Purification and Properties of a Pig Heart Thiolase with Broad Chain Length Specificity and Comparison of Thiolases from Pig Heart and Escherichia coli*

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A thiolase (acetyl-CoA acyltransferase, EC 2.3.1.16) which acts on substrates of various chain lengths (thiolase I) has been purified from pig heart muscle 366-fold to near homogeneity as judged by gel electrophoresis. Its molecular weight was estimated to be 180,000 in the absence and 46,000 in the presence of sodium dodecyl sulfate. Kinetic measurements with acetoacetyl-CoA, 3-ketohexanoyl-CoA, 3-ketoctanoyl-CoA, and 3-ketodecanoyl-CoA yielded apparent K_m values of 16, 8.3, 2.4, and 1.5 μM, respectively, whereas apparent V_max values of 65 to 69 μmol/min/mg were obtained with all substrates except for acetoacetyl-CoA, with which a value of 28.5 μmol/min/mg was observed. Antibodies prepared against this thiolase were used to demonstrate that thiolase I and acetoacetyl-CoA thiolase (thiolase II) from pig heart mitochondria are immunologically unrelated. The antibodies cross-reacted, however, with thiolase I from beef heart.

Kinetic constants (K_m, V_max) were also determined for thiolases I and II from Escherichia coli, as were the native and subunit molecular weights of E. coli thiolase II. Although the E. coli thiolases were found to be immunologically distinct from the pig heart enzymes, their physical and kinetic properties are strikingly similar to those of the heart thiolases. In view of this finding and in view of the known physiological roles of the latter two enzymes.

EXPERIMENTAL PROCEDURES

Materials—Fresh pig hearts were purchased from Max Insel Cohen Co., N. J. New Zealand white rabbits were bought from Marland Breeding Farms, N. J. Immunodiffusion plates and Freund’s complete adjuvant were obtained from Cappel Laboratories. Crotonase was prepared from beef liver by following the procedure of Steinman and Hill (4) and thiolases I and II were prepared from Escherichia coli B cells as previously described (9). All other enzymes, except for 3-hydroxyacyl-CoA dehydrogenase, which was purchased from Boehringer Mannheim, were obtained from Sigma Chemical Co. The sources, respectively, of the syntheses of trans-Δ^2-enoic acids have been previously described (6). The CoA derivatives of the Δ^2-enoic acids were prepared by the mixed anhydride method of Goldman and Vagelos (7) as previously described (6). Acetoacetyl-CoA was synthesized according to the method of Seubert (8). Since the extinction coefficients of the Mg^2+ 3-ketoacyl-CoA complexes are dependent on pH, the 3-ketoacyl-CoA, and Mg^2+ concentrations, all routine assays were performed in Tris/HCl (pH 8.3) in the presence of 25 mM MgCl_2 and 10 μM 3-ketoacyl-CoA (33 μM in the case of acetoacetyl-CoA). The extinction coefficients under these conditions were determined by measuring the total change of absorbance at 308 nm in the presence of 70 μM CoASH and thiolase (5 milliunits/0.6 ml) and by measuring the concentration of the 3-ketoacyl-CoA substrates with 3-hydroxyacyl-CoA dehydrogenase (10 milliunits/0.6 ml) as previously described (5). The molal extinction coefficients (ε) thus obtained were: acetoacetyl-CoA, 21,400 M^−1.
**Thiolases from Pig Heart and E. coli**

cm⁻¹; 3-ketobutanoyl-CoA, 16,600 m⁻¹ cm⁻¹; 3-ketoacetonyl-CoA, 14,400 m⁻¹ cm⁻¹; 3-ketodecanoyl-CoA, 13,900 m⁻¹ cm⁻¹; 3-ketodecanoyl-CoA, 11,000 m⁻¹ cm⁻¹; 3-ketotetradecanoyl-CoA, 11,600 m⁻¹ cm⁻¹; 3-ketohexadecanoyl-CoA, 9,800 m⁻¹ cm⁻¹. Enol-CoA hydratase and 3-hydroxyacyl-CoA dehydratase were assayed as described (11). Units of enzyme activity are expressed as moles of substrates utilized or products formed per min.

**Purification of Thiolase with Broad Chain Length Specificity (Thiolase I) and Acetoacetyl-CoA Thiolase (Thiolase II) from Pig Heart** — All operations were performed at 4°C. Fresh pig heart (579 g) was minced with a meat grinder and homogenized together with 2.2 liters of 0.05 M potassium phosphate (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol. The resulting homogenate was centrifuged at 11,900 x g for 45 min. The supernatant was applied to a phosphocellulose column (5 x 50 cm) which had been previously equilibrated with 0.05 M potassium phosphate (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol. The column was washed with the same buffer until material absorbing light at 280 nm ceased to be eluted. Another thiolytic and 3-hydroxyacyl-CoA dehydrogenase were assayed as described (11). Thiolase II was further purified by following in principle the procedure above.

