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β-Ketothiolase from *Hydrogenomonas eutropha* H16 and its Significance in the Regulation of Poly-β-hydroxybutyrate Metabolism

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1. β-Ketothiolase was purified 49-fold from fructose-grown cells of *Hydrogenomonas eutropha* H16 with a yield of 27%; the purification procedure involved precipitation by cetyltrimethylammonium bromide, DEAE-cellulose chromatography and exclusion chromatography on Sephadex G-200; the freeze-dried enzyme is stable. The molecular weight determined by sucrose-gradient centrifugation (8.2S) and by gel filtration is 147000–150000. The optimum pH for the cleavage reaction is 8.1, that for the condensation reaction 7.8, both measured in Tris–HCl buffer. 2. The kinetics of the cleavage reaction are described. Substrate-saturation curves were measured with both acetoacetyl-CoA and CoA as the variable substrates. The concentration of the second substrate was kept constant and was varied during successive experiments. The cleavage reaction is characterized by substrate inhibition by acetoacetyl-CoA, which is partially relieved by free CoA. Hill plots indicate two acetoacetyl-CoA-binding sites. 3. The substrate(acetyl-CoA)-saturation curve for the condensation reaction is hyperbolic. The K_m was 3.9×10^{-4} M-acetyl-CoA. In the presence of CoA sigmoidal curves were obtained, with an increasing sigmoidicity from 0.03 to 0.30 mM-CoA. The inhibitory action of CoA on the β-ketothiolase condensation reaction and its possible involvement in the regulation of poly-β-hydroxybutyrate synthesis and degradation are discussed.

Poly-β-hydroxybutyrate is synthesized by the hydrogen-oxidizing bacterium *Hydrogenomonas eutropha* H16 at a high rate and is accumulated intracellularly up to the extent of 65% of its dry weight.

The accumulation starts when growth is limited by the exhaustion of the nitrogen source (Schlegel *et al.*, 1961) or by limiting concentrations of O_2 (Schuster & Schlegel, 1967). The pathway of poly-β-hydroxybutyrate synthesis commences with acetyl-CoA; the carbon resulting from autotrophic CO_2 fixation as well as from organic acids enters the pathway of poly-β-hydroxybutyrate synthesis via acetyl-CoA. The intermediates involved are acetoacetyl-CoA and β-hydroxybutyryl-CoA and the conversions are catalysed by β-ketothiolase and acetoacetyl-CoA reductase (Schindler, 1964).

The degradation of poly-β-hydroxybutyrate occurs via β-hydroxybutyric acid, which is the first depolymerization product, followed by acetoacetic acid, acetoacetyl-CoA and acetyl-CoA. β-Ketothiolase is therefore also involved in the reutilization of poly-β-hydroxybutyrate.

Since β-ketothiolase is apparently the key enzyme in poly-β-hydroxybutyrate metabolism, the present investigation aimed at the isolation of this enzyme from *H. eutropha* H16 and a study of its kinetics and of potential effectors.

Experimental

Methods

Culture of organism. The organism was *Hydrogenomonas eutropha* H16 (Wilde, 1962; A.T.C.C. 17699). The basal medium was prepared from solutions of the following compositions (g/l, final volume). Solution A: $Na_2HPO_4,12H_2O$, 9.0; KH_2PO_4, 1.5. Solution B: NH_4Cl, 1.0; $MgSO_4,7H_2O$, 0.2. Solution C: ferric ammonium citrate, 0.005; $CaCl_2,2H_2O$, 0.02. Solution D: fructose, 5.0. Solution E: trace elements (SL 4; Pfennig & Lippert, 1966), 1 ml/l. The solutions were autoclaved separately (121°C for 20 min) and combined after cooling. The pH of the complete medium was between 7.0 and 7.2.

Liquid cultures (1 litre) were grown in culture vessels (2 litre) with magnetic stirring, according to the method of Schlegel *et al.* (1961), at 30°C with a 5% inoculum of stationary-phase cells approx. 24 h old. Larger quantities of cells were cultured in a 10 litre fermenter with an aeration rate of 10 litres/min (Braun-Biostat, Braun, Melsungen, Germany).

Growth was determined turbidimetrically at 436nm in a Zeiss PM-4 spectrophotometer.

Harvesting of cultures. The cultures were centrifuged at the end of the logarithmic phase at 5000*g* for 30min at 3–5°C (Christ IV KS), and then the supernatant was decanted. Larger volumes were collected by continuous-flow centrifugation at 30000rev./min (Cepa-Schnellzentrifuge; Carl Padberg, Lahr/Baden).

Preparation of cell-extracts. Harvested cells were washed twice with 50mM-potassium phosphate buffer, pH7.0, resuspended in a small volume of the same buffer and stored at −20°C. Cell extracts were prepared by disruption of cells by ultrasonic treatment. A sonifier (20kH, 600W) from Firma Schoeller and Co., Frankfurt am Main, Germany was used in combination with a cooled (Ultrakryomat Tk 30 D, Meßgerätewerk Lauda) flow-through vessel of 30ml capacity. The exposure time was 50s/ml of cell suspension. The temperature was maintained between +4°C and +10°C.

The crude cell extracts were centrifuged at 20000*g* for 30min at 0°C (Phywe Kühlzentrifuge) and the supernatant was centrifuged again at 120000*g* for 60min at 0°C (Omikron Zentrifuge, Firma Heraeus-Christ, Osterode) to remove the particulate NADH-oxidase.

