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The Purification and Characterization of Acetoacetyl-Coenzyme A Reductase from *Azotobacter beijerinckii*

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A soluble acetoacetyl-CoA reductase (EC 1.1.1.36) was purified 54-fold from *Azotobacter beijerinckii* N.C.I.B. 9067 and the reaction product identified as D(−)-β-hydroxybutyryl-CoA. The Michaelis constants for acetoacetyl-CoA, NADPH and NADH were determined and the reaction rate was found to be some fivefold greater with NADPH than with NADH. At neutral pH the equilibrium greatly favours the formation of the reduced product. Substrate specificity was in the order: acetoacetyl-CoA > acetoacetylpantetheine > acetoacetyl-(acyl-carrier protein). The enzyme possesses a functional thiol group, suffers inactivation by oxygen and is inhibited by thiol-blocking reagents. Inhibition by *p*-chloromercuribenzoate is reversed by excess of dithiothreitol, which also protects the enzyme from inactivation by oxygen.

Our interest in the biosynthesis of the storage compound poly-β-hydroxybutyrate in bacteria has led us to purify and characterize the enzyme acetoacetyl-CoA reductase [D(−)-β-hydroxybutyryl-CoA–NADP oxidoreductase, EC 1.1.1.36] found in *Azotobacter beijerinckii*, an organism capable of accumulating the polymer to the extent of 70% of its dry weight (Stockdale, Ribbons & Dawes, 1968).

Nicotinamide nucleotide-linked acetoacetyl-CoA dehydrogenases have been detected in several micro-organisms (Schindler, 1964; Kominek & Halvorson, 1965; Carr & Lascelles, 1961; Stern, del Campillo & Raw, 1956; Overath, Raufuss, Stoffel & Ecker, 1967). The reduction of acetoacetyl-CoA or oxidation of β-hydroxybutyryl-CoA by crude extracts of *Hydrogenomonas* H16 proceeded more rapidly with NAD than with NADP but the stereospecificity of the product, β-hydroxybutyryl-CoA, was not determined (Schindler, 1964).

The enzyme from *Bacillus cereus* was found to be NADH-specific and induced coincidently with the accumulation of poly-β-hydroxybutyrate before sporulation. The reaction product of this enzyme was not determined (Kominek & Halvorson, 1965).

The biosynthesis of poly-β-hydroxybutyrate in *Rhodospirillum rubrum* has been shown to proceed by a pathway involving the reduction of acetoacetyl-CoA to L(+)-β-hydroxybutyryl-CoA, which was then converted into D(−)-β-hydroxybutyryl-CoA via crotonyl-CoA, the conversion being catalysed by two stereospecific enoyl-CoA hydratases. There was no evidence for the presence of a specific D(−)-β-hydroxybutyryl-CoA dehydrogenase (Moskowitz & Merrick, 1969). The present work demonstrates the occurrence of a specific D(−)-β-hydroxybutyryl-CoA dehydrogenase in *A. beijerinckii* and indicates a major difference between the routes of poly-β-hydroxybutyrate synthesis in this organism and *R. rubrum*.

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MATERIALS AND METHODS

Culture of organism. The organism was *Azotobacter beijerinckii* N.C.I.B. 9067. The basal medium (nitrogen-free) was prepared from two solutions of the following composition (g/l). Solution A: glucose, 20.0; $MgSO_4,7H_2O$, 0.4; $CaCl_2$, 0.11; $FeSO_4,7H_2O$, 0.012; $Na_2MoO_4,2H_2O$, 0.01. Solution B: K_2HPO_4, 2.0; NaCl, 0.40. The two solutions were autoclaved separately (15lb/in^2 for 20min) and combined in equal volumes after cooling. The pH of the complete medium was 7.7.

Liquid cultures (1 litre) were grown in 4-litre wide-neck Erlenmeyer flasks on a Gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 30°C by using a 1% inoculum of stationary-phase cells approx. 24h old. Large quantities of the organism were cultured in a 20-litre fermenter with an aeration rate of 20 litres/min and automatic pH control at 7.4. Growth was determined turbidimetrically at 500nm in a Unicam SP.600 spectrophotometer. The dry weight of the cells was determined from curves relating turbidity and dry weight.

Harvesting of cultures. The organism was difficult to harvest since the cells possessed thick slime-capsules. Small volumes were centrifuged at 5000*g* for 45min at

0°C (MSE Mistral 6L, rotor no. 69167), after which most of the supernatant could be decanted. Larger volumes were collected by continuous-flow centrifugation at 9000*g* with a flow rate of 70ml/min (Griffin Christ Junior 15000, with 300ml-capacity rotor).

