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Purification and Properties of an Acetoacetyl Coenzyme A-Reacting Phosphotransbutyrylase from *Clostridium beijerinckii* ("*Clostridium butylicum*") NRRL B593

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During the study of acetoacetyl coenzyme A (CoA)-reacting enzymes of *Clostridium beijerinckii* NRRL B593, a phosphate-dependent acetoacetyl-CoA-utilizing activity was detected in protein fractions devoid of thiolase and phosphotransacetylase. Further purification of this acetoacetyl-CoA-utilizing activity yielded an enzyme which may be designated as phosphotransbutyrylase (PTB; phosphate butyryltransferase [EC 2.3.1.19]). PTB from *C. beijerinckii* NRRL B593 was purified 160-fold with a yield of 14% and, with the best fractions, purified 190-fold to near homogeneity. It showed a native M_r of 205,000 and a subunit M_r of 33,000. PTB activity was sensitive to pH changes within the physiological range of 6 to 8. PTB exhibited a broad substrate specificity. The K_m values at pH 7.5 for butyryl-CoA, acetoacetyl-CoA, and acetyl-CoA were 0.04, 1.10, and 3.33 mM, respectively. The V_{max} values with butyryl-CoA and acetoacetyl-CoA were comparable, but the V_{max}/K_m was higher for butyryl-CoA than for acetoacetyl-CoA. An apparent K_m of 6.5 mM for phosphate was obtained with butyryl-CoA as the cosubstrate, whereas it was 12.9 mM with acetoacetyl-CoA as the cosubstrate. It remains to be established whether the putative compound acetoacetyl phosphate is produced in the PTB-catalyzed reaction with acetoacetyl-CoA.

During our study of acetoacetyl coenzyme A (CoA)-reacting enzymes of *Clostridium beijerinckii* NRRL B592 and NRRL B593, a phosphate-dependent acetoacetyl-CoA-utilizing activity was detected in protein fractions devoid of thiolase and phosphotransacetylase (PTA) activities. Further purification of the phosphate-dependent acetoacetyl-CoA-utilizing activity yielded an enzyme which may be designated as phosphotransbutyrylase (PTB).

PTB (phosphate butyryltransferase [EC 2.3.1.19]) catalyzes the interconversion of butyryl-CoA and butyryl phosphate with orthophosphate or CoA, respectively, as the cosubstrate for the two directions. The formation of butyryl phosphate from butyryl-CoA is the first specific reaction in the pathway leading to butyrate production in saccharolytic clostridia (9, 22, 24). Both PTB and butyrate kinase play important roles in the energy metabolism of butyric acid clostridia, since ATP is generated when the high-energy phosphodiester bond of butyryl phosphate is cleaved via kinase to form butyrate. PTB and butyrate kinase may also be responsible for the reutilization of butyrate for butanol production under certain conditions (15).

PTB activity has been detected in various *Clostridium* species (1, 7, 9, 23, 24; this work). The existence of PTB was first reported by Gavard et al. (7), who partially purified the enzyme from *C. acetobutylicum* and showed it to be distinct from PTA. Valentine and Wolfe (23) partially purified PTB from *C. beijerinckii* ATCC 6014 (previously "*Clostridium butyricum*" ATCC 6014), and they performed a limited study of the catalytic properties of the enzyme. Wiesenborn et al. (24) were the first to purify PTB to homogeneity and to characterize the enzymes extensively. These researchers reported that PTB from *C. acetobutylicum* ATCC 824 exhibited a broad substrate specificity in the acyl phosphate-forming direction, with *n*-butyryl-CoA, isovaleryl-CoA, and *n*-valeryl-CoA giving the highest relative activities (100: 95:78, respectively). However, Wiesenborn et al. (24) did not report whether PTB from *C. acetobutylicum* also reacts with acetoacetyl-CoA. The PTB gene of *C. acetobutylicum* ATCC 824 has been cloned and expressed in *Escherichia coli* (5).

In solvent-producing clostridia, acetoacetyl-CoA is a key metabolic intermediate in both acid (butyrate) and solvent (acetone-isopropanol and butanol) production (Fig. 1 in reference 25). The conversion of acetoacetyl-CoA to acetoacetate, for example, is the first specific reaction in the pathway leading to the formation of acetone in *C. acetobutylicum* or acetone and isopropanol in *C. beijerinckii*. Since acetoacetyl-CoA is at the branch point for the acetone-isopropanol pathway and the butyrate-butanol pathways, enzymes reacting with this key metabolic intermediate may have a role in determining the product ratio during solvent formation. This regulation can have practical importance, because acetone, butanol, and isopropanol have different values as feedstock chemicals, solvents, and fuel additives.

