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Kinetics and Properties of β-Ketothiolase from *Clostridium pasteurianum*

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Abstract. 1. β-Ketothiolase of *Clostridium pasteurianum* was purified 130-fold by ammonium sulphate fractionation and by column chromatography using DEAE-Sephadex A-50 and hydroxylapatite. Subjected to gel electrophoresis β-ketothiolase revealed two distinct bands; by isoelectric focusing two enzymes with isoelectric points at pH 4.5 and 7.6 were separated. As established by sucrose density gradient centrifugation the molecular weight of both enzymes was found to be 158000.

2. The condensation reaction was measured by a coupled optical test using β-hydroxybutyryl-CoA dehydrogenase as auxiliary enzyme and either acetyl-CoA or free coenzyme A plus acetyl-phosphate and phosphotransacetylase (regenerating system) or acetyl-CoA plus regenerating system as substrates. β-Ketothiolase from *C. pasteurianum* used only 20% of the chemically synthesized acetyl-CoA; the enzyme from *Alcaligenes eutrophus H 16* used 25%. When the regenerating system was added the condensation reaction continued. The enzyme from *C. pasteurianum* was inactivated by free coenzyme A, while the enzyme from *A. eutrophus* was inhibited.

When acetyl-CoA was added as the substrate the initial velocity determination was impeded by the lack of linearity. With acetyl-CoA as the substrate the K_m-value was found to be 2.5 mM acetyl-CoA. If free CoASH (or acetyl-CoA) plus regenerating system was added the K_m was 0.44 mM (0.42 mM) acetyl-CoA.

3. The β-ketothiolase activity was measured in the direction of acetoacetyl-CoA cleavage by an optical assay following the decrease of the enol and chelate form of acetoacetyl-CoA by absorption measurement at 305 nm. The activity was maximal at 24 mM $MgCl_2$. The apparent K_m values for acetoacetyl-CoA were 0.133 mM and 0.105 mM with 0.065 and 0.016 mM CoASH, respectively. The K_m-values as calculated for only the keto form of acetoacetyl-CoA were 0.0471 and 0.0372 mM, respectively. The cleavage reaction was inhibited by high acetoacetyl-CoA concentrations; the inhibition was partially relieved by CoASH. In the range of low concentrations of acetoacetyl-CoA only a slight inhibition by CoASH was observed. The K_m for CoASH was found to be 0.0288 and 0.0189 mM with 0.09 and 0.045 mM acetoacetyl-CoA, respectively. High concentrations of CoASH exerted an inhibitory effect on the cleavage reaction. With respect to enzyme kinetics and sensitivity to inhibitors and metabolites the β-ketothiolases of *C. pasteurianum* and *A. eutrophus* were rather similar.

Key words: β-Ketothiolase — *Clostridium pasteurianum* — *Alcaligenes eutrophus H 16* — Isoenzymes — Inhibition/Inactivation by Coenzyme A.

β-Ketothiolase is involved in fatty acid degradation, in synthesis of mevalonic acid, synthesis and reutilization of poly-β-hydroxybutyrate as well as in butyric acid fermentation. The β-ketothiolases involved in fatty acid degradation and ketogenesis were well characterized (Gehring and Riepertinger, 1968; Huth *et al.*, 1974; Kornblatt and Rudney, 1971; Mazzai *et al.*, 1970). The β-ketothiolase of *Azotobacter beijerinckii* (Senior and Dawes, 1973) and of *Alcaligenes eutrophus H 16* (Oeding and Schlegel, 1973) were recently analyzed, especially with respect to their regulatory properties. The tested enzymes are involved in the synthesis and reutilization of poly-β-hydroxybutyrate.

The present investigation aimed at the isolation and purification of β-ketothiolase from *Clostridium pasteurianum* and at a comparison of the kinetic and regulatory properties of this enzyme to that of *A. eutrophus H 16*. In cells of both bacteria β-ketothiolase *in vivo* is mainly involved in the condensation reaction and converts large amounts of acetyl-CoA to acetoacetyl-CoA for the synthesis of poly-β-hydroxybutyrate in *A. eutrophus H 16* and for butyrate production in *C. pasteurianum*. While in the first case the enzyme served the accumulation of a storage material, in *C. pasteurianum* the enzyme is involved in a process to remove excess reduction equivalents during fermentative energy generation.

Methods

Culture of Organism. The organism used was *Clostridium pasteurianum* (ATCC 6013). The β-ketothiolase of *Alcaligenes eutrophus (Hydrogenomonas eutropha) H 16* (DSM 428, ATCC 17699) was prepared according to the method

of Oeding and Schlegel (1973). The modified synthetic medium of Carnahan and Castle (1958) was prepared from solutions of the following compositions (per l): Solution A: glucose, 20 g; KH_2PO_4, 0.95 g; K_2HPO_4, 5.75 g; $(NH_4)_2SO_4$, 1.0 g; NaCl, 0.1 g. Solution B: $CaCO_3$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.247 g; $MnCl_2 \cdot 4H_2O$, 1.07 mg; $Na_2MoO_4 \cdot 2H_2O$, 12 mg; $FeSO_4 \cdot 7H_2O$, 5.6 mg; biotin, 1 µg; p-aminobenzoic acid 5 µg. *C. pasteurianum* was grown anaerobically in a fermenter (180 l). The glucose-solution plus 35 mg $Na_2S_2O_4$/l were autoclaved separately, while solution B was sterilized by filtration. The combined solutions had a volume of 150 l. A culture of 8 l (optical density at 546 nm about 10) served as inoculum. After a lag phase of about 35 hrs the cells grew within 10 hrs to an optical density of 9. After 6 hrs 250 ml KOH (20%, w/v) and 500 ml of a solution containing 500 g glucose and 2.5 g $(NH_4)_2SO_4$ were added under sterile conditions. The pH value shifted from 7.8 to 4.5 during the growth period.

Preparation of Cell Extracts. The suspension volume was reduced by means of a separator (Westphalia) and then centrifuged at 8000 g for 30 min. The harvested cells were stored at −20° C. The disruption of the cells was carried out with the French-press at approximately 60 kg/cm². The crude cell extracts were centrifuged at 20000 g for 30 min, and the supernatant was centrifuged again at 120000 g for 60 min. All steps were carried out at 0−4° C.

Preparations. Acetyl-CoA was prepared by the method of Simon and Shemin (1953) with acetic anhydride and CoASH. The remaining free CoASH was oxidized by the method described by Oeding and Schlegel (1973). The oxidized CoA did not affect the activity of β-ketothiolase in the condensation reaction. The concentration of the acetyl-CoA preparation was determined according to Bergmeyer (1970). The molar extinction coefficient of 5.5′-dithiobis-(2-nitrobenzoic acid) (DTNB) of 11400 was used (Robyt *et al.*, 1971). Free CoASH was measured employing the DTNB-test: 0.05 M Tris-HCl buffer plus 3.9 mg DTNB per 100 ml buffer and 0.02 ml CoASH solution in a total volume of 1.00 ml. Another test with phosphotransacetylase (Bergmeyer, 1970) was used to test the commercial CoASH preparation for none-CoASH sulfhydryl groups.