Preparation of cell extracts. Cell extracts were prepared by disruption of cells in a French pressure cell (Milner, Lawrence & French, 1950). Harvested cells were resuspended in 67mM-Na_2HPO_4–KH_2PO_4 buffer, pH7.2, at 4°C and passed twice through the precooled pressure cell in 20ml portions. Large quantities of cells were disrupted by ultrasonic treatment with a Soniprobe type 1130A (Dawe Instruments Ltd., London W.3, U.K.) in combination with a cooled all-glass flow-through vessel of 25ml capacity. Washed cells were suspended in 0.2M-KH_2PO_4–K_2HPO_4 buffer, pH7.2, containing 10mM-2-mercaptoethanol, and passed through the ultrasonic vessel at 20ml/min and at 8A current. The crude cell extracts were centrifuged at 30000*g* for 20min at 0°C (Sorvall RC-2B, rotor SS-34) and the resulting supernatant used as starting material for enzyme purification.

Preparations. Acetoacetyl-CoA was prepared by treating CoA with diketen. CoA (20mg) was dissolved in 2ml of cold 0.2M-$KHCO_3$ through which a stream of N_2 was passed. Diketen (0.1ml) in 1ml of diethyl ether was added over 30min. Excess of diketen was removed in a stream of N_2, and the reaction mixture was adjusted to pH4.0 with 1.0M-HCl and extracted four times with 3ml of diethyl ether. Ether was removed from the aqueous layer in a stream of N_2. The yield based on added CoA was 90–95% by spectrophotometric assay at 303nm in 0.1M-tris–HCl buffer, pH8.8, containing 20mM-$MgCl_2$, and by using the value $\epsilon = 2.2 \times 10^7 \cdot mol^{-1} \cdot cm^{-1}$ (Beinert, 1953); however, the yield was considerably lower (50–70%) when based on acetoacetyl-CoA measured with L-β-hydroxybutyryl-CoA dehydrogenase as described below. The discrepancy was presumably due to side reactions with diketen, and the enzymically determined concentrations have been used throughout.

Acetoacetylpantetheine was synthesized by treating a threefold excess of diketen with pantetheine at room temperature for 1.5h before extraction and assay at 303nm in the way described above for acetoacetyl-CoA. Pantetheine was prepared by reduction of D-pantethine with KBH_4.

D(−)-β-Hydroxybutyryl-CoA was prepared by a procedure described by Ritchie & Dawes (1969).

The preparation of acetoacetyl-(acyl-carrier protein) was analogous to the method of Alberts, Majerus, Talamo & Vagelos (1964). Freeze-dried acyl-carrier protein (1.0μmol, 11.0mg) was dissolved in 2ml of 10mM-KH_2PO_4–K_2HPO_4 buffer, pH6.3, containing 10mM-2-mercaptoethanol and allowed to stand at 4°C for 6h. The protein solution was applied to a DEAE-cellulose column (1cm × 4cm) previously equilibrated with 10mM-KH_2PO_4–K_2HPO_4 buffer, pH6.3, at room temperature. The column was washed with the same buffer until all free thiol material had been eluted. The reduced acyl-carrier protein was eluted with the above buffer containing 0.5M-LiCl and monitored by measurement of extinction at 280nm by using microcuvettes (1cm light-path) and a Unicam SP.500 spectrophotometer. Fractions containing protein were combined and the pH was adjusted to 8.0 with 0.2M-$KHCO_3$. The reduced acyl-carrier protein was treated at 2°C for 45min with diketen (40μl) in 1ml of ether in a stream of O_2-free N_2. The reaction mixture was acidified to pH3.5–4.0 with 0.1M-HCl and the acyl-carrier protein derivative precipitated. After centrifugation, the supernatant was removed and the derivative resuspended in 0.1M-HCl and centrifuged again. The washing procedure was repeated three times and the acyl-carrier protein derivative redissolved in 2.0ml of 0.25M-imidazole–HCl buffer, pH6.2.

