Phosphatidylinositol Synthase from Canine Pancreas: Solubilization by *n*-Octyl Glucopyranoside and Stabilization by Manganese†

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ABSTRACT: Phosphatidylinositol synthase (CDP-1,2-diacyl-*sn*-glycerol:*myo*-inositol 3-phosphatidyltransferase) is active in mammalian pancreas, where it plays a role in the resynthesis of phosphatidylinositol (PI) during agonist-stimulated inositol–phospholipid metabolism. The enzyme was found to be present in relatively high specific activity [30 nmol of PI formed min^{-1} (mg of protein)$^{-1}$] in dog pancreas microsomal membranes, and its activity in these membranes was partially characterized. The K_m for *myo*-inositol was 0.76 mM, and the apparent K_m for cytidine(5′)diphospho-1,2-diacylglycerol (CDP-diacylglycerol) was 18 μM. The apparent K_a values for activation by Mn^{2+} and Mg^{2+} were respectively 42 μM and 2.5 mM. The pH optimum was 8.5–9.0. The enzyme was solubilized in stable form and in nearly quantitative yield with 40 mM *n*-octyl glucopyranoside (OG), with 4–6 mg of OG/mg of microsomal protein. In the presence of solubilizing levels of OG, the enzyme exhibited less than maximal activity, but full activity was restored by dilution of the OG to below its critical micelle concentration of 20–25 mM. The presence of Mn^{2+} was essential for stabilization of the OG-solubilized enzyme, with half-maximal stabilization at 40 μM Mn^{2+}. The stability of the OG-solubilized enzyme was sufficient to facilitate purification of the enzyme in the presence of this detergent, with 67% of the activity remaining after 3 days at 4 °C. The enzyme was partially purified by OG extraction and DEAE-cellulose chromatography, in 98% yield, to a specific activity of 290 nmol of PI formed min^{-1} (mg of protein)$^{-1}$. A mixture of PI (0.5 mM) and crude egg yolk phospholipids (5 μg of phospholipid–P_i/mL) stabilized the enzyme during chromatography.

Phosphatidylinositol synthase (DCP-1,2-diacyl-*sn*-glycerol: *myo*-inositol 3-phosphatidyltransferase, EC 2.7.8.11) catalyzes the final step in the biosynthesis of PI,[1] the reaction of CDP-diacylglycerol and *myo*-inositol to form PI and CMP; with a requirement for either Mn^{2+} or Mg^{2+} as a metal ion cofactor (Agranoff et al., 1958; Paulus & Kennedy, 1960). The enzyme is membrane-associated and, in animal tissues, is localized primarily in the endoplasmic reticulum, where it functions to maintain normal levels of PI. In addition, the enzyme is an integral part of the well-known cycle of receptor-stimulated inositol–phospholipid metabolism that occurs in many tissues in response to a wide variety of hormones, neurotransmitters, and other agents [reviewed by Michell (1975) and Irvine et al. (1982)].

In this laboratory, we have a long-standing interest in the regulation of the events associated with stimulated inositol–phospholipid metabolism in the exocrine pancreas. In this tissue, agonist occupancy of muscarinic cholinergic of cholecystokinin receptors induces a net hydrolysis of PI by PI phosphodiesterase activity (Hokin-Neaverson, 1974; Hokin-Neaverson & Sadeghian, 1984) followed by a net resynthesis of PI after removal of the agonist or introduction of antagonists (Hokin, 1974; Geison et al., 1976). Presumably, this resynthesis of PI involves the action of PI synthase. Characterization of the molecular properties and regulatory parameters of PI synthase may provide insight into the role of this enzyme in the control of the resynthesis phase of the cycle.

PI synthase activity in mammalian pancreas has been described in microsomes from guinea pig pancreas (Prottey & Hawthorne, 1967) and mouse pancreas (Hokin-Neaverson et al., 1977). However, detailed biochemical characterization of the properties of the enzyme from mammalian tissues has been hampered by difficulty in the purification of substantial amounts of enzyme. Due to the tight association of the enzyme with the microsomal membrane, its purification requires that it must first be solubilized from the membrane by a suitable detergent. PI synthase, from *Saccharomyces cerevisiae*, has been solubilized with Triton X-100 and recently purified to near homogeneity (Fischl & Carman, 1983). However, attempts to solubilize and purify the enzyme from animal tissues have met with difficulty due to the instability of the enzyme in the presence of the various detergents used to solubilize the enzyme, which include a number of nonionic poly(oxyethylene) derivatives (including Triton X-100), bile salts, and alkyl ionic detergents (Rao & Strickland, 1974; Takenawa & Egawa, 1977). In a preliminary paper, Eichberg et al. (1983) appeared to have some success in purification of the enzyme from rat brain, using Triton X-100 to solubilize the enzyme.

n-Octyl glucopyranoside (OG), a nonionic alkyl saccharide detergent, has a number of properties that make it a detergent of choice for the study of membrane proteins. OG is a chemically homogeneous compound and is relatively nondenaturing to proteins (Stubbs et al., 1976). It has been successfully used in the solubilization of a variety of membrane-bound proteins (Gould et al., 1979; Schneider et al. 1979; Matsushita et al., 1983). In addition, OG has an exceptionally high cmc, which facilitates its removal from detergent–protein micelles (Jackson et al., 1982) and which has resulted in its use in the reconstitution of some membrane proteins (Mimms et al., 1981; Jackson & Litman, 1982).

