STUDIES ON RAT BRAIN ACYL-COENZYME A HYDROLASE (SHORT CHAIN)

Jack B. Robinson, Jr.*, Donald E. Mahan† & Roger E. Koeppe

Department of Biochemistry
Oklahoma Agricultural Experiment Station
Oklahoma State University, Stillwater, Oklahoma 74074

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Summary

A mitochondrial short chain acyl-CoA hydrolase, purified 1375-fold from rat brain, has a molecular weight of approximately 1.55×10^5, a pH optimum of 8.1, an ionic strength optimum for activity and stability of 100-300 mM, is product activated by acetate and inhibited by DL-lipoic acid ($K_i \cong 5$ μM) and 0.1 M orthophosphate (>50%). Acetyl, propionyl, butyryl, succinyl, acetoacetyl, malonyl and octanoyl-CoA are substrates. The highest maximum velocity and product activation was observed with acetyl-CoA as substrate.

An acetyl-CoA hydrolase is an important contaminant during much of the purification of rat brain pyruvate carboxylase (1). Because this enzyme may interfere with other enzyme assays using short chain thioesters and understanding of the biochemical role of acyl-CoA hydrolases is limited, we report herein the partial purification of acetyl-CoA hydrolase (EC 3.1.2.1) from rat brain mitochondria and describe some of its properties.

Since short chain acyl-CoA hydrolases were first described in 1952 (2) they have been found in porcine brain (3), reported as a contaminant of purified choline acetyl transferase (4) of rabbit brain and mentioned in studies with rat brain acyl-CoA hydrolases (5). Acetyl-CoA hydrolase is widely distributed in rat and sheep tissues, the highest activity being in liver mitochondria (6).

*Present address: Basic Biochemistry Unit, Veterans Administration Hospital, Dallas, Texas 75216.

†Present address: Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C.

Methods

Preparation of reagents - Acetyl-CoA was prepared by the action of acetic anhydride on free CoA in 0.1 M potassium bicarbonate using a 15% excess of the anhydride as suggested by Utter (personal communication). Other thioesters were prepared in a similar manner except acetoacetyl-CoA, which was prepared from lithium-CoA and diketene.

Enzyme assays - During purification the assay medium used was 20 µl of enzyme (up to 2 units/ml) in 0.96 ml of Buffer 1 (Table 1) with 100 µM 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB, from Sigma Chemical Company). An initial absorbance, due to reaction of 2-mercaptoethanol with DTNB, of about 0.6 was allowed to develop fully before the hydrolase reaction was started by addition of 20 µl of 12 mM acetyl-CoA. The mM absorbtivity of the DTNB chromagen at 412 nm was assumed to be 13.6 (7). All assays were run at 25°.

In assays where reduced sulfhydryl groups had to be maintained (e.g. CoA inhibition) absorbance of the thioester group at 232 nm (3) was measured.

Tissue distribution studies - Male Holtzmann rats, weighing 150-250 g, were used. After decapitation tissues were excised and homogenized in 10 volumes of Buffer 1 (Table 1). To 1 ml of homogenate was added 0.1 ml of 10% Tergitol-NPX (from Sigma Chemical Company), pH 7.4, and the mixture placed in ice for 30 min. The 1% concentration of Tergitol used was shown to be optimal (0.5-2.5%) for the detection of hydrolase activity in brain tissue.

Enzyme Purification

Mitochondria - Mitochondria from the cerebral hemispheres of 32 rats were prepared (8), lyophilized and stored overnight in vacuo at -29° (1). The enzyme is not stable; in one instance storage at this stage for three weeks resulted in a 95% loss of activity.

