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Purification and Properties of 3-Hydroxybutyryl-Coenzyme A Dehydrogenase from *Clostridium beijerinckii* ("*Clostridium butylicum*") NRRL B593

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The enzyme 3-hydroxybutyryl-coenzyme A (CoA) dehydrogenase has been purified 45-fold to apparent homogeneity from the solvent-producing anaerobe *Clostridium beijerinckii* NRRL B593. The identities of 34 of the N-terminal 35 amino acid residues have been determined. The enzyme exhibited a native M_r of 213,000 and a subunit M_r of 30,800. It is specific for the (*S*)-enantiomer of 3-hydroxybutyryl-CoA. Michaelis constants for NADH and acetoacetyl-CoA were 8.6 and 14 μM, respectively. The maximum velocity of the enzyme was 540 μmol min^{-1} mg^{-1} for the reduction of acetoacetyl-CoA with NADH. The enzyme could use either NAD(H) or NADP(H) as a cosubstrate; however, k_{cat}/K_m for the NADH-linked reaction was much higher than the apparent value for the NADPH-linked reaction. Also, NAD(H)-linked activity was less sensitive to changes in pH than NADP(H)-linked activity was. In the presence of 9.5 μM NADH, the enzyme was inhibited by acetoacetyl-CoA at concentrations as low as 20 μM, but the inhibition was relieved as the concentration of NADH was increased, suggesting a possible mechanism for modulating the energy efficiency during growth.

3-Hydroxyacyl-coenzyme A (CoA) dehydrogenase (EC 1.1.1.35) and acetoacetyl-CoA reductase (AcAcCoAR; EC 1.1.1.36) in both eukaryotic and prokaryotic systems have been studied. These enzymes catalyze analogous reactions and are common to several metabolic pathways including β-oxidation of fatty acids (8, 24, 27), short-chain fatty acid elongation (10, 13), ketone body formation (18), and polyhydroxybutyrate synthesis (5, 9, 17, 19, 21). Since polyhydroxybutyrate is a polymer of (*R*)-3-hydroxybutyrate, the enzymes catalyzing the interconversions of polyhydroxybutyrate and acetoacetyl-CoA (AcAcCoA) are specific for that enantiomer. The enzymes of the other pathways may be specific for one enantiomer or the other (9, 13, 14, 16, 18, 21, 27) or may lack stereospecificity (9).

3-Hydroxybutyryl-CoA dehydrogenase (3HBDH; EC 1.1.1.35 or 1.1.1.157) in clostridia (10, 13, 22, 32; this work) catalyzes the reduction of AcAcCoA by NAD(P)H. This reaction is the first committed step toward production of butyrate and butanol. Although there is a high degree of similarity in the amino acid sequences of 3-hydroxyacyl-CoA dehydrogenases (32), there is less similarity in kinetic properties and quaternary structures (9, 13–16, 21, 31).

The acid- and solvent-producing pathways of the clostridia are shown in Fig. 1 of reference 30. As evidenced by its position at a branch point in the fermentation, AcAcCoA is an important intermediate in the metabolism of these organisms. The metabolic fate of this compound directly affects the product pattern of the fermentation. Thiolase (28) and CoA transferase (29), which react with AcAcCoA, have been isolated from a solvent-producing organism, *Clostridium acetobutylicum* ATCC 824. Thompson and Chen (26) have purified and characterized phosphotransbutyrylase, which also reacts with AcAcCoA, from *C. beijerinckii* NRRL B593. Characterization of the other enzymes reacting with this intermediate should yield insights into the metabolic control mechanisms for the fermentation. A thorough understanding of the control mechanisms will make it possible to customize the product pattern of the fermentation for various industrial uses.

Youngleson et al. (32) have cloned and sequenced the *hbd* gene encoding 3HBDH from *C. acetobutylicum* P262 and expressed it in *Escherichia coli*. The *hbd* gene is located upstream from the *adh-1* gene (encoding an alcohol dehydrogenase) and was identified as the structural gene for 3HBDH because its encoded amino acid sequence has a high similarity to that of 3-hydroxyacyl-CoA dehydrogenases. Although 3HBDH activity was observed at a high level in *E. coli* harboring a plasmid containing the *hbd* gene, the enzyme has not been purified or extensively studied. In this paper, we describe the purification and characterization of 3HBDH from *C. beijerinckii* NRRL B593.