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[1] Abbreviations: PI, phosphatidylinositol; OG, *n*-octyl β-D-glucopyranoside; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; DEAE, diethylaminoethyl; cmc, critical micelle concentration; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography; CDP-diacylglycerol, cytidine(5′)diphospho-1,2-diacylglycerol.

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Because OG is a well-suited detergent for the solubilization and study of membrane proteins, we examined its usefulness in the solubilization of PI synthase from dog pancreas microsomal membranes. We report here that OG, in the presence of micromolar concentrations of Mn^{2+}, effectively solubilizes PI synthase, in a stable form, and we describe some properties of the OG-solubilized enzyme.

Experimental Procedures

Materials. Sources of materials were as follows: *n*-octyl β-D-glucopyranoside, Calbiochem-Behring, San Diego, CA; phosphatidylinositol and CDP-1,2-dioleoylglycerol, Serdary Research Laboratories, London, Ontario, Canada; scintillation Triton X-100, Research Products International, Elk Grove Village, IL; *myo*-[2-^{3}H]inositol, New England Nuclear, Boston, MA; [5-^{3}H]cytidine 5′-monophosphate, Amersham, Arlington Heights, IL; silica gel 60 precoated TLC plates, E. Merck, Darmstadt, West Germany; silicic acid impregnated ITLC sheets, Gelman Instrument Co., Ann Arbor, MI; other reagents, Sigma Chemical Co., St. Louis, MO.

Preparation of Lipid Substrates. CDP-1,2-diacyl-*sn*-glycerol was synthesized from cytidine 5′-(monophosphomorpholidate) and phosphatidic acid (prepared by phospholipase D hydrolysis of egg yolk phospholipids) according to the method of Agranoff & Soumi (1963) and purified by silicic acid column chromatography essentially as described by Raetz & Kennedy (1973). The purified CDP-diacylglycerol (Tris salt) migrated as a single UV-absorbing spot on silicic acid impregnated ITLC sheets in chloroform/methanol/water (65:37:5 v/v/v) and had the same A_{260} to A_{280} ratio as standard CMP. The molar ratio of cytidine to phosphorous to fatty acid was close to the theoretical ratio of 1:2:2. The fatty acid composition of the preparation was determined by gas–liquid chromatography of fatty acid methyl esters on a column of 15% diethyleneglycol succinate on 80/100-mesh chromosorb W (Hewlett-Packard, Avondale, PA) and was found to contain 33% palmitate, 29% oleate, 18% linoleate, 15% stearate, 4% arachidonate, and 1% palmitoleate (mole percent). CDP-diacylglycerol was stored at −80 °C in chloroform/methanol (2:1, v/v), which contained 0.01% (w/v) butylated hydroxytoluene.

[5-^{3}H]Cytidine(5′)diphospho-1,2-diacylglycerol was synthesized enzymatically from PI (porcine liver) and [5-^{3}H]-cytidine 5′-monophosphate (40 μCi/μmol) via the reverse reaction of PI synthase, using mouse pancreas microsomes, essentially as described by Hokin-Neaverson et al. (1977). For use as a substrate for PI synthase, the [^{3}H]CDP-diacylglycerol was diluted with unlabeled CDP-1,2-dioleoylglycerol to a specific activity of 0.5 μCi/μmol.

Preparation of Dog Pancreas Microsomal Membranes. Dog pancreas tissue was obtained from Professor Larry D. Davis, Department of Physiology, University of Wisconsin Medical School, Madison, WI. In his experiments, mongrel dogs (25–30 kg, fed ad libitum) were anesthetized by an intravenous injection of pentobarbital and then killed by exsanguination. The pancreas (typically 20–30 g wet wt) was quickly removed and trimmed free of major blood vessels, fat, and mesentery tissue. All subsequent operations were carried out at 0–4 °C. The tissue was minced with scissors and then homogenized with a Thomas Teflon pestle tissue grinder in 8 volumes (w/v) of 0.25 M sucrose, which contained 2.5 mM Tris–maleate, pH 6.0, 0.1 mM EDTA, 0.1 mM DTT, and 50 μM PMSF. The homogenization medium was adjusted to pH 6.0 to minimize leakage of lipases and other digestive enzymes from secretory granules (Hokin, 1955). The pancreas homogenate was centrifuged at $1000g_{max}$ for 10 min to remove heavy particulate matter (cell debris, erythrocytes, nuclei, and secretory granules) and then twice at $9000g_{max}$ for 15 min to sediment mitochondria. The postmitochondrial supernatant was collected and centrifuged at $100000g_{max}$ for 1 h. The microsomal pellet was resuspended in 10 mM Tris-HCl (pH 8.4 at 4 °C) and recentrifuged at $100000g_{max}$ for 1 h. The washed microsomes were then resuspended in 10 mM Tris-HCl (pH 8.4 at 4 °C) to a concentration of 5–8 mg of microsomal protein/mL. The microsomes were either used immediately or were divided into 1-mL aliquots and stored at −80 °C. While stored at −80 °C, no appreciable loss of PI synthase activity was detected for up to at least 6 months.

