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Thiolases of *Escherichia coli*: Purification and Chain Length Specificities

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The presence of only one thiolase (EC 2.3.1.9) in wild-type *Escherichia coli* induced for enzymes of beta oxidation was demonstrated. A different thiolase was shown to be present in a mutant constitutive for the enzymes of butyrate degradation. The two thiolases were purified to near homogeneity by a simple two-step procedure and were found to be associated with different proteins as shown by gel electrophoresis. The thiolase isolated from induced wild-type *E. coli* cells was active on β-ketoacyl-coenzyme A derivatives containing 4 to 16 carbons, but exhibited optimal activity with medium-chain substrates. In contrast, the thiolase isolated from the constitutive mutant was shown to be specific for acetoacetyl-coenzyme A.

The induction of enzymes involved in the beta oxidation of fatty acids in *Escherichia coli* has enabled an extensive study of these bacterial enzymes and has led to the isolation of constitutive mutants (3, 9-11, 16). Thiolase (acetyl-CoA acetyltransferase, EC 2.3.1.9), as the last enzyme in this pathway, functions in the thiolytic cleavage of the β-ketoacyl-coenzyme A (CoA) derivatives in the presence of CoA as a cofactor. Recent studies (19, 11) have shown the presence of two thiolase activities, thiolase I and II, which were mapped at different points on the *E. coli* chromosome map. Thiolase I is induced by oleic acid and is necessary for the growth of *E. coli* on long-chain fatty acids as the sole source of carbon. This enzyme has previously been partially purified (6). Thiolase II is induced by acetoacetate and is involved in the breakdown of butyrate. However, neither the number of enzymes associated with these thiolase activities nor their substrate specificities are known. These questions, and recent reports on the multiplicity of thiolases in higher organisms (7, 8), have prompted this study of thiolases in *E. coli* and their substrate specificities.

MATERIALS AND METHODS

Chemicals. CoA and nicotinamide adenine dinucleotide in its reduced form and in its oxidized form were purchased from PL-Biochemicals. Ethyl chloroformate, triethylamine, diketene, and *trans*-$\Delta^{2,3}$-decenoic acid were obtained from Aldrich Chemical Co. *trans*-$\Delta^{2,3}$-hexadecenoic acid was purchased from Miles Laboratories, Inc. L-3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) was bought from Boehringer Mannhein Corp. Lactate dehydrogenase (EC 1.1.1.27) was purchased from Worthington Biochemicals Corp. All other chemicals were of reagent grade. Crotonase (EC 4.2.1.17) was prepared by the method of Stern et al. (15). *trans*-$\Delta^{2,3}$-hexenoic acid and *trans*-$\Delta^{2,3}$-tetradecenoic acid were synthesized by reacting malonic acid in the presence of pyridine with *n*-butyraldehyde and *n*-dodecanal, respectively, by the procedure of Linestead et al. (4).

Synthesis of substrates. The CoA derivatives of $\Delta^{2,3}$-hexenoic acid and its longer-chain homologues were synthesized by reacting their mixed anhydrides with CoA, as described by Schulz (12). The concentrations of the resulting $\Delta^{2,3}$-enoyl-CoA substrates were determined by the method of Ellman (2) after cleaving the thioester bond with hydroxylamine at pH 7. The β-ketoacyl-CoA derivatives, other than acetoacetyl-CoA, were synthesized enzymatically by the method of Seubert et al. (14). In a standard preparation, 1.1 μmol of $\Delta^{2,3}$-decenoyl-CoA was dissolved in a mixture of 500 μmol of tris(hydroxymethyl)aminomethane (pH 9.7), 500 μmol of NaCl, 2 mg of bovine serum albumin, 1.8 μmol of pyruvate, 1.12 μmol of NAD^+, and 20 μmol of $MgCl_2$ in a total volume of 2 ml. The reaction was started by the addition of 27.5 U of crotonase, 1.8 U of 3-hydroxyacyl-CoA dehydrogenase, and 21.9 U of lactate dehydrogenase. After 30 min at room temperature, the same amounts of enzymes were added again to the reaction mixture. The reaction was terminated after a total reaction time of 60 min by the addition of 6 M HCl to bring the mixture to approximately pH 4.5. The precipitate was discarded after centrifugation, and the concentration of the β-ketoacyl-CoA derivatives was estimated with 3-hydroxyacyl-CoA dehydrogenase by following a decrease in absorbance at 340 nm. A standard assay mixture contained 50 μmol of potassium phosphate (pH 7), approximately 10 nmol of β-ketoacyl-CoA, 60 nmol of nicotinamide adenine dinucleotide, and 1.8 mU of 3-hydroxyacyl-CoA dehydrogenase in a total volume of 0.6 ml. A molar extinction coefficient of

E_{340} = 6,200 $cm^{-1}M^{-1}$ was used for calculating the substrate concentrations. Acetoacetyl-CoA was prepared from diketene and CoA, as described by Seubert (13).