Preparations. Acetyl-CoA was prepared by treating CoASH with acetic anhydride by the method of Ochoa (1955). Since not all CoASH had been transformed into the thioester the preparation was treated with iodine-saturated ethanol (0.1ml for 5ml of acetyl-CoA solution) for 5min with shaking. The remaining iodine was removed by extracting four times with a tenfold excess of diethyl ether. Ether was removed from the aqueous layer under a stream of N_2. The concentration of the acetyl-CoA preparation was determined enzymically by the condensation reaction with oxaloacetate in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid). The assay mixture contained (final concentrations): 100mM-Tris–HCl buffer, pH8.0; 39μg of 5,5′-dithiobis-(2-nitrobenzoic acid); 0.4mM-oxaloacetate; 20μl of citrate synthase; 20μl of acetyl-CoA solution containing 20–50nmol in a final volume of 1.0ml. The reaction was started by the addition of the enzyme and monitored spectrophotometrically until equilibrium was reached. The increase in E_{412} was measured and this value was used to calculate the initial concentration of acetyl-CoA.

Acetoacetyl-CoA was synthesized by treating CoASH with diketen by the method of Simon & Shemin (1953). Free CoA was removed from the preparations by iodine treatment as described for acetyl-CoA. The concentration of the acetoacetyl-CoA preparations was determined enzymically with L-β-hydroxyacyl-CoA dehydrogenase. The assay mixture contained (final concentrations): 24mM-potassium citrate–potassium phosphate buffer, pH5.5; 0.25mM-NADH; 20μg of L-β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35); 10μl of acetoacetyl-CoA solution containing 60–120nmol; final volume was 1.0ml. 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 was used to calculate the initial concentration of acetoacetyl CoA.

Oxidized CoA, present in the preparations of both these CoA thioesters, does not affect the activity of β-ketothiolase in either the condensation reaction or the cleavage reaction.

Protein measurement. Protein was determined either by the method of Beisenherz *et al.* (1953) or, if the concentration was too low, by the method of Lowry *et al.* (1951) with crystallized serum albumin as a standard.

Assay of β-ketothiolase. For assay of enzyme activity the condensation reaction of two molecules of acetyl-CoA forming acetoacetyl-CoA and free CoA was followed by measuring the oxidation of NADH concomitant with the reduction of acetoacetyl-CoA to L(+)-β-hydroxybutyryl-CoA with β-hydroxyacyl-CoA dehydrogenase as an auxiliary enzyme (Lynen *et al.*, 1952). The assay mixture contained (final concentrations): 80mM-Tris–HCl buffer, pH7.8; 1.25mM-GSH; 2.25mM-acetyl-CoA; 4mM-$MgCl_2$; 0.125mM-NADH; 1.25 units of β-hydroxyacyl-CoA dehydrogenase; 10μl of β-ketothiolase preparation; final volume was 1.0ml. The auxiliary enzyme was used in a more than 30-fold excess. It has a high affinity for acetoacetyl-CoA ($K_m = 5.0\times10^{-5}$M; Bergmeyer, 1970) and the equilibrium constant ($K = 2.17\times10^{-10}$; Lynen & Wieland, 1955) strongly favours the formation of β-hydroxybutyryl-CoA. The linear decrease in E_{340} was measured in a Zeiss PM-4 recording spectrophotometer at 20°C. When starting with acetyl-CoA there was no lag and initial rates were recorded for 40s. A linear relationship between protein concentration and initial velocity was observed up to 0.04μmol of NADH oxidized/min per ml. One unit of enzyme catalyses the condensation of 2μmol of acetyl-CoA/min at pH7.8.

The cleavage (thiolysis) reaction of β-ketothiolase was measured by following the linear decrease of the extinction at 305nm (Stern & Del Campillo, 1955) with a Zeiss PMQ-II recording spectrophotometer at 20°C. Each assay mixture contained (final concentration) 67mM-Tris–HCl buffer, pH8.1, and 265mM-$MgCl_2$, in a final volume of 1.5ml. The various concentrations of acetoacetyl-CoA and CoASH are indicated in the legends to the figures. When the reaction was started with the enzyme there was no lag, and the reaction was followed for 3min.

Enzyme purification. All procedures were done at 4°C. The buffers used throughout were K_2HPO_4–KH_2PO_4 mixtures at pH7.4. Cetyltrimethylammonium bromide solution (2%, w/v; 0.25vol.) was added with stirring to the cell-free supernatant containing approx. 20mg of protein/ml, and after being left for 10min on ice the mixture was centrifuged at 20000*g* for 30min and the precipitate was discarded.

Chromatography on DEAE-cellulose. The cetyltrimethylammonium bromide supernatant was dialysed against two changes of 5mM-potassium phosphate buffer, adsorbed on a DEAE-cellulose column (Whatman DE-52 microgranular grade, 2.5cm×40cm) and washed with 50ml of the same buffer. The enzyme was eluted with a linear gradient of a chloride solution supplied by mixing 250ml of 5mM-potassium phosphate buffer, containing 0.2M-KCl, into 250ml of 5mM-potassium phosphate buffer (Fig. 1). Protein elution was monitored by measurement of E_{280}. The enzyme was eluted at about 0.11M-KCl. The most active fractions were combined and concentrated by freeze-drying.

Chromatography on Sephadex G-200. The freeze-dried enzyme preparation was dissolved in a small volume of 50mM-potassium phosphate buffer and chromatographed on a Sephadex G-25 column to remove the salt. The effluent enzyme solution was layered on top of a Sephadex G-200 column (fine grade, 2.5cm×100cm) and eluted with 50mM-potassium phosphate buffer. Active fractions were concentrated as before. The preparation resulted in a 49-fold purification with a yield of 27% (Table 1). The freeze-dried enzyme could be stored at 4°C for several months without detectable loss of activity.

