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SOLUBILIZATION OF MICROSOMAL PHOSPHOETHANOLAMINETRANSFERASE BY OCTYL GLUCOSIDE

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SUMMARY: Phosphoethanolaminetransferase of high specific activity was solubilized from rat liver microsomes with the non-ionic detergent octyl glucoside. The solubilization method is fast and simple, allowing for processing of large amounts of material. The solubilized enzyme is stable. It contains virtually no phosphocholinetransferase activity. A preliminary characterization of the enzyme, with both diacyl- and alkylacylglycerol as substrate, is given. For the reaction, the lipid substrates were incorporated into artificial phospholipid bilayers (liposomes).

INTRODUCTION: CDP-ethanolamine : 1, 2-diradylglycerol phosphoethanolaminetransferase (EC 2.7.8.1) and CDP-choline : 1, 2-diradylglycerol phosphocholinetransferase (EC 2.7.8.2) catalyze the de novo synthesis of ethanolamine and choline diacyl glycerophospholipids, quantitatively the most important phospholipid in animal cellular membranes (1, 2). The de novo synthesis of alkyl glycerophospholipids in mammals is catalyzed by microsomal enzymes in a manner analogous to that established for diacyl glycerophospholipids (3, 4, 5, 6, 7). The question whether phosphoethanolamine- and phosphocholinetransferase activities reside in one or more enzymes is still a matter of controversy (2, 4, 8, 9, 10, 11, 12, 13, 14). Also, the number of transferases responsible for the synthesis of different types of phospholipids (diacyl-, alkylacyl-, and alkenylacyl-) is not known. The presence of endogenous diradylglycerols in microsomal membranes

Abbreviations: AAG: alkylacylglycerol; DAG: diacylglycerol; "radyl" denotes alkyl or acyl.

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and the possibility of further conversion of alkylacyl to alkenylacyl glycerophospholipids (plasmalogens) makes the study of the biosynthesis of various types of phospholipids difficult; only apparent activities can be observed.

Numerous approaches have been made in order to solubilize and separate the phosphoethanolamine- and phosphocholinetransferase activities. They were mostly unsuccessful due to the fact that the detergents used for solubilization were inhibitory (15, 16). The first solubilization and partial co-purification of both enzymes from liver microsomal membranes was described by Kanoh and Ohno (14). However, only limited evidence on the separation of the two activities was presented by these authors; after separation, the enzymes had a low specific activity and were unstable.

In the present paper, we describe a method of solubilization of phosphoethanolaminetransferase of high specific activity and with good yield. The solubilized enzyme is stable for long periods of time and contains virtually no phosphocholinetransferase activity. A preliminary characterization of the enzyme, with both diacyl- and alkylacylglycerol as substrate, is given.

The preparation of water-insoluble substrates for reactions carried out in aqueous media presents many difficulties. In the case of diradylglycerols, emulsions of the lipid in non-ionic detergents are commonly used. A disadvantage of this method is the susceptibility of some enzymes to inactivation by detergents and, possibly, a limited availability of the substrate. In the present paper we introduce the use of artificial membranes containing the diradylglycerol, as substrates. In our opinion, such membranes approximate the *in vivo* situation better than lipid emulsions. A more detailed comparison of both substrate preparations in the phosphoethanolaminetransferase reaction will be published elsewhere.

MATERIALS AND METHODS: Cytidine-5'-diphosphate-[Me-^{14}C]choline and cytidine-5'-diphosphate-[1, 2-^{14}C]ethanolamine were prepared according to (17). Octyl glucoside (octyl-β-D-glucopyranoside) was synthesized essentially according to (18). Its purity was checked by silica gel TLC in acetone : methanol 2:1 and by proton NMR. An approximately 0.5 M stock solution was prepared and the actual concentration of the detergent was determined by the anthrone reaction (19) with glucose as standard. *Chimera monstrosa* (ratfish) liver oil was subjected to lipolysis as pre-

