Coenzyme A Transferase from Clostridium acetobutylicum ATCC 824 and Its Role in the Uptake of Acids

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Coenzyme A (CoA) transferase from Clostridium acetobutylicum ATCC 824 was purified 81-fold to homogeneity. This enzyme was stable in the presence of 0.5 M ammonium sulfate and 20% (vol/vol) glycerol, whereas activity was rapidly lost in the absence of these stabilizers. The kinetic binding mechanism was Ping Pong Bi Bi, and the $K_m$ values at pH 7.5 and 30°C for acetate, propionate, and butyrate were, respectively, 1,200, 1,000, and 660 μM, while the $K_m$ value for acetoacetyl-CoA ranged from about 7 to 56 μM, depending on the acid substrate. The $K_m$ values for butyrate and acetate were 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 was able to convert valerate, isobutyrate, and crotonate; however, the conversion of formate, $n$-caproate, and isovalerate was not detected. The acetate and butyrate conversion reactions in vitro were inhibited by physiological levels of acetone and butanol, and this may be another factor in the in vivo regulation of enzyme activity. The optimum pH of acetate conversion was broad, with at least 80% of maximal activity from pH 5.9 to greater than 7.8. The purified enzyme was a heterotetramer with subunit molecular weights of about 23,000 and 25,000.

Coenzyme A transferases activate carboxylic acids to the respective CoA thioesters 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 for Clostridium kluyveri (26). 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 (24), Peptostreptococcus elsdenii (28), and Clostridium sp. strain SB4 (3). 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 that transfers CoA from acetoacetyl-CoA to butyrate or to acetate has been found in crude extracts (1, 10, 30). 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 that in other bacteria for 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 (3, 24, 28). 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 (10, 11, 17). This provides acetoacetate for decarboxylation to acetone and provides 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 increases gradually, reaching peak activity during solventogenesis. Hartmanis et al. (10) 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. (30) 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 was measured in combination with acetoacetyl-CoA hydrolase as an indication of total acetoacetate-forming (acetoacetyl-CoA-utilizing) activity. This combined activity showed only a two- to threefold increase at the onset of solventogenesis, in contrast to more dramatic increases in the levels of other enzymes also 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. A better understanding of the regulatory features of CoA transferase and other key metabolic enzymes will help guide the improvement of this fermentation. This paper reports the purification to homogeneity and the physical and kinetic characterization of CoA transferase in C. acetobutylicum.

MATERIALS AND METHODS

Organism and growth conditions. C. acetobutylicum ATCC 824 was grown in a 14-liter fermentor (Microferm; New Brunswick Scientific Co., Inc., Edison, N.J.) sparged with nitrogen and controlled at 37°C and pH 4.5. Maintenance of the organism, including storage, transfer, and heat shocking, has been described previously (20). The medium contained the following (per liter of distilled water): KH$_2$PO$_4$, 0.75 g; K$_2$HPO$_4$, 0.75 g; MgSO$_4$·7H$_2$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; (NH₄)₂SO₄, 2.0 g; antifoam C, 0.2 ml; and glucose, 50 g. Cells were harvested by centrifugation approximately 3 h after the fermentation reached the stationary growth phase and then were stored under nitrogen at ∼20°C.

CoA transferase assay. CoA transferase was routinely assayed at room temperature in the butyrate conversion direction by monitoring the decrease in A₃₄⁵₄ as an indication of the disappearance of the enolate form of acetoacetyl-CoA (24). The assay mixture contained, in a final volume of 1.0 ml, 100 mM Tris hydrochloride (pH 7.5), 100 mM potassium butyrate (pH 7.5), 20 mM MgCl₂, 0.10 mM acetoacetyl-CoA, 5% (vol/vol) glycerol, and less than 0.05 U 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 crude extracts were used. One unit of activity is defined as the amount of enzyme which converts 1 μmol of acetoacetyl-CoA per min under these conditions. The extinction coefficient is 8.0 M⁻¹ cm⁻¹ (27).

Other assays. Protein was assayed by the method of Bradford (4) with bovine serum albumin as the standard.

The concentration of acetoacetyl-CoA used in the standard assay and in kinetic and substrate specificity studies was determined by incubating the acetoacetyl-CoA with neutral hydroxylamine for 1 min to release the reduced form of CoA (CoASH) (7). CoASH was then measured by adding DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] and measuring the total change in A₄₁₂. The extinction coefficient is 13.6 M⁻¹ cm⁻¹ (7). The calculated concentration was corrected for the presence of CoASH in the stock solution of acetoacetyl-CoA by adding DTNB to stock solution that had not been hydrolyzed with neutral hydroxylamine and then measuring the initial change in A₄₁₂.

