Solubilization of Microsomal-Associated Phosphatidylinositol Synthase from Germinating Soybeans

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ABSTRACT

CDP-1,2-diacyl-sn-glycerol (CDP-diacylglycerol):myo-inositol phosphatidylinositol transferase (EC 2.7.8.11, phosphatidylinositol synthase) catalyzes the final step in the de novo synthesis of phosphatidylinositol in the endoplasmic reticulum fraction of germinating soybeans (Glycine max L. var Cutler 71). A variety of solubilization agents were examined for their ability to release phosphatidylinositol synthase activity from the microsome fraction. The most effective agent to solubilize the enzyme was the nonionic detergent Brij W-1. A 2.1-fold increase in specific activity was achieved using 1% Brij W-1 with 69% activity solubilized.

Maximal solubilization of phosphatidylinositol synthase was completely dependent on Brij W-1 (1%), potassium ions (0.3 m), and manganese ions (0.5 mm). Solubilization of the enzyme was not affected by the protein concentration of microsomes between 3 to 20 milligrams per milliliter. Solubilization was not affected by the pH of solubilization buffer between 6.5 to 8.5. To our knowledge, this is the first phospholipid biosynthetic enzyme solubilized from plant membranes. The Brij W-1-solubilized phosphatidylinositol synthase remained at the top of a glycerol gradient, whereas the membrane-associated enzyme sedimented to the bottom of the gradient. Maximal activity of the Brij W-1-solubilized phosphatidylinositol synthase was dependent on manganese (5 mm) or magnesium (30 mm) ions, and Triton X-100 (3.6 mm) at pH 8.0 with Tris-HCl buffer. The apparent K_m values for CDP-diacylglycerol and myo-inositol for the solubilized enzyme was 0.1 mm and 46 mm, respectively. Solubilized phosphatidylinositol synthase activity was thermally inactivated at temperatures above 30°C.

The enzyme responsible for the biosynthesis of PI is CDP-1,2-diacyl-sn-glycerol (CDP-diacylglycerol):myo-inositol phosphatidylinositol transferase (EC 2.7.8.11, PI synthase). PI synthase catalyzes the formation of PI and CMP from CDP-diacylglycerol and myo-inositol (15). PI synthase has been identified from the membranes of a variety of plant tissues (5, 20, 22). However, studies concerning the mode of regulation and kinetics of PI synthase have been hampered by the difficulty in obtaining an active purified enzyme from membrane preparations. As a result, most work on PI synthase from plants has been conducted in systems with intact membranes. The first step in the purification of a membrane-associate enzyme is the release of the enzyme in a soluble form from the membrane. Solubilization agents such as detergents and bile salts have been employed to release membrane-associated enzymes from cell membranes (11). In this communication, we report the conditions for the solubilization of PI synthase from germinating soybeans in reasonably high yield with good stability. PI synthase is localized in the endoplasmic reticulum of this seed (4). To our knowledge, this is the first report of the successful solubilization of a plant phospholipid biosynthetic enzyme. Therefore, we have paid particular attention to those factors required for the optimal release of PI synthase from soybean membranes.

MATERIALS AND METHODS

Materials. All materials were reagent grade or better. PI, myo-inositol, BSA, diginton, sodium cholate, sodium deoxycholate, and Brij W-1 (a mixture of 60–65% 20-cetyl ether and 30–35% 10-cetyl ether) were purchased from Sigma Chemical Co. Triton X-100 (octylphenoxypolyethoxyethanol) was a product of Rohm and Haas Co. Renex 690 (polyoxyethylenealkylary) ether was obtained from ICI. myo-[2-3H]inositol was purchased from Amer sham Corp. Precoated silica gel analytical thin layer plates were obtained from E. Merck. Soybean lecithin-derived CDP-diacylglycerol was prepared by the method of Carman and Fischel (6).

Preparation of Microsomes. The microsome fraction of germinating soybeans was used as the source of PI synthase. Microsomes were prepared from the whole seed of germinated (24 h) soybeans (Glycine max L., var Cutler 71) as described by Carman and Felder (5).