viously described (6). The 1,2-diacyl-sn-glycerols were prepared from lecithin obtained from asolectin (crude soybean phospholipids, from Associated Concentrates, Woodside, NY, partially purified according to (20)) by treatment of phospholipase C (Clostridium welchii, Calbiochem, Los Angeles, Ca.) according to (21). For preparation of liposomes containing the substrate, the purified diacyl- or alkylacylglycerol in benzene was mixed with a ninefold excess (by weight) of asolectin. After drying under a stream of nitrogen, 1 ml water per 30 mg lipid was added and the lipids were allowed to swell. Liposomes were formed by sonication (3 x 1 min) in a MSE probe type sonicator at 0°C. Emulsions of diradylglycerol in Tween 20 were prepared as described previously (6). Rat liver microsomes (White Wistar rats, 200-220 g) were prepared according to (6). The solubilization of microsomes was carried out in a final volume of 2 or 3 ml of 10 mM Tris-HCl pH 7.5, 0.5 M KCl, 10% glycerol (w/v), 5 mg microsomal protein/ml, and the required concentration of octyl glucoside, at 0°C. The detergent was added last with vigorous stirring. The mixture was incubated in ice for 1 hour with occasional mixing and centrifuged for 2 hours at 150.000 x g_{av}. The pellets were suspended in 0.25 M sucrose, 1 mM Tris-HCl pH 7.5. In some experiments, detergent was removed from the supernatants by passing through a Sephadex G-50 column. Phosphoethanolamine- and phosphocholinetransferase activities were assayed in a final volume of 0.15 ml of 50 mM Tris-HCl pH 8.0, 13 mM $MnCl_2$, 1.33 mM diacyl- or alkylacylglycerol in the form of liposomes prepared as described above, 0.88 mM [^{14}C]CDP-ethanolamine or 0.1 mM [^{14}C]CDP-choline (0.5 - 1.0 Ci/mole), and 50 µg of microsomes or solubilized enzyme. In the latter case, about 3 mM octyl glucoside and 50 mM KCl were introduced into the assay. Eventual changes in assay conditions are indicated in the figures and tables. The reactions were stopped and total incorporated radioactivity determined as described previously (7). Protein was measured by the method of Lowry et al. (22) after dissolving the samples in 2% Na-deoxycholate, 2 mM NaOH.

RESULTS AND DISCUSSION: Phosphoethanolaminetransferase activity could be solubilized from microsomal membranes by treatment with the synthetic non-ionic detergent octyl glucoside. Lack of sedimentation after 2 hours centrifugation at 150.000 x g_{av} was taken as the criterion of solubilization. Although no further characterization of the physico-chemical state of the enzyme was undertaken, the existence of mixed protein-phospholipid-detergent micelles seems to be a reasonable assumption. The solution is optically clear and becomes turbid after removal of detergent.

The amount of solubilized activity and protein, as well as the total recovered activity, are shown in Fig. 1. At low detergent concentrations, less activity than protein was solubilized and the yield of solubilization was poor. However, at 31 mM octyl glucoside, some 80% of the recovered activity and 70% of total protein were found in the supernatant, and no significant

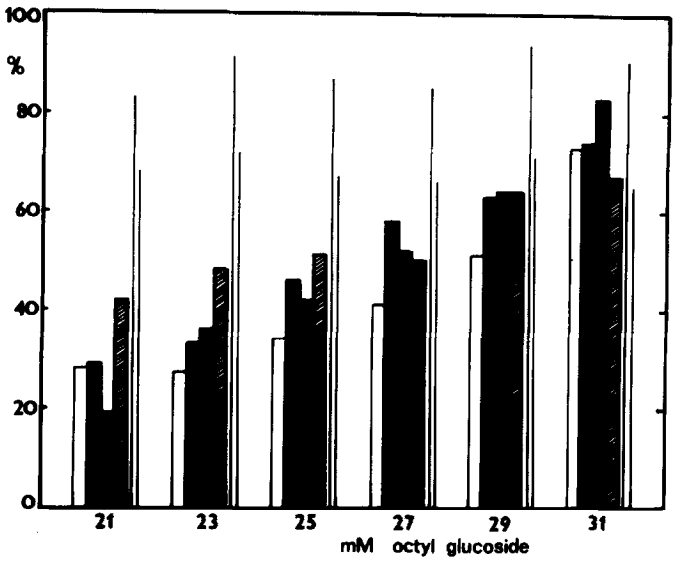

Fig. 1 Dependence of the percentage of solubilized protein and phosphoethanolaminetransferase activity on the concentration of octyl glucoside.