Solubilization of PI Synthase with n-Octyl Glucopyranoside. An aliquot of dog pancreas microsomal membrane suspension, in 10 mM Tris-HCl (pH 8.4), was centrifuged at $105000g_{max}$ for 1 h, followed by resuspension of the microsomal membranes in a medium that contained 25 mM KCl, 1.0 mM $MnCl_2$, 0.5 mM DTT, and 10 mM Tris-HCl (pH 8.4). OG was then added to give a final concentration of 40 mM, with 4–6 mg of OG/mg of microsomal protein. Other additions or deletions are as indicated in the table and individual figures. After 20 min on ice, the samples were centrifuged at $105000g_{max}$ for 1 h. Solubilized enzyme activity was defined as that which remained in the supernatant after centrifugation at $105000g$ for 1 h. In some experiments, where PI synthase activity was determined in the unsolubilized fraction, the pellet that remained after centrifugation was resuspended in a medium identical with that indicated above except it contained no OG.

Assay of PI Synthase Activity. PI synthase activity was determined by measurement of the rate of CDP-diacylglycerol-dependent incorporation of *myo*-[2-^{3}H]inositol into PI. The standard assay system for PI synthase from dog pancreas microsomal membranes contained 100 μM CDP-diacylglycerol (fatty acid cmposition of egg yolk phosphatidylcholine), 1.0 mM $MnCl_2$, 5.0 mM *myo*-[2-^{3}H]inositol (1.0–4.0 μCi/μmol), 50 mM Tris-HCl, pH 8.5 at 37 °C, 100 mM KCl, and approximately 15 μg of microsomal protein in a final volume of 250 μL. After a 5-min preincubation (at 37 °C) of the enzyme sample with the liponucleotide substrate, the reaction was initiated by the addition of $MnCl_2$ and *myo*-[2-^{3}H]inositol (preequilibrated to 37 °C). The reaction was allowed to proceed for 5 min at 37 °C and then terminated by the rapid addition of 3.0 mL of an ice-cold solution of chloroform/methanol/12 N HCl (200:100:1 v/v/v). The phospholipid products were partitioned into the chloroform phase by extraction with 5 mL of 2 M KCl (pH 3). After a brief centrifugation to separate phases, the chloroform layer was washed twice with 5 mL of 0.1 N HCl. To identify the radioactive product as PI, an aliquot of the chloroform phase was collected, mixed with 50 nmol of carrier PI, and subjected to two-dimensional TLC on precoated silica gel 60 plates. The chromatograms were developed in the first dimension in chloroform/methanol/glacial acetic acid/water (100:30:35:3 v/v/v/v) followed by chloroform/methanol/28% ammonium hydroxide/water (130:70:6:4 v/v/v/v) in the second dimension (Thomas et al., 1983). The dried plates were exposed to iodine vapor to stain the phospholipids, and the area that corresponded to standard PI (R_f of 0.4 and 0.8 in the first and second dimensions, respectively) was scraped into a glass minivial, mixed with 0.2 mL of water and 4 mL of toluene [containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis(5-phenyloxazol-2-yl)benzene]/Triton X-100 (2:1 v/v) scintillation fluid, and then quantitated for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer equipped with a

Texas Instrument data terminal. Under these assay conditions, the radioactivity in PI accounted for at least 97% of the total radioactivity. Thus, for routine measurement of [^{3}H]PI formation, an aliquot of the chloroform phase was evaporated to dryness, mixed with scintillation fluid, and directly counted for radioactivity. With up to 25 μg of microsomal protein, PI formation proceeded at a linear rate for at least 10 min. In all assays, conversion of the limiting substrate (CDP-diacylglycerol) to product was less than 10%.

Alternatively, PI synthase activity was determined by quantitation of [5-^{3}H]cytidine 5'-monophosphate release from [5-^{3}H]cytidine(5')diphospho-1,2-diacylglycerol. [^{3}H]CDP-diacylglycerol was incubated with the enzyme sample under conditions identical with those for the assay of PI formation, and [^{3}H]CMP release was determined essentially as described by Raetz et al. (1976). PI synthase activity in the reverse direction was determined by assay of [^{3}H]CDP-diacylglycerol formation from PI and [^{3}H]CMP, essentially as described by Hokin-Neaverson et al. (1977).

For assay of PI synthase activity in OG-solubilized membranes, the standard incubation procedure was modified to contain a final concentration of 20 mM OG. $MnCl_2$ was added prior to the 5-min preincubation, and the reaction was then initiated by the addition of *myo*-[2-^{3}H]inositol. All other conditions were identical with those outlined under the standard assay procedure.

Enzyme activities are expressed as the means of duplicate determinations that were in close agreement. One unit of PI synthase activity is defined as that amount of enzyme that catalyzes the formation of 1 nmol of PI/min under the standard conditions.

Other Procedures. Protein concentration was determined either by the method of Lowry et al. (1951) or, where appropriate, by a modification by Peterson (1977), which is applicable to solutions that contain detergents. Cytidine concentration was determined by its optical density at 280 nm in methanol that contained 0.1 N HCl. SDS–polyacrylamide gel electrophoresis was as described by Laemmli (1975).