Acetoacetyl-(acyl-carrier protein) was determined as acetoacetate by the method of Walker (1954) after alkaline hydrolysis under the following conditions. Acetoacetyl-(acyl-carrier protein) (50nmol of protein) was incubated with 0.1ml of 1M-NaOH for 90min at 30°C. Acyl-carrier protein was precipitated by addition of 0.40ml of 3M-HCl and the supernatant solution taken for determination of acetoacetate. Standard solutions of lithium acetoacetate were treated in the same way and compared with untreated standards. Acetoacetate was not destroyed by the hydrolytic procedure.

Preparation of the Sepharose 4B column. Sepharose 4B (Pharmacia, Uppsala, Sweden) was obtained as a preswollen gel. The gel was diluted with the eluting buffer (5mM-KH_2PO_4–K_2HPO_4 buffer, pH7.4, containing 0.01M-2-mercaptoethanol) and loaded in one volume on to a column (2.7cm × 47cm). During preparation of the column a pressure head of 10cm was maintained and this was increased to 25cm before use. A circle of Whatman no. 54 paper was placed on top of the column. The flow rate during chromatography was 4.7ml/h per cm^2 and the void volume, determined with 0.5% Blue Dextran 2000 immediately after chromatography, was 88ml for the column bed volume of 270ml.

Electrophoresis. Electrophoresis was carried out in a disc electrophoresis apparatus (Shandon Scientific Co. Ltd., London N.W.10, U.K.) by using the standard procedures given by the manufacturers. Gels contained 7.5% (w/v) acrylamide and protein samples were retained on the large-pore gel before electrophoresis with a small amount of dry Sephadex G-75. Electrophoresis was conducted at 2°C for 60min at 5mA/running tube.

Protein measurement. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystallized bovine serum albumin, dried to constant weight, as a standard.

Assay of acetoacetyl-CoA reductase. For assay of enzyme activity the corrected linear decrease or increase in E_{340} was measured in a Unicam SP.800 recording spectrophotometer at 30°C. For the reduction reaction each assay mixture contained (final concentrations) 60mM-KH_2PO_4–K_2HPO_4 buffer, pH5.5, 12mM-$MgCl_2$, 0.5mM-dithiothreitol, 0.24mM-NADPH, 28–40μM-acetoacetyl-CoA and 0.1ml of cell extract in a total volume of 2.5ml. The buffer, Mg^{2+}, dithiothreitol and extract were preincubated for 5min before addition of NADPH. The decrease in E_{340} was recorded for 1min before addition of acetoacetyl-CoA. There was no lag and linear initial rates were recorded for 2min.

At pH values below 6.5 there was a significant linear rate of decrease in E_{340} due to the instability of both NADH and NADPH at acid pH. For each enzyme assay it was necessary to correct the initial reaction rates for this destruction. The extinction of NADPH at 340nm was measured at both pH5.5 and 8.5. The values were identi-

cal, indicating that the published value of $\epsilon = 6.22 \times 10^3 \text{l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Horecker & Kornberg, 1948) was applicable at low pH values.

The assay mixture for the oxidation reaction contained (final concentrations) 30mM-tris–HCl buffer, pH8.5, 12mM-$MgCl_2$, 0.5mM-dithiothreitol, 0.4mM-$NADP^+$, 0.2mM-β-hydroxybutyryl-CoA and 0.1ml of cell extract in a total volume of 2.5ml.

Unit of enzyme activity. One unit of enzyme is that amount of protein which catalyses the reduction or oxidation of 1μmol of substrate/min at pH5.5 or 8.5 and at 30°C.

Enzyme purification. All procedures were carried out at 2°C. The buffers used throughout were KH_2PO_4–K_2HPO_4 mixtures at appropriate pH values and, in view of the oxygen-sensitivity of the enzyme, 2-mercaptoethanol was added to all buffers to give a final concentration of 0.01M. To the cell-free supernatant prepared from 150g dry wt. of cells was added 0.1vol. of protamine sulphate solution (10mg/ml), pH5.5, and after standing on ice for 2h the mixture was centrifuged at 30000*g* for 5min and the precipitate discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant to 30% saturation (16.4g/100ml), the suspension was held overnight and then centrifuged at 30000*g* for 10min. The small precipitate was discarded. The supernatant was increased to 60% saturation by the addition of a further 18.1g of solid $(NH_4)_2SO_4$/100ml and held for 2h at 2°C. The heavy tan-coloured precipitate was separated by centrifuging at 10000*g* for 10min, redissolved in 350ml of 0.14M buffer, pH7.2, and dialysed against two changes of 5 litres of the same buffer. The 30–60%-satd.-$(NH_4)_2SO_4$ fraction (7mg of protein/ml) was dispensed in 10ml portions and frozen at −15°C. Such fractions retained their activity for several months.