Purification of CoA transferase. All of the steps described below were carried out at room temperature, and anaerobic conditions were not used; however, between steps the enzyme preparation was stored under nitrogen at 4°C. Buffer A contained 25 mM MOPS (4-morpholinepropanesulfonic acid) (pH 7.0), 0.5 M (NH₄)₂SO₄, and 20% (vol/vol) glycerol. Buffer B contained 25 mM MOPS (pH 7.0) and 15% (vol/vol) glycerol. Buffer C was buffer B with 0.75 M (NH₄)₂SO₄. The results of a typical preparation are presented in Table 1.

Preparation of cell extract. Frozen cells (95 g) were thawed in two volumes of buffer A and then passed once through a French pressure cell (model PA-073; SLM Instruments, Inc., Urbana, Ill.) at 15,000 lb/in². Unlysed cells were removed by centrifugation, resuspended in buffer A to a total volume of 40 ml, and passed through the pressure cell a second time at 15,000 lb/in². The disrupted-cell suspensions were combined and centrifuged for 45 min at 30,000 × g to remove cell debris.

(i) Ammonium sulfate precipitation. Recrystallized ammonium sulfate was added to a final concentration of 1.8 M. After 30 min of stirring, the suspension was centrifuged at 20,000 × g for 15 min and the pellet was discarded. Ammonium sulfate was then added to give the supernatant a final concentration of 2.2 M. After being stirred for 30 min, the suspension was centrifuged at 20,000 × g for 15 min and the supernatant was discarded. Pellets from this second precipitation were resuspended in buffer A to a final volume of about 40 ml.

(ii) Octyl Sepharose CL-4B chromatographies. 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 Fine Chemicals, Piscataway, N.J.) column (2.5 by 63 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 give 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 (2.5 by 14 cm) equilibrated with 1.5 M (NH₄)₂SO₄ in buffer B at 80 ml/h. The column was then washed with equilibration buffer until the A₂₈₀ was less than 0.8, and 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.

(iv) Phenyl Sepharose CL-4B chromatography. The combined sample from the previous step, totalling 90 ml, was applied directly to a Phenyl Sepharose CL-4B (Pharmacia) column (2.5 by 38 cm) equilibrated with buffer C at 110 ml/h. The column was then washed with buffer C until the A₂₈₀ was less than 0.4, and then activity was eluted by a linear gradient from 100% buffer C to 75% (vol/vol) buffer C plus 25% (vol/vol) 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 with type PM10 ultrafiltration membranes in a stirred cell (Amicon, Danvers, Mass.) and then concentrated further to 2.8 ml with filtering cones (Amicon type CF25 ultrafiltration membrane).

(v) Sephacryl S-300 chromatography. The concentrated sample was applied to a Sephacryl S-300 (Pharmacia) column (1.6 by 90 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 with filtering cones (Amicon type CF25 ultrafiltration membranes), and then crystalline ammonium sulfate was added to a final concentration of 1.5 M.

(vi) High-pressure liquid chromatography with hydrophobic-interaction column. A polyPROPYL A (Custom LC, Inc., Houston, Tex.) column (4.6 by 200 mm) was used with a high-pressure liquid chromatography system (Laboratory Control Data, Riviera Beach, Fla.). 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 of equilibration buffer, and 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 was added to a final concentration of 20% (vol/vol).

Determination of native and subunit molecular weights. The native molecular weight was determined by measuring elution volume on a calibrated gel filtration column (2). The column (1.6 by 90 cm) contained Sephacryl S-300 equilibrated with buffer A. Samples of 2.0 ml were applied and then eluted at 15 ml/h with collection of 5-min 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 subunit molecular weight was determined by gel electrophoresis on a 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (18).

Kinetic studies. The following kinetic studies were conducted at 30°C and were initiated by addition of acetocetetyl-CoA. With the exception of studies of optimum pH and relative rate of simultaneous conversion with acetate and butyrate, the standard assay for CoA transferase was used with modifications as noted below. All measurements were done in duplicate.

The conversion of butyrate was studied with potassium butyrate concentrations ranging from 33 to 300 mM and acetocetyl-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 acetocetyl-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 acetocetyl-CoA concentrations ranging from 2.8 to 25 μM.