Results

Characterization of Phosphatidylinositol Synthase Activity in Dog Pancreas Microsomal Membranes. Microsomal membranes isolated from dog pancreas exhibit considerable PI synthase activity. Under the standard assay conditions, the specific activity of PI synthase in these membranes averaged 30 nmol of PI formed min^{-1} (mg of protein)$^{-1}$. A similar specific activity was obtained when [5-^{3}H]CMP release from [5-^{3}H]cytidine(5')diphospho-1,2-diacylglycerol was measured. No product formation was detected in the absence of added substrates or metal ion cofactors. A Lineweaver–Burk plot of enzyme velocity vs. *myo*-inositiol concentration was linear with a K_m for *myo*-inositol of 0.76 mM. Lineweaver–Burk analysis of reaction velocity vs. CDP-diacylglycerol concentration resulted in a hyperbolic curve (Figure 1). The apparent K_m for CDP-diacylglycerol (fatty acid composition of egg phosphatidylcholine) was estimated to be 18 μM by inspection of the velocity vs. substrate concentration plot. Although such non-Michaelis–Menton kinetics can be indicative of cooperative ligand interactions, the hyperbolic nature of the double-reciprocal plot for CDP-diacylglycerol may be best explained by the high membrane solubility of the liponucleotide, such that the enzyme may be exposed to higher local CDP-diacylglycerol concentrations than indicated. The apparent K_a values for activation by Mn^{2+} and Mg^{2+} were 42 μM and 2.5 mM, respectively, as determined by Lineweaver–Burk analysis of reaction velocity vs. metal ion concentration. The maximum velocities obtained with saturating concentrations of either divalent cation were similar. Replacement of Mn^{2+} or Mg^{2+} with 1 mM Co^{2+} gave approximately 10% of maximal activity. Zn^{2+}, Ni^{2+}, Cu^{2+}, Ca^{2+}, and Fe^{2+}, at 1 mM concentrations, did not activate the enzyme.

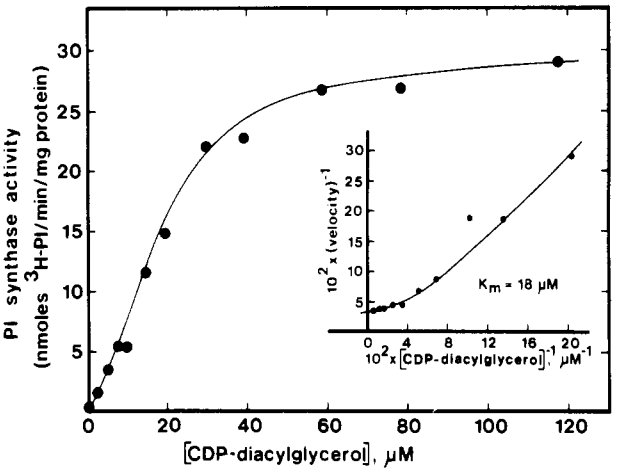

FIGURE 1: CDP-diacylglycerol concentration curve for phosphatidylinositol synthase in dog pancreas microsomal membranes. PI formation was determined at various CDP-diacylglycerol concentrations as described under Experimental Procedures. (Inset) Lineweaver–Burke analysis of enzyme velocity vs. CDP-diacylglycerol concentration.

PI synthase in dog pancreas microsomes was also active in the reverse direction, as indicated by the formation of CDP-diacylglycerol from PI and [5-^{3}H]CMP (data not shown). CDP-diacylglycerol formation was stimulated by Mn^{2+} and inhibited by *myo*-inositol and CDP-diacylglycerol, which are the products of the reaction. The pH optima in the forward and reverse directions were 8.5–9.0 and 6.5–7.0, respectively.

n-Octyl Glucopyranoside Solubilization of Dog Pancreas Phosphatidylinositol Synthase. Treatment of dog pancreas microsomal membranes with 40 mM *n*-octyl glucopyranoside (OG), a concentration used to solubilize a variety of membrane proteins (Stubbs et al., 1976), resulted in a 95–100% recovery of PI synthase activity. Centrifugation of the OG-treated microsomes indicated that virtually all of the enzyme activity was present in the 105000*g* 1-h supernatant. Solubilization of PI synthase activity started to occur at an OG concentration of 20 mM (Figure 2A). This corresponds closely to the cmc of OG, which is approximately 22 mM (Jackson et al., 1982). Solubilization of PI synthase activity by increasing concentrations of OG was accompanied by a corresponding loss of enzyme activity from the 105000*g* membrane pellet. With 40 mM OG, there was complete solubilization of the enzyme, in nearly quantitative yield. Since OG causes an apparent activation of the enzyme (see below), all PI synthase activities were determined in the presence of the same OG concentration (20 mM).

Solubilization of PI synthase by OG was dependent on the ratio of detergent to microsomal membrane (Figure 2B). At 40 mM OG, a ratio of less than 1 mg of OG/mg of microsomal protein resulted in very little solubilization of the enzyme, but as the ratio of OG to microsomal protein was increased, there was an increase in the amount of enzyme activity in the supernatant and a corresponding decrease in the amount of enzyme activity in the 105000*g* membrane pellet. Optimal solubilization of the enzyme occurred with 40 mM OG, at an OG to microsomal protein ratio (w/w) of 4–6 to 1.

