Phosphotransbutyrylase from *Clostridium acetobutylicum* ATCC 824 and Its Role in Acidogenesis

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Phosphotransbutyrylase (phosphate butyryltransferase [EC 2.3.1.19]) from *Clostridium acetobutylicum* ATCC 824 was purified approximately 200-fold to homogeneity with a yield of 13%. Steps used in the purification procedure were fractional precipitation with \((NH_4)_2SO_4\), Phenyl Sepharose CL-4B chromatography, DEAE-Sephadex chromatography, high-pressure liquid chromatography with an anion-exchange column, and high-pressure liquid chromatography with a hydrophobic-interaction column. Gel filtration and denaturing gel electrophoresis data were consistent with a native enzyme having eight 31,000-molecular-weight subunits. Within the physiological range of pH 5.5 to 7, the enzyme was very sensitive to pH change in the butyryl phosphate-forming direction and showed virtually no activity below pH 6. This finding indicates that a change in internal pH may be one important factor in the regulation of the enzyme. The enzyme was less sensitive to pH change in the reverse direction. The enzyme could use a number of substrates in addition to butyryl coenzyme A (butyryl-CoA) but had the highest relative activity with butyryl-CoA, isovaleryl-CoA, and valeryl-CoA. The \(K_m\) values at 30°C and pH 8.0 for butyryl-CoA, phosphate, butyryl phosphate, and CoASH (reduced form of CoA) were 0.11, 14, 0.26, and 0.077 mM, respectively. Results of product inhibition studies were consistent with a random Bi Bi binding mechanism in which phosphate binds at more than one site.

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Phosphotransbutyrylase (PTB; phosphate butyryltransferase [EC 2.3.1.19]) carries out the intersubunit conversion of butyryl coenzyme A (butyryl-CoA) and butyryl phosphate: butyryl-CoA + \(\text{PO}_4\) \(\rightleftharpoons\) butyryl phosphate + CoASH, where CoASH denotes the reduced form of CoA. PTB was first reported by Gavard et al. (7) in *Clostridium acetobutylicum*. Those workers partially purified the enzyme and showed that it is an enzyme distinct from phosphotransacytase (PTA), which catalyzes a similar reaction with acetyl-CoA. Valentine and Wolfe (27) partially purified PTB from *Clostridium butyricum* and detected PTB in several other species of clostridia. PTB has not previously been isolated in homogenous form, and there have been no detailed reports of its physical or kinetic properties.

PTB and butyrate kinase 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-butyrate kinase pathway is inactive, producing little or no butyrate, and the available butyryl-CoA is channeled toward 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 (11) observed the almost complete loss of PTB activity coinciding with cessation of butyrate production; however, they also noted an increase in butyrate kinase activity which was contrary to expectations. In contrast, Andersch et al. (1) found that specific activities of PTB and butyrate kinase 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, 15).

In addition to the documented involvement in butyrate synthesis, PTB and butyrate kinase may also have an important role in butyrate uptake in specific metabolic states. This pathway was previously shown to be reversible in vitro (27). Meyer et al. (21) 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 (12). It is likely that butyrate uptake under these conditions was catalyzed by the reversal of the PTB-butyrate kinase pathway, because no other pathways for butyrate uptake have been found in *C. acetobutylicum* (12).

A more complete understanding of PTB is required to better understand the regulation and multiple roles of the PTB-butyrate kinase pathway. Isolation to homogeneity and partial characterization of butyrate kinase have recently been reported (10). As was noted by Rogers (24), understanding the properties of these economically useful biocatalysts is vital for improving or altering butanol fermentation by methods of recombinant DNA technology. This paper reports the isolation to homogeneity and the physical and kinetic characterization of PTB.

**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 6.0. Maintenance of the organism, including storage, transfer, and heat shocking, has been described previously (20). The medium...
contained, per liter of distilled water: KH₂PO₄, 0.75 g; K₂HPO₄, 0.75 g; MgSO₄·7H₂O, 0.4 g; MnSO₄·H₂O, 0.01 g; FeSO₄·7H₂O, 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 when the optical density at 600 nm reached 8.0 and were then frozen in an acetone-dry ice bath and stored at −20°C.