Acetoacetyl-CoA was prepared with diketen and CoASH by the method of Senior and Dawes (1973, modified). CoASH (16 µmoles) was dissolved in 2 ml H_2O and adjusted to pH 7.0 with 3 M $KHCO_3$. The following steps were carried out as described by Senior and Dawes (1973). The preparation contained no free CoASH. The concentration of acetoacetyl-CoA was measured enzymatically with L-β-hydroxyacyl-CoA dehydrogenase (see Oeding and Schlegel, 1973). Protein was determined according to Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Enzyme assays. β-ketothiolase. The *condensation reaction* of two molecules of acetyl-CoA forming acetoacetyl-CoA and CoASH was followed measuring the oxidation of NADH, coupled with the reduction of acetoacetyl-CoA by β-hydroxyacyl-CoA dehydrogenase (Lynen *et al.*, 1952). Kinetic studies of the condensation reaction were carried out using three different enzyme assays: a) with acetyl-CoA. A cuvette contained: 100 mM Tris-HCl buffer, pH 8.2; 2.1−2.3 mM acetyl-CoA; 0.30 mM NADH; 2.5 U of β-hydroxyacyl-CoA dehydrogenase; 10 µl of β-ketothiolase preparation; the final volume was 1.00 ml. The decrease in E_{334} was measured in an Eppendorf-spectrophotometer at 25° C with 1 cm-cuvettes. Protein concentration and initial velocity of the reaction showed a linear relationship up to 0.03 mM NADH oxidized per min. b) With the regenerating system acetyl-phosphate and phosphotransacetylase plus CoASH. A cuvette contained: 100 mM Tris-HCl buffer, pH 8.2; 0.63 mM CoASH; 20 mM acetyl-phosphate and 5 µl of phosphotransacetylase (2 mg/ml). The mixture was incubated for 10 min. Then the reaction was started by adding 0.30 mM NADH; 5 µl of β-hydroxyacyl-CoA dehydrogenase (2 mg/ml) and 10 µl of the thiolase preparation. The enzyme reaction was recorded as described above. The determination of the acetyl-CoA concentration was performed with DTNB by measuring the free CoASH not transformed to acetyl-CoA. The reaction mixture contained: 100 mM Tris-HCl, pH 8.2; 20 mM acetyl-phosphate; 5 µl of phosphotransacetylase (2 mg/ml) and CoASH in different concentrations. After 10 min of incubation the protein was precipitated by 0.1 ml of trichloroacetic acid (20% w/v) and centrifuged at 4000 rpm for 15 min; 0.02 ml of the supernatant were used for the determination of CoASH. c) With the regenerating system (acetyl-phosphate and phosphotransacetylase) plus acetyl-CoA. A cuvette contained: 100 mM Tris-HCl pH 8.1; 2.1−2.3 mM acetyl-CoA; 20 mM acetyl-phosphate; 5 µl of phosphotransacetylase. The mixture was incubated for 10 min; then 0.30 mM NADH, 5 µl of β-hydroxyacyl-CoA dehydrogenase (2 mg/ml) and 10 µl of β-ketothiolase were added. The reaction was recorded as described above.

The *cleavage reaction* (thiolysis) of β-ketothiolase was measured at 305 nm and 25° C. A cuvette contained: 100 mM Tris-HCl buffer, pH 8.2; 24 mM $MgCl_2$; 0.09 mM acetoacetyl-CoA; 0.06 mM CoASH; 10 µl of β-ketothiolase in a total volume of 1.00 ml. A linear relationship between decrease of extinction and time as well as protein concentration and initial velocity was observed. The apparent extinction coefficient of acetoacetyl-CoA was 19.0×10^3 litre $\cdot$ mol^{-1} $\cdot$ cm^{-1}. The magnesium ions interact at pH values greater than 7.5 with the enol form of acetoacetyl-CoA to form a chelate (Stern, 1956). The keto form decreases with increasing concentrations of magnesium. The amount of the three forms of acetoacetyl-CoA in the presence of 24 mM $MgCl_2$, at a pH of 8.2, a wave length of 305 nm and at different concentrations of acetoacetyl-CoA was calculated according to the method of Stern (1956).

The enzyme *β-hydroxybutyryl-CoA dehydrogenase* was measured using an optical test at 334 nm. A cuvette contained: 85 mM $K_2HPO_4-KH_2PO_4$ buffer, pH 7.0; 1.66 mM acetoacetyl-CoA; 0.125 mM NADH; 5 µl of crude extract. The total volume was 1.00 ml. In the case of NADH-oxidase activity the values of dehydrogenase activity were corrected.

The *reference substances* used for molecular weight determinations were measured by the following procedures: aldolase was determined according to the method of Racker (1947), catalase after Bergmeyer (1955). Ferritin was estimated at 400 nm, dextranblue at 612 nm and crystalline bovine serum albumin as well as chymotrypsinogen at 280 nm.

Purification of β-Ketothiolase. All steps were done at 0−4° C. The buffers used throughout were $K_2HPO_4-KH_2PO_4$ mixtures at pH 7.4, plus 1 mM mercaptoethanol. *Protamine sulphate* (1% w/v; 0.3 vol %) was added with stirring to the crude extract (approx. 20 mg of protein/ml). After 15 min the mixture was centrifuged at 20000 g for 30 min. The precipitate was discarded and the supernatant was then fractionated by adding powderized *ammonium sulphate* at pH 7.4 with stirring to give a 65% saturated solution. During addition of ammonium sulphate the pH

