Kinetic Studies on Purified Thiolase from

Blakeslea trispora

LARS BJÖRK and HALINA Y. NEUJAHR

Department of Pure and Applied Biochemistry, Royal Institute of Technology, S-100 44 Stockholm 70, Sweden

Thiolase, (EC 2.3.1.9) was purified 190-fold from the β-carotene producing mould, *Blakeslea trispora*. The activity of the enzyme was studied for the reverse reaction using acetoacetyl-CoA as substrate. With the most active preparations the K_m for the purified enzyme was 4.5×10^{-5} M with respect to acetoacetyl-CoA. The V_{max}, at saturating substrate concentrations was 6.7 μmoles acetoacetyl-CoA turned over per min and mg protein.

The effect on the reaction velocity of potassium salts of several organic acids was insignificant in most cases studied. It could be demonstrated that the strong inhibition of the enzyme by citrate was due to its complex formation with Mg^{2+}.

The extensive synthesis of β-carotene in mated cultures of *Blakeslea trispora* [1-5] is stimulated by several types of compounds, *e.g.* lipids,[6] hydrocarbons,[7] terpenes,[8,9] β-ionone,[10,11] and also by certain mono-, di- and tricarboxylic acid anions.[12,13]

We have earlier observed that pyruvate and members of the tricarboxylic acid cycle significantly stimulate β-carotene synthesis in growing cultures of *B. trispora*.[13] This gave an incentive to study the possible effect of these stimulators on the enzyme, generally considered to initiate isoprenoid synthesis from acetyl-CoA, *i.e.* acetyl-CoA:acetyl-CoA C-acetyltransferase (thiolase, EC 2.3.1.9).[14] The occurrence of this enzyme in cell-free systems from organisms with a pronounced ability to synthesize carotenoids has, to our knowledge, not been studied earlier (*cf.* Ref. 13). The present paper demonstrates that thiolase is present in *B. trispora*. None of the organic acid anions that have been shown to stimulate β-carotene synthesis in growing cultures [13] affects the activity of purified thiolase. Preliminary results of this work were reported.[15]

EXPERIMENTAL

Materials. Coenzyme A, reduced form (CoASH), reduced glutathione (GSH), and acetoacetyl-CoA, 87 % pure, were purchased from Sigma Chemical Co., St. Louis, Mo., USA.

Procedure

Organisms, growth media and conditions of fermentation were similar to those described elsewhere.[9,13] Mycelium from mated cultures of *B. trispora* NRRL 2456(+) and 2457(−) grown for 6 days in 100 ml cultures, was used in all experiments.

Enzyme assay was carried out for the reverse reaction (acetoacetyl-CoA + CoASH ⇌ 2 acetyl-CoA) with acetoacetyl-CoA and CoASH as substrates. The reaction velocity was measured by the disappearance of acetoacetyl-CoA from the reaction mixture essentially as described by Stern,[19] but using 1 cm standard quartz cuvettes and without acetoacetate in the blank. The acetoacetyl-CoA was omitted in the control mixture. The optical density was measured in a Hitachi-Perkin Elmer Spectrophotometer, Model 139, at 20°C, immediately before, and at 10 sec intervals after the reaction has been initiated by enzyme addition (3.5−9.0 μg protein/ml). The amount of the substrate transformed was calculated using the extinction coefficient for acetoacetyl-CoA, $E(1 \%, 1$ cm$) = 14\ 000$, as given by Stern *et al.*[16]

One unit of the enzyme is defined as the amount which transforms 1 μmole acetoacetyl-CoA per min. Protein determinations during the purification procedure were carried out by the method of Warburg and Christian.[17] The protein content of the final preparations was also tested by the method of Lowry *et al.*[18]

Acetate, pyruvate, citrate, and oxaloacetate were added as potassium salts. To eliminate a possible interference from potassium and carbonate the effect of these two ions was studied in separate control experiments.

Preparation of crude extracts. Method A. Mycelium from 2 litres of culture, containing about 100 g solids, was filtered under suction and washed twice with 2 l ice-cold 0.05 M potassium phosphate buffer, pH 7.4, containing 0.2 M potassium chloride. The filter cake was re-suspended in four 200 ml portions of the same buffer. Each portion was homogenized for 10 min with 30 g 1 mm glass beads in an Ato-Mix (MSE) at highest speed while cooled by successively added dry ice. The homogenate was filtered through five layers gauze tissue and centrifuged at 20 000 *g* for 7 min. The supernatant liquid was filtered through 10 layers gauze tissue to remove floating lipid material. The filtrate was used as crude preparation for the fractionation procedure described below.

