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SOLUBILIZATION OF ALKYLDIHYDROXYACETONE-P SYNTHASE FROM EHRLICH ASCITES CELL MICROSOMAL MEMBRANES*

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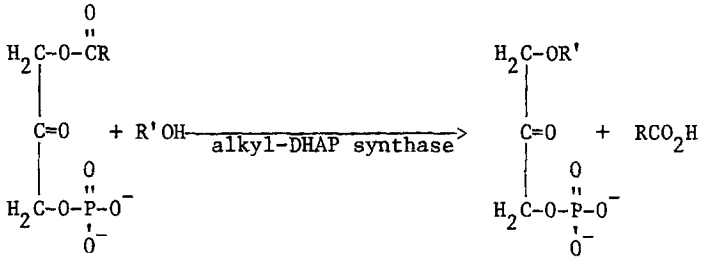
SUMMARY

The enzyme, alkyldihydroxyacetone-P synthase, has been solubilized and partially purified from microsomal preparations of Ehrlich ascites cells after treatment with Triton X-100 and phospholipase C, followed by chromatography on Sepharose 4B. When the Triton X-100 was removed after solubilization the enzyme was still active but eluted in the void volume of the Sepharose 4B column, whereas in the presence of detergent it eluted much later as a single peak of activity, indicating that the solubilized enzyme tends to aggregate unless detergent is present. The lower molecular weight form of alkyldihydroxyacetone-P synthase (in detergent) had an estimated molecular mass of 250,000-300,000 daltons.

Acyl-DHAP[1], formed by the acylation of DHAP, is enzymatically converted to alkyl-DHAP by alkyl-DHAP synthase (1-4). In this reaction, which is unique in higher biological systems, the ester-linked fatty acid of acyl-DHAP is replaced by a fatty alcohol to form the first detectable ether-linked intermediate in the biosynthetic pathway that forms O-alkyl glycerolipids and plasmalogens.



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1 DHAP represents dihydroxyacetone-P

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The membrane-bound form of this enzyme has been well characterized in microsomal preparations from mouse Ehrlich ascites cells (5-7), rabbit harderian glands (6, 8) and Tetrahymena (9, 10), and in mitochondrial preparations from guinea pig liver (4). Although the precise mechanism of catalysis is not yet known, isotope studies have shown that the hydroxyl oxygen of the fatty alcohol is incorporated into alkyl-DHAP (11). Also, one of the hydrogens attached to the carbon of acyl-DHAP that forms the ether linkage is exchanged with water (9, 10). Interpretation of kinetic data is limited due to the asymmetric localization of this enzyme on the lumenal surface of the microsomal vesicles (6). However, recent reports have suggested that the enzyme is located in the peroxisomes of mouse brain and guinea pig liver (12). Attempts to remove the enzyme from its membrane environment have been unsuccessful until now. In this communication we describe an effective procedure for solubilizing the enzyme to a low molecular weight form using Triton X-100 and phospholipase C. Under these conditions, the alkyl-DHAP synthase is fully active, is obtained in high yields, and has a molecular mass of less than 300,000 daltons as judged by Sepharose 4B chromatography.

MATERIALS AND METHODS

Materials - [1-^{14}C]Hexadecanol was synthesized from [1-^{14}C]palmitate (New England Nuclear, spec. act. = 50.2 Ci/mol) by reduction with Vitride (13). Palmitoyl-DHAP was obtained as described by Piantadosi et al. (14); Triton X-100 was purchased from Fisher Scientific Co.; Bio-Beads SM-2 from BioRad; phospholipase C of Bacillus cereus and phospholipase A_2 of Naja Naja from Sigma; and Sepharose 4B from Pharmacia.

Enzyme Source - Ehrlich ascites cells, transplanted in Swiss albino mice (HA/ICR) by intraperitoneal innoculation, were harvested after 7 days of growth. The cells were washed extensively with 0.1 M Tris-HCl (pH 7.4) containing 0.25 M sucrose and 0.1 M KCl, and the microsomal fraction was prepared by differential centrifugation as described previously (15).