First chromatography on DEAE-cellulose. A portion of the 30–60%-satd.-$(NH_4)_2SO_4$ fraction (200mg of protein) was dialysed against 0.5mM buffer, pH7.4, adsorbed on a DEAE-cellulose column (Whatman DE32 microgranular, 2.4cm × 30cm) and washed with 100ml of the same buffer. The enzyme was eluted with a linear gradient of chloride supplied by mixing 400ml of 0.5mM buffer, pH7.4, containing 0.3M-KCl, into 400ml of 0.5mM buffer, pH7.4. The flow rate was 0.5ml/min. Protein elution was monitored by measurement of E_{280}. The enzyme was eluted in the range 0.120–0.145mM-KCl. The most active fractions were combined and concentrated in dialysis tubing covered with Aquacide (Calbiochem, Los Angeles, Calif., U.S.A.).

Second chromatography on DEAE-cellulose. Concentrated fractions containing enzyme were dialysed overnight against a 200-fold excess of 12mM buffer, pH8.0, adsorbed on a DEAE-cellulose column (1.3cm × 30cm) and washed with 50ml of the same buffer. The enzyme was eluted in a linear chloride gradient supplied by mixing 300ml of 12mM buffer, pH8.0, containing 0.25M-KCl, into 300ml of 12mM buffer, pH8.0. Enzyme was eluted in the range 0.085–0.130M-KCl. Active fractions were combined and concentrated as before.

Chromatography on Sepharose 4B. The concentrated enzyme was dialysed against two changes of 500ml of 5mM buffer, pH7.4. A sucrose solution, 0.25M, was added to the enzyme to give a final concentration of 50mM, and the solution (4.0ml) was carefully loaded on the top of a column of Sepharose 4B prepared as described above. The enzyme was chromatographed with 5mM buffer solution as used for dialysis at a flow rate of 0.5ml/min. Active fractions were combined and concentrated to 2mg of protein/ml. Volumes of 0.2ml were dispensed and stored at −15°C.

Table 1 summarizes the progress of a large-scale purification of the enzyme.

Product of the reaction. An assay mixture for the reduction reaction with increased amounts of acetoacetyl-CoA (1.36mM) and NADPH (1.44mM) together with a control mixture without acetoacetyl-CoA were incubated with purified enzyme at 30°C. On completion of the reaction the decrease of E_{340} continued due to destruction of NADPH at pH5.5. After completion of the reaction the test and control incubation mixtures were adjusted to pH12, by addition of 0.06ml of 3.5M-NaOH, to hydrolyse CoA esters and then heated at 90°C for 10min to destroy residual NADPH. After cooling, the mixtures were centrifuged and the supernatants readjusted to pH8.5 with 2M-HCl. Portions were taken as substrates for the enzymic determination of D(−)-β-hydroxybutyrate by the procedure of Williamson, Mellanby & Krebs (1962).

Table 1. *Summary of purification of acetoacetyl-CoA reductase from* A. beijerinckii

Only the most active fractions were taken at steps (5), (6) and (7).

Step	Total volume (ml)	Total activity (m-units)	Total protein (mg)	Specific activity (units/mg)	Recovery %
(1) Crude sonicated extract (150g dry wt. of cells)	2760	27.80	14960	1.86	100
(2) Supernatant after centrifuging at 29000*g* for 10min	2410	25.93	5590	4.64	93
(3) Protamine sulphate treatment	2500	19.54	4920	3.97	70
(4) $(NH_4)_2SO_4$ fractionation	340	13.59	2520	5.40	49
(5) First chromatography on DEAE-cellulose	—	8.27	200	41.30	30
(6) Second chromatography on DEAE-cellulose	—	3.27	76	43.00	12
(7) Chromatography on Sepharose 4B	—	2.00	22	90.70	7

Calcium phosphate gel. The gel was prepared by adding 150ml of $CaCl_2$ solution (132g of $CaCl_2,6H_2O$/l) to 150ml of Na_3PO_4 solution (152g of $Na_3PO_4,12H_2O$/l). The mixture was adjusted to pH7.4 with 2M-acetic acid and the precipitate washed by decantation four times with water (15–20 litres). It was stored under water in the dark.

