blue Sepharose CL-6B Chromatography.** Concentrated fractions from the DEAE-Sephadex column were applied to a Blue Sepharose CL-6B (Pharmacia) column (1.5 x 3 cm) equilibrated with 20 mM Tris-HCl, pH 7.5. The column was then washed with 25 mL of this same buffer before elution of the activity with a gradient. A linear gradient from 0.1 to 0.6 M KCl in a total of 300 mL of 20 mM Tris-HCl, pH 7.5, was used. Fractions of 7 mL were collected, and those with high activity were combined and concentrated to dryness by dialysis overnight against 500 mL 10% (w/v) PEG in 10 mM potassium phosphate, pH 7.2, then resuspended in 2 mL of fresh dialysis buffer without PEG.

**Step 5: HPLC Anion Exchange Chromatography.** An HPLC system (Laboratory Control Data, Riviera Beach, FL) with a SynChropak AX300 anion exchanger (SynChrom, Inc., Linden, IN) was used for
the final step. The column (4.1x250 mm) was equilibrated with 10 mM potassium phosphate, pH 7.2. Enzyme was loaded onto the column in 1.0 mL injections, then eluted with a linear gradient from 10 to 100 mM potassium phosphate, pH 7.2 in a total of 60 mL. Fractions of 1.0 mL were collected and those with high activity combined and concentrated to dryness by dialysis overnight against 10% (w/v) PEG in 1.0 M (NH₄)₂SO₄, 50 mM Mops, pH 7.0, and 10% (v/v) glycerol, then resuspended in 2 mL of fresh dialysis buffer without PEG.

4.2.2 Enzyme Assays

The three assays used to study this thiolase were all performed with a final reaction volume of 1.0 mL. The first two assays were used to study kinetics, optimum pH, inhibition, and inactivation, with temperature controlled at 30°C.

(a) The condensation reaction was coupled to the oxidation of NADH using β-hydroxyacyl CoA dehydrogenase (HOADH). Unless otherwise noted, each assay contained 100 mM Tris-HCl, pH 7.4; 1.0 mM acetyl CoA; 0.2 mM NADH; 1 mM dithiothreitol; and 2 units of HOADH. After equilibration of the cuvette contents at 30°C for two minutes, the reaction was initiated by the addition of about 125 ng thiolase in 10 μL. The absorbance decrease at 340 nm due to oxidation of NADH was measured, and an extinction coefficient of 6.22 mM⁻¹cm⁻¹ used (49). A baseline to account for oxidation of NADH in the absence of acetoacetyl CoA was obtained by omitting acetyl CoA and was generally small. With
the baseline correction, the assay was linear for the first 20
to 40 seconds. Since the condensation reaction is favored over
the thiolysis reaction in vivo, this assay was modified to study
pH dependence, inactivation by iodoacetamide and DTNB, and inhi-
bition by metabolites. To determine pH dependence, a cuvette
contained in 1 mL: 10 mM Mes, 10 mM Hepes, and 10 mM Ches
buffers with pH ranging from 5.5 to 9.0; 0.5 mM acetyl CoA;
0.2 mM NADH; 1 mM dithiothreitol; 5 units of HOADH; and
homogeneous thiolase. The coupling enzyme was not limiting
within this range of pH values.

(b) The kinetics of the thiolysis reaction were studied by
monitoring the absorbance of the enolate form of acetoacetyl CoA
at 303 nm. Each assay contained 100 mM Tris-HCl, pH 8.0; 10 mM
MgSO$_4$; 1 mM dithiothreitol; 3.5 to 17.5 μM CoASH; and 5.1 to
25.6 μM acetoacetyl CoA. All reagents were pre-equilibrated at
30°C and the assay initiated by addition of 18 ng enzyme in 10
μL. An extinction coefficient of 14.0 mM$^{-1}$cm$^{-1}$ was used (64).
A baseline to account for spontaneous hydrolysis of acetoacetyl
CoA in the presence of alkaline pH and magnesium ions was ob-
tained by omitting CoASH. Hydrolysis of one molecule of
acetoacetyl CoA liberates one molecule of CoASH which will in
turn cause the thiolytic cleavage of an additional molecule of
acetoacetyl CoA. Therefore the slope of the baseline was
divided by two before it was subtracted from the slope of the
assay. This adjusted baseline was 5-20% of the slope of the
traces for the various studies of this reaction.
(c) A second assay in the thiolysis direction was used for the measurement of thiolase during its purification and in crude extracts of cells from continuous fermentations. The thiolysis reaction was coupled at room temperature to the arsenolysis of acetyl CoA with the aid of phosphotransacetylase. Each assay contained 67 mM Tris-HCl, pH 8.1; 0.2 mM CoASH; 0.2 mM acetoacetyl CoA; 25 mM potassium arsenate, pH 8.1; 2 units of phosphotransacetylase; and less than 0.05 units of thiolase. The reaction was initiated by the addition of acetoacetyl CoA. The absorbance decrease at 232 nm due to the cleavage of the acyl CoA bond was measured and an extinction coefficient of 4.5 mM\(^{-1}\) cm\(^{-1}\) used (68). One unit of enzyme activity is defined as the amount of enzyme catalyzing the thiolytic cleavage of 1 \(\mu\)mole acetoacetyl CoA per minute under these conditions.

4.2.3 Activity in Continuous Fermentations

Operation of continuous fermentations, measurement of biomass concentration, gas chromatography analysis of liquid products and calculation of the specific product rate have all been described previously (46,47,58). The specific product rate (SPR) is defined as the rate of production of a metabolite per gram (dry weight) of cells. Since butyrate, butanol, and acetone are all derived from the condensation reaction effected by thiolase, the specific product rates of all three of these metabolites are summed to give a measure of the in vivo net specific rate of condensation. Although some intermediates derived from
acetoacetyl CoA are used for synthesis of lipids and PHB, the amount is negligible (55). Cells were collected after steady state conditions had been maintained for at least 48 hours. In the case of CO-sparging, cells were collected 5-6 hours after initiation of CO-sparging. Crude extracts were prepared from freshly-collected cells and immediately assayed for thiolase activity. The method of preparation of crude extract was similar to the procedure described in Section 4.2.1 for purifying thiolase.

4.3 Results

4.3.1 Purification of Thiolase

The results of a typical purification of thiolase are presented in Table 4-1. The purified enzyme lost less than 10% of its activity after one month of storage in 1 mM dithiothreitol and 10% glycerol under a nitrogen atmosphere at 4°C.

Denaturing gel electrophoresis showed a single band corresponding to a molecular weight of 44,000 (Figure 4-1). The elution volume of thiolase on a calibrated gel filtration column corresponded to a native enzyme having four subunits of equal molecular weight, which has been reported for thiolase from other sources (13, 22, 74). The amino acid composition of this thiolase is given in Appendix A.
4.3.2 Kinetic Studies

On double-reciprocal plots for the thiolysis reaction (Appendix B.1.1), the data fell on parallel lines, which is consistent with a Ping Pong binding mechanism (20). All other thiolases previously described in the literature have a Ping Pong binding mechanism (22,62). The $K_m$ values for CoASH and acetoacetyl CoA were determined from replots of the intercepts (20) and are 4.1 $\mu$M and 35 $\mu$M, respectively, at 30°C and pH 8.0.

A double-reciprocal plot of the condensation reaction is shown in Figure 4-2. In the absence of CoASH, the data on this plot can be fitted with a straight line and thus follow Michaelis-Menten kinetics. The $K_m$ of acetyl CoA is 0.27 mM at 30°C and pH 7.4. In contrast, at each fixed concentration of CoASH, the data are best fitted with a parabolic curve. This phenomenon has been shown with other thiolases (49,62). The inhibition pattern is consistent with a Ping Pong Bi Bi binding mechanism (20; see Appendix B.1.2). The parabolic shape of the CoASH-inhibition plots is observed partly because the two reactant molecules are of the same species (acetyl CoA). It is noteworthy that this thiolase is sensitive even to very low ratios of CoASH to acetyl CoA.

The pH dependence of the condensation reaction was studied over the range of pH 5.5 to 9.1 (Figure 4-3). There is a broad peak, with high activity from at least pH 5.5 to about 8.0.
4.3.3 Inactivation by Iodoacetamide and DTNB

Thiolase was incubated at 30°C with 0.3 mM iodoacetamide or 0.3 mM DTNB and assayed in the condensation direction. As shown in Table 4-2, iodoacetamide caused almost complete inactivation within 30 minutes, while DTNB caused a lower but significant inactivation. Thiolase was incubated in the presence of an equal proportion of distilled water as a control and did not show a detectable loss of activity.

4.3.4 Effect of Metabolites on Condensation Reaction

The following metabolites were tested for their effect on the condensation reaction in 100 mM potassium phosphate at pH 6.2: acetate (100 mM), butyrate (100 mM), ethanol (100 mM), butanol (100 mM), acetone (100 mM), acetoacetate (10 mM), pyruvate (10 mM), phosphoenolpyruvate (10 mM), butyryl CoA (1 mM and 0.2 mM), ATP (10 mM and 2 mM), ADP (5 mM), AMP (5 mM), and NAD (10 mM). The concentration of acetyl CoA equalled 3.7 times its $K_m$ value. Relative to the control, activity with butyryl CoA was 58% at 1 mM and 85% at 0.2 mM, and with ATP was 59% at 10 mM and 90% at 2 mM. Therefore, if butyryl CoA or ATP accumulated to high internal concentrations, they may act as feedback inhibitors. In the case of all other metabolites tested, activity ranged from 89 to 108% of the control.

4.3.5 Effect of Continuous Fermentations on Thiolase

Table 4-3 shows the specific activity of thiolase and the SPR measured under different types of continuous fermentations.
Results are paired to contrast results of an acid-forming and solvent-forming fermentation. For example, the first pair of experiments in Table 4-3 contrasts the results of an acid-forming fermentation, obtained by using a low ratio of glucose-to-ammonium, with a solvent-forming fermentation obtained with a higher ratio (58). Likewise, the CO-sparging dramatically shifts an acid-forming fermentation to one that produces butanol (47). The highest specific activities were associated with high glucose-to-ammonium ratio, cell-recycle, and CO-sparging, conditions which favor formation of solvents.

4.4 Discussion

During acidogenesis, the branch-point enzyme thiolase competes with phosphotransacetylase for the available pool of acetyl CoA, with the two branch pathways leading to formation of butyric and acetic acids respectively. Compared to formation of butyric acid, formation of acetic acid yields twice as much ATP per mole of acetyl CoA. Thus, the organism's capacity to regulate thiolase, and thus the ratio of these two end products, is vital to controlling the yield of ATP. The CoASH-inhibition data presented in Figure 4-2 suggest that the ratio of CoASH to acetyl CoA may be an important factor in modulating the net rate of condensation of acetyl CoA to acetoacetyl CoA by thiolase. Decker and co-workers measured acetyl CoA and CoASH in extracts of C. kluyveri and estimated the intracellular concentrations to be 0.9 mM and 0.24 mM respectively (14). Such a level of
CoASH in *C. acetobutylicum* would be expected to be very inhibitory to the condensation reaction, given the sub-saturating level of acetyl CoA. It should be noted that in *Clostridium butyricum* the ratio of acetyl CoA to CoASH has been shown to be important in regulating the oxidation of NADH, and this is also thought to be an important mechanism of regulating the ratio of acetic to butyric acid (11).

It was noted earlier that others have suggested change in internal pH might be an important factor in regulating thiolase in *C. acetobutylicum*, since the activity of thiolase in *C. pasteurianum* falls sharply with decreasing pH from an optimum at pH 8.1. Figure 4-3 shows that this thiolase has high activity over a broad range of pH values from at least pH 5.5 to greater than pH 7.0. It has been shown in typical batch *C. acetobutylicum* fermentations that the internal pH remains within the range of about pH 5.5 to 7.0 (33). Thus, change in internal pH does not appear to be important for the regulation of this thiolase. This is not surprising, because thiolase is essential to the formation of the solvents acetone and butanol, as well as formation of butyric acid. It should be noted that, during solventogenesis, thiolase competes with acetaldehyde dehydrogenase for available acetyl CoA, and this influences the ratio of butanol plus acetone to ethanol.

With respect to number of subunits, subunit size, and kinetic binding mechanism, this thiolase is somewhat similar to other bacterial thiolases that have been described. Values of
$K_m$ and other properties are summarized in Table 4-4, together with values reported for thiolase from \textit{C. pasteurianum}. We found only one thiolase enzyme in \textit{C. acetobutylicum}, whereas two such enzymes were found in \textit{C. pasteurianum} (6).

The rapid inactivation of thiolase by iodoacetamide has also been reported for other thiolases and is evidence for a sulfhydryl group at the active site (13,22). The relatively slow inactivation of this thiolase by DTNB was not expected; thiolase from \textit{Z. ramigera} is rapidly inactivated by DTNB (48). It may be that the sulfhydryl group at the active site is less accessible to DTNB.

The association of highest thiolase specific activity with conditions that favor solvent formation agrees with studies of thiolase in batch fermentations of \textit{C. acetobutylicum}, in which an increase in thiolase specific activity coincided with the onset of solvent formation (27). The variation in specific product rate is different from the variation in specific activity. This is best shown with the CO-sparging data in Table 4-3, where CO-sparging resulted in a 60-80 percent decrease in the specific product rate despite more than a two-fold increase in thiolase specific activity.

