PURIFICATION OF CHOLINEPHOSPHATE CYTIDYLYLTRANSFERASE FROM RAT LIVER BY AFFINITY CHROMATOGRAPHY

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SUMMARY

A procedure is reported for the rapid purification of choline phosphate cytidylyltransferase (E.C. 2.7.7.15) from rat liver by affinity chromatography. The affinity column was prepared by reaction of glycerolphosphorylcholine with epoxy-activated Sepharose 6B. Unless we added CTP and magnesium acetate to the cytosol prior to chromatography, the enzyme did not bind to the column. In combination with ammonium sulfate precipitation, this procedure yielded 687-fold purification from rat liver cytosol with a 59% recovery of total activity.

INTRODUCTION

Cholinephosphate cytidylyltransferase (E.C. 2.7.7.15) catalyzes the reversible formation of CDP-choline and PPi from CTP and phosphorylcholine and may control the rate of phosphatidylcholine biosynthesis (1). Schneider and co-workers have shown that a large percentage of the enzyme activity from rat liver homogenate prepared in isotonic saline remains in the cytosol (2). Despite the soluble nature of this enzyme, attempts at purification by conventional methods have been only partially successful. Severe loss of enzyme activity from rat liver 105,000 x g supernatant was reported by Fiscus and Schneider during ion-exchange chromatography (3) and Radominska-Pyrek et al. during Sephadex G-200 chromatography (4).

In this communication, we wish to describe the purification of cholinephosphate cytidylyltransferase by affinity chromatography. A combination of ammonium sulfate fractionation and affinity chromatography enabled us to purify rapidly the choline-

phosphate cytidylyltransferase from rat liver cytosol 687-fold with a 59% yield.

MATERIALS AND METHODS

Chemicals– Epoxy-activated Sepharose 6B was the product of Pharmacia. [^{14}C]Glycerolphosphorylcholine was obtained from ICN Pharmaceuticals and [^{3}H]phosphorylcholine was synthesized enzymatically from [^{3}H]choline and ATP with a highly purified preparation of choline kinase (5) and the product was purified by thin layer chromatography. CTP, CDP-choline and glycerolphosphorylcholine were purchased from Sigma Chemical Company. All other chemicals were of reagent grade and used without further purification.

Ligand Coupling– The epoxy-activated Sepharose 6B powder (15 g) was washed with distilled water and suspended in 100 ml aqueous solution that contained 20 mM glycerolphosphorylcholine. The pH of the reaction mixture was adjusted to 13.0 by 1 N NaOH. The reaction mixture was shaken for 20 hours at 45° after which the gel was washed successively with distilled water, 0.1 M sodium borate-0.5 M NaCl, pH 8.0, and 0.1 M sodium acetate-0.5 M NaCl, pH 4.0. The washed gel was allowed to equilibrate with 20 mM Tris-HCl-1 mM dithiothreitol, pH 7.0, overnight and subsequently packed into a 2 x 15 cm column. Experiments with [^{14}C]glycerolphosphorylcholine indicated that 1.5 µmoles of ligand was immobilized per ml of epoxy-activated Sepharose 6B.

Enzyme Assays– Cholinephosphate cytidylyltransferase activity was assayed by incubation of 1 mM [^{3}H]phosphorylcholine (20 µCi/µmole), 2 mM CTP, 12 mM magnesium acetate, 20 mM Tris-succinate and enzyme sample at 37° for 15 min. The reaction mixture had a final volume of 0.1 ml and a final pH of 6.3. The reaction was stopped by immersion of the tubes in boiling water and the CDP-choline formed was separated by thin layer chromatography, eluted from the silica gel with 2 ml 0.5 N NaOH and the radioactivity was determined with a liquid scintillation counter. Choline kinase activity was assayed as described by Weinhold and Rethy (6) and ethanolaminephosphate cytidylyltransferase activity was determined by the method of Sundler (7). Protein was determined by the method of Lowry et al. (8).