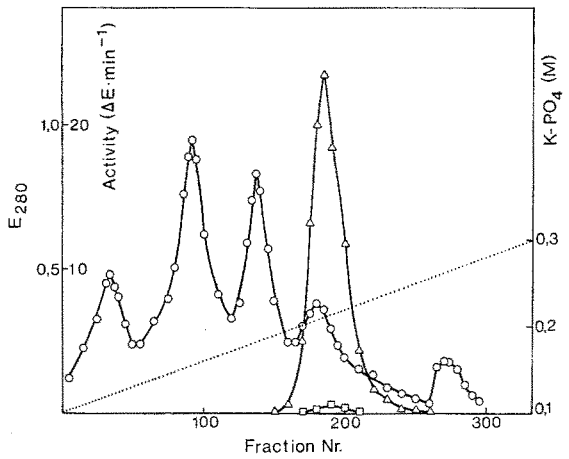


Fig. 1. Elution diagram for purification of β-ketothiolase on DEAE-Sephadex A-50. The column (2.5×27.5 cm) was equilibrated with 0.1 M potassium phosphate buffer, pH 7.4, plus 1 mM mercaptoethanol and started with 19 ml of enzyme solution (18.6 mg protein/ml). Elution was carried out with 1000 ml of a linear gradient of potassium phosphate buffer (0.1−0.3 M), pH 7.4, plus 1 mM mercaptoethanol. The flow rate was 30 ml/h and the fraction volume 3 ml. (○) protein at 280 nm; (△) β-ketothiolase activity; (□) β-hydroxybutyryl-CoA dehydrogenase; (.....) 0.1−0.3 M $K\text{-}PO_4$ buffer

value was adjusted to pH 7.4 with 1 N KOH. After centrifugation at 20000 g for 20 min the pellet containing the main portion of β-hydroxybutyryl-CoA dehydrogenase was removed. Then the supernatant was brought to 75% saturation ammonium sulphate. After the last addition of salt a period of 60 min was allowed for the completion of precipitation before centrifugation at 20000 g for 20 min was commenced. The pellet was dissolved in 100 mM $K\text{-}PO_4$ buffer, pH 7.4, plus 1 mM mercaptoethanol and dialyzed against the same buffer overnight.

The dialyzed β-ketothiolase was adsorbed on a *DEAE-Sephadex* A-50 column (2.5×27.5 cm) previously equilibrated with 0.1 M $K\text{-}PO_4$ buffer, pH 7.4, plus 1 mM mercaptoethanol. After application of the enzyme solution (19 ml; 18.6 mg protein/ml) the column was washed with 1 l of a linear gradient of 0.1−0.3 M $K\text{-}PO_4$ buffer, pH 7.4, plus 1 mM mercaptoethanol. The flow rate was 30 ml/h; fractions with a volume of 3 ml were collected. The β-ketothiolase was eluted between 0.2 and 0.23 M $K\text{-}PO_4$ buffer (Fig. 1). The combined active fractions were concentrated by ultrafiltration (Diaflo cell, Amicon) and dialyzed overnight against 10 mM $K\text{-}PO_4$ buffer. The solution was then applied to a *hydroxylapatite* column (1.6×15 cm); the protein was eluted with a linear gradient (500 ml) of 0.01−0.1 M $K\text{-}PO_4$ buffer, pH 7.4, plus 1 mM mercaptoethanol. Fractions of 3 ml were collected with a flow rate of approx. 30 ml/h. The active fractions were combined, concentrated by ultrafiltration (Diaflo cell, Amicon) and dialyzed against 50 mM $K\text{-}PO_4$ buffer, pH 7.4, plus 1 mM mercaptoethanol. The purified β-ketothiolase was stored at −20°C in glycerol (one part of enzyme to three parts of glycerol).

Determination of Molecular Weight. The molecular weight of β-ketothiolase was determined by two methods, by gel filtration (Gelotte, 1960) and by sucrose density gradient centrifugation according to the method of Martin and Ames (1961). For *sucrose density gradient centrifugation*, partially purified β-ketothiolase plus catalase and aldolase as reference proteins were allowed to sediment in 13 ml of a linear sucrose gradient between 7% and 30% sucrose in 50 mM $K\text{-}PO_4$ buffer, pH 7.4, plus 1 mM mercaptoethanol. The gradients were formed by a gradient former (Isco, model 570). Centrifugation was performed in the Omega II 70000 (Heraeus-Christ) using a swing-out rotor (6×15 ml) at 40000 rpm and 4°C for 17.5 hrs. Enzyme activities were measured after perforating the centrifuge tubes and collecting fractions of 4 drops each. *Gelfiltration* with Sephadex G-200 was performed with the following proteins (molecular weights in parantheses): ferritin (540000), aldolase (158000), crystalline bovine serum albumin (67000) and chymotrypsinogen A (25000) in a column (2.5×60 cm) equilibrated with 50 mM $K\text{-}PO_4$ buffer, pH 7.4, plus 1 mM mercaptoethanol. A sample of 0.5 ml was eluted with the same $K\text{-}PO_4$ buffer as above at a flow rate of 10 ml/h, fractions of 1 ml were collected.

Isoelectric Focusing. The isoelectric focusing of the β-ketothiolase was performed in a column of 110 ml (LKB) with ampholytes pH 3.5−10. The experiment was mainly carried out as recommended in the LKB manual. For focusing 0.5 ml of the purified enzyme (716 E/mg) was dialyzed against a solution of ampholytes (0.5% v/v; pH 3.5−10), plus 0.1 mM mercaptoethanol. With the anode at the top, the enzyme solution was focused for 28 hrs at 310 volt (constant) and 6°C. The collected fractions (3 ml) were provided with 5 µl of mercaptoethanol to reactivate the β-ketothiolase.

Polyacrylamide-Gel Electrophoresis. The purified enzyme was subjected to polyacrylamide-gel electrophoresis following the procedure of Davis (1964). The electrophoresis (50 µg of protein) was performed for 4 hrs at 2.5 mA per tube. Staining of the protein was carried out with coomassie brilliant blue (Diezel *et al.*, 1972).

Chemicals. Aldolase, catalase, β-hydroxyacyl-CoA dehydrogenase, phosphotransacetylase, citrate synthase, acetylphosphate, ferritin, crystalline bovine serum albumin, chymotrypsinogen, CoASH, NAD, NADH, NADPH, ATP, ADP, AMP, pyruvate, phosphoenolpyruvate, oxalacetate and α-oxoglutarate were obtained from Boehringer, Mannheim. Another aldolase preparation was purchased from Worthington, Freehold, DTNB from Serva, Heidelberg.

Results

Properties of β-Ketothiolase

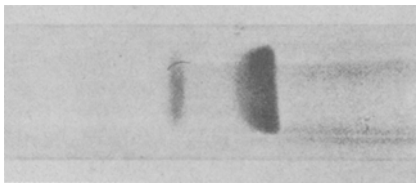
β-Ketothiolase was purified from glucose grown cells of *Clostridium pasteurianum*. After disruption of the cells by the French-press the extract was treated with protamine sulphate and the enzyme was purified by ammonium sulphate fractionation and by chromatography with DEAE-Sephadex A-50 and hydroxylapatite 130-fold with a yield of 67% (Table 1). The specific activity was measured throughout the purification procedure by the condensation reaction with the regenerating system (acetyl-phosphate and phosphotransacetylase) plus CoASH.