Calcium phosphate-gel treatment. The enzyme preparation subjected to calcium phosphate-gel treatment was one that had been treated with protamine sulphate followed by $(NH_4)_2SO_4$ fractionation. The precipitate from 30–50% saturation was dissolved in a small volume of 70mM-phosphate buffer, pH7.0, and dialysed overnight against 10mM-phosphate buffer, pH6.0. To the dialysed enzyme were added small portions of gel until no activity could be detected in the supernatant. The suspension was kept on ice for 10min, centrifuged at 5000*g* for 5min and the supernatant discarded. The gel was washed by suspension in a volume of water equal to the volume of supernatant and recentrifuged. The enzyme was eluted from the gel by two successive resuspensions in equivalent volumes of 0.14M-buffer, pH6.8, and the supernatants were combined. The sevenfold-purified enzyme recorded in Table 2 was prepared in this way.

Assay of acetoacetyl-CoA. Acetoacetyl-CoA was determined enzymically at 25°C with L(+)-β-hydroxybutyryl-CoA dehydrogenase. The assay mixture contained (final concentrations): 40mM-potassium phosphate buffer, pH7.5; 0.4mM-dithiothreitol; 22μM-NADPH; 50μg of L(+)-β-hydroxybutyryl-CoA dehydrogenase (approx. 4 units); 10–50μl of acetoacetyl-CoA solution in a total volume of 2.5ml. The reaction was started by the addition of the enzyme and monitored spectrophotometrically until equilibrium was reached. The decrease in E_{340} was measured and this value used to calculate the initial concentration of acetoacetyl-CoA.

Chemicals. NADPH, CoA, diketen and D-pantethine were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.); NAD^+, NADH, L(+)-β-hydroxybutyryl-CoA dehydrogenase and D(−)-β-hydroxybutyrate dehydrogenase were from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); $NADP^+$ and crystalline bovine albumin were from British Drug Houses Ltd. (Poole, Dorset, U.K.); dithiothreitol was from Calbiochem; 2-mercaptoethanol and protamine sulphate (pure, from herring) were from Koch–Light Laboratories Ltd., (Colnbrook, Bucks., U.K.); palmitoyl-CoA was from P-L Biochemicals Inc. (Milwaukee, Wis., U.S.A.)

RESULTS

Relationship between growth and specific activity of acetoacetyl-CoA reductase. The variation of specific activity of acetoacetyl-CoA reductase as a function of the growth cycle is recorded in Fig. 1. A 4.5-fold increase in the specific activity was observed during exponential growth. From the time (11h) corresponding to the transition to a lower growth rate there was an abrupt fall which continued until the cell mass had reached a maximum in the stationary phase, when constant specific activities were recorded. Cultures used for enzyme purification were harvested at the end of the exponential phase.

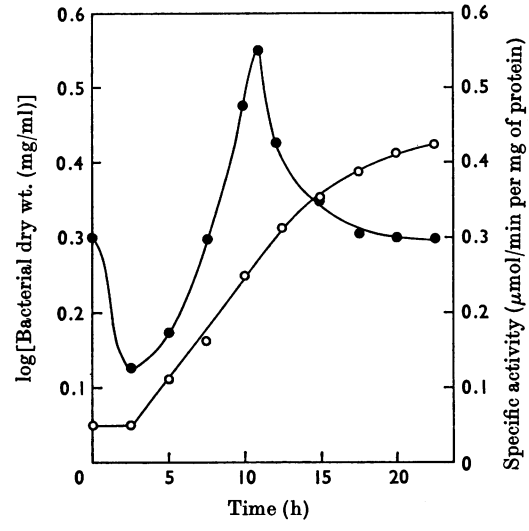

Fig. 1. Specific activity of acetoacetyl-CoA reductase during batch culture growth in nitrogen-free 1% glucose medium (1 litre). The inoculum was 100ml of a 16h culture and samples for enzyme assays were withdrawn with aseptic precautions during growth. Cells were centrifuged, washed and resuspended in 5ml of 0.1M-KH_2PO_4–K_2HPO_4 buffer, pH7.5, containing 0.1M-2-mercaptoethanol. Extracts were prepared by sonication at 0°C for 1min at 1A. Cell debris was removed by centrifuging at 6200*g* for 20min at 0°C and the supernatants were assayed under the standard conditions described in the Materials and Methods section. ○, Cell density; ●, enzyme specific activity.

Purification of acetoacetyl-CoA reductase. Enzyme obtained after the second DEAE-cellulose chromatographic step was 39-fold purified. Samples were checked for purity by disc gel electrophoresis at pH9.5. After staining, two intense bands of protein were seen very close together and, in addition, several small faintly stained bands migrating at a faster rate.