Organisms. *E. coli* B (ATCC 11775) was grown in M-9 mineral salts medium with oleate as the sole carbon source, as previously described (9). A mutant constitutive for thiolase II (*fadR16-fad-5-atoC49*), obtained from P. Overath, was grown in the same salts medium with acetate as the sole carbon source (9). These cells were grown with shaking to the late exponential growth phase (absorbance at 420 nm of 1.7 to 1.8, measured on a Gilford spectrophotometer, model 240), harvested, and stored at -20 C. Growth conditions have been previously described (9).

Purification of thiolases I and II. Both thiolases were purified by the same procedure. All operations were performed at 0 to 5 C unless otherwise stated. The crude homogenate was prepared by suspending cells (2 g) in 4 ml of 10 mM potassium phosphate (pH 7.0) containing 10 mM 2-mercaptoethanol and 25% (vol/vol) glycerol, sonically treating it for 2 min, and centrifuging it for 30 min at 31,300 × *g*. The resulting crude homogenate was subjected to a 10-min heat treatment at 60 C, centrifuged at 31,300 × *g* for 30 min, and dialyzed overnight at 4 C against 1 liter of 20 mM potassium phosphate (pH 6.6) containing 25% (vol/vol) glycerol and 10 mM 2-mercaptoethanol. This dialysate was applied to a phosphocellulose column (1.2 by 45 cm) previously equilibrated with the dialysis buffer at pH 6.6. The column was washed with the same buffer until no more ultraviolet-absorbing material was eluted, and thiolase was eluted with a phosphate gradient prepared from 250 ml of 20 mM potassium phosphate (pH 6.6) containing 10 mM mercaptoethanol and 25% (vol/vol) glycerol, and from 250 ml of 500 mM potassium phosphate (pH 6.6) containing 10 mM mercaptoethanol and 25% (vol/vol) glycerol. Fractions (5 ml) were collected and assayed for thiolase activity. The fractions with thiolase activity were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane to approximately 5 ml. The protein was stored at 5 C. Data for this purification procedure are presented in Table 1.

Protein and enzyme determinations. Protein concentrations were determined by the method of Lowry et al. (5). The enzymes were assayed by following the decrease in absorbance at 303 nm due to the disappearance of the Mg^{2+} enolate complex of the β-ketoacyl-CoA substrates at 25 C on a Gilford recording spectrophotometer, model 240. A typical assay contained, in a total volume of 0.6 ml, 67 μmol of tris(hydroxymethyl)aminomethane buffer (pH 8.1), 0.134 μmol of dithiothreitol, 15 μmol of $MgCl_2$, 58 nmol of CoA, 12 nmol of β-ketoacyl-CoA, and thiolase to give absorbancy values (per minute) of between 0.015 and 0.025. The reaction was started by the addition of CoA. The extinction coefficients of β-ketoacyl-CoA compounds used in calculating thiolase activities were: acetoacetyl-CoA, 16.9 × 10^3 cm^{-1} M^{-1}; β-ketohexanoyl-CoA, 15.6 × 10^3 cm^{-1} M^{-1}; β-ketodecanoyl-CoA, 13.5 × 10^3 cm^{-1} M^{-1} (8). The latter value was also used as the extinction coefficient for β-ketotetradecanoyl-CoA and β-ketohexadecanoyl-CoA.

Disc-gel electrophoresis. Electrophoresis of thiolase I and II was performed with a standard 7.5% acrylamide gel and tris(hydroxymethyl)aminomethane-glycine buffer (oH 8.5) containing 10 mM 2-mercaptoethanol at 15 C by the procedure of Davis (1). Gels were stained for protein in Coomassie brilliant blue, destained with 7% acetic acid, and scanned at 600 nm. Duplicate unstained gels were sliced, and each slice was kept overnight in 0.5 ml of 0.05 M tris-(hydroxymethyl)aminomethane buffer (pH 8.1) containing 25% (vol/vol) glycerol, bovine serum albumin (1 mg/ml), and 2 mM dithiothreitol. Samples were assayed for thiolase activities with acetoacetyl-CoA and β-ketodecanoyl-CoA as described above.

RESULTS AND DISCUSSION

Purification of thiolases I and II. Thiolase I, isolated from induced wild-type *E. coli*, proved to be unstable under conditions used for purification even in the presence of thiol reagents. However, the presence of 25% glycerol in all buffers during the purification procedure greatly increased the stability of the enzyme.