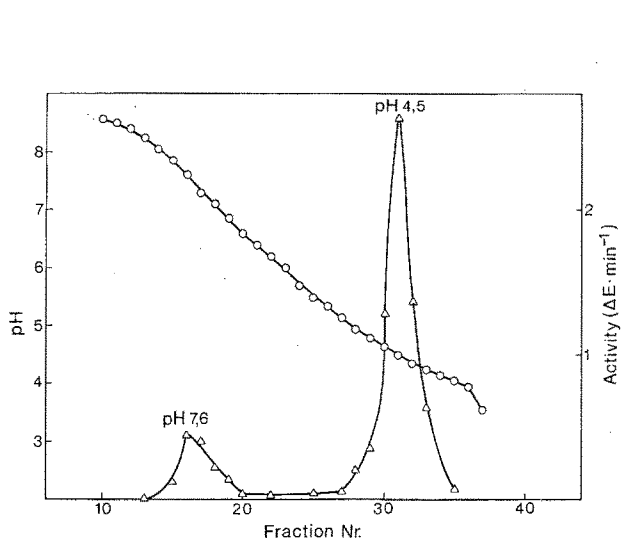
Table 1. Purification of β-ketothiolase from *Clostridium pasteurianum*

Procedure	Volume (ml)	Protein (mg/ml)	Specific activity (U/mg)	Yield %
Crude extract [a]	160	18.8	5.5	100
Protamine sulphate fractionation	204	6.4	11.1	87
Ammonium sulphate fractionation	19	18.6	27.9	59.7
DEAE-Sephadex column	116	0.4	210.1	54.3
Hydroxylapatite column	4.2	3.8	715.5	69.4

[a] 120000 g supernatant.

1 mM mercaptoethanol by sucrose density gradient centrifugation. Both enzymes had the same molecular weight of 158000. Running a polyacrylamide-gel electrophoresis two protein staining bands were detected (Fig. 3). Thiolase activity was measured in gels which were cut in pieces of 1 mm using a gel fractionator (Gilson) and eluted with 50 mM K-PO_4, pH 7.4, plus 1 mM mercaptoethanol. Two peaks with β-keto-





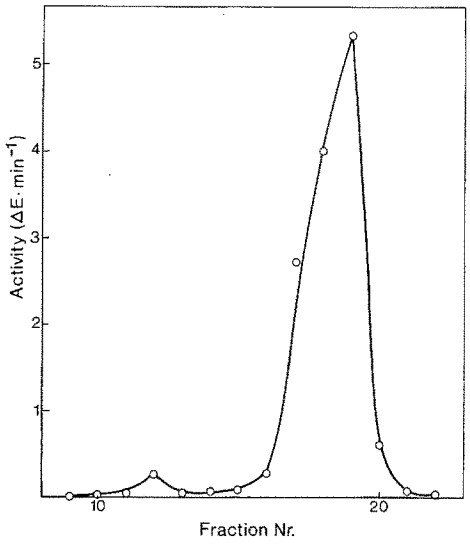


Fig. 2. Isoelectric focusing of β-ketothiolase. Isoelectric focusing was carried out with a 110 ml column (LKB). The pH range of ampholytes was 3.5—10. A sample of 0.5 ml enzyme solution (716 U/mg) was subjected to 310 volts for 38 hrs. Fractions of 3 ml were collected. Before measuring the activity 5 μl of mercaptoethanol were added to each fraction. (○) pH gradient; (△) β-ketothiolase (cleavage reaction, E_{305})

Fig. 3. Polyacrylamide-gel electrophoresis of β-ketothiolase. Migration of β-ketothiolase in polyacrylamide gel (7.5% acrylamide) is shown in the photograph. As electrode buffer, Tris-glycine, pH 8.9 was used. 50 μg of protein (716 U/mg) were subjected to 2.5 mA per tube for 4 hrs. Protein staining was performed with coomassie brilliant blue. Active β-ketothiolase bands were eluted as exhibited in the elution diagram. Gel tubes were cut into pieces of 1 mm using a gel fractionator (Gilson) and eluted with 50 mM K-PO_4 buffer, pH 7.4, plus 1 mM mercaptoethanol. (○) β-ketothiolase, as measured by cleavage reaction (E_{305})

The purified enzyme was subjected to polyacrylamide-gel electrophoresis and isoelectric focusing. The distribution of the β-ketothiolase in a pH gradient is shown in Fig. 2. Two forms of β-ketothiolase were found, one with an isoelectric point (IEP) of 4.5, the other with an IEP of 7.6. The molecular weights of the two thiolases (fraction 16 and 31) were determined after dialysis against 50 mM K-PO_4 buffer, pH 7.4, plus thiolase activity were found corresponding to the stained bands (Fig. 3).

The effect of pH on enzyme activity measured by the condensation and cleavage reaction is shown in Fig. 4. The optimum pH for the synthesis was about 8.1 that for the thiolysis 8.2.

The estimation of the molecular weight by sucrose density gradient centrifugation yielded a value of

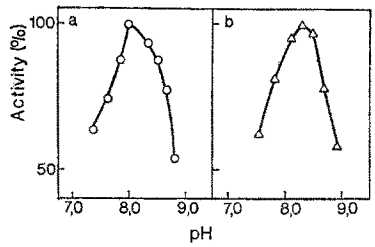


Fig. 4a and b. Dependence of β-ketothiolase activity on pH. Reaction mixtures were used as given in methods. Condensation reaction was carried out with acetyl-CoA. (a) Synthesis; (b) thiolysis

158000. This value corresponds to a Svedberg constant of s_{20} S = 7.82 as it is given for aldolase (MW = 158000) by Kawahara and Tanford (1966). With catalase (MW = 240000, Boehringer) as reference protein a molecular weight of 160000 was determined. When using Sephadex G-200 the molecular weight was found to be 158000. The column was run with aldolase (Worthington), ferritin, cristalline bovine serum albumin and chymotrypsinogen A (Boehringer) as reference proteins.

Condensation Reaction

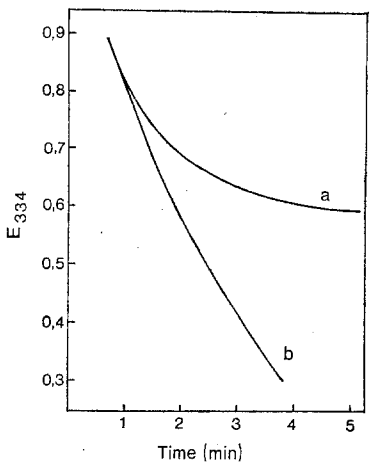
In the direction of synthesis β-ketothiolase catalyses the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA and CoASH. The reaction rate was measured by three optical tests with β-hydroxyacyl-CoA dehydrogenase as auxiliary enzyme (see "Methods"). Although the oxidized coenzyme NAD exerts an inhibitory effect on the auxiliary enzyme, impairment of the gross reaction rate was prevented by an excess of β-hydroxyacyl-CoA dehydrogenase in the reaction mixture. Free CoASH had no effect on the activity of β-hydroxyacyl-CoA dehydrogenase.