In this paper, we describe the purification and characterization of PTB from *C. beijerinckii* NRRL B593.

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MATERIALS AND METHODS

Materials. Tryptone and yeast extract were obtained from Difco Laboratories, Detroit, Mich. Trizma base (Tris), sodium phosphate (monobasic), TES (*N*-tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid), CoA (sodium salt), diketene, DNase I, acetyl-CoA (lithium salt), *n*-butyryl-CoA (lithium salt), acetyl phosphate (potassium-lithium salt), 5,5′-dithiobis-[2-nitrobenzoic acid] (DTNB), dithiothreitol, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, ferritin, aldolase, yeast alcohol dehydrogenase, conalbumin, bovine serum

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albumin, α-chymotrypsinogen A, RNase, blue dextran, Sephacryl S-300, Cibacron Blue 3GA-agarose, and phenol reagent were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium dodecyl sulfate (SDS), silver staining kit, dye-binding protein assay kit, and hydroxy(l)apatite (Bio-Gel HTP) were obtained from Bio-Rad Laboratories, Richmond, Calif. α-Toluenesulfonyl fluoride, acrylamide, methylene-bis-acrylamide, ammonium peroxydisulfate, and bromophenol blue were obtained from Eastman Kodak Co., Rochester, N.Y.

Organism and growth conditions. Stock cultures of *C. beijerinckii* VPI 13437 ("*C. butylicum*" NRRL B593) were stored as frozen spore suspensions in tryptone-yeast extract-sucrose (TYS) medium at −70°C (8). To grow batch cultures, 6 ml of a spore suspension was heated in boiling water for 2 min. Then the heat-treated spore suspension was transferred to 50 ml of a chopped-meat–carbohydrate (meat removed) medium prepared under N_2 (11) and incubated at 35°C with shaking. Growth was monitored at 550 nm with a Hitachi 100-40 spectrophotometer. The chopped-meat–carbohydrate culture was transferred to 1 liter of TYS medium when the A_{550} was between 4 and 5. The 1-liter culture was used to inoculate 7 liters of TYS medium. All batch cultures were incubated at 30°C with stirring. TYS medium was kept anaerobic by flushing with N_2 before inoculation.

Cells from 8-liter TYS cultures were harvested by centrifugation at 13,000 × *g* for 20 min at 4°C when the A_{550} was between 4 and 5, washed once in 50 mM Tris-chloride buffer (pH 8), and centrifuged again under similar conditions. Cell paste was first frozen in liquid nitrogen and then stored frozen at −70°C.

Preparation of crude extracts. Cell paste was thawed under argon in 50 mM Tris-acetate buffer (pH 7.0) (3 ml/g of cell paste). Lysozyme (2.0 mg/ml), DNase I (0.1 mg/ml), and the protease inhibitor α-toluenesulfonyl fluoride (0.3 mg/ml) were added to the cell suspension. The cell suspension was incubated for 1.5 to 2 h at room temperature with gentle stirring. To remove cell debris, the lysate was centrifuged at 37,000 × *g* for 30 min at 4°C. The supernatant (crude extract) was stored as frozen pellets in liquid nitrogen if not immediately used.

Preparation of acetoacetyl-CoA. Acetoacetyl-CoA was prepared by a modified version of the Senior and Dawes (18) method. The reaction was carried out in a closed vial under argon. A 20-fold molar excess of diketene was added to the CoA solution in 1- to 2-μl portions over a period of 1 to 2 h at 0°C. Synthesized acetoacetyl-CoA was stored at −20°C and was stable for at least 1 month.

Analytical procedures. Protein was determined by the method of Bradford (4) or Lowry et al. (14). The protein standards were bovine plasma gamma globulin for the dye-binding assay and bovine serum albumin for the Lowry assay. When the protein concentration was <40 μg/ml, the procedure described by Sargent (17), which gives a 50-fold amplification of the Lowry protein assay, was used, with bovine serum albumin as the standard. Chloride concentration was determined with the Sigma chloride determination kit.

Enzyme assays. Phosphate-dependent acetoacetyl-CoA-utilizing activity was initially measured in an assay mixture (1.0-ml total) containing 50 mM Tris-chloride buffer (pH 7.5), 0.1 mM acetoacetyl-CoA, 20 mM $MgCl_2$, and 5 mM sodium phosphate (pH 7.5). The reaction was initiated by the addition of enzyme or phosphate and monitored at 310 nm. An extinction coefficient of 6.4 mM^{-1} cm^{-1}, instead of 8 mM^{-1} cm^{-1} (20), was used to correct for a sodium (monovalent cation) effect. (PTB-containing fractions from the hydroxyapatite column carried phosphate into the assay to a final concentration of about 5 mM, and no additional phosphate was added when assaying these fractions.) Phosphate-dependent acetoacetyl-CoA-utilizing activity of PTB was also assayed with DTNB (see below).