After chromatography on Sepharose 4B the fraction containing enzyme with highest specific activity represented a 54-fold purification. The disc-gel-electrophoretic pattern at pH9.5 was essentially the same as that for enzyme after DEAE-cellulose chromatography; the enzyme activity coincided with both of the main bands.

Effect of pH *on enzyme activity.* The pH optima for the reduction and oxidation reactions with purified enzyme are shown in Fig. 2. The optimum pH for reduction was 5.5 for both purified enzyme and crude extracts. The activity of the purified enzyme in potassium phthalate–sodium hydroxide

buffer was 80% of the activity in potassium phosphate buffer. The oxidation reaction showed a rather broad optimum activity at pH 8.0–9.0, but above pH 9.0 the enzyme was denatured. Activities were identical in potassium phosphate buffer and tris–hydrochloric acid buffer at pH 7.0 and 7.5.

Effect of temperature on enzyme activity. Increasing the temperature from 20°C to 45°C produced a linear fourfold increase in the initial reaction rate at pH 5.5. At 50°C there was a decrease in activity although no enzyme precipitation was observed; this may reflect the instability of NADPH at this temperature and at acid pH.

Coenzyme specificity. The reaction rates with NADPH were consistently greater than with NADH at all stages of purification (Table 2). With 54-fold purified enzyme the NADPH/NADH reaction-rate ratio was 5 at four different substrate concentrations.

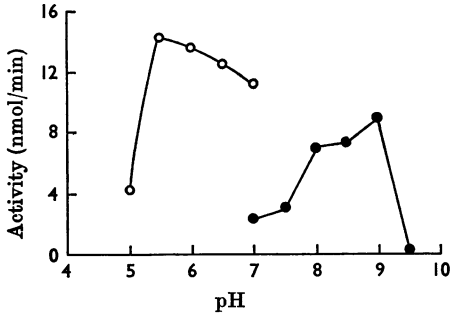

Fig. 2. pH optima of acetoacetyl-CoA reductase. Buffer solutions (0.1 M) were: pH 5.0–6.0, potassium phthalate–NaOH; pH 5.5–7.5, KH_2PO_4–K_2HPO_4; pH 7.5–9.5, tris–HCl. Assay conditions were as described in the Materials and Methods section and reaction rates were corrected for buffer changes where appropriate. ○, Reduction reaction; ●, oxidation reaction.

Michaelis constants. The enzyme showed normal Michaelis–Menten kinetics. Some difficulty was experienced in obtaining reproducible values of K_m and values were therefore calculated both by Lineweaver & Burk (1934) plots and by plots of substrate concentration versus the value obtained by dividing the substrate concentration by the reaction rate. The slopes of the straight lines were obtained by the method of least squares. For purified acetoacetyl-CoA reductase with acetoacetyl-CoA and either NADH or NADPH as coenzyme K_m values for acetoacetyl-CoA were in the range 2.92×10^{-6}–21.1×10^{-6} M whereas K_m values for NADPH and NADH were 1.19×10^{-4}–7.65×10^{-4} M. The maximum initial velocities for the same batch of purified enzyme were within approximately the same range for acetoacetyl-CoA (140–220 μmol/min per mg of protein) and for coenzyme (144–160 μmol/min per mg of protein).

Equilibrium constant. The equilibrium of the reaction: Acetoacetyl-CoA + NADPH + H^+ ⇌ D(−)-β-hydroxybutyryl-CoA + $NADP^+$ was approached from the left-hand side at pH 7.5 (Table 3). At equilibrium the rates of extinction decrease in both test and control incubations were equal. The equilibrium constant, K, was determined from the equation:

$$K = \frac{[\text{Acetoacetyl-CoA}]\,[\text{NADPH}]\,[H^+]}{[\text{D(−)-β-Hydroxybutyryl-CoA}]\,[NADP^+]}$$

The average value for $K = 3.45\pm0.10\times10^{-10}$ M (5).

Product of the reaction. In three experiments with 0.394 μmol of acetoacetyl-CoA reduced in the reaction medium at pH 7.5, the yields of D(−)-β-hydroxybutyrate in the hydrolysed incubation mixture on the completion of the reaction were respectively 0.363, 0.335 and 0.379 μmol representing recoveries of 92.2, 85.1 and 96.2%. The reaction product was therefore identified as D(−)-β-hydroxybutyryl-CoA.