**RESULTS**

**Purification and Stabilities of Thiolases I and II from Pig Heart Muscle** — Chromatography of a pig heart homogenate on phosphocellulose led to the separation of two thiolases, one of which acted on both acetoacetyl-CoA and 3-ketodecanoyl-CoA, whereas the second one was specific for acetoacetyl-CoA (data not shown). No evidence for the existence of yet another thiolase was obtained. This finding is in general agreement with a previous report in which it was additionally shown that all thiolases of heart muscle, including those of pig heart, are located in mitochondria (1). Since only one acetoacetyl-CoA specific thiolase (thiolase II) was found in pig heart, this thiolase must be the one which had previously been purified to homogeneity and extensively studied by Gehring et al. (15-17).

The thiolase, which was active with acetoacetyl-CoA as well as with longer chain substrates (thiolase I), was purified 366-fold with a recovery of 32% of the original activity by chromatography on phosphocellulose and CM-cellulose as summarized in Table I and as described in detail under "Experimental Procedures." Polyacrylamide gel electrophoresis demonstrated the presence of one intensive protein band which coincided with both the acetoacetyl-CoA thiolytic and 3-ketododecanoyl-CoA thiolytic activities detected on an identical gel (see Fig. 1). When the same preparation was subjected to electrophoresis on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (13), approximately 90% of the protein banded in one position, a finding which is indicative of the presence of small amounts of impurities. Assays for other enzymes of β oxidation showed that this preparation contained 0.65 units/mg of 3-hydroxyacyl-CoA dehydratase (assayed with acetoacetyl-CoA) and 5.8 units/mg of crotonase (assayed with crotonyl-CoA) which, because of their high specific activities (18, 19), can account for only 1% of the protein of the purified thiolase I preparation.

While studying the stabilities of pig heart thiolases I and II in dilute solution (1 μg/ml), it was observed that thiolase I remained active for several days in 0.75 M Tris/HCl (pH 8.1) containing 10 mM mercaptoethanol. However, at a lower Tris/HCl concentration or in the absence of mercaptoethanol, thiolase II showed a strong immunoprecipitation of pig heart thiolase I by serum taken on these days. The rabbit was terminally bled at the 14 days after giving the booster injection. Eighty-five milliliters of whole blood were collected and stored overnight at 4°C. The clotted cells formed during storage were loosened from the walls of the tube with a wooden spatula and precipitated by centrifugation for 20 min. Purification of the antibodies to thiolase I on DEAE-cellulose followed an established procedure (14). Antibodies to thiolase II from E. coli were raised, isolated, and purified as described above for the antibodies to pig heart thiolase I. Additionally, unfractionated serum of a rabbit immunized with lactate dehydrogenase from beef heart was prepared following the above procedure.

**TABLE 1**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (g)</th>
<th>Total activity (units/g)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>20.24</td>
<td>3900</td>
<td>0.163</td>
<td>1</td>
</tr>
<tr>
<td>Phosphocellulose (batch)</td>
<td>1.468</td>
<td>2935</td>
<td>2.0</td>
<td>12</td>
</tr>
<tr>
<td>Phosphocellulose (gradient)</td>
<td>0.110</td>
<td>1494</td>
<td>12.8</td>
<td>79</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>0.016</td>
<td>1056</td>
<td>59.6</td>
<td>366</td>
</tr>
</tbody>
</table>

For details of the purification see "Experimental Procedures." Assays were performed as described under "Experimental Procedures" with 3-ketodecanoyl-CoA as the substrate.
Yielded subunit molecular weights for these two thiolases of *Escherichia coli* pig heart and *Escherichia coli* units as has been established for pig heart thiolase II (16), for cytoplasmic thiolases (2, 31, and recently for these thiolases are composed of four possibly identical subunits of 46,000 and 41,500, respectively. Hence, it is concluded that thiolase I and thiolase II values of 200,000 and 175,000 were obtained for pig heart thiolase too has an essential sulfhydryl group at its active site.

Electrophoresis in the presence of sodium dodecyl sulfate (13) led to 84% of its activity when reacted for 15 min with 10^{-4} M iodoacetamide but only 14% when in addition 1 mM acetoacetate-CaO was present, it is concluded that this thiolase too has an essential sulfhydryl group at its active site.

