Purification and characterization of phosphatidylinositol synthase from human placenta

Bruno E. ANTONSSON
Glaxo Institute for Molecular Biology, 14 chemin des Aulx, CH-1228 Plan-les-Ouates/Geneva, Switzerland

Phosphatidylinositol synthase (CDP-1,2-diacyl-*sn*-glycerol:*myo*-inositol 3-phosphatidyltransferase, EC 2.7.8.11) was purified from the microsomal fraction of human placenta. The Triton X-100-extracted enzyme was purified 8300-fold over the microsomal fraction by affinity chromatography on CDP-diacylglycerol–Sepharose followed by ion-exchange chromatography on Mono Q. The purified enzyme had a molecular mass of 24000 Da on SDS/PAGE. The enzyme had a pH optimum at 9.0, required Mn^{2+} or Mg^{2+}, and was inhibited by Ca^{2+} and Zn^{2+}. The K_m for *myo*-inositol was determined to be 0.28 mM. Optimal activity was obtained at 0.2–0.4 mM CDP-diacylglycerol; higher concentrations of the lipid substrate inhibited the enzyme reaction. The enzyme was inhibited by nucleoside di- and tri-phosphates, P_i and PP_i. CDP competitively inhibited the enzyme reaction with a K_{is} of 4 mM. The optimal temperature for the PtdIns synthase reaction was 50 °C.

INTRODUCTION

Phosphatidylinositol (PtdIns) is an essential phospholipid in cell membranes [1]. A key enzyme in its biosynthesis is PtdIns synthase (EC 2.7.8.11). This enzyme catalyses the final reaction step whereby the addition of *myo*-inositol to CDP-diacylglycerol results in the formation of PtdIns and CMP [2]. Besides being a component of the cell membrane, PtdIns plays an important role in protein membrane anchoring as well as being a precursor to polyphosphoinositides and diacylglycerol (DAG). A wide range of membrane-associated proteins, including enzymes, coat proteins and adhesion molecules, are attached to cell membranes via glycosyl-PtdIns anchors (for reviews, see [3,4]). A derivative of PtdIns, its 4,5-bisphosphate, is hydrolysed to InsP_3 and DAG in response to agonist stimulation of various hormone and neurotransmitter receptors (for reviews, see [5,6]). These second messengers are involved in regulation of cell metabolism, contraction, secretion and proliferation.

PtdIns synthase is a membrane-bound enzyme. In mammalian tissues it is predominantly located in the endoplasmic reticulum and the Golgi [7,8]. PtdIns synthase activity has also been found in the plasma membrane of rat pituitary tumour cells (GH_3) [9]. This might indicate the existence of two different PtdIns synthases, since the plasma-membrane enzyme shows different kinetic characteristics compared with the endoplasmic-reticulum enzyme [10].

Despite the increasing interest in PtdIns derivatives, little is known about the enzymes involved in their biosynthesis. PtdIns synthase from *Saccharomyces cerevisiae* has been purified and characterized [11,12]. The gene has been cloned, and *S. cerevisiae* PtdIns synthase has been expressed in *Escherichia coli* [13,14]. The cDNA clone codes for a protein with 220 amino acids and a molecular mass of 24823 Da. From mammalian sources, the enzyme has been partially purified from dog pancreas [15] and rat brain [16]. In the present study PtdIns synthase from human placenta has been purified to near homogeneity, as judged by silver-stained SDS/PAGE, and the enzyme has been characterized.

MATERIALS AND METHODS

Materials

CDP-DAG (derived from egg lecithin), deoxy-CDP-DAG (derived from egg lecithin) and 1,2-DAG were purchased from Serdary Research Laboratories, London, Ontario, Canada. CNBr-activated Sepharose 4B, Sephadex G-25 and the Mono Q column were from Pharmacia, Uppsala, Sweden. *myo*-[^{3}H]-Inositol was from New England Nuclear. Adipic acid dihydrazide, *myo*-inositol, nucleotides and phosphatidylcholine were from Sigma. Triton X-100 was purchased from Boehringer-Mannheim. All other chemicals were reagent grade or better.

Human placentae were kindly provided by Professor F. Beguin, University Hospital, Geneva.

Preparation of CDP-DAG–Sepharose affinity resin

CDP-DAG was covalently attached to Sepharose 4B as described by Larson et al. [17], with the following modifications. CNBr-activated Sepharose 4B was incubated with adipic acid dihydrazide at 4 °C for 50 h. The CDP-DAG was oxidized with $NaIO_4$ for 50 h at 4 °C, and subsequently coupled to the adipic acid–Sepharose by incubation with rotation at 4 °C for 4 days. The extended incubation periods increased the binding capacity of the affinity resin. At the end of the incubation the resin was extensively washed and stored in 10 mM NaN_3 at 4 °C.