Substrate specificity of the enzyme. The enzyme

Table 2. *Coenzyme specificity for acetoacetyl-CoA reductase*

Assay conditions were standard and 0.24 mM-coenzymes were used. Enzyme preparations: 1, sevenfold purified after calcium phosphate-gel treatment (3.6 μg of protein); 2, 20-fold purified after DEAE-cellulose chromatography at pH 7.4 (5.7 μg of protein); 3, 54-fold purified after Sepharose 4B chromatography (0.82 μg of protein).

Enzyme preparation	Coenzyme	Initial reaction rate (ΔE_{340}/min)	NADPH/NADH rate ratio
1	NADH	0.050	8
	NADPH	0.420	
2	NADH	0.135	5
	NADPH	0.685	
3	NADH	0.030	5
	NADPH	0.150	

Table 3. *Equilibrium constant of the acetoacetyl-CoA reductase*

The reaction mixtures contained (final concentrations) 40mM-potassium phosphate buffer, pH7.5, 131μM-NADPH, 127μM-acetoacetyl-CoA, 2mM-dithiothreitol and 15μg of partially purified enzyme (sp. activity 67.0μmol/min per mg of protein) in a total volume of 2.5ml. The temperature was 25°C. The E_{340} was measured against a distilled-water reference. The enzyme solution was added and, after mixing, extinctions were recorded continuously until equilibrium was reached. The pH of the reaction mixture was then measured. The concentrations of all components at equilibrium were calculated from the change in extinction at 340nm.

		Concentration at equilibrium					
Expt. no.	pH	H^+ (μM)	Acetoacetyl-CoA (μM)	NADPH (μM)	D-(−)-β-Hydroxybutyryl-CoA (μM)	$NADP^+$ (μM)	$10^{10}K$ (M)
1	7.5	0.0316	10.50	14.45	116.5	116.5	3.54
2	7.5	0.0316	10.50	14.45	116.5	116.5	3.54
3	7.5	0.0316	9.60	13.65	117.4	117.4	3.26

Mean of K: 3.45 (S.D. $\pm$0.10) $\times 10^{-10}$ M

Table 4. *Inhibition of acetoacetyl-CoA reductase*

Each inhibitor was preincubated for 5min at 30°C with the enzyme before addition of coenzyme and substrate. Assay conditions were standard except for the omission of dithiothreitol. Enzyme preparations: A, sevenfold-purified enzyme, 20μg of protein/assay; B, 54-fold purified enzyme, 0.33μg of protein/assay. The rates in the absence of inhibitor were: A, 50nmol/min; B, 14.1nmol/min.

		Inhibition (%)	
Inhibitor	Concn. (mM)	Prep. A	Prep. B
Iodoacetamide	1.00		0
Iodoacetate	2.00	45	
	8.00	94	
N-Ethylmaleimide	0.50		36
	1.00		40
	4.00	100	
Sodium arsenite	1.00		8
	2.00	95	
p-Chloromercuribenzoate	0.03		73
	0.05		100
	1.00	85	100

utilized acetoacetylpantetheine as a substrate, but the Michaelis constant was 2.6×10^{-4} M indicating a lower affinity of the enzyme for this compound. However, the maximum velocity ($V_{max.}$ 113μmol reduced/min per mg of protein) was similar to that with acetoacetyl-CoA.

Acetoacetyl-(acyl-carrier protein) prepared for this investigation contained 2mol of acetoacetate/mol of acyl-carrier protein but the assays were based on the assumption that only one residue of acetoacetate was reactive in the enzyme-catalysed reaction. With purified enzyme, activity was detected only at very high enzyme concentrations and activity with acetoacetyl-CoA was some 40-fold greater than with acetoacetyl-(acyl-carrier protein). Experiments to check whether the acyl-carrier protein ester might be reduced more rapidly by a crude cell extract revealed that this was not the case: e.g. initial rates of 0.18, 0.25 and 0.33μmol/min per mg of protein were recorded with acetoacetyl-(acyl-carrier protein) concentrations of 5, 10 and 15μM respectively compared with 6.25μmol/min per mg of protein for acetoacetyl-CoA at the highest concentration (15μM).

The enzyme was inactive with acetoacetate or palmitoyl-CoA as substrate. No inhibition was recorded in the presence of added palmitoyl-CoA at 40 and 80μM.