4.5 Summary

The following inferences are made regarding regulation of thiolase in \textit{C. acetobutylicum}: (a) The condensation reaction is sensitive to competitive inhibition by CoASH, and the
relative amounts of acetyl CoA and CoASH may be the most important factor in regulating the flux through this branch-point of the fermentation. This in turn helps determine the ratio of \( C_3 + C_4 \) products (butanol, acetone and butyrate) to \( C_2 \) products (ethanol and acetate). (b) Change in internal pH does not directly alter the net rate of condensation by thiolase. (c) The specific activity is significant in all fermentations, but is highest in solvent fermentations. (d) The variation in specific activity can be significantly different from the variation in specific product rate, thus key regulation of flux through this branch of the metabolism occurs at the enzyme level.
Table 4-1. Purification of thiolase from *C. acetobutylicum*.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Purif. (fold)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1090</td>
<td>3420</td>
<td>3.1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>530</td>
<td>3060</td>
<td>5.8</td>
<td>1.9</td>
<td>89</td>
</tr>
<tr>
<td>fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephacel column</td>
<td>32</td>
<td>1830</td>
<td>57</td>
<td>18</td>
<td>54</td>
</tr>
<tr>
<td>Blue Sepharose column</td>
<td>7.0</td>
<td>780</td>
<td>111</td>
<td>36</td>
<td>23</td>
</tr>
<tr>
<td>AX300 column</td>
<td>3.2</td>
<td>690</td>
<td>216</td>
<td>70</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 4-2. Inactivation of thiolase by iodoacetamide and DTNB.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>ND</td>
<td>100</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>87</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>DTNB</td>
<td>108</td>
<td>94</td>
<td>79</td>
</tr>
</tbody>
</table>

ND = not determined

Enzyme was incubated at 30°C in the presence of either distilled water (control), 0.3 mM iodoacetamide or 0.3 mM DTNB. Activity was assayed in the condensation direction without dithiothreitol. The initial assay was performed after addition of thiolase to the incubation solution. Results are presented as percentages relative to the control at 0 min.
Table 4-3. Variation of specific activity of thiolase under different continuous fermentations. All fermentations were controlled at pH 4.5.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Specific Activity of Thiolase (U/mg)</th>
<th>SPR&lt;sup&gt;a&lt;/sup&gt; (mmole/g·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical acid-fermentation&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Typical solvent-fermentation&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.2</td>
<td>7.5</td>
</tr>
<tr>
<td>Without cell-recycle&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.5</td>
<td>7.5</td>
</tr>
<tr>
<td>With 41% cell-recycle&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Before CO-sparge (D=0.18 h&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.5</td>
<td>4.3</td>
</tr>
<tr>
<td>End of CO-sparge (D=0.18 h&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>3.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Before CO-sparge (D=0.25 h&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>4.4</td>
<td>5.2</td>
</tr>
<tr>
<td>End of CO-sparge (D=0.25 h&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;g,h&lt;/sup&gt;</td>
<td>10.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

D is the dilution rate.

<sup>a</sup> SPR = specific production rate of butanol, acetone, and butyrate combined.

<sup>b</sup> [glucose] = 44 mM, [NH<sub>4</sub><sup>+</sup>] = 120 mM, D = 0.25 h<sup>-1</sup>

<sup>c</sup> [glucose] = 130 mM, [NH<sub>4</sub><sup>+</sup>] = 114 mM, D = 0.25 h<sup>-1</sup>

<sup>d</sup> [glucose] = 208 mM, [NH<sub>4</sub><sup>+</sup>] = 57 mM, D = 0.30 h<sup>-1</sup>

<sup>e</sup> [glucose] = 50 mM, [NH<sub>4</sub><sup>+</sup>] = 30 mM

<sup>f</sup> CO-sparge was 54 mmole/h for 5.5 h.

<sup>g</sup> [glucose] = 44 mM, [NH<sub>4</sub><sup>+</sup>] = 60 mM

<sup>h</sup> CO-sparge was 40 mmole/h for 5 h.
Table 4-4. Properties of thiolase from *C. acetobutylicum* and *C. pasteurianum* (6).

<table>
<thead>
<tr>
<th></th>
<th><em>C. acetobutylicum</em></th>
<th><em>C. pasteurianum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Native molecular weight</td>
<td>158,000</td>
<td>158,000</td>
</tr>
<tr>
<td>Subunit molecular weight</td>
<td>44,000</td>
<td>NR</td>
</tr>
<tr>
<td>Binding mechanism</td>
<td>ping-pong</td>
<td>ping-pong</td>
</tr>
<tr>
<td>$K_m$ for acetyl CoA (mM)</td>
<td>0.27</td>
<td>0.42</td>
</tr>
<tr>
<td>$K_m$ for CoASH (mM)</td>
<td>0.0041</td>
<td>0.029$^a$</td>
</tr>
<tr>
<td>$K_m$ for acetoacetyl CoA (mM)</td>
<td>0.035</td>
<td>0.133$^b$</td>
</tr>
<tr>
<td>Optimum pH (condensation reaction)</td>
<td>at least 5.5 to 8.1 (narrow)</td>
<td>about 8.0</td>
</tr>
<tr>
<td>Inhibitors of condensation reaction</td>
<td>ATP, butyryl CoA, butyryl phosphate</td>
<td>NR</td>
</tr>
</tbody>
</table>

$a$ apparent $K_m$ at 0.09 mM Acetoacetyl CoA

$b$ apparent $K_m$ at 0.065 mM CoASH

NR not reported
Figure 4-1. Thiolase from *C. acetobutylicum* and molecular weight standards on a denaturing gel after electrophoresis. Left lane: molecular weight standards (molecular weight is given in parentheses and is marked beside the pertinent band in thousands) are phosphorylase B (95,500), glutamate dehydrogenase (55,000), lactate dehydrogenase (36,000), carbonic anhydrase (29,000), lactoglobulin (18,400), and cytochrome C (12,400). Right lane: purified thiolase from *C. acetobutylicum*. 
Figure 4-2. Kinetics of condensation reaction with CoASH inhibition. Symbols denote the following concentrations of CoASH: □, 0.0 μM; Δ, 9.7 μM; +, 19.3 μM; and ×, 38.6 μM.
Figure 4-3. pH dependence of condensation reaction by thiolase from *C. acetobutylicum*. 
CHAPTER 5

PHOSPHOTRANSBUTYRYLASE AND ITS ROLE IN THE SYNTHESIS AND UPTAKE OF BUTYRATE

5.1 Background

PTB carries out the interconversion of butyryl CoA and butyryl phosphate:

\[ \text{butyryl CoA + phosphate} \rightarrow \text{butyryl phosphate + CoASH} \]

PTB was first reported by Gavard et al. (21) in Clostridium acetobutylicum. Those workers partially purified the enzyme and showed that it is a distinct enzyme from phosphotransacetylase (PTA) which catalyzes a similar reaction with acetyl CoA. Valentine and Wolfe (77) partially purified PTB from Clostridium butyricum and detected PTB in several other species of clostridia. PTB has not previously been isolated in homogeneous form, and there have been no detailed reports of its physical or kinetic properties.

PTB and butyrate kinase (BK) together form a pathway that enables butyric acid clostridia to convert butyryl CoA to butyrate, a conversion which is an important source of ATP. This pathway is of particular interest in C. acetobutylicum, because under certain fermentation conditions, the PTB-BK pathway is inactive, producing little or no butyrate, and the available butyryl CoA is channeled towards the formation of butanol.

Several observations have helped to explain how this shift from the butyrate pathway to the butanol pathway is effected.
Hartmanis and Gatenbeck (27) observed the almost complete loss of PTB activity coinciding with cessation of butyrate production; however, they also noted an increase in BK activity which was contrary to expectations. In contrast, Andersch et al. (1) found that specific activities of PTB and BK decreased at the end of butyrate formation by about 30% and 60%, respectively, relative to their peak activities. The specific activities of enzymes of the butanol pathway increased. The shift to the butanol pathway also has been linked to the loss of hydrogenase activity occurring at about the same time. Consequently, the metabolic shift to butanol production allows for the dissipation of reduction equivalents in the form of NADH and NADPH (1,39). More recent studies by Huesemann and Papoutsakis showed that activity of PTB and BK did not change at the onset of solventogenesis (manuscript submitted).

In addition to the documented involvement in butyrate synthesis, PTB and BK may also have an important role in butyrate uptake in specific metabolic states. This pathway was previously shown to be reversible in vitro (77). Meyer et al. (47) found that when continuous steady-state fermentations producing high levels of butyrate were sparged with carbon monoxide, there was a transient high rate of uptake of butyrate with concomitant formation of butanol but not of acetone. The absence of acetone production rules out involvement of a CoA transferase, contrary to previous suggestions that butyrate uptake in this organism occurs only through the CoA transferase
It is likely that butyrate uptake under these conditions was catalyzed by the reversal of the PTB-BK pathway, because no other pathways for butyrate uptake have been found in C. acetobutylicum (28).

A more complete understanding of PTB is required to better understand the regulation and multiple roles of the PTB-BK pathway. An isolation to homogeneity and partial characterization of BK has recently been reported (26).

5.2 Methods

5.2.1 PTB Assay

PTB was routinely assayed at room temperature in the butyryl phosphate-forming direction by monitoring the formation of a complex between CoASH and DTNB at 412 nm (1). The assay contained in a final volume of 1.0 mL: 0.1 M potassium phosphate, pH 7.4; 0.2 mM butyryl CoA; 0.08 mM DTNB and less than 0.02 U PTB. Addition of enzyme initiated the reaction. A unit of activity is defined as the amount of enzyme which converts 1 μmole butyryl CoA per minute under these conditions. The extinction coefficient is 13.6 mM⁻¹cm⁻¹ (19).

5.2.2 Purification of PTB

All buffers used to isolate PTB contained 2 mM dithiothreitol. Buffer A referred to below contained 25 mM Tris-HCl, pH 7.6. Buffer B is buffer A plus 2% (v/v) glycerol. Buffer C contained variable concentrations of potassium phosphate, pH 7.2; 1 mM EDTA and 2% (v/v) glycerol. Buffer D
contained 0.1 M potassium phosphate, pH 7.0 and 1 mM EDTA. The enzyme was stored or dialyzed under a nitrogen atmosphere.

Cells were grown in a 14-liter fermentor at pH 6.0 as described in Section 3.1. Cells were harvested by centrifugation when the optical density at 600 nm reached 8.0, then frozen in an acetone-dry ice bath and stored at -20°C.

Step 1: Preparation of Cell-Free Extract. Frozen cells (85 g wet weight) were thawed in two volumes of buffer A then passed once through a French pressure cell at 11,000 psi. The disrupted cell suspension was centrifuged for 70 minutes at 33,000 x g to remove cell debris. The cell-free extract was stored at -20°C.

Step 2: Ammonium Sulfate Precipitation. Recrystallized ammonium sulfate was added to a final concentration of 1.0 M in the extract, at 4°C. After 30 minutes, this was centrifuged at 32,000 x g for 30 minutes and the pellet discarded. Ammonium sulfate was then added to 1.6 M. After 30 minutes, this was centrifuged at 32,000 x g for 15 minutes and the supernatant discarded. Pellets were resuspended in buffer A to a final volume of about 50 mL then stored at -20°C.

Step 4: DEAE-Sephacel Chromatography. Dry enzyme from the previous step was dissolved in 25 mL buffer B, then applied to a DEAE-Sephacel column (10.5x2.5 cm) equilibrated with buffer B at 4°C. A flow rate of 50 mL/h was maintained during application, washing and elution. After application of the enzyme, the column was washed with 250 mL buffer B. Then PTB activity was
eluted with a linear gradient from 0 to 0.2 M (NH₄)₂SO₄ in a total of 400 mL buffer B. Fractions with high PTB activity (totalling 50 mL) were combined and concentrated to dryness with 1 liter of buffer C containing 25 mM potassium phosphate and 20% (w/v) polyethylene glycol.

**Step 5: HPLC Anion Exchange Chromatography.** A SynChropak AX1000 anion exchange column (SynChrom, Inc., Linden, IN) was used with an HPLC system. The column (250x4.6 mm) was equilibrated at room temperature with buffer C containing 25 mM potassium phosphate. Dry PTB from the previous step was dissolved in 2 mL of the equilibration buffer, then applied to and eluted from the column in four 0.5 mL injections. The PTB activity was eluted with a linear gradient from 25 to 100 mM potassium phosphate in buffer C over 45 minutes at 1.0 mL/min. The column was washed with 10 mL 250 mM potassium phosphate in buffer C between injections. Fractions with high PTB activity (totalling about 24 mL) were combined and concentrated to 1.5 mL using Centriflo membrane cones (Type CF25, Amicon, Danvers, MA).