PTB activity was measured by monitoring the liberation of CoA as butyryl-CoA was converted to butyryl phosphate. The assay mixture (1.0-ml total) contained 150 mM potassium phosphate buffer (pH 7.4), 0.2 mM butyryl-CoA, and 0.08 mM DTNB. The reaction was initiated with the addition of enzyme and monitored at 412 nm. The extinction coefficient used was 13.6 mM^{-1} cm^{-1} (6). Acetoacetyl-CoA-linked activity of PTB was assayed similarly, with acetoacetyl-CoA (0.2 mM) replacing butyryl-CoA. All enzyme assays were performed under aerobic conditions with a Beckman Acta M6 spectrophotometer. One unit equals the amount of enzyme which consumes 1 μmol of substrate or forms 1 μmol of product per min.

PTA activity was determined spectrophotometrically by following the formation of acetyl-CoA from acetyl phosphate and CoA, which caused an increase in A_{233}. The assay mixture (1.0 ml), a modification of the method of Hartmanis and Gatenbeck (10), consisted of 100 mM Tris-chloride buffer (pH 8.0), 100 mM potassium chloride, 0.1 mM CoA, and 10 mM acetyl phosphate. Addition of enzyme initiated the reaction. The extinction coefficient (ε_{233}, 4.44 mM^{-1} cm^{-1}) used in the PTA assay was the difference between the extinction coefficients for acetyl-CoA and CoA (3).

Acetoacetyl-CoA thiolase activity was measured by monitoring at 310 nm the thiolysis of acetoacetyl-CoA in the presence of CoA. The assay mixture contained 105 mM Tris-chloride buffer (pH 7.5), 0.1 mM acetoacetyl-CoA, 24 mM $MgCl_2$, and enzyme in a total volume of 1 ml. The reaction was initiated by the addition of 60 μM CoA. The extinction coefficient used was 8 mM^{-1} cm^{-1} (20).

Purification of PTB. All chromatographic columns were run at room temperature (about 23°C). Fractions were stored at 4°C between purification steps, since PTB activity was found to be stable under these conditions.

(i) Step 1. Preparation of crude extracts. Crude extracts of *C. beijerinckii* NRRL B593 were prepared as described above.

(ii) Step 2. Hydroxyapatite chromatography. An 80-ml portion of a crude extract (about 500 mg of protein) was applied to a Bio-Gel HTP column (2.5 by 18 cm) equilibrated anaerobically with 20 mM potassium phosphate buffer (pH 7.0) containing 2 mM dithiothreitol. Protein was eluted at a flow rate of 80 ml/h with a linear gradient of potassium phosphate (0.1 to 0.6 M in a total volume of 500 ml) containing 2 mM dithiothreitol. Fractions of 10 ml were collected. This step effectively separated PTB from PTA and thiolase activities, with PTB being eluted at a higher phosphate concentration. Earlier preparations used aerobic conditions and sodium phosphate buffer for this step, which were adequate for PTB but gave a lower yield of PTA activity.

(iii) Step 3. Gel filtration. Fractions containing PTB activity from the HTP column were pooled and concentrated in an Amicon ultrafiltration cell with a PM30 Diaflo membrane under argon (60 lb/in^2). A 5-ml amount of the concentrated sample (about 20 mg of protein) was loaded onto a Sephacryl S-300 column (2.5 by 48 cm) equilibrated with 50 mM sodium-TES buffer (pH 7.5) containing 0.1 M NaCl. The flow rate was 50 ml/h. Fractions of 3 ml were collected.

