Phosphatidic Acid Synthesis in Castor Bean Endosperm

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ABSTRACT

Enzyme assays on organelles isolated from the endosperm of castor bean (Ricinus communis var. Hale) by sucrose density gradient centrifugation showed that palmitoyl-CoA sn-glycerol 3-phosphate acyltransferase (EC 2.3.1.15) was localized in the membranes of the endoplasmic reticulum. Mn²⁺ was required for activity, but Ca²⁺ and Mg²⁺ could substitute for Mn²⁺ at higher concentrations. The apparent Km was 170 µM for sn-glycerol 3-phosphate and approximately 8 µM for palmitoyl-CoA. The optimum pH range was 7 to 7.5 and the principal reaction product was diacyl-sn-glycerol 3-phosphate (phosphatic acid). Monoacyl-sn-glycerol 3-phosphate (lysophosphatic acid) was not released as a free intermediate in the reaction. The maximum activity of the enzyme occurred immediately after imbibition, preceding the development of mitochondria and glyoxysomes.

The acylation of sn-glycerol-3-P is a fundamental reaction in the synthesis of complex lipids in animals (9), plants (3, 13, 22), and bacteria (19). Acyl-CoA is the acyl donor in animals and plants, in some bacteria the acyl donor is acyl-ACP (12), and in Escherichia coli (21) either donor may function. In rat liver microsomal preparations (23) and in E. coli (18), the acylation has been shown to occur as two distinct reactions, with 1-acyl-sn-glycerol-3-P as the product of the first acylation, and 1,2-diacyl-sn-glycerol-3-P (phosphatic acid) as the product of the second. The reactions are catalyzed by acyltransferase(s) and are of particular interest because the final product, phosphatic acid, serves as a precursor to all of the phospholipid constituents of membranes as well as the di- and triacylglycerols.

Because of its importance in membrane biogenesis, the intracellular site of acyltransferase activity has been examined by several investigators. All of these used differential centrifugation to separate subcellular fractions. Acytransferase enzymes from rat liver (14, 23), rabbit lung (6), and rabbit heart (8) were shown to be localized largely in the microsomal fraction (10,000–100,000 g pellet), but were also present in the mitochondrial preparations. The mitochondrial enzyme usually differed from the microsomal enzyme by catalyzing a single acylation of sn-glycerol-3-P to form monoacyl-sn-glycerol-3-P (lysophosphatic acid), while the microsomal fractions were able to acylate both hydroxyls to give phosphatic acid. In plants, Cheniae (3) demonstrated that “microsomal-like particles” from spinach leaves were able to catalyze the incorporation of sn-glycerol-3-P into phosphatic acid. Sastry and Kates (22), also working with spinach leaves, found essentially the same results, but acknowledged that their microsomal preparation may have contained some mitochondria and grana. Macher et al. (13) used crude extracts of etiolated cucumber cotyledons to study sn-glycerol-3-P incorporation into phosphatic acid. Except for these reports, little is known about the properties of the acyltransferase enzyme in plants.

In the present paper, subcellular organelles of castor bean endosperm were separated by sucrose density gradient centrifugation. Palmitoyl-CoA sn-glycerol-3-P acyltransferase was localized predominantly in a discrete membrane fraction comprised of vesicles of the ER. In addition, the products of the acyltransferase reaction, the requirements for acyltransferase activity, and the pattern of acyltransferase activity from dry seed through 7 days of growth were investigated.

MATERIALS AND METHODS

Palmitoyl-CoA was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. sn-Glycerol-3-P, di-monocyclohexylamine salt, and dipalmitoyl phosphatid acid, sodium salt were obtained from Sigma Chemical Co. Palmitoyl lyso phosphatidic acid was purchased from Serdary Research Laboratories, Inc., London, Ontario. [14C(U)]sn-Glycerol-3-P, disodium salt (130.5 mCi/mmol) and [methyl-14C]cytidine diposphocholine (49 mCi/mmol) were supplied by New England Nuclear.

Germination Conditions. Castor bean seeds (Ricinus communis var. Hale) were soaked for 24 hr in running tap water, planted in moist vermiculite, and germinated in the dark at 30 C in a humidified growth chamber. The time of planting was considered to be time zero in developmental studies.