**Step 6: HPLC Hydrophobic-Interaction Chromatography.** A Poly-PROPYL A (Custom LC, Inc., Houston, TX) column (200x4.6 mm) was equilibrated at room temperature with buffer D containing 1.8 M (NH₄)₂SO₄. PTB from the previous step was mixed with an equal volume of buffer D containing 1.8 M (NH₄)₂SO₄, then applied to and eluted from the column in three 1.0 mL injections. PTB activity was eluted with a linear gradient from 1.8 to 0 M (NH₄)₂SO₄ in buffer D over 40 minutes at 1.0 mL/min. Fractions
with high activity were combined and brought to 20% (v/v) glycerol prior to storage at 4°C.

5.2.3 Kinetic Studies

All kinetic studies were conducted at 30°C and were initiated by addition of 10–20 µL PTB to a final volume of 1.0 mL.

Studies in the butyryl phosphate-forming direction were similar to the standard PTB assay described above, except that each cuvette contained 100 mM Hepes, pH 8.0; variable butyryl CoA (ranging from 0.067 to 0.60 mM); variable potassium phosphate at pH 8.0 (ranging from 5.0 to 45 mM); 0.08 mM DTNB and 0.008 units of PTB. Studies in the acetyl phosphate-forming direction were performed in essentially the same way, with a cuvette containing 100 mM HEPES, pH 8.0; variable acetyl CoA (ranging from 0.12 to 0.61 mM); variable potassium phosphate at pH 8.0 (ranging from 29 to 200 mM); 0.08 mM DTNB and 0.6 units of PTB.

Studies in the butyryl CoA-forming direction were performed by measuring the increase in absorbance at 232 nm (40). A cuvette contained 120 mM Tris, pH 8.0; variable CoASH (ranging from 0.085 to 0.68 mM); variable butyryl phosphate at pH 8.0 (ranging from 0.10 to 0.81 mM) and 0.004 units of PTB. The extinction coefficient is 4.5 mM⁻¹cm⁻¹, which represents the difference between the extinction coefficients of the butyryl CoA product and the CoASH substrate.
pH dependence was studied in both directions. The butyryl phosphate-forming reaction was carried out with 10 mM Mes, 10 mM Hepes, 10 mM Ches, 140 mM potassium phosphate, 0.08 mM DTNB, 0.2 mM butyryl CoA and 0.008 units of PTB. The butyryl CoA-forming reaction was carried out with 10 mM Mes, 10 mM Tris, 10 mM Ches, 8 mM butyryl phosphate, 0.5 mM CoASH, and 0.004 units of PTB.

Product inhibition studies were performed in both the butyryl CoA-forming and butyryl phosphate-forming directions. In the butyryl CoA-forming direction, the inhibitory effect of each product (butyryl CoA and potassium phosphate) was tested by alternately fixing the concentration of one substrate (CoASH and butyryl phosphate) at twice the value of its $K_m$ while using variable concentrations of the other (1, 1.33, 2 and $\frac{1}{2}$ times the $K_m$ of the varied substrate). Inhibition by potassium phosphate was tested at concentrations equal to 0, 1 and 2 times the value of its $K_m$ for fixed concentrations of CoASH and at 0, 2 and 4 times the value of its $K_m$ for fixed concentrations of butyryl phosphate. Inhibition by butyryl CoA was tested at concentrations equal to 0 and 1 times the value of its $K_m$.

In the butyryl phosphate-forming direction, only the inhibitory effect of butyryl phosphate was tested, because DTNB present in the assay immediately reacts with any added CoASH. The range of concentrations of substrate was the same as those used for inhibition studies in the opposite direction. Inhibition by butyryl phosphate was tested at concentrations of 0, 1 and 2 times the value of its $K_m$. 
5.3 Results

5.3.1 Purification of PTB

The results of a typical purification of PTB are presented in Table 5-1. The procedures for each step are described under Methods. Purified PTB showed no detectable loss of activity when stored for six months under nitrogen at 4°C in 25 mM Tris-HCl, pH 7.6, with 20% (v/v) glycerol, 2 mM dithiothreitol and 100 mM (NH₄)₂SO₄.

The final purification step, HPLC hydrophobic-interaction chromatography, was used to ensure that the PTB preparation was homogeneous for raising antibodies. The slight decrease in activity associated with the last step indicates the presence of some inactive PTB. The last step also yielded two peaks of PTB activity. The first peak was sharp and contained 56 percent of the eluted activity. The second peak was broader and irregular in shape. Polyacrylamide gel electrophoresis with sodium dodecyl sulfate showed a single band for each peak, with both corresponding to a molecular weight of 31,000. Probably the second peak resulted from dissociation into subunits in the high-salt environment and later reassociation into active enzyme in the low-salt buffer. The homogeneity of the PTB preparation as determined by denaturing gel electrophoresis is shown in Figure 5-1.

The elution volume of PTB on a calibrated gel filtration column corresponded to a native molecular weight of 264,000.
Assuming that PTB does not have an irregular shape, then it consists of eight subunits of equal molecular weight. The amino acid composition of PTB is given in Appendix A.

5.3.2 Kinetic Studies

Double-reciprocal plots in the butyryl phosphate-forming direction with variable concentrations of butyryl CoA and phosphate, and in the butyryl CoA-forming direction with variable concentrations of butyryl phosphate and CoASH intersected to the left of the 1/v axis (see Appendix B.2.1 and B.2.2) consistent with either an Ordered or Random Bi Bi binding mechanism (59). The 1/phosphate plots exhibit a slight nonlinearity at the concentrations of substrates used consistent with phosphate binding at more than one substrate site. The apparent $K_m$ values for butyryl CoA, phosphate, butyryl phosphate and CoASH are, respectively, 0.11 mM, 14 mM, 0.26 mM and 0.077 mM; and the dissociation constants are, respectively, 0.22 mM, 28 mM, 0.54 μM and 0.16 μM.

To distinguish between the Ordered and Random Bi Bi binding mechanisms, the product inhibition effects of phosphate and butyryl CoA on the butyryl CoA-forming reaction and of butyryl phosphate on the butyryl phosphate-forming reaction were studied. Inhibition by phosphate is noncompetitive with respect to butyryl phosphate and CoASH, whereas butyryl CoA inhibits competitively in each case. Butyryl phosphate is a competitive inhibitor with respect to butyryl CoA, but a noncompetitive
inhibitor with respect to phosphate. An interesting feature of inhibition by butyryl phosphate with respect to phosphate is that the data are best fitted by a family of parabolic curves, rather than straight lines (Figure 5-2). Double-reciprocal plots of product inhibition data are presented in Appendix B.2.3.

The pattern of competitive inhibition by butyryl CoA, together with the complex inhibition shown in Figure 5-2, is not consistent with an Ordered Bi Bi binding mechanism (59). The nonlinearity of Figure 5-2 and the noncompetitive inhibition by phosphate are atypical for a classical Random Bi Bi binding mechanism; however, a likely explanation for both phenomena is that phosphate influences each catalytic site at more than one binding site as indicated above, although nonlinearity from a steady state random mechanism cannot be excluded.

Product inhibition constants are summarized in Table 5-2. These constants are similar to the $K_m$ values and suggest that butyryl phosphate and butyryl CoA are relatively important inhibitors of the reactions that form them. In contrast, phosphate is a relatively weak inhibitor of the butyryl CoA-forming reaction.

5.3.3 pH Dependence

The pH dependence of the reaction in both directions is shown in Figure 5-3. In both directions, the percent activity is very sensitive to pH changes within the pH range of 5.5 to
7.0, the range of internal pH in typical fermentations (33). This is most markedly true in the butyryl phosphate-forming direction, in which the enzyme is virtually inactive at a pH of about 6.

5.3.4 Activity with Other Acyl CoA Substrates

Table 5-3 shows that PTB has a fairly broad substrate specificity in the acyl phosphate-forming direction. The activity with acetyl CoA is negligible compared to PTA activity in the crude extract, and small relative to activity with butyryl CoA.

The formation of acetyl phosphate by pure PTB was studied in more detail over a range of concentrations of acetyl CoA and phosphate. The $K_m$ values for acetyl CoA and phosphate are 6.2 mM and 610 mM, respectively. The dissociation constants are 0.028 mM and 2.8 mM, respectively. On double-reciprocal plots, the kinetic data could nearly be fitted with a family of parallel lines, in contrast to the converging lines observed for plots of data obtained in the butyryl phosphate-forming reaction.

Other enzymes have been previously described which exhibit parallel lines with one substrate and converging lines with an alternate substrate (54b). The appearance of parallel lines in these cases does not indicate a ping pong binding mechanism, rather it is the result of the small dissociation constants relative to the $K_m$ value corresponding to the same substrate.
5.3.5 Effect of Metabolites

The effects of various metabolites on the butyryl phosphate-forming reaction were tested at pH 8.0 with initial concentrations of both butyryl CoA and phosphate equal to 1.5 x their respective $K_m$ values. The following metabolites were tested: acetate (100 mM), butyrate (100 mM), ethanol (100 mM), butanol (100 mM), acetone (100 mM), acetoacetate (10 mM), pyruvate (10 mM), acetyl CoA (0.2 mM), acetoacetyl CoA (0.2 mM), ATP (2 and 10 mM), ADP (5 mM), AMP (5 mM), NAD (10 mM), NADH (10 mM), NADP (10 mM) and NADPH (10 mM). None of the metabolites showed any significant activation of PTB. Those metabolites which inhibited the reaction by greater than 10% are summarized in Table 5-4.

5.4 Discussion

The physical and kinetic properties of PTB from C. acetobutylicum are summarized in Table 5-5. PTB has not previously been characterized in detail for any organism. Although partial-purification of PTB has been reported by others (77), those workers assayed the enzyme in the butyryl CoA-forming direction, and thus a direct comparison of specific activity was not attempted.

It might be expected that PTB would resemble PTA in such respects as size and number of subunits and binding mechanism. In fact, with respect to size and number of subunits, this PTB is not similar to clostridial PTA that has been characterized.
PTA has not been isolated from *C. acetobutylicum*, but has been isolated and at least partially characterized in three other species of clostridia. Molecular weights for PTA were found to be 88,000 in *Clostridium thermoaceticum* (16), 63,000 and 75,000 in *Clostridium acidurici* (56), and 60,000 in *Clostridium kluyveri* (63), in contrast to 264,000 for PTB in *C. acetobutylicum*. PTA in *C. thermoaceticum* appears to be a tetramer (16), whereas PTB in *C. acetobutylicum* is probably an octamer.

Detailed studies of binding mechanism in PTA were reported only by Kyrtopolous and Satchell (41), who used PTA from *C. kluyveri*. They reported that a Random Bi Bi binding mechanism was likely, but it should be noted that they used Dixon's method which may not distinguish between Random and Ordered Bi Bi binding mechanisms (59). They also observed some complex product inhibition features which, they suggested, could best be explained by adsorption of phosphate and acetyl phosphate at up to three sites adjacent to the binding site for the CoA species. Our product inhibition studies with PTB are also consistent with a Random Bi Bi binding mechanism. Although the complex product inhibition reported for PTA from *C. kluyveri* was not observed, the inhibition does have features which suggest multiple binding sites for phosphate.

Perhaps one of the most interesting properties of this PTB is the sensitivity of its relative activity to pH change within the range of physiologic pH. The decrease in internal pH during a typical batch fermentation of *C. acetobutylicum* and the
concomitant shift from butyrate formation to butanol formation is well documented (25,33). The internal pH can decrease from 7 to as low as 5.5 during a fermentation, because the acetic and butyric acids produced decrease the external pH and the ΔpH across the cell membrane. Figure 5-3A shows that the attendant decrease in relative activity of PTB within this range should act as a feedback control to slow or halt the formation of butyric acid by the PTB-BK pathway, and is thus in agreement with in vivo observations described above. Therefore, the pH dependence of PTB, and probably BK (26), is likely a factor in regulation of the flux through this pathway.

Additional factors are likely involved in reducing the flux through this pathway. Butyrate can accumulate rapidly within the cell, and since reactions in this pathway are reversible, it has been suggested that the level of the precursor, butyryl phosphate, also increases (25). The relatively low inhibition constant for butyryl phosphate of 0.35 mM (Table 5-2) indicates that an accumulation of butyryl phosphate should also significantly reduce the net rate of phosphorylation of butyryl CoA.

It is noteworthy, however, that the relative activity of PTB in the butyryl CoA-forming direction is not as sensitive to pH change. The relative activity at pH 5.5, although low, is significant and does not preclude the possibility that this pathway sometimes operates in the butyrate-uptake direction under conditions of low external pH. In fact, this appears to be the most likely pathway under some conditions.
Other interesting features of PTB are its inhibition by ATP and its broad substrate specificity. The ATP inhibition of the butyryl phosphate-forming reaction suggests another feedback control mechanism, since ATP is a product of the PTB-BK pathway. CoA transferase and BK from \textit{C. acetobutylicum} also exhibit broad specificities (26; this work, Chapter 6). Jewell et al. (37) demonstrated that propionic, valeric and 4-hydroxybutyric acids can be taken up by \textit{C. acetobutylicum} and converted to their respective alcohols. This broad specificity could be the basis for other novel fermentations.

