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Purification and Properties of 4-Hydroxybutyrate Coenzyme A Transferase from *Clostridium aminobutyricum*

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A new coenzyme A (CoA)-transferase from the anaerobe *Clostridium aminobutyricum* catalyzing the formation of 4-hydroxybutyryl-CoA from 4-hydroxybutyrate and acetyl-CoA is described. The enzyme was purified to homogeneity by standard techniques, including fast protein liquid chromatography under aerobic conditions. Its molecular mass was determined to be 110 kDa, and that of the only subunit was determined to be 54 kDa, indicating a homodimeric structure. Besides acetate and acetyl-CoA, the following substrates were detected (in order of decreasing k_{cat}/K_m): 4-hydroxybutyryl-CoA, butyryl-CoA and propionyl-CoA, vinylacetyl-CoA (3-butenoyl-CoA), and 5-hydroxyvaleryl-CoA. In an indirect assay the corresponding acids were also found to be substrates; however, DL-lactate, DL-2-hydroxybutyrate, DL-3-hydroxybutyrate, crotonate, and various dicarboxylates were not.

The fermentation of 4-aminobutyrate to ammonia, acetate, and butyrate in *Clostridium aminobutyricum* proceeds via 4-hydroxybutyrate (Fig. 1A) (7, 8). Recently, the key step of the pathway, the unusual dehydration of this hydroxy acid, has been measured in cell extracts by the release of tritiated water from 4-hydroxy[3-^{3}H]butyrate (25). This reaction is of considerable mechanistic interest, since the C-^{3}H bond which has to be cleaved is too far away to be activated by the carboxyl group. The requirement of acetyl coenzyme A (acetyl-CoA) for this reaction has suggested the formation of 4-hydroxybutyryl-CoA (Fig. 1B) prior to the dehydration (25). Therefore, the availability of 4-hydroxybutyryl-CoA is a prerequisite for the purification of the dehydratase and for studying the dehydration mechanism. However, attempts to synthesize this CoA-ester by the mixed-anhydride method (11, 24) failed, most likely because of the facile formation of γ-butyrolactone. Thus, an enzymatic approach was developed. This paper describes the purification of a new CoA-transferase from *C. aminobutyricum* which was successfully applied in the preparation of 4-hydroxybutyryl-CoA (Fig. 1B) as well as vinylacetyl-CoA (Fig. 1C).

MATERIALS AND METHODS

Assays. 4-Hydroxybutyrate CoA-transferase (EC 2.8.3.–) was routinely assayed with acetate and butyryl-CoA as substrates (25). This analog was used instead of 4-hydroxybutyryl-CoA because of the instability and laborious preparation of the latter (see below). In the assay the formed acetyl-CoA was condensed with oxaloacetate, thereby liberating CoASH, which reacted with 5,5′-dithio-bis-(2-nitrobenzoate) to a yellow thiophenolate anion. The cuvette contained, in a total volume of 1 ml at 25°C, 100 mM potassium phosphate (pH 7.0), 200 mM sodium acetate, 1 mM oxaloacetate, 1 mM 5,5′-dithio-bis-(2-nitrobenzoate), 0.1 mM butyryl-CoA, and 7.5 nkat of citrate synthase (EC 4.1.3.7; Boehringer, Mannheim, Germany). The reaction was initiated by the addition of CoA-transferase (up to 2 nkat). The initial rates were determined at 412 nm. The dependence of the activity on the pH was measured with the universal buffer system (15). Acetyl-, butyryl-, and crotonyl-CoA were prepared by the method of Simon and Shemin (20). Vinylacetyl-CoA Δ-isomerase (EC 5.3.3.3) was assayed as described in reference 14, with 140 μM vinylacetyl-CoA (Fig. 1C; see below) instead of 3-hexenoyl-CoA as the substrate. Protein was determined either by the biuret method (6) (cell extract) or by the Lowry method (16) using bovine serum albumin standards.

Cultivation of bacteria. *C. aminobutyricum* DSM 2643 was grown on a modified 4-aminobutyrate–yeast extract medium (7, 25). One liter of medium contained 10 g of 4-aminobutyric acid, 3 g of yeast extract (Merck, Darmstadt, Germany), 2 g of $NaHCO_3$, 50 mM potassium phosphate (pH 7.4), 200 mg of $MgCl_2 \cdot 6H_2O$, 10 mg of $FeCl_3 \cdot 6H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 1 mg of $MnSO_4 \cdot H_2O$, 1 mg of $Na_2MoO_4 \cdot H_2O$, 500 mg of cysteine-HCl (neutralized), and 350 mg of sodium dithionite (pH 7.4). Cultures (up to 1 liter) were shaken under an atmosphere of nitrogen. Larger cultures (up to 100 liters) were stirred (160 rpm) under an atmosphere of 80% N_2 and 20% CO_2. No dithionite had to be added to large cultures.

