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

(Harold Staack, Judith F. Binstock, and Horst Schulz)

From the Department of Chemistry, City College of the City University of New York, New York, New York 10031

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 200,000 in the absence and 46,000 in the presence of sodium dodecyl sulfate. Kinetic measurements with acetoacetyl coenzyme A, 3-ketohexanoyl-CoA, 3-ketoctanoyl-CoA, and 3-ketodecanoyl-CoA yielded apparent $K_m$ values of 16, 8.3, 2.4, and 1.8 $\mu$M, respectively, whereas apparent $V_{max}$ values of 65 to 69 $\mu$mol/min/mg were obtained with all substrates except for acetoacetyl-CoA, with which a value of 29.5 $\mu$mol/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 functions of the *E. coli* thiolases, it is proposed that thiolase I from pig heart is only involved in fatty acid metabolism, whereas thiolase II functions solely in ketone body degradation.

Thiolases (acetyl-CoA acyltransferase, EC 2.3.1.9 and acetyl-CoA acyltransferase, EC 2.3.1.16) are ubiquitous enzymes which occur in multiple forms within one tissue. Mammalian liver, for example, contains three types of thiolases which differ in intracellular location or substrate specificity (or both) (1). In the cytoplasmin of liver, an acetoacetyl coenzyme A-specific thiolase is present which is believed to function in cholesterol biosynthesis (1-3), whereas in heart, which ordinarily does not synthesize cholesterol, this enzyme was not observed (1). In mitochondria, two types of thiolase have been identified, one (thiolase I) which acts on substrates of various chain lengths and which therefore must be involved in $\beta$ oxidation, and an acetoacetyl-CoA-specific thiolase (thiolase II) which may function in ketone body metabolism (1).

As part of our study aimed at elucidating the regulation of fatty acid metabolism in heart, we have purified and studied the physical as well as kinetic properties of thiolase I from pig heart. Antibodies raised against this thiolase were used to evaluate the immunological relationships of various thiolases. Additionally, the two functionally well-defined thiolases from *Escherichia coli* were studied for the purpose of comparing their properties with those of the pig heart thiolases and with the aim of thereby obtaining further evidence concerning the 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 method of Steinman and Hill (4) and thiolases I and II were purified from *Escherichia coli* B cells as previously described (5). 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-$\alpha$-enolic acids have been previously described (6). The CoA derivatives of the $\Delta^2$-enolic 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), while all other 3-ketoacyl-CoA compounds were prepared enzymatically as described by Seubert et al. (9).

Protein and Enzyme Determinations—Protein concentrations were determined by the method of Lowry et al. (10). Thiolase activities were routinely measured as described by Lynen and Ochoa (11). 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$_2$, 10 $\mu$M 3-ketoacyl-CoA (52 $\mu$M in the case of acetoacetyl-CoA). The extinction coefficients under these conditions were determined by measuring the total change of absorbance at 380 nm in the presence of 70 $\mu$M 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 molar extinction coefficients ($\epsilon$) thus obtained were: acetoacetyl-CoA, 21,400 $M^{-1}$

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Thiolases from Pig Heart and E. coli

Heart - All operations were performed at 4°C. Fresh pig heart (579 g) was minced with a meat grinder and homogenized together with 2.5 liters of 0.05 M potassium phosphate (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol. This eluate was collected in batches of 200 ml and the purification of thiolase II which was eluted with a higher ionic strength buffer was described (11). Units of enzyme activity were expressed as μmol 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 2.5 liters of 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. Thiolase activity was assayed with [14C]-acetoceto-CoA and assayed with 0.15 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.

For details of the purification see "Experimental Procedures." The purity of this preparation was assessed by polyacrylamide gel electrophoresis (18) and presence of sodium dodecyl sulfate (13) was 50%. No significant loss of activity was observed when this preparation was stored at -76°C for more than a year.

Acetoacetoyl-CoA thiolase (thiolase II) was eluted from the first phosphocellulose column with 1 liter of 0.4 M potassium phosphate (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol after thiolase I had been removed with 0.15 M potassium phosphate 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.

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 acetoacetoyl-CoA and 3-ketodecanoyl-CoA, whereas the second one was specific for acetoacetoyl-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). Sixty-one acetoacetoyl-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 acetoacetoyl-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 acetoacetoyl-CoA thiolytic and 3-ketodecanoyl-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 dehydrogenase (assayed with acetoacetoyl-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 I was unstable.

Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification yield</th>
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<tbody>
<tr>
<td>g</td>
<td>units/mg</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
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<td>3300</td>
<td>0.163</td>
<td>1</td>
</tr>
<tr>
<td>Phosphocellulose</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>0.018</td>
<td>1056</td>
<td>59.6</td>
<td>366</td>
</tr>
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</table>
yielded subunit molecular weights for these two thiolases of *Escherichia coli*—Pig Heart and *Escherichia coli*—cytoplasmic thiolases (2, 31, and recently for these thiolases are composed of four possibly identical subunits having molecular weights of 46,000 and 41,500, respectively. Hence, it is concluded that thiolase I and thiolase II lost 84% of its activity when reacted for 15 min with 10 mM iodoacetamide but only 14% when in addition 1 mM acetoacetyl-CoA 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 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 acetoacetyl-CoA at 10 µM 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 acetoacetyl-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 (ε) is a function of the 3-ketoacyl-CoA concentration, values of ε 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 µM resulted in a decrease of ε from 18 to 12 mmol·cm$^{-1}$·µmol$^{-1}$. Only in the case of the Mg$^{2+}$-acetoacetyl-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 for several substrates were determined.
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.

**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-ketoyl-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

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**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Apparent $K_a$</th>
<th>Apparent $V_{max}$</th>
<th>Relative $V_{max}$</th>
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<tbody>
<tr>
<td>Acetoacetyl-CoA</td>
<td>17</td>
<td>29</td>
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</tr>
<tr>
<td>3-Ketohexanoyl-CoA</td>
<td>8.3</td>
<td>69</td>
<td>2.4</td>
</tr>
<tr>
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<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>
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</table>

**Table III**

<table>
<thead>
<tr>
<th>Chain length specificity</th>
<th>Activity ratio $K_a$ values</th>
<th>Molecular weight</th>
<th>Physiological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart mitochondria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiolase I</td>
<td>$C_2$-$C_{10}$</td>
<td>1:6</td>
<td>$C_2$-$C_{10}$</td>
</tr>
<tr>
<td>Thiolase II</td>
<td>$C_3$</td>
<td></td>
<td>$C_3$-$C_{10}$</td>
</tr>
<tr>
<td>E. coli</td>
<td>$C_2$-$C_{10}$</td>
<td>1:20</td>
<td>$C_2$-$C_{10}$</td>
</tr>
<tr>
<td>Thiolase II</td>
<td>$C_2$</td>
<td></td>
<td>$C_2$-$C_{10}$</td>
</tr>
</tbody>
</table>

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* See Ref. 16.
* See Ref. 5.
* See Ref. 22.
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 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.

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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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