5.5 Summary

The following inferences are made regarding regulation of PTB in \textit{C. acetobutylicum}: (a) Based on data presented by others, the amount of PTB enzyme present in the cell is probably fairly constant; thus, in \textit{vivo} activity of this step is probably not closely regulated at the genetic level. (b) In the direction of butyrate synthesis, PTB activity decreases significantly with decreasing pH within the range of physiologic pH, and this may serve as a sort of feedback control to halt the formation of butyrate. (c) The inhibition constant of butyryl phosphate is 0.35 mM; accumulation of butyryl phosphate to high levels could also act as a feedback control. (d) The kinetic, thermodynamic, and pH-dependence characteristics do not preclude the possibility that the PTB-BK pathway operates in the direction of butyrate uptake under some fermentation conditions.
Table 5-1. Purification of PTB from \textit{C. acetobutylicum}.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Purif. (fold)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4235</td>
<td>30,500</td>
<td>7.2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation</td>
<td>840</td>
<td>23,800</td>
<td>28.3</td>
<td>3.9</td>
<td>78</td>
</tr>
<tr>
<td>Phenyl Sepharose column</td>
<td>102</td>
<td>18,500</td>
<td>182</td>
<td>25</td>
<td>61</td>
</tr>
<tr>
<td>DEAE-Sephacel column</td>
<td>21.8</td>
<td>15,200</td>
<td>694</td>
<td>96</td>
<td>50</td>
</tr>
<tr>
<td>AX-1000 column</td>
<td>6.2</td>
<td>9,070</td>
<td>1460</td>
<td>200</td>
<td>30</td>
</tr>
<tr>
<td>PolyPROPYL A column</td>
<td>2.9</td>
<td>4,010</td>
<td>1380</td>
<td>192</td>
<td>13</td>
</tr>
</tbody>
</table>
Table 5-2. Product inhibition constants for PTB

<table>
<thead>
<tr>
<th>Product Inhibitor</th>
<th>Varied Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Butyryl phosphate</td>
</tr>
<tr>
<td>Phosphate</td>
<td>$K_{is} = 27$ mM</td>
</tr>
<tr>
<td></td>
<td>$K_{ii} = 28$ mM</td>
</tr>
<tr>
<td>Butyryl CoA</td>
<td>$K_{is} = 0.17$ mM</td>
</tr>
<tr>
<td>Butyryl CoA</td>
<td></td>
</tr>
<tr>
<td>Butyryl phosphate</td>
<td>$K_{is} = 0.35$ mM</td>
</tr>
</tbody>
</table>

$K_{is}$ = slope inhibition constant

$K_{ii}$ = intercept inhibition constant

ND = not determined because of nonlinear behaviour
Table 5-3. Substrate specificity of PTB with respect to various acyl CoA compounds.

<table>
<thead>
<tr>
<th>Acyl CoA Compound</th>
<th>Relative Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-butyryl CoA</td>
<td>100 %</td>
</tr>
<tr>
<td>acetyl CoA</td>
<td>1.6</td>
</tr>
<tr>
<td>n-propionyl CoA</td>
<td>23</td>
</tr>
<tr>
<td>n-valeryl CoA</td>
<td>78</td>
</tr>
<tr>
<td>isobutyryl CoA</td>
<td>30</td>
</tr>
<tr>
<td>isovaleryl CoA</td>
<td>95</td>
</tr>
</tbody>
</table>
Table 5-4. Inhibition of PTB (butyryl phosphate-forming reaction) by cofactors.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Assay Conc. (mM)</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>ATP</td>
<td>10</td>
<td>53</td>
</tr>
<tr>
<td>ADP</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>AMP</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>NADPH</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>NADP</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 5-5. Physical and kinetic properties of PTB from *C. acetobutylicum*.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity of purified enzyme</td>
<td>1400 U/mg</td>
</tr>
<tr>
<td>Native molecular weight</td>
<td>264,000</td>
</tr>
<tr>
<td>Subunit molecular weight</td>
<td>31,000</td>
</tr>
<tr>
<td>$K_m$ (butyryl CoA)</td>
<td>0.11 mM</td>
</tr>
<tr>
<td>$K_m$ (phosphate)</td>
<td>14 mM</td>
</tr>
<tr>
<td>$K_m$ (butyryl phosphate)</td>
<td>0.26 mM</td>
</tr>
<tr>
<td>$K_m$ (CoASH)</td>
<td>0.077 mM</td>
</tr>
<tr>
<td>Probable binding mechanism</td>
<td>Random Bi Bi</td>
</tr>
<tr>
<td>Substrates besides butyryl CoA</td>
<td>isovaleryl CoA (95%)</td>
</tr>
<tr>
<td></td>
<td>n-valeryl CoA (78%)</td>
</tr>
<tr>
<td>Inhibitors (butyryl phosphate-forming reaction)</td>
<td>ATP</td>
</tr>
</tbody>
</table>
Figure 5-1. PTB from *C. acetobutylicum* and molecular weight standards on a denaturing gel after electrophoresis. Left lane: molecular weight standards (molecular weight is given in parentheses and is marked beside the pertinent band in thousands) are bovine albumin (66,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100) and α-lactalbumin (14,200). Right lane: purified PTB from *C. acetobutylicum*.
Figure 5-2. Competitive inhibition of PTB (butyryl phosphate formation) by butyryl phosphate with a fixed concentration of butyryl CoA. The concentration of butyryl phosphate was: □, 0 mM; △, 0.26 mM; and +, 0.52 mM.
Figure 5-3. Dependence of PTB activity on pH in (A) the butyryl phosphate-forming direction, and (B) the butyryl CoA-forming direction.
CHAPTER 6

COA TRANSFERASE AND ITS ROLE
IN ACID UPTAKE AND SOLVENT FORMATION

6.1 Background

Coenzyme A (CoA) transferases activate carboxylic acids to the respective CoA thioester at the expense of the CoA thioester of another species of carboxylic acid. The reaction is easily reversed. The presence of CoA transferase in cell extracts was first demonstrated by Stadtman in *Clostridium kluyveri* (70). Different types of CoA transferase have been subsequently shown to be present in several other species of bacteria. These enzymes have been prepared in very pure form from *Escherichia coli* (68), *Peptostreptococcus elsdenii* (76), and *Clostridium SB4* (4). Some physical and kinetic properties of these enzymes have been characterized. All studied CoA transferases show relatively broad substrate specificities, but each enzyme has characteristic preferred substrates.

Among clostridia that are used to carry out the acetone-butanol fermentation, a CoA transferase activity has been reported in crude extracts that transfers CoA from acetoacetyl CoA to butyrate or to acetate (1,28,83). However, the direct transfer of CoA from acetyl CoA to butyrate has not been detected (1). The role of CoA transferase in butanol-forming clostridia is fundamentally different from other bacteria in which it has been characterized. Generally, in this
latter group of bacteria the enzyme is involved in the uptake of substrates for energy and structural use (4,68,76). In butanol-forming clostridia, however, the CoA transferase acts mainly to detoxify the medium by removing the acetate and butyrate excreted earlier in the fermentation (28,31,38). This provides acetoacetate for decarboxylation to acetone and butyryl CoA for subsequent conversion to butanol during solventogenesis. Consequently, CoA transferase plays a key role in this economically important fermentation.

Andersch et al. (1) found in batch solvent fermentations with Clostridium acetobutylicum DSM 1732 that CoA transferase activity measured with butyrate or acetate increased gradually, reaching peak activity during solventogenesis. Hartmanis et al. (28) determined the relative activity with various carboxylic acids, using crude extracts of C. acetobutylicum ATCC 824. They reported that a wide variety of carboxylic acids, including formate, straight chain acids up to heptanoate, and several branched chain and unsaturated carboxylic acids were used as substrates.

More recently, Yan et al. (83) studied the change in levels of CoA transferase in batch fermentations of Clostridium beijerinckii NRRL-B592 and NRRL-B593. The transferase activity was not measured separately, but in combination with acetoacetyl-CoA hydrolase as an indication of total acetooacetate-forming (acetoacetyl CoA-utilizing) activity. This combined activity showed only a 2 to 3-fold increase at the
onset of solventogenesis, in contrast to more dramatic increases in the levels of other enzymes involved in solventogenesis. Those workers suggested that expression of acetoacetate-forming activity might be an early event in the switch from acidogenesis to solventogenesis.

A more complete understanding of CoA transferase in butanol-forming clostridia is required to better understand the regulation of acid-uptake and solvent-formation.

6.2 Methods

6.2.1 CoA Transferase Assay

CoA transferase was routinely assayed at room temperature in the butyrate-conversion direction by monitoring the disappearance of the enolate form of acetoacetyl CoA at 310 nm (68). The assay contained in a final volume of 1.0 mL: 100 mM Tris, pH 7.5; 100 mM potassium butyrate, pH 7.5; 20 mM MgCl₂; 0.10 mM acetoacetyl CoA; 5% (v/v) glycerol; and less than 0.05 unit of CoA transferase. Addition of acetoacetyl CoA initiated the reaction. The hydrolysis of acetoacetyl CoA was measured by omitting potassium butyrate and was subtracted from the slope obtained when butyrate was included in the cuvette. This hydrolysis was less than 10% of the uncorrected slope when using crude extracts. A unit of activity is defined as the amount of enzyme which converts 1 μmole acetoacetyl CoA per minute under these conditions. The extinction coefficient is 8.0 mM⁻¹ cm⁻¹ (72).
6.2.2 Purification of CoA Transferase

All steps were carried out at room temperature without anaerobic conditions; however, between steps the enzyme preparation was stored under nitrogen at 4°C. Buffer A referred to below contained 25 mM Mops, pH 7.0, 0.5 M $\text{(NH}_4\text{)}_2\text{SO}_4$, and 20% (v/v) glycerol. Buffer B contained 25 mM Mops (4-morpholinepropanesulfonic acid), pH 7.0 and 15% (v/v) glycerol. Buffer C was Buffer B with 0.75 M $\text{(NH}_4\text{)}_2\text{SO}_4$.

Cells were grown in a 14 L fermentor at pH 4.5 as described in Section 3.1. Cells were harvested by centrifugation approximately 3 hours after the fermentation reached stationary growth phase, then stored under nitrogen at -20°C. A typical enzyme preparation was performed as follows:

Step 1: Preparation of Cell-Free Extract. Frozen cells (95 g) were thawed in two volumes of buffer A then passed once through a French pressure cell (Model FA-073, SLM Instruments, Inc., Urbana, IL) at 15,000 psi. Unlysed cells were removed by centrifugation, resuspended to a total volume of 40 mL, and passed through the pressure cell a second time at 15,000 psi. The disrupted-cell suspensions were combined and centrifuged for 45 minutes at 30,000 x g to remove cell debris.

Step 2: Ammonium Sulfate Precipitation. Recrystallized ammonium sulfate was added to a final concentration of 1.8 M. After 30 minutes of stirring, the suspension was centrifuged at 20,000 x g for 15 minutes and the pellet discarded. Ammonium
sulfate was then added to give the supernatant a final concentration of 2.2 M. After stirring for 30 minutes, the suspension was centrifuged at 20,000 x g for 15 minutes and the supernatant discarded. Pellets from this second precipitation were resuspended in Buffer A to a final volume of about 40 mL.

**Step 3: Octyl Sepharose CL-4B Chromatography.** The resuspended ammonium sulfate fraction was diluted with Buffer B containing 2.0 M (NH₄)₂SO₄ to a final concentration of 0.8 M (NH₄)₂SO₄. This enzyme preparation was then applied directly to an Octyl Sepharose CL-4B (Pharmacia) column (6.3 x 2.5 cm) equilibrated with 1.0 M (NH₄)₂SO₄ in Buffer B. The column was then washed with equilibration buffer. A flow rate of about 80 mL/h was maintained at all times. The CoA transferase does not bind to the column at this level of ammonium sulfate, but a large amount of contaminating protein is removed. Fractions containing high activity were combined to a total volume of about 80 mL. Crystalline ammonium sulfate was added to a final concentration of 1.5 M in the combined fractions. This preparation was then applied directly to a second Octyl Sepharose CL-4B column (14 x 2.5 cm) equilibrated with 1.5 M (NH₄)₂SO₄ in Buffer B at 80 mL/h. The column was washed with equilibration buffer until the absorbance at 280 nm was less than 0.8, then activity was eluted by a linear gradient from 1.5 to 1.0 M (NH₄)₂SO₄ in a total volume of 240 mL. Washing and elution were performed at 160 mL/h.

**Step 4: Phenyl Sepharose CL-4B Chromatography.** The combined
sample from the previous step totaling 90 mL was applied directly to a Phenyl Sepharose CL-4B (Pharmacia) column (3.8 x 2.5 cm) equilibrated with Buffer C at 110 mL/h. The column was washed with Buffer C until the absorbance at 280 nm was less than 0.4, then activity was eluted by a linear gradient from 100% Buffer C to 75% (v/v) Buffer C plus 25% (v/v) ethylene glycol, in a total volume of 240 mL. Washing and elution were performed at 160 mL/h. Fractions with high activity totalling 190 mL were concentrated to about 40 mL using type PM10 ultrafiltration membranes in a stirred cell (Amicon, Danvers, MA), and then concentrated further to 2.8 mL using filtering cones (type CF25 ultrafiltration membrane, Amicon).

