PING-PONG CHROMATOGRAPHY
A NOVEL PURIFICATION OF CoA-TRANSFERASE

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SUMMARY: Succinyl-CoA:acetoacetate CoA transferase, which forms a covalent intermediate with CoA, can be readily purified from rat kidney, heart, brain, and skeletal muscle using an acetoacetyl-CoA-agarose column. Significant purification (20-180 fold) with low losses in activity was achieved in each case, although minor contaminants were detected by SDS-gel electrophoresis. The isolated material appears to have a mol. wt. of 55-58,000 daltons, suggesting a dimeric structure for the native enzyme. Thus, ping-pong chromatography, based on the formation of a covalent enzyme-substrate intermediate on a solid support, can be used in the selective purification of certain enzymes.

Covalent catalysis is demonstrable with a number of different enzymes (1), most notably those that manifest a ping pong mechanism from steady state kinetic data (2). This involvement of a covalent enzyme-substrate intermediate should be exploitable for purposes of enzyme purification using a variation of "affinity chromatography" (3). Any enzyme capable of forming a covalent intermediate should bind covalently to a solid support, to which the enzyme's substrate has been previously attached, and, following elution of most contaminants, should be removable with a solution that decomposes the chemical bond between enzyme and substrate. In the following we demonstrate the utility of "ping-pong chromatography" in achieving a simple and rapid purification of succinyl-CoA:acetoacetate CoA transferase (EC 2.8.3.5). This mitochondrial enzyme (4) displays parallel line plots of initial velocity kinetic data (4-6), characteristic of a ping pong mechanism, and forms an enzyme-CoA intermediate (5, 7).

MATERIALS AND METHODS

The isolation and partial purification of CoA transferase from Buffalo

rat kidney, heart, brain, and skeletal muscle have been detailed elsewhere (4) and involve gel chromatography (using Sephadex G-100) of the supernatant following centrifugation of the sonicated particulate fraction from the different tissues. CoA transferase activity was measured using the spectrophotometric procedures of Hersh and Jenck (5); standard spectrophotometric procedures were employed in measuring other enzyme activities. Protein concentrations were determined by the Lowry method with bovine serum albumin as a standard. Acetoacetyl-CoA, succinyl-CoA and lithium acetoacetate were prepared as described previously (4). All other reagents were the highest purity available.

SDS-gel electrophoresis was performed by the method of Weber and Osborn (8); gels were destained according to Fairbanks et al. (9). Marker proteins for molecular weight determinations were obtained from Sigma Chemical Corp. (St. Louis, Mo.).

A ping-pong chromatographic column was prepared using a commercially available agarose support bearing coenzyme A (with free thiol groups) that is linked to the support via the caproyl moiety (P-L Biochemicals, Inc., Milwaukee, Wisc.). In order to prepare acetoacetyl-CoA-agarose the support (25 ml with 0.5 μmole CoA per ml) was washed in a 60 ml fritted glass filter funnel (having a disc of coarse porosity) with β-mercaptoethanol (5 mM; ca. 50 ml) and 200 ml of cold water deaerated with nitrogen. A suspension of the agarose in 10 ml cold water was transferred to a 30 ml test tube, the pH was adjusted to 7-7.5 with solid $NaHCO_3$, and nitrogen was gently bubbled through the suspension chilled on ice. Diketene (freshly distilled) was added in two 10 μl portions over 10 min; after a total reaction time of 20 min the suspension was poured back into the funnel and washed sequentially with cold water (50 ml), diethyl ether (50 ml), cold water (200 ml), and cold 0.02 M phosphate buffer (pH 7.0, 250 ml). The solution containing CoA transferase (ca. 1.2 I.U./ml of column packing) was added to the moist support, the mixture was transferred to a chromatographic column (1.5 cm diam.), and after 2-5 min at 4°C the solution was drained off. Elution of the column was begun using 0.02 M phosphate buffer (pH 7.0; 75-200 ml); CoA transferase was removed using 100 ml of 0.02 M phosphate buffer (pH 7.0) containing 10 mM acetoacetate and 0.25 M ammonium sulfate. Following this elution the column was washed with ca. 500 ml of 4 M urea solution containing 5 mM β-mercaptoethanol and stored at 4°C in a 5 mM β-mercaptoethanol solution. The column could then be recycled for use as described above.