(iv) Step 4. Dye-ligand chromatography. Fractions (2 to 3

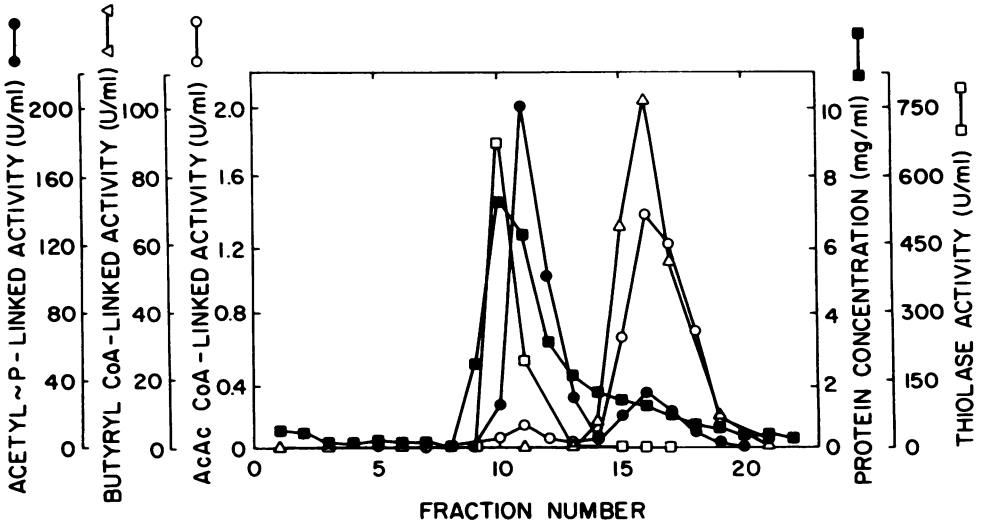

FIG. 1. Elution of an acetoacetyl (AcAc)-CoA-linked activity and activities attributable to thiolase, PTA, and PTB of *C. beijerinckii* NRRL B593 from a hydroxyapatite column. A crude extract (291 mg of protein) was loaded onto the column (2.5 by 15.1 cm). Acetoacetyl-CoA-linked activity was measured by A_{310} changes. Other conditions were as described in Materials and Methods, except that sodium phosphate buffers containing 0.1 M NaCl, a gradient of 0.1 to 1.0 M phosphate, and aerobic conditions were used.

mg of protein) with PTB activity from the gel filtration step were pooled and loaded onto a Cibacron Blue 3GA-agarose column (1.4 by 6.5 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0). Protein was eluted at a flow rate of 25 ml/h with a linear gradient of sodium chloride (0.5 to 3.0 M in a total volume of 90 ml) in 50 mM sodium phosphate buffer (pH 7.0). Fractions of 3 ml were collected. PTB activity was eluted at a chloride concentration of 1.7 M.

SDS-polyacrylamide gel electrophoresis. To examine the purity and determine the subunit molecular weight of PTB, electrophoresis was performed on a 10% polyacrylamide gel slab (63 by 92 by 1.5 mm) in the presence of SDS by the method of Laemmli (13). Fractions with PTB activity from a Cibacron Blue 3GA-agarose column were electrophoresed at a constant current of 60 mA. Protein bands were detected by silver staining.

Determination of native and subunit molecular weight. The native molecular weight of PTB was estimated by gel filtration on a Sephacryl S-300 column (2.5 by 44.0 cm) equilibrated with 50 mM sodium-TES buffer (pH 7.5) containing 0.1 M NaCl. A partially purified sample of PTB was used. The column was calibrated with the following molecular weight markers (molecular weights in parentheses): RNase A (13,700), α-chymotrypsinogen A (25,000), conalbumin (77,000), yeast alcohol dehydrogenase (150,000), aldolase (158,000), and ferritin (440,000). The subunit molecular weight of PTB was determined by SDS-polyacrylamide gel electrophoresis (13). Molecular weight markers were bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), and trypsinogen (24,000).

Kinetic studies. For determination of the K_m and V_{max} of PTB for butyryl-CoA, acetoacetyl-CoA, and acetyl-CoA, enzyme activities were measured in 150 mM potassium phosphate buffer (pH 7.5) with 0.08 mM DTNB. The reaction was monitored at 412 nm. The concentrations of butyryl-CoA, acetoacetyl-CoA, and acetyl-CoA varied, respectively, from 0.03 to 0.13 mM, 0.10 to 0.50 mM, and 0.09 to 0.87 mM. The apparent K_m for phosphate was obtained with butyryl-CoA or acetoacetyl-CoA as the cosubstrate by measuring enzyme activities at 412 nm in 50 mM Tris-chloride buffer (pH 7.5)–0.08 mM DTNB–0.1 mM butyryl-CoA or acetoacetyl-CoA. The concentration of potassium phosphate ranged from 5 to 150 mM. All kinetic studies were performed at ambient temperature (about 23°C). Reactions were initiated with the addition of PTB to a final assay volume of 1.0 ml.

RESULTS

Phosphate-dependent acetoacetyl-CoA-utilizing activity. When crude extracts of *C. beijerinckii* NRRL B593 were chromatographed on a hydroxyapatite column, a major acetoacetyl-CoA-utilizing activity was observed at the position of PTB, which was separated from thiolase and PTA (Fig. 1). The PTB-containing fractions were further chromatographed on a Sephacryl S-300 column, and the acetoacetyl-CoA-linked activity was again eluted with PTB (Fig. 2). The phosphate dependence of the acetoacetyl-CoA-utilizing activity of PTB was demonstrated by the following experiments: (i) PTB-containing fractions from the Sephacryl S-300 column (Fig. 2) required the addition of phosphate to show acetoacetyl-CoA-linked activity; (ii) after the removal of phosphate by buffer exchanges through ultrafiltration, PTB-containing fractions from the hydroxyapatite column also required added phosphate for acetoacetyl-CoA-linked activity (data not shown); and (iii) a K_m for phosphate was obtained for PTB with acetoacetyl-CoA as a cosubstrate (see below).