With acetyl-CoA as the substrate no linear relationship between decrease of extinction and time appeared (Fig. 5, a); after starting the reaction by addition of β-ketothiolase ΔE/min decreased immediately. Higher initial velocities were achieved by using the condensation test with acetyl-phosphate and phosphotransacetylase plus CoASH as the acetyl-CoA forming and regenerating system (Fig. 5, b). A similar time course of the reaction was observed when the regenerating system plus acetyl-CoA was added (see Fig. 5, b).

The kinetics of the condensation reaction were studied using two optical test systems. With acetyl-CoA (0.07—2.3 mM) as the substrate and in the absence of a regenerating system β-ketothiolase followed Michaelis-Menten kinetics (Fig. 6). The apparent K_m value was found to be 2.5 mM. With the regenerating system plus CoASH substrate saturation curves showed an inhibitory effect at high substrate concentrations (Fig. 7). This inhibition is apparently due to increasing free CoASH concentrations in the test system (see "Methods"). In these experiments the regenerating system was preincubated with increasing concentrations of free CoASH. When the initial concentration of CoASH in the preincubation mixture was 0.79 mM only 0.52 mM was transformed to acetyl-CoA while 0.27 mM CoASH remained non-acetylated. As will be shown later this is already a highly inhibitory concentration of free coenzyme A. The apparent K_m value for acetyl-CoA was 0.44 mM. Employing a coupled optical test with regenerating system plus acetyl-CoA the apparent K_m was found to be 0.42 mM.

Since CoASH is known to be a vigorous inhibitor of β-ketothiolase, inhibition experiments were carried out employing the condensation reaction with acetyl-CoA. As shown in Fig. 8, β-ketothiolase activity decreased within 10 min about 90% with 33 μM and 80% with 3.3 μM CoASH. A different inhibition pattern was obtained with the β-ketothiolase of *Alcaligenes eutrophus H 16* (Fig. 9). In *H 16* the inhibition was not dependent on the duration of the incubation period with CoASH. In modifying the inhibition experiment performed by Oeding and Schlegel (1973; Fig. 6) β-ketothiolase of *C. pasteurianum* was not incubated in the presence of the substrate acetyl-CoA.

Since the initial rates and the time course of the reaction were extremely different when synthetic or commercial acetyl-CoA or the regenerating system plus CoASH were added as a substrate of β-ketothiolase, the following experiment was done (Fig. 10). If acetyl-CoA was the substrate, the rate of the condensation reaction decreased very quickly and reached zero when only a small portion of the acetyl-CoA had been consumed. The curve indicates that only 20% of that acetyl-CoA concentration, which reacts with citrate synthase in the quantitative determination of acetyl-CoA (0.4 mM acetyl-CoA) had been used and transformed to acetoacetyl-CoA. Upon the addition of phosphotransacetylase and acetyl-phosphate the reaction continued. In this case the free coenzyme A was reacetylated. If the regenerating system was added after a longer time interval the reaction continued, too, however, at a diminished rate. The longer the enzyme was incubated in the absence of the regenerating system the lower was the enzyme activity measured following the addition of the regenerating system. The addition of a further sample of β-ketothiolase made sure that it was β-ketothiolase which became affected during prolonged incubation. From this experiment no unequivocal conclusion can be drawn. The early decline of the reaction rate with acetyl-CoA as a sub-



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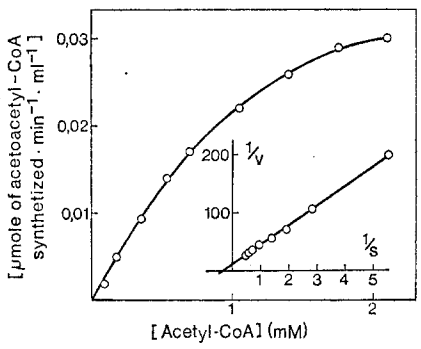

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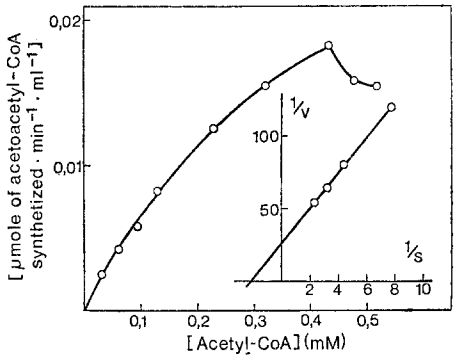

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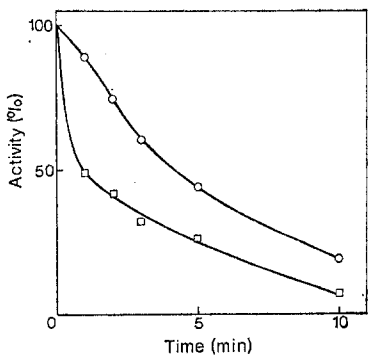
Fig. 5. Dependence of the reaction rate of β-ketothiolase on time. *a* Condensation reaction with acetyl-CoA in the absence of a regenerating system. The reaction mixture contained in 1.00 ml: 100 mM Tris-HCl, pH 8.2; 2.1 mM acetyl-CoA; 0.30 mM NADH; 5 µl of β-hydroxybutyryl-CoA dehydrogenase (2 mg/ml) and 10 µl of partially purified β-ketothiolase. *b* Condensation reaction in the presence of acetyl-phosphate and phosphotransacetylase plus CoASH as regenerating system. The reaction mixture contained in 1.00 ml: 10 mM Tris-HCl, pH 8.2; 0.63 mM CoASH; 20 mM acetyl-phosphate; 5 µl of phosphotransacetylase (2 mg/ml). After 10 min of preincubation the following compounds were added: 0.30 mM NADH; 5 µl of hydroxybutyryl-CoA dehydrogenase (2 mg/ml) and 10 µl of β-ketothiolase

Fig. 6. Substrate (acetyl-CoA)-saturation curve of β-ketothiolase. The reaction mixture contained in 1.00 ml; 151 mM Tris-HCl, pH 8.2; 0.30 mM NADH; 5 µl of β-hydroxybutylryl-CoA dehydrogenase (2 mg/ml) and 10 µl of β-ketothiolase. The acetyl-CoA was variied as indicated in the figure