RESULTS

A typical separation using a preparation of the sonicated particulate fraction from rat kidney that previously had been chromatographed through Sephadex G-100 is shown in Figure 1. If CoA-agarose only is used as a column support, no separation is noted. If crude material from the total homogenate of rat kidney is used, a similar purification results, yielding a CoA transferase preparation of comparable specific activity. However, a treatment of the column more severe than a urea wash is needed to prepare the column for reuse if the Sephadex chromatographic step was omitted. Thus, the data included in Table 1 were collected using the rat tissue preparations that had been chromatographed through Sephadex G-100 prior to

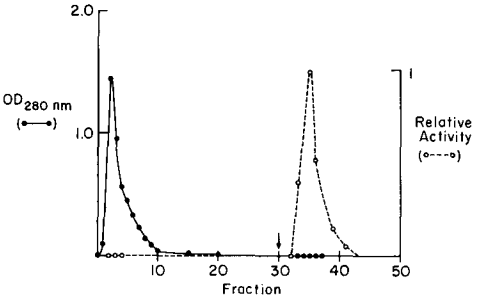

Figure 1. Elution Diagram of Rat Kidney CoA Transferase Activity from a Ping-Pong Column. Gel-filtered material from sonicated kidney particulate fractions (total activity, 10.0 I.U.) was placed on an acetoacetyl-CoA-agarose column (9 ml) as described in Materials and Methods. Fractions (1 ml) were collected over approx. 1 min using first 0.02 M potassium phosphate buffer (pH 7.0) alone and then adding at the arrow phosphate buffer containing 10 mM lithium acetoacetate and 0.25 ammonium sulfate. Total recovered activity, 8.1 I.U.

ping-pong chromatography. These indicate that an overall 20-180 fold purification can be achieved using the acetoacetyl-CoA-agarose column with generally low losses of activity. Recoveries of transferase activity for kidney, brain and skeletal muscle preparations ranged from 60-80%; greater

TABLE 1. Purification of CoA Transferase from Various Rat Tissues using Ping-Pong Chromatography

The specific experimental details are contained in Materials and Methods. The values are averages of two separate experiments.

	TISSUE			
SPECIFIC ACTIVITY[a]	Kidney	Heart	Brain	Skeletal Muscle
Total Homogenate	0.82	0.50	0.23	0.11
G-100 Chromatography	1.55(90)[b]	1.26(95)	0.46(93)	(c)
Ping-pong Chromatography	24.1 (77)	10.9 (38)	15.6 (74)	19.5(66)
Overall Fold Purification	29 (70)	22 (36)	65 (69)	180 (66)

(a) Specific activity is measured in terms of μmoles acetoacetate formed per min (or I.U.) per mg protein.

(b) The number in parentheses refers to the percent recovery for the purification step.

(c) Due to low total CoA transferase activity in the skeletal muscle homogenate, the gel filtration step was omitted.

losses (of 50-60%) were encountered when preparations of heart CoA transferase were employed. No attempts were made at altering chromatographic conditions with each transferase preparation in order to maximize recovery of activity.

One noteworthy feature of the transferase preparations isolated by ping-pong chromatography is that >90% of the activity is retained in samples stored frozen for over two months. CoA transferase from any of the four rat tissues purified by an alternative procedure involving cellulose phosphate chromatography (10) generally lost over 70% of its activity after an overnight storage in the freezer.

The purity of the kidney CoA transferase was further investigated by examining the material for the presence of contaminating enzymatic activities The effectiveness of the ping-pong chromatographic purification is shown in Table 2, which lists the ratios of various enzymatic activities in the pooled transferase fractions off of the ping-pong column to those in the solutions placed on the column. Significant amounts of citrate synthase activity were found in the transferase fractions; this result might be due to a high affinity for acetoacetyl-CoA, as an analog of the citrate synthase

TABLE 2. Comparison of Various Enzyme Activities Before and After Ping-Pong Chromatography of a Sonicated Rat Kidney Particulate Fraction.

The indicate enzyme activities were measured in the sonicate from a rat kidney particulate fraction before ping-pong chromatography and in the pooled fractions containing CoA transferase activity after elution from the column. R is the ratio of recovered activity to initial activity.

Enzyme	R
CoA transferase (EC 2.8.3.5)	0.61
Citrate synthase (EC 4.1.3.7)	0.39
β-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)	0.07
Acetoacetyl-CoA deacylase (EC 3.1.2._)	<0.01
Acetoacetyl-CoA thiolase (EC 2.3.1.9)	<0.01
Carnitine acetyltransferase (EC 2.3.1.7)	<0.01
Lactate dehydrogenase (EC 1.1.1.28)	0.016
Malate dehydrogenase (EC 1.1.1.37)	0.012
Glutamate dehydrogenase (EC 1.4.1.3)	<0.01