PtdIns synthase assay

PtdIns synthase was assayed essentially as previously described [18]. A standard assay mixture contained 100 mM glycylglycine/NaOH, 0.2 mM CDP-DAG, 2 mM Triton X-100, 5 mM *myo*-[^{3}H]inositol (5000 c.p.m./nmol), 2 mM $MnCl_2$, 50 mM $MgCl_2$ and enzyme extract in a total volume of 100 μl at pH 9.0 (37 °C). After 10 min incubation at 37 °C, the reaction was stopped by addition of 2 ml of chloroform/methanol/10 M HCl (200:100:1, by vol.). After phase separation, 1 ml of the chloroform phase was dried and counted for radioactivity to determine the in-

Abbreviations used: DAG, diacylglycerol; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; K_{is}, competitive inhibition constant.

corporation of *myo*-[^{3}H]inositol into PtdIns. An enzyme unit is defined as the amount of enzyme that catalyses the formation of 1 nmol of PtdIns in 1 min.

PtdIns synthase purification

Human placentae were placed at 4 °C as soon as possible, cut into small pieces and frozen in liquid N_2. The tissue was kept at −80 °C until further processing. All subsequent manipulations were carried out at 4 °C unless stated otherwise.

Preparation of crude extract

The frozen placental tissue was mixed with 2 vol. of buffer A [50 mM Tris/HCl, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM phenylmethanesulphonyl fluoride (PMSF), pH 7.5] in a Waring blender. The mixture was further homogenized in a Potter–Elvehjem homogenizer and subsequently centrifuged at 16000 *g* for 10 min. The supernatant was collected and stored at 4 °C, and the pellet was rehomogenized and centrifuged as before. The supernatant was combined with that from the first homogenization, and the whole was centrifuged at 100000 *g* for 1.5 h. The microsomal pellet was resuspended in buffer B (50 mM Tris/HCl, 20% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, pH 7.5), centrifuged (100000 *g* for 1.5 h) and the washed pellet was subsequently used for detergent extraction.

Detergent extraction

The washed pellet was suspended in buffer C [20 mM Tris/HCl, 5% glycerol, 0.5 mM dithiothreitol (DTT), 10 μM PMSF, pH 8.5] at a protein concentration of 10 mg/ml. To the sample were added 1 mM $MnCl_2$, 30 mM $MgCl_2$ and 0.9% (w/v) Triton X-100. After incubation for 1 h at 4 °C, the sample was centrifuged at 150000 *g* for 1.5 h. The solubilized extract containing PtdIns synthase was used directly for further purification, or frozen in liquid N_2 and stored at −80 °C.

Affinity-column chromatography

A column of CDP-DAG–Sepharose (2.5 cm × 2.0 cm) was equilibrated in buffer D (50 mM Tris/HCl, 10% glycerol, 0.5 mM DTT, 20 mM $MgCl_2$, 0.2 M NaCl, 0.5% Triton X-100, 0.3 mg/ml phosphatidylcholine, 0.2 mg/ml 1,2-DAG, pH 8.5). The lipids were dried under a stream of N_2 and subsequently incorporated into the buffer by sonication for 4 × 30 s. A 60 ml portion of Triton extract containing 2300 units of PtdIns synthase was applied to the column (0.5 ml/min). The column was washed with 150 ml of buffer E (50 mM Tris/HCl, 10% glycerol, 0.5 mM DTT, 20 mM $MgCl_2$, 1.3 M NaCl, 0.5% Triton X-100, 0.2 mg/ml phosphatidylcholine, 0.1 mg/ml 1,2-DAG, pH 7.5) at a flow rate of 1 ml/min. Bound PtdIns synthase was eluted with 60 ml of buffer E containing 1 mM CDP-DAG at 0.5 ml/min. Fractions containing PtdIns synthase activity were pooled, frozen in liquid N_2 and stored at −80 °C.

Anion-exchange chromatography

The pool from the affinity column was desalted into buffer F (20 mM glycylglycine/NaOH, 5% glycerol, 0.1 mM DTT, 0.1% Triton X-100, 0.3 mg/ml phosphatidylcholine, pH 8.9, and conductivity 1.00 mS, adjusted with 0.1 M NaCl, at 23 °C) on a Sephadex G-25 column before the sample was submitted to anion-exchange chromatography on a f.p.l.c. Mono Q HR 5/5 column equilibrated in buffer F. After sample application the column was washed with 6 ml of buffer F, developed with a 6 ml linear gradient of 0–1 M NaCl in buffer F, and finally washed with buffer F containing 1 M NaCl. PtdIns synthase activity was recovered in the flow-through fractions and in the middle of the gradient. The flow-through fractions containing PtdIns synthase were saved. The PtdIns synthase eluted with the gradient was not pure and, after desalting into buffer F, was resubmitted to chromatography on the Mono Q column under the same conditions as described above. The flow-through fractions from the two runs containing PtdIns synthase activity were pooled, concentrated using an Amicon 8010 concentrator with a PM10 membrane, frozen in liquid N_2 and stored at −80 °C.