Step 5: Sephacryl S-300 Chromatography. The concentrated sample was applied to a Sephacryl S-300 (Pharmacia) column (90 x 1.6 cm) equilibrated with Buffer A and eluted at 15 mL/h. Fractions with high activity totalling about 10 mL were concentrated to about 3 mL using filtering cones (type CF25 ultrafiltration membranes, Amicon), and then crystalline ammonium sulfate was added to a final concentration of 1.5 M.

Step 6: HPLC Hydrophobic-Interaction Chromatography. A Poly-PROPYL A (Custom LC, Inc., Houston, TX) column (200 x 4.6 mm) was used with an HPLC system (Laboratory Control Data, Riviera Beach, FL). The column was equilibrated with 1.1 M (NH₄)₂SO₄ in Buffer B. After application of the sample, the column was washed with 15 mL equilibration buffer, then activity was eluted with a linear gradient from 1.1 to 0.9 M (NH₄)₂SO₄ in Buffer B
in a total volume of 15 mL. The flow rate was 1.0 mL/min throughout this step. Fractions with high activity were combined into three groups corresponding to the beginning, middle and end of the activity peak, and then glycerol added to a final concentration of 20% (v/v).

6.2.3 Kinetic Studies

The following kinetic studies were conducted at 30°C and were initiated by addition of acetoacetetyl CoA. With the exception of studies of optimum pH and of acetate and butyrate as alternative substrates, the standard assay for CoA transferase was used with modifications as noted below.

The conversion of butyrate was studied with potassium butyrate concentrations ranging from 33 to 300 mM and acetoacetyl CoA concentrations ranging from 3.9 to 100 μM. The conversion of propionate was studied with potassium propionate concentrations ranging from 60 to 300 mM and acetoacetyl CoA concentrations ranging from 3.9 to 35 μM. The conversion of acetate was studied with potassium acetate concentrations ranging from 60 to 300 mM and acetoacetyl CoA concentrations ranging from 2.8 to 25 μM.

The substrate specificity was studied at 100 mM of each carboxylic acid tested. Stock solutions of the free acids were first adjusted to pH 7.5 by the addition of KOH.

The effect of metabolites was studied using 300 mM potassium butyrate or 300 mM potassium acetate. The addition of NAD,
ADP and ATP, and to a lesser extent CoASH, acetyl CoA and butyryl CoA caused a marked reduction of the extinction coefficient of acetoacetyl CoA, probably by chelating the magnesium ions which are added to the assay. The extinction coefficient is very sensitive to the concentration of magnesium ions and was measured in each case for use in calculating the enzyme activity.

The optimum pH of acetate conversion was studied by adding the coupling enzyme phosphotransacetylase to convert acetyl CoA to acetyl phosphate (68). The rate of breakage of acyl CoA bonds was determined by monitoring the decrease in absorbance at 232 nm. The cuvette contained in a final volume of 1.0 mL: 15 mM Mes and 15 mM Mops buffers, 100 mM potassium phosphate, 5% (v/v) glycerol, 0.1 mM acetoacetyl CoA, 3 units of phosphotransacetylase, 100 mM potassium acetate, and sufficient CoA transferase to cause a maximal rate of absorbance change of about 0.3 min⁻¹. Phosphotransacetylase was not limiting over the range of pH 5.4 to 7.8. The hydrolysis of acetoacetyl CoA was measured by omitting acetate from the cuvette and was less than 3% of the rate of absorbance change when acetate was present.

The simultaneous conversion of the alternative substrates acetate and butyrate was studied by measuring the rate of conversion of acetoacetyl CoA (and thus the total rate of formation of acetyl CoA and butyryl CoA) by monitoring the decrease in absorbance at 310 nm. Then, under identical assay conditions,
solely the rate of acetyl CoA formation was measured, by monitoring the decrease in absorbance at 232 nm (68). The rate of butyryl CoA formation was calculated by subtracting the rate of acetyl CoA formation from the rate of acetoacetyl CoA conversion. Each assay contained in a final volume of 1.0 mL: 100 mM Tris-HCl, pH 7.5; 5% (v/v) glycerol; 20 mM MgCl₂; 25 mM potassium arsenate, pH 7.5; 0.1 mM acetoacetyl CoA; potassium acetate ranging in concentration from 0 to 200 mM, pH 7.5; potassium butyrate ranging in concentration from 0 to 200 mM, pH 7.5; 3 units phosphotransacetylase; and about 0.02 units of pure CoA transferase. The phosphotransacetylase, which was purified from Clostridium kluyveri (Sigma), hydrolyzes acetyl CoA but not butyryl CoA in the presence of arsenate.

6.3 Results

6.3.1 Purification of CoA Transferase

The results of the purification of CoA transferase are presented in Table 6-1. The procedures for each step are described under Methods. The high concentration of ammonium sulfate and glycerol which was added to buffers was necessary to stabilize activity both in crude and pure preparations of the enzyme. Omitting either salt or glycerol or both led to rapid loss of activity. EDTA and dithiothreitol apparently are not needed to protect enzyme activity. Activity was somewhat cold labile, but full activity was restored by allowing the enzyme preparation to stand at room temperature for at least one hour.
Pure and partially pure enzyme preparation stored for one week at 4°C under nitrogen showed less than 5% loss in activity. Cell-free extracts could be stored at room temperature for up to four days without detectable loss in activity.

The high concentration of salt and glycerol required to stabilize activity prevented CoA transferase and other proteins from binding to ion exchange and dye-ligand affinity columns. Consequently, the purification procedure reported here relies mainly on various hydrophobic-interaction chromatographies. No dialysis is required at any step. This purification clearly illustrates the potential of new chromatography techniques for purification of proteins with unusual stability requirements.

Fractions from the HPLC hydrophobic-interaction chromatography were electrophoresed on a polyacrylamide gel in the presence of sodium dodecyl sulfate (Figure 6-1). Fractions corresponding to the beginning, middle, and end of the activity peak all showed two bands of essentially equal intensity corresponding to molecular weights of 23,000 and 25,000. The elution volume of CoA transferase on a calibrated gel filtration column corresponded to a native molecular weight of about 93,000. Thus, this CoA transferase likely is a heterotetramer of two α and two β subunits.

6.3.2 Kinetic Studies

The butyrate-conversion data as analyzed by double-reciprocal plots are best fitted by a family of lines
that are approximately parallel at moderate concentrations of both substrates, but become hyperbolic concave-up at high concentrations of the varied substrate (Figure 6-2). At the highest concentration of the fixed substrate, the fitted line intersects the other lines to the right of the 1/v axis. This behavior, known as double competitive substrate inhibition, is consistent only with a Ping Pong Bi Bi kinetic binding mechanism (9,20,61). The inhibitory effect of high concentrations of one substrate is relieved by increasing the level of the other substrate. The general bisubstrate kinetic equation presented in Section 3.4 does not allow for double substrate inhibition. The appropriate equation is presented in Appendix B.3.1, together with a brief explanation of how $K_m$ values were calculated.

On double-reciprocal plots for acetate-conversion and propionate-conversion (Appendix B.3.2) the data were best fitted by a family of parallel lines, and did not exhibit significant substrate inhibition at the levels of substrates used. $K_m$ values for acetoacetyl CoA, butyrate, acetate, and propionate, and inhibition constants ($K_i$) for acetoacetyl CoA and butyrate are presented in Table 6-2. The $K_m$ values for the carboxylic acids are remarkably high.

The pH dependence of the acetate-conversion reaction from pH 5.4 to 7.5 is shown in Figure 6-3. The activity is at least 80% of the maximal activity from pH 5.9 to at least 7.8, and relative activity is still greater than 50% at pH 5.4.
6.3.3 Substrate Specificity

Table 6-3 shows the relative activity of CoA transferase with 100 mM of the potassium salt of various carboxylic acids. Relative activity is highest with acetate, propionate, and butyrate, while activity with valerate, isobutyrate, and crotonate is significant but low. Activity with the other carboxylic acids tested was below the level of detection. Thus, while the substrate specificity is fairly broad, it is not as broad as was indicated by the results reported previously by Hartmanis et al. (28), which are presented in Table 6-3 for comparison. Those workers used the same strain of \textit{C. acetobutylicum} and essentially the same assay with the same pH and concentrations of substrates and magnesium ions. One difference in their procedure is their use of crude extract as opposed to purified enzyme; however, we measured the relative activity in crude extract with respect to acetate, propionate, and butyrate and found approximately the same ratio of activity of about 4:2:1, respectively, that was measured using pure enzyme. Also, the presence of ammonium sulfate and glycerol did not affect the rate of conversion of butyrate relative to acetate.

6.3.4 Studies of Acetate and Butyrate as Alternative Substrates

Measurements of the relative rate of conversion of acetate and butyrate when both acids were present (see Table 6-4) confirmed the general trend predicted for a Ping Pong Bi Bi mechanism when two alternative substrates are present (60).
Addition of acetate depressed the rate of butyrate conversion, and addition of butyrate depressed the rate of acetate conversion. When equimolar acetate and butyrate were present together, the rate of conversion of butyrate relative to acetate was 27% at 100 mM of each and 32% at 200 mM. In comparison, when the two acids were present separately, the rate of conversion of butyrate relative to acetate was 26% at 100 mM of each (Table 6-3) and 25% at 200 mM of each. When 100 mM acetate and 200 mM butyrate were present together, the rate of conversion of butyrate was 71% that of acetate.

6.3.5 Effect of Metabolites, Cofactors, and Inorganic Salts on CoA Transferase Activity

Table 6-5 summarizes the in vitro effect of various metabolites and cofactors on activity with acetate and butyrate. 200 mM butanol and acetone resulted in significant inhibition; the highest physiologic levels of these products are 200 mM and 100 mM, respectively (38,45). Significant inhibition by 200 mM 1-propanol and 2-butanone, which are not found in this fermentation, suggests that this CoA transferase is inhibited by solvents in general. Inhibition by acetoacetate, acetyl CoA, and butyryl CoA was to be expected, because those metabolites are products of acetate and butyrate conversion. Inhibition by pyruvate is comparable to that of acetoacetate, and may be a result of the somewhat similar structure of these two compounds. Unfortunately, there is little or no data on in vivo levels of
these intermediate metabolites and cofactors in butanol-producing clostridia. The cofactors ADP, ATP, and NAD did not cause significant inhibition, even at the relatively high levels that were tested. Acetate conversion was more sensitive than butyrate conversion to inhibition by CoASH, acetyl CoA, and butyryl CoA, but generally the two reactions showed similar degrees of inhibition by the other metabolites and cofactors tested.

In addition to the results presented in Table 6-5, the in vitro effect of KCl, NaCl, LiCl, and (NH₄)₂SO₄ on butyrate activation was tested. These inorganic salts caused less than 10% inhibition at 100 mM concentration. Furthermore, there was no difference in activity when sodium butyrate was used in place of potassium butyrate.

6.4 Discussion

The CoA transferase purified in this study shows only superficial similarities to some other bacterial CoA transferases which have been purified and characterized. The size is similar, consisting of two subunits with molecular weights of about 23,000 and 25,000, compared to 23,000 and 26,000 in E. coli (68) and 23,000 and 25,000 in Clostridium SB4 (4). Also, like other CoA transferases studied, the kinetic binding mechanism is Ping Pong Bi Bi (36,69,76). The metabolic role of CoA transferase in C. acetobutylicum is, however, fundamentally different from that of other CoA transferases which have been
characterized, and this is reflected in the much higher $K_m$ values for the carboxylic acid and different specificities. With the * Clostridium acetobutylicum* CoA transferase, the $K_m$ values for the preferred carboxylic acid substrates butyrate, propionate, and acetate are very high, ranging from 660 mM to 1200 mM. In contrast, for CoA transferase in *Clostridium SB4*, the apparent $K_m$ values for eight preferred carboxylic acid substrates including butyrate, propionate, and acetate ranged from 0.8 to 29 mM (4). Similarly, with *E. coli* CoA transferase, a preliminary $K_m$ value for acetate of about 20 mM was reported (69). All of these $K_m$ values were determined using acetoacetyl CoA as the acyl CoA substrate. In addition, other CoA transferases which have been purified are not highly unstable in the absence of salt and glycerol, unlike this CoA transferase.

As with thiolase and PTB, a major objective in purifying CoA transferase was to carry out *in vitro* experiments regarding the effect on enzyme activity of substrate concentration, pH, and various metabolites and cofactors to assess what factors are important *in vivo* for the metabolic regulation of this enzyme. The intracellular concentrations of acetate, butyrate, butanol, ethanol, acetone, and some cofactors (55; C. L. Meyer, Ph.D. thesis, Rice University, 1987), and the pH (25,33,34) are known to vary over a significant range under batch fermentation conditions.

The intracellular concentrations of acetate and butyrate do not exceed about 300 mM and 700 mM respectively (25,34,35), and
are generally much lower than the apparent $K_m$ values of 1200 mM and 660 mM, respectively. At such subsaturating levels, CoA transferase activity in vivo should be very sensitive to changes in the intracellular acetate and butyrate concentration. The high $K_m$ values suggest that the enzyme has evolved in such a way that the uptake of acetate and butyrate from the media is a graded response to the progressive toxic effects of increasing levels of these carboxylic acids.

The intracellular concentration of acetoacetyl CoA in butanol-forming clostridia has not been reported. Thus, it is not possible to use the $K_m$ values reported here to predict the effect of fluctuations of levels of that intermediate on in vivo activity.