The separation of PTB from thiolase and PTA is an important step in establishing an acetoacetyl-CoA-linked activity in PTB. In the presence of a catalytic amount of CoA (which can be from the spontaneous decomposition of acetoacetyl-CoA), the combined actions of thiolase (acetoacetyl-CoA + CoA $\rightleftharpoons$ 2 acetyl-CoA) and PTA (acetyl-CoA + phosphate $\rightleftharpoons$ acetyl phosphate + CoA) can also generate a significant level of phosphate-dependent acetoacetyl-CoA-utilizing activity (based on A_{310} decreases; data not shown). It is thus necessary to separate both thiolase and PTA from PTB before the phosphate-dependent acetoacetyl-CoA-utilizing activity of PTB can be established. Hydroxyapatite column chromatography (Fig. 1) is very effective in achieving this separation.

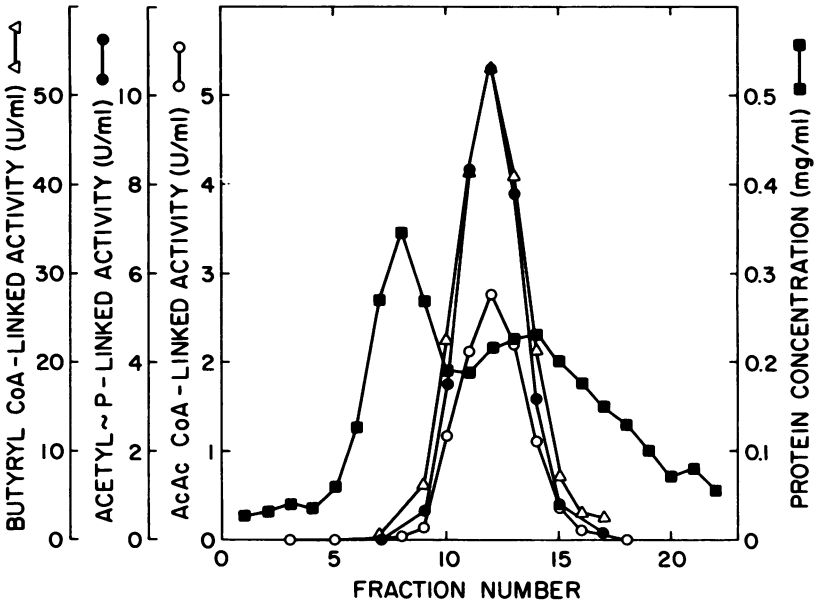

FIG. 2. Elution profile of PTB of *C. beijerinckii* NRRL B593 from a Sephacryl S-300 column. Fractions containing butyryl-CoA-linked activity from a hydroxyapatite column were pooled and concentrated. The concentrated sample (5.6 ml, 15.3 mg of protein) was loaded onto the Sephacryl S-300 column (2.5 by 44.2 cm). Fractions of 5 ml were collected. Acetoacetyl (AcAc)-CoA-linked activity was measured by A_{310} changes. See Materials and Methods for other conditions.

Purification of PTB. A summary of the purification of PTB from *C. beijerinckii* NRRL B593 is presented in Table 1. The procedures for each purification step are described in Materials and Methods. The elution profiles of PTB activity from hydroxyapatite, Sephacryl S-300, and Cibacron Blue 3GA-agarose columns are shown, respectively, in Fig. 1, 2, and 3. This scheme achieved 160-fold purification to an average specific activity (with butyryl-CoA) of 967 U/mg of protein and a yield of 14%. The highest specific activity obtained was 1,160 U/mg of protein, which is a purification of 190-fold. The ratio of butyryl-CoA-utilizing activity to acetoacetyl-CoA-utilizing activity remained about 6 (with the DTNB assay) throughout purification. In comparison, 200-fold-purified PTB from *C. acetobutylicum* ATCC 824 had a specific activity of 1,460 U/mg (24).

An activity loss of 92 to 100% was observed when purified PTB (6 to 12 μg of protein per ml in 50 mM sodium phosphate buffer [pH 7.0] containing various NaCl concentrations) was frozen in liquid nitrogen for storage purposes. A purified PTB preparation was also stored at −20°C in the presence of 50% (vol/vol) glycerol to investigate alternative storage methods. A 25% loss of PTB activity resulted after 5 days of storage under such conditions. However, less purified PTB (0.22 mg of protein per ml in 50 mM sodium-TES buffer [pH 7.5] containing 0.1 M NaCl) could be stored in liquid nitrogen without activity loss. When stored at 4°C in 50 mM sodium phosphate buffer (pH 7.0) containing high salt concentrations (1.3 to 3.0 M), activity of purified PTB declined by approximately 10% from the initial activity after storage for 3 months.