Solubilization and determination of enzymatic activity were as described in "Materials and Methods". Open bars represent endogenous activity (without added diradylglycerols), and black bars the activity in presence of alkylacylglycerol (left) and diacylglycerol (right bar). The values were calculated from the ratio of activity in the supernatant to the sum of activities in supernatant and pellet. Shaded bars represent protein. Recovery of activity (sum of activities in supernatant and pellet compared with untreated microsomes) is indicated by vertical lines (left for alkylacyl-, right for diacylglycerol).

loss of total activity was observed. Although virtually no purification over microsomes was achieved, a fractionation of the supernatant should be easily possible by a variety of methods. Lowering the ionic strength during detergent treatment significantly reduced the amount of solubilized protein and activity (data not shown). No separation of phosphoethanolaminetransferase activities measured in the presence of diacylglycerol or in the presence of alkylacylglycerol was observed under our solubilization conditions.

The highest specific activity of soluble phosphoethanolaminetransferase was obtained when 31 mM octyl glucoside was used for solubilization. The preparation seems to be partially depleted of endogenous diradylglycerols, since its activity without addition of this substrate was less than half of that of control microsomes. It could be greatly stimulated by externally

Table I. Specific activities of phosphoethanolaminetransferase in microsomes and in the solubilized preparation.

Solubilization of the enzyme (with 31 mM octyl glucoside) and assay were as described in "Materials and Methods". AAG and DAG denotes liposomes containing alkylacyl- and diacylglycerol, respectively.

protein	additions to assay	specific act. mU/mg	stimulation factor
microsomes	-	0.69	1
	AAG	5.05	7
	DAG	7.58	11
solubilized enzyme	-	0.30	1
	AAG	5.06	17
	DAG	6.09	20

added diradylglycerol (Table I). Therefore, uncontrollable side reactions were supressed after solubilization.

Almost no phosphocholinetransferase could be found in the solubilized preparation. This activity was strongly inhibited by octyl glucoside under our solubilization conditions, especially in the presence of high salt concentrations (at 25 - 31 mM octyl glucoside by more than 97%). The remaining activity was roughly equally distributed between the supernatant and the pellet. The specific activity of the enzyme was therefore usually less than 1% of control microsomes regardless of assay conditions (Mg^{2+} or Mn^{2+} as cofactor, diradylglycerol in form of liposomes or emulsion with Tween 20, ±added phospholipids, before and after removal of detergent), whereas in the case of phosphoethanolaminetransferase no loss of specific activity was observed (Table I). At the moment, we are not able to determine whether the inactivated enzyme is solubilized or not.

The solubilized phosphoethanolaminetransferase could be stored at -15°C for at least several weeks. Removal of the detergent caused a slight (about 30%) increase in specific activity, but precipitation and decay of the enzyme occured upon subsequent freezing and thawing. In contrast, in presence of octyl glucoside a more than twofold increase in specific activity was observed after seven days of storage at -15°C with several

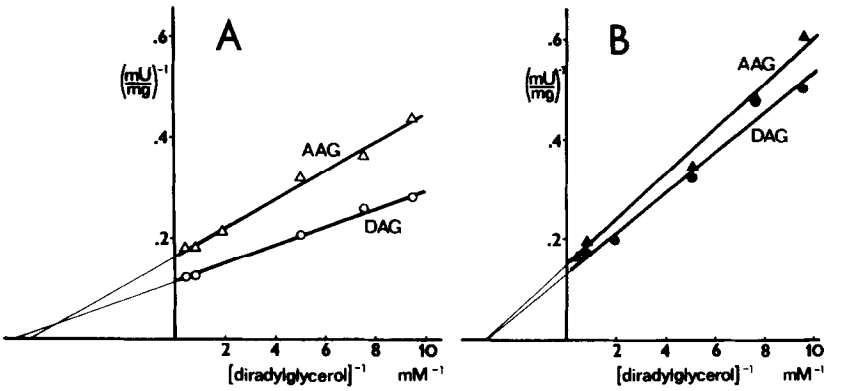

Fig. 2 Double-reciprocal plots of the dependence of phosphoethanolaminetransferase reaction rate on the concentration of diradylglycerol.