Enzyme Assay. PI synthase activity was measured at 30°C for 20 min as described by Carman and Felder (5) by following the incorporation of 0.5 mm myo-[2-3H]inositol. (2000 cpm/nmol) into chloroform-soluble material in the presence of 50 mm Tris-HCl buffer (pH 8.0), 5 mm MnCl_2, 0.2 mm CDP-diacylglycerol, 3.6 mm Triton X-100, and protein (0.05 to 0.15 mg) in a total volume of 0.1 ml. A unit of enzymic activity is defined as the amount of enzyme that catalyzes the formation of 1 mmol chloroform-soluble product/min under the assay conditions described above. The specific activity is defined as units per mg protein. The phospholipid product of the reaction, PI, was identified by chromatography on activated (110°C, 30 min) silica gel thin layer plates. Radioactive profiles on thin layer plates were determined by counting 1-cm strips in scintillation fluid as described by Carman and Felder (5). Standard PI was visualized with phosphate spray reagent (9). The radioactive product co-chromatographed with standard PI with an R_f of 0.18 in a solvent system containing chloroform-methanol-water (65:24:4) and an R_f of 0.40 in a solvent system containing chloroform-methanol-glacial acetic acid-water (50:30:48).

Solubilization. A 3.75-mg aliquot of microsome protein was suspended in 0.25 ml solubilization buffer (50 mm Tris-HCl [pH

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3 Abbreviation: PI, phosphatidylinositol.
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8.0], 2 mM MnCl₂, 0.5 M KCl, and 10% [w/v] glycerol] and 1% [w/v] of various solubilization agents. After incubation for 60 min at 5°C, the suspension was centrifuged at 100,000g for 60 min to obtain the solubilized fraction. The pellet fraction was resuspended in solubilization buffer. To obtain a solubilized preparation of PI synthase to study the properties of the enzyme, the solubilization procedure was scaled up 20-fold using 1% Brij W-1.

**Glycerol Gradient Centrifugation.** Gradients were prepared in a 5-ml cellulose nitrate tubes (1.3 × 5.1 cm). A 0.5-ml sample of microsome or solubilized protein was layered on top of a 20 to 50% [w/v] glycerol gradient (4.2 ml) with a 70% [w/v] sucrose shelf (0.3 ml) at the bottom. The buffer system used throughout the gradient was 50 mM Tris-HCl (pH 8.0). The gradient to which the solubilized enzyme was applied also contained 0.5% Brij W-1. The gradients were centrifuged at 200,000g for 90 min at 5°C in a Beckman L ultracentrifuge equipped with an SW 50.1 rotor. After centrifugation, fractions (0.34 ml) were collected from the gradient by puncturing the bottom of the gradient tube.

**Protein Determination.** Protein was determined by the method of Bradford (2) using bovine serum albumin as a standard.

**RESULTS**

**Solubilization of PI Synthase.** The microsome fraction was suspended in solubilization buffer with 1% variety of solubilization agents (Table I). The nonionic detergent, Brij W-1, solubilized PI synthase activity from the microsome fraction with a yield of 69% and an increase in specific activity of 2.1-fold. Diginiton also solubilized the enzyme, however with only a yield of 44%. The other solubilizing agents tested were less effective in releasing PI synthase activity and probably caused enzyme inactivation, as little activity was recovered in supernatant and pellet fractions. The conditions for the solubilization of PI synthase with Brij W-1 were further investigated.

**Effect of Brij W-1, Microsome Protein and pH on Solubilization of PI Synthase.** The conditions for the solubilization of PI synthase were varied with respect to the concentration of Brij W-1, the microsome protein concentration, and the pH of the solubilization buffer. The release of the enzyme from microsome membranes was completely dependent on the presence of the detergent. Maximal solubilization of PI synthase was achieved with 1% Brij W-1 with a yield of 69% (Fig. 1A). Concentrations above 1% Brij W-1 were either less effective in solubilizing the enzyme or were inhibitory to the enzyme. Solubilization of PI synthase with Brij W-1 was not significantly affected by the protein concentration of the microsomes in the experiment between 3 to 20 mg/ml (Fig. 1B). PI synthase activity was solubilized with Brij W-1 in buffers ranging from pH 6.5 to 8.5. The pH of the solubilization buffer had little effect on the yield of solubilized enzyme (Fig. 1C).

**Effect of Salts on Solubilization of PI Synthase.** The conditions for the solubilization of PI synthase with Brij W-1 were varied with respect to the presence of salts in the solubilization buffer. When KCl was omitted from the solubilization buffer, negligible PI synthase activity was released from the microsome membranes (Fig. 2A). Maximal solubilization was achieved with 0.3 to 0.5 M KCl. In the presence of 0.5 M KCl, manganese ions (the preferred cofactor for the enzyme [5]) were not necessary to solubilize PI synthase. However, the addition of 0.5 to 2 mM MnCl₂ increased the yield of solubilized enzyme to 69% (Fig. 2B). Magnesium ions (an alternate cofactor for the enzyme [5]) also increased the yield of solubilized enzyme, but not to the extent of that obtained with manganese (Fig. 2C). The presence of glycerol in the solubilization buffer did not affect the degree of solubilization. However, glycerol was included to stabilize PI synthase activity to storage at −80°C.

