Purification and Characterization of an Extramitochondrial Acetyl Coenzyme A Hydrolase from Rat Liver*

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A rat liver acetyl-coenzyme A hydrolase has been purified to homogeneity. The enzyme, which appears to have an extramitochondrial location, has escaped previous detection presumably due to its extreme cold lability. Enzymatic activity has a half-life of 10 min at 4°C in 0.3 M sucrose but is stable at room temperature or in 1.5 M sucrose. Tissue activity levels and final specific activity are high, ~20 μmol/min/g wet weight of liver and 930 μmol/min/mg of protein for the purified enzyme at 30°C, respectively. The protometric molecular weight is about 64,000, and the overall molecular weight is 240,000 to 340,000. The reaction with acetyl-CoA produces stoichiometric amounts of acetate and CoASH. Attempts to demonstrate that the enzyme has any function other than a hydrolytic one have been unsuccessful. Acetyl-CoA hydrolysis is highly regulated by nucleotides. The presence of 2 mM ATP lowers the apparent Km, from 1.33 mM (no nucleotides) to 60 μM, while 2 mM ADP completely inhibits enzymatic activity. In the absence of ATP, acetyl-CoA kinetics exhibit negative cooperativity (nH = 0.74) but is linear (nH = 1.0) with 2 mM ATP. Hydrolytic activity is relatively specific for short chain acyl-CoA, while longer chain acyl-CoA compounds are hydrolyzed at rapidly diminishing velocities. Enzyme affinity increases with chain length which suggests that longer chain CoAs may be effective competitive inhibitors of acetyl-CoA hydrolysis. CoASH is possibly also an inhibitor. Inhibition by palmitoyl-CoA (Ki = 7 μM) is probably nonspecific in nature, while malonyl-CoA is neither a substrate nor an inhibitor. Species distribution of ATP-stimulated, ADP-inhibited acetyl-CoA hydrolyase activity appears to be limited to rodents, although a minor amount was found in monkey. The role of this enzyme is not known but is speculated to be related to the maintenance of cytosolic CoASH or acyl-CoA levels.

Present knowledge of the number, properties, and function of acyl-coenzyme A hydrolases is very limited. Only recently have papers appeared which address the subject in more than very limited. Only recently have papers appeared which address the subject in more than

MATERIALS AND METHODS

Materials and Tissue Sources—CoASH, malonyl-CoA, valeryl-CoA, acetocetyl-CoA, palmitoyl-CoA, and ATP-agarose were obtained from P-L Biochemicals. DEAE-Bio-Gel A and DE52 ion exchange resins were purchased from Bio-Rad Laboratories and Whatman Laboratories, respectively. Lactate dehydrogenase, pyruvate kinase, and citrate synthase were obtained from Boehringer Mannheim. Partially purified β-ketothiolase, obtained by the method of Kornblatt and Rudney (6), was a gift from Dr. Gregory Barritt. [1-14C]Acetyl-CoA and [1-14C]acetate were purchased from Amersham/Searle. [32P]ATP was a gift from Dr. David Goldthwait. Acetyl-L-carnitine and L-carnitine were generous gifts from Dr. Charles Hoppel, and palmitate/albumin mixtures were obtained as a gift from Dr. Herman Miesner. All other chemicals were of the highest grade available.

Male Sprague-Dawley rats were obtained from the Zivic-Miller Co. Rhesus monkey liver was obtained fresh at investigative surgery while the animal was under pentothal anesthesia. Fresh liver samples of calf and hybrid White Rock chickens were obtained from local butcher merchants. Hamsters, mice, and guinea pigs were obtained from Charles River Breeding Laboratories.

Preparation of Acetyl-CoA and Propionyl-CoA—Propionyl-CoA and acetyl-CoA were prepared by the method of Simon and Shemin...
(7) from the appropriate redistilled anhydrides. The concentrations of propionyl-CoA solutions were determined at 299 nm using the molar extinction coefficient of 15.4 M⁻¹ cm⁻¹ × 10⁻³. Concentrations of acetyl-CoA solutions were determined via the citrate synthase method (8).

**Protein Determinations**—Wherever ample protein was available, protein content was determined by a biuret method (9). Crude preparations obtained by deoxycholate precipitation were solubilized prior to addition of reagent. Protein samples containing ammonium sulfate were precipitated in 5% trichloroacetic acid and centrifuged in an Eppendorf model 3200 centrifuge. The resulting pellet was resuspended in 0.1 M NaOH, often requiring gentle heating, and biuret reagent was added. The rest of the procedure was as discussed in Ref. 9. Bovine serum albumin was used as the protein standard and reagent blanks were used throughout.

When only limited amounts of sample were available, protein content was determined by a modification of the method of Bradford (10). The assays (0.5 ml) were read in glass cuvettes with bovine serum albumin as protein standard. The assay was linear over a range of 1 to 10 μg of protein at a sensitivity of approximately 0.0074 A/μg.

**Assay of Acetyl-CoA Hydrolase**—The liberation of free CoASH from acetyl-CoA was routinely assayed spectrophotometrically through the use of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) or 2,2'-dipyridylamine (2-PDS) with extinction coefficients of 1.36 × 10⁴ (412 nm (8)) and 7.06 × 10³ M⁻¹ cm⁻¹ (343 nm (11)), respectively. The minimum assay contained 100 mM buffer, acetyl-CoA and 0.2 mM DTNB or 2-PDS to which modifiers, potential modifiers, and detergents were added as indicated. Reactions were initiated routinely by addition of enzyme. The progress of these and all other spectrophotometric assays was monitored with a Gilford spectrophotometer at 30°C.