A variety of other detergents were examined for their effectiveness in the solubilization of PI synthase from dog pancreas microsomal membranes and were all found to be less

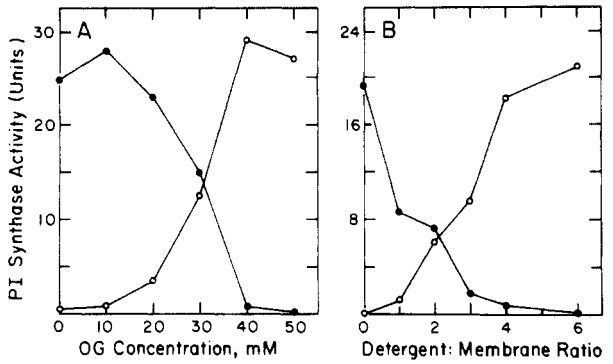

FIGURE 2: *n*-Octyl glucopyranoside solubilization of phosphatidylinositol synthase. (A) Solubilization of PI synthase with increasing OG concentrations. Dog pancreas microsomal membranes were treated with various concentrations of OG, at 4 mg of OG/mg of microsomal protein, as described under Experimental Procedures. PI synthase activity was measured in the OG supernatants (○) and pellets (●) after centrifugation at 105000*g*. (B) OG solubilization of PI synthase with increasing detergent to membrane ratios. Microsomal membranes were treated with 40 mM OG, at various ratios of OG to microsomal protein (mg of OG/mg of protein), as described under Experimental Procedures. After centrifugation at 105000*g*, PI synthase activity was determined in the OG supernatants (○) and pellets (●). All activities were determined in the presence of a final concentration of OG of 20 mM.

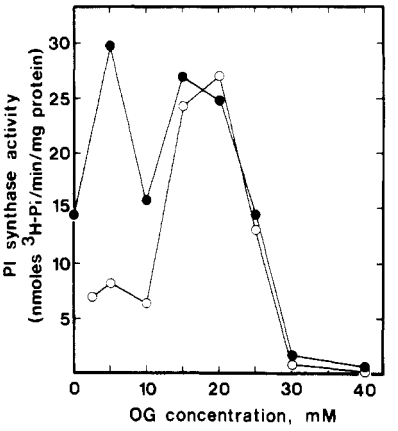

FIGURE 3: Phosphatidylinositol synthase activity in the presence of varying *n*-octyl glucopyranoside concentrations. Dog pancreas microsomal membranes were treated with a solubilization buffer in either the absence or the presence of 40 mM OG, at 4 mg of OG/mg of microsomal protein, as described under Experimental Procedures. After centrifugation at 105000*g* for 1 h, PI synthase activity was measured in the intact microsomal membranes (●) and OG-solubilized microsomes (○) in the presence of the indicated OG concentrations.

effective than OG. Treatment of microsomal membranes with sodium deoxycholate, digitonin, Triton X-100, Triton X-114, Tergitol NP-10, Tergitol NP-27, Tergitol NP-40, poly(oxyethylene) 10-lauryl ether poly(oxyethylene) 10-cetyl ether, or poly(oxyethylene) 20-stearyl ether [at 1% (w/v) concentrations] resulted in large losses (70–100%) of PI synthase activity. In the presence of 1% Triton X-100, 21% of the enzyme activity remained after 1 h at 4 °C, but prolonged exposure of the enzyme to this detergent resulted in a gradual loss of the remaining activity. Sodium cholate (1%) partially solubilized the enzyme, but the yield of soluble activity was always less than 30%.

Effect of n-Octyl Glucopyranoside on the Activity of Phosphatidylinositol Synthase. It is known that a number of enzymes become activated in the presence of some detergents (Raetz & Kennedy, 1974; Hirabayashi et al., 1974). It was therefore of interest to investigate the effect of OG on PI synthase activity and to determine if the high yield of OG-solubilized PI synthase might be a result of enzyme activation in the presence of this detergent. When PI synthase activity in intact microsomes was measured in the presence of various OG concentrations, enzyme activity increased 77 ± 24% (n = 3) at 5 mM OG and increased 78 ± 8% (n = 2) and 63 ± 9% (n = 2) at 15 and 20 mM OG, respectively. In the presence of 10 mM OG, enzyme activity decreased 23 ± 16% (n = 3). In comparison, when an identical quantity of PI synthase activity was first solubilized with 40 mM OG and then subsequently assayed in the presence of various OG concentrations, the rate of product formation was nearly identical with that in intact microsomes at 15 mM OG and above (Figure 3). Assuming that OG activates both the microsomal and OG-solubilized enzyme preparations to the same extent, these results suggest that OG solubilization of the enzyme occurred with nearly quantitative yield.

When the OG-solubilized PI synthase was diluted to below 15 mM OG, the amount of enzyme activity was less than that in intact microsomes with equivalent OG concentrations (Figure 3). This partial loss of enzyme activity may be due to suboptimal conditions for reconstitution of the solubilized enzyme into lipid vesicles since, under these conditions, no additional phospholipid was added to the OG-solubilized enzyme for vesicle formation during dilution of the OG to below its cmc.

The apparent activation of the enzyme at 15–20 mM OG may be due to an increase in substrate (CDP-diacylglycerol) accessibility to the enzyme as a result of increased fluidization of the membrane. Jackson et al. (1982) have shown that OG (at 16–22 mM) produces an increased fluidization of phosphatidylcholine vesicles as measured by fluorescence anisotropy with diphenylhexatriene. Since maximal enzyme activity was obtained in the presence of 20 mM OG, this final concentration of detergent was included in all assays of OG-treated enzyme.