Protein determination

Protein concentrations were determined by the modified Lowry method [19]. The proteins were precipitated with deoxycholate and trichloroacetic acid to minimize interference by the detergent [20]. Purified PtdIns synthase was quantified on silver-stained SDS/PAGE, with Bio-Rad SDS/PAGE molecular-mass marker mixture as standard [21].

SDS/PAGE

PAGE was performed in the PhastSystem from Pharmacia. The samples were analysed on 10–15% gradient gels in a SDS buffer system.

RESULTS

Purification of PtdIns synthase

PtdIns synthase is a membrane enzyme which can be extracted by detergent from the cell microsomal fraction in the presence of $MgCl_2$. When 0.9% Triton X-100 and 30 mM $MgCl_2$ were used, 40–60% of the microsomal activity was recovered in the detergent extract, with a 1.5-fold increase in the specific activity. The presence of a low concentration of $MnCl_2$ in the solubilization buffer stabilized the extracted enzyme. Triton X-100 concentrations greater than 1.0% (1 mg of Triton/mg of protein) resulted in severe losses of activity.

The Triton extract (60 ml) was submitted to affinity chromatography on a CDP-DAG–Sepharose 4B column (Figure 1). Binding of PtdIns synthase to the affinity column was dependent on the presence of both Triton X-100 and $MgCl_2$ or $MnCl_2$. In order to saturate all affinity-binding sites and obtain optimal purification, the column was overloaded. However, when only 20 ml of Triton extract was loaded on the column, over 95% of PtdIns synthase bound to the affinity resin. Elution of PtdIns synthase from the column required CDP-DAG, $MgCl_2$, Triton X-100, high salt (1.3 M NaCl) and lipids (phosphatidylcholine and DAG). The recovery of PtdIns synthase activity was 20–40%, with 150–220-fold purification. Densitometric scanning after SDS/PAGE showed that PtdIns synthase constituted 3–5% of the total protein in the sample (Figure 2).

Newly prepared column resin showed very low recovery of PtdIns synthase, even after incubation with elution buffer for 3 h at 25 or 37 °C. However, recovery of activity was greatly increased after 60 ml of Triton extract had been passed over the column 3–4 times. The affinity column could subsequently be used for 10–15 purifications, without any detectable decrease in binding capacity.

Increased enzyme recovery from the column did not result in higher purification, suggesting that the contaminating proteins

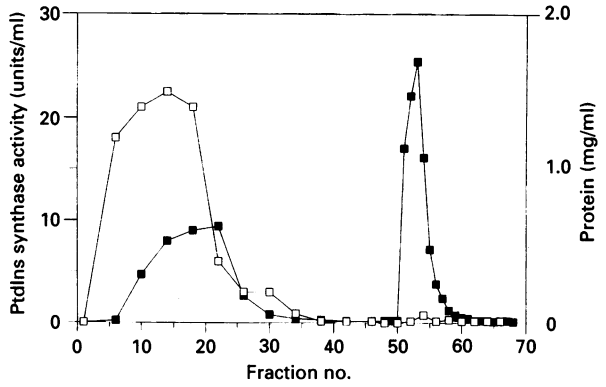

Figure 1 CDP-DAG–Sepharose affinity chromatography of Triton X-100-extracted PtdIns synthase

The sample was applied and the column was washed with buffer E. Bound PtdIns synthase was eluted with buffer E containing 1 mM CDP-DAG (applied at fraction 47); 4 ml fractions were collected. □, Protein concn. (mg/ml); ■, PtdIns synthase activity (units/ml). Fractions 51–54 were pooled, and this pool was used for the further purification.