In crude preparations, such as with homogenates and initial supernatants, acetyl-CoA hydrolase activity was estimated by subtracting the activity measured in 2 mM ADP from that observed in 0.2 mM ATP. In purer systems, the nonenzymatic rate was subtracted from the rate in 2 mM ATP. The nonenzymatic rate increased linearly with acetyl-CoA concentration. The slope was characteristic for a given buffer and pH. In most instances, the nonenzymatic rates contributed a negligible fraction of the enzymatically catalyzed rate, and were measured directly or taken from standard curves constructed for each set of conditions. All assays for the routine estimation of acetyl-CoA hydrolase activity were done at 0.5 mM acetyl-CoA.

**Measurement of Acetyl-CoA Hydrolase Activity in the Absence of DTNB**—Acetyl-CoA hydrolase activity was determined in the absence of DTNB by a modification of the spectrophotometric method. The reaction mixture contained 100 mM Tris-Cl (pH 7.8), 0.5 mM acetyl-CoA, and 2 mM ATP. Reactions were begun by addition of purified enzyme and terminated after incubation at 30°C for specified time intervals by the addition of perchloric acid. The resulting solution was neutralized with 2 M K₂CO₃ and 2 M NaOH. The resulting pellets were respun. The supernatants were combined, adjusted to a 2% final concentration of 5% (w/v) and centrifuged 35 min at 41,000 × g. The homogenate was centrifuged 35 min at 41,000 × g. The combined initial supernatant solution was made 50% in ammonium sulfate by pouring it into an appropriate slurry of Buffer B and solid ammonium sulfate. The protein 2-PDS dispersion was stirred 10 min and centrifuged 10 min at 31,700 × g. Buffer D: Buffer A with 15 mM KP, adjusted to a conductivity of approximately 3.6 mS with distilled water. Buffer E: Buffer A without ATP and benzamidine HCl.

**Summary of Buffer Solutions**—Buffer A: 0.3 M sucrose, 50 mM KP, (pH 7.4), 5 mM EDTA, 1 mM benzamidine HCl, 0.02% NaN₃, and 0.2 mM benzamidine HCl. Buffer B: Buffer A with 0.5 M sucrose. Buffer C: Buffer A + 0.5 M sucrose. Buffer D: Buffer A with 15 mM KP, adjusted to a conductivity of approximately 3.6 mS with distilled water. Buffer E: Buffer A without ATP and benzamidine HCl. The 18 and 32% ammonium sulfate solutions also contained 50 mM KP, (pH 7.4), 5 mM EDTA, 1 mM benzamidine HCl, 0.02% NaN₃ and 0.2 mM benzamidine HCl. The high (1.5 M) sucrose storage buffer also contained all of these ingredients except ATP.

**Purification of Acetyl-CoA Hydrolase**—Except where otherwise indicated, all manipulations were carried out at room temperature. Livers from 28-48-h-starved male Sprague-Dawley rats were excised, minced, and homogenized 1:3 (w/v) in Buffer A. The homogenate was centrifuged 35 min at 41,000 × g. The combined initial supernatant solution was made 50% in ammonium sulfate by pouring it into an appropriate slurry of Buffer B and solid ammonium sulfate. The protein 2-PDS dispersion was stirred 10 min and centrifuged 10 min at 31,700 × g. The pellet was extracted twice with 400-ml volumes of 32% ammonium sulfate solution and twice with 200-ml volumes of 18% ammonium sulfate solution. The two 18% ammonium sulfate extracts were combined and made 50% in ammonium sulfate by addition of an ammonium sulfate slurry as before. The protein suspension was quick-frozen on a dry ice/ethanol bath and stored at −70°C.

The frozen suspension was thawed in warm water and centrifuged 10 min at 31,700 × g. The pellet was resuspended in Buffer A and dialyzed 24 h against the same buffer. The dialysate was made 10% in ATP, brought to 50°C in a boiling water bath, and incubated 10 min at 52°C. The suspension was cooled to 20°C in an ice water bath and centrifuged 20 min at 41,000 × g. The resulting pellet was resuspended in Buffer A, and both supernatant and resuspended pellet were respun. The supernatants were combined, adjusted to a conductivity at or below that of Buffer C with glass-distilled water, and applied to a 250-ml DEAE-Bio-Gel A column (3.2 X 30 cm) pre-equilibrated with Buffer C. The column was washed with 500 ml of Buffer D and then with a 1-liter linear gradient from 0 to 0.09 M KCl in the same buffer. Collected samples containing activity were pooled (150 ml), precipitated by addition of saturated ammonium sulfate to a concentration of 50%, and centrifuged 10 min at 31,700 × g. The pellet was resuspended in 20 to 30 ml of Buffer A, reprecipitated by addition of 30 ml of saturated ammonium sulfate, quick-frozen on a dry ice/ethanol bath, and stored at −80°C.

The pellets were weighed, suspended in warm water and centrifuged 10 min at 31,700 × g. The resulting pellet was resuspended in 23 ml of Buffer E and applied to a Sephadex G-25 column (1.5 X 25 cm) pre-equilibrated with Buffer E. Activity-containing fractions, eluted with Buffer E, were combined (approximately 16 ml) and applied in 3-ml aliquots (approximately 50 units of enzyme) to two 2.5-ml ATP-agarose columns (7 X 70 mm) pre-equilibrated with Buffer E. Once loaded, the columns were washed with 15 ml of Buffer E followed by 15 ml of Buffer E containing 5 mM AMP. The enzyme was then specifically eluted with Buffer E containing 0.5 mM ATP. Activity-containing fractions were quickly pooled and made 50% in ammonium sulfate by addition of saturated solution. Benzamidine was also added to a concentration of 0.1% in 0.1 M Tris-Cl (pH 7.8). The resulting suspension was centrifuged 20 min at 27,000 × g in a swinging bucket rotor. The pellet (not visible) was resuspended in a small amount of 1.5 M sucrose (for storage at −90°C) or Buffer A (for equilibrium centrifugation). A typical purification procedure which resulted in an approximate 14,000-fold purification of the enzyme will be discussed in connection with Table 1.