**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 5.5′-dithiobis-(2-nitrobenzoic acid) (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 of PTB. Addition of enzyme initiated the reaction. One unit of activity is defined as the amount of enzyme that converts 1 μmol of butyryl-CoA per minute under these conditions. The extinction coefficient was 13.6 M⁻¹ cm⁻¹ (6).

**Protein assay.** Protein was assayed by the method of Bradford (4). Bovine serum albumin was the standard.

**Other assays.** CoASH in stock solutions of both CoASH and acyl-CoA compounds used in kinetic and substrate specificity studies was determined by adding DTNB and measuring the initial change in A₄₁₂ (8). The extinction coefficient again was 13.6 M⁻¹ cm⁻¹ (1). The assay contained 70 mM potassium phosphate (pH 8.0), 0.08 mM DTNB, and CoASH diluted to give an absorbance change of less than 1.0.

Concentrations of acyl-CoA compounds in stock solutions used in kinetic and substrate specificity studies were determined by incubating the acyl-CoA compound with neutral hydroxylamine for 1 min to release CoASH and then measuring the released CoASH as described above (8). The true acyl-CoA concentration was derived by subtracting the concentration of CoASH present in the stock solution. Stock solutions of CoASH and acyl-CoA could be stored at −20°C and pH 4 for at least 1 week with no detectable loss.

Butyryl phosphate was assayed by the method of Lipmann and Titte (19) modified by Avram (3). Acetohydroxamic acid was the standard. Solutions of butyryl phosphate were assayed before and after kinetic studies and showed no more than a 2% loss.

**Purification of PTB.** All buffers used to isolate PTB contained 2 mM dithiothreitol. Buffer A contained 25 mM Tris hydrochloride (pH 7.6). Buffer B was buffer A plus 2% (vol/vol) glycerol. Buffer C contained variable concentrations of potassium phosphate (pH 7.2), 1 mM EDTA, and 2% (vol/vol) 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.

(i) **Step 1. Preparation of cell extract.** Frozen cells (85 g [wet weight]) were thawed in 2 volumes of buffer A and then passed once through a French pressure cell (model FA-073; SLM Instruments, Inc., Urbana, Ill.) at 11,000 lb/in². The disrupted cell suspension was centrifuged for 70 min at 33,000 × g to remove cell debris. The cell extract was stored at −20°C.

(ii) **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 min, this preparation was centrifuged at 32,000 × g for 30 min, the pellet was discarded, and ammonium sulfate was added to 1.6 M. After 30 min, this preparation was centrifuged at 32,000 × g for 15 min, and the supernatant was discarded. Pellets were suspended in buffer A to a final volume of about 50 ml and then stored at −20°C.

(iii) **Step 3. Phenyl Sepharose CL-4B chromatography.** Enzyme from the previous step was thawed in 1 volume of buffer A containing 1.5 M (NH₄)₂SO₄. The resulting volume of about 100 ml containing 0.75 M (NH₄)₂SO₄ was applied to a Phenyl Sepharose CL-4B (Pharmacia, Inc., Piscataway, N.J.) column (2.5 by 15 cm) equilibrated at room temperature with buffer A containing 0.75 M (NH₄)₂SO₄. The sample was applied at 120 ml/h, and then the column was washed with 425 ml of equilibration buffer at 180 ml/h to remove protein that did not bind. PTB activity was eluted with a linear gradient from 0.75 to 0 M (NH₄)₂SO₄ in a total of 350 ml of buffer A at 180 ml/h. Fractions with high PTB activity (totaling 125 ml) were combined and concentrated essentially to dryness by dialysis against 1 liter of buffer A containing 20% (wt/vol) polyethylene glycol.