The substrate specificity was studied at 100 mM concentrations 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 by using 300 mM potassium butyrate or 300 mM potassium acetate. The addition of NAD, ADP, and ATP, and to a lesser extent CoASH, acetocetyl-CoA, and butyryl-CoA, caused a marked reduction of the extinction coefficient of acetocetyl-CoA, probably by chelating the magnesium ions which are added to the assay. The extinction coefficient is 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 (24). The rate of breakage of acyl-CoA bonds was determined by monitoring the decrease in A254. The cuvette contained, in a final volume of 1.0 ml, 15 mM MES (morpholineethanesulfonic acid) buffer, 15 mM MOPS buffer, 100 mM potassium phosphate, 5% (vol/vol) glycerol, 0.1 mM acetocetyl-CoA, 3 U 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 acetocetyl-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 use of acetate and butyrate as alternative substrates was studied by measuring the rate of conversion of acetocetyl-CoA and thus the total rate of formation of acetyl-CoA from butyryl-CoA by monitoring the decrease in A310. Then, under identical assay conditions, only the rate of acetyl-CoA formation was measured, by monitoring the decrease in A325 (24). The rate of butyryl-CoA formation was calculated by subtracting the rate of acetyl-CoA formation from the rate of acetocetyl-CoA formation.

The conversion of acetate was studied with acetocetyl-CoA concentrations ranging from 60 to 300 mM and propionate concentrations ranging from 60 to 300 mM (pH 7.5). 3.1 U of phosphotransacetylase, about 0.02 U of pure CoA transferase. The phosphotransacetylase, which was purified from C. kluyveri (Sigma Chemical Co., St. Louis, Mo.), hydrolyzes acetocetyl-CoA but not butyryl-CoA in the presence of arsenate.

Source of chemicals. The dye-binding protein assay kit was purchased from Bio-Rad Laboratories, Richmond, Calif. All other chemicals were purchased from Sigma Chemical Co., except where noted.

RESULTS

Purification of CoA transferase. The results of the purification of CoA transferase are presented in Table 1. The procedures for each step are described in Materials and Methods. The high concentrations of ammonium sulfate and glycerol added to buffers were necessary to stabilize activity in both 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 preparations to stand at room temperature for at least 1 h. Pure and partially pure enzyme preparations stored for 1 week at 4°C under nitrogen showed less than 5% loss in activity. Cell extracts could be stored at room temperature for up to 4 days without detectable loss in activity.

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

Kinetic studies. The butyrate conversion data as analyzed by double-reciprocal plots were best fitted by a family of lines that are approximately parallel at moderate concentrations of both substrates but that become hyperbolic concave-up at high concentrations of the varied substrate (Fig. 2). At the highest concentration of the fixed substrate, the fitted line intersects the other line to the right of the reciprocal-velocity axis. This behavior, known as double competitive substrate inhibition, is consistent only with a Ping Pong Bi Bi kinetic binding mechanism (5, 6, 23). The inhibitory effect of high concentrations of one substrate is relieved by increasing
the level of the other substrate. Values of $K_m$ and inhibition constants ($K_i$) for both butyrate and acetoacetyl-CoA were calculated from intercept and slope replots by using the procedure described by Segel (23). Those values are presented in Table 2.

On double-reciprocal plots for acetate-conversion and propionate conversion (not shown), 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, acetate, and propionate were calculated from intercept and slope replots (Table 2). The $K_m$ values for the carboxylic acids were remarkably high.

Relative-activity measurements for the acetate conversion reaction in the range of pH 5.4 to 7.8 indicated that relative activity was greater than 90% from at least pH 6.4 to 7.8. The relative activity was 82% at pH 5.9 and 56% at pH 5.4.

Substrate specificity. Table 3 shows the relative activity of CoA transferase with a 100 mM concentration of the potassium salt of various carboxylic acids. The relative activity was highest with acetate, propionate, and butyrate, while the activity with valerate, isobutyrate, and crotonate was significant but low. The activity with the other carboxylic acids tested was below the level of detection.

Studies of acetate and butyrate as alternative substrates. Measurements of the relative rate of conversion of acetate and butyrate when both acids were present confirmed the general trend predicted for a Ping Pong Bi Bi mechanism when two alternative substrates are present (22). Addition of acetate depressed the rate of conversion of butyrate, and addition of butyrate depressed the rate of conversion of acetate. When equimolar acetate and butyrate were present together, the rate of conversion of butyrate relative to acetate was 27% with each substrate at 100 mM 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% with each substrate at 100 mM and 25% at 200 mM. When 100 mM acetate and 200 mM butyrate were present simultaneously, the rates of conversion of acetate and butyrate were 79% and 21%, respectively.