$(NH_4)_2SO_4$ precipitation - The freeze-dried mitochondrial residue was homogenized with 30 ml of Buffer 1 (Table 1) and centrifuged at 5.4×10^4g at 4° for 1 hour. The supernatant was brought to 50% saturation (291 g/l) by addition of solid $(NH_4)_2SO_4$ over a one hour period at 0-4°; the pellet was extracted by suspension in Buffer 2 and recentrifugation. This washing process did not solubilize any hydrolase activity. The washed pellet was resuspended in 2 ml of Buffer 2 and placed on a 1 x 7 cm Celite column, prepared by a slight modification (1) of the procedure of King (9), previously washed extensively with Buffer 2. This column was eluted at 0.25 ml/min with a linear gradient (50 ml) of decreasing $(NH_4)_2SO_4$ concentration from Buffer 2 to Buffer 1 at 4°. The elution profile was very similar to that previously described for brain pyruvate carboxylase (1). The fractions (1.5 ml) containing activity by DTNB assay were pooled, concentrated by the collodion bag technique (10) to about 5 ml and dialyzed 10 hours against Buffer 3. Stability was marginal in this buffer; hence longer dialysis times were avoided.

Table 1. Buffer Systems Used

Buffer 1	100 mM Tris, 100 mM Tris Acetate, 150 mM Na Acetate, 10% glycerol (v/v), 5 mM 2-mercaptoethanol, 5 mM Mg SO_4, 1 mM Na_2 EDTA, pH 8.1.
Buffer 2	Buffer 1 plus $(NH_4)_2SO_4$ (221 g/l) to 38% saturation.
Buffer 3	25 mM KH_2PO_4 to pH 7.4 with KOH, 150 mM $(NH_4)_2SO_4$, 5 mM 2-mercaptoethanol.
Buffer 4	Buffer 3 with $(NH_4)_2SO_4$ at 200 mM.

Hydroxyapatite - This preparation was placed on a 6 ml Bio-Gel HTP hydroxyapatite column poured in a 10 ml syringe barrel over pyrex wool at room temperature and washed with 5 volumes of Buffer 3. In all steps of this procedure the buffer was kept on ice and the column held at room temperature. Attempts to run this column in the cold room, to use buffer at room temperature and to use a gradient system led to poor yield of enzyme. The Bio-Gel column was eluted with 10 ml of Buffer 3, 10 ml of an equal mixture of Buffers 3 & 4, and 5 ml portions of Buffer 4 until all enzyme activity was eluted. Characteristically the enzyme emerged in the second 5 ml Buffer 4 wash and continued until approximately the sixth portion. The best results were obtained with freshly prepared columns. Fractions containing activity were pooled, brought to 10% glycerol immediately and concentrated to 2 ml by the collodion bag technique using Buffer 1.

Sephadex - When 5 units of enzyme were available at this stage from a single preparation or the combination of two successive batches they were applied to a 2.3 x 71.5 cm Sephadex G-200 column equilibrated with Buffer 1 at 4°. This column, whose void volume was 99 ml, was developed with Buffer 1 at about 5 ml/hr. The hydrolase was eluted at a V_e/V_o of 1.1 to 1.3. The active fractions were pooled and concentrated as before. Data for one of the better purifications are presented in Table 2.

Purification problems - Prior to the development of the acetate-glycerol buffer system, yields and stabilities were highly variable. This system gives fairly reproducible enzyme yields. Heating at 58°, polyethyleneglycol or ethanol precipitation, and DEAE-cellulose chromatography resulted in large losses of activity with little gain in enzyme purity. Affinity columns were tried in which CoA was linked to Sepharose 4B through the adenine amino group with hexane spacer or by a thioester bond with a hexanoic acid spacer; the former bound the enzyme but activity was not eluted by pH change, ionic strength

Table 2. Purification of Rat Brain Acetyl-CoA Hydrolase

Fraction	Units/mg protein*	Total units
Homogenate (0.9% Tergitol)	0.04	250
Mitochondria (Extract of)	1.8	32
Celite	5.0	14
Hydroxyapatite	30	6
Sephadex G-200	55	4.2

*By method of Lowry et al. (12)

gradients or substrate. The thioester column proved to be a substrate for the enzyme.

Results and Discussion

Purity - The activity isolated from hydroxyapatite shows at least four bands on polyacrylamide disc gel electrophoresis (11), one band predominating. The preparation has slight lactate dehydrogenase activity, not present after Sephadex G-200 treatment. Gel electrophoresis was not attempted with the small amount of enzyme obtained from G-200. During the enzymatic hydrolysis of acetyl-CoA by this brain hydrolase no substrate for β-hydroxyacyl-CoA dehydrogenase was formed, indicating a lack of thiolase activity in the purified preparation.