(iv) **Step 4. DEAE-Sephalac chromatography.** Dry enzyme from the previous step was dissolved in 25 ml of buffer B and then applied to a DEAE-Sephalac (Pharmacia) column (2.5 by 10.5 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 of 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 of buffer B. PTB activity was eluted with a linear gradient from 0.2 to 0 M (NH₄)₂SO₄ in buffer C over 45 min at 1.0 ml/min. The column was washed with 10 ml of 250 mM potassium phosphate in buffer C between injections. Fractions with high PTB activity (totaling about 24 ml) were combined and concentrated to 1.5 ml by using Centriflo membrane cones (type CF25; Amicon Corp., Danvers, Mass.).

(v) **Step 5. High-pressure liquid chromatography with an anion-exchange column.** A SynChropak AX1000 anion-exchange column (SynChrom, Inc., Linden, Ind.) was used with a high-pressure liquid chromatography system (Laboratory Control Data, Riviera Beach, Fla.). The column (4.6 by 250 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 and 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 min at 1.0 ml/min. The column was washed with 10 ml of 250 mM potassium phosphate in buffer C between injections. Fractions with high PTB activity (totaling about 24 ml) were combined and concentrated to 1.5 ml by using Centriflo membrane cones (type CF25; Amicon Corp., Danvers, Mass.).

(vi) **Step 6. High-pressure liquid chromatography with a hydrophobic-interaction column.** A PolyPROPYL A (Custom LC, Inc., Houston, Tex.) column (4.6 by 200 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 and 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 min at 1.0 ml/min. Fractions with high activity were combined and brought to 20% (vol/vol) glycerol before storage at 4°C.

**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 (1.5 by 90 cm) was packed with Sephacryl S-300 (Pharmacia) equilibrated with 50 mM potassium phosphate (pH 7.0), 100 mM (NH₄)₂SO₄, 5% (vol/vol) glycerol, and 2 mM dithiothreitol. Samples of 2.0 ml were applied and then eluted with buffer at 24 ml/min and with 1.6-ml fractions. The following standards were used to calibrate the column (molecular weights in parentheses): carbonic anhy-
drase (29,000), serum albumin (66,000), alcohol dehydrogenase (150,000), β-amylase (200,000), apoferritin (443,000), and thyroglobulin (669,000). Subunit molecular weight was determined by gel electrophoresis on a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate (18).

**Kinetic studies.** All kinetic studies were conducted at 30°C and were initiated by addition of 10 to 20 μl of 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 (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 8.0, variable butyryl-CoA (from 0.067 to 0.60 mM), variable potassium phosphate at pH 8.0 (from 5.0 to 45 mM), 0.08 mM DTNB, and 0.008 U 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 (from 0.12 to 0.61 mM), variable potassium phosphate at pH 8.0 (from 29 to 200 mM), 0.08 mM DTNB, and 0.6 U of PTB.

Studies in the butyryl-CoA-forming direction were performed by measuring the increase in A232 nm. A cuvette contained 120 mM Tris hydrochloride (pH 8.0), variable CoASH (from 0.065 to 0.68 mM), variable butyryl phosphate at pH 8.0 (from 0.10 to 0.81 mM), and 0.004 U of PTB. The extinction coefficient was 4.5 M⁻¹ cm⁻¹, which represents the difference between the extinction coefficients of the butyryl-CoA product and the CoASH substrate.

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 at twice the value of its Kₘ (0.15 mM CoASH and 0.52 mM butyryl phosphate) while using variable concentrations of the other (1.133, 2, and 4 times the Kₘ of the varied substrate, that is, 0.075 to 0.3 mM CoASH and 0.26 to 1.04 mM butyryl phosphate). Inhibition by potassium phosphate was tested at concentrations of 0, 14, and 28 mM (equal to 0, 1, and 2 times the value of its Kₘ) for the fixed concentration of CoASH and at 0, 28, and 56 mM (0, 2, and 4 times the value of its Kₘ) for the fixed concentration of butyryl phosphate. Inhibition by butyryl-CoA was tested at concentrations of 0 and 0.11 mM (equal to 0 and 1 times the value of its Kₘ) for fixed concentrations of both substrates.