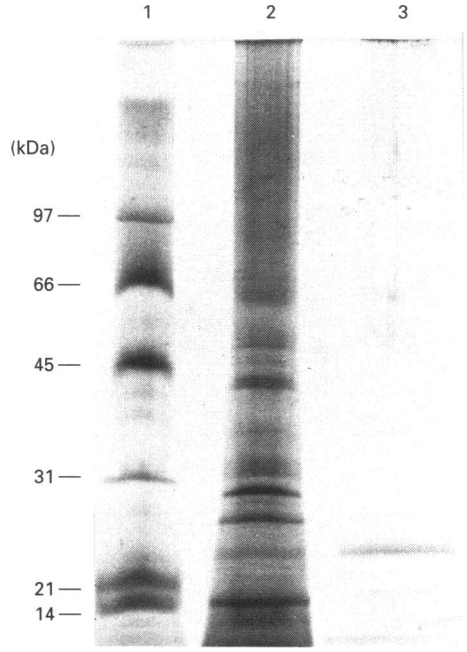

Figure 2 PtdIns synthase samples analysed on gradient 10–15% Phastgel SDS/PAGE

The samples were denatured at 95 °C for 5 min in buffer containing 2% SDS and 5 mM DTT before electrophoresis. Proteins were detected by silver staining. Lanes: 1, molecular-mass markers (kDa); 2, pool from the affinity column; 3, pool of the flow-through fractions from the Mono Q column.

were not binding directly to the affinity column, but were interacting with the bound PtdIns synthase and so were co-eluted. The existence of such interactions between PtdIns synthase and the contaminants is supported by the finding that when an affinity-purified sample was submitted to gel filtration the contaminating proteins (10000–100000 Da) co-migrated with the PtdIns synthase Triton micelles (results not shown).

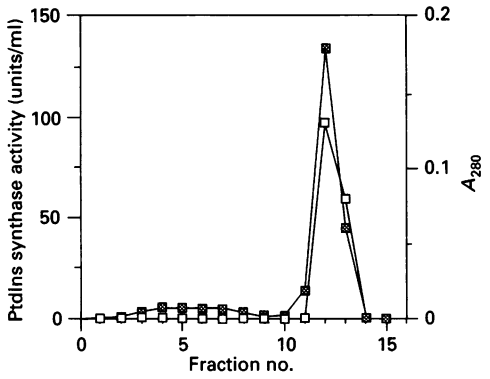

Figure 3 Ion-exchange chromatography on f.p.l.c. Mono Q of the PtdIns synthase pool from the affinity column

After the sample (14 ml) was applied, the column was washed with 6 ml of buffer F, subsequently developed with a 6 ml linear gradient of 0–1 M NaCl in buffer F, and finally washed with buffer F containing 1 M NaCl. The gradient started at fraction 11. Fractions of volume 2 ml were collected. □, A_{280}; ■, PtdIns synthase activity (units/ml). Fractions 3–8 were pooled and contained pure PtdIns synthase. Fractions 11–13 were pooled, desalted and re-chromatographed on Mono Q.

The affinity-purified sample was further purified by ion-exchange chromatography. DEAE-Sepharose, chromatofocusing PBE 94 and f.p.l.c. Mono S and Mono Q resins were tried. With DEAE-Sepharose there were large losses of activity, and no significant purification was experienced. On Mono S and chromatofocusing PBE 94 (the latter was used in the purification of PtdIns synthase from *S. cerevisiae* [11]), no activity could be recovered from the columns. On Mono Q 5–10% of the applied PtdIns synthase activity did not bind to the column and emerged with the flow-through fractions, fractions 3–8 in Figure 3. The flow-through fractions containing PtdIns synthase were saved. Of the remaining PtdIns synthase activity, some 70–80% was recovered, together with the impurities, in the middle of the 0–1 M NaCl gradient. The active fractions eluted with the gradient were desalted and re-applied to the Mono Q column under the same conditions as before. In the second run a further 5–10% of the PtdIns synthase activity was recovered in the flow-through fractions. The elution profile of the second Mono Q run was identical with the first Mono Q run, except that the PtdIns synthase activity peak eluted with the gradient was considerably smaller, only 30–40% of the applied activity. The flow-through fractions from the two Mono Q runs were pooled and contained the pure PtdIns synthase. This purification step resulted in a 20–30-fold purification and gave a specific activity of 19000–35000 units/mg for the purified PtdIns synthase. The purified sample showed a major protein band (over 90% of total protein, determined by densitometry scanning) with a molecular mass of 24000 Da on silver-stained SDS/PAGE (Figure 2). The identity of the 24000 Da protein as PtdIns synthase is supported by several lines of evidence. The protein was present in all preparations, with protein amount correlating with PtdIns synthase activity, whereas the pattern of the trace bands varied between preparations. At lanes 2 and 3 in Figure 2 the same amount of PtdIns synthase activity was loaded. The only protein present at approximately the same concentration in the two samples is the 24000 Da protein.