**Physical and Kinetic Properties of Thiolases I and II from Pig Heart and Escherichia coli**—The native molecular weights of thiolase I from pig heart and thiolase II from *E. coli* were estimated by gel filtration on a standardized Sephadex G-200 column according to the procedure of Andrews (20). Values of 200,000 and 175,000 were obtained for pig heart thiolase I and *E. coli* thiolase II, respectively. Polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (13) yielded subunit molecular weights for these two thiolases of 46,000 and 41,500, respectively. Hence, it is concluded that these thiolases are composed of four possibly identical subunits of 46,000 and 41,500, respectively. Hence, it is concluded that these thiolases are composed of four possibly identical subunits as has been established for pig heart thiolase II (16), for cytoplasmic thiolases (2, 3), and recently for *E. coli* thiolase II (21).

The chain length specificity of pig heart thiolase I was determined by measuring the rates of the thiolytic cleavage of a number of CoA derivatives of even-numbered 3-keto acids having 4 to 14 carbons. As shown in Fig. 2, the rate observed with acetoacetate-CaO at 10 {\mu}M concentration was approximately one-fifth of those measured with longer chain substrates for all of which nearly equal rates were determined. The decrease in rate seen with 3-ketotetradecanoyl-CoA is most likely due to its nonspecific inhibition of thiolase I because this inhibition was found to be dependent on the bovine serum albumin concentration in the assay mixture. In order to further analyze the rate difference between the cleavage of acetoacetate-CoA and those of longer chain substrates, the apparent {K_m} and {V_{max}} values for several substrates were determined. Since the thiolase assay is based on the disappearance of the Mg^{2+}-3-ketoacyl-CoA complex, whose extinction coefficient (\(\epsilon\)) is a function of the 3-ketoacyl-CoA concentration, values of \(\epsilon\) were determined at various substrate concentrations as described under "Experimental Procedures." The results obtained with 3-ketohexanoyl-CoA are shown in the inset of Fig. 3. An increase in the substrate concentration from approximately 4 to 40 {\mu}M resulted in a decrease of \(\epsilon\) from 18 to 12 mmo1^-1 cm^-1. Only in the case of the Mg^{2+}-acetoacetate-CoA complex did the extinction coefficient not change dramatically. When rates, measured in the presence of various concentrations of 3-ketohexanoyl-CoA, were corrected for the concentration dependence of the extinction coefficient and were plotted according to Lineweaver and Burk, they fell on a straight line (see Fig. 3). However, when the values were calculated using a constant extinction coefficient, a complex result was obtained which could be taken as evidence for substrate inhibition (see Fig. 3). The {K_m} and {V_{max}}
values obtained by the above outlined approach and in the presence of a saturating level of CoA (70 μM) are listed in Table II. Kinetic parameters for longer chain substrates then listed were not determined because these compounds severely inhibited the enzyme. For comparative purposes, several kinetic constants were determined with pig heart thiolase II and with thiolases I and II from E. coli (see Table III).

Immunological Studies – Antibodies prepared against purified pig heart thiolase I were used to study the structural relationships between the two thiolases of pig heart and between E. coli and pig heart thiolases. Since antibodies to pig heart thiolase I inhibited this enzyme maximally only 60%, it was necessary to remove the thiolase-antibody complex by either ultrafiltration or centrifugation before determining the remaining activity. The results of such an experiment with purified thiolase I are shown in Fig. 4. The smooth immunotitration curve obtained is indicative of the specific and complete precipitation of thiolase I. This experiment also demonstrates that the antibodies to thiolase I did not cross-react with pig heart thiolase II even at 2500 times higher concentration than required to achieve 50% inhibition of thiolase I. This finding leads to the conclusion that the two mitochondrial thiolases differ greatly in their structures. Not surprising is the observation that antibodies prepared against E. coli thiolase II did not react with either of the two pig heart thiolases. The above mentioned conclusions were confirmed by double immunodiffusion experiments (data not shown) according to Ouchterlony (23). Antibodies prepared against pig heart thiolase I were found to cross-react with thiolase I from beef heart (data not shown), a finding which conforms to the rule that functionally identical enzymes of closely related species have similar structures and antigenic sites. The antibodies to pig heart thiolase I precipitated optimally 100% of the 3-ketodecanoyl-CoA thiolase activity and 35% of the acetoacetyl-CoA activity present in an acetone powder extract of pig heart mitochondria. This observation confirms that only one thiolase, which can act on substrates of various chain lengths, is present in pig heart mitochondria and that one-third of the total acetoacetyl-CoA thiolase activity of pig heart mitochondria is due to the thiolase I, whereas two-thirds are due to thiolase II.