In both the intact and OG-solubilized microsomes, PI synthase activity decreased when assayed in the presence of OG concentrations above its cmc (Figure 3). However, this decrease in activity was readily reversible since dilution of the 40 mM OG-solubilized enzyme to below 25 mM resulted in a complete recovery of enzyme activity. This decrease in activity in the presence of high levels of OG is presumably due to the formation of OG micelles, which might effectively reduce the amount of liponucleotide substrate available to the enzyme under these assay conditions.

As shown in Figure 3, in the absence of added OG, PI synthase in microsomal membranes exhibited a specific activity of approximately 15 nmol of PI formed min^{-1} (mg of protein)$^{-1}$, which is lower than the specific activity of 30 observed for the enzyme in intact microsomes (See Figure 1). This apparent decrease in enzyme activity is presumably a result of treatment of these membranes with solubilization buffer and subsequent centrifugation procedures prior to assay of enzyme activity.

Manganese Requirement for Stabilization of n-Octyl Glucopyranoside Solubilized Phosphatidylinositol Synthase. In finding conditions in which the OG-solubilized PI synthase was most stable, it was observed that recovery of enzyme activity was dependent on the presence of manganese ion in the solubilization medium. When dog pancreas microsomal membranes were pretreated with various concentrations of OG, in the presence of Mn^{2+}, there was no loss of enzyme activity with OG concentrations up to 50 mM. However, pretreatment of the membranes with OG in the absence of Mn^{2+} resulted in a drastic loss of enzyme activity when the OG concentration was increased to 20 mM and higher (Figure 4). The loss of activity in the absence of Mn^{2+} appeared to be irreversible since

Table I: Partial Purification of Phosphatidylinositol Synthase from Dog Pancreas Microsomal Membranes[a]

enzyme fraction	vol (mL)	protein (mg)	tot act. (units)	sp act. (units/mg)	yield (%)	x-fold purification factor
microsomes	1.0	3.8	112	29	100	1.0
15 mM OG-washed microsomes	1.2	3.0	96	32	86	1.1
40 mM OG supernatant	2.0	1.1	124	113	110	3.8
DEAE-cellulose (40–150 mM KCl)	2.0	0.38	110	290	98	10

[a] Microsomal membranes were suspended in buffer A (10 mM Tris-HCl, pH 8.4 at 4 °C, 0.5 mM DTT, 2.0 mM $MnCl_2$, 40 mM KCl, and 15 mM OG), and after 20 min at 4 °C, the membranes were centrifuged at 105000*g* for 1 h. The resulting pellet was resuspended in buffer A, except it contained 40 mM OG (at 4 mg of OG/mg of protein), and then recentrifuged at 105000*g* for 1 h. The supernatant was collected and PI (porcine liver) was added to give a final concentration of 0.5 mM. The solubilized enzyme was then added batchwise to DEAE-cellulose (Whatman DE-52, 0.15 g/mL of sample), which was equilibrated with buffer A, which also contained 40 mM OG, 0.5 mM PI, and 5 μg of egg yolk phospholipid–P_i/mL. After 30 min at 4 °C, the resin was rapidly filtered, and the enzyme was released by resuspending the resin in the same equilibration buffer, except it contained 150 mM KCl. Enzyme activity in all fractions was determined in the presence of 20 mM OG.

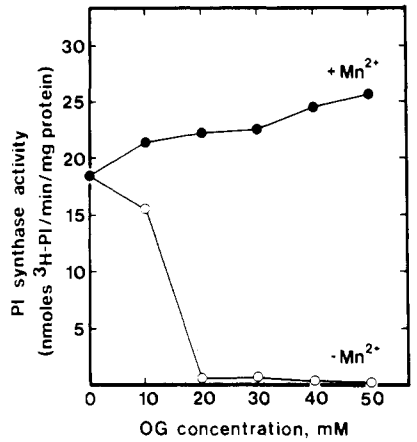

FIGURE 4: Mn^{2+} stabilization of phosphatidylinositol synthase activity in the presence of *n*-octyl glucopyranoside. Dog pancreas microsomal membranes were treated with various concentrations of OG, at 4 mg of OG/mg of microsomal protein, in the absence or presence of 1.0 mM $MnCl_2$. After 1 h at 4 °C, PI synthase activity was measured in the with Mn^{2+} (●) and without Mn^{2+} (○) samples. The enzyme assay mixture contained final concentrations of Mn^{2+} and OG of 1.0 and 20 mM, respectively.

there was no recovery of enzyme activity when the OG-treated membranes were subsequently assayed in the presence of 1.0 mM Mn^{2+}. The loss of enzyme activity occurred at essentially the same OG concentration that results in solubilization of the enzyme, which suggests that Mn^{2+} plays a role in stabilization of the enzyme during OG solubilization.

To determine the specificity of Mn^{2+} in the stabilization of OG-solubilized PI synthase, various other metal ions were examined for their ability to replace Mn^{2+}. Treatment of microsomal membranes with 40 mM OG under conditions identical with those in Figure 4, indicated that Ca^{2+} could partially replace Mn^{2+}, with 1 mM Ca^{2+} resulting in 9% of the amount of stabilization afforded by 1 mM Mn^{2+}. Mg^{2+}, at concentrations up to 10 mM, resulted in only 5% stabilization. All other metal ions examined, which included Co^{2+}, Fe^{2+}, Fe^{3+}, Ag^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Al^{3+}, and Li^+ at 1 mM concentrations, resulted in less than 4% stabilization. The concentration of Mn^{2+} required to give half-maximal stabilization of the OG-solubilized enzyme was approximately 40 μM, which is similar to the concentration of Mn^{2+} (42 μM) that results in half-maximal enzyme activation. Maximal stabilization occurred with Mn^{2+} concentrations of 0.1 mM and higher.