MATERIALS AND METHODS

Source of chemicals. $NAD(P)^+$, NAD(P)H, CoA, 3-hydroxybutyryl-CoA, glycine, chloride determination kit, 2-(*N*-morpholino)ethanesulfonic acid (MES), Tris, Sephacryl S-300 (Pharmacia), Coomassie brilliant blue R-250, (*S*)-3-hydroxyacyl-CoA dehydrogenase (porcine heart), lysozyme (chicken egg white), DNase I, ferritin (horse spleen), alcohol dehydrogenase (baker's yeast), conalbumin (chicken egg white), thyroglobulin (bovine), bovine serum albumin, ovalbumin (chicken), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), trypsin inhibitor (soybean), carbonic anhydrase (bovine erythrocyte), and β-lactoglobulin (bovine milk) were obtained from Sigma Chemical Co.; α-toluenesulfonyl fluoride (PMSF), ammonium peroxydisulfate, acrylamide, and *N*,*N*′-methylenebisacrylamide were obtained from Eastman Kodak Co.; diketene was obtained from Aldrich Chemical Co.; tryptone and yeast extract were obtained from Difco Laboratories; Matrex Gel Red A, ultrafiltration membranes, and Centricon 3 and Centricon 30 ultrafiltration units were obtained from Amicon Corp.;

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Q-Sepharose (fast flow) was obtained from Pharmacia, Inc.; and the dye-binding protein assay kit, *N,N,N',N'*-tetramethylenediamine, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories.

Organism and growth conditions. Spores of *C. beijerinckii* NRRL B593 (VPI 13437), produced in potato extract-glucose medium (6), were stored in the same medium at −70°C. Heat shock and growth conditions were as previously described (26), except that glucose was used in place of sucrose in 600-ml and 8-liter cultures incubated at 34°C. Cells were allowed to grow until the A_{550} (1-cm path length) of the culture, as measured with a Hitachi model 100-40 spectrophotometer, increased by less than 0.5 over a 2-h period (typically about 10 h after inoculation of the 8-liter culture). Cells were harvested by centrifugation at 13,000 × *g* for 30 min at 4°C. The packed cells were resuspended in about 1 liter of 50 mM potassium phosphate buffer (pH 7.0) and centrifuged again for 45 min to remove remaining growth medium and fermentation products. Crude extracts (see below) were made from freshly prepared cell paste. However, crude extracts could also be made from cell paste stored at −70°C with no apparent adverse effects on the activity of the enzyme.

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

Enzyme assays. The activity of 3HBDH was routinely assayed in air at room temperature (about 23°C) in the physiological (3-hydroxybutyryl-CoA-forming) direction by monitoring the decrease in A_{340} of NADH. The standard assay mixture contained 50 mM Na-MES (pH 6.0), 200 μM NADH, 200 μM AcAcCoA, and as much as 0.2 U of 3HBDH in a total volume of 1 ml. The reaction was initiated by addition of AcAcCoA. Activities were corrected for oxygen-linked oxidation of NAD(P)H when crude preparations were assayed. One unit of activity is defined as the amount of enzyme which results in the oxidation of 1 μmol of NAD(P)H per min. The extinction coefficient used (340 nm) for NAD(P)H was 6.22 mM^{-1} cm^{-1}.

To determine whether the inhibition observed with AcAc CoA was caused by free CoA, a possible contaminant of the AcAcCoA preparation, 3HBDH was assayed in the presence and absence of CoA. The reaction mixture contained 50 mM Na-MES (pH 5.5), 60 μM AcAcCoA, 100 μM NADH, 0.2 U of 3HBDH, and either 0 or 200 μM CoA in a total volume of 1 ml. The suitability of the CoA preparation for the enzyme assay was confirmed by assaying for a previously observed (30) CoA-dependent AcAcCoA thiolase activity in a crude extract prepared from *C. beijerinckii* NRRL B592. The reaction mixture for thiolase contained 100 mM Tris-Cl (pH 7.5), 100 μM AcAcCoA, 20 mM $MgCl_2$, 60 μM CoA, and 2 μl of crude extract (about 40 μg of protein) in a final volume of 1 ml. The reaction was initiated by the addition of CoA. The activity was determined by monitoring the decrease in A_{310} of the Mg:AcAcCoA chelate; the extinction coefficient used was 8.0 mM^{-1} cm^{-1} (23).

Protein assays. Protein concentrations were determined by the dye-binding assay (3), with bovine gamma globulin as the standard.

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

Chloride concentrations were determined with the Sigma chloride determination kit.