Method B. Mycelium was treated as in Method A, but employing 0.05 M tris·HCl, pH 7.4, instead of phosphate. The filter cake was re-suspended in two 500 ml portions of the same buffer. 2500 ml of acetone (−15°C) was added to each portion slowly under continuous stirring and the resulting precipitate was filtered off under suction. Each filter cake was re-suspended in 2500 ml acetone (−15°C) and homogenized for 2 min in an Ato-Mix (MSE) at highest speed. The homogenate was filtered and washed once by re-suspension in 2500 ml acetone (−15°C), whereafter it was filtered again. The final filter cake was dried for 30 min under suction and then for 3 h in a freeze drier. The resulting acetone powder was suspended in 2000 ml 0.05 M tris·HCl, pH 9, cooled in ice. The pH was adjusted with 3 M HCl to 7.0 and the suspension was centrifuged for 30 min at 3000 *g* and 0°C. The supernatant liquid was used as a crude preparation for the fractionation procedure described below.

Purification of the enzyme from crude extracts. The purification procedure was essentially similar to that employed by Stern[19] for the purification of thiolase from pig heart, but modified as described below. Dialysis steps were exchanged for desalting on Sephadex G-25 columns (2.5 × 100 cm). The acetic acid precipitation step, proposed by Stern,[19] was found superfluous and therefore omitted.

The enzyme was collected between 35−65 % ammonium sulphate saturation and then fractionated with acetone. The fraction precipitating between 0 and 40.5 % (v/v) acetone at −5°C was fractionated again with ammonium sulphate and the enzyme collected between 55 and 70 % saturation. In earlier experiments using acetic acid treatment and dialysis, the enzyme occasionally precipitated between 0 and 55 % saturation instead of between 55 and 70 % as described for the corresponding enzyme preparation from pig heart.[19] The active (55−70 %) fraction was further fractionated with ethanol and zinc acetate at different temperatures.[19] The enzyme was collected between 25 and 40 % (v/v) ethanol at −15°C and 0.007 M zinc acetate.

Gel filtration experiments were carried out with Sephadex G-100 and G-200 to test the homogeneity of the enzyme and to determine its approximate molecular weight.

RESULTS AND DISCUSSION

The effectiveness of the two methods employed for the preparation of crude enzyme extracts is shown in Table 1. It is seen that acetone treatment

Table 1. Extraction of thiolase from *B. trispora* by two different methods.

Method	Volume ml	Enzyme units/ml	Enzyme units, total	Protein mg/ml	Spec. activity units/mg protein
A	2×10^3	4.05×10^{-2}	81	1.13	3.60×10^{-2}
B	2×10^3	10.2×10^{-2}	204	2.95	3.46×10^{-2}

of the homogenized mycelium (method B) released considerably more protein into solution than mechanical disruption (method A). The specific activity of the crude enzyme preparations obtained by method B was only slightly lower than that obtained by method A. For this reason method B was employed for the subsequent preparations described here.

The purification of the enzyme from crude extracts is summarized in Table 2. The enzyme activities of the successive fractions were determined as maxi-

Table 2. Purification of thiolase from crude extracts of *B. trispora*.

Fraction	Volume ml	Enzyme units/ml	Enzyme units, total	Protein mg/ml	Spec. activity units/mg protein	Yield %	Purification fold
Crude extract	2000	0.12	238	3.4	3.5×10^{-2}	100	1
First ammonium sulphate precipitate 35−65 %	140	0.88	123	5.9	0.15	52	4
Acetone precipitate 0−40.5 %	65	1.75	114	6.6	0.27	48	8
Second ammonium sulphate precipitate 55−70 %	45	1.76	79.2	1.6	1.1	33	31
(Zn)-ethanol precipitate 25−40 %	35	1.44	50.3	0.75	6.7	21	190

mum reaction velocities by extrapolation from initial reaction velocities using different concentrations of acetoacetyl-CoA. The specific activity of the purified enzyme (best preparations) was 6.7 units/mg protein which corresponds to a 190-fold purification from the crude extract.

Properties of the enzyme upon gel filtration. The enzyme was excluded from Sephadex G-100, but included into Sephadex G-200, whereby a single peak of protein was obtained. The molecular weight of the enzyme thus seems to be in the range 100 000 − 200 000.