The ionic strength and pH of the buffer were crucial for the Mono Q purification step. When the ionic strength was decreased, all PtdIns synthase bound to the column. However, the bound PtdIns synthase could not be separated from the contaminating

Table 1 Purification of PtdIns synthase from human placenta

Summary of a preparation starting with 100 g of placenta tissue. One enzyme-activity unit corresponds to the amount of enzyme that catalyses the formation of 1 nmol of phosphatidylinositol in 1 min.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Microsomes	60	564	2340	4.1	1	100
Triton X-100 extract	56	196	1232	6.3	1.5	53
CDP-DAG–Sepharose	14	0.28	378	1350	329	16
Mono Q f.p.l.c.	25	0.002	68	34000	8290	3

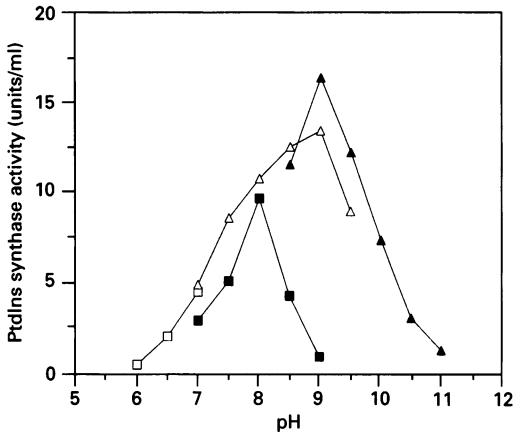

Figure 4 Effect of pH on the PtdIns synthase activity

A PtdIns synthase sample was incubated in 100 μl of assay mixture as described in the Materials and methods section, except that the following buffers (100 mM) were used: □, Bistris/HCl; ■, Tris/HCl; △, glycylglycine/NaOH; ▲, glycine/NaOH. After 10 min incubation at 37 °C, the reaction was stopped by addition of 2 ml of chloroform/methanol/10 M HCl (200:100:1, by vol). A portion of the chloroform phase was dried and counted for radioactivity in a liquid-scintillation counter. One unit corresponds to the formation of 1 nmol of PtdIns in 1 min. Each data point is the mean of duplicate determinations

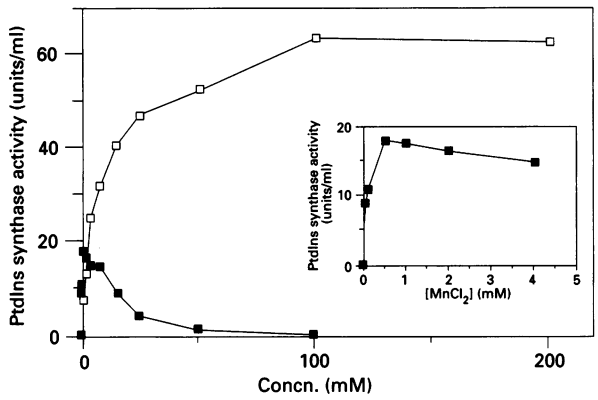

Figure 5 Effect of $MgCl_2$ and $MnCl_2$ on the PtdIns synthase activity

PtdIns synthase samples were incubated under standard assay conditions as described in the Materials and methods section in the presence of increasing concentrations of $MgCl_2$ (□) or $MnCl_2$ (■). After 10 min incubation at 37 °C, the reaction was stopped by addition of 2 ml of chloroform/methanol/10 M HCl (200:100:1, by vol.). A portion of the chloroform phase was dried and counted for radioactivity in a liquid-scintillation counter. One unit corresponds to the formation of 1 nmol of PtdIns in 1 min. Each data point is the mean of duplicate determinations. The insert shows the low concentration range for $MnCl_2$.

proteins. Conversely, the recovery of PtdIns synthase in the flow-through fractions could be increased by either decreasing pH or increasing the ionic strength. However, this resulted in co-elution of contaminating proteins. A typical purification is summarized in Table 1.

Enzyme characterization

pH-dependence

The enzyme activity was not only strongly pH-dependent, but was also affected by the buffer system used (Figure 4). In Tris/HCl the pH optimum was 8.0, whereas in glycylglycine/NaOH or glycine/NaOH buffers optimal activity was obtained at pH 9.0. The maximal activity obtained in Tris/HCl buffer was only 60% of the activity in glycylglycine/NaOH at pH 9.0. At pH 9.0 in Tris/HCl buffer the enzyme showed almost no activity.