Stability - The enzyme obtained from Sephadex G-200 was stable for 4 months in Buffer 1 at 4°. Sulfhydryl compounds, ionic strength in the 0.2-0.4 M range, and glycerol enhance stability during purification. Dialysis against cold 0.25 M sucrose in 10 mM Tris-Cl causes total loss of activity, implying that ionic strength rather than osmolarity is critical for sta-

bility without affecting catalysis. Alkali metal ions have little effect on the hydrolase.

Molecular Weight - Previously we (1) reported an $S_{20,W}$ value for acetyl-CoA hydrolase of about 8.0, determined by sedimentation velocity in a sucrose gradient, corresponding to a molecular weight of about 1.6×10^5. During purification acetyl CoA hydrolase eluted from Sephadex G-200 at V_e/V_o of peak activity of 1.1, similar to that observed for yeast alcohol dehydrogenase which has a molecular weight of 1.5×10^5.

Inhibition - Whereas a variety of compounds have little or no effect on the hydrolase activity (using acetyl-CoA as substrate), 1-10 mM ADP or ATP inhibits the enzyme; their Mg^{++} complexes do not inhibit. 100 μM CoA causes over 50% inhibition. Orthophosphate and DL-lipoic acid are noncompetitive inhibitors with apparent K_is of about 40 mM and 5 μM respectively. Lipoic acid was also a potent inhibitor of hydrolysis when substrates (see below) other than acetyl-CoA were used.

Activation - The rate of hydrolysis of acetyl-CoA by this hydrolase is increased approximately 3-fold by 100-250 mM acetate, and about 50-70% by 100 mM flouroacetate or DL-β-hydroxybutyrate or 250 mM propionate. Butyrate, acetoacetate and acetaldehyde had little or no effect. Although the activation by acetate of acetyl-CoA hydrolysis shows irregular kinetics, not readily explained, it permits a more sensitive assay for acetyl-CoA hydrolase in tissue preparations. The hydrolysis of succinyl-CoA is slightly (~25%) but reproducibly inhibited by 250 mM acetate.

Substrate specificity - Although only acetyl-CoA was used to follow the purification of this hydrolase, the purified enzyme can hydrolyze propionyl, butyryl, acetoacetyl, succinyl,

Table 3. Acetyl-CoA Hydrolase in Rat Tissues

Tissue	Units/g tissue
Cerebrum	10
Cerebellum and Pons	4
Liver	12
Kidney	6
Heart	0
Leg Muscle	3
Lung	3
Testes	7

malonyl and octanoyl-CoA. Michaelis constants for these substrates are below 100 μM (not determined for malonyl and octanoyl-CoA). Palmityl-CoA, benzoyl-CoA and acetylthiocholine are not substrates.

Tissue distribution studies - The distribution of acetyl-CoA hydrolase activity in several rat and sheep tissues has been reported (6) using an assay buffer 100 mM in phosphate, pH 7.0, without acetate activation of the enzyme; enzyme activities were released by sonication. Using Buffer 1 (Table 1) and the nonionic detergent, Tergitol-NPX, an excellent solubilizer for this enzyme at concentrations of 0.5-1.5%, we find (Table 3) substantially greater amounts of acetyl-CoA hydrolase activity in rat tissue than those reported by Knowles, et al, (6). Whereas our value of 4 units per gram tissue in rat liver is similar to theirs, our data for cerebrum, kidney and heart are 10 to 20-fold higher. Also, we find much more acetyl-CoA

hydrolase in rat brain than did other investigators (5, 13) using phosphate buffer without glycerol. It should be noted that the figures in Table 3 are for total tissue and do not give insight concerning subcellular distribution. Fasting for 24 h had little or no effect on the hydrolase activity values for cerebrum, liver, kidney and testes.

Costa and Snoswell (14) have recently demonstrated that the acetyl-CoA hydrolase of rat and sheep liver is an artifact caused by the combined action of carnitine acetyltransferase and acetylcarnitine hydrolase. All the data in Table 3 (except those for brain) could result from a similar artifact. Our partial purification of the acetyl-CoA hydrolase from rat brain clearly demonstrates that this activity is not due to the carnitine related enzymes.

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