Stability of the enzyme. The enzyme was stored at −15°C in 0.01 M potassium phosphate containing 10^{-3} M L-cysteine. The activity decreased slowly indicating a half-life time of approximately three weeks. Some of the kinetic and activation studies were carried out with preparations exhibiting approximately 30 % of the activity of freshly purified enzyme.

Kinetic studies with the purified enzyme. The progress curves for the activity of the purified enzyme as a function of the reaction time using several initial substrate concentrations is shown in Fig. 1. The time-velocity relationship was
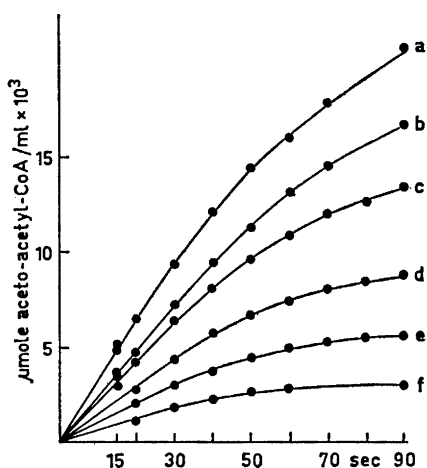

Fig. 1. Progress curves for the activity of purified thiolase from *B. trispora* as a function of time using different initial substrate concentrations (μM): a, 39.6; b, 29.2; c, 20.9; d, 13.6; e, 7.92; f, 3.96.
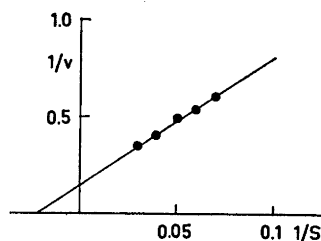
Fig. 2. Lineweaver-Burk plot of reciprocal reaction velocity ($v = \mu$moles/min, mg protein) *versus* reciprocal μmoles concentration of acetoacetyl-CoA (S) using a 190-fold purified thiolase preparation from *B. trispora.*

linear within 0 − 30 sec. The variation of the initial reaction velocity with substrate concentration is given in Fig. 2 as a Lineweaver-Burk plot of reciprocal reaction velocity ($v = \mu$moles acetoacetyl-CoA transformed/min, mg protein) *versus* the reciprocal μmolar concentration of acetoacetyl-CoA originally added to the reaction mixture (reference plot). From Fig. 2 the Michaelis constant with respect to acetoacetyl-CoA and the maximum reaction velocity can be calculated to $K_m = 4.5 \times 10^{-5}$ M and $V_{max} = 6.7$ μmoles/min,

mg protein. The last figure corresponds to a turnover number of 670 moles of acetoacetyl-CoA per minute per 100 000 g of enzyme at 20°C. Stern reports a corresponding turnover number for a thiolase preparation from pig heart to be 6500 at 25°C.[19] The preparations from *B. trispora* thus seem to have lower activity, although a part of the difference may be attributed to the difference in the assay temperature. After this work had been completed a paper by Gehring *et al.*[20] came to our knowledge. It reports on thiolase-preparations from pig heart, which, after a 600 – 700 fold purification, had a turnover number corresponding to 10 800 at 20°C.

Studies on the effect of organic anions. The plots of reciprocal reaction velocities *versus* reciprocal substrate concentrations in the absence and in the presence of certain organic acid anions, added as potassium salts, are given in Fig. 3, A-E. It can be seen that the addition of 0.03 – 30 mM acetate (Fig. 3B), oxaloacetate (Fig. 3D) or pyruvate (Fig. 3E) did not affect the reaction.
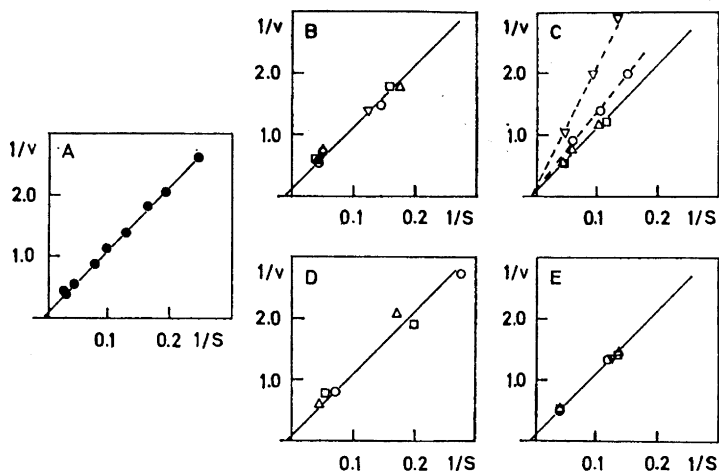

Fig. 3. Lineweaver-Burk plots as in Fig. 2, but using a 180-fold purified thiolase preparation *B. trispora* and K-salts of carboxylic acid anions in the reaction mixture. A, Reference plot (no anion added); B, acetate; C, citrate; D, oxaloacetate, E, pyruvate.