Purification of 4-hydroxybutyrate CoA-transferase. All of the following steps were performed at 4°C under aerobic conditions. Frozen cells (30 g) of *C. aminobutyricum* were suspended in 60 ml of 50 mM potassium phosphate, pH 6.8, containing 10 mM $MgCl_2$ and 0.6 mg of DNase I. The bacteria were lysed by three passes through a French press at 70 MPa and centrifuged at 130,000 × *g* for 60 min. The clear supernatant (cell extract) was fractionated by addition of a saturated ammonium sulfate solution (55 to 80% saturation). The precipitate was dissolved in 50 mM potassium phosphate (pH 6.8)–1.5 M ammonium sulfate (22 ml) and was applied on a phenyl-Sepharose column (5 by 4 cm; Pharmacia, Freiburg, Germany) equilibrated with 50 mM potassium phosphate (pH 6.8)–1.5 M ammonium sulfate. The enzyme was eluted with a decreasing linear ammonium sulfate gradient (1.5 to 0 M) in 360 ml of 50 mM potassium phosphate, pH 6.8, followed by 370 ml of 50 mM potassium phosphate, pH 6.8 (flow rate, 3 ml/min). The fractions containing enzymatic activity (97 ml) were concentrated to 40 ml by ultrafiltration (PM 30 membrane; Amicon, Witten, Germany).

In the following step, the enzyme solution was applied on a DEAE-Sephacel column (5 by 7.5 cm; Pharmacia) in 50

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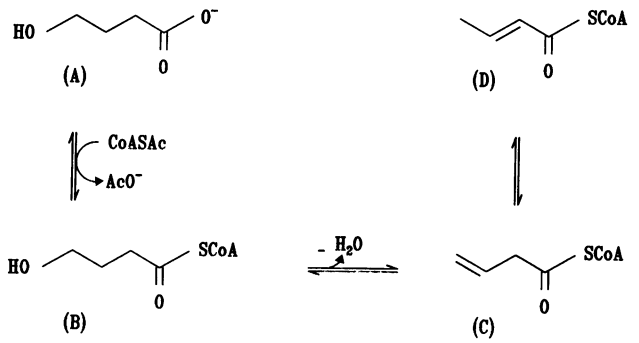

FIG. 1. Proposed conversion of 4-hydroxybutyrate (A) to crotonyl-CoA (D) by *C. aminobutyricum*. The formation of vinylacetyl-CoA (C) from 4-hydroxybutyryl-CoA (B) remains to be established.

mM potassium phosphate, pH 6.8. After washing with 100 ml of this buffer, the enzyme was eluted by linearily increasing the potassium phosphate concentration to 400 mM (360 ml) followed by 370 ml of 1 M potassium phosphate, pH 6.8 (flow rate, 3 ml/min). The concentrated active fractions were chromatographed on the molecular sieve Superdex 200 HiLoad 26/60 column (Pharmacia) which was equilibrated with 20 mM potassium phosphate (pH 6.8)–100 mM NaCl and connected to a fast protein liquid chromatography (FPLC) system (Pharmacia) at a flow rate 2 ml/min. The final purification of the CoA-transferase was achieved by FPLC on Mono Q HR 10/10 equilibrated with 50 mM potassium phosphate, pH 6.8 (flow rate, 2 ml/min). The enzyme was eluted by a linear KCl gradient from 0 to 150 mM (50 ml) and 150 to 300 mM (150 ml) in the same buffer. After concentration of the active fractions (150 to 200 mM KCl, 32 ml) to 7 ml, the enzyme was frozen in liquid nitrogen and stored at −80°C.

Determination of the molecular mass. The molecular mass of the native enzyme was determined by chromatography on a Superose 12 column (Pharmacia) in 50 mM potassium phosphate (pH 6.8)–100 mM NaCl (flow rate, 0.4 ml/min). The following standards were used: catalase (240 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and cytochrome *c* (13 kDa). The molecular mass of the subunit was determined by polyacrylamide gel electrophoresis (PAGE) (13% acrylamide) in the presence of sodium dodecyl sulfate (SDS) (13). The standards were obtained from Pharmacia.