**Molecular Weight Determinations**—The subunit molecular weight of rat liver acetyl-CoA hydrolase was determined by SDS-polyacrylamide electrophoresis relative to molecular weight standards: phosphorylase a (95,000), glutamate dehydrogenase (58,000), pyruvate kinase (57,000), ovalbumin (44,000), aldolase (40,000), lactate dehydrogenase (36,000), and carbonic anhydrase (31,000). All samples were prepared and run separately on 8% SDS-polyacrylamide gels as described in Ref. 14. Molecular weight was estimated by interpolation from a standard plot of log molecular weight versus Rₑ. The molecular weight of the intact protein was estimated by its

The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); 2-PDS, 2,2'-dipyridylamine; SDS, sodium dodecyl sulfate; MOPS, 3-(N-morpholino)propanesulfonic acid.

J. R. Panuska and D. A. Goldthwait, unpublished results.
migration through Bio-Gel A-1.5 relative to glutamate dehydrogenase (350,000), pyruvate kinase (240,000), lactate dehydrogenase (140,000), and hexokinase (102,000). A column (7 x 355 mm) containing 13 ml of Bio-Gel A-1.5 was washed 20 h continuously with Buffer A. Molecular weight standards (0.5 mg/ml) were dialyzed against Buffer A and ular weight standards (0.5 mg/ml) were dialyzed against Buffer A and centrifuged 10 min in an Eppendorf model 3200 centrifuge. Aliquots (100 μl) were applied to the column and eluted at a rate of 6 ml/h. Collected fractions were mixed with Coomassie blue reagent B and peak fractions were determined at 595 nm on a Zeiss spectrophotometer. Acetyl-CoA hydrolase, stored in 1.5 M sucrose, was diluted 1:5 with Buffer B and applied to the column as above. Fractions were collected in vials containing saturated ammonium sulfate, and peak fractions were determined by activity measurements. Molecular weight was estimated by interpolation from a standard plot of log molecular weight versus elution volume.

Molecular weight determination of the intact protein made by equilibrium centrifugation will be discussed in connection with Fig. 3.

RESULTS

Extramitochondrial acetyl-CoA hydrolase from rat liver was first observed in experiments involving the measurement of pyruvate carboxylase in crude homogenates of rat liver. The assay for pyruvate carboxylase activity is based on the reaction of DTNB with CoASH liberated when the oxalacetate formed by this enzyme is converted to citrate in the presence of exogenous acetyl-CoA and citrate synthase. The assay is such that any acetyl-CoA hydrolase activity contributes to the apparent pyruvate carboxylase activity. Appropriate controls involving the omission of pyruvate or ATP, or both, are necessary to distinguish the two reactions. During these experiments, it was noted that rat liver homogenates prepared in 1.5 M sucrose hydrolyzed acetyl-CoA rapidly (12 units/g wet weight of liver) in the presence of ATP.1 Pyruvate was not required for the reaction and no significant activity was detected if the homogenate was prepared in 0.3 M sucrose rather than 1.5 M sucrose. These results suggested the presence of an ATP-dependent or ATP-stimulated acetyl-CoA hydrolase in the 1.5 M sucrose homogenates.

Cold Lability—In order to understand the effect of high sucrose concentrations on the observed acetyl-CoA hydrolase activity, the possibility of cold lability was investigated. The results are shown in Fig. 1. Homogenate incubated in 0.3 M sucrose at 4°C rapidly lost activity with a half-time of approximately 10 min. In contrast, the activity was perfectly stable over the length of the experiment at room temperature in the same buffer. The enzyme was stabilized at 4°C in 1.3 M sucrose, but it could not be stabilized in 0.3 M sucrose at 4°C by the addition of acetyl-CoA, ATP, or ADP. These results suggested that high sucrose concentrations prevent a very rapid cold inactivation process that occurs under isotonic conditions. The high rate of activity loss under the usual conditions of homogenization appears to explain the failure to detect activity previously.

Results of Purification—That the observed catalytic properties of acetyl-CoA hydrolase were due to the characteristics of a single protein rather than a combined effect of two or more different proteins was established by purification of the enzyme to apparent homogeneity as described under "Materials and Methods." The purification of acetyl-CoA hydrolase from 25 male Sprague-Dawley rats (48 h fasted) is summarized in Table 1. The enzyme's extreme cold lability necessitated the use of room temperature conditions for fractionating procedures. The combined crude homogenate contained about 20 units/g wet weight of liver. This apparent increase in activity over that observed in fed rats (12 units/g wet weight of liver) can be accounted for by a 40 to 50% reduction of liver weight in the fasted state. The final recovery after the ATP-agarose step was approximately 0.32 mg of protein with a specific activity of 930 units/mg representing a 14,000-fold purification over the starting material. After concentration of the pooled ATP-agarose eluate by ammonium sulfate precipitation, the yield was typically to 50 to 70% of this amount (protein) and specific activities were reduced to about 400 to 700 units/mg. As conservatively judged by SDS-gel electrophoresis, the enzyme was found to be greater than 90% pure showing essentially only one protein band (Fig. 2).

The limited yield prohibited the use of the biuret method of protein determination in the case of the pure enzyme. Final specific activities were based upon protein determinations made by a modification of the method of Bradford (10), the results of which may be influenced by the dye-binding characteristics of the protein under study. Greater than 95% of the protein loaded onto ATP-agarose, as determined by the biuret method, was recovered in the wash prior to specific elution of acetyl-CoA hydrolase. Thus, the final specific activity estimate appears to be a reasonable one despite the uncertain dye-binding characteristics of this particular enzyme.