In the butyryl phosphate-forming direction, only the inhibitory effect of butyryl phosphate was tested because DTNB present in the assay immediately reacted with any added CoASH. The inhibitory effect of butyryl phosphate was tested at 0, 0.26, and 0.52 mM (equal to 0, 1, and 2 times its Kₘ). The concentration of each substrate was alternately fixed at twice the value of its Kₘ while the concentration of the other substrate was varied (1, 1.33, 2, and 4 times the Kₘ value of the varied substrate). Thus, phosphate was fixed at 28 mM while butyryl-CoA was varied from 0.11 to 0.44 mM, and then butyryl-CoA was fixed at 0.22 mM while phosphate was varied from 14 to 56 mM.

**Source of materials.** Butyryl phosphate was synthesized by the method of Avison (3). All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., except where noted above.

### RESULTS

**Purification of PTB.** The results of a typical purification of PTB are presented in Table 1. The procedures for each step are described in Materials and Methods. Purified PTB showed no detectable loss of activity when stored for 6 months under nitrogen at 4°C in 25 mM Tris hydrochloride (pH 7.6) with 20% (vol/vol) glycerol, 2 mM diithiothreitol, and 100 mM (NH₄)₂SO₄.

The final purification step, high-pressure liquid chromatography with a hydrophobic-interaction column, 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. This step also yielded two peaks of PTB activity. The first peak was sharp and contained 56% of the eluted activity. The second peak was broader and irregular in shape. Sodium dodecyl sulfate-gel electrophoresis showed a single band for each peak, 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 sodium dodecyl sulfate-gel electrophoresis is shown in Fig. 1.

The elution volume of PTB on a calibrated gel filtration column corresponded to a native molecular weight of 264,000. If, as assumed, PTB does not have an irregular shape, then it consists of eight subunits of equal molecular weight.

**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, consistent with either an ordered or a random Bi Bi binding mechanism (25). The 1/v plots exhibited a slight nonlinearity at the concentrations of substrates used, consistent with phosphate binding at more than one substrate site. The Kₘ values at 30°C and pH 8.0 for butyryl-CoA, phosphate, butyryl phosphate, and CoASH were, respectively, 0.11, 14, 0.26, and 0.077 mM, and the dissociation constants were, 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 was noncompetitive with respect to butyryl phosphate and CoASH, whereas butyryl-CoA inhibited competitively in each case. Butyryl phosphate was 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

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**Table 1. Purification of PTB from C. acetobutylicum**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4,235</td>
<td>30,500</td>
<td>7.2</td>
<td>1</td>
<td>100</td>
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<tr>
<td>Ammonium sulfate fractionation</td>
<td>840</td>
<td>23,800</td>
<td>28.3</td>
<td>3.9</td>
<td>78</td>
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<tr>
<td>Phenyl Sepharose</td>
<td>102</td>
<td>18,500</td>
<td>182</td>
<td>25</td>
<td>61</td>
</tr>
<tr>
<td>CL-4B column</td>
<td>21.8</td>
<td>15,200</td>
<td>694</td>
<td>96</td>
<td>50</td>
</tr>
<tr>
<td>DEAE-Sephal column</td>
<td>AX-1000 column</td>
<td>6.2</td>
<td>9,070</td>
<td>1,460</td>
<td>200</td>
</tr>
<tr>
<td>PolyPROPYL A column</td>
<td>2.9</td>
<td>4,010</td>
<td>1,380</td>
<td>192</td>
<td>13</td>
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fitted by a family of parabolic curves rather than straight lines (Fig. 2).