Determination of sedimentation coefficient. The sedimentation behaviour of β-ketothiolase was determined by the method of Martin & Ames (1961). Linear gradients of sucrose were prepared from equal amounts (2.7ml) of solution of two different concentrations (7 and 30%, w/v, in 20mM-potassium phosphate buffer, pH7.0) with a two-cylinder mixing device. The gradients were stored for 4h at 0°C to ensure smooth gradients, and then 0.15ml of a mixture of β-ketothiolase and the reference protein, catalase (11.3S), was layered on top of the gradient. The procedure is based on the assumption that the partial specific volumes of the proteins are similar (0.715ml/g). The Svedberg constant was calculated by

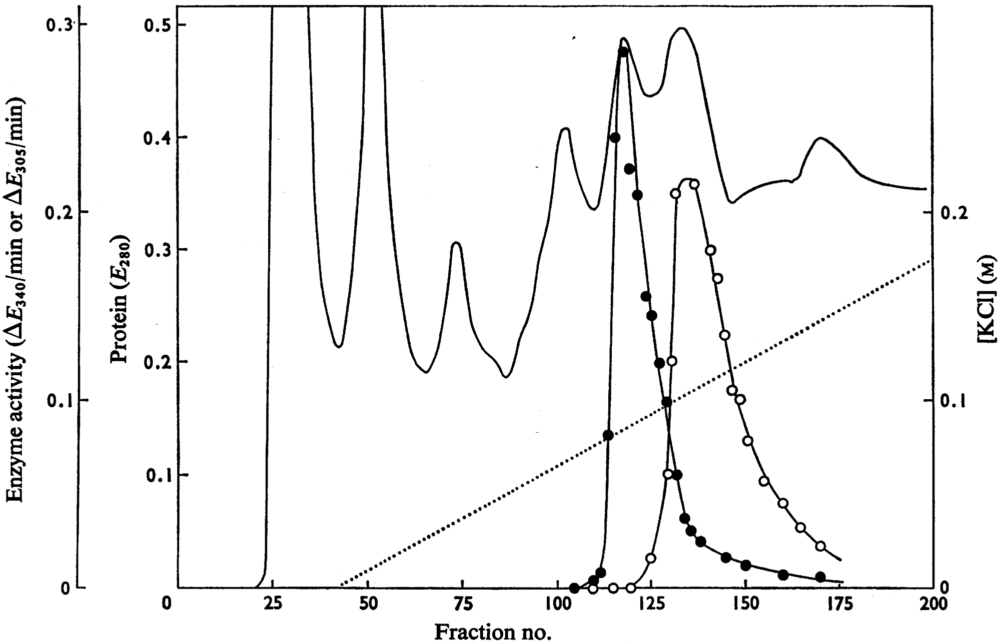


Fig. 1. *Elution diagram for the separation of β-ketothiolase from acetoacetyl-CoA reductase by chromatography on a DEAE-cellulose column*

The DEAE-cellulose column (diameter 2.5cm; length 37cm) was equilibrated with 5mM-potassium phosphate buffer (pH7.4). The enzyme solution (40ml, 7.8mg of protein/ml) was layered on top of the column. Elution was started with 50ml of the phosphate buffer and continued with a linear KCl gradient (0.0–0.2M-KCl in a 5mM-phosphate buffer). Fractions of 2ml were collected. ○, Relative activity of β-ketothiolase; ●, acetoacetyl-CoA reductase; ——, protein (E_{280}); · · · ·, KCl (M).

Table 1. *Partial purification of β-ketothiolase*

Fraction	Volume (ml)	Protein (mg/ml)	Sp. activity (units/mg of protein)	Total activity (units)	Yield (%)	Purification factor
Crude extract*	70	21.3	0.097	149	100	1.0
Cetyltrimethylammonium bromide supernatant	78	5.9	0.29	133	89	2.9
DEAE-cellulose	48	1.03	1.82	89.5	60	18.7
Sephadex G-25	5.2	8.6	1.81	80.5	54	18.6
Sephadex G-200	23	0.37	4.78	40.6	27	49

* Supernatant after centrifugation at 120000*g* for 60min.

comparing the distances travelled from the meniscus of the gradient. Centrifugation was performed in a preparative ultracentrifuge (Omega II 70000, Firma Heraeus-Christ, Osterode) at 5°C by using a 3×5ml swinging-bucket rotor with the geometry: bottom of the centrifuge tube–axis of rotation, 9.8cm; meniscus of the gradient–axis of rotation, 5.02cm. The centrifugation velocity was 38800rev./min, corresponding to 4063rads/s. The centrifugation time (16.5h) was chosen according to the work of McEwen (1967), so that the reference protein catalase migrated nearly to the middle of the gradient. After the run the centrifuge tubes were punctured and fractions of 4 drops each were collected.

Determination of Stokes radius. The Stokes radius and molecular weight were determined by gel filtration on a Sephadex G-200 column (fine grade, 2.5cm×41cm). The gel was diluted in the elution buffer (K_2HPO_4–KH_2PO_4, 50mM, pH7.0) and loaded in one volume on the column. During preparation of the column a pressure head of 10cm was maintained and this was increased to 15cm before use. The freeze-dried reference proteins ferritin (mol.wt. 540000), catalase (mol.wt. 240000), aldolase (mol.wt. 147000), chymotrypsinogen A (mol.wt. 25000) and cytochrome *c* (mol.wt. 14500), as well as the purified β-ketothiolase, were dissolved in 1ml of 50mM-potassium phosphate buffer, pH7.0, and layered on top of the column. Fractions of 1ml each were collected. The void volume, determined with Blue Dextran 2000 immediately after chromatography, was 70.5ml for a bed volume of 201.3ml.

