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Characterization of reactions catalysed by yeast phosphatidylinositol synthase

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The nature of reactions catalysed by yeast phosphatidylinositol synthase expressed in *E coli* has been investigated. The single enzyme is shown to carry both CDP-diacylglycerol-dependent incorporation of inositol into phosphatidylinositol (K_m for inositol of 0.090 mM) and a CDP-diacylglycerol-independent exchange reaction between phosphatidylinositol and inositol (K_m for inositol of 0.066 mM). The exchange reaction and reversal of phosphatidylinositol synthase were both stimulated by CMP, but had different optimum pH and requirements for substrates. These results suggest that CMP-stimulated exchange and CMP-dependent reverse reactions are distinct processes catalysed by the same enzyme, phosphatidylinositol synthase.

CDP-diacylglycerol, Phosphatidylinositol synthase; Exchange reaction; *Saccharomyces cerevisiae*

1. INTRODUCTION

Phosphatidylinositol (PI) plays a very important role in signal transduction. Receptor-stimulated cleavage of its phosphorylated derivative, PI-4,5-biphosphate, leads to the formation of two messengers, inositol-1,4,5-trisphosphate and diacylglycerol [1].

Free inositol (Ins) can be incorporated into PI by two different reactions. One reaction is de novo synthesis of PI from CDP-diacylglycerol (CDPdG) and free Ins catalysed by PI synthase (CDPdG-myo-Ins-3-phosphatidyltransferase, EC 2.7.8.11) [2]. The other reaction is a CDPdG-independent reaction, presumed to represent the exchange of the Ins head group of pre-existing PI with exogenous Ins [2–8].

It is well established that there are two kinds of exchange processes: CMP-stimulated [7,9–11] and nucleotide-independent [11,12]. It has been suggested that the CMP-dependent exchange reaction might be catalysed by PI synthase [10,11] and represents the net result of the reverse and subsequent forward activities, whereas the nucleotide-independent reaction is catalysed by a different enzyme [11,12].

In the present work we investigated the activity(ies) of PI synthase from *Saccharomyces cerevisiae* expressed in *E. coli* in order to identify the reactions that could be catalysed by this enzyme. Its structural gene (PIS) has been isolated by means of genetic complementation [13] and expressed in *E. coli* [14]. This model *E. coli*–PIS system has several advantages. *E. coli* cells do not possess PI synthase and their membranes are absolutely devoid of PI. Therefore successful expression of the yeast PIS can be easily detected by analysis of the phospholipid composition of membranes. Another advantage is that PI synthase is the only enzyme produced by expression of PIS gene.

Abbreviations Ins, inositol; PI, phosphatidylinositol; CDPdG, cytidine diphosphate diacylglycerol; DOC-Na, Sodium deoxycholate; DTT, dithiothreitol.

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2. MATERIALS AND METHODS

2.1. *Bacterial strain and plasmid*

E. coli K12 strain HB101 was used as the host strain. Plasmid pUC8 was used as cloning vector. pUC-PI synthase gene (pUC-PIS) construction was a generous gift of Dr. S. Yamashita and was the same as in [14].

2.2. *Bacterial cultures*

E. coli transformants were grown at 37°C in either rich LB medium [15], or M9 glucose minimal medium [15], supplemented with vitamin-free acid hydrolysed casein (2 g/l). Both media were supplemented with 100 μg of ampicillin per ml. Cell growth was monitored by measuring the absorbance of the culture at 550 nm.

2.3. *Enzyme assays*

Bacterial cells were suspended in 50 mM HEPES buffer (pH 8.0) containing 8% glycerol and 2 mM DTT, disrupted by sonication and centrifuged at 1,000×*g* for 3 min to remove intact cells. The membrane fraction was then obtained by centrifugation of supernatant at 100,000×*g* for 45 min. The pellet was resuspended in solubilisation buffer (50 mM Tris-HCl, pH 8.0, 20% glycerol, 10 mM mercaptoethanol, 1% Triton X-100), and the membranes were either used directly for enzyme activity assays or frozen in liquid nitrogen and stored at −80°C.

The CDPdG-dependent incorporation was assayed in a medium containing 0.1 mM Ins (50,000 cpm/nmol), 30 mM Tris-HCl buffer (pH 8.0), 3 mM $MnCl_2$, 2 mM DTT, 0.26% (w/v) Triton X-100, 0.3 mM CDPdG and 600–800 μg of protein in a total volume of 0.33 ml.