Partial Purification and Stability of Phosphatidylinositol Synthase Solubilized with n-Octyl Glucopyranoside. Extraction of dog pancreas microsomal membranes with 40 mM OG resulted in an apparent 3.8-fold enrichment in PI synthase activity (Table I). Since low OG concentrations can permeabilize membranes (Dean & Blankenship, 1983), the extraction procedure for the purification shown in Table I included washing the membranes with 15 mM OG prior to solubilization to facilitate the removal of soluble degradative enzymes that may have been trapped within the intravesicular spaces of the microsomes during the initial homogenization of the pancreas tissue. This washing procedure removed 21% of the total microsomal protein and nearly doubled the stability of the preparation. When microsomes were first washed and then solubilized with OG, as described in Table I, the OG-solubilized enzyme was stable during storage at 4 °C; 97% of the enzyme activity remained after 1 day, and 67% remained after 3 days. The stability of the solubilized enzyme was approximately the same as in intact microsomal membranes and is sufficient to allow further purification of the enzyme in the presence of this detergent.

The OG-solubilized PI synthase was subjected to further purification by ion-exchange chromatography on DEAE-cellulose. In a batch procedure, the enzyme was absorbed to DEAE-cellulose at low ionic strength and released with a 40–150 mM KCl step gradient. A mixture of PI (0.5 mM) and crude egg yolk phospholipids (5 μg of phospholipid–P_i/mL) stabilized the enzyme during the chromatography step. This procedure resulted in an overall purification of the enzyme of 10-fold from the microsomal fraction, in 98% yield, with a specific activity of 290 nmol of PI formed min^{-1} (mg of protein)$^{-1}$. SDS–polyacrylamide gel electrophoresis (7–14% gradient gel) of the active fraction from the ion-exchange resin indicated the presence of two major protein bands with M_r's of approximately 30 000 and 60 000, with a number of minor protein bands also present.

Discussion

We have used dog pancreas microsomes as a source of PI synthase for several reasons. These include our overall interest in PI metabolism in the mammalian pancreas, the availability of large quantities of fresh pancreas tissue, and the finding that the specific activity of the enzyme in dog pancreas microsomes [approximately 30 nmol of PI formed min^{-1} (mg of protein)$^{-1}$] is among the highest of the values reported for other tissues, so that these microsomes represent a relatively enriched source of enzyme. Also, since the endoplasmic reticulum is the major membrane system of the pancreas acinar cell, there is a high total content of enzyme per cell.

The general characteristics of PI synthase activity in dog pancreas microsomes are similar to those reported in microsomes from guinea pig pancreas (Prottey & Hawthorne, 1967), although the dog pancreas enzyme exhibits a pH optimum of 8.5–9.0 as compared to 7.5 in guinea pig pancreas. The K_m for *myo*-inositol of 0.76 mM corresponds closely to the concentration of free *myo*-inositol of 0.7 mM (0.7 μmol/g of

tissue) in mouse pancreas tissue (Hokin-Neaverson et al., 1975). Previously reported K_m values for CDP-diacylglycerol range from 1.8 mM in rat brain (Bishop & Stickland, 1970) to 40 μM in rabbit mammary tissue (Wooton & Kinsella, 1977). The apparent K_m for CDP-diacylglycerol of 18 μM in dog pancreas microsomes is lower than this range, but it is unknown how accurately this K_m reflects the actual in vivo concentration of CDP-diacylglycerol exposed to the enzyme within the microsomal membrane. The effect of CDP-diacylglycerol concentration on enzyme velocity did not fit Michaelis–Menton kinetics as indicated by the hyperbolic double-reciprocal plot of substrate concentration vs. enzyme velocity. A similar non-Michaelis–Menton plot for CDP-diacylglycerol has been observed with *Escherichia coli.* phosphatidylserine synthetase in the presence of Triton X-100 (Larson & Dowhan, 1976). PI synthase activity in the reverse direction appears to be similar to that described in microsomes from mouse pancreas (Hokin-Neaverson et al., 1977) and rabbit lung (Bleasdale et al., 1979).

The selection of a detergent for the solubilization of a membrane-bound enzyme is generally based on the detergents ability to preserve enzymatic activity. Of a variety of detergents examined for the solubilization of PI synthase from dog pancreas microsomes, OG is the most effective detergent since solubilization with OG results in a very high recovery of enzyme activity. Solubilization of the enzyme from rat brain or rat liver by a variety of detergents, which included a number of nonionic poly(oxyethylene) derivatives such as Cutscum, Miranol H2M, Nonidet P-40, Lubrol W, and Tween 20 and other ionic detergents such as sodium deoxycholate, sodium dodecyl sulfate, and cetyltrimethylammonium bromide, resulted in large losses of enzyme activity (Rao & Strickland, 1974; Takanawa & Egawa, 1977). The properties of OG that afford preservation of enzyme activity are unknown, but presumably, the structure of this detergent facilitates solubilization of the enzyme while also maintaining various structural characteristics of the enzyme that are essential for activity. A major difference between OG and the nonionic alkyl poly(oxyethylene) type detergents described here is in the length of their respective hydrocarbon chains. The C_8-alkyl chain of OG is the shortest of the detergents examined, and this structural characteristic may play an important role in the efficacy of this detergent in the solubilization of PI synthase.