Chemicals

NADH, NADPH, ADP, AMP, ATP, sodium pyruvate, phosphoenolpyruvate (potassium salt), CoASH and 3 hydroxyacyl-CoA dehydrogenase were obtained from C.F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany, Diketen was from Schuchardt, München, Germany. The materials for column chromatography were obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K. (Whatman DE-52) and from Pharmacia, Uppsala, Sweden (Sephadex G-25 and G-200).

Results

Properties of the enzyme

Effect of pH on enzyme activity. The pH optima for the condensation and cleavage reactions with the purified enzyme are shown in Fig. 2. The optimum pH for the cleavage reaction was 8.1, that for the condensation reaction 7.8, both reactions measured in Tris–HCl buffer. The activities were almost identical in potassium phosphate buffer and Tris–HCl buffer at pH7.4 and 7.1 respectively.

Physical parameters of β-ketothiolase. The sedimentation behaviour of β-ketothiolase was studied by sucrose-gradient centrifugation by the method of Martin & Ames (1961). A Svedberg constant of 8.2 S was determined. A crude estimate of molecular weight can be obtained from the sedimentation constant alone (Martin & Ames, 1961):

$$s_1/s_2 = (M_1/M_2)^{\frac{2}{3}}$$

By using the known molecular weight (*M*) of catalase (240000), a molecular weight of 148000 was obtained for β-ketothiolase. To verify these determinations the sedimentation results were compared with data from gel filtration on Sephadex G-200. The K_D values of the reference proteins and of β-ketothiolase were calculated by the equation of Gelotte (1960). With the exception of cytochrome *c* all values showed a linear relationship to log mol.wt. The discrepancy observed with cytochrome *c* may be due to the basic nature of this protein (Flodin & Killiander, 1962; Andrews, 1962), leading to adsorption to the gel matrix.

By graphical determination a molecular weight of 147000 was obtained. From these experiments also a Stokes radius of 4.61nm for β-ketothiolase was determined by the method of Siegel & Monty (1966).

With a Stokes radius measured by the chromatographic method and a sedimentation coefficient determined by density-gradient centrifugation, reasonable estimates for both the molecular weight and the frictional ratio (f/f_0) of β-ketothiolase are obtainable. The equations:

$$M = 6\pi\eta Nas/(1-\bar{v}\rho)$$

and

$$f/f_0 = A/(3\bar{v}M/4\pi N)^{\frac{1}{3}}$$

where M = molecular weight, a = Stokes radius, s = sedimentation coefficient, $\bar{v}$ = partial specific volume (assumed to be 0.715ml/g), f/f_0 = frictional ratio, η = viscosity of the medium, ρ = density of the medium and N = Avogadro's number, were used for the calculations and a molecular weight of 150000 and a frictional ratio of 1.29 were obtained for β-ketothiolase from *Hydrogenomonas eutropha* H16. The calculated molecular weight is in good agreement with the values obtained by each of the physical methods.

Cleavage and condensation reactions

Kinetics of the cleavage reaction. The cleavage of acetoacetyl-CoA catalysed by β-ketothiolase is a two-substrate reaction. The first substrate bound by the enzyme is acetoacetyl-CoA and the second substrate is CoASH. The enzyme kinetics have been determined by measuring the variation of the initial velocity as a function of the variation of the concentration of the first substrate and then of the second one.

Substrate (acetoacetyl-CoA)-saturation curves. Acetoacetyl-CoA saturation curves were measured at various concentrations from 0.0016 to 0.0326mM-acetoacetyl-CoA. Owing to the high molar extinction coefficient of acetoacetyl-CoA it was not possible to measure the reaction rates at higher substrate concentrations. The concentrations of the second substrate, CoA, were kept constant at 0.037, 0.073 and 0.184mM during successive experiments. The velocity versus substrate concentration profile (Fig. 3*a*) does not coincide with simple Michaelis–Menten kinetics. The sigmoidicity of the curves becomes apparent in the Lineweaver–Burk plot (Fig. 3*b*); the values do not fit a straight line and indicate paraboloid curves. With increasing concentrations of acetoacetyl-CoA and after maximal activity had been reached, an increasing inhibition of the reaction rate by the first substrate became apparent. The extent of this inhibition is dependent also on the concentration of the second substrate CoA; with increasing CoA concentrations the inhibition is shifted towards higher acetoacetyl-CoA concentrations. At 0.185mM-CoASH the maximal activity of the enzyme and the inhibitory range was not reached under the conditions used. When the data were replotted according to the Hill equation (Changeux, 1963), straight lines were obtained with