Fig. 7. Substrate-saturation curve of β-ketothiolase employing the regenerating system acetyl-phosphate and phosphotransacetylase plus CoASH. The reaction mixture contained in 1.00 ml: 100 mM Tris-HCl buffer, pH 8.2; 20 mM acetyl-phosphate; 5 µl of phosphotransacetylase (2 mg/ml) and different CoASH concentrations. The mixture was incubated for 10 min and then 5 µl of β-hydroxyacyl-CoA dehydrogenase (2 mg/ml), 0.30 mM NADH and 10 µl of β-ketothiolase were added. The concentration of resulting acetyl-CoA was estimated as described in "Methods"



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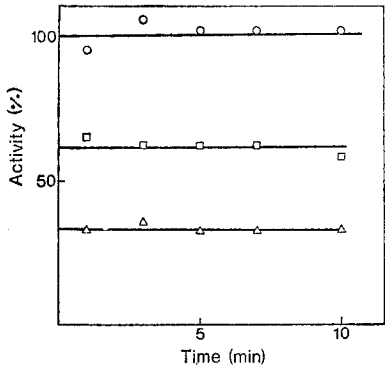

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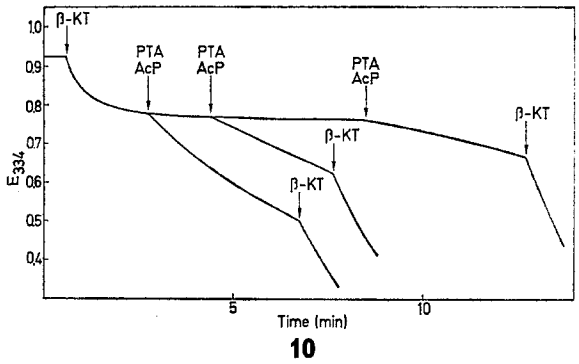
Fig. 8. Inactivation of β-ketothiolase by free CoASH with condensation reaction (acetyl-CoA). The reaction mixture contained in 1.00 ml: 151 mM Tris-HCl, pH 8.2; 0.30 mM NADH; 2.1 mM acetyl-CoA; 5 µl of β-hydroxyacyl-CoA dehydrogenase (2 mg/ml); 10 µl of β-ketothiolase. The concentrations of CoASH were: (○) 0.0033 mM and (□) 0.033 mM. The reaction was started with acetyl-CoA

Fig. 9. Inhibition of β-ketothiolase of *Alcaligenes eutrophus H 16* by CoASH with condensation reaction (acetyl-CoA). The reaction mixture contained in 1.00 ml: 100 mM Tris-HCl, pH 8.2; 0.30 mM NADH; 2 mM acetyl-CoA; 5 µl of β-hydroxyacyl-CoA dehydrogenase (2 mg/ml); 10 µl of β-ketothiolase and different concentrations of CoASH: (△) 0.126 mM; (□) 0.0128 mM; (○) without CoASH. The reaction was started with acetyl-CoA



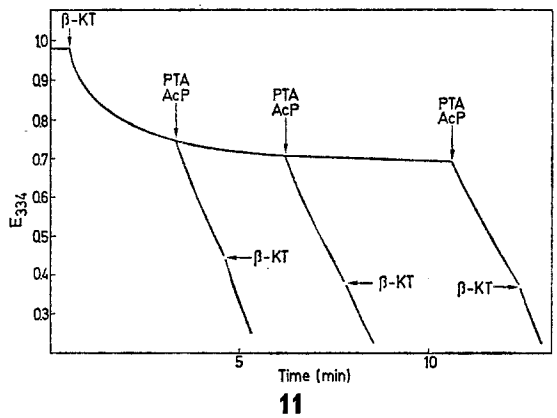


Fig. 10. Temporal course of the condensation reaction with acetyl-CoA as the initial substrate and after the addition of the regenerating system added after different time intervals. The reaction mixture contained in 1.00 ml; 100 mM Tris-HCl, pH 8.2; 0.40 mM acetyl-CoA; 0.30 mM NADH; 5 µl of β-hydroxyacyl-CoA dehydrogenase (2 mg/ml) and 10 µl of β-ketothiolase. After different time intervals 5 µl of phosphotransacetylase (2 mg/ml) and 20 mM acetyl-phosphate were added. The abbreviations are: *β-KT* β-ketothiolase; *AcP* acetyl-phosphate; *PTA* phosphotransacetylase. As a control in each instance 10 µl β-ketothiolase were finally added to check the integrity of the auxiliary components of the test system. The concentration of the acetyl-CoA preparation was determined with 5.5′-dithiobis-(2-nitrobenzoic acid) (DTNB) using a molar extinction coefficient of 14140 (Collier, 1973)

Fig. 11. Temporal course of the condensation reaction with acetyl-CoA as the initial substrate and after the addition of the regenerating system added after time intervals using the enzyme β-ketothiolase from *Alcaligenes eutrophus H 16*. Reaction mixtures and test conditions were the same as described in Fig. 10

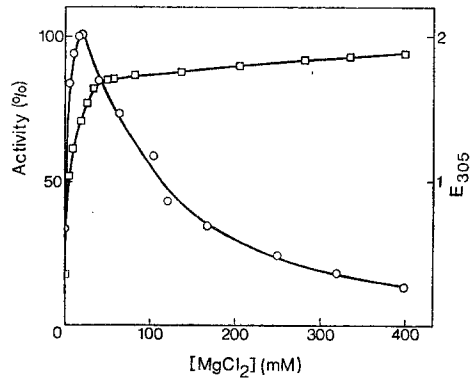


Fig. 12. Dependence of β-ketothiolase activity (thiolysis) on the magnesium ion concentration. The enzyme test was performed with standard assay conditions (see "Methods"). Estimation of the absorbance of enol and chelate form of acetoacetyl-CoA was carried out in 1.00 ml containing 100 mM Tris-HCl, pH 8.2, and 0.09 mM acetoacetyl-CoA. (○) β-ketothiolase; (□) enol and chelate form of acetoacetyl-CoA

strate may be due to the unacceptability of the major part of the chemically synthesized acetyl-CoA to the enzyme. It may be tentatively assumed that acetyl-CoA consists of different stereoisomeric forms when synthesized with acetic anhydride. An influence of effectors on the rate of β-ketothiolase reaction as demonstrated for phosphofructokinase by Ramaiah and Tejwani (1973) was not observed. All components introduced into the enzyme assay with the regenerating system were tested separately. No effect was observed with acetyl-phosphate, inorganic phosphate, lithium ions, phosphotransacetylase and ammonium ions.

Furthermore, prolonged incubation resulted in an increasing loss of enzyme activity. This experiment was repeated using the purified preparation of β-ketothiolase from *A. eutrophus H 16* (Fig. 11). While the decrease of the reaction rate was similarly pronounced as in the case of the *Clostridium* enzyme an inactivation effect during prolonged incubation was not observed.