Metal-ion requirement

PtdIns synthase had an absolute requirement for either Mn^{2+} or Mg^{2+} (Figure 5). With Mn^{2+} as the bivalent cation, optimal activity was obtained between 0.5 and 2 mM. Higher concentrations resulted in enzyme inhibition, and at 50 mM the enzyme was completely inhibited. In the presence of $MgCl_2$, enzyme activity increased with increasing $MgCl_2$ concentration, with maximal activity at 100 mM. Including 2 mM $MnCl_2$ did not affect the $MgCl_2$ activation. The maximal activity with $MgCl_2$ was approx. 4 times higher than the maximal activity obtained with $MnCl_2$. Other bivalent cations inhibited the enzyme; $CaCl_2$ and $ZnCl_2$ showed K_i of 0.6 mM and 2 mM respectively (results not shown).

Substrates

All determinations of substrate specificity were performed under standard assay conditions in 100 mM glycylglycine buffer, pH 9.0, as described in the Materials and methods section. The reaction mixtures contained saturation concentrations of the substrates, except the one for which the dependence was to be determined.

From a Lineweaver–Burk plot the apparent K_m for *myo*-inositol was determined as 0.28 ± 0.05 mM. When CDP-DAG was used as the limiting substrate, optimal activity was obtained between 0.2 and 0.4 mM. Higher concentrations inhibited the enzyme; at 2 mM the inhibition was 28%. From the initial

Table 2 Inhibition of the PtdIns synthase reaction by nucleotides and phosphate

The enzyme was assayed with 0.2 mM CDP–DAG as described in the Materials and methods section in the presence of increasing concentration of the inhibitors. K_{is} is the competitive inhibition constant. Abbreviations: n.i., no inhibition detected up to 40 mM inhibitor; n.d., not determined.

Inhibitor	K_{is} (mM)
CMP	n.i.
CDP	4
CTP	1.8
UMP	n.i.
UDP	3.8
UTP	1.4
TMP	n.i.
TDP	n.d.
TTP	1.4
AMP	n.i.
ADP	4.1
ATP	1.4
GMP	n.d.
GDP	n.d.
GTP	1.6
P_i	0.6
PP_i	0.2

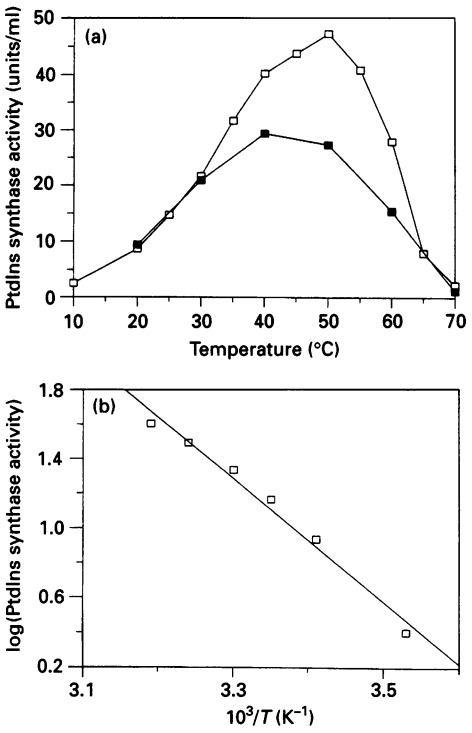

Figure 6 Effect of temperature on the PtdIns synthase activity

(**a**) A PtdIns synthase sample was incubated in 100 μl of standard assay mixture as described in the Materials and methods section at various temperatures. The assay mixtures and the PtdIns synthase sample were preincubated at the various temperatures for 3 min. At the end of the preincubation period, a portion of the PtdIns synthase sample was transferred to the assay mixture, and the incubation was continued for 10 min (□) or 20 min (■). The reaction was stopped by addition of 2 ml of chloroform/methanol/10 M HCl (200:100:1, by vol.). A portion of the chloroform phase was dried and counted for radioactivity in a liquid-scintillation counter. One unit corresponds to the formation of 1 nmol of PtdIns in one minute. Each data point is the mean of duplicate determinations. (**b**) The values from 10 min incubations at temperatures between 10 and 40 °C were used to construct an Arrhenius plot. The activation energy was found to be 67 kJ (16 kcal)/mol.

reaction rate K_m was calculated as 36 ± 3 μM. With 2′-deoxy-CDP-DAG as substrate, optimal activity was reached at 0.7 mM and the inhibitory effect was less pronounced; at 2 mM the inhibition was only 4%. K_m was determined to be 45 μM. The optimal activity obtained with the deoxy derivative was 25% higher than with CDP-DAG.