TABLE 1. Purification of PTB from *C. beijerinckii* NRRL B593

Step	Protein (mg)	Activity (U)[a]	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	539	3,240	6.01	1	100
Hydroxylapatite	77.6	2,400	30.9	5	74
Sephacryl S-300	7.80	1,630	209	35	50
Cibacron Blue 3GA-agarose	0.453	438	967[b]	160	14

[a] One unit of activity is the amount of enzyme that converts 1 μmol of butyryl-CoA to product per min.

[b] The highest specific activity obtained was 1,160 U/mg.

SDS-polyacrylamide gel electrophoresis was used to examine the purity of PTB. A common intense band was observed at the same migration position for different fractions containing highly purified PTB (Fig. 4). Fractions following the activity peak from a Cibacron Blue 3GA-agarose column (see Fig. 3 for elution profile) showed a higher purity, since minor bands appearing in earlier (fractions 16 to 18) and later (fraction 22) fractions were not seen with fractions 19 to 21 eluted immediately after the activity peak. The highest and nearly constant specific activities for purified PTB were measured in fractions 19 (1,120 U/mg), 20 (1,080 U/mg), and 21 (1,160 U/mg), which eluted immediately after the peak fraction from a Cibacron Blue 3GA-agarose column. Fractions 19 (lanes 7 and 8 in Fig. 4) and 20 (lane 9 in Fig. 4) each gave an intense protein band on SDS-polyacrylamide gel.

Native and subunit molecular weight. The elution volume of PTB from *C. beijerinckii* NRRL B593 on a calibrated Sephacryl S-300 gel filtration column corresponded to a native molecular weight of 205,000 (data not shown). SDS-polyacrylamide gel electrophoresis showed a subunit molecular weight of 33,000 (data not shown). Partially purified PTB from *C. beijerinckii* NRRL B592 (which produces acetone and butanol but not isopropanol) gave a molecular weight of 240,000 (M. Walker and J.-S. Chen, unpublished results). In comparison, Wiesenborn et al. (24) reported a molecular weight of 264,000 for PTB purified from *C. acetobutylicum* ATCC 824; it was proposed as an octamer of identical subunits with a subunit molecular weight of 31,000. Because of the broad criteria for the genus *Clostridium*, it is difficult to evaluate the structural relatedness of PTB from

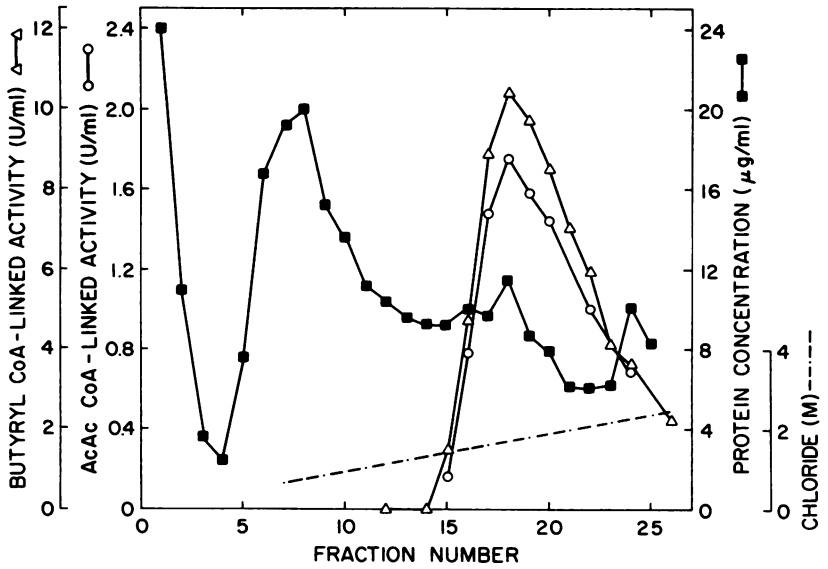

FIG. 3. Elution profile of PTB of *C. beijerinckii* NRRL B593 from a Cibacron Blue 3GA-agarose column. Fractions (2.52 mg of protein) with butyryl-CoA-linked activity from a Sephacryl S-300 column were pooled and loaded onto a Cibacron Blue 3GA-agarose column (1.4 by 6.5 cm). Acetoacetyl (AcAc)-CoA-linked activity was measured with DTNB. See Materials and Methods for other conditions.