**Table II**

**Apparent kinetic constants of pig heart thiolase I with substrates of various chain lengths**

Thiolase was assayed as described under "Experimental Procedures," except that molar extinction coefficients were determined for each Mg**2+**-3-ketoacyl-CoA complex as a function of 3-ketoacyl-CoA concentrations in the range of 5 to 40 μM. The concentration of CoASH in each assay was 70 μM.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Apparent Kₐ</th>
<th>Apparent Vₘₐₓ</th>
<th>Relative Vₘₐₓ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetoacetyl-CoA</td>
<td>17</td>
<td>29</td>
<td>1</td>
</tr>
<tr>
<td>3-Ketohexanoyl-CoA</td>
<td>8.3</td>
<td>69</td>
<td>2.4</td>
</tr>
<tr>
<td>3-Ketoctanoyl-CoA</td>
<td>2.4</td>
<td>65</td>
<td>2.2</td>
</tr>
<tr>
<td>3-Ketodecanoyl-CoA</td>
<td>1.8</td>
<td>67</td>
<td>2.3</td>
</tr>
</tbody>
</table>

**Table III**

**Comparison of thiolases from pig heart and Escherichia coli**

<table>
<thead>
<tr>
<th>Chain length specificity</th>
<th>Activity ratio</th>
<th>Kₐ values</th>
<th>Molecular weight</th>
<th>Physiological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiolase I</td>
<td></td>
<td>C₅₃-C₁₀₃</td>
<td>μM</td>
<td></td>
</tr>
<tr>
<td>Thiolase II</td>
<td></td>
<td>C₁₀</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Es. coli</td>
<td></td>
<td>C₅₃-C₁₀₃</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>Thiolase I</td>
<td></td>
<td>C₁₀</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiolase II</td>
<td></td>
<td>C₁₀</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* See Ref. 16.
† See Ref. 5.
‡ See Ref. 22.

**DISCUSSION**

The pig heart thiolase which acts on substrates of various chain lengths (thiolase I) is the second mammalian thiolase of its type to be purified to near homogeneity and it is the first of its type to be studied with respect to its native and subunit molecular weights and with respect to its kinetic parameters with longer chain substrates. Since the acetoacetyl-CoA-specific thiolase (thiolase II) from pig heart has previously been purified and studied (15–17), this report completes the in depth investigation of the mitochondrial thiolases present in one mammalian organ. Of great importance to a full understanding of the regulation of β oxidation is the possible cooperation of thiolases I and II which, if effective, would assure a high rate of degradation of all 3-ketocoyl-CoA compounds formed during fatty acid oxidation. Although no definite evidence for or against this cooperation is available, a comparison of the *Escherichia coli* fatty acid oxidation enzymes with those found in animals might provide further insight into this problem. Most important in this context is the recent demonstration
that the *E. coli* fatty acid oxidation system exists as a multienzyme complex which includes thiolase, 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase (22). Although it has been suggested that the enzymes of fatty acid oxidation exist in mitochondria in a highly organized arrangement (24), *E. coli* is the first organism in which this was shown to be the case. This finding adds weight to the contention that a similar situation may exist in mitochondria except that the enzymes would be more loosely bound to one another. Since thiolase II of *E. coli* was shown to be a separate enzyme (51, it is suggested that also in mitochondria thiolase II is not part of the putative multienzyme arrangement of β oxidation. Further suggestive evidence concerning the physiological functions of thiolases I and II of heart is derived from a comparison of *E. coli* and pig heart thiolases. A compilation of properties of both thiolases from *E. coli* and pig heart is presented in Table III. Clearly, the two thiolases I are strikingly similar in that they both act on substrates of various chain lengths and both act more efficiently on longer chain substrates than on acetoacetyl-CoA. Additionally, their kinetic parameters are very similar, specifically the low $K_{m}$ values for the longer chain substrates. Since the *E. coli* thiolase I is part of a multienzyme complex, its true molecular weight and subunit structure are still unknown. Also, the two thiolases II are nearly identical with respect to the listed properties except for their $K_{m}$ values for CoASH which differ by a factor of 7. It is thus concluded that the two types of thiolases present in heart mitochondria and *E. coli*, which are strikingly similar, may have identical functions. Since thiolases I and II of *E. coli* are induced by, and thus function in, the degradation of fatty acids and acetoacetate, respectively, we conclude that thiolase I of pig heart mitochondria is the sole thiolase required for β oxidation of fatty acids, whereas thiolase II functions only in ketone body degradation.

REFERENCES

Purification and properties of a pig heart thiolase with broad chain length specificity and comparison of thiolases from pig heart and Escherichia coli.

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