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

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

(ii) Q-Sepharose column. The crude extract (about 1.2 g of protein) was applied directly to a Q-Sepharose column (2.5 by 17 cm) which had been equilibrated with phosphate buffer. The column was washed with 500 ml of phosphate buffer, and activity was eluted with a linear gradient of KCl (0 to 0.4 M in phosphate buffer; total volume, 2,000 ml). The flow rate was maintained at 300 ml/h, and 50-ml fractions were collected.

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

(iv) Sephacryl S-300 column. Fractions (about 35 ml) from the Matrex Gel Red A column containing high 3HBDH activity were pooled and concentrated by ultrafiltration through a YM30 membrane in a stirred cell to about 4 ml. The concentrated sample was applied to a Sephacryl S-300 column (2.5 by 52 cm) which had been equilibrated with phosphate buffer. The flow rate was maintained at 50 ml/h, and 2.5-ml fractions were collected.

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

The purity and the subunit molecular weight of the enzyme were determined by polyacrylamide gel electrophoresis (PAGE) on a 12.5% gel in the presence of SDS (12). Before being applied to the gel, purified 3HBDH and protein standards were equilibrated with SDS-PAGE stacking gel buffer (0.5 M Tris-Cl [pH 6.8]) through extensive washing in Centricon 3 ultrafiltration units and then heated at 100°C for 2 min in the presence of SDS and 2-mercaptoethanol. Protein was stained with Coomassie brilliant blue R-250.

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

The substrates and concentrations used during the determination of Michaelis constants for the reaction in the physiological direction, with NADH as the cosubstrate, were NADH (9.5 to 70 μM) and AcAcCoA (10 to 100 μM). The apparent K_m and V_{max} values for NADPH were measured at 100 μM AcAcCoA and 35 to 1,000 μM NADPH. The apparent K_m value for AcAcCoA with NADPH as the cosubstrate was measured at 400 μM NADPH and 9 to 100 μM AcAcCoA. Apparent K_m values for 3-hydroxybutyryl-CoA were measured at 100 μM NAD^+ or 10 mM $NADP^+$ and 17 to 100 μM 3-hydroxybutyryl-CoA. Apparent K_m values for NAD^+ and $NADP^+$ were measured at 200 μM 3-hydroxybutyryl-CoA and either 7.5 to 75 μM NAD^+ or 1 to 10 mM $NADP^+$.

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

Determination of the stereospecificity of 3HBDH. The stereospecificity for 3-hydroxybutyryl-CoA of 3HBDH from *C. beijerinckii* was determined by comparison with the pig heart enzyme, which is specific for the (*S*)-enantiomer (14). In one set of assays, a reaction mixture (1.5 ml) containing 50 mM Na-MES (pH 5.5), 200 μM AcAcCoA, and 200 μM NADH was allowed to react in air in the presence of either the pig heart enzyme (5 U) or the enzyme from *C. beijerinckii* (5 U) until no further change in A_{340} was observed (about 2 min; about 75% of AcAcCoA was reduced). After the initially present enzyme had been removed by passing the solution through a Centricon 30 ultrafiltration unit, the other enzyme was added to the filtrate along with 3 mM NAD^+. The oxidation of 3-hydroxybutyryl-CoA was monitored by observing the increase in A_{340} as a result of production of NADH. In a second set of assays, a reaction mixture (1.5 ml) containing 200 mM Tris-Cl (pH 8.0), 100 μM (*RS*)-3-hydroxybutyryl-CoA, 3 mM NAD^+, and 8 U of the pig heart enzyme was allowed to react in air until no further change in A_{340} was observed (about 3 min; about 50% of 3-hydroxybutyryl-CoA was oxidized). The reaction mixture was then passed through a Centricon 30 ultrafiltration unit to remove the pig heart enzyme. 3HBDH from *C. beijerinckii* (5 U) was added to the filtrate, and the A_{340} of the reaction mixture was monitored.

Determination of the N-terminal amino acid sequence. The N-terminal amino acid sequence of purified 3HBDH was determined with an Applied Biosystems Model 477A sequencer (35 cycles by C. L. Rutherford and R. Peery of the Protein Sequencing Facility, Virginia Polytechnic Institute and State University, Blacksburg). The enzyme sample was washed extensively with distilled water in a Centricon 30 ultrafiltration unit before analysis. Approximately 50 μg of protein was used for the analysis.