these strains without additional information, such as the amino acid sequence of the enzymes.

pH dependence of PTB activity. The effect of varying pH on PTB activity was measured with butyryl-CoA and acetoacetyl-CoA as the substrates. PTB attained a maximal activity at pH 7.5 (Fig. 5), whereas acetoacetyl-CoA-utilizing activity increased from pH 6 to 8. (Acetoacetyl-CoA-linked activity was not examined above pH 8 because of the instability of this compound in alkaline solutions.) The parallel response, in the pH range of 6 to 7.5, of *C. beijerinckii* PTB activities toward butyryl-CoA and acetoacetyl-CoA suggests that the acetoacetyl-CoA-linked activity of PTB will not be selectively enhanced during the solvent-producing phase of growth (at which a lowering in intracellular pH is expected [21; and references cited in reference 12]), unless factors other than pH can regulate the activity of PTB toward its alternate substrates.

Kinetic studies. The K_m values at 23°C and pH 7.5 for butyryl-CoA, acetoacetyl-CoA, and acetyl-CoA were, respectively, 40 μM, 1.10 mM, and 3.33 mM (Table 2). An apparent K_m of 6.5 mM for phosphate was obtained with butyryl-CoA as the cosubstrate, whereas, when acetoacetyl-CoA was the cosubstrate, an apparent K_m of 12.9 mM for phosphate was obtained. The K_m values (at 30°C and pH 8) of the *C. acetobutylicum* PTB for butyryl-CoA and phosphate are 0.11 and 14 mM, respectively (24).

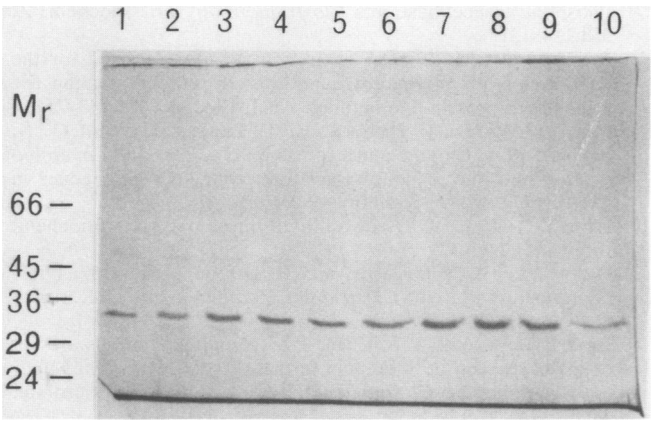

FIG. 4. Examination of purity of PTB prepared from *C. beijerinckii* NRRL B593 by SDS-polyacrylamide gel electrophoresis. Fractions with PTB activity from a Cibacron Blue 3GA-agarose column (see Fig. 3 for elution profile) were analyzed (lanes 1 and 2, fraction 16; lanes 3 and 4, fraction 17; lanes 5 and 6, fraction 18; lanes 7 and 8, fraction 19; lane 9, fraction 20; and lane 10, fraction 22). A 0.3- to 0.6-μg amount of protein was loaded. Protein bands were detected by silver staining. The migration positions of molecular weight markers and their molecular weights (10^3) are indicated.

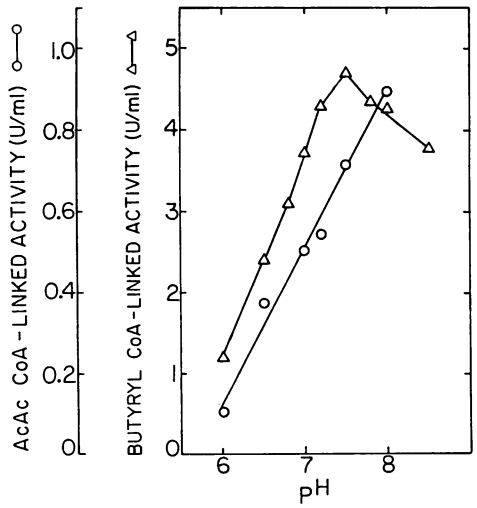

FIG. 5. Effect of pH on PTB activity. Butyryl-CoA-linked activity and acetoacetyl (AcAc)-CoA-linked activity of PTB were measured in an assay mixture (1.0-ml total) which contained 150 mM potassium phosphate at the indicated pHs, 0.08 mM DTNB, and 0.2 mM butyryl-CoA or acetoacetyl-CoA. Purified PTB (4 ng of protein) was added to initiate the reaction.