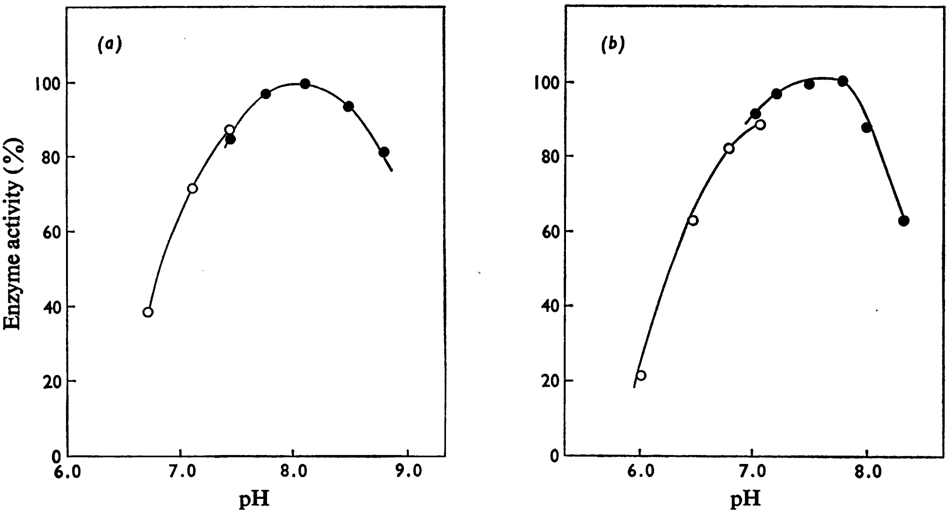


Fig. 2. *Dependence of the activity of β-ketothiolase on pH*

(*a*) pH optimum of the cleavage reaction. The reaction mixture contained 100μmol of buffer, 400μmol of $MgCl_2$, 0.016μmol of acetoacetyl-CoA, 0.1μmol of CoASH and β-ketothiolase (0.82μg of protein) in a final volume of 1.5ml. (*b*) pH optimum of the condensation reaction. The reaction mixture contained 80μmol of buffer, 1.25μmol of GSH, 2.25μmol of acetyl-CoA, 4μmol of $MgCl_2$, 0.005ml of β-hydroxyacyl-CoA dehydrogenase and β-ketothiolase (6.2μg of protein) in a final volume of 1.0ml. ○, Potassium phosphate buffer; ●, Tris–HCl buffer.

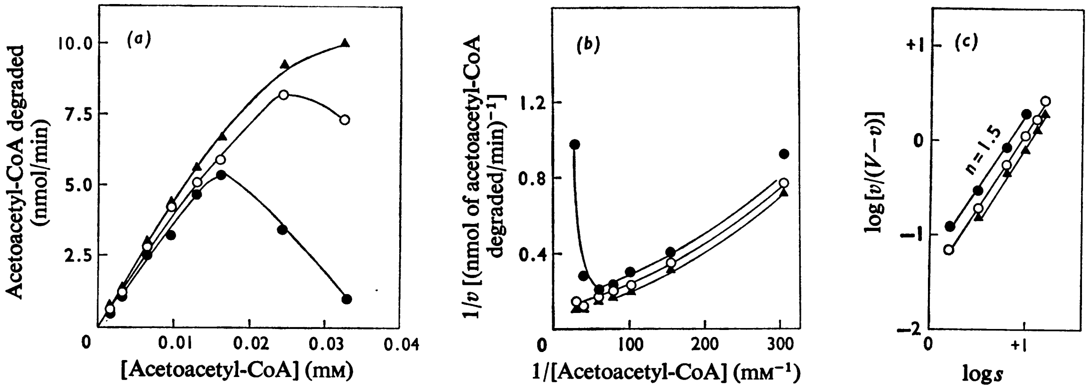


Fig. 3. *Dependence of the activity of β-ketothiolase on the concentration of acetoacetyl-CoA at three different concentrations of CoASH*

(*a*) Substrate(acetoacetyl-CoA)-saturation curves. The reaction mixture contained 100 μmol of Tris–HCl buffer, pH 8.1, 400 μmol of $MgCl_2$ and β-ketothiolase (0.63 μg of protein) in 1.5 ml final volume. The concentration of acetoacetyl-CoA was varied as indicated. (*b*) Lineweaver–Burk plot. (*c*) Hill plot. Concentration of CoASH (μmol/ml): 0.037 (●); 0.073 (○); 0.184 (▲).

slopes of $n = 1.45-1.53$ (Fig. 3*c*). The kinetic data fit a high-substrate inhibition by acetoacetyl-CoA, which is competitive with respect to CoASH.

Certain conclusions with respect to the mechanism of the β-ketothiolase cleavage reaction can be drawn from Fig. 3(*b*). In the Lineweaver–Burk plot a family of parallel lines is apparent. Each of them represents a saturation curve obtained at a different concentration of the second substrate. Parallel lines indicate that the two-substrate reaction follows a Ping-Pong (Cleland, 1967) rather than a sequential mechanism. This result is in accordance with the reaction mechanism discussed for the β-ketothiolase of yeast on the basis of the parallel straight lines obtained in the reciprocal velocity versus substrate-concentration plot (Kornblatt & Rudney, 1971).

Substrate (*CoA*)-*saturation curves*. Saturation curves for CoASH as a substrate at constant concentrations of acetoacetyl-CoA were measured by varying the CoASH concentration from 0.018 to 0.366 mM. In successive experiments three different concentrations of acetoacetyl-CoA were employed (0.0065, 0.0162 and 0.0327 mM). The saturation curves shown in Fig. 4(*a*) exhibit a sigmoidal nature. The sigmoidicity is most significant at the highest concentration of acetoacetyl-CoA (0.0327 mM), which is evident from the Lineweaver–Burk plot (Fig. 4*b*). At the lowest concentration of CoASH (0.02 mM) the reaction rate was zero and with increasing CoASH concentrations approached a rate of 7 nmol of acetoacetyl-CoA cleaved/min per ml. The sigmoidal nature of the substrate saturation curve was also apparent when only half of the concentration of acetoacetyl-CoA was used. If, however, the acetoacetyl-CoA concentration is decreased to 0.0065 mM the curve cannot be analysed any more. The series of sigmoidal saturation curves indicates that the rate of the thioclastic cleavage is limited by the concentration of acetoacetyl-CoA in such a way that at low CoASH concentration the rate decreases with increasing concentrations of acetoacetyl-CoA, possibly caused by competitive interaction with the acetyl–enzyme complex. When the data are plotted according to the Hill equation (Fig. 4*c*), straight lines with slopes of $n = 3.3–3.4$ result. These high values are indicative of co-operative interactions of CoA-binding sites.