**Properties of Solubilized PI Synthase Activity.** Glycerol gradient centrifugation was used as a method to show the difference in the sedimentation properties of membrane-bound and solubi-
Solubilized PI synthase activity (Fig. 3). Under the conditions of the experiment, the membrane-associated enzyme would be expected to sediment to the bottom of the gradient with the membrane fraction. The Brij W-1 solubilized enzyme, presumably as a protein-detergent mixed micelle (11), should remain at the top of the glycerol gradient. The recovery of membrane-bound activity from the gradient was 51% relative to the applied sample, while the recovery of solubilized activity was 45% relative to the applied sample. The majority of the membrane-bound PI synthase activity was present at the bottom of the gradient in fractions 1 to 4. A second smaller peak of membrane-bound enzyme was present in fractions 9 to 11. This peak of activity may be due to the presence of smaller membrane fragments which, by virtue of their size and composition, did not sediment with the bulk of the microsomal membranes. The Brij W-1-solubilized PI synthase activity was present at the top of the glycerol gradient in fractions 10 to 13. It was necessary to add 0.5% Brij W-1 to the glycerol gradient to which the solubilized enzyme was applied to avoid enzyme aggregation and irreversible loss of activity.

Solubilized PI synthase activity was linear with time for 20 min and with protein concentration under assay conditions. PI synthase was dependent on CDP-diacylglycerol, myo-inositol, manganese, and Triton X-100 for activity. The optimum pH for solubilized PI synthase activity was 8.0 with Tris-HCl buffer. Activity was dependent on manganese ions (5 mM) or magnesium ions (30 mM). Maximal activity was also dependent on the addition of Triton X-100 (3.6 mM) to the assay system. Solubilized PI synthase exhibited normal saturation kinetics toward CDP-diacylglycerol ($K_m$ of 0.1 mM) and myo-inositol ($K_m$ of 46 μM). The assay requirements described above for solubilized PI synthase activity are essentially identical with those reported for the membrane-bound enzyme (3, 5).

Solubilized preparations of PI synthase were stable to storage at −80°C for at least 3 months and were stable to at least two cycles of freezing and thawing with no loss in enzyme activity. The solubilized enzyme was examined for its stability to temperature (Fig. 4). PI synthase activity was thermally unstable when incubated for 20 min at temperatures above 30°C with negligible activity present at 45°C.

**DISCUSSION**

PI synthase catalyzes the final step in the *de novo* synthesis of PI by displacing CMP from CDP-diacylglycerol by myo-inositol.
(15). PI synthase has been identified from the microsome fraction of germinating soybeans (5) and is localized in the ER (4).

A variety of solubilizing agents have been used to solubilize several membrane-associated enzymes of animal and bacterial phospholipid metabolism. Triton X-100 has been used to solubilize the majority of these enzymes (8, 10, 12, 17–19, 21) while digitonin (1, 14), cholate (23), and octylglucoside (16) have been used to a less extent. PI synthase has been solubilized from rat brain (18) and rat liver (23) microsomes with Triton X-100 or cholate. The enzyme has also been released from yeast microsomes with Triton X-100 or Renex 690 (7). We have examined a variety of solubilizing agents to release PI synthase activity from the microsome fraction of germinating soybeans. The most effective agent to solubilize PI synthase was the nonionic detergent Brij W-1. Unlike the animal and yeast systems, Triton X-100 was ineffective in solubilizing PI synthase from soybean microsomes. Triton X-100 caused almost complete inactivation of the soybean enzyme. This is not surprising since many enzymes bind large quantities of Triton X-100 after solubilization which results in enzyme inactivation (24). Solubilization of the enzyme with Brij W-1 was dependent on the addition of potassium ions to the solubilization buffer. The dependence on potassium may be explained by the fact that salts such as KCl at concentrations between 0.1 to 1 M increases the solubilizing power of nonionic detergents such as Brij W-1 (13). The salt decreases the critical micelle concentration and lowers the aggregation number of mixed micelles (13). Although the manganese ions were not necessary for solubilization, the presence of the enzyme’s cofactor afforded a higher degree of solubilization than in the absence of cofactor. The presence of cofactor (manganese or magnesium) may have exerted a protective effect of the enzyme during solubilization.

In summary, we have developed a procedure to solubilize PI synthase from the microsome fraction of germinating soybeans. Maximal solubilization of the enzyme was achieved with the nonionic detergent Brij W-1 and was dependent on the presence of potassium and manganese ions.

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LITERATURE CITED