FIG. 1. Sodium dodecyl sulfate-polyacrylamide electrophoresis gel showing CoA transferase and standards. Proteins were stained with Coomassie blue. The left lane contained molecular weight standards (molecular weight is given below in parentheses and is marked beside the pertinent band in thousands) 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). The right three lanes contained purified CoA transferase from three different fractions within the activity peak from the last step of purification.

FIG. 2. Double-reciprocal plots of butyrate conversion data. (A) Butyrate was varied with the following fixed concentrations of acetoacetyl-CoA: ○, 3.9 μM; □, 5.0 μM; ×, 7.0 μM; ⋄, 11.7 μM; Δ, 35 μM; and □, 100 μM. (B) Acetocetyl-CoA was varied with the following fixed concentrations of butyrate: ○, 33.3 μM; ×, 42.9 mM; +, 60 mM; Δ, 100 mM; and □, 300 mM. The dashed lines correspond to 100 μM acetocetyl-CoA (A) and 300 mM butyrate (B).
present together, the rate of conversion of butyrate was 71% of that of acetate.

**Effects of metabolites, cofactors, and inorganic salts on CoA transferase activity.** Table 4 summarizes the in vitro effects of various metabolites and cofactors on activity with acetate and butyrate. Butanol and acetone at 200 mM resulted in significant inhibition; the highest physiological levels of these products are 200 and 100 mM, respectively (17, 19). Significant inhibition by 200 mM 1-propanol and 2-butanol, which are not found in this fermentation, suggests that the CoA transferase is inhibited by solvents in general. Inhibition by acetocetate, acetyl-CoA, and butyryl-CoA was to be expected, because those metabolites are products of acetate and butyrate conversion. Inhibition by pyruvate was comparable to that of acetocetate and may be a result of the somewhat similar structure of these two compounds. Unfortunately, there are 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 tested. Acetate conversion was more sensitive to inhibition by CoASH, acetyl-CoA, and butyryl-CoA than was butyrate conversion, 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 4, the in vitro effects of KCl, NaCl, LiCl, and (NH₄)₂SO₄ on butyrate activation were tested. These inorganic salts caused less than 10% inhibition at 100 mM. Furthermore, there was no difference in activity when sodium butyrate was used in place of potassium butyrate.

**DISCUSSION**

The CoA transferase purified in this study showed only superficial similarities to other bacterial CoA transferases which have been purified and characterized. The size was similar, consisting of two subunits with molecular weights of about 23,000 and 25,000, compared with 23,000 and 26,000 in E. coli (24) and 23,000 and 25,000 in Clostridium sp. strain SB4 (3). Also, as with all other CoA transferases studied, the kinetic binding mechanism was Ping-Pong Bi Bi (15, 23, 28). The metabolic role of CoA transferase in C. acetobutylicum, however, was fundamentally different from that of other CoA transferases which have been characterized, and this was reflected in the much higher $K_m$ values for the carboxylic acid and different specificities. With the C. acetobutylicum CoA transferase, the $K_m$ values for the preferred carboxylic acid substrates butyrate, propionate, and acetate were very high, ranging from 660 to 1,200 mM. In contrast, for CoA transferase in Clostridium sp. strain SB4, the apparent $K_m$ values for eight preferred carboxylic acid substrates, including butyrate, propionate, and acetate, ranged from 0.8 to 29 mM (3). Similarly, with E. coli CoA transferase, a preliminary $K_m$ value for acetate of about 20 mM was reported (25). All of these $K_m$ values were determined with 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.

A major objective in purifying this CoA transferase was to carry out in vitro experiments to investigate 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 (14, 21; L. Meyer, Ph.D. thesis, Rice University, Houston, Tex., 1987) and the pH (8, 12, 13) are known to vary over a significant range under batch fermentation conditions.

The intracellular concentrations of acetate and butyrate do not exceed about 300 and 700 mM, respectively, and are generally much lower than the apparent $K_m$ values of 1,200 and 660 mM, respectively (8, 12, 14). At such subsaturating levels, CoA transferase activity in vivo should be very sensitive to changes in the intracellular acetate and butyrate concentrations. The high $K_m$ values suggest that the enzyme has evolved in such a way that the uptake of acetate and butyrate from the medium 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

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**Table 2. Kinetic constants of conversion of acetate, propionate, and butyrate by CoA transferase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (mmole/min/mg)</th>
<th>Relative activity (% with acetate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>1,200</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Propionate</td>
<td>1,000</td>
<td>48</td>
<td>10</td>
</tr>
<tr>
<td>Butyrate</td>
<td>660</td>
<td>26</td>
<td>10</td>
</tr>
</tbody>
</table>

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**Table 4. In vitro effects of metabolites and cofactors on CoA transferase**