The pattern of competitive inhibition by butyryl-CoA, together with the complex inhibition shown in Fig. 2, is not consistent with an ordered Bi Bi binding mechanism (25). The nonlinearity of Fig. 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 (Table 2) were similar to the \( K_m \) values and suggested that butyryl phosphate and butyryl-CoA are relatively important inhibitors of the reactions that form them. In contrast, phosphate was a relatively weak inhibitor of the butyryl-CoA-forming reaction.

**pH dependence.** The pH dependence of the reaction in both directions is shown in Fig. 3. In both directions, the percent activity was very sensitive to pH changes within the pH range of 5.5 to 7.0, the range of internal pH in typical fermentations (13). This result was most markedly true in the butyryl phosphate-forming direction, in which the enzyme was virtually inactive at a pH of about 6.

**Activity with other acyl-CoA substrates.** PTB had a fairly broad substrate specificity in the acyl phosphate-forming direction (Table 3). Activity with acetyl-CoA was negligible compared with 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. At 30°C and pH 8.0, the \( K_m \) values for acetyl-CoA and phosphate were 6.2 and 610 mM, respectively. The dissociation constants were 0.028 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 that exhibit parallel lines with one substrate and converging lines with another have been previously described (22). The appearance of parallel lines in these cases does not indicate a ping-pong binding mecha-

![FIG. 1. Polyacrylamide electrophoresis gel with sodium dodecyl sulfate showing PTB and standards. Proteins were stained with Coomassie blue. Lane 1, Molecular weight standards (in thousands): bovine albumin (66,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), and \( \alpha \)-lactalbumin (14,200). Lane 2, Purified PTB.](image)

![FIG. 2. Competitive inhibition of PTB (butyryl phosphate formation) by butyryl phosphate with a fixed concentration of 0.22 mM butyryl-CoA. Butyryl phosphate concentrations were 0 (□), 0.26 (△), and 0.52 (+) mM.](image)

**TABLE 2. Product inhibition constants**

<table>
<thead>
<tr>
<th>Product inhibitor</th>
<th>( K_m ) (mM)</th>
<th>( K_i ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyryl phosphate</td>
<td>27</td>
<td>18</td>
</tr>
<tr>
<td>Butyryl phosphate</td>
<td>0.17</td>
<td>0.36</td>
</tr>
<tr>
<td>Butyryl phosphate</td>
<td>0.35</td>
<td>ND</td>
</tr>
</tbody>
</table>

\( K_m \), Slope inhibition constant; \( K_i \), intercept inhibition constant; ND, not determined because of nonlinear behavior.
TABLE 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>Isovaleryl-CoA</td>
<td>30</td>
</tr>
<tr>
<td>N-Isobutyryl-CoA</td>
<td>95</td>
</tr>
</tbody>
</table>

* Determined at 30°C and pH 8.0, using 0.2 mM acyl-CoA in place of butyryl-CoA in the standard assay described in Materials and Methods.

FIG. 3. Dependence of PTB activity on pH. The assays described in the text were modified as follows: the butyryl phosphate-forming reaction was carried out with 10 mM MES [2-(N-morpholino)ethanesulfonic acid], 10 mM HEPES, 10 mM CHES [2-(N-cyclohexylamino)ethanesulfonic acid], 140 mM potassium phosphate, 0.08 mM DTNB, 0.2 mM butyryl-CoA, and 0.008 U of PTB (2); 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 U of PTB (Δ).

TABLE 4. Inhibition of PTB (butyryl phosphate-forming reaction) by cofactors

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Assay concn (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>
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<tr>
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<td>19</td>
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<tr>
<td>NADPH</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>NADP</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

DISCUSSION

PTB has not previously been characterized in detail for any organism. Although partial purification of PTB has been reported by others (7, 27), 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 (5), 63,000 and 75,000 in Clostridium acidiflavum (23), and 60,000 in Clostridium kluyveri (26), in contrast to 264,000 for PTB in C. acetobutylicum. PTA in C. thermoaceticum appears to be a tetramer (5), whereas PTB in C. acetobutylicum is probably an octamer.