Inhibitors

The inhibitory effect of CDP (0, 5, 10, 20 mM) against increasing concentrations of CDP-DAG (0.02–0.5 mM) was analysed by using a double-reciprocal plot; the lines intersected, showing competitive inhibition. K_{is} was calculated to be 4 mM, from $K_m^{app.} = K_m(1 + [I]/K_{is})$, where $K_m^{app.}$ is the apparent K_m at inhibitor concentration [I]. Assuming that the inhibition by the other nucleotides and phosphate was also of competitive nature, the model $v = V_{max.}[S]/K_m(1 + [I]/K_{is}) + [S]$ would apply. The inhibition determinations were done at 0.2 mM CDP-DAG, i.e. $5.5 \times K_m$. Thus, at 50% enzyme inhibition the inhibitor concentration [I] is $6.5 \times K_{is}$. The K_{is} values for the various inhibitors were determined from this relationship (Table 2). PP_i, and then P_i, were the most efficient inhibitors. The nucleoside monophosphates, including CMP, did not inhibit the enzyme reaction. Nucleoside diphosphates inhibited the reaction, and the triphosphates were even more inhibitory. The base in the nucleotides had no effect on the inhibition. This suggests that the enzyme inhibition produced by the nucleoside di- and tri-phosphates is due to inhibition through the phosphate groups.

Temperature-dependence of the enzyme reaction

PtdIns synthase was incubated in standard reaction mixtures as described in the Materials and methods section at various temperatures for 10 and 20 min (Figure 6a). The optimal temperature was 50 °C. At temperatures above 30 °C the apparent activity after 20 min incubation was lower compared with 10 min incubation. The discrepancy presumably reflects thermal denaturation of the enzyme. To ensure that no components in the assay mixture were destroyed at higher temperatures, an assay mixture, without protein, was incubated for 10 min at 70 °C, cooled to 37 °C and, after addition of PtdIns synthase, incubated for 10 min at 37 °C. No difference in enzyme activity was detected compared with the same sample incubated with a reaction mixture not submitted to preincubation. When incubated in buffer F without substrates, the enzyme had a half-life of 20 min at 50 °C.

The values from 10 min incubations at temperatures between 10 and 40 °C were used to construct an Arrhenius plot (Figure 6b). The activation energy was 67 kJ (16 kcal)/mol.

Enzyme stability

The enzyme preparations were regularly frozen in liquid N_2 and stored at −80 °C. No detectable loss of activity was experienced during storage for up to 2 months. However, when the samples were frozen and stored at −20 °C, or frozen in liquid N_2 and subsequently stored at −20° or −60 °C, over 50% of the PtdIns

synthase activity was lost after 24 h. When stored at 4 °C on ice 10% of the activity was lost during the same time period. These results were reproduced with three independent preparations.

DISCUSSION

Microsomal PtdIns synthase from human placenta was purified to near homogeneity, as judged by silver-stained SDS/PAGE. The main purification step was affinity chromatography on CDP-DAG–Sepharose. This affinity resin has been used in the purification of several enzymes [17,22,23], including PtdIns synthase from *S. cerevisiae* [11] and rat brain [16]. Enzymes have been reported to bind extremely strongly to the CDP-DAG affinity resin [11,22]. When newly prepared affinity resin was used, human PtdIns synthase could not be eluted from the affinity column. However, after the column had been exposed to the Triton extract, the recovery sharply increased. This is most likely due to oxidation or exchange of the fatty acid moieties on the affinity CDP-DAG. The human PtdIns synthase eluted from the affinity column required phosphatidylcholine to retain activity. The requirement for phospholipids is similar to the rat brain enzyme [16], but contrasts with *S. cerevisiae* enzyme, where phospholipids were not required [11].

The only other PtdIns synthase that has been purified to homogeneity and extensively characterized is that from *S. cerevisiae* [11,12]. The human PtdIns synthase required 23–50-fold higher purification than the yeast enzyme. The *S. cerevisiae* enzyme was over 50% pure after the affinity column. Human PtdIns synthase was purified approx. 200-fold on the affinity column; however, PtdIns synthase constituted only 3–5% of the total protein. The specific activity of the purified human enzyme was 19000–35000 units/mg, whereas the pure yeast enzyme had 800 units/mg.