Preparation of 4-hydroxybutyryl-CoA and vinylacetyl-CoA. The incubation mixture contained, in a total volume of 1 ml, 125 mM potassium phosphate (pH 7.0), 42 mM 4-hydroxybutyrate or vinylacetate (3-butenoate), 4.2 mM acetyl-CoA, and 40 nkat of 4-hydroxybutyrate CoA-transferase. After 20 min at 37°C, the enzyme was removed by ultrafiltration (Centricon 30; Amicon). The product was purified by high-performance liquid chromatography (HPLC) on a LiChrosorb column (250 by 10 mm, RP-18 100, 7 μm; Merck) with 10 mM HCl and linear methanol gradients (0 to 5 min, 15 to 20% methanol; 5 to 20 min, 20 to 30% methanol) as solvents (flow rate, 4 ml/min). The fractions containing the desired CoA-ester were combined, concentrated under vacuum to about 50 μl, and dissolved in 1 ml of water. In the case of 4-hydroxybutyryl-CoA the residual acetyl-CoA was hydrolyzed after ultrafiltration by incubation with 13 mM oxaloaceate and 12 μg of citrate synthase for 20 min at 37°C. The enzyme was then precipitated with 100 mM HCl, and the product was purified as described above. The retention times on an analytical HPLC column (250 by 5 mm; flow rate, 1 ml/min) operated with 50 mM KH_2PO_4 and methanol gradients (same as before) were determined to be 2.6 min (CoASH), 4.3 min (acetyl-CoA and 4-hydroxybutyryl-CoA) (Fig. 1B) 9.2 min (vinylacetyl-CoA) (Fig. 1C), and 10.6 min (crotonyl-CoA) (Fig. 1D). The concentrations of vinylacetyl-CoA, 4-hydroxybutyryl-CoA, propionyl-CoA, and butyryl-CoA were also determined by end-point analysis with the standard assay. 5-Hydroxyvaleryl-CoA was prepared as recently described (5). It was purified as described above.

TABLE 1. Purification of 4-hydroxybutyrate CoA-transferase

Step	Protein (mg)	Activity (μkat)	Sp act (μkat/g of protein)	Yield (%)
Cell extract	1,137	81	71	100
Ammonium sulfate	383	49	129	61
Phenyl-Sepharose	97	28	290	35
DEAE-Sephacel	79	26	327	32
Superdex 200	29	20	680	24
Mono Q	7.7	17	2,210	21

RESULTS AND DISCUSSION

The purification of 4-hydroxybutyrate CoA-transferase is summarized in Table 1. A homogeneous enzyme was obtained with 21% yield and a specific activity of 2.2 μkat/mg of protein (butyryl-CoA as the substrate) or 4.5 μkat/mg (4-hydroxybutyryl-CoA as the substrate), which compared well with that of other CoA-transferases (see, e.g., reference 3). The DEAE-Sephacel step of the purification procedure, although not very efficient, was necessary to obtain a pure preparation by the final anion-exchange chromatography on Mono Q. The 31-fold purification already leading to a homogeneous enzyme shows that the CoA-transferase represents 3% of the soluble protein of the organism, which one might expect for a component of the energy-conserving fermentation pathway. By gel filtration the molecular mass of the native enzyme was determined to be 110 kDa, whereas PAGE in the presence of SDS revealed a band of 54 kDa. Hence, a homodimeric quaternary structure appeared most likely. The same quaternary structure was found in 3-oxoacid CoA-transferase from pig heart (EC 2.8.3.5) (10) and in acrylate CoA-transferase from *Megasphaera elsdenii* (23), whereas other bacterial CoA-transferases have different structures, such as a monomer (1), a homotetramer (4, 18), and a heterotetramer (2, 21), or even a heterooctamer (3).

4-Hydroxybutyrate CoA-transferase is a colorless enzyme with no additional absorbance between 400 nm and the protein peak at 280 nm. The highest rates of the CoA-transferase activity were obtained at around pH 9.5; at pH 7, the enzyme was only half as active. In order to ascertain the substrate specificity of the enzyme, competition experiments were performed. An assay was used in which 7.5 mM acetate competed with the acid of interest (at 7.5 mM) for the CoAS moiety of butyryl-CoA. Thus, a good substrate should decrease the rate of acetyl-CoA formation, the measure of enzymatic activity. A strong apparent inhibition was observed with 4-hydroxybutyrate (Fig. 1A), followed by butyrate, propionate, and vinylacetate, whereas 5-hydroxyvalerate only had a small effect (Table 2). All other compounds were not inhibitory, notably crotonate, 2- and 3-hydroxybutyrate, and dicarboxylates.

The formation of 4-hydroxybutyryl-CoA (Fig. 1B) from

TABLE 2. Inhibition of the formation of acetyl-CoA from butyryl-CoA by various potential substrates of 4-hydroxybutyrate CoA-transferase[a]

Acid added	Relative activity (%)[b]
None	100
4-Hydroxybutyrate	12
Propionate	31
Butyrate	31
Vinylacetate (3-butenoate)	48
5-Hydroxyvalerate	79
Acrylate	88
n-Valerate	92

[a] Each assay contained 100 mM potassium phosphate (pH 7.0), 7.5 mM sodium acetate, 7.5 mM of the indicated acid, 1 mM oxaloacetate, 1 mM 5,5'-dithio-bis-(2-nitrobenzoate), 0.1 mM butyryl-CoA, 7 nkat of citrate synthase, and 2 nkat of 4-hydroxybutyrate CoA-transferase. The initial rates were determined at 412 nm.