Of the metabolites and cofactors tested for their effect on enzyme activity, butanol and acetone are of particular interest, because they caused significant inhibition at physiologic levels. Butanol and acetone accumulate in the medium during the solventogenic phase of the fermentation, up to maximum concentrations of about 200 mM and 100 mM, respectively (45). These nonpolar solvents passively diffuse through the cell membrane as they are excreted into the medium such that the internal concentration is slightly higher than the external concentration. Acetone is a direct product of the CoA transferase-acetoacetate decarboxylase pathway, and butanol is an indirect product; therefore, inhibition by these two solvents may act as a feedback control to slow the accumulation of toxic levels of
butanol. It is interesting that high levels of ethanol, which is a relatively minor fermentation product, had little effect on enzyme activity.

The remaining metabolites that were tested for their effect on acetate and butyrate conversion either caused no inhibition, or there are not yet sufficient data on their concentrations in vivo to determine whether the inhibition is physiologically important.

The relative flux in vivo of acetate versus butyrate through the CoA transferase pathway has not yet been determined, because both species may be simultaneously produced or taken up by two other pathways (55), namely the acetate kinase-phosphotransacetylase and butyrate kinase-phosphotransbutyrylase pathways, respectively. During solventogenesis in batch fermentations, the level of butyrate often drops much more than that of acetate, suggesting that some regulatory mechanism may cause CoA transferase to convert butyrate in preference to acetate (1,28,55). The in vitro studies reported here show that this CoA transferase converts acetate at a higher rate than butyrate when both substrates are present together at equal levels; however, the relative rate of conversion in vivo should be sensitive to the relative concentrations of acetate and butyrate, and to the levels of CoASH, acetyl CoA, and butyryl CoA, which tend to inhibit acetate conversion more than butyrate conversion. The intracellular level of butyrate is often higher than acetate in vivo (25,34,35). Nevertheless,
the in vitro results suggest that butyrate is probably not usually converted by CoA transferase in preference to acetate. Therefore, the greater decrease in butyrate concentration described above probably does not result solely from the CoA transferase reaction; at least one of the other two pathways mentioned above is probably also involved.

The intracellular pH in C. acetobutylicum has been shown to decrease from pH 7.0 to as low as 5.5 in a batch fermentation (33), and this CoA transferase apparently has fairly high activity throughout this range. The decrease in activity at the lower end of this range was not expected, because low internal pH is generally the result of high levels of acetate and butyrate. It seems unlikely that this characteristic of the enzyme is beneficial to the metabolism of the organism. This may be of little consequence, or it may partly explain the observation that, when internal pH falls below 5.5, the organism is unable to initiate solvent formation and dies (25).

Substrate specificity was studied, because of the potential application of C. acetobutylicum in novel fermentations. For example, Jewell et al. (37) showed that propionate and valerate, but not isobutyrate, could be taken up by C. acetobutylicum and converted to the respective alcohol. Although this CoA transferase has broad substrate specificity, like other CoA transferases described in the literature, it should be noted that carboxylic acids may also be activated by the butyrate
kinase-phosphotransbutyrylase pathway (77). Both of those enzymes also have broad specificities (26; Section 5.3.4).

The substrate specificity results presented in Table 6-3 point to some inconsistencies between this work and results previously presented by others. In addition to a much broader substrate specificity for this CoA transferase, Hartmanis et al. (28) reported that this enzyme is activated by the potassium salts of carboxylic acids and shows no activity with the sodium salts. We found no difference in activity between equimolar sodium Acetylpyrate and potassium butyrate.

The results presented by Hartmanis et al., as well as those presented by Andersch et al. for C. acetobutylicum DSM 1732 (1), and by Yan et al. for C. beijerinckii (83) are based on work with crude extracts that did not contain ammonium sulfate or glycerol to stabilize activity. We found little or no activity in such extracts, although some activity is recovered if ammonium sulfate and glycerol are added after the crude extract is prepared. Even though Andersch et al. reported specific activities with acetate of up to 1.47 U/mg (1) that are comparable to the highest activities we have observed, we calculated from sample spectrophotometric data which they include in their paper that their specific activities are more probably on the order of about 0.01 U/mg. Furthermore, if only low levels of CoA transferase are present, measurement is complicated, because thiolase together with enzymatic and non-enzymatic hydrolysis of acetoacetyl CoA create a relatively high background that cannot
be accurately accounted for. Consequently, experiments performed on CoA transferase in crude extracts of butanol-forming clostridia that has not been stabilized with salt and glycerol are liable to give artifactual results.

Evidence that this has been the case can be seen when studying the profiles of CoA transferase and acetoacetate decarboxylase in solvent-producing batch fermentations. Andersch et al. (1) and Yan et al. (83) both found that the increase in CoA transferase activity was relatively small and/or gradual in contrast to the abrupt and significant induction of the acetoacetate decarboxylase activity. CoA transferase and acetoacetate decarboxylase form a two-enzyme pathway in many butanol-forming clostridia that might be expected to be coordinately induced. When CoA transferase activity was stabilized with salt and glycerol, it was found that CoA transferase showed an induction pattern that was very similar to the induction of acetoacetate decarboxylase (M.H.W. Huesemann and E.T. Papoutsakis, personal communication).

6.5 Summary

The following inferences are made regarding regulation of CoA transferase in _C. acetobutylicum_: (a) The _K_m_ values for acetate and butyrate are high; therefore, in *vivo* activity should be sensitive to the levels of those acids. (b) Acetate and butyrate are activated to the respective acyl CoA compound simultaneously, and the relative rate of activation depends upon
the relative concentrations of these acids, and possibly upon the levels of acyl CoA's and CoASH. (c) The amount of active enzyme increases sharply at about the same time that initiation of solventogenesis occurs, in the same way shown for other enzymes of the solvent pathways; thus, regulation at the level of induction of enzyme synthesis is very important. (d) Physiologic concentrations of butanol and acetone inhibit the enzyme, and accumulation of these products during fermentation may act as a feedback control on the enzyme.
Table 6-1. Purification of CoA transferase from *C. acetobutylicum*.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Purif. (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>3710</td>
<td>1320</td>
<td>0.36</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>855</td>
<td>720</td>
<td>0.84</td>
<td>2.3</td>
<td>55</td>
</tr>
<tr>
<td>fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octyl Sepharose columns</td>
<td>55.4</td>
<td>322</td>
<td>5.8</td>
<td>16.1</td>
<td>24</td>
</tr>
<tr>
<td>Phenyl Sepharose column</td>
<td>33.7</td>
<td>229</td>
<td>6.8</td>
<td>18.9</td>
<td>17</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>9.8</td>
<td>171</td>
<td>17.4</td>
<td>48.3</td>
<td>13</td>
</tr>
<tr>
<td>PolyPROPYL A column</td>
<td>3.4</td>
<td>99</td>
<td>29.1</td>
<td>80.8</td>
<td>7.5</td>
</tr>
</tbody>
</table>
Table 6-2. Kinetic constants of activation of acetate, propionate, and butyrate by CoA transferase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (acid)</th>
<th>$K_m$ (AACoA)</th>
<th>$K_i$ (acid)</th>
<th>$K_i$ (AACoA)</th>
<th>$V_{max}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetate</td>
<td>1200 mM</td>
<td>0.021 mM</td>
<td>----</td>
<td>----</td>
<td>100</td>
</tr>
<tr>
<td>propionate</td>
<td>1000 mM</td>
<td>0.007 mM</td>
<td>----</td>
<td>----</td>
<td>32</td>
</tr>
<tr>
<td>butyrate</td>
<td>660 mM</td>
<td>0.056 mM</td>
<td>410 mM</td>
<td>0.21 mM</td>
<td>13</td>
</tr>
</tbody>
</table>

AACoA = acetoacetyl CoA
Table 6-3. Substrate specificity of CoA transferase with respect to 100 mM of various carboxylic acids.

<table>
<thead>
<tr>
<th>Carboxylic Acid</th>
<th>Relative Rate I&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relative Rate II&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>formate</td>
<td>&lt; 1</td>
<td>87</td>
</tr>
<tr>
<td>acetate</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>propionate</td>
<td>48</td>
<td>89</td>
</tr>
<tr>
<td>n-butyrate</td>
<td>26</td>
<td>85</td>
</tr>
<tr>
<td>n-valerate</td>
<td>2.7</td>
<td>73</td>
</tr>
<tr>
<td>n-caproate</td>
<td>&lt; 1</td>
<td>56</td>
</tr>
<tr>
<td>isobutyrate</td>
<td>2.5</td>
<td>77</td>
</tr>
<tr>
<td>isovalerate</td>
<td>&lt; 1</td>
<td>57</td>
</tr>
<tr>
<td>isocaproate</td>
<td>&lt; 1</td>
<td>48</td>
</tr>
<tr>
<td>(DL) 3-methyl-n-valerate</td>
<td>&lt; 1</td>
<td>--</td>
</tr>
<tr>
<td>crotonate</td>
<td>3.9</td>
<td>61</td>
</tr>
</tbody>
</table>

<sup>a</sup> this work (procedure described under Section 6.2.3)

<sup>b</sup> reported by Hartmanis and co-workers (28)
Table 6-4. Acetate and butyrate as alternative substrates.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Substrate Conc. (mM)</th>
<th>Relative Rate of Conversion (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
<td>Butyrate</td>
</tr>
<tr>
<td>A</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>E</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

<sup>a</sup> All rates of conversion are expressed relative to acetate in experiment A.
Table 6-5. *In vitro* effects of metabolites and cofactors on CoA transferase.

<table>
<thead>
<tr>
<th>Metabolite/Cofactor</th>
<th>Assay Conc. (mM)</th>
<th>Relative Activity (%) with Acetate</th>
<th>Relative Activity (%) with Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>----</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Butanol</td>
<td>200</td>
<td>63</td>
<td>53</td>
</tr>
<tr>
<td>Ethanol</td>
<td>200</td>
<td>95</td>
<td>98</td>
</tr>
<tr>
<td>Acetone</td>
<td>200</td>
<td>70</td>
<td>71</td>
</tr>
<tr>
<td>1-Propanol&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200</td>
<td>81</td>
<td>78</td>
</tr>
<tr>
<td>2-Butanone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200</td>
<td>48</td>
<td>50</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>20</td>
<td>61</td>
<td>59</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>20</td>
<td>61</td>
<td>64</td>
</tr>
<tr>
<td>ATP</td>
<td>5</td>
<td>92</td>
<td>99</td>
</tr>
<tr>
<td>ADP</td>
<td>5</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>NAD</td>
<td>5</td>
<td>91</td>
<td>89</td>
</tr>
<tr>
<td>CoASH</td>
<td>0.9</td>
<td>75</td>
<td>94</td>
</tr>
<tr>
<td>Acetyl CoA</td>
<td>1.0</td>
<td>73</td>
<td>80</td>
</tr>
<tr>
<td>Butyryl CoA</td>
<td>1.0</td>
<td>62</td>
<td>77</td>
</tr>
</tbody>
</table>

<sup>a</sup> not a metabolite of this fermentation
Figure 6-1. CoA transferase from C. acetobutylicum and molecular weight standards on a denaturing gel after electrophoresis. Left three lanes: purified CoA transferase from three different fractions within the activity peak from the last step of purification. Right lane: molecular weight standards (molecular weight is given in parentheses and is marked beside the pertinent band in thousands) are bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100) and α-lactalbumin (14,200).
Figure 6-2. Activation of butyrate to butyryl CoA. (A) Acetoacetyl CoA was varied with the following fixed concentrations of butyrate: ◻, 33.3 mM; ×, 42.9 mM; +, 60 mM; Δ, 100 mM; and □, 300 mM. (B) Butyrate was varied with the following fixed concentrations of acetoacetyl CoA: O, 3.9 μM; ◻, 5.0 μM; ×, 7.0 μM; +, 11.7 μM; Δ, 35 μM; and □, 100 μM. The dashed lines correspond to 300 mM butyrate in A and 100 μM acetoacetyl CoA in B.
Figure 6-3. pH dependence of activation of acetate to acetyl CoA by CoA transferase from C. acetobutylicum.
CHAPTER 7

CONCLUDING REMARKS

7.1 Use of the Three Purified Enzymes in Genetic Studies

Samples of purified PTB and CoA transferase prepared in this work are being used at Rice University in the laboratory of Dr. George Bennett for genetic studies.