Homogenization and Fractionation of Cellular Components. Although efforts were made to keep germination conditions constant, growth rates occasionally varied between experiments. Therefore, the average weight of the endosperm from one seed was used as a guide to the metabolic age of the seedling (see Fig. 8C). The castor beans were harvested after 3 to 4 days when the average weight of the endosperm, with cotyledons and testae removed, was in the range of 0.75 to 0.90 g. Forty endosperm halves were chopped with razor blades for 20 min in 17 ml of a medium containing 1 mm EDTA and 13% (w/w) sucrose in 150 mm Tricine buffer (pH 7.5). The extract was passed through nylon curtain material, centrifuged at 270g for 10 min to remove cellular debris, and pipetted directly onto a sucrose gradient. The sucrose gradient was prepared in centrifuge tubes (2.5 × 7.5 cm) with a 3-mm cushion of 60% (w/w) sucrose, 30 ml of a linear gradient ranging from 60% to 15% sucrose, 10 ml of 15% sucrose, and 17 ml of the homogenate. All sucrose solutions contained 1 mm EDTA (pH 7.5). The gradients were centrifuged at 21,000 rpm for 90 min in a Beckman SW 25.2 rotor. Following centrifugation and removal of lipid bodies from the top of the gradient, 1.2-ml fractions were collected using a sucrose density gradient fractionator. The specific gravity of each fraction was determined by refractometry.

Organelles were separated by differential centrifugation for the investigation of changes in enzyme activity during germination. At each selected stage of germination, the endosperms of 20 seeds were removed and ground with a mortar and pestle in
15 ml of the above extraction medium, strained through nylon curtain material, and centrifuged for 10 min at 270g. The supernatant was brought to 25 ml with extraction medium and a 0.2-ml aliquot was withdrawn and diluted 10-fold for the assay of mitochondrial and glyoxysomal enzymes. The remaining supernatant was centrifuged at 12,000g for 15 min, the pellet discarded, the supernatant brought to 25 ml with extraction medium, and the microsomal pellet obtained by centrifugation at 140,000g for 1 hr. The microsomal pellet was resuspended in 6 ml of extraction medium and assayed for enzyme activity.

Enzyme Assays. Fumarase was measured spectrophotometrically by the appearance of fumarate (20), catalase by the disappearance of \( \text{H}_2\text{O}_2 \) (11), and malate synthetase by the formation of CoASH (7).

The phosphorylcholine glyceride transferase assay was a modification of that of Lord et al. (10). The 0.5-ml reaction mixture contained 100 \( \mu \)M tris buffer (pH 7), 20 \( \mu \)M MgCl\(_2\), 0.1 \( \mu \)Ci [methyl\(^{14}\)C]cytidine diphosphocholine (49 mCi/mmol), and 50 \( \mu \)l of enzyme. After incubating the mixture for 15 min at 30 C, the reaction was stopped by the addition of 4 ml of absolute ethanol, and the precipitated protein was removed by centrifugation. After the addition of 3 ml of chloroform and 5 ml of 2 \( \mu \)M KCl, the solution was shaken for 5 min and the chloroform phase was washed sequentially with 5 ml of 2 \( \mu \)M KCl and two 5-ml portions of water. The chloroform phase was transferred to liquid scintillation vials, evaporated to dryness, and assayed for \(^{14}\)C. All radioactive analyses were made using 0.5% PPO in toluene appropriate for use in a Beckman LS-230 counter and were corrected for instrument efficiency.