We have shown here that treatment of dog pancreas microsomal membranes with OG, in the presence of micromolar concentrations of Mn^{2+}, results in solubilization of PI synthase in nearly quantitative yield and in relatively stable form. In addition to the effectiveness of OG in the solubilization of PI synthase, a major advantage of OG over other nonionic detergents is that it can be rapidly removed by dialysis (Helenius et al., 1979). This property of OG has resulted in its use in the reconstitution of several membrane proteins into liposomes (Mimms et al., 1981; Jackson & Litman, 1982), and it may be possible to use OG to reconstitute purified PI synthase into lipid vesicles for further characterization of its properties.

It was of interest to note the dramatic stabilization of the OG-solubilized enzyme by micromolar concentrations of manganese. The finding that Mg^{2+} was unable to replace Mn^{2+} in stabilization of the enzyme, even though Mg^{2+} can readily substitute for Mn^{2+} as an essential cofactor for enzymatic activity, suggests that the role of Mn^{2+} in stabilization may be different from the role Mn^{2+} plays in enzyme catalysis. Although the mechanism for Mn^{2+} stabilization has not been investigated, it is possible that Mn^{2+} might interact with a secondary site (e.g., structural or regulatory) on the enzyme, which results in stabilization during OG solubilization. Alternatively, Mn^{2+} might act to inhibit enzymes that inactivate PI synthase upon OG solubilization. The role of Mn^{2+} in stabilization of the enzyme could be readily investigated with the availability of a highly purified enzyme preparation.

The relatively high stability of the OG-solubilized PI synthase is sufficient to facilitate purification of the enzyme in the presence of this detergent. We have purified the enzyme 10-fold from dog pancreas microsomal membranes by detergent extraction and DEAE-cellulose chromatography. Although this partially purified preparation was clearly not homogeneous, as determined by SDS–polyacrylamide gel electrophoresis, it had a specific activity [expressed as nmol of PI formed min^{-1} (mg of protein)$^{-1}$] of 290, which is much higher that the specific activity of 11.3 reported for the enzyme purified from rat liver (Takenawa & Egawa, 1977) and is comparable to the specific activity of 270 reported for the enzyme purified from rat brain (Eichberg et al., 1983). We are currently engaged in further purification of the dog pancreas enzyme by affinity chromatography on CDP-diacylglycerol-derivatized Sepharose.

Currently, little is known about the properties of the membrane-associated enzymes of inositol–phospholipid metabolism, including PI synthase. An increased understanding of the parameters necessary to maintain the integrity of these membrane enzymes in the presence of detergents should facilitate their purification and characterization, which may aid in elucidating possible modes of regulation of inositol–phospholipid metabolism.

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Registry No. OG, 29836-26-8; Mn, 7439-96-5; Mg, 7439-95-4; *myo*-inositol, 87-89-8; phosphatidylinositol synthase, 9027-01-4.

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Local Structural Changes in Tropomyosin Detected by a Trypsin-Probe Method†

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ABSTRACT: Structural changes in tropomyosin from rabbit skeletal muscle were studied by the tryptic digestion method, which is an application of the quantitative enzyme-probe method recently developed by Ueno and Harrington [Ueno, H., & Harrington, W. F. (1984) *J. Mol. Biol. 173*, 35–61]. Effects of ionic strength, temperature, and an interchain disulfide bond at Cys-190 on the structure of tropomyosin were examined. A region of high susceptibility to trypsin was found to be localized in the middle portion of the molecule, and its susceptibility increased on lowering ionic strength and/or raising temperature. With the introduction of a disulfide bond at Cys-190, cleavage on the N-terminal side of Cys-190 was accelerated. The results suggest that skeletal muscle tropomyosin is flexible in the middle of the molecule in contrast to the flanking N- and C-terminal trypsin-resistant segments.

In a recent study (Ueno & Harrington, 1984), proteolytic enzymes were used to quantitatively probe local conformational changes within the α-helical region of the myosin rod. In this enzyme-probe method, the kinetic analysis of proteolytic digestion and the sites of cleavage in the myosin rod were determined by following the time course of fragmentation on SDS[1]-containing gels. Since the method can be applied to any other system as well and is particularly powerful to detect local conformational changes, it was extended in this study to probe local conformational changes in tropomyosin.

Tropomyosin plays an important role in the regulation of vertebrate skeletal muscle together with troponin (Ebashi & Endo, 1968), as well as in various cell motile systems. Rabbit skeletal muscle tropomyosin is an α-helical coiled-coil molecule composed of 284 amino acid residues per polypeptide chain (Stone & Smillie, 1978; Sodek et al., 1978). Although an exact regulatory mechanism of this protein is still in controversy, it is likely that some conformational changes in tropo-

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[1] Abbreviations: TM, rabbit skeletal α-tropomyosin; TM_{SH}^{SH} or reduced TM, TM without an interchain disulfide bond; TM_{S}^{S} or oxidized TM, TM with an interchain disulfide bond at Cys-190; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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