[b] No inhibition was observed with DL-3-hydroxybutyrate, DL-2-hydroxybutyrate, DL-lactate, acetoacetate, crotonate, fumarate, succinate, and glutarate.

acetyl-CoA and 4-hydroxybutyrate catalyzed by CoA-transferase was indicated by the rapid cleavage of the γ-hydroxy thiolester at pH 7.0 (Table 3) due to the facile formation of γ-butyrolactone (tetrahydrofuran-2-on). Thus, addition of the enzyme to the complete reaction mixture resulted in the formation of CoASH as measured with 5,5'-dithio-bis-(2-nitrobenzoate). If 4-hydroxybutyrate (Fig. 1A) was replaced by butyrate, no CoASH was liberated, since the stable butyryl-CoA was formed. The third experiment (Table 3) also demonstrated that only 4-hydroxybutyrate, which was added last, caused the liberation of CoASH.

4-Hydroxybutyrate CoA-transferase was applied to prepare 4-hydroxybutyryl-CoA as well as vinylacetyl-CoA (Fig. 1C) free of crotonyl-CoA (Fig. 1D) as analyzed by HPLC. This was possible since the enzyme was completely free of vinylacetyl-CoA Δ-isomerase, which was present in large amounts in the cell extract (38 nkat/mg) (also see reference 9). Table 4 shows the K_m and K_{cat} values of the various CoA-ester substrates of 4-hydroxybutyrate CoA-transferase. The data clearly demonstrate that 4-hydroxybutyryl-CoA is by far the best substrate for the enzyme, which was also indicated by the competition experiments (Table 2).

4-Hydroxybutyrate CoA-transferase was absolutely necessary for the assay of purified 4-hydroxybutyryl-CoA dehydratase in which the formation of ^{3}HOH was measured in an incubation containing 4-hydroxy[3-^{3}H]butyrate, acetyl-CoA, CoA-transferase, and dehydratase. Omission of either acetyl-CoA or CoA-transferase resulted in a complete loss of

TABLE 3. Formation and hydrolysis of 4-hydroxybutyryl-CoA[a]

First addition	Activity (pkat)	Second addition	Activity (pkat)
4-Hydroxybutyrate (1 mM)	0	CoA-transferase[b] (11 nkat)	430
Butyrate (1 mM)	0	CoA-transferase (11 nkat)	0
CoA-transferase (11 nkat)	0	4-hydroxybutyrate (1 mM)	470

[a] Each assay contained, in a total volume of 0.5 ml at 25°C, 50 mM morpholinepropanesulfonic acid (MOPS) (pH 7.5), 10 mM $MgCl_2$, 1 mM 5,5'-dithio-bis-(2-nitrobenzoate), 0.2 mM acetyl-CoA, and the indicated additions. The initial rates were determined at 412 nm.

[b] CoA-transferase, pure 4-hydroxybutyrate CoA-transferase.

TABLE 4. Kinetic constants of CoA-ester substrates of 4-hydroxybutyrate CoA-transferase[a]

CoA-ester	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
4-Hydroxybutyryl-CoA	100	241	2.4
Butyryl-CoA	240	119	0.50
Propionyl-CoA	240	120	0.50
Vinylacetyl-CoA	260	68	0.26
5-Hydroxyvaleryl-CoA	500	30	0.06
Crotonyl-CoA	—[b]	0	—

[a] The second substrate was acetate at a concentration of 200 mM.

[b] —, not measurable.

activity (17). Obviously, dehydration of any hydroxymonocarboxylic acid requires activation to the CoA-ester; examples are 2-hydroxyglutaryl-CoA dehydratase (19), crotonase (3-hydroxyacyl-CoA dehydratase, EC 4.2.1.17), and 5-hydroxyvaleryl-CoA dehydratase (5).

In recent years there has been increasing scientific as well commercial interest in biodegradable polyhydroxy fatty acids which are synthesized by several bacteria from the corresponding CoA esters (for a review, see reference 22). Although the most commonly used monomer is (*R*)-3-hydroxybutyric acid, there is a report on the improved properties of copolymers with 4-hydroxybutyric acid (12). Thus, 4-hydroxybutyrate CoA-transferase may become an useful tool for the synthesis of such plastics starting from 4-hydroxybutyrate. An alternative route would be the conversion of the common intermediate crotonyl-CoA to 4-hydroxybutyryl-CoA (Fig. 1).

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