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The mixture was incubated for 30 min at 30°C. The reaction was terminated by the addition of 5 ml of chloroform-methanol-HCl (100:50:1, by volume) solution. The chloroform phase was washed twice with 0.4 ml of a 2% NaCl/1% Ins solution. A 150 μl aliquot of the washed lower phase was then mixed with 10 ml of Toluene/PPO (5 g/l) and radioactivity was determined by scintillation counting. Exchange activity was measured without addition of CDPdG. Reverse activity was measured by following the release of radioactive free Ins in the upper aquous phase from ^{3}H-labeled PI added to the reaction mixture (3,500 cpm/nmol), or the decrease of the level of radiolabeled PI in the lower organic phase.

2.4. *Synthesis of radiolabeled PI*

^{3}H-Labeled PI was prepared by the forward reaction of yeast PI synthase, i.e. by CDPdG-dependent incorporation of *myo*-[2^{3}H]Ins into PI. PI purification was performed by TLC on silica gel 60 (Merck, Darmstadt) plates with chloroform-methanol-water-acetone-acetic acid (40:13:7.15:13, by volume). The ^{3}H-labeled spot containing PI was scraped off and the lipid was extracted with chloroform-methanol-water (65:35:8, by volume). The extract was taken to dryness under argon and redissolved in chloroform-methanol (2·1, v/v).

2.5. *Materials*

Myo-[2^{3}H]Ins (18 Ci/mmol) was obtained from Amersham International (UK), and *myo*-Ins from Fluka (Buchs, Switzerland). CDPdG was supplied by Serdary Research Laboratories (London, Ont., Canada). PI was obtained from Sigma (St. Louis, MO, USA). Vitamin-free acid hydrolysed casein was from Difco (Michigan, USA).

3. RESULTS

It has been shown that addition of Ins to the LB culture medium of *E. coli*–PIS cells allows the synthesis and accumulation of PI [14]. Moreover, we have observed that the accumulation of PI in cell membranes took place without Ins added to the LB medium. Thus, PI synthase can catalyse the formation of PI using endogenous CDPdG and Ins already present in the medium.

When membranes from *E. coli*–PIS cells grown in LB medium were used for the PI synthase activity test we observed both CDPdG-dependent and -independent incorporation of labeled Ins into PI (Table I). Addition of 0.2% DOC-Na (w/v) to the reaction mixture greatly inhibited the CDP-independent reaction of PI synthase, whereas CDP-dependent incorporation of Ins was slightly decreased (Table I). There are two possible explanations of CDP-independent synthesis of labeled PI in these conditions. First, pre-existing PI, made from Ins present in LB medium, may react with added labeled Ins to re-form PI, and thus an exchange reaction takes place. Secondly, there may be no such exchange and PI synthase catalyses only the forward reaction using pre-existing endogenous CDPdG and the labeled Ins added to the reaction mixture.

In order to show whether the exchange reaction really exists it was necessary to avoid the presence of any PI in cell membranes. For this reason, *E. coli* cells were grown in M9 glucose minimal medium without Ins. The results of the activity test on those membranes are shown in Table II. It was found that in the absence of added PI there was no CDPdG-independent incorporation of labeled Ins into lipids. When 0.36 mM PI was added to the reaction mixture the exchange reaction took place and a slight inhibition of the forward reaction (8%) occured.

Some exchange reaction did take place without added CMP, however, addition of cytidine nucleotides (either CMP or CTP) stimulated the incorporation of labeled Ins into PI during the exchange process (CMP>CTP) (Table II). The exchange activity was maximally stimulated by 4 μM of CMP but did not follow hyperbolic Michaelis–Menten kinetics.

The Ins concentration dependence was studied for the different PI synthesis reactions. The curves obtained showed typical Michaelis–Menten kinetics. The apparent K_m for Ins for CMP-independent and CMP-stimulated exchange reactions were different, 0.066 mM and 0.277 mM, respectively (Fig. 1). The K_m for Ins for the forward reaction was estimated to be 0.090 mM (not shown). The optima pH for exchange and forward reactions were also different, 7.0 and 8.0, respectively (Fig. 2).

In order to demonstrate the existence of the reverse reaction of PI synthase, ^{3}H-labeled PI was added to the reaction mixture and the activity test was performed in the absence of Ins. CMP stimulated the formation of free ^{3}H-labeled Ins in a concentration-dependent manner with apparent K_m of 0.022 mM. The optimum pH for the reverse reaction was estimated to be 8.5 (Fig. 3).

4. DISCUSSION

Although the environment of yeast PI synthase ex-

Table I

Incorporