Phosphatidylethanolamine Synthesis in Castor Bean Endosperm

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ABSTRACT

Phosphatidylethanolamine synthesis by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1) from the endoplasmic reticulum of castor bean (Ricinus communis L. var. Hale) endosperm was characterized. The Michaelis-Menten constant of the enzyme for CDP-ethanolamine was approximately 8.0 micromolar. The pH optimum was 6.5 and a divalent cation was an absolute requirement for activity, with Mg\(^{2+}\) giving the greatest stimulation at 3 millimolar. Sulphydryl reagents variously affected enzyme activity. No discernible differences were detected between the responses of the ethanolaminephosphotransferase and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) to a variety of treatments. CDP-choline and CDP-ethanolamine were competitive inhibitors of the ethanolaminephosphotransferase and cholinephosphotransferase reactions, respectively.

Many of the ER enzymes involved in phospholipid synthesis in castor bean (Ricinus communis L. var. Hale) endosperm have been characterized. These include enzymes responsible for the synthesis of phosphatic acid (25), phosphatidylcholine by both the cholinephosphotransferase and methylation reactions (18), phosphatidylglycerol (16), phosphatidylserine (17), phosphatidylinositol (21), and diglyceride (19). On the other hand, the synthesis of phosphatidylethanolamine has received little attention, even though it is one of the major phospholipids present in the organelles of this tissue (4, 6). There are three potential biochemical reactions by which both plants (24) and animals (10) may synthesize phosphatidylethanolamine. These reactions are as follows:

I. Diglyceride + CDP-ethanolamine → Phosphatidylethanolamine + CMP
II. Phosphatidylserine → Phosphatidylethanolamine + CO\(_2\)
III. Phosphatidylserine + ethanolamine → Phosphatidylethanolamine + serine

Among these, reaction I catalyzed by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1) generally predominates (10, 24). This enzyme has been the subject of several investigations by workers using rat systems (1, 5, 7–9, 11, 20, 23) and also has been characterized in spinach leaves (14, 15). Characterization of the castor bean enzyme would provide a valuable comparison to the spinach enzyme and contribute to a fuller understanding of phospholipid metabolism in the castor bean endosperm. This paper describes such a characterization and compares some characteristics of the ethanolaminephosphotransferase activity to the analogous CDP-choline:1,2-diacylglycerol cholinephosphotransferase. A preliminary report of this investigation was presented elsewhere (26).

MATERIALS AND METHODS

Materials. All salts were obtained from the J. T. Baker Chemical Co. and were of reagent grade. The organic compounds (except sucrose, phospholipid standards, and diglyceride) were purchased from Sigma. Sucrose was from Mallinkrodt Chemical Works; phospholipids and diglyceride were purchased from Serday Research Laboratories (London, Ontario, Canada). [Ethanolamine-1,2-\(^{14}\)C]CDP-ethanolamine (60 mCi/mmol) was purchased from ICN Chemical and Radioisotope Division. [Methyl-\(^{14}\)C]CDP-choline (52.5 mCi/mmol) was from New England Nuclear.

Seeds of castor bean (Ricinus communis L. var. Hale), obtained from Mcnaire Seed Co., were soaked overnight in running tap water, planted in moist vermiculite, and germinated in the dark at 30°C for 4 to 5 days in a humidified growth chamber.

Tissue Homogenization and Cell Fractionation. Castor bean endosperm halves were homogenized and the organelles separated on modified sucrose density gradients as described elsewhere (21). Protein bands corresponding to the ER were collected with a syringe equipped with a hooked needle (21).