Pure PTB was used to show that C. acetobutylicum PTB cloned into and expressed in E. coli has the same subunit molecular weight as PTB prepared directly from C. acetobutylicum, indicating that the complete PTB gene, not just a fragment, was cloned (8). Incidentally, the transformed E. coli were selected for their ability to grow on butyrate, requiring that the butyrate kinase gene be simultaneously cloned and expressed. It was found that the PTB and BK genes are in close proximity and may represent an operon. Currently, attempts are being made to determine the N-terminal amino acid sequence of the purified PTB. The position on the cloned DNA fragment at which coding for PTB begins is only approximately known. Given the nucleotide sequence in this region and the N-terminal amino acid sequence, the exact location at which coding begins can be identified. This will in turn identify the nucleotide sequence of the region which influences transcription and translation. Unfortunately, it has not yet been possible to determine the N-terminal amino acid sequence for PTB, probably because it is blocked (J. W. Cary, personal communication).
Attempts to clone the CoA transferase gene into *E. coli* by selecting for transformants that can grow on acetoacetate have not been successful. Since this enzyme apparently consists of two different subunits, it was expected that a different gene exists for each subunit. If only one of the two genes was cloned into *E. coli*, there would be no active enzyme; however, the presence of the gene could be detected with a suitable probe. Such probes were constructed by determining the N-terminal amino acid sequence for each subunit, then using the sequences to design radiolabelled oligonucleotides that hybridize with the cloned genes. These probes were used to show that the two subunits were successfully cloned into a phage, and the expression of those genes was verified using the Western blotting technique. Polyclonal anti-CoA transferase was obtained from white sheep immunized with CoA transferase. The cloning and expression of the two CoA transferase genes into *E. coli* was subsequently accomplished (J. W. Cary, personal communication).

No attempts have yet been made to clone the thiolase gene. It probably would not be difficult to insert the thiolase gene into an *E. coli* strain that is deficient in thiolase. Such a strain is available. If this were pursued, the purified thiolase already available could augment the study of the gene in the same way as described above for PTB.
7.2 Recommendations

There remains a great deal to be learned about regulation of product formation in the acetone-butanol fermentation. There is an obvious need for more knowledge of the genetics and enzymology; this knowledge has been and continues to be pursued in a number laboratories.

A need that perhaps is not as well-appreciated is for data on the internal concentrations of cofactors and intermediate metabolites in butanol-forming clostridia. In this work, it has been noted that such data is needed to determine whether a cofactor or metabolite might be present at sufficient levels to significantly activate or inhibit an enzyme reaction. Some data on internal concentrations of NAD, NADH, ADP, and ATP was recently reported (C. L. Meyer, Ph.D. thesis, Rice University, 1987). Nevertheless, there is extremely little if any data in the literature on internal concentrations in butanol-forming clostridia of CoASH, acyl CoA compounds, acetyl and butyryl phosphate, NADP, NADPH, acetoacetate, acetaldehyde and butyraldehyde. Knowledge of the internal concentrations of intermediates of the glycolytic pathway is also desirable.

Methods for preparing extracts without destruction of the desired metabolite or cofactor, and for assaying the compound of interest, appear to be available for most of the cofactors and metabolites of interest (5). Some exceptions might be butyryl CoA, butyryl phosphate, and butyraldehyde; it might be necessary to develop assays specific for these compounds. The
internal concentrations of some intermediates and cofactors can be determined by methods such as NMR which do not require the destruction of the cell sample.

With detailed knowledge of internal concentrations, it should be possible to identify steps in the metabolic pathway that are most likely to be rate-limiting. Such steps are usually relatively far from equilibrium. Rate-limiting enzymes may also be identified using the cross-over theorem which states that "following a perturbation of a metabolic steady state the variations in the concentrations of the metabolites before and after a controlling enzyme have different signs" (54).

Enzyme assays are typically performed with saturating levels of substrate(s), with a pH outside the physiologic range, and without products or other metabolites and cofactors that alter the activity in vivo. Thus it is hardly surprising that in vitro enzyme activities often greatly exceed the in vivo activity (27). It would be interesting to measure enzyme activity in vitro under conditions which match in vivo conditions of pH and levels of substrates, products, and other metabolites and cofactors that were previously shown to be important for the regulation of the enzyme. The results of this type of assay could then be compared with the in vivo enzyme activity, and thereby give an indication as to whether the most important factors in the regulation of the activity have really been identified. A close correlation of this sort of in vitro activity with in vivo activity over a variety of conditions would suggest
that the most important factors for enzyme-level regulation have been identified.

7.3 Impact of this Research on Commercial Development of the Acetone-Butanol Fermentation.

The ultimate goal of studying the regulation of this mixed-product fermentation is to make the acetone-butanol fermentation economically attractive by improving the productivity and selectivity for the most desirable product(s). This could be accomplished by a combination of manipulating the environment and the genetic constituents of the microorganism.

Before productivity can be enhanced, one or more rate-limiting enzymes should be identified. Then the pathway may be "debottlenecks" by increasing the activity of those controlling enzymes. The fermentation conditions might be optimized to maximize the activity of those controlling enzymes; or the expression of those enzymes amplified by introducing the relevant genes on a plasmid or manipulating the mechanisms of induction or derepression. Unfortunately, as was pointed out in section 7.2, there has not yet been a systematic attempt to identify the rate-limiting enzymes of this fermentation.

Selectivity for the most desirable product(s) could be improved by selectively inhibiting or blocking enzymes involved in the formation of undesirable products. Since direct addition of enzyme inhibitors could be prohibitively expensive and also adversely affect desirable reactions, the best approach might be
to use site-specific mutagenesis to block the synthesis of the first enzyme of the branch leading to formation of the undesirable product. Site-specific mutagenesis requires the identification of the gene coding for the enzyme to be blocked. Blocking formation of undesirable product at the first enzymatic step in its synthesis prevents the accumulation of undesirable intermediates.

In light of this general framework for improving the fermentation, the results presented here regarding purification and characterization of three branch-point enzymes of the acetone-butanol fermentation are admittedly only a modest contribution. Perhaps the chief value of the results presented in this study lies in increased understanding of some factors that govern the formation of acids and their subsequent uptake, as opposed to any immediate practical applications. Nevertheless, some speculation on practical applications is of interest.

The recent successful cloning of the CoA transferase gene, achieved as a result of the purification of the enzyme described here, opens the door to the site-specific mutagenesis of that gene. That would block the formation of acetone, and thereby improve selectivity for the more desirable product, butanol. On the other hand, if improved CoA transferase activity is desirable, synthesis of CoA transferase could be enhanced by reintroducing the gene on a high-expression plasmid. This not only would be of interest for the synthesis of acetone, but also for
the synthesis of isopropanol, which is obtained by enzymatic hydrogenation of acetone.

The striking, high \( K_m \) values with respect to acetate and butyrate for this CoA transferase may spur research into the structure of that enzyme. Such a contribution to our understanding of how structure determines functional properties of enzymes may guide the engineering of enzymes to enhance their commercial value.

The interesting pH dependence of PTB only confirms what was previously observed \textit{in vivo}: that conditions of low pH favor formation of butanol over butyrate. However, as in the case of CoA transferase, manipulation of the gene could result in a microorganism with useful fermentative properties. For example, site-specific mutagenesis of the PTB gene together with amplification of the two genes of the butanol pathway might permit good butanol formation at neutral pH. Butanol formation at neutral pH might improve the yield, because without maintenance of a large pH gradient in the face of a falling external pH, the demand for ATP would be less. By taking the opposite approach of amplifying both the PTB and butyrate kinase genes and driving that pathway in the direction of acid uptake, the recombinant microorganism might convert novel carboxylic acids to their respective alcohols.

Since the genes coding for enzymes of both acid-formation and solvent-formation have now been identified, perhaps it will be possible to identify the factors that cause the latter group
of genes to be inducible when the former group is apparently constitutive. This in turn might permit the conversion of the solvent-formation genes to constitutive genes, eliminating the need to introduce the genes on plasmids and the corresponding problems of plasmid stability.

Finally, the acetone-butanol fermentation provides a model for mixed-product fermentations in general; advances in this fermentation should suggest a strategy for improving this class of fermentations in general.
REFERENCES


APPENDIX A

AMINO ACID COMPOSITION OF THIOLASE AND PTB
Table A-1. Amino acid composition of thiolase from *C. acetobutylicum* and *C. kluyveri*.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th><em>C. acetobutylicum</em></th>
<th><em>C. kluyveri</em>(28)</th>
</tr>
</thead>
<tbody>
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**MW (subunit)** 39,500 38,000

ND  not determined
Table A-2. Amino acid composition of PTB from *C. acetobutylicum*.

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<td>Cys</td>
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</table>

MW (subunit) 31,000

ND = not determined
APPENDIX B

DETAILED TREATMENT OF KINETIC DATA

B.1 Kinetic Studies of Thiolase

B.1.1 Thiolysis Reaction

Data for the thiolysis reaction are shown on double-reciprocal plots in Figure B-1. The data are best fitted by a family of parallel lines, which is consistent with a Ping Pong Bi Bi kinetic binding mechanism. The data were fitted by linear regression, and the fitted lines were used to construct replots of the intercepts and slopes, shown in Figure B-2 and B-3, respectively. The replotted intercepts were then also fitted by linear regression. The lines shown in the slope replots represent an average slope instead of a linear regression. The lines appearing in Figure B-1 are not the lines determined by the initial linear regression; rather the slopes and intercepts of these lines were back-calculated from the slope and intercept replots.

$K_m$ values for acetoacetyl CoA and CoASH were calculated from the intercept and slope replots as described in Section 3.4.; however, since the kinetic mechanism appears to be ping pong, the slope is assumed to be constant, and therefore $K_A$ equals 0. Each of the two double-reciprocal plots in Figure B-1 yields an estimate for $V_{max}$, $K_m$(acetoacetyl CoA), and $K_m$(CoASH). The average values are 0.0106 µmole/min, 35 µM and 4.1 µM,
respectively. The percent differences for each pair of estimates were 15, 23 and 7, respectively. Of the 25 points shown in each plot in Figure B-1, three points were omitted from the calculation of kinetic constants. Those three points indicate some substrate inhibition effect and correspond to [CoA] = 17.5 μM and [acetoacetyl CoA] = 5.1 μM, 6.4 μM and 8.5 μM.

B.1.2 Condensation Reaction

In section 4.3.2, it was reported that the product inhibition data shown in Figure 4-2 are consistent with a Ping Pong Bi Bi kinetic binding mechanism. A more detailed proof is given here.

The rate equation for the Ping Pong Bi Bi mechanism in the presence of product P is as follows (57):

\[
\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_A}{V_{max}[A]} + \frac{K_B}{V_{max}[B]} \left[ 1 + \frac{[P]}{K_{IP}} \right] + \frac{K'_A K_B [P]}{V_{max} K_{IP} [A] [B]}
\]

(B-1)

where \([P]\) = concentration of product P, \(K_{IP}\) = product inhibition constant, and the other symbols are defined in Section 3.4. Since both substrate molecules are identical (acetyl CoA), let \([A] = [B]\) and \(K_A = K_B\):

\[
\frac{1}{v} = \frac{1}{V_1} + \frac{K_A}{V_{max}[A]} \left[ 2 + \frac{[P]}{K_{IP}} \right] + \frac{K'_A K_A [P]}{V_{max} K_{IP} [A]^2}
\]

(B-2)
Essentially the same equation is derived for the presence of the other product Q. Equation B-2 predicts that, on a double-reciprocal plot (1/v versus 1/[A]), the y-intercept will be the same for all values of [P], and when [P] \neq 0, the data should be fitted with a parabolic curve. Thus, the data in Figure 4-2, where [CoASH] \neq 0, are qualitatively consistent with this equation. No attempt was made to estimate $K_A'$ or $K_{1P}$ by fitting the data to this equation.

In the absence of P, the preceding equation reduces to:

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{2K_A}{V_{\text{max}}[A]}$$  \hspace{1cm} (B-3)

This equation predicts that, when [CoASH] = 0, data will be linear on a double-reciprocal plot, which again agrees with the data in Figure 4-2. In the case of other two-substrate mechanisms in which the two substrate molecules are identical and [P] = 0, the rate equations retain a term that has [A]$^2$ in the denominator; thus, those equations predict that such data will be parabolic on a double-reciprocal plot. This can be derived from the rate equations for two-substrate systems as given by Rudolph (59). Equation B-3 is identical to the Michaelis-Menten equation for a one-substrate reaction except that equation B-3 contains a factor of 2 in the numerator of the last term. The
$K_m$ value of acetyl CoA reported in Table 4-4 was calculated from the Michaelis-Menten equation.

B.2 Kinetic Studies of PTB

B.2.1 Formation of Butyryl Phosphate

Kinetic data for the formation of butyryl phosphate by PTB are shown on double-reciprocal plots in Figure B-4. The data can be fitted by a family of lines which intersect at a single point left of the y-axis. The data were fitted by linear regression, and the fitted lines were used to construct replots of the intercepts and slopes, shown in Figure B-5 and B-6, respectively. The lines shown in Figure B-4 were constructed on the basis of back-calculations from the replots.

The replots were used to calculate $V_{max}$, $K_m$(butyryl CoA), $K_m$(phosphate) and $K_A^'$, as described in Section 3.4. The average values are $0.011$ mM/min, $0.11$ mM, $14$ mM and $0.22$ mM. The percent differences for the pairs of estimates are $9$, $26$, $19$ and $21$, respectively. Some of the data corresponding to $[\text{butyryl CoA}] = 0.067$ mM were not consistent with the bulk of the data, and therefore data for this concentration were not included in the replots in Figures B-5B and B-6B.