Subcellular Distribution—During purification, nearly all of the acetyl-CoA hydrolase activity remained in the supernatant fraction following the initial centrifugation. This would suggest that the enzyme might be localized in an extramitochondrial compartment. We were concerned, however, that mitochondrial integrity and sedimentation properties might be different at room temperature than at the low temperatures routinely used in fractionation studies. Thus, the subcellular

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1. F. Isohashi and M. F. Utter, unpublished results.
Acetyl-CoA hydroxalase was purified from livers of 28 male 48-h-fasted Sprague-Dawley rats. See "Materials and Methods" for details of procedure. The combined total body and wet liver weights were 7,206 and 206 g, respectively. Acetyl-CoA hydroxalase activity measurements were made in the presence of 2 mm ATP or 2 mm ADP at 0.5 mm acetyl-CoA (30°C). Activity values reported above are the net difference of these two determinations. Protein determinations were made with the biuret procedure except in the case of the ATP-agarose fraction where the Coomassie blue method was used.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity protein</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>4,320</td>
<td>66,460</td>
<td>0.065</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant (39,100 x g, 35 mm)</td>
<td>4,060</td>
<td>39,420</td>
<td>0.103</td>
<td>2</td>
<td>94</td>
</tr>
<tr>
<td>Reverse (NH₄)₂SO₄ (extract 18-32%)</td>
<td>1,923</td>
<td>2,160</td>
<td>0.89</td>
<td>14</td>
<td>45</td>
</tr>
<tr>
<td>52°C, 10 min</td>
<td>1,330</td>
<td>830</td>
<td>1.6</td>
<td>25</td>
<td>31</td>
</tr>
<tr>
<td>DEAE-Bio-Gel</td>
<td>790</td>
<td>29</td>
<td>27.0</td>
<td>415</td>
<td>18</td>
</tr>
<tr>
<td>ATP-agarose</td>
<td>290</td>
<td>0.32</td>
<td>930.0</td>
<td>14,307</td>
<td>7</td>
</tr>
</tbody>
</table>

**Table I**

Purification of acetyl-CoA hydroxalase from rat liver

**Table II**

Subcellular distribution of acetyl-CoA hydroxalase and marker activities at room temperature

Liver homogenate was prepared 1:10 (w/v) in buffer ("Materials and Methods") from one male 48-h-fasted Sprague-Dawley rat and centrifuged successively: 20 min at 600 x g, 20 min at 15,000 x g, and 35 min at 41,000 x g. Aliquots of homogenate, supernatant fractions, and resuspended pellets (in Buffer A) were assayed as follows: Assay of net acetyl-CoA hydroxalase activity (ATP minus ADP) was done in the usual spectrophotometric manner except for the addition of detergent in some of the assays (see below). Lactate dehydrogenase was assayed spectrophotometrically at 340 nm. Assay mixtures contained 100 mM MOPS (pH 7.4), 20 mM pyruvate, and 0.4 mM NADH. Citrate synthase assays contained 100 mM MOPS (pH 7.4), 0.2 mM 2-PDS, 15 mM oxalacetate, 2 mM ADP, and 0.5 mM acetyl-CoA and were followed at 343 nm. In the case of the homogenate, first supernatant, and all resuspended pellets, Triton X-100 was added to all assay mixtures at a ratio of 1.75 µg/µg of protein. In these instances, the protein fraction was incubated 4 min in the reaction mixture prior to initiation by addition of a pertinent substrate.

| Protein                 | Activity in subcellular fractions obtained by sequential centrifugation
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>Crude ho-mogenate</td>
<td>Nuclei</td>
<td>Mitochondria</td>
<td>Microsome</td>
</tr>
<tr>
<td>Acetyl-CoA hydrolase</td>
<td>74</td>
<td>14</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>109</td>
<td>87</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>5,126</td>
<td>220</td>
<td>166</td>
<td>76</td>
</tr>
</tbody>
</table>

All activities are expressed as micromoles per min per mg of protein.

in that most of the activity remained in the supernatant fraction following centrifugation at 15,000 x g. The distribution was more similar, but not necessarily identical with that of lactate dehydrogenase. Acetyl-CoA hydroxalase activity was partially inhibited in the presence of Triton X-100, added in the assay of certain fraction (see Table II), which affected the apparent recovery. More evidence is needed to rule out other possibilities such as lysosomal or peroxisomal locations, but it is clear that the rat liver acetyl-CoA hydroxalase is not located in the mitochondrial matrix.

**Molecular Weight Determinations**—The molecular weight of the native protein was estimated by equilibrium centrifugation. The results, plotted as ln deflection versus (radius)² are given in Fig. 3. The linearity of the plot (correlation coefficient, 0.9987) implies that the enzyme preparation was still essentially homogeneous at the end of the 16-h run. From the slope, the molecular weight was determined to be 340,000 assuming a partial specific volume of 0.74 and correcting for the density of the 0.3 m sucrose solution used in the run.