TABLE 1. *Summary of the purification of thiolases I and II*

Purification step	Total activity[a] (μmol/min)	Total protein (mg)	Sp act (μmol/min per mg of protein)	Purification (fold)	Ratio[b] C_4-CoA: C_{10}-CoA
Thiolase I					
Crude homogenate	69.3	174	0.4	1	1:12.2
Heat treated	57.7	50.1	1.2	2.9	1:12.9
Phosphocellulose	5.3	1.7	3.1	7.8	1:14.2
Thiolase II					
Crude homogenate	1,207	101	12.0	1	
Heat treated	1,155	73.7	15.7	1.3	
Phosphocellulose	498	0.98	506	42.4	

[a] Enzyme activity was determined by using β-ketodecanoyl-CoA as a substrate for thiolase I and acetoacetyl-CoA for thiolase II.

[b] Ratio of specific activity using acetoacetyl-CoA to specific activity using β-ketodecanoyl-CoA.

Thiolase II, isolated from the *E. coli* mutant, was also stabilized by the presence of glycerol, although this enzyme was less susceptible to inactivation in dilute solution. Both thiolases were purified by the same two-step procedure which included heat treatment and column chromatography on phosphocellulose. They were eluted from phosphocellulose with approximately 0.2 M potassium phosphate (Fig. 1, thiolase I). In the case of thiolase I, which in contrast to thiolase II is also active on long-chain substrates, activities with acetoacetyl-CoA and β-ketodecanoyl-CoA were associated with the same fractions as seen in Fig. 1. The results of these purification procedures are summarized in Table 1. The instability of thiolase I, even in the presence of glycerol, reduces the specific activity of the purified enzyme, and thus the fold purification may be underestimated.

The purities of the two thiolases were evaluated by polyacrylamide disc-gel electrophoresis. Thiolase II was virtually homogeneous and the acetoacetyl-CoA thiolase activity coincided with the main protein band which migrated at a relative mobility of 0.40 (Fig. 2A). The thiolase I preparation showed several bands, but the thiolytic activities with acetoacetyl-CoA and β-ketodecanoyl-CoA were both associated with the main protein band whose relative mobility was 0.23 (Fig. 2B). When thiolases I and II were examined on the same gel, a good separation of these two enzymes was achieved (Fig. 2C), and the relative mobilities observed agreed well with those determined when these enzymes were subjected to electrophoresis on separate gels (Fig. 2A-C). Because all acetoacetyl-CoA thiolytic activity of thiolase I was associated with one band (Fig. 2B) which migrated differently from thiolase II, it is concluded that no thiolase II was present in this thiolase I preparation. This fact, as well as the observation that the ratios of activities with acetoacetyl-CoA and β-ketodecanoyl-CoA remained constant throughout the purification, indicates that only one thiolase (thiolase I) is present in *E. coli* B cells induced for enzymes of beta oxidation and that this enzyme is active on short- and long-chain substrates.

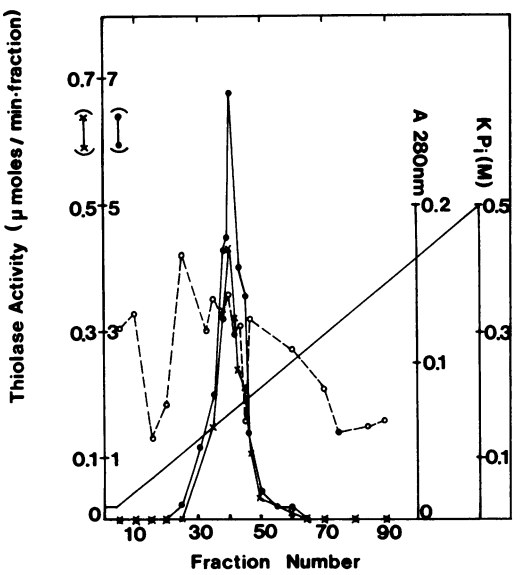

FIG. 1. *Purification of thiolase I by chromatography on a phosphocellulose column. Symbols: ●, enzyme activity with β-ketodecanoyl-CoA; X, enzyme activity with acetoacetyl-CoA; ○, absorbance at 280 nm.*