Cleavage Reaction

The cleavage of acetoacetyl-CoA was measured at 305 nm in the presence of magnesium ions by following the decrease of the absorbance of the enol and chelate form of acetoacetyl-CoA. Since the true substrate of β-ketothiolase is assumed to be the ketoform of acetoacetyl-CoA (Stern, 1956; Middleton, 1974), the change of absorbance at 305 nm includes the reinstatement of the equilibrium between keto, enol and chelate form of the acetoacetyl-CoA during the cleavage reaction.

The rate of thiolysis was influenced by the magnesium content, too. When the concentration of $MgCl_2$ was raised the rate of the enzyme reaction reached a maximum value at 24 mM $MgCl_2$ (Fig. 12). Over a range of 0—0.128 mM acetoacetyl-CoA the optimum was always at 24 mM $MgCl_2$. When the substrate concentration was increased to 0.16 mM acetoacetyl-CoA the highest enzyme activity appeared with 8 mM $MgCl_2$.

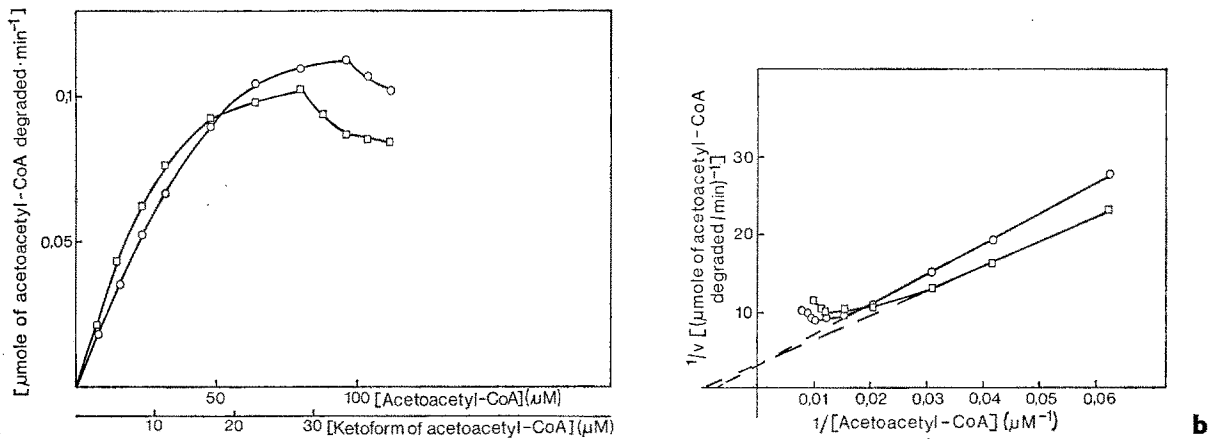


Fig. 13a and b. Substrate-saturation curves (acetoacetyl-CoA) of β-ketothiolase at two CoASH concentrations measured by the cleavage reaction. (a) Substrate-saturation curves. The reaction mixture contained in 1 ml: 100 mM Tris-HCl, pH 8.2; 24 mM $MgCl_2$; 10 µl of β-ketothiolase. Acetoacetyl-CoA concentration was variied as indicated. (b) Lineweaver-Burk plot. Concentrations of CoASH: (○) 0.065 mM; (□) 0.016 mM

The concentration of the ketoform of acetoacetyl-CoA in the substrate preparation was determined at 24 mM $MgCl_2$, pH 8.2, and 305 nm employing the method of Stern (1956). The molar extinction coefficient of the enol and chelate form was found to be 28400. The proportion of keto form in the total acetoacetyl-CoA was estimated to be 35.4%; this value was reasonably constant with increasing concentrations of acetoacetyl-CoA.

The kinetics of thiolysis as a two-substrate reaction have been determined by varying the concentration of the first substrate and then of the second one. While the concentration of CoASH was kept constant at 0.065 and 0.016 mM, acetoacetyl-CoA concentrations were increased from 0.008 to 0.112 mM. As is evident from Fig. 13a and b the enzyme followed Michaelis-Menten kinetics at low substrate concentrations. With increasing substrate concentrations the enzyme was inhibited. The second substrate CoASH influenced the reaction rate in two ways. At low concentrations of acetoacetyl-CoA the free CoASH caused a slight inhibition; with increasing CoASH (0.065 mM) the inhibition by acetoacetyl-CoA was shifted towards higher acetoacetyl-CoA concentrations. The K_m values were found to be 0.133 mM and 0.105 mM with 0.65 and 0.016 mM CoASH, respectively. The K_m values considering only the concentration of the keto form of acetoacetyl-CoA were 0.0471 and 0.0372 mM, respectively. With different concentrations of CoASH and constant amounts of acetoacetyl-CoA (0.09 and 0.045 mM) saturation curves were obtained as shown in Fig. 14a and b. At high substrate levels inhibition of β-ketothiolase occurred. As is evident from the Lineweaver-Burk plot substrate saturation curves gave parallel lines in the range of low CoASH concentrations (Fig. 14b).

Table 2. Inhibition of β-ketothiolase (cleavage reaction) by metabolites

Substance	Concentration (mM)	Enzyme activity (% of control)
NAD	1	98
	5	83
	10	66
α-Oxoglutarate	1	98
	5	76
	10	55

Parallel lines indicate that the two-substrate reaction follows a Ping-Pong mechanism (Cleland, 1963). The K_m values for CoASH were found to be 0.0288 and 0.0189 mM with 0.09 and 0.045 mM acetoacetyl-CoA, respectively.

Influence of Metabolites on β-Ketothiolase. As possible effectors of β-ketothiolase the following metabolites were tested at concentrations up to 10 mM: adenosine phosphates (ATP, ADP and AMP), nicotinamide nucleotides (NAD, NADP, NADPH) as well as pyruvate, phosphoenolpyruvate, acetate, α-oxoglutarate and ferredoxin in the reduced and oxidized form. The two substances with a considerable inhibitor effect are listed in Table 2. Besides β-ketothiolase β-hydroxybutyryl-CoA dehydrogenase in crude extracts of *C. pasteurianum* was inhibited by NAD. In the presence of 5 mM NAD the activity amounted to 47%.