<table>
<thead>
<tr>
<th>Metabolite or cofactor</th>
<th>Assay concn (mM)</th>
<th>Relative activity (% with acetate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Butanol</td>
<td>63</td>
<td>53</td>
</tr>
<tr>
<td>Ethanol</td>
<td>95</td>
<td>98</td>
</tr>
<tr>
<td>Acetone</td>
<td>70</td>
<td>71</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>81</td>
<td>78</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>48</td>
<td>50</td>
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<td>59</td>
</tr>
<tr>
<td>Pyruvate</td>
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<td>64</td>
</tr>
<tr>
<td>ADP</td>
<td>92</td>
<td>97</td>
</tr>
<tr>
<td>NAD</td>
<td>91</td>
<td>89</td>
</tr>
<tr>
<td>CoASH</td>
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<td>94</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>73</td>
<td>80</td>
</tr>
<tr>
<td>Butyryl-CoA</td>
<td>62</td>
<td>77</td>
</tr>
</tbody>
</table>

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**Table 3. Substrate specificity of CoA transferase with respect to various carboxylic acids at 100 mM**

<table>
<thead>
<tr>
<th>Carboxylic acid</th>
<th>Relative rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Acetate</td>
<td>100</td>
</tr>
<tr>
<td>Propionate</td>
<td>48</td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>26</td>
</tr>
<tr>
<td>n-Valerate</td>
<td>2.7</td>
</tr>
<tr>
<td>n-Caproate</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>2.5</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Isocaproate</td>
<td>&lt;1</td>
</tr>
<tr>
<td>(2Z)-3-Methyl-n-valerate</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Crotonate</td>
<td>3.9</td>
</tr>
</tbody>
</table>
possible to use the $K_m$ values reported here to predict the effect of fluctuations in levels of that intermediate on in vivo activity.

Of the metabolites and cofactors tested for their effects on enzyme activity, butanol and acetate are of particular interest, because they caused significant inhibition at physiological levels. Butanol and acetate accumulate in the medium during solvent-producing phase of the fermentation, up to maximum concentrations of about 200 and 100 mM, respectively (19). These nonpolar solvents passively diffuse through the cell membrane so 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.

Of the remaining metabolites that were tested for their effects on acetate and butyrate conversion, either they showed 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 (21), 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, 10, 21). 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 that of acetate in vivo (8, 12, 14). 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 7.0 to as low as 5.5 in a batch fermentation (13), 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, and it may be of little consequence.

Substrate specificity was studied, because of the potential application of C. acetobutylicum in novel fermentation. For example, Jewell et al. (16) showed that propionate and valerate, but not isobutyrate, could be taken up by C. acetobutylicum and converted to the respective acyl-CoA by the butyrate kinase-phosphotransbutyrylase pathway (29). Both of those enzymes also have broad specificities (9, 29a).

The properties of the CoA transferase described here are different from those reported by Hartmanis et al., who studied this enzyme in crude extracts of C. acetobutylicum ATCC 824. Hartmanis et al. reported significantly higher relative activity with propionate, butyrate, n-valerate, isobutyrate, and crotonate, and they also reported significant relative activity with formate, n-caproate, isovalerate, and isocaproate (10). Those workers used the same assay conditions with respect to pH and concentrations of substrates and magnesium ions as we did. The difference is not the result of their use of crude extracts; we measured in a crude extract the relative activity with acetate, propionate, and butyrate and observed the same ratio of activity of about 4:2:1, respectively, that was measured by using pure enzyme. Also, at least in the cases of acetate and butyrate, the presence of ammonium sulfate and glycerol in the assay did not affect the relative rate. Hartmanis et al. reported that the enzyme is activated by potassium salts of carboxylic acids and shows no activity with the sodium salts (10), whereas we found no difference in activity between equimolar sodium butyrate 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 (30), 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 of C. acetobutylicum ATCC 824, although some activity was recovered if ammonium sulfate and glycerol were added after the crude extract was prepared. Even though Andersch et al. reported specific activities with acetate of up to 1.47 U/mg (1), which are comparable to the highest activities we have observed, we calculated from sample spectrophotometric data included 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, in the case of crude extracts of butanol-forming clostridia that have not been stabilized with salt and glycerol, the CoA transferase assay may 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. (30) 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. In many butanol-forming clostridia, CoA transferase and acetoacetate decarboxylase form a two-enzyme pathway that might be expected to be coordinately induced. When CoA transferase activity was stabilized with salt and glycerol, the induction of CoA transferase paralleled that of acetoacetate decarboxylase (unpublished data; M. H. W. Hüsemann, personal communication).

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