Assay conditions were as described in "Materials and Methods" except for the variation in the concentration of diradylglycerol. A: microsomes measured with diacyl- (○) and alkylacylglycerol (△); B: solubilized enzyme measured with diacyl- (●) and alkylacylglycerol (▲).

freeze-thaw cycles. A similar increase - although less pronounced - occured in intact microsomes. A possible explanation of this phenomenon would be an inactivation of a hypothetical regulatory subunit of the enzyme. The existence of such a subunit would be consistent with a behavior deviating from the Michaelis-Menten kinetics (see below).

The phosphoethanolaminetransferase reaction was linear with time for at least 30 min for both microsomes and solubilized enzyme, except for a transient burst of activity at 1 - 2 min. We used routinely an incubation time of 15 min, thus ignoring this initial burst. The dependence of the reaction rate on the concentration of the diradylglycerol is shown in Fig. 2. The resulting K_m values for this substrate were 0.16 - 0.18 mM for microsomes and 0.30 - 0.31 mM for solubilized microsomes; in both cases, there were no significant differences between the constants for diacyl- and alkylacylglycerol. In contrast to the K_m values, the reaction rates were different, namely higher for diacyl- than for alkylacylglycerol. However, this difference largely disappeared after solubilization. Fig. 3 shows double-reciprocal plots of the dependence of the reaction velocity on the CDP-ethanolamine concentration. If diacylglycerol was used as the acceptor, a slightly curved line was observed for both microsomes and solubi-

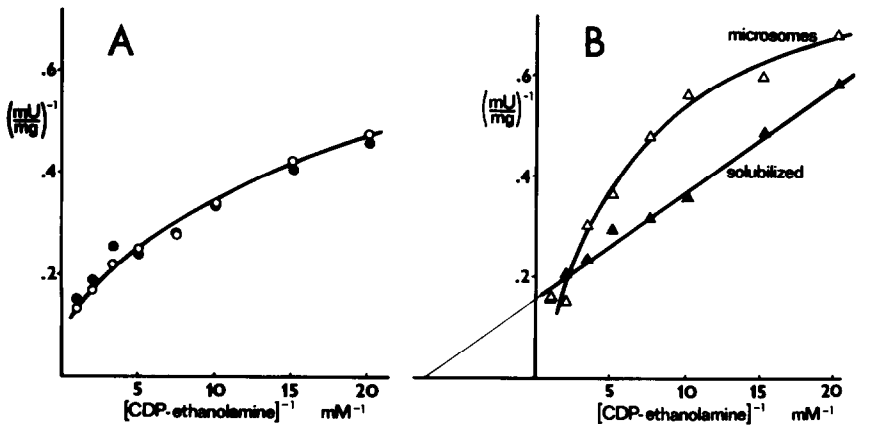

Fig. 3 Double-reciprocal plots of the dependence of phosphoethanolaminetransferase reaction rate on the concentration of CDP-ethanolamine.

Assay conditions were as described in "Materials and Methods" except for the variation in the concentration of CDP-ethanolamine. A: diacylglycerol was used as substrate with microsomes (○) and solubilized enzyme (●); B: alkylacylglycerol was used as substrate with microsomes (△) and solubilized enzyme (▲). AAG and DAG denotes liposomes containing alkylacyl- and diacylglycerol, respectively.

lized enzyme (Fig. 3 A). The situation was, however, different for alkylacylglycerol (Fig. 3 B). Here, the deviation from the Michaelis-Menten kinetics was very strong, but disappeared after solubilization. The K_m of the solubilized alkylacylglycerol-dependent phosphoethanolaminetransferase activity for CDP-ethanolamine is 0.14 mM. Although the available data do not allow for an unambigous interpretation, the possibility appears attractive that there is a regulatory subunit of the enzyme, which is lost after solubilization and, eventually, after ageing. Such a regulation would be not surprising in the case of an enzyme located at a branch point of metabolic pathways. The fact that only the alkylacylglycerol-dependent activity is affected by the solubilization is striking. Although it could indicate the existence of two separate enzymes for the two diradylglycerols, it is also consistent with both activities being located in one enzyme. More work will be required to clarify this point.

In summary, we developed a method for solubilization of phosphoethanolaminetransferase free of phosphocholinetransferase activity. This represents, in our view, strong evidence that these two reactions are catalyzed

by separate enzyme entities. However, the alternative assumption of both activities being located in one protein, although remote, will be definitely ruled out only after their separation without inactivation. Work on the solu bilization of the phosphocholinetransferase is in progress in our laboratory.

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