Kinetics of the condensation reaction. In its synthetic function β-ketothiolase catalyses the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA and free CoASH. The reaction rate is measured by a coupled optical test system; the product acetoacetyl-CoA is reduced by the auxiliary enzyme β-hydroxyacyl-CoA dehydrogenase and in this way is continuously removed from the equilibrium. The auxiliary enzyme is not affected by free CoASH.

Substrate (*acetyl-CoA*)-*saturation curve*. The substrate-saturation curve was measured by varying the acetyl-CoA concentration from 0.037 to 2.36 mM. In successive experiments three concentrations of CoASH (0.03, 0.15 and 0.3 mM) were employed (Fig. 5*a*). In the absence of CoASH the velocity versus substrate-concentration plot exhibits normal Michaelis-Menten kinetics. This impression is confirmed by the Lineweaver–Burk plot; the values form

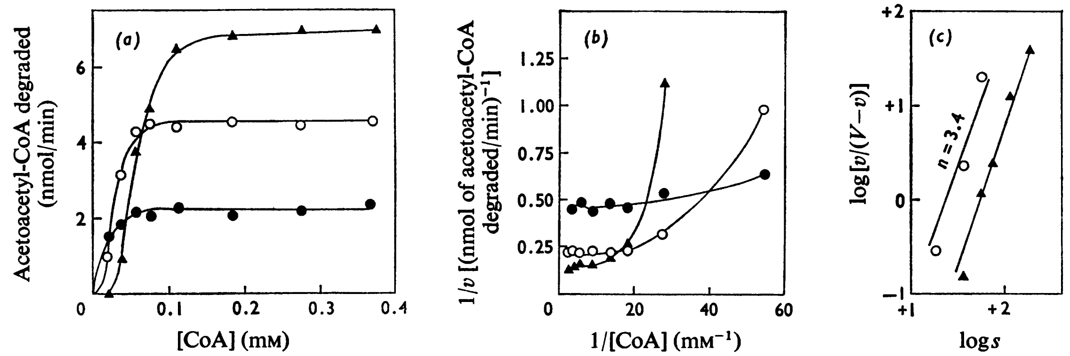


Fig. 4. *Dependence of the activity of β-ketothiolase on the concentration of CoA at three different concentrations of acetoacetyl-CoA*

(*a*) Substrate(coenzyme A)-saturation curves. The reaction mixture contained 100μmol of Tris–HCl buffer, pH 8.1, 400μmol of $MgCl_2$ and β-ketothiolase (0.82μg of protein) in 1.5ml final volume. The CoASH concentration was varied as indicated. (*b*) Lineweaver–Burk plot. (*c*) Hill plot. Concentration of acetoacetyl-CoA (μmol/ml): 0.0065 (●); 0.0162 (○); 0.0327 (▲).

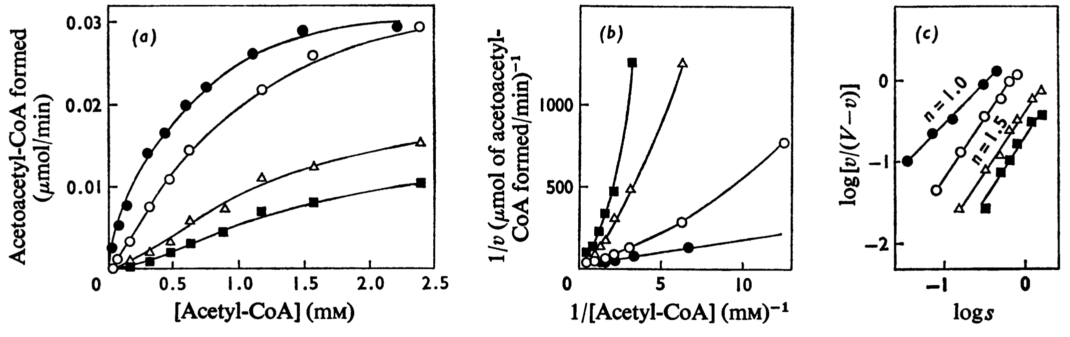


Fig. 5. *Substrate(acetyl-CoA)-saturation curves of β-ketothiolase in the absence and in the presence of CoA*

(*a*) Substrate-saturation curves. The reaction mixture contained 80μmol of Tris–HCl buffer, pH 7.8, 1.25μmol of GSH, 4μmol of $MgCl_2$, 0.125μmol of NADH, 0.005ml of β-hydroxyacyl-CoA dehydrogenase (2mg/ml), acetyl-CoA as indicated and β-ketothiolase (6.2μg of protein) in a final volume of 1 ml. (*b*) Lineweaver–Burk plot. (*c*) Hill plot. Concentration of CoASH (mM): 0 (●); 0.03 (○); 0.15 (△); 0.30 (■).

a straight line crossing the abscissa at an apparent K_m value of 3.9×10^{-4}M (Fig. 5*b*).

In the presence of free CoASH the reaction rate is less. With increasing concentrations of CoASH the substrate-saturation curves become increasingly sigmoidal. In the Lineweaver–Burk plot the values deviate from a straight line (Fig. 5*b*); however, the curves intersect at one point on the ordinate, indicating that V is reached at high concentrations of acetyl-CoA even in the presence of increasing concentrations of free CoASH. These kinetics are in accordance with our statement made above assuming a Ping-Pong mechanism. In the presence of the product (CoA) a binary term is introduced into the initial rate equation, leading to non-linear curves in the Lineweaver–Burk plot. The Hill coefficient $n = 1$ in the absence of CoA and approaches 2 in its presence (Fig. 5*c*).