Discussion

In living cells of *Clostridium pasteurianum* β-ketothiolase catalyses the synthesis of acetoacetyl-CoA and CoASH by transforming two molecules of acetyl-CoA. The enzyme starts the reductive part in glucose fermen-

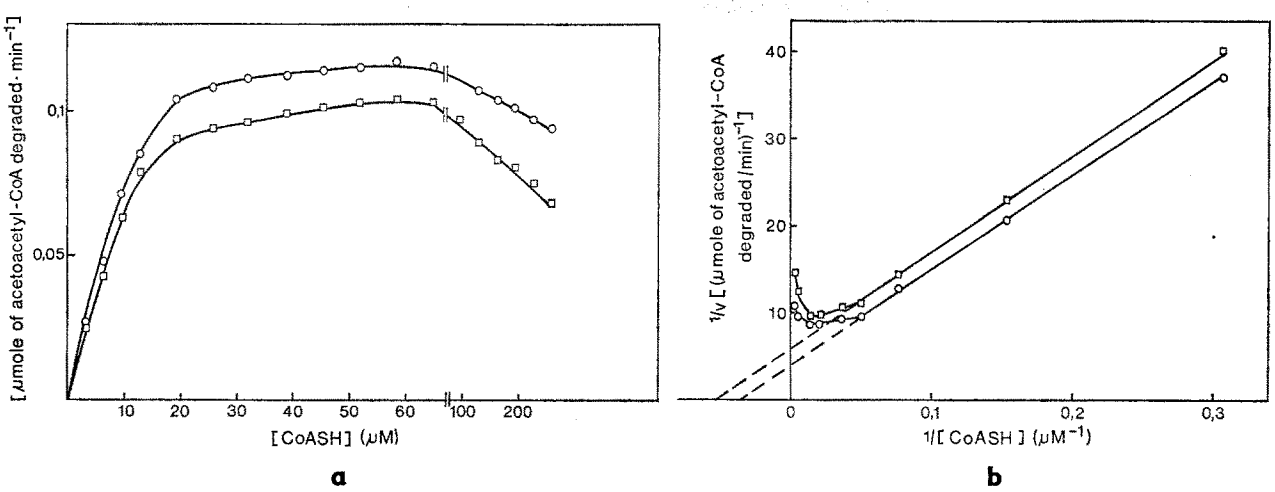


Fig. 14a and b. Substrate-saturation curves (CoASH) of β-ketothiolase at two concentrations of acetoacetyl-CoA. (a) Substrate-saturation curves. The reaction mixture was the same as described in Fig. 13. CoASH concentration was variied as indicated. (b) Lineweaver-Burk plot. Concentrations of acetoacetyl-CoA: (○) 0.09 mM; (□) 0.045 mM

tation. As established by v. Hugo *et al.* (1972) and Jungermann *et al.* (1973) butyrate, acetate, CO_2 and H_2 are the main products of glucose fermentation in *C. pasteurianum*.

β-Ketothiolase was purified 130-fold from *C. pasteurianum*. Isoelectric focusing revealed two enzymes with isoelectric points at 4.5 and 7.6. This observation is in accordance with the results of Kornblatt and Rudney (1971) who described two thiolases in yeast with isoelectric points at pH 5.4 and 7.8 and molecular weights of 140000 and 65000, respectively. Discussing the question whether thiolase 5.3 is a dimer and thiolase 7.8 a monomer of the same protein the authors left the problem undecided. In the case of thiolase from *C. pasteurianum* the molecular weights of both thiolases were found to be 158000. Assuming that both enzymes are coded by different genes one may call them isoenzymes. Their role in living cells may be supposed to be a response to different pH values during cellular growth. The initial pH value of the synthetic medium of 7.8 shifted to 4.5 while cultivating the cells in the fermenter.

Investigating the inhibitory and kinetic properties of the two thiolases separately no dissimilarities of condensation reactions were observed. CoASH inactivated both enzymes (see Figs. 8 and 10), the reaction rate was established to be the same as indicated in Fig. 5 and both enzymes utilized synthetic acetyl-CoA partly (see Fig. 10). The study is continued in order to elucidate the role of the two thiolases in butyrate fermentation of *C. pasteurianum*.

As mentioned above the β-ketothiolase enzymes of strictly aerobic bacteria, e.g. *Alcaligenes eutrophus H 16* and of anaerobic clostridia are involved in quite different metabolic pathways. While in *A. eutrophus H 16* the enzyme catalyzes the first reaction of storage material (PHB) synthesis, in *C. pasteurianum* it is involved in butyrate formation serving to remove excess hydrogen equivalents. These different metabolic roles might have been reflected by enzyme kinetics and sensitivity to metabolic effectors or inhibitors. The differences between the characteristics of the β-ketothiolase from *A. eutrophus H 16* (Oeding and Schlegel, 1973) and *Azotobacter beijerinckii* (Senior and Dawes, 1973) on the one hand and that of *C. pasteurianum* on the other hand are, however, surprisingly small. Although the kinetics are not identical, the enzymes from *H 16* and *C. pasteurianum* resemble each other with respect to the Michaelis-Menten kinetics of the condensation and the cleavage reaction, to substrate (acetoacetyl-CoA) inhibition in the cleavage reaction, and to the partial relief of this substrate inhibition by the second substrate, coenzyme A, as well as to the unacceptability of the major protein of chemically synthesized acetyl-CoA to the enzyme in the condensation reaction.

The *Clostridium* enzyme differed from the *H 16* enzyme with respect to the inhibition by free coenzyme A. While the *H 16* enzyme is instantaneously inhibited when CoASH is added to the enzyme assay (Fig. 9), the *Clostridium* enzyme is increasingly inactivated during prolonged incubation in the presence of free coenzyme A (Figs. 8 and 10). Another dissimilarity was the inhibition of the β-ketothiolase of *C. pasteurianum* by CoASH at low concentrations of acetoacetyl-CoA (Fig. 13).

The cleavage reaction was found to be affected by the magnesium concentration (Fig. 12). With increasing

concentrations of $MgCl_2$ the initial velocity of β-ketothiolase from rat liver decreased (Middleton, 1974); this effect was found to be due to the decrease of the substrate (acetoacetyl-CoA) concentration since in the presence of magnesium the equilibrium is shifted towards the enol and chelate form, and the concentration of the ketoform is diminished. Both the β-ketothiolases of *C. pasteurianum* and *A. eutrophus H 16* differed with respect to the response to increasing magnesium concentrations compared with the rat liver enzyme. While with the rat liver enzyme the initial rate decreased and the rate versus magnesium concentration curve resembled the ketoform versus magnesium concentration curve, with the bacterial enzymes the curves exhibited a maximum at 24 mM $MgCl_2$. Apparently there is an additional effect of magnesium on the enzyme in the cleavage reaction which is not observed with the rat liver enzyme. The rate of the condensation reaction was not affected by magnesium ions neither with the rat liver enzyme (Middleton, 1974) nor with the bacterial enzymes.

The clostridial enzyme is not inhibited by physiological concentrations of metabolites to a significant extent, nor exerts free coenzyme A in vivo an instantaneous inhibition. Apparently pyruvate oxidation is sufficiently fast and keeps the level of CoASH low.

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