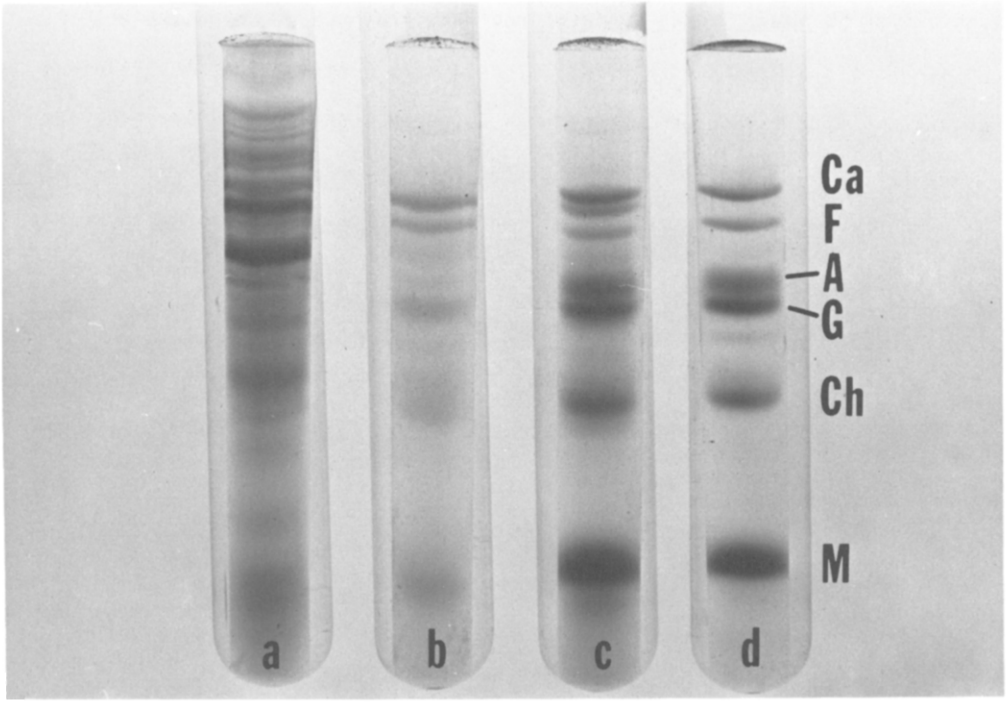

Figure 2. SDS-Gels of Different Preparations of Rat Kidney CoA Transferase. a) Sonicate of the particulate fraction. b) Pooled fractions from ping-pong column containing CoA transferase activity. c) Pooled fractions plus all marker proteins except fumarase. d) Marker proteins: catalase (Ca), fumarase (F), aldolase (A), glyceraldehyde phosphate dehydrogenase (G), chymotrypsinogen (Ch), and myoglobin (M).

substrate, acetyl-CoA. On the other hand, only trace amounts of the activity of other acetoacetyl-CoA utilizing enzymes, the deacylase (11) and thiolase (12), could be detected.

SDS-gel electrophoretic patterns (Fig. 2) also indicate the extent of purity of the kidney transferase preparation. The ping-pong chromatographed material contains a major component of about 5.8×10^4 daltons and a minor one at 5.5×10^4 daltons. These results, considered along with our earlier report of an approximate molecular weight of 10×10^4 for the native enzyme from these rat tissues (4), suggest an enzyme composition of two identically sized subunits; this would be consistent with the published description of the pig heart enzyme (native enzyme, $9.2\text{-}11.3 \times 10^4$ daltons;

subunit, 4.8-5.5 x 10^4 daltons) (6, 10). Rat brain, heart, and skeletal muscle CoA transferases provide SDS-gel electrophoretic patterns similar to those shown for the kidney enzyme, augmenting the list of structural similarities noted earlier for the transferases from these rat tissues (4).

DISCUSSION

In this paper we have described a novel and mild means for purifying succinyl-CoA:acetoacetate CoA transferase in which the formation of a stable enzyme-CoA intermediate is capitalized upon by the use of ping-pong chromatography. The column and eluting conditions described did not effect complete purification of the enzyme from four different rat tissues, minor contaminants still being detectable on gel electrophoresis. This is due in part to the ability of other enzymes to bind selectively (either covalently or non-covalently) to the acetoacetyl-CoA and CoA ligands attached to agarose. Nevertheless, it is clear that a large purification factor can be obtained in all cases. Due to the simplicity of this technique it is possible to achieve within a day better than a 20 fold purification of CoA transferase with low losses in total activity. Further purification of the enzyme should be afforded by additional steps, such as adsorption on calcium phosphate gel (13) or alumina gel (14).

The results indicate that ping-pong chromatographic procedures can be applied in the purification of enzymes that form stable covalent intermediates. In this instance purification can be achieved via the formation of a covalent bond between enzyme and solid support (as opposed to the non-covalent interactions that occur during a typical affinity column separation). The enzyme can then be eluted by breaking this bond using addition of a substrate or a chemical reagent, a pH change, etc. Thus, a reasonable purification scheme can be devised from limited kinetic or mechanistic information obtained on impure enzyme preparations.

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