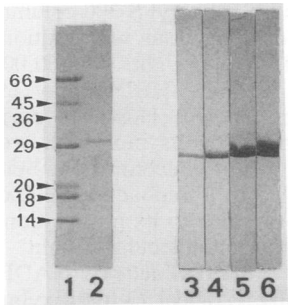

FIG. 1. Examination of purity and estimation of subunit molecular weight of 3HBDH from *C. beijerinckii* NRRL B593 by SDS-PAGE. Lanes 1 and 2 contain protein standards and purified 3HBDH, respectively. Lanes 3, 4, 5, and 6 contain approximately 4, 8, 16, and 24 μg, respectively, of purified 3HBDH. Protein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

RESULTS

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

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

When crude extracts were fractionated by successive gel

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

Step	Amt of protein (mg)	Total activity[a] (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	1,180	9,050	7.65	1.0	100
Q-Sepharose	156	6,780	43.5	5.7	75
Matrex Gel Red A	18.7	4,560	244	32	50
Sephacryl S-300	6.4	2,060	323[b]	42	23

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

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

filtration steps on a Sephacryl S-300 column, 3HBDH activity was recovered in fractions with elution volumes corresponding to molecular weights of 360,000, 235,000, and 120,000, suggesting that 3HBDH may exist as different multimers (data not shown). However, purified 3HBDH did not show any change in its molecular weight (213,000 ± 6,000) when samples at concentrations between 3 and 0.05 mg/ml were applied to a Sephacryl S-300 column. It is not clear why 3HBDH, prior to its purification, would undergo apparent changes in its molecular weight.

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

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

Stereospecificity of 3HBDH. (*S*)-3-Hydroxybutyryl-CoA, produced by the pig heart 3-hydroxyacyl-CoA dehydrogenase, was oxidized by the *C. beijerinckii* enzyme (data not shown). 3-Hydroxybutyryl-CoA produced by 3HBDH from *C. beijerinckii* was also oxidized by the pig heart enzyme. Furthermore, when 3HBDH from *C. beijerinckii* was added to an assay solution that originally had contained a mixture of the (*R*)- and (*S*)-enantiomers of 3-hydroxybutyryl-CoA but had reacted with the (*S*)-specific pig heart enzyme [leaving primarily (*R*)-3-hydroxybutyryl-CoA], no further reaction was observed. The 3HBDH from *C. beijerinckii* was therefore specific for the (*S*)-enantiomer of 3-hydroxybutyryl-CoA.

Kinetic studies. Values of the kinetic constants for the physiological reaction (3-hydroxybutyryl-CoA formation) involving NADH were obtained by using replots of slopes and intercepts of double-reciprocal plots (Fig. 2). The maximal velocity of the enzyme was 540 U/mg, with a k_{cat} value of 115,000 min^{-1}. The K_m for NADH was 8.6 μM, and that for AcAcCoA was 14 μM.

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

The presence of 200 μM CoA in the assay mixture caused less than 10% inhibition of 3HBDH activity when the concentrations of NADH and AcAcCoA were 100 and 60 μM, respectively. Therefore, the inhibition of 3HBDH by AcAcCoA was not caused by the possible contamination of the AcAcCoA preparation with CoA.

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

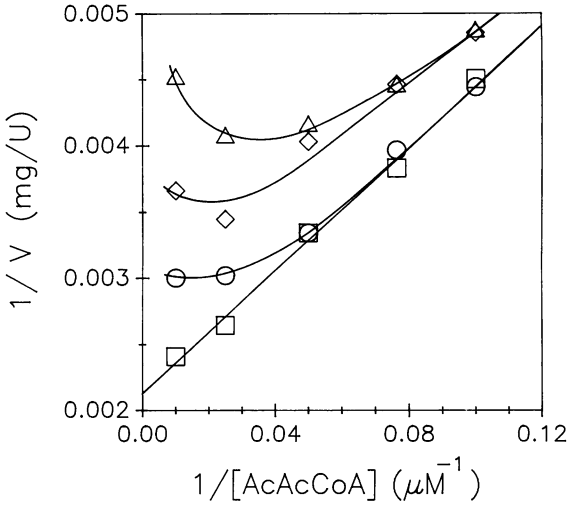

FIG. 2. Double-reciprocal plot of initial velocity data in the physiological direction with AcAcCoA as the variable substrate and NADH as the changing fixed substrate. Details are described in Materials and Methods. NADH concentrations were 70 μM (□), 28 μM (○), 14 μM (◇), and 9.5 μM (△). AcAcCoA concentrations were 100, 40.1, 20.0, 13.1, and 10.0 μM.