Acyltransferase was measured by the incorporation of \(^{14}\)C\(\text{sn}\)-glycerol-3-P into phosphatidic acid. The reaction mixture contained 30 \( \mu \)M tris buffer (pH 7.5), 1 \( \mu \)M EDTA, 100 \( \mu \)M unlabeled dipalmitoyl phosphatidic acid as a carrier, 1 \( \mu \)M \(^{14}\)C\(\text{sn}\)-glycerol-3-P (made to 0.225 mCi/mmol with unlabeled substrate), 2 \( \mu \)M MnCl\(_2\), 30 \( \mu \)M palmitoyl-CoA, and enzyme in a final volume of 0.8 ml. The reaction was initiated by the addition of enzyme and allowed to proceed at 25 C. After 30 min, the reaction was stopped and the lipids were extracted according to Bligh and Dyer (1) by adding 3 ml of methanol-chloroform (2:1, v/v) to the reaction mixture, followed by 1 ml each of chloroform and water. The chloroform phase was washed with 1.5 ml of water, dried over anhydrous sodium sulfate, and concentrated to 0.1 ml under a stream of \( \text{N}_2 \). The extract was applied to TLC plates (E. Merck Silica Gel 60) and chromatographed in chloroform-methanol-10 N ammonium hydroxide (65:35:4.5). This solvent was found to give a good separation of phosphatidic acid from other phospholipids. The spot corresponding to a co-chromatographed phosphatidic acid standard was scraped into a scintillation vial and counted for \(^{14}\)C.

Protein was determined by a modified Lowry method (4).

RESULTS

Intracellular Location. Figure 1 shows the separation of subcellular organelles of castor bean endosperm on a sucrose density gradient. Phosphorylcholine glyceride transferase marked the ER at 1.12 g/cm\(^3\), fumarase identified the mitochondria at 1.18 g/cm\(^3\), and catalase located the glyoxysomes at 1.23 g/cm\(^3\). The data showed clearly that palmitoyl-CoA:sn-glycerol-3-P acyltransferase was localized almost exclusively in the ER of germinating castor bean endosperm. A minor amount, about 10% of the total activity, was associated with the mitochondrial fraction. This activity probably represented contamination of the mitochondrial fraction by ER since phosphorylcholine glyceride transferase, a marker enzyme reported to be specific for the ER (10) was also associated with the mitochondrial fraction to the same minor degree in this preparation.

Properties of the Acyltransferase Reaction. Fractions from the sucrose gradient which represented ER were pooled and used as a source of acyltransferase activity. The acyltransferase reaction in Figure 2 shows the incorporation of \( \text{sn}\)-glycerol-3-P into phosphatidic acid with time. When the ER fraction was treated at 100 C for 10 min, there was no incorporation of \( \text{sn}\)-glycerol-3-P, demonstrating the enzymic nature of the reaction. A separate experiment verified that the major product of the acylation reaction was phosphatidic acid. Unlabeled palmitoyl lysophosphatidic acid and dipalmitoyl phosphatidic acid were added to an acyltransferase reaction mixture to trap newly synthesized radioactive intermediates. When the reaction products were separated by TLC (Fig. 3A) and an autoradiograph prepared (Fig. 3B), it was evident that virtually all of the \(^{14}\)C was present in dipalmitoyl phosphatidic acid and less than 8% was recovered in palmitoyl lysophosphatidic acid. The evidence indicated that the acyltransferase of castor bean esterified both the 1 and 2 positions of \( \text{sn}\)-glycerol-3-P in a concerted mechanism to
range, the relation between protein and acyltransferase activity was linear, but at higher levels of protein, the reaction was severely suppressed. For this reason, the amount of ER protein in all subsequent assays of the acyltransferase reaction was kept less than 40 μg/ml. The inhibitory effect was shown to be caused by a soluble factor which partially contaminated the ER fraction on the sucrose gradient (Fig. 4B). When several fractions from the soluble region in the upper gradient were pooled and then added in increasing portions to an active acyltransferase reaction mixture, the reaction rate declined. Treatment of the soluble fraction at 100 C for 10 min partially relieved the inhibition.

The pH optimum for acyltransferase from castor bean was in the range of 7 to 7.5 (Fig. 5). The enzyme had an absolute metal cation requirement for activity. The highest activity was obtained in the presence of 2 mM Mn2+ (Fig. 6A). Other cations, Ca2+ and Mg2+, also stimulated activity (Fig. 6B) but were effective only at higher concentrations, 4 mM and 7 mM, respectively. When Co2+ was used, only slight stimulation occurred. It is recognized that the optimum metal ion concentrations are actually lower than those recorded, since 1 mM EDTA was present in the reaction mixture. When EDTA was omitted from the reaction mixture, the optimum Mn2+ concentration was lowered by 1 mM (data not shown), but considerable loss in total acyltransferase activity occurred. Table I demonstrates the effect on enzyme activity when certain components of the reaction mixture were omitted.