The data were supplemented by measuring the reaction rate at varied concentrations of CoASH (0.01–0.05mM) and at two concentrations of acetyl-CoA (0.7 and 2.5mM). If the percentage reaction rate compared with the maximal rate (measured in the absence of CoASH) is plotted versus the concentration of free CoASH, curves of a weakly sigmoidal nature are obtained (Fig. 6*a*). From the straight lines in the

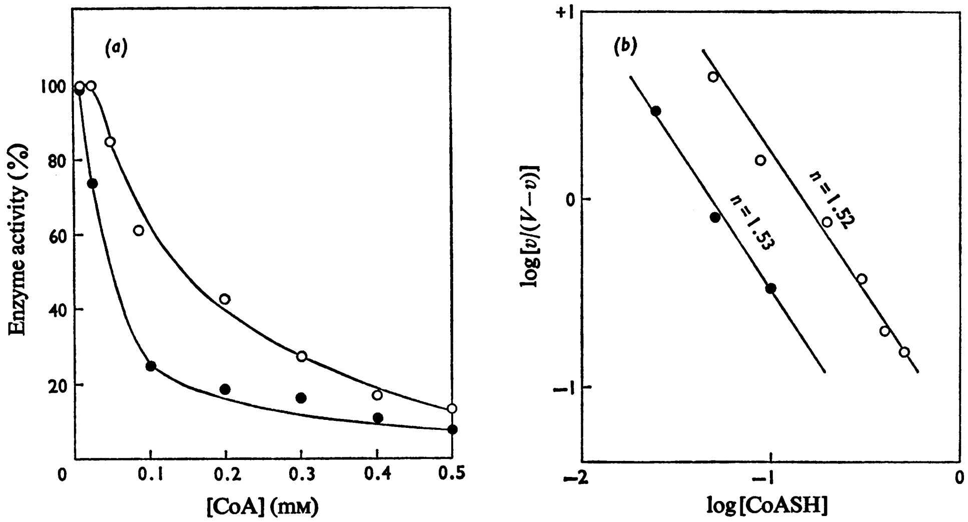


Fig. 6. *Inhibition of the condensation reaction by free CoA*

(*a*) Dependence of the inhibition on the concentration of CoA. The reaction mixture contained 80 μmol of Tris–HCl buffer, pH 7.8, 1.25 μmol of GSH, 4 μmol of $MgCl_2$, 0.125 μmol of NADH, 0.005 ml of β-hydroxyacyl-CoA dehydrogenase (2 mg/ml) and CoA as indicated in a final volume of 1.0 ml. ○, 2.5 mM-Acetyl-CoA; ●, 0.7 mM-acetyl-CoA. (*b*) Hill plot. The enzyme content was 6.2 μg at 2.5 mM-acetyl-CoA and 31 μg at 0.7 mM-acetyl-CoA.

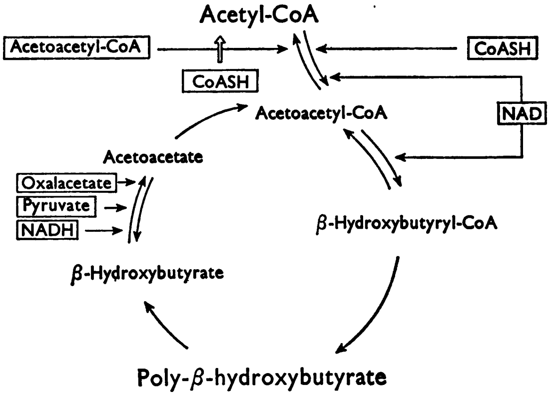
Hill diagram (Fig. 6*b*) *n* values of −1.53 and −1.52 were obtained.

Influence of metabolites on β-ketothiolase. The results obtained from investigating the cleavage and condensation reactions coincided in so far as β-ketothiolase was recognized as a regulatory enzyme. The question arose whether the activity of the enzyme is influenced by metabolites other than substrate and reaction products. Among those metabolites that would possibly function as effectors for β-ketothiolase condensation reaction, four groups were tested: adenosine phosphates, nicotinamide nucleotides, intermediates of the tricarboxylic acid cycle and precursors of acetyl-CoA. The concentration of acetyl-CoA was 2.25 mM and therefore within the range of saturation of the enzyme. The following activities were measured (as % of control): 10 mM-ATP, 48; 5 mM-ADP, 122; 5 mM-AMP, 97; 10 mM-NAD, 47; 5 mM-NAD, 78; 10 mM-NADP, 107; 3 mM-NADPH, 105; 20 mM-oxaloacetate, 93; 20 mM-citrate, 105; 10 mM-phosphoenolpyruvate, 73; 10 mM-pyruvate, 103. The 100% enzyme activity of the control is equivalent to 0.029 μmol of acetoacetyl-CoA formed/min per ml. With the substances tested, significant effects were only observed at high, non-physiological concentrations. The effects are relatively small and a regulatory function of these substances is highly improbable.

Discussion

The β-ketothiolase of *Hydrogenomonas eutropha* H16 catalyses the reversible condensation of two molecules of acetyl-CoA to acetoacetyl-CoA and free CoASH. There is no evidence for isofunctional or parallel enzymes either in strain H16 or in other prokaryotic cells. Therefore β-ketothiolase is not only the first enzyme of the pathway for poly-β-hydroxybutyrate biosynthesis but is also involved in the re-utilization of poly-β-hydroxybutyrate (Scheme 1). The enzyme is not inhibited by physiological concentrations of metabolites to a significant extent. Only one product of the condensation reaction, free CoASH, exerts an inhibitory effect on the enzyme catalysing the condensation reaction. The inhibitory effect is very marked at a concentration of 0.1 mM-CoASH. The kinetic data are in accordance with the mathematical treatment of a Ping-Pong mechanism with free CoASH as an early product diminishing the reaction rate by fostering the reverse reaction. With respect to the high Hill coefficients ($n > 3$) obtained from Fig. 4(*c*), an allosteric-type interaction cannot be ruled out.