The molecular weight of the intact protein was also estimated by gel filtration on Bio-Gel A-1.5 relative to molecular
concentration

radius obtained

3200

containing 10

pended enzyme was dialyzed

volume of

tyl-coA ammonium sulfate and suspended as usual in Buffer

obtained by gel filtration is lower than that observed by

tion states of the enzyme at different ATP concentrations.

mM ATP, while gel filtration was run at

acetyl-coA hydrolase was approximately 240,000. Both esti-

ated of identical molecular weight. The fact that the estimate

higher molecular weight (approximately 350,000) and another

weight standards (see “Materials and Methods”). From inter-

oration from a standard curve, the molecular weight of intact

acytetyl-CoA hydrolase. Fresh ATP-agarose eluate was precipitated with

ammonium sulfate and suspended as usual in Buffer A. The resus-

suspended enzyme was dialyzed 3 h against 1000 volumes of Buffer A

containing 10 mM ATP and centrifuged 10 min in an Eppendorf model

2800 centrifuge. The sample was loaded into a Beckman rotor at a

concentration of approximately 0.2 mg/ml as determined by the

Coomassie blue method (10). Equilibrium was obtained by 16 h at

14,000 rpm (19°C). Calculations of molecular weight were derived

from the slope of the above plot (15) assuming a partial specific

volume of 0.74 and correcting for a solution density of 1.04 g/cm³.

Identification of Products and Stoichiometry—Acetyl-CoA

hydrolase was purified on the basis of its ability to release

CoASH from acetyl-CoA without certainty as to the identity of

the other product of the reaction. For example, the action of

β-ketothiolase would result in the release of CoASH from

acetyl-CoA. It is also possible that the enzyme might transfer

an acyl group to an acceptor provided by the presence of

buffer or DTNB (or 2-PDS) used in the assay. Purified enzyme

fractions contained no demonstrable thiolase activity when

tested with acetocacetyl-CoA and CoASH for acetyl-CoA forma-

tion (see “Materials and Methods”). Partially purified thiolase

produced a rapid reaction rate with this procedure. The

enzyme was about equally active in phosphate, Tris-C1, and

MOPS buffers, which argues against critical involvement in

the reaction of the buffer. In order to eliminate the possibility

that DTNB (or 2-PDS) is an essential participant, the reaction

was run in the absence of DTNB. After termination of the

reaction, DTNB was added and the production of CoASH

was determined spectrophotometrically. Production of

CoASH was linear over the first 4 min of the reaction but fell

off rapidly thereafter. The rate of the linear region (0.0063

μmol/min) compared closely to the rate measured with DTNB

present throughout (0.0071 μmol/min). This observation elimi-

nates the possibilities that DTNB acts as an acceptor or that it

alters enzyme conformation. However, the reaction did not

proceed as far in the absence of DTNB as in its presence.

Increasing the amount of enzyme, or the incubation time, or

both, did not result in any significant increase in the final

CoASH concentration which seemed to reach a maximum of

approximately 30 μM. Thus, in the absence of DTNB, accumu-

lation of CoASH may inhibit enzyme activity.

Although the results of the above experiments are consist-

ent with the existence of a true acetyl-CoA hydrolase, the

production of acetate and its stoichiometric relation to CoASH

liberation had yet to be demonstrated. A partition method

was used for the identification of acetate. Its sensitivity is

illustrated in Fig. 4. An aqueous mixture of a relatively large

amount of acetate, propionate, or formate, and a minor

amount of [1-14C]acetate was acidified and extracted with ether.

The movement of the bulk acid and [1-14C]acetate from

aqueous to ether phases was followed as described in the

legend (Fig. 4). As expected, extraction of a mixture of bulk

acetate and [1-14C]acetate resulted in a relatively constant

specific activity of extracted acid with successive extractions

while specific activities increased and decreased, respectively,

when propionate and formate were used as the bulk acids.

In order to facilitate comparison, the ordinate is represented as

the inverse of specific activity. In a similar experiment, the

accumulated product of the reaction of acetyl-CoA hydrolase

with [1-14C]acetyl-CoA was mixed with a bulk amount of

acetate and extracted as before. These results (also Fig. 4)

show that the product of the acetyl-CoA hydrolase reaction

behaves identically with known [1-14C]acetate.

The stoichiometric relation among the formation of acetate,

the liberation of CoASH, and the consumption of acetyl-CoA was

also investigated. Amounts of CoASH liberated from [1-

14C]acetyl-CoA were calculated at specific times from a con-

tinuous tracing using the extinction coefficient of 2-PDS (7.06

× 10⁻⁴ M⁻¹ cm⁻¹). In parallel assays, reactions were terminated

by addition of ethanol, and [1-14C]acetyl-CoA and [1-14C-acet-

tate were separated on DE52 as described in the legend to

Table III. These results show that the formation of acetate is

related to the liberation of CoASH and the disappearance

of acetyl-CoA in an approximate 1:1:1 ratio. The assay was not

linear over the 5 min period of the experiment, but the
The presence of nucleotides, the enzyme hydrolyzed at a rate well above the nonenzymatic level. In the presence of ATP, however, there was a 5.5-fold increase in the hydrolytic rate. With 2 mM ADP, the hydrolytic rate was necessarily low [1-14C]acetyl-CoA concentration used, in order to assure that the amount of radioactivity in the product would represent a substantial proportion of the total, was well below saturation. This would explain the fall in activity as the reaction progressed.

Regulatory Effects of Nucleotides—The effects of ATP and other possible effectors on the activity of acetyl-CoA hydrolase were investigated using the purified enzyme. In the absence of nucleotides, the enzyme hydrolyzed 0.5 mM acetyl-CoA at a rate well above the nonenzymatic level. In the presence of 2 mM ATP, however, there was a 5.5-fold increase in the hydrolytic rate. With 2 mM ADP, the hydrolytic rate was reduced essentially to that of the nonenzymatic rate (Table IV). The addition of acetate, NADH, NAD, NADPH, NADP, AMP, Fru-2,6-P, citrate, L-carnitine, acetyl-L-carnitine, pyruvate, or CAMP had no apparent effect on the rate of acetyl-CoA hydrolysis. The essentially complete inhibition of enzyme activity by 2 mM ADP provided a useful control in estimating net enzymatic activity in crude systems. Interestingly, the effects of ATP and ADP did not require the presence of Mg2++. In fact, high Mg2+ (>10 mM) concentrations inhibited ATP activation. The nature of this interaction is being investigated. Adenosine triphosphatase activity might explain or be related to the observed effects of ATP and ADP on acetyl-CoA hydrolysis. However, in separate experiments, the production of neither ADP nor inorganic phosphate was detected with purified enzyme under conditions where acetyl-CoA was being rapidly hydrolyzed. Both methods would have detected activities <1% of the rate of acetyl-CoA hydrolysis. Thus, ATP does not appear to be a substrate, and its effect is probably due to allosteric interaction.