The difference seen in pH optimum between the mammalian enzymes (pH 9.0) and the yeast enzyme (pH 8.0) could be a secondary effect of the buffer systems used. The rat enzyme was assayed in glycylglycine buffer [16], whereas the yeast enzyme was assayed in Tris buffer [11]. When the human enzyme was assayed in glycylglycine buffer, pH optimum was at pH 9.0, whereas in Tris buffer the optimum was at pH 8.0. The mammalian enzymes are better activated by $MgCl_2$ than by $MnCl_2$; the yeast enzyme shows the reverse pattern. However, the detergent-extracted human enzyme was stabilized by Mn^{2+}, whereas Mg^{2+} showed no stabilizing effect. This suggests that the enzyme has several binding sites for the bivalent cations, stabilizing the enzyme and regulating activity, respectively. Alternatively, the enzyme is binding only Mn^{2+}, and the effect of Mg^{2+} on PtdIns synthase activity is due to interactions between the liponucleotide substrate and the ion. Whether the inhibition of PtdIns synthase by Ca^{2+} is of significance *in vivo* in the regulation of the PtdIns pool after receptor stimulation is unclear. Inhibition by Ca^{2+} has also been reported for other PtdIns synthases [9,10,24]. K_m values for *myo*-inositol between 4.6 and 0.76 mM have been reported for mammalian PtdIns synthase [15,16,25]. The human enzyme described here displayed a K_m of 0.28 mM. The yeast enzyme had a K_m of 0.08 mM, which is considerably lower than for the mammalian enzymes [12].

Since CDP is a part of the lipid substrate, the effect of various nucleotides on PtdIns synthase activity was examined in order to determine the influence of the nucleotide base. PtdIns synthase was competitively inhibited by nucleoside di- and tri-phosphates. Surprisingly, the nucleotide base had no effect (Table 2). This contrasts with the *S. cerevisiae* enzyme, where nucleotides had no effect on the enzyme activity [12]. Removing the 2′ hydroxyl group on the ribose increased the K_m for the liponucleotide substrate by 25%, and the substrate inhibition seen with CDP-DAG disappeared. Combined, these observations suggest that interactions between the enzyme molecule and the liponucleotide substrate involve the phosphate groups and the ribose, but not the nucleotide base.

I thank Dr. Lisa Klig and Dr. Timothy Wells for many helpful discussions. I am grateful to Dr. Timothy Wells for critically reading the manuscript.

REFERENCES

1 Zambrano, F., Fleischer, S. and Fleischer, B. (1975) Biochim. Biophys. Acta **380**, 357–369
2 Paulus, H. and Kennedy, E. P. (1960) J. Biol. Chem. **235**, 1303–1311
3 Low, M. G. and Saltiel, A. R. (1988) Science **239**, 268–275
4 Doering, T. L., Masterson, W. J., Hart, G. W. and Englund, P. T. (1990) J. Biol. Chem. **265**, 611–614
5 Berridge, M. S. (1987) Annu. Rev. Biochem. **56**, 159–193
6 Berridge, M. J. and Irvine, R. F. (1989) Nature (London) **341**, 197–204
7 Batenburg, J. J., Klazinga, W. and Van Golde, L. M. G. (1985) Biochim. Biophys. Acta **833**, 17–24
8 Bleasdale, J. E., Tyler, N. E. and Snyder, J. M. (1985) Lung **163**, 345–359
9 Imai, A. and Gershengorn, M. C. (1987) Nature (London) **325**, 726–728
10 Cubitt, A. B. and Gershengorn, M. C. (1989) Biochem. J. **257**, 639–644
11 Fischl, A. S. and Carman, G. M. (1983) J. Bacteriol. **154**, 304–311
12 Fischl, A. S., Homann, M. J., Poole, M. A. and Carman, G. M. (1986) J. Biol. Chem. **261**, 3178–3183
13 Nikawa, J., Kodaki, T. and Yamashita, S. (1987) J. Biol. Chem. **262**, 4876–4881
14 Nikawa, J., Kodaki, T. and Yamashita, S. (1988) J. Bacteriol. **170**, 4727–4731
15 Parries, G. S. and Hokin-Neaverson, M. (1984) Biochemistry **23**, 4785–4791
16 Ghalayini, A. and Eichberg, J. (1985) J. Neurochem. **44**, 175–182
17 Larson, T. J., Hirabayashi, T. and Dowhan, W. (1976) Biochemistry **15**, 974–979
18 Rao, R. H. and Strickland, K. P. (1974) Biochim. Biophys. Acta **348**, 306–314
19 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
20 Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356
21 Blum, H., Beier, H. and Gross, H. J. (1987) Electrophoresis **8**, 93–99
22 Hirabayashi, T., Larson, T. J. and Dowhan, W. (1976) Biochemistry **15**, 5205–5211
23 Cousminer, J. J., Fischl, A. S. and Carman, G. M. (1982) J. Bacteriol. **151**, 1372–1379
24 Takenawa, T. and Egawa, K. (1977) J. Biol. Chem. **252**, 5419–5423
25 Takenawa, T. and Nagai, Y. (1982) J. Biochem. (Tokyo) **91**, 793–799

Received 19 July 1993/17 September 1993; accepted 24 September 1993