Symbols: □ 0.03 mM; △ 0.3 mM; ○ 3 mM; ▽ 30 mM.

There is considerable uncertainty in the spectrophotometric determinations in the presence of oxaloacetate because of the light absorption by this acid anion at the wave length (305 mμ) employed in the enzyme assay.

In contrast to the above three organic acid anions, the addition of 3 – 30 mM citrate seemed to inhibit the reaction (see Fig 3C). Lower concentrations of citrate (0.03 – 0.3 mM) were without effect. The observed inhibition by citrate was further studied using higher concentrations of magnesium in the reaction mixture. Mg^{2+} is usually added to the assay mixture in order, to

enhance the absorption of the enolate ion of the β-keto-thioester, which gives the characteristic band at 305 mμ. Citrate, however, is known to form complexes with Mg^{2+}. The equilibrium constant for the formation of Mg^{2+}-citrate complex is 1.58×10^3.[21] Using this value it can be calculated that in a 30 mM citrate solution 32.1 mM Mg^{2+} is required to give a 5.3 mM concentration of free Mg^{2+}. It can be seen in Fig. 4 that when the magnesium concentration
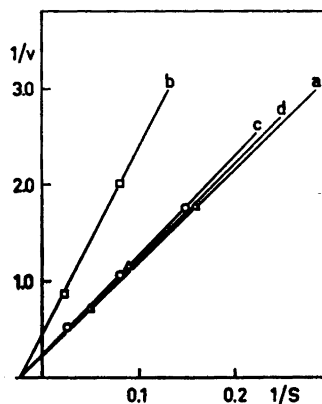

Fig. 4. Reciprocal Lineweaver-Burk plots as in Fig. 2 but using a 180-fold purified thiolase from B. trispora and different concentrations of Mg^{2+} and citrate in the assay medium. a, 5 mM Mg^{2+}, no citrate; b, no Mg^{2+}, 30 mM citrate; c, 32 mM Mg^{2+}, 30 mM citrate; d, 32 mM Mg^{2+}, no citrate.

in the assay mixture is 32 mM, instead of the ususally employed 5.3 mM, the inhibition by 30 mM citrate, observed at the lower magnesium concentration, is almost completely relieved.

The conclusion that citrate inhibits the reaction by removal of free Mg^{2+} ions can further be confirmed by use of the equation for partial non-competitive inhibition according to Dixon and Webb:[22]

$$v = \frac{ke}{(1+K_s/s)\dfrac{(1+i/K_i)}{[1+(i/K_i)(k'/k)]}} \tag{1}$$

The numerator and the denominator of the right side of eqn. (1) are multiplied by the expression $\left(1+\dfrac{i}{K_i}\dfrac{k'}{k}\right)$ and following substitutions are made:

$$ke = V_{max} \tag{2}$$
$$k'e = V_i \tag{3}$$

This gives a following equation:

$$v = \frac{V_{max} + \dfrac{iV_i}{K_i}}{\left(1+\dfrac{K_s}{s}\right)\left(1+\dfrac{i}{K_i}\right)} \tag{4}$$

where:

v	initial reaction velocity
V_{max}	maximum reaction velocity in the absence of inhibitor
V_i	maximum reaction velocity in the presence of an excess of inhibitor
s	substrate concentration
i	inhibitor concentration

K_s, K_i constants; K_s is, in our terminology, equal to the Michaelis constant K_m; K_i was obtained by a graphic method according to Dixon and Webb.[23] V_i was obtained from eqn. (4) above. In this equation the constants K_s and V_{max} were calculated from Fig. 3A. When V_{max} is calculated from the assay medium without Mg^{2+} (Fig. 4, curve b) a value of 2.3 μmole/min, mg protein is obtained. Thus, a value very close to the $V_i = 2.1$ μmole/min, mg protein. These results give a fairly good support for the conclusion that citrate inhibits the reaction through complex binding of Mg^{2+}.

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