In the absence of nucleotides, the kinetics of acetyl-CoA hydrolysis with varying acetyl-CoA concentration, showed negative cooperativity with a Hill coefficient of approximately 0.74 for concentrations less than 0.2 mM acetyl-CoA. In the presence of 2 mM ATP, the kinetics was converted to that of the linear Michaelis-Menten type and the K_{0.5} for acetyl-CoA, as determined by Hill plots, was decreased from 1.38 mM for no nucleotide to 60 μM with a negligible change in V_{max}. Thus, ATP appears to have a profound effect on the catalytic properties of acetyl-CoA hydrolyase.

Regulatory Effects of Nucleotides—The effects of ATP and other possible effectors on the activity of acetyl-CoA hydrolase were investigated using the purified enzyme. In the absence of nucleotides, the enzyme hydrolyzed 0.5 mM acetyl-CoA at a rate well above the nonenzymatic level. In the presence of 2 mM ATP, the kinetics was converted to that of the linear Michaelis-Menten type and the K_{0.5}, for acetyl-CoA, as determined by Hill plots, was decreased from 1.38 mM for no nucleotide to 60 μM with a negligible change in V_{max}. Thus, ATP appears to have a profound effect on the catalytic properties of acetyl-CoA hydrolyase. The kinetics of propionyl-CoA also showed negative cooperativity in the absence of nucleotides and were converted to the Michaelis-Menten type in the presence of 2 mM ATP. While the maximal hydrolytic properties of acetyl-CoA hydrolase were investigated using the purified enzyme. In the absence of nucleotides, the enzyme hydrolyzed 0.5 mM acetyl-CoA at a rate well above the nonenzymatic level. In the presence of 2 mM ATP, the kinetics was converted to that of the linear Michaelis-Menten type and the K_{0.5} for acetyl-CoA, as determined by Hill plots, was decreased from 1.38 mM for no nucleotide to 60 μM with a negligible change in V_{max}. Thus, ATP appears to have a profound effect on the catalytic properties of acetyl-CoA hydrolyase. The kinetics of propionyl-CoA also showed negative cooperativity in the absence of nucleotides and were converted to the Michaelis-Menten type in the presence of 2 mM ATP. While the maximal hydrolytic properties of acetyl-CoA hydrolase were investigated using the purified enzyme. In the absence of nucleotides, the enzyme hydrolyzed 0.5 mM acetyl-CoA at a rate well above the nonenzymatic level. In the presence of 2 mM ATP, the kinetics was converted to that of the linear Michaelis-Menten type and the K_{0.5} for acetyl-CoA, as determined by Hill plots, was decreased from 1.38 mM for no nucleotide to 60 μM with a negligible change in V_{max}. Thus, ATP appears to have a profound effect on the catalytic properties of acetyl-CoA hydrolyase. The kinetics of propionyl-CoA also showed negative cooperativity in the absence of nucleotides and were converted to the Michaelis-Menten type in the presence of 2 mM ATP. While the maximal hydrolytic.

**Table III**

**Stoichiometry of the acetyl-CoA hydrolysis reaction**

To a 0.4-ml assay mixture, containing 100 mM MOPS (pH 7.4), 0.2 mM 2-PDS, and 0.07 mM [1-14C]acetate (6 x 10^6 cpm/nmol) was added 0.15 unit of purified acetyl-CoA hydrolase. The reaction was followed continuously via the usual spectrophotometric method at 343 nm and the production of CoASH was calculated at specific time points. In parallel experiments, the reaction was terminated at fixed times by addition of 0.08 M K_{2}CO_{3}, 0.5 mM 2-PDS, 0.5 mM ATP, and 2.75 mM [1-14C]acetate-CoA (6 x 10^6 cpm/nmol). The mixture was incubated 10 min at 30°C, after which the reaction was terminated by addition of HCl. Bulk acetate was added and the extraction procedure was carried out as before.

**Table IV**

**Effect of ATP and ADP on the activity of purified acetyl-CoA hydrolase**

Effect of ATP and ADP on the activity of purified acetyl-CoA hydrolase. Reaction mixtures contained 100 mM MOPS (pH 7.4), 0.2 mM 2-PDS, 0.5 mM acetyl-CoA, and 2 mM ATP (2 mM ADP, or H2O). Reactions were initiated by addition of enzyme, incubated at 30°C, and monitored at 343 nm.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>ΔA/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ATP</td>
<td>0.383</td>
</tr>
<tr>
<td>+H2O</td>
<td>0.071</td>
</tr>
<tr>
<td>+ADP</td>
<td>0.008</td>
</tr>
<tr>
<td>-Enzyme</td>
<td>0.006</td>
</tr>
</tbody>
</table>
rate observed in the presence of 2 mM ATP was one-half that observed for acetyl-CoA, the affinity was 3-fold greater ($K_{d,5} = 19 \mu M$).