N-terminal amino acid sequence. The N-terminal amino acid sequence of 3HBDH from *C. beijerinckii* NRRL B593 was Met-Lys-Lys-Ile-Phe-Val-Leu-Gly-Ala-Gly-Thr-Met-Gly-Ala-Gly-Ile-Val-Gln-Ala-Phe-Ala-Gln-Lys-Gly-(xxx)-Glu-Val-Ile-Val-Arg-Asp-Ile-Lys-Glu-Glu-. The identity of residue 25 has not been determined. Of 34 amino acid residues identified in the *C. beijerinckii* enzyme, 32 are identical to the N-terminal amino acid sequence deduced from the gene encoding 3HBDH in *C. acetobutylicum* P262 (32). In the *C. beijerinckii* enzyme, residues 2 and 34 were lysine and glutamate, respectively, whereas residues 2 and 34 of the *C. acetobutylicum* enzyme are glutamate and aspartate, respectively. According to the rules of Chou and Fasman (4), these sequence differences should not disrupt the predicted β-α-β secondary structure in this region for pyridine nucleotide binding (32).

DISCUSSION

Purification of *C. beijerinckii* 3HBDH to homogeneity resulted in a 45-fold increase in specific activity over that of the crude extract. The NADP-specific 3HBDH was previously purified from *C. kluyveri* by two research groups (13, 22), and they achieved 14- and 18-fold purification. These results suggest that 3HBDH is present at high levels, which may be a characteristic of the butyrate-producing clostridia.

3HBDH from *C. beijerinckii* shares a nearly identical N-terminal region with the enzyme from *C. acetobutylicum*, including a putative dinucleotide-binding site. The subunit size of the *C. beijerinckii* enzyme (M_r, 30,800) is comparable

to the deduced size of the *C. acetobutylicum* enzyme (M_r, 31,435). Youngleson et al. (32) noted that significant sequence similarities are present among the *C. acetobutylicum* 3HBDH, the pig heart muscle mitochondrial 3-hydroxyacyl-CoA dehydrogenase, and the 3-hydroxyacyl-CoA dehydrogenase portion of the rat peroxisomal bifunctional enzyme. The subunit size and kinetic properties (14, 15) of the pig heart muscle mitochondrial 3-hydroxyacyl-CoA dehydrogenase are similar to those of the *C. beijerinckii* enzyme, and both are inhibited by AcAcCoA at concentrations near their K_m values. This is further evidence of a possible common evolutionary origin of the two enzymes.

Birktoft et al. (1) have determined the three-dimensional structure of the pig heart mitochondrial enzyme and proposed a model for acyl-CoA binding in which the CoA moiety is not specifically bound by the enzyme. Our observations that the enzyme is inhibited by AcAcCoA, but not by CoA, lend support to this model. The kinetic and physical properties of the rat peroxisomal enzyme are not as similar (16) to those of 3HBDH from *C. beijerinckii* as are those of the pig heart enzyme.

Although 3HBDH from *C. beijerinckii* is very similar in subunit size and kinetic properties to the NADH-linked AcAcCoAR of *Alcaligenes eutrophus* (9), 3HBDH was specific for (*S*)-3-hydroxybutyryl-CoA, whereas the AcAc CoAR lacks stereospecificity. The kinetic constants and subunit sizes of the *C. beijerinckii* 3HBDH and several other AcAcCoARs (5, 9, 17–19, 21) did not show any significant similarity.

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

The broad pH profile of the enzyme activity suggests that it remains active throughout the internal pH range (6.7 to 5.5) reported for the *C. acetobutylicum* cell (2, 7, 11, 25). Although the in vivo levels of AcAcCoA have not been determined for *C. beijerinckii* or reported for any other solvent-producing clostridia, the fact that inhibition by AcAcCoA was observed at concentrations at or above the Michaelis constant when the NADH concentration was near the Michaelis constant suggests that the metabolic intermediate plays some role in the regulation of 3HBDH activity in vivo. It is possible that the inhibition of 3HBDH by AcAc CoA when there is not a great excess of NADH allows the cell to generate more ATP from acetyl-CoA and to maintain a redox balance. It is known that 1 mol of ADP may be phosphorylated per mol of acetyl-CoA if the phosphate is derived from acetylphosphate, whereas only 0.5 mol of ADP may be phosphorylated per mol of acetyl-CoA if the phosphate is derived from butyrylphosphate. Inhibition of 3HBDH by AcAcCoA may therefore serve to increase the energetic efficiency. Under conditions where the NADH concentration rises, the inhibition by AcAcCoA is relieved so that more 3-hydroxybutyryl-CoA may be formed to lead to the production of butyrate or butanol, thereby allowing the oxidation of more NADH. A more complete understanding of the role of AcAcCoA in the regulation of metabolism in *C. beijerinckii* awaits the characterization of the other AcAcCoA-reacting enzymes from this organism.

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