In the direction of poly-β-hydroxybutyrate biosynthesis the inhibition of β-ketothiolase by CoA has the following consequences: in cells kept under optimum growth conditions, the substrate will be mainly used for biosynthetic reactions. Acetyl-CoA



Scheme 1. *Regulation of poly-β-hydroxybutyrate metabolism in Hydrogenomonas eutropha H16*

Inhibition is indicated by horizontal straight arrows. The vertical outlined arrow for CoASH indicates relief of inhibition.

will mainly enter the tricarboxylic acid cycle for energy generation and formation of aspartate, glutamate and other amino acids. The citrate synthase reaction results in the liberation of free CoASH. Under such growth conditions one would expect the intracellular concentration of acetyl-CoA to be low and consequently that of free CoASH to be high. The high CoA concentration results in an inhibition of the β-ketothiolase condensation reaction and of poly-β-hydroxybutyrate synthesis. If protein synthesis is impaired or inhibited, pyruvate and the intermediates of the tricarboxylic acid cycle do not flow into anabolic pathways. Ahrens (1970) has observed that in the absence of a nitrogen source the intracellular pyruvate concentration is five times as high as under optimum growth conditions. As a consequence the concentration of acetyl-CoA will be high and the concentration of free CoASH will be low; consequently β-ketothiolase will not be inhibited and acetoacetyl-CoA synthesis will proceed unimpaired.

If growth is limited by a shortage of oxygen the nicotinamide nucleotides are not reoxidized. This will result in a decrease of the effectiveness of the tricarboxylic acid cycle, since the activity of the citrate synthase (Weitzman & Dunmore, 1969; Eidels & Preiss, 1970; Senior & Dawes, 1971) and of isocitrate dehydrogenase (Glaeser & Schlegel, 1972) is decreased by NADH. Therefore deprivation of oxygen will also result in the accumulation of acetyl-CoA and in a low intracellular concentration of free CoASH. The sensitivity of β-ketothiolase to free CoASH is in accordance with the results obtained with intact cells and explains these observations. Obviously the determination of intracellular concentrations of acetyl-CoA and free CoASH is required for further confirmation of these conclusions.

In the direction of the reutilization and degradation of poly-β-hydroxybutyrate, acetoacetyl-CoA is formed by the activation of acetoacetic acid, which is derived from poly-β-hydroxybutyrate by hydrolysis and dehydrogenation via β-hydroxybutyric acid. This has been shown for *Rhodospirillum rubrum* (Merrick & Doudoroff, 1964) and *H. eutropha* H16 (Schindler & Schlegel, 1963; Hippe & Schlegel, 1967). In strain H16 poly-β-hydroxybutyrate is reutilized when the carbon or carbon and energy source is lacking. The degradation is a slow process *in vivo* (Hippe, 1967). Under these conditions the intracellular concentrations of acetyl-CoA and free CoASH will be shifted in favour of CoASH, and the condensation of acetyl-CoA to form acetoacetyl-CoA will be inhibited.

The thioclastic cleavage of acetoacetyl-CoA by β-ketothiolase is inhibited by acetoacetyl-CoA (see Fig. 3). This substrate inhibition, which is due to an impairment of the second co-ordinated step of the enzyme reaction, the binding of CoA to the acetyl–enzyme complex, is relieved by increasing concentrations of the second substrate, CoA. High intracellular concentrations of CoA therefore favour the formation of acetyl-CoA from acetoacetyl-CoA. The maintenance of a high CoA concentration may be guaranteed by the tricarboxylic acid cycle, which functions as a sink for the acetyl residues of acetyl-CoA.

From these observations it can be deduced that the kinetic data obtained for β-ketothiolase are also in accordance with the biological data on the reutilization of poly-β-hydroxybutyrate. The control mechanism of β-ketothiolase favours the same trend that became apparent when investigating two other enzymes involved in poly-β-hydroxybutyrate metabolism, acetoacetyl-CoA reductase and β-hydroxybutyrate dehydrogenase (Oeding, 1972). It is, however, conceivable that the degradation of poly-β-hydroxybutyrate could be additionally controlled at the hydrolytic cleavage of the polymer.

The information obtained with respect to the kinetics of β-ketothiolase from *Azotobacter beijerinckii* by Senior & Dawes (1973) is very similar and suggests a control mechanism of poly-β-hydroxybutyrate synthesis that is almost identical with that in *H. eutropha* H16.

The β-ketothiolase isolated from strain H16 has a medium affinity for acetyl-CoA; the apparent K_m is 0.39mM for acetyl-CoA. The molecular weight determined by sucrose-gradient centrifugation (8.2S) and by gel filtration amounts to 147000–150000 and is significantly higher than that of the pH7.8 isoenzyme from *Saccharomyces cerevisiae* (mol.wt. 65000). It is in the same order of magnitude as the molecular weight of the pH5.3 thiolase from *S. cerevisiae* (mol.wt. 140000; Kornblatt & Rudney, 1971) and of the thiolase from *Escherichia coli* (mol.wt. 140000±5000; Mazzai *et al.*, 1970) as well as the thiolase from pig heart (mol.wt. 168000; Gehring & Riepertinger, 1968). Since enzymes with molecular weights higher than 100000 are usually composed of subunits (Sund & Weber, 1966), the thiolase from strain H16 probably consists of several subunits; it is, however, not yet possible to draw conclusions on the number of subunits.

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