**Substrate Specificity**—Several acyl-CoA derivatives of varying chain length were tested as substrates for acetyl-CoA hydrolase. Rates of hydrolysis were determined at substrate concentrations of 0.5, 6.0, and 1.0 mM. Oxalacetate and citrate synthase were added to the reaction mixture prior to addition of acetyl-CoA hydrolase to eliminate acetyl-CoA contamination which was found to be as high as 6% for acetocacetate-CoA. Except for malonyl-CoA, which appeared to be a special case, the rates were essentially constant over the three concentrations and were taken to be the $V_{max}$ values for the respective substrates (Table V). These rates showed a marked decrease with increasing chain length. Valeryl-CoA was hydrolyzed at a rate of only 5% of that of acetyl-CoA. Despite the lower hydrolytic rates, the longer chain CoA derivatives showed increasing affinities toward acetyl-CoA hydrolase with increasing chain length as determined by the method of Dixon and Webb (17). All assays were done in the presence of 2 mM ATP to satisfy the requirement of linear kinetics. The kinetics of acetyl-CoA and propionyl-CoA is linear under these conditions, and it was assumed that the kinetics of the other substrates was similarly affected. The affinity of malonyl-CoA was determined relative to acetyl-CoA, using 10 mM concentration of each substrate. Due to the much lower affinity constants of the longer chain CoA derivatives, affinities of acyl-CoAs longer than C4 were determined with propionyl-CoA as the substrate of known kinetics (0.5 mM substrate concentrations) for better accuracy. These results suggest that while longer chain CoA derivatives are not efficient substrates for hydrolysis, they may be effective competitive inhibitors of acetyl-CoA hydrolysis. Malonyl-CoA, the only compound tested with a free carboxyl group, appears to be neither a substrate nor an inhibitor. It was hydrolyzed at a negligible rate as compared to propionyl-CoA (also C4) and was not saturating at 1.0 mM levels.

Palmityl-CoA was not included in the above series due to the problem of micelle formation at concentrations above 3 to 5 mM (18). Bernson (4) reported that acetyl-CoA hydrolysis by the hamster brown adipose tissue was inhibited by 10 mM palmityl-CoA. Preliminary experiments indicated that this was true as well for the rat liver enzyme, and the effect was investigated further. Palmityl-CoA was not hydrolyzed to a measurable extent over a concentration range of 1 to 1000 mM with or without ATP. Increasing palmityl-CoA concentrations strongly inhibited acetyl-CoA hydrolysis. This experiment was repeated several times at several different fixed acetyl-CoA concentrations with no significant differences in the inhibition patterns observed. This would not be expected for a competitive process. In each case, acetyl-CoA hydrolysis began decreasing at approximately 3 mM palmityl-CoA and rapidly progressed to complete inhibition ($K_{I} = 3.0$). Since the $K_{d,5}$ for the inhibitor ($\sim 7 \mu M$) is above the critical micelle concentration, it must be considered nonspecific in nature. Neither palmate nor palmitate/albumin mixtures had any effect on enzymatic activity.

**Species Distribution**—In the hope of finding a more abundant source of the enzyme, a limited survey was undertaken to measure the level of acetyl-CoA hydrolase activity in liver from a number of laboratory and slaughterhouse animals which were available. Net hydrolyase activity was measured as the difference between activities in the presence of 2 mM concentrations of ATP and ADP, respectively. In order to facilitate comparison among the various species, activity values were given with respect to total body weight, as well as with respect to liver weight. The former means of expression was far less sensitive to variations of nutritional state of the animals. These results, summarized in Table VI, suggest that ATP-stimulated, ADP-inhibited acetyl-CoA hydrolase activity appears to be limited primarily to rodents, i.e. rats, hamsters, and mice. Hamsters and mice appeared to have significantly higher levels of activity than rat, no matter which expression of activity was used. Monkeys had activity which was about one-tenth of that of rat. All other species showed no detectable difference between the ATP and ADP assays. Monkey, hamster, and mouse hydrolase activities were also investigated for cold lability. Mouse hydrolase activity showed a pattern of cold lability similar to that of the rat enzyme with a half-life of approximately 10 min at 4°C. However, the hamster enzyme had a half-life at this temperature of about 85 min, and monkey enzyme was stable over the entire 250-min period of the experiment.

**DISCUSSION**

The evidence presented here further supports the existence of a true acetyl-CoA hydrolase. Purification of extramitochondrial acetyl-CoA hydrolase to homogeneity has eliminated, at least in this case, the possibility that such activity is due to the artificial combination of two or more activities as was suggested by Costa and Snoswell (2). Our continuing skepticism that the complex ATP-stimulated and ADP-inhibited

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**Table V**

<table>
<thead>
<tr>
<th>CoA Derivative</th>
<th>$K_{d,5} \mu M$</th>
<th>Relative $V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>Propanoyl-CoA</td>
<td>0.05</td>
<td>0.48</td>
</tr>
<tr>
<td>Butyryl-CoA</td>
<td>0.16</td>
<td>0.90</td>
</tr>
<tr>
<td>Acetoacetyl-CoA</td>
<td>0.16</td>
<td>0.90</td>
</tr>
<tr>
<td>Valeryl-CoA</td>
<td>0.05</td>
<td>0.80</td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>&gt;500</td>
<td>0.80</td>
</tr>
</tbody>
</table>