Figure 7A shows acyltransferase activity when the concentration of palmitoyl-CoA substrate was varied. At higher concent-

form phosphatidic acid, and did not release lysophosphatidic acid as an intermediate after the first acylation. Previously studied plant systems have also shown that phosphatidic acid is the major product of the acyltransferase reaction (3, 13, 22).

The effect of increasing the amount of ER protein on phosphatidic acid formation is shown in Figure 4A. In the lower
trations, substrate inhibition occurred, making a determination of $K_m$ difficult. The $K_m$ was estimated from the half-maximum velocity to be about 8 $\mu M$, but the estimate should be taken with caution in view of the surfactant nature of the substrate, which can result in micelle formation and nonspecific binding of palmitoyl-CoA to proteins. The other substrate of the acyltransferase reaction, $sn$-glycerol-3-P, showed typical Michaelis-Menten kinetics (Fig. 7B). A Lineweaver-Burk plot (inset) gave an apparent $K_m$ of 170 $\mu M$.

**Development of Enzyme Activity in Germinating Castor Bean Endosperm.** Microsomal pellets from germinating castor bean endosperm, which ranged in age from dry seed to 7 days were assayed for acyltransferase activity (Fig. 8A). Very low acyltransferase activity was present in the dry seed, but immediately upon imbibition a rapid increase in activity occurred, reaching a maximum level after only 24 hr of soaking. The high activity was maintained for 3 days, then declined sharply. The enzyme synthesizing phosphatidicholine (phosphorylcholine acyltransferase) followed a similar pattern of development, but did not reach its maximum activity until the 3rd day, after which it too decreased rapidly. For comparative purposes, Figure 8B shows the development of mitochondria, represented by the activity of fumarase, and glyoxysomes, represented by the activity of malate synthetase. The cellular content of mitochondria reached a maximum by the 3rd day and glyoxysomes by the 4th day. The results show that high rates of synthesis of phospholipids can occur during the earlier stages of germination before the peak of development of mitochondria and glyoxysomes.

**DISCUSSION**

The evidence presented in this paper clearly established that acyltransferase was localized in the ER of germinating castor bean endosperm. The enzyme had a metal ion requirement which was best satisfied by Mn$^{2+}$ and had an apparent $K_m$ of 170
measured the activities of some phospholipid-synthesizing enzymes during castor bean germination. Their data showed a more gradual increase in phosphorylchoine glyceride transferase activity which peaked on the 3rd day just ahead of the mitochondrial and glyoxysomal enzymes. In contrast to the studies on castor bean, Machet et al. (13) showed that in crude homogenates of cucumber cotyledons, the maximum levels of sn-glycerol-3-P incorporation into lipid did not occur until the 5th through the 8th day of germination, and actually lagged behind the glyoxysomal enzymes, malate synthetate and catalase. 

Thus, in castor bean endosperm, nearly all of the enzymes of phospholipid biosynthesis outlined by Kennedy (9) have now been shown to occur in the membranes of the ER (2, 10, 15-17). As the only cellular fraction capable of producing the major phospholipids of membranes, the ER clearly plays an obligatory role in the biogenesis of the membranes of organelles such as glyoxysomes which are synthesized de novo in the castor bean endosperm. Our current view is that this occurs directly by the differentiation of portions of the ER but the transport of individual phospholipids to different sites of membrane assembly cannot be ruled out.

LITERATURE CITED

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Fig. 8. Effect of age of castor bean seedling on enzyme activity of the endosperm. A: ER enzymes, acyltransferase (●) and phosphorylcholine glyceride transferase (○); B: mitochondrial enzyme, fumarase (●), and glyoxysomal enzyme, malate synthetase (□); C: variation in average weight (▲) of a castor bean endosperm with age. Reaction conditions were those described under "Materials and Methods," except that in the acyltransferase reaction, 0.2 ml of the microsomal preparation (70-300 μg protein) was used.