Effect of thiol compounds. A sevenfold-purified enzyme was protected by dithiothreitol, cysteine, GSH, thioglycollate, 2-mercaptoethanol and 2,3-dimercaptopropan-1-ol, all at 1mM. In the absence of a thiol compound activity was rapidly lost but could be fully restored by incubation for 2min at 30°C with any of these thiol compounds at 1mM. The 54-fold-purified enzyme was not inactivated

so rapidly under these conditions and 0.5mM-dithiothreitol either protected completely or slightly stimulated the enzyme.

Inhibition studies. The enzyme was inhibited by various thiol reagents (Table 4). At the concentrations tested, *N*-ethylmaleimide and *p*-chloromercuribenzoate brought about significant inhibition of the purified enzyme (iodoacetate was not tested). Reversal of inhibition by *p*-chloromercuribenzoate was achieved by an excess of dithiothreitol, e.g. 0.05mM-*p*-chloromercuribenzoate effected 100% inhibition on enzyme with an initial reaction rate of 50μmol/min per mg of protein and 1.0mM-dithiothreitol restored about half the initial activity.

DISCUSSION

Although acetoacetyl-CoA reductase appears to be a constitutive enzyme in *A. beijerinckii*, a marked variation in specific activity is observed during growth in batch culture, a peak being reached before the onset of the massive accumulation of poly-β-hydroxybutyrate that occurs towards the end of exponential growth. At this time the oxygen concentration of the medium falls to zero (Ritchie, Senior & Dawes, 1969). High specific activities were detected in cell extracts and the enzyme was found to react with NADPH at five times the initial rate with NADH. This increased rate was also observed with the purified enzyme and is not attributable to the activity of a transhydrogenase.

The properties of this reductase are similar to those of acetoacetylCoA reductase purified from pigeon liver (Wakil & Bressler, 1962). The pH optimum, coenzyme specificity, reaction product and physiological equilibrium are all analogous to the bacterial reductase. Differences were evident, however, for values of the Michaelis constants and inhibitor characteristics. The bacterial reductase has an affinity for acetoacetyl-CoA 100-fold greater (K_m 2.9×10^{-6}–21.0×10^{-6}M) than that of the avian enzyme. The bacterial enzyme is protected from oxygen inactivation by thiol compounds and inhibited by the thiol-blocking reagents *N*-ethylmaleimide and *p*-chloromercuribenzoate, whereas the avian enzyme is unaffected by these compounds.

The product of enzymic reduction is D(−)-β-hydroxybutyryl-CoA, the immediate precursor of poly-β-hydroxybutyrate. The reaction is readily reversible at alkaline pH with authentic D(−)-β-hydroxybutyryl-CoA.

Over the range of pH investigated (6.3–7.1) the equilibrium of the reaction favours the formation of β-hydroxybutyryl-CoA, the substrate for poly-β-hydroxybutyrate synthetase, and hence facilitates synthesis of the polymer. At pH7.5, $K = 3.45\times10^{-10}$M. Consequently, in the presence of acetoacetyl-CoA reductase and NADPH at this pH, acetyl-CoA is rapidly converted into acetoacetyl-CoA by the action of β-ketothiolase (Ritchie, 1968), the specific activity of which enzyme undergoes a sequence of change during batch growth similar to that observed with acetoacetyl-CoA reductase (Ritchie *et al.* 1969).

The possibility that the reductase may be involved in fatty acid biosynthesis was investigated by comparing the relative activities of acetoacetyl esters of CoA, pantetheine and acyl-carrier protein in the enzymic reaction. The affinity of acetoacetylpantetheine for the enzyme is significantly less than that of the CoA ester and the relative rates of reduction of acyl-carrier protein ester and CoA ester are about 1:40. It is concluded therefore that the enzyme reacts primarily with the CoA ester and a lack of specificity towards acetoacetyl thiol esters permits the acyl-carrier protein and pantetheine esters to serve as substrates in this reaction. Acetoacetyl-CoA reductase is unaffected by the presence of palmitoyl-CoA, although this ester inhibits the individual enzyme activities of fatty acid biosynthesis in *Escherichia coli* (Majerus & Vagelos, 1967). These findings suggest that the enzyme is not involved in fatty acid biosynthesis.

The available evidence thus indicates that poly-β-hydroxybutyrate biosynthesis proceeds via a different sequence of reactions in *A. beijerinckii* and *R. rubrum*, possibly reflecting the different modes of existence of these bacteria in nature.

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