Substrate specificities. The substrate specificities of thiolases I and II were determined by the optical assay described above. Thiolase I proved to be active with β-ketoacid derivatives containing 4 to 16 carbons but was most active with medium-chain substrates (Fig. 3). Since long-chain substrates at higher concentrations were found to inhibit thiolase I, the activities with β-ketotetradecanoyl-CoA and β-ketohexadecanoyl-CoA were measured at 10 μM concentrations, whereas assays with short- and medium-chain substrates were performed at 20 μM concentrations. The decrease in activities found with longer-chain substrates may have been at least partially due to the above-mentioned substrate inhibition. Noteworthy is the observation that the activity of thiolase I with acetoacetyl-CoA is only 7% of that found with β-ketodecanoyl-CoA. In view of the established chain length specificity of thiolase I, and in view of the reported high activities of crotonase and 3-hydroxylacyl-CoA dehydrogenase as compared to that of thiolase (9), it is possible that the thiolytic cleavage of acetoacetyl-CoA is the rate-limiting step in beta oxidation in *E. coli*, unless the dehydrogenation of the acyl-CoA intermediates proceeds at an even slower rate. It thus seems possible that acetoacetyl-CoA accumulates and may induce the synthesis of thiolase II. However, during the course of our study we were not able to detect measurable amounts of thiolase II in wild-type *E. coli* B induced for fatty acid oxidation. Since the activity of thiolase II towards β-ketohexanoyl-CoA was found to be less than 1% of that observed with acetoacetyl-CoA, it is concluded that thiolase II is an acetoacetyl-CoA thiolase.

Based on studies of wild-type *E. coli* K-12 and fatty acid degradation mutants, Overath has suggested that there are at least two types of

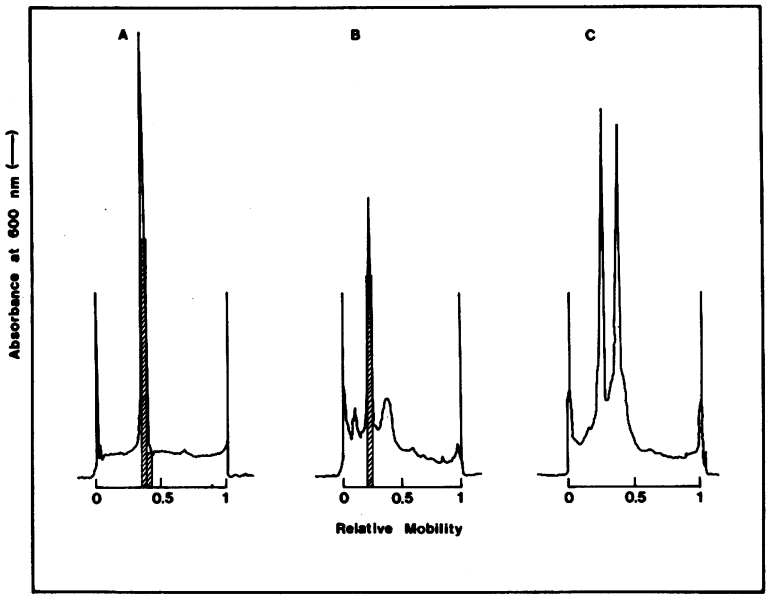

FIG. 2. *Disc-gel electrophoresis of thiolase I and thiolase II. (A), Thiolase II; (B), thiolase I; (C), thiolases I and II. The positions of the thiolytic activities are indicated by hatched bars. (A) Activity with acetoacetyl-CoA; (B) activity with acetoacetyl-CoA and β-ketodecanoyl-CoA.*

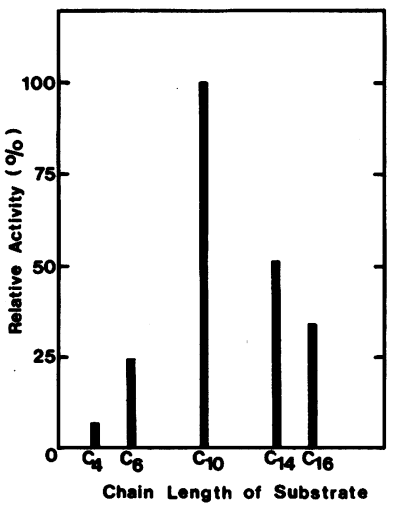

FIG. 3,. *Chain length specificities of thiolase I. C_4, Acetoacetyl-CoA; C_6, β-ketohexanoyl-CoA; C_{10}, β-ketodecanoyl-CoA; C_{14}, β-ketotetradecanoyl-CoA; C_{16}, β-ketohexadecanoyl-CoA.*

thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, which differ in their chain length specificities present in *E. coli*. However, our results, which indicate the presence of only one thiolase (thiolase I) in induced *E. coli* B cells, cast doubt on this suggestion. The known function of thiolase I in fatty acid oxidation and that of thiolase II in butyrate and acetoacetate degradation agree well with the broad chain length specificity of thiolase I and the high specificity of thiolase II for acetoacetyl-CoA. This good correlation between functions and chain length specificities of the two thiolases in *E. coli* leads us to suggest that similarly in higher organisms thiolases with broad chain length specificities are active in fatty acid oxidation, whereas thiolases specific for acetoacetyl-CoA are involved in either ketone body degradation, ketone body formation, or cholesterol synthesis.

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