TABLE 2. K_m, V_{max}, and V_{max}/K_m values of PTB from *C. beijerinckii* NRRL B593

Substrate	K_m (mM)	V_{max} (U/mg)[a]	V_{max}/K_m
Butyryl-CoA	0.04	1,560	39,000
Acetoacetyl-CoA	1.10	1,300	1,180
Acetyl-CoA	3.33	560	170

[a] One unit of activity is the amount of enzyme that converts 1 μmol of substrate to product per min.

Although PTB of *C. beijerinckii* showed different K_m values for butyryl-CoA and acetoacetyl-CoA, comparable V_{max} values were obtained for these substrates. PTB reacted with butyryl-CoA, acetoacetyl-CoA, and acetyl-CoA in the V_{max}/K_m range of 100:3:0.44. Partially purified PTB from *C. beijerinckii* NRRL B592 gave an almost identical V_{max}/K_m range (100:3:0.4) for butyryl-CoA, acetoacetyl-CoA, and acetyl-CoA (Walker and Chen, unpublished results). The V_{max}/K_m value was 33-fold higher with butyryl-CoA than with acetoacetyl-CoA for PTB. When phosphate is not limiting and the intracellular steady-state levels of acetoacetyl-CoA and butyryl-CoA are comparable and well below their respective K_m values, PTB can exhibit some acetoacetyl-CoA-linked activity. However, possible formation of enzyme complexes (19) between thiolase and PTB or between butyryl-CoA dehydrogenase and PTB (for which there is as yet no experimental evidence) may exert further control on the relative activities of PTB toward acetoacetyl-CoA and butyryl-CoA. If expressed in vivo, acetoacetyl-CoA-linked activity of PTB would be interesting since acetoacetyl-CoA is a metabolic precursor of butyryl-CoA in the fermentative pathways of *C. beijerinckii*.

DISCUSSION

Since PTB and PTA catalyze analogous reactions, it might be expected that these acid-producing enzymes show similarities in their physical and catalytic properties. On the contrary, PTB and PTA from *C. beijerinckii* showed significant differences in substrate specificity and native molecular weight. Whereas PTB showed a broad substrate specificity, PTA showed activity towards acetyl-CoA but not towards acetoacetyl-CoA and butyryl-CoA (data not shown). Furthermore, the apparent molecular weight of PTA from *C. beijerinckii* NRRL B593 was 56,000 (unpublished data), a value intermediate between those estimations reported for *C. kluyveri* (38,000 to 41,000 [3]) and *C. acidiurici* (63,000 and 75,000 [16]). PTB, with an apparent molecular weight of 205,000, is a much larger protein than PTA.

PTB purified in this study resembled PTB purified from *C. acetobutylicum* ATCC 824 (24). Broad substrate specificity and a sensitivity of activity to pH changes in the 6 to 8 range were among the most striking characteristics shared by the enzyme from the two clostridial species. However, it is not known whether PTB from *C. acetobutylicum* also reacts with acetoacetyl-CoA.

The relatively high K_m of PTB for phosphate (24; this study) suggests that a possible decrease in the availability of phosphate in the cell, especially when active solvent production begins during the late growth phase, may serve to limit the activity of PTB towards the formation of butyric acid, thus making more butyryl-CoA available for butanol formation. Phosphate limitation has been shown to favor the production of solvents by *C. acetobutylicum* (2).

PTB from *C. beijerinckii* NRRL B593 showed activity towards three physiologically relevant substrates: butyryl-CoA, acetoacetyl-CoA, and acetyl-CoA. These three compounds are at the branch points of the complex metabolic pathways of solvent-producing clostridia (see Fig. 1 in reference 25). The most interesting feature of PTB from *C. beijerinckii* is its activity towards acetoacetyl-CoA in the presence of phosphate, and free CoA has been identified as one of the end products by the DTNB assay. Since acetoacetyl-CoA is at the branch point for the acetone-isopropanol pathway and the butyrate-butanol pathways, enzymes reacting with this metabolic intermediate may have a role in determining the product pattern during solvent formation.

It is known that PTB catalyzes the interconversion of butyryl-CoA and butyryl phosphate (reaction 1). As an analogy to reaction 1, reaction 2 is postulated as a probable mechanism for acetoacetyl-CoA utilization by PTB:

reaction 1: Butyryl-CoA + $P_i \rightleftharpoons$ butyryl-P + CoASH
reaction 2: Acetoacetyl-CoA + $P_i \rightleftharpoons$ [acetoacetyl-P] + CoASH

The postulated compound acetoacetyl phosphate has not been reported previously. The reaction suggested above cannot be confirmed until acetoacetyl phosphate is synthesized and identified as a reaction product. It would also be interesting to determine whether acetoacetyl phosphate, if it is indeed a reaction product, can be further converted to acetoacetate via a kinase with the formation of ATP.

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