Phospholipid Synthesis Assays. CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase was assayed at 37°C for 30 min in a final volume of 0.5 ml containing 10 mm Mers buffer (pH 6.5), 3 mm MgCl\(_2\), 1 mm DTT, and 50 μM [ethanolamine-1,2-\(^{14}\)C]CDP-ethanolamine (3 mCi/mmol). CDP-choline:1,2-diacylglycerol cholinephosphotransferase was assayed as previously described (18). Reactions were initiated by the addition of 100 μl (50–75 μg) ER protein and terminated by the addition of 3.5 ml of chloroform:methanol:water (1:2:0.3, v/v/v). The reaction mixture was subsequently extracted by the method of Bligh and Dyer (2). Radioactive phosphatidylethanolamine and phosphatidylcholine were identified by thin layer co-chromatography with known standards on commercially prepared (Analtech) plates of 250-μm thick silica gel developed with chloroform:methanol:7 N NH\(_4\)OH (65:35:4, v/v/v) (21). The radioactivities of the chloroform fractions were measured with a Beckman model LS-8000 liquid scintillation counter in a scintillation cocktail consisting of 5 g PPO and 0.3 g dimethyl-POPOP/l toluene. Experiments designed to compare the thermal stabilities of the phosphatidylcholine and phosphatidylethanolamine-synthesizing enzymes were performed as described elsewhere (22).

Protein Determination. Protein of the ER fractions was measured according to the method of Lowry et al (15).

RESULTS

CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase activity under the standard assay conditions was slightly curvilinear with respect to time (Fig. 1). The rate of phosphatidylethanolamine synthesis increased linearly up to 150 μg of ER protein, the highest concentration tested (Fig. 2).

The ethanolaminephosphotransferase was most active at pH 6.5 using both Mes and Hepes buffers (Fig. 3), the total activity being consistently higher with the Mes buffer. A divalent cation was an absolute requirement for activity (Fig. 4), with Mg\(^{2+}\) strongly
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FIG. 1. Incorporation of ethanolamine from CDP-ethanolamine into phosphatidylethanolamine by the CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase reaction as a function of time.

FIG. 2. Effect of increasing amounts of ER protein on the rate of phosphatidylethanolamine synthesis by the CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase reaction.

FIG. 3. CDP-ethanolamine:1,2-diacylglycerol ethanolamine phosphotransferase activity as a function of pH in Mes and Heps (10 mM) buffers.

FIG. 4. CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase activity as a function of Mg\(^{2+}\) and Mn\(^{2+}\) concentrations. Metals were in the form of their chloride salts.

FIG. 5. Effect of increasing CDP-ethanolamine concentrations on CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase activity.

preferred and optimal at approximately 3 mM. Mn\(^{2+}\), which partially substituted for Mg\(^{2+}\) at lower concentrations, was optimal at 0.7 mM and appeared to inhibit at higher concentrations. Other divalent cations showed little or no ability to stimulate enzyme activity.

The ethanolaminephosphotransferase reached a maximum velocity of about 40 nmol/h.mg ER protein at a substrate concentration of approximately 30 \(\mu\)M (Fig. 5). A double reciprocal plot (Fig. 5, inset) gave an apparent Michaelis-Menten constant of 8.0 \(\mu\)M.

Sulphydryl reagents affected the ethanolaminephosphotransferase variously. At 0.1 mM, p-chloromercuribenzoate inhibited 100%, N-ethylmaleimide 50%, and reduced glutathione 25%. Higher concentrations gave greater inhibitions. \(\beta\)-Mercaptoethanol, iodoacetate, iodoacetamide, oxidized glutathione, and DTT (0.1 mM) gave little or no effect. DTT, when preincubated with the enzyme, seemed to protect enzyme activity and so was routinely included in the reaction mixture.

Comparisons Between Phosphatidylethanolamine and Phosphatidylcholine Synthesis. CDP-choline and CDP-ethanolamine competitively inhibited the ethanolamine- and cholinephosphotrans-
ferase, respectively (Fig. 6). There was no discernible difference between the stabilities of these enzymes at 37 °C, each losing about 40 to 60% of its initial activity after 6 h. There were also no significant differences between the responses of these two enzymes to the inclusion of 0.1 mM palmityl-CoA, 0.1 mM ATP, 1 mM Ca$^{2+}$, and a mixture of diglyceride and oleate (both 0.8 mM) in the standard reaction mixture. Both enzymes were inhibited approximately 65% by the addition of palmityl-CoA and 100% by the addition of Ca$^{2+}$. ATP alone inhibited slightly (10–20%). Diglyceride and oleate mixtures inhibited both enzymes greater than 95%. Lyophilization inhibited both enzymes by approximately 40%.