Enzyme Assay - The assay system was a slight modification of one described recently (6). A substrate solution was prepared by mixing 100 nmol of [1-^{14}C]-hexadecanol and 300 nmol acyl-DHAP in chloroform. After evaporating the solvent under nitrogen, 1 ml of assay buffer (0.1 M Tris-HCl at pH 8.2, 0.25 M sucrose, 0.1 M KCl) containing 0.5 M NaF was added and the lipid substrates were dispersed by sonication. The reaction was initiated by adding 0.05 ml of substrate solution to 0.45 ml of assay buffer that contains the enzyme. After incubation at 37°C for 60 min, the reaction was terminated by extraction of total lipids (16). The lipids were resolved on Silica Gel HRB layers developed in chloroform/methanol/acetic acid (90:10:10, v/v). Distribution of radioactivity was determined by zonal profile scanning or area scraping utilizing a liquid scintillation spectrometer.

Solubilization - Ehrlich ascites cell microsomes (10 mg protein/ml) were solubilized in the assay buffer containing 0.5% Triton X-100. The mixture was homogenized with five strokes of a loose fitting Kontes glass homogenizer and immediately centrifuged at 100,000 x g for 60 min. The supernatant, which contained over 90% of the enzyme activity, was then incubated at 37°C for 10 min with either phospholipase C or phospholipase A_2 (0.2 units/mg protein). Lipid extraction (16) of the sample and resolution of the lipids on HRB plates developed in chloroform:methanol:acetic acid:water (50:25:8:3, v/v) followed by H_2SO_4 charring verified that the degradation of phosphoglycerides was nearly quantitative under these conditions. Protein was determined by the method of Lowry et al. (17) or by absorbance at 280 nm.

Gel Filtration - A 1-ml sample of the enzyme preparation from the various steps of solubilization was applied to a Sepharose 4B column (1.5 x 24 cm) previously equilibrated with the assay buffer or with the buffer containing 0.5% Triton X-100. A flow rate of 2 ml/h was maintained. Sephacryl S-200 and Sephadex G-200 columns of the same dimensions were used under identical conditions. When Triton X-100 was absent from the column buffer, protein eluting from the column was measured spectrophotometrically at 280 nm.

Removal of Triton X-100 with Bio-Beads SM-2 - Bio-Beads SM-2, washed with methanol and H_2O, were used in the batch procedure described by Holloway (18). The washed Bio-Beads were pipetted carefully into a screw-top tube and the aqueous supernatant was removed and replaced with the protein solutions (0.3 g Bio-Beads/ml protein solution). After the suspension was mixed gently on a rotary wheel for 1 h at 4°C, the protein solution was removed and filtered through glass wool. We determined the solution was free of Triton X-100 by extracting a portion of the sample (16), resolving the lipids on Silica Gel HRB layers developed in chloroform/methanol/acetic acid/water (50:25:8:3, v/v), and visualizing the lipids after H_2SO_4 charring. Triton X-100 had an R_f of 0.85 in this system.

RESULTS AND DISCUSSION

Previous results from our laboratory (6) indicated that the alkyl-DHAP synthases of rabbit harderian gland and Ehrlich ascites cells were inhibited by high concentrations of deoxycholate, but were stimulated by concentrations of Triton X-100 from about 0.025 to 0.05%. This maintenance of activity in the presence of Triton X-100 served as a successful starting point for our attempts to solubilize the enzyme from the microsomal fraction of Ehrlich ascites cells. We found that higher levels of Triton X-100 (> 0.5%) released over 90% of the alkyl-DHAP synthase activity into the supernatant after centrifugation at 100,000 x g for 60 min (Fig. 1). In order to establish an approximate molecular weight of this form of the enzyme, we chromatographed the material solubilized by Triton X-100 on Sephadex G-200 and Sepharose 4B (exclusion limits: 8×10^5 and 2×10^7 daltons, respectively). The enzyme activity eluted with the excluded components in the void volumes of both