The slight nonlinearity of the $1/[\text{phosphate}]$ plots which was shown in Figure 5-2 is not significant in the absence of the product butyryl phosphate. The nonlinearity was ignored for the purpose of analyzing the data in Figure B-4B.
B.2.2 Formation of Butyryl CoA

Kinetic data for the formation of butyryl CoA by PTB are shown on double-reciprocals plots in Figure B-7. The data can be fitted by a family of lines which intersect at a single point left of the y-axis, similar to the data in Figure B-4. Thus, the data in Figures B-4 and B-7 are consistent with both a Random Bi Bi and an Ordered Bi Bi kinetic binding mechanism. The data were fitted by linear regression, and the fitted lines were used to construct replots of intercepts and slopes, shown in Figures B-8 and B-9, respectively. The lines shown in Figure B-7 were constructed on the basis of back-calculations from the intercept replots.

$V_{max}$, $K_m$(butyryl phosphate), $K_m$(CoASH) and $K_A$ were calculated from the replots as described in Section 3.4. The average values are 0.017 mM/min, 0.26 mM, 0.077 mM and 0.54 mM, respectively. The percent differences for the pairs of estimates are < 1, < 1, 4 and 2, respectively.

B.2.3 Product Inhibition Studies

In Section 5.3.2 it was said that product inhibition studies were carried out to distinguish the kinetic binding mechanism. Results of these studies are presented in Figure B-10 for inhibition by phosphate, in Figure B-11 for butyryl CoA, and in Figure B-12 for butyryl phosphate. Inhibition by CoASH was not studied; the assay used to study formation of butyryl phosphate contains DTNB which immediately reacts with
CoASH to form a colored complex. On double-reciprocal plots, the intersection of fitted lines to the left of the y-axis indicates noncompetitive inhibition, whereas intersection of lines on the y-axis indicates competitive inhibition. Note that Figure B-12B contains the same data shown in Figure 5-2.

The product inhibition constants presented in Table 5-2 were determined from the data in Figures B-10 to B-12 together with the following equation:

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} \left[ 1 + \frac{[I]}{K_{ii}} \right] + \frac{K_m}{V_{\text{max}}[S]} \left[ 1 + \frac{[I]}{K_{is}} \right] \quad (B-4)
\]

where \([I]\) = concentration of inhibitor, \(K_{ii}\) = intercept inhibition constant, \(K_{is}\) = slope inhibition constant, and \([S]\) = substrate concentration. This equation treats the reaction as a one-substrate reaction; \(K_m\) and \([S]\) are based on the substrate which is present in variable concentration, and the other substrate which is present at fixed concentration does not enter into equation B-4 directly. In the case of competitive inhibition, the intercept is not affected and the term containing \(K_{ii}\) is omitted.

B.3 Kinetic Studies of CoA Transferase

B.3.1 Double Substrate Inhibition with Butyrate

In Section 6.3.2 the kinetic data for butyrate presented in Figure 6-2 were said to be consistent with a Ping Pong Bi Bi
kinetic binding mechanism with double substrate inhibition. The rate equation that applies to this kinetic mechanism is (19):

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_A}{V_{\text{max}}[A]} \left[ 1 + \frac{[B]}{K_{iB}} \right] + \frac{K_B}{V_{\text{max}}[B]} \left[ 1 + \frac{[A]}{K_{iA}} \right] \tag{B-5}
\]

The five kinetic constants in this equation, namely \( V_{\text{max}} \), \( K_A \), \( K_B \), \( K_{iA} \) and \( K_{iB} \), could be estimated by fitting the data to this equation by the method of least squares; however, that approach proved unsatisfactory, because of limitations in the amount and quality of data. Instead, the equation was simplified by assuming that the term \([A]/K_{iA}\) is negligible. Equation B-5 then reduces to:

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_B}{V_{\text{max}}[B]} + \frac{K_A}{V_{\text{max}}} \left[ 1 + \frac{[B]}{K_{iB}} \right] \frac{1}{[A]} \tag{B-6}
\]

Therefore, at fixed \([B]\), a plot of \(1/v\) versus \(1/[A]\) should be linear for sufficiently small \([A]\). Also, replots of the slopes and intercepts should be consistent with the following equations:

\[
\text{intercept} = \frac{1}{V_{\text{max}}} + \frac{K_B}{V_{\text{max}}[B]} \tag{B-7}
\]

\[
\text{slope} = \frac{K_A}{V_{\text{max}}} \left[ 1 + \frac{[B]}{K_{iB}} \right] \tag{B-8}
\]
Equation B-8 shows that a slope replot should be linear with respect to [B], whereas the general bisubstrate rate equation given in Section 3.4 shows that the slope replot will be linear with respect to 1/[B] in the absence of substrate inhibition. The intercept replot should be linear with respect to 1/[B] in either case. Equations analogous to equations B-6 to B-8 can be derived from the assumption that [B]/K_{1B} is negligible.

Figure B-13 shows that the intercept replots are in fact linear with respect to reciprocal substrate concentration, and these plots were used to estimate $V_{\text{max}}$, $K_m$(butyrate) and $K_m$(acetoacetyl CoA). Figure B-14 shows that the slope replots are not truly linear with respect to substrate concentration, although the plots do approach linearity with increasing butyrate concentration and with increasing acetoacetyl CoA concentration in Figure B-14A and B-14B, respectively. The discrepancy between the behaviour predicted in equation B-8 and the behaviour observed in Figure B-14 likely results from the assumption that [A]/K_{1A} is negligible. Consequently, the plots in Figure B-14 are of somewhat limited value, but they were used to estimate $K_i$ values for butyrate and acetoacetyl CoA. Lines shown in Figure B-14 were fitted to the right two points in each plot, and were used to estimate $K_i$ values.
B.3.2 CoA Transferase Reaction with Propionate and with Acetate

It was stated in Section 6.3.2 that double-reciprocal plots of kinetic data using propionate and acetate do not show significant substrate inhibition (see Figures B-15 and B-18). Instead, the kinetic behaviour appears more consistent with the same Ping Pong Bi Bi equation described in the discussion of thiolase. The absence of significant substrate inhibition with propionate and acetate is not surprising. Substrate inhibition generally becomes apparent only under conditions of high substrate concentrations relative to the $K_m$ values. Even in the case of butyrate, substrate inhibition would not have been apparent if the initial-rate measurements at the highest concentrations of acetoacetyl CoA and butyrate had been omitted.

Calculation of $K_m$ values in the case of propionate and acetate was performed in essentially the same way as thiolase, as described in Section 3.4 and Appendix B.1.1. Replots for the propionate data are shown in Figures B-16 and B-17, and for the acetate data in Figures B-19 and B-20. Accurate calculation of $V_{\text{max}}$ is made difficult because the intercepts of the intercept replots (Figures B-16 and B-19) are very close to the origin. Consequently, a small change in these intercepts results in a large change in the estimated values of $V_{\text{max}}$, which in turn results in a large change in the estimated $K_m$ values. This problem might have been circumvented by using much higher concentrations of propionate and acetate, but the essential
conclusion remains unchanged: the $K_m$ values for these two acids are strikingly high.

The $K_m$ values of 1000 mM and 1200 mM reported in Table 6-2 for propionate and butyrate, respectively, are conservatively low estimates determined by using personal judgement to fit lines through the data. The data were also analyzed using linear regression, and the plots shown in Figures B-15 to B-20 are based on linear regressions.

In the case of propionate, analysis using linear regression yielded average $K_m$ values of 1760 mM and 0.015 mM for propionate and acetoacetyl CoA, respectively, and the percent difference for each pair of estimates (based on each series of replots) was $< 1$ and 3, respectively. The average $V_{max}$ was 0.18 μmole/min with a percent difference less than 1. No data in Figure B-15 were excluded from this analysis.

In the case of acetate, linear regression yielded average $K_m$ values of 3600 mM and 0.038 mM for acetate and acetoacetyl CoA, respectively, with percent differences of 7 and 2, respectively. The average $V_{max}$ was 0.28 μmole/min with a percent difference of 3. The two points corresponding to [acetate] = 300 mM and [acetoacetyl CoA] = 2.8 μM and 3.6 μM were omitted from the linear regression in Figure B-18A, and intercept points corresponding to [acetoacetyl CoA] = 2.8 μM and 3.6 μM were omitted from the linear regression in Figure B-19B. Without those deletions, the estimated $K_m$ values were either much higher or undefined (negative $V_{max}$).
Figure B-1. Double-reciprocal plots showing kinetic data for the thiolysis reaction. (A) CoASH was varied with the following fixed concentrations of acetoacetyl CoA: □, 25.6 µM; Δ, 12.8 µM; +, 3.5 µM; X, 6.4 µM; and O, 5.1 µM. (B) Acetoacetyl CoA was varied with these fixed concentrations of CoASH: □, 17.5 µM; Δ, 8.8 µM; +, 5.8 µM; X, 4.4 µM; and O, 3.5 µM.
Figure B-2. Thiolytic intercept replots based on (A) the double-reciprocal plot in Figure B-1A, and (B) the plot in Figure B-1B.
Figure B-3. Thiolyis slope repots based on (A) the double-reciprocal plot in Figure B-1A, and (B) the plot in Figure B-1B.
Figure B-4. Double-reciprocal plots showing kinetic data for the PTB reaction in the direction of butyryl phosphate formation. (A) Butyryl CoA was varied with the following fixed concentrations of phosphate: Δ, 45 mM; X, 15 mM; ◇, 9.0 mM; :, 6.4 mM; and □, 5.0 mM. (B) Phosphate was varied with the following fixed concentrations of butyryl CoA: Δ, 0.60 mM; X, 0.20 mM; ◇, 0.12 mM; :, 0.086 mM; and □, 0.067 mM.
Figure B-5. PTP (butyryl phosphate formation) intercept plots based on (A) the double-reciprocal plot in Figure B-4A, and (B) the plot in Figure B-4B.
Figure B-6. PTB (butyryl phosphate formation) slope replots based on (A) the double-reciprocal plot in Figure B-4A, and (B) the plot in Figure B-4B.
Figure B-7. Double-reciprocal plots showing kinetic data for the PTB reaction in the direction of butyryl CoA formation. (A) Butyryl phosphate was varied with the following fixed concentrations of CoASH: □, 0.68 mM; +, 0.34 mM; ◊, 0.17 mM; ×, 0.11 mM; and Δ, 0.085 mM. (B) CoASH was varied with the following fixed concentrations of butyryl phosphate: □, 0.81 mM; +, 0.41 mM; ◊, 0.20 mM; ×, 0.135 mM; and Δ, 0.100 mM.
Figure B-8. PTB (butyryl CoA formation) intercept replots based on (A) the double-reciprocal plot in Figure B-7A, and (B) the plot in Figure B-7B.
Figure B-9. PTB (butyryl CoA formation) slope replots based on (A) the double-reciprocal plot in Figure B-7A, and (B) the plot in Figure B-7B.
Figure B-10. Inhibition of PTB (butyryl CoA formation) by phosphate. (A) Butyryl phosphate was varied with the following fixed concentrations of phosphate: □, 0 mM; △, 28 mM; and ◊, 56 mM. (B) CoASH was varied with the following fixed concentrations of phosphate: □, 0 mM; △, 14 mM; and ◊, 28 mM.
Figure B-11. Inhibition of PTB (butyryl CoA formation) by butyryl CoA.
(A) Butyryl phosphate and (B) CoASH were varied with □, no butyryl CoA and △,
0.11 mM butyryl CoA.
Figure B-12. Inhibition of PTB (butyryl phosphate formation) by butyryl phosphate. (A) Butyryl CoA and (B) phosphate were varied with the following fixed concentrations of butyryl phosphate: □, 0 mM; △, 0.26 mM; and ◇, 0.52 mM.
Figure B-13. CoA transferase intercept replots with butyrate based on (A) the double-reciprocal plot in Figure 6-2A, and (B) the plot in Figure 6-2B.
Figure B-14. CoA transferase slope replots with butyrate based on (A) the double-reciprocal plot in Figure 6-2A, and (B) the plot in Figure 6-2B.
Figure B-15. Double-reciprocal plots showing kinetic data for the CoA transferase reaction with propionate. (A) Acetoacetyl CoA was varied with the following fixed concentrations of propionate: □, 300 mM; Δ, 100 mM; and †, 60 mM. (B) Propionate was varied with the following fixed concentrations of acetoacetyl CoA: □, 24.9 μM; Δ, 8.3 μM; †, 5.0 μM; X, 3.6 μM; and ◇, 2.8 μM.
Figure B-16. CoA transferase intercept replots with propionate based on (A) the double-reciprocal plot in Figure B-15A, and (B) the plot in Figure B-15B.
Figure B-17. CoA transferase slope replots with propionate based on (A) the double-reciprocal plot in Figure B-15A, and (B) the plot in Figure B-15B.
Figure B-18. Double-reciprocal plots showing kinetic data for CoA transferase reaction with acetate. (A) Acetoacetyl CoA was varied with the following fixed concentrations of acetate: □, 300 mM; Δ, 100 mM; and +, 60 mM. (B) Acetate was varied with the following fixed concentrations of acetoacetyl CoA: □, 35.0 μM; Δ, 11.7 μM; +, 7.0 μM; ×, 5.0 μM; and ◊, 3.9 μM.
Figure B-19. CoA transferase intercept replot with acetate based on (A) the double-reciprocal plot in Figure B-18A, and (B) the plot in Figure B-18B.
Figure B-20. CoA transferase slope replots with acetate based on (A) the double-reciprocal plot in Figure B-18A, and (B) the plot in Figure B-18B.