**DISCUSSION**

The ethanolaminephosphotransferase of the ER of castor bean endosperm compares variously with the enzyme from other sources. Generally, ethanolaminephosphotransferase activity can be subdivided into Mg$^{2+}$- and Mn$^{2+}$-stimulated activities, with Mn$^{2+}$ most often being more effective than Mg$^{2+}$, especially at low concentrations (1, 8, 11–15). Concentrations of Mn$^{2+}$ yielding optimal enzyme activity range from 0.6 and 2 mM for the spinach leaf enzyme (11, 13) to 1 to 10 mM for the various rat systems (1, 8, 11); for Mg$^{2+}$, the optimal concentrations are 8 to 25 mM in spinach (14, 15) and 20 to 40 mM for the animal systems (1, 8, 11).

With ER from castor bean endosperm, Mg$^{2+}$ is the preferred cation, being optimal at concentrations of 3 mM and above. Mn$^{2+}$ is effective only at lower concentrations, and at the optimal concentration of 0.7 mM is only 50% as effective as the Mg$^{2+}$-stimulated activity.

Reported pH optima for the ethanolaminephosphotransferase vary from 7.0 (15) and 7.5 (14) for the Mg$^{2+}$-stimulated enzyme of spinach leaf microsomes to 8.4 to 9.0 for rat brain microsomes (1). The castor bean enzyme is optimal at a lower pH of 6.5.

Michaelis-Menten constants for CDP-ethanolamine, using the Mg$^{2+}$-stimulated enzyme, range from 20 μM for both spinach (14) and the partially purified enzyme from rat liver (8) to 250 μM for rat liver (11) and brain (1) microsomal activity. The Km for CDP-ethanolamine in the castor bean system is 8.0 μM.

In castor bean, CDP-choline is a competitive inhibitor of PE synthesis (12) and CDP-ethanolamine is a competitive inhibitor of PC synthesis. The same is true of spinach leaf (14), rat brain (1), and rat liver (8) enzymes. Such studies generally indicate that the substrate and inhibitor bind to the same site in the same enzyme. This, along with parallel development in castor bean (3) and the similarity of the responses of the two plant transferases to lyophilization, inhibitors, etc., as reported here and elsewhere (12, 14), suggests that there may be only one enzyme responsible for both phosphatidylcholine and phosphatidylethanolamine synthesis. Investigations with mammalian enzymes describing differential susceptibilities of phosphatidylcholine and phosphatidylethanolamine synthesis to lyophilization (9), acyl-CoA esters (5), and mixtures of diglyceride and oleate (23) led to suggestions that two distinct transferases may be involved. More recently, the two Mg$^{2+}$-requiring enzymes from rat liver have been partially separated during purification (8). These partially purified enzymes responded differently to increasing amounts of microsomal phospholipids, Triton X-100 and sodium deoxycholate (8). Thus, it seems that at least two separate enzymes are present in rat liver microsomes. Conclusive evidence regarding the existence of one or more enzymes in plants or other animals awaits purification of the transferases(s).

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Fig. 6. Effects of CDP-choline and CDP-ethanolamine on CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (A) and CDP-choline:1,2-diacylglycerol choline phosphotransferase (B) activities, respectively. (A), CDP-choline was used as an inhibitor of phosphatidyl-ethanolamine synthesis at 5, 25, and 50 μM. (B), CDP-ethanolamine was used as an inhibitor of phosphatidylcholine synthesis at 5, 15, and 25 μM.
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