**Table VI**

<table>
<thead>
<tr>
<th>Species Distribution</th>
<th>ATP-stimulated, ADP-inhibited acetyl-CoA hydrolase among various animal species</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Total body weight</td>
</tr>
<tr>
<td>units/g</td>
<td>min</td>
</tr>
<tr>
<td>Hamster, fed 9</td>
<td>1 0.9</td>
</tr>
<tr>
<td>Hamster, fasted 9</td>
<td>1 1.7</td>
</tr>
<tr>
<td>Mouse, fed 9</td>
<td>2 0.9</td>
</tr>
<tr>
<td>Rat, fed 9</td>
<td>9 0.5</td>
</tr>
<tr>
<td>Rat, fasted 9</td>
<td>25 0.7</td>
</tr>
<tr>
<td>Monkey, adult</td>
<td>1 0.06</td>
</tr>
<tr>
<td>Monkey, infant</td>
<td>1 0.08</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1 0.00</td>
</tr>
<tr>
<td>Chicken</td>
<td>1 0.00</td>
</tr>
</tbody>
</table>
enzyme was a "mere" hydrolase prompted us to pursue other possibilities and to identify the reaction products. These results provide no support for the hypothesis that the enzyme has any action other than catalyzing the hydrolysis of acetyl-CoA to acetate and CoASH. As one of a small but possibly growing number of short chain acyl-CoA hydrolases, the enzyme presents a difficult dilemma in that a thioester is dissociated with no apparent gain. At its maximally stimulated level (20 units/g wet weight of liver), the rat liver enzyme may hydrolyze acetyl-CoA at a rate which is 10-fold greater than the capacity of the cell to convert it to fatty acid (19).

In this perspective, it is not surprising that extramitochondrial rat liver acetyl-CoA hydrolase appears to possess a high degree of regulation. Although the only activators of which we are aware are nucleoside triphosphates, hydrolysis of acetyl-CoA is inhibited by ADP, longer chain acyl-CoAs, and possibly CoASH. Recent evidence, which will be presented elsewhere, seems to suggest that Mg$^{2+}$ may also have an inhibiting influence on enzyme activity, probably through its interaction with ATP. In addition, because the $K_{m}$ for acetyl-CoA is high relative to estimates of cytosolic concentrations (20, 21), substrate availability may play an important role in limiting enzyme activity. Thus, subject to a preponderance of negative influence on reaction rate, the enzyme may not work at anywhere near maximum efficiency. It is conceivable that inhibition might occur only under conditions where acetyl-CoA is unusually elevated.

The implications as to the physiological role, or the evolutionary demand, or both, for this active and highly controlled acetyl-CoA hydrolase are not yet clear. Due to its limited distribution, it seems likely that the function of ATP-stimulated, ADP-inhibited extramitochondrial acetyl-CoA hydrolase is a species-specific one. In that levels of cytosolic free CoASH have been estimated to be quite low (20, 21), it is possible that the enzyme may play a part in maintaining this important metabolite pool. CoASH availability, for example, might be threatened in instances of acute overproduction of acetyl-CoA through ATP:citrate lyase action or with production of metabolically inert acetyl-CoA derivatives during the catabolism of toxic substances. The three species found to have the highest levels of hydrolysis activity, rat, hamster, and mouse, share the peculiar 90:10 (cytosol/mitochondria) distribution of phosphoenolpyruvate carboxykinase in liver. This enzyme utilizes the regulatory step in the production of glucose (22). It has been suggested, since they may have a lesser capacity to produce mitochondrial P-enolpyruvate than other species, that these animals might use alternate metabolic means to produce cytosolic P-enolpyruvate (23). One possibility is that citrate may be synthesized in mitochondria and transported to cytosol as a source of oxalacetate which could then be converted to P-enolpyruvate through the action of phosphoenolpyruvate carboxykinase. Generation of oxalacetate in this manner, by ATP:citrate lyase, would also produce acetyl-CoA from CoASH as a by-product. In order for the process to proceed meaningfully, free CoASH would have to be regenerated. In the absence of fatty acid synthesis this might be accomplished through the action of hydroxyster. It might be noted, however, that the activity of ATP:citrate lyase is much reduced under prolonged fasting conditions where gluconeogenesis might be expected to be maximal. Gibson et al. (24) have reported values for this enzyme in rat liver of slightly less than 1 unit/g wet weight after 48 h of fasting. This level of activity would not be sufficient to maintain gluconeogenesis. With shorter fasting times, the level of activity of this enzyme is considerably higher and it is possible that the pathway may play a significant role under these latter conditions which more closely approximate the expected feeding patterns of rats, hamsters, and mice. Interestingly, although malonyl-CoA is a short chain, acyl-CoA, it is not a substrate and has low affinity for the enzyme. It would appear that once acetyl-CoA has been committed to fatty acid synthesis by its conversion to malonyl-CoA, it is no longer "seen" as a potential source of free CoASH.

Circumstantial evidence seems to support, also, the possibility that the enzyme might be involved in a detoxification function. The species distribution of activity parallels remarkably closely to that of hypoglycin A tolerance (25, 26). The toxic effects of hypoglycin A are considered to be the result of the production of a metabolically inert acyl-CoA, methylene cyclopropyl-CoA, although there has been some disagreement as to whether the mechanism is through CoASH sequestration or by direct inhibition of certain key enzymes (26, 27). It is possible that acetyl-CoA hydrolase might confer tolerance by directly releasing CoASH from methylene cyclopropyl-CoA or by making free CoASH available at the expense of the acetyl-CoA pool. The possession of hydrolase may provide an evolutionary advantage in certain animals which are routinely exposed to toxic substances in their diet.

The failure of earlier studies to demonstrate extramitochondrial acetyl-CoA hydrolase activity in rat liver (1, 2, 5) is probably due to the common use of isotonic buffers and low temperatures (4°C) during homogenization and fractionation procedures. In this regard, it is difficult to assess what implications might be associated with the continued widespread use of the above-mentioned conditions during tissue manipulations. To our knowledge, this enzyme is the first reported extramitochondrial acetyl-CoA hydrolase. The intracellular distribution appears to be intermediate between that of citrate synthase and lactate dehydrogenase. A similar distribution has been recently suggested for ATP:citrate lyase (28). The possibility that these two enzymes might have similar distributions is intriguing in light of the previous discussion.

REFERENCES

8. Ellman, L. G. (1959) Arch. Biochem. Biophys. 82, 70-77