Detailed studies of the binding mechanism in PTA were reported only by Kyrtopoulos and Satchell (17), 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 the method of Dixon, which may not distinguish between random and ordered Bi Bi binding mechanisms (25). 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 did have features suggesting 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 physiological 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 (9, 13). 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 aPH across the cell membrane. The attendant decrease in relative activity of PTB within this range could act as a feedback control to slow or halt the formation of butyric acid by the PTB-butyrate kinase pathway (Fig. 3), which is in agreement with in vivo observations described above. Therefore, the pH dependence of PTB, and probably butyrate kinase (10), is likely a factor in regulation of the flux through this pathway.

It is likely that additional factors are involved in reducing the flux through this pathway. Butyrate can accumulate rapidly within the cell, and since the reactions in this
pathway are reversible, it has been suggested that the level of
the butyryl phosphate precursor also increases (9). The
relatively low inhibition constant for butyryl phosphate
shown in Table 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 direc-
tion under conditions of low external pH.

Other interesting features of PTB are its inhibition by ATP
and broad substrate specificity. ATP inhibition of the butyryl
phosphate-forming reaction suggests another feedback con-
trol mechanism, since ATP is a product of the PTB-butyrate
kinase pathway. Since PTB is also somewhat inhibited by
AMP and ADP, and the in vivo level of ATP appears to be
generally less than 2 mM (C. L. Meyer, Ph.D. thesis, Rice
University, Houston, Tex., 1987), the inhibitory effect of
ATP may not be physiologically significant. CoA transferase
and butyrate kinase from C. acetobutylicum also exhibit
broad specificities (10, 11). Jewell et al. (14) demonstrated
that propionic, valeric, and 4-hydroxybutyric acids can be
taken up by C. acetobutylicum and converted to their
respective alcohols. This broad specificity could be the basis
for other novel fermentations.

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LITERATURE CITED

1. Andersch, W., H. Bahl, and G. Gottschalk. 1983. Level of
enzymes involved in acetate, butyrate, acetone and butanol
formation by Clostridium acetobutylicum. Eur. J. Appl. Micro-

2. Andrews, P. 1964. Estimation of the molecular weight of pro-

3. Avison, A. W. D. 1955. The synthesis of acyl phosphates in

the quantitation of microgram quantities of protein utilizing

cinental components from Clostridium thermocellum which cata-
yze synthesis of acetate from pyruvate and methyltetrahydro-

Biophys. 82:70–77.

7. Gavard, R., B. Hautoer, and H. Descourteix. 1957. Phos-
photransbutyrylase de Clostridium acetobutylicum. C. R. Acad.
Sci. 244:2322–2326.

1981. The relation of acyl transfer to the overall reaction of

Clostridium acetobutylicum and its effect on the shift from acid


metabolism in Clostridium acetobutylicum: levels of enzymes
involved in the formation of acetate and butyrate. Appl. Envi-

Uptake and activation of acetate and butyrate in Clostridium

13. Huang, L., L. N. Gibbins, and C. W. Forsberg. 1985. Trans-
membrane pH gradient and membrane potential in Clostridium
acetobutylicum during growth under acetogenic and solvento-

Bicarbonation of propionic, valeric, and 4-hydroxybutyric acids
into the corresponding alcohols by Clostridium acetobutylicum

metabolism in regulation of solventogenesis by Clostridium


with phosphotransacetylase. IV. Inhibition by products. Bio-

the assembly of the head of bacteriophage T4. Nature (London)


Gas chromatography and gateway sensors for on-line state
estimation of complex fermentations (butanol-acetone ferme-

monoxide gassing leads to alcohol production and butyrate
uptake without acetone formation in continuous cultures of
159–167.

hexokinases: kinetic, physical, and regulatory properties. p.


24. Rogers, P. 1986. Genetics and biochemistry of Clostridium
Microbiol. 31:1–60.


Phosphotransacylase of Escherichia coli B, purification and

27. Valentine, R. C., and R. S. Wolfe. 1960. Purification and role of