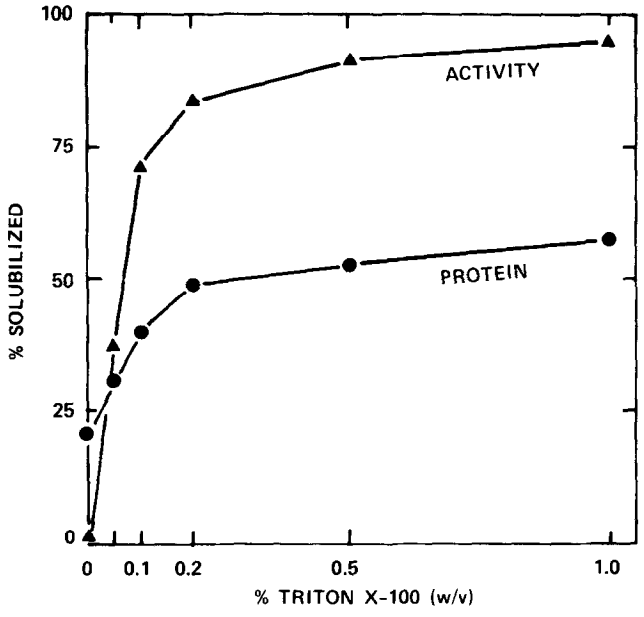

Fig. 1 Release of total protein and alkyl-DHAP synthase activity into the supernatant by Triton X-100 treatment after a 6,000,000 x g-min centrifugation. Triton X-100 treatment of the microsomal fraction of Ehrlich ascites cells was performed as described under experimental procedures. Symbols represent total protein (●) and alkyl-DHAP synthase activity (▲).

columns; this indicated the enzyme was still in a form with a molecular mass of several million daltons. Thus, even though the enzyme activity was not associated with the pellet after high speed centrifugation, it was not completely solubilized. This high molecular weight form was still active even when the Triton X-100 was removed with Bio-Beads SM-2 as described under Experimental Procedures.

In an effort to obtain a smaller molecular weight component, several other methods, by themselves and in combination with Triton X-100, were tried. The list of unsuccessful treatments included sonication, trypsin, urea, high salt, low salt, lyophilization, delipidation, freeze-thawing, other nonionic detergents such as Tweens and Lubrol, and various cholate derivatives. Success was finally achieved by treatment of the Triton X-100 solubilized material with phospholipases. Both phospholipases A_2 and C yielded lower molecular weight forms of the enzyme. The form obtained with phospholipase A_2 eluted from