RESULTS

Rat liver cytosol (2 ml) in 0.145 M NaCl was diluted with an equal volume of water and applied to the affinity column. The chromatogram in Figure 1 shows that less than 1% of the total cholinephosphate cytidylyltransferase activity was retained by the column. However, when 2 µmoles of CTP and 10 µmoles of magnesium acetate were added to the diluted cytosol and the pH was adjusted to 7.0 prior to chromatography, more than 60% of

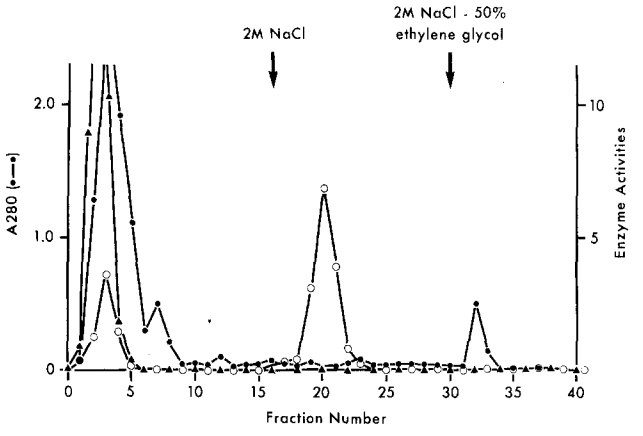

Figure 1 - Purification of Cholinephosphate Cytidylyltransferase by Affinity Chromatography. Rat liver cytosol was chromatographed on a column equilibrated with 20 mM Tris-HCl-1 mM dithiothreitol, pH 7.0, and the cholinephosphate cytidylyltransferase was assayed (▲———▲); in a subsequent experiment 2 µmoles of CTP and 10 µmoles magnesium acetate was added to the cytosol prior to chromatography (o———o). Enzyme activity is expressed as µmoles of CDP-choline formed per min per ml of enzyme. Flow rate of the eluent was 0.5 ml/min.

the enzyme activity was retained by the column and subsequently eluted by 2 M NaCl.

In order to improve on the purification, we fractionated 5 ml of rat liver cytosol with ammonium sulfate. As shown in Table I, 79% of the enzyme activity was precipitated between 25% and 50% ammonium sulfate saturation at 0°. The precipitate was dissolved in 5 ml of 20 mM Tris-HCl-1 mM dithiothreitol, pH 7.0, and dialyzed against 2 x 100 ml of the same buffer overnight. The omission of dithiothreitol in the dialysis buffer caused a severe loss of enzyme activity. After dialysis, 5 µmoles of CTP and 25 µmoles of magnesium acetate were added to the enzyme preparation and the final volume was adjusted to 10 ml by the same buffer. This enzyme preparation was applied to the affinity column and after the bulk of the protein was eluted, the enzyme

Table I

Purification of Cholinephosphate Cytidylyltransferase

	Total Activity (units*)	Total Protein (mg)	Specific Activity (units/mg)	Yield (%)	Relative Specific Activity
Cytosol	165	185	0.89	100	1
Ammonium sulfate fraction (25-50%)	130	54	2.40	79	2.7
Affinity Chromatography	98	0.16	612	59	687

* One unit is equal to 1 nmole CDP-choline formed per min at pH 6.3.

was removed by a 160 ml linear gradient of 0-2 M NaCl. Cholinephosphate cytidylyltransferase activity was measured after removal of the NaCl from the fractions by dialysis. The results of a typical purification scheme are summarized in Table I. The affinity column was washed with 2 bed volumes of a 50% mixture of 4 M NaCl and ethylene glycol. The column was subsequently re-equilibrated with 20 mM Tris-HCl-1 mM dithiothreitol, pH 7.0.

Neither ethanolaminephosphate cytidylyltransferase nor choline kinase activities were detected in the enzyme preparations after affinity chromatography. The highly purified cholinephosphate cytidylyltransferase was unstable - 80% of the enzyme activity was lost when placed at 0° for 6 days. The instability of the enzyme was partially overcome by addition of 1 mM dithiothreitol and 1% bovine serum albumin or 0.5 mM CTP and 2.5 mM magnesium acetate.

DISCUSSION

Glycerolphosphorylcholine was selected as a potential ligand for immobilization of the cholinephosphate cytidylyltransferase since the hydroxyl groups of glycerol should be readily available for reaction with the epoxy-activated Sepharose 6B. In addition, it was anticipated that the enzyme would bind to the phosphorylcholine moiety of the ligand. In the presence of the other substrate (CTP), the enzyme was apparently bound to the ligand specifically, which resulted in an approximately 700-fold purification from the rat liver cytosol. In addition, non-specific binding of proteins also occurred as indicated in Figure 1 by a peak of absorption at 280 nm when the column was eluted with an equal volume mixture of 4 M NaCl and ethylene glycol. This might be attributed to interaction between hydrophobic sites on these proteins and the spacer arm of the ligand (9).

The availability of a rapid purification procedure for cholinephosphate cytidylyltransferase will enable us and other workers to purify large quantities of this enzyme. We will then be able to evaluate the regulatory properties of this enzyme and the role it plays in phosphatidylcholine biosynthesis.

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