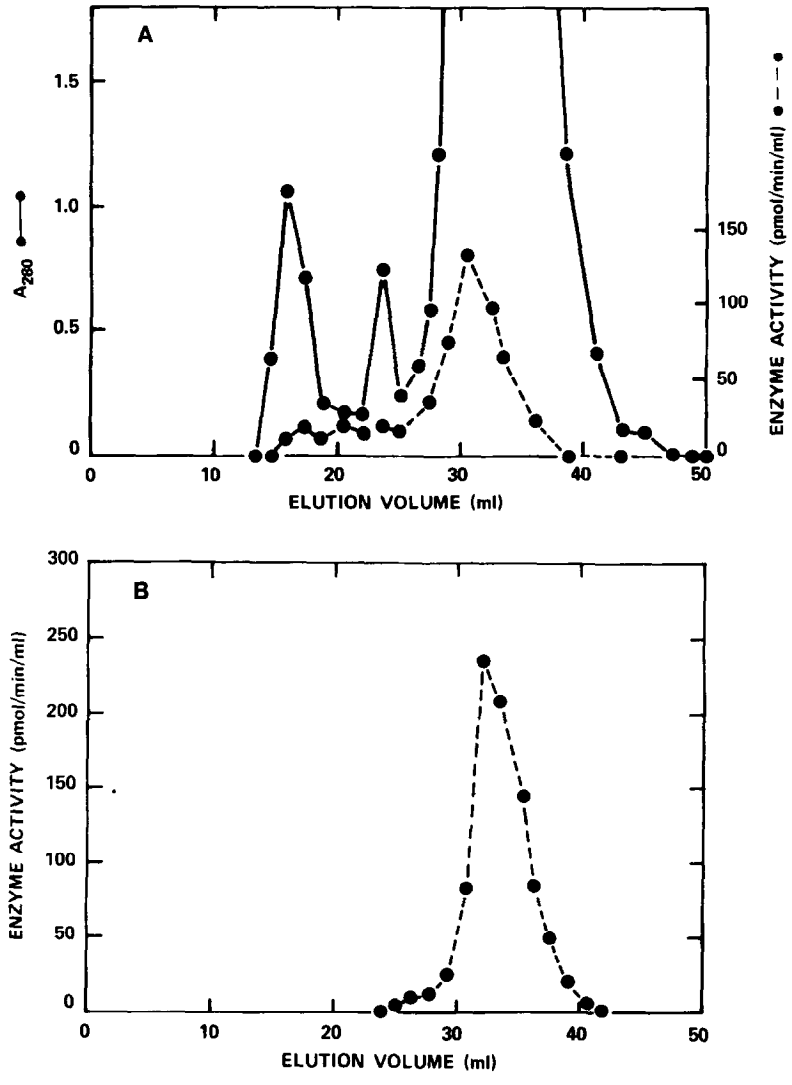

Fig. 2 Sepharose 4B chromatography of the Triton X-100/phospholipase C-treated microsomal preparation. Panel A shows the protein (●——●) and enzyme activity (●- -●) profiles on Sepharose 4B (1.5 x 24 cm) equilibrated in 0.25 M sucrose, 0.1 M KCl, 0.1 M Tris-HCl at pH 8.2. Panel B shows the enzyme activity profile on the same Sepharose 4B column equilibrated with the above buffer containing 0.5% Triton X-100.

Sepharose 4B with an approximate molecular mass of 500,000 daltons, whereas the form obtained by phospholipase C treatment was estimated to be from 250,000 to 300,000 daltons. The lower value was obtained by the elution of the enzyme activity in the void volume of Sephacryl S-200 (exclusion limit: 2.5 x 10^5 daltons). The upper value was estimated by measuring the elution volume of the enzyme from Sepharose 4B. Figure 2a shows the elution profile of the

TABLE I

Summary of protein and enzyme activity during different steps of solubilization of alkyl-DHAP synthase from Ehrlich ascites cells

Step	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity (nmol/min·mg)	% Yield Protein	% Yield Activity
Microsomal fraction	15.6	0.827	0.052	100	100
Triton X-100 treatment	9.3	0.818	0.088	60	99
Phospholipase C treatment	9.0	0.747	0.083	58	90
Sepharose 4B chromatography	4.9	0.482	0.098	31	58

enzyme from Sepharose 4B equilibrated with the assay buffer and Fig. 2b shows a profile from an identical Sepharose 4B column equilibrated with assay buffer containing 0.5% Triton X-100. The elution volumes of the enzyme activity from the two columns were nearly identical. The small amount of activity eluting earlier from the column in Fig. 2a was due to incomplete degradation of phospholipid by the phospholipase C treatment; in Fig. 2b, complete degradation of the phosphoglycerides was achieved. The large A_{280} peak with an elution volume of 30 to 40 ml is due to Triton X-100, which absorbs strongly at this wavelength.

The data obtained at various stages of solubilization are shown in Table I. The Triton X-100 solubilization consistently gave a 2-fold purification since approximately one half of the protein and nearly all of the alkyl-DHAP synthase activity was solubilized as evidenced by their appearance in the supernatant. The phospholipase treatments did not affect the enzyme activity while converting it to lower molecular weight forms. Analysis of the lipids in the sample showed that the phospholipase C treatment quantitatively degraded all of the phospholipids present, except sphingomyelin, to diacylglycerols. A small amount of monoacylglycerols was also detected, indicating some additional hydrolysis by other lipases. The fatty acids produced by this latter hydrolysis, when appreciable, inhibited the enzyme slightly as observed previously (19). Removal of the Triton X-100 after phospholipase C treatment had little effect on the enyzme activity, but when this material was chromatographed on Sepharose 4B

all of the activity eluted in the void fraction. It appears, then, that the enzyme must remain in detergent to prevent aggregation after hydrolysis of the lipids. The procedure developed by us for preparing the partially purified alkyl-DHAP synthase is an important prelude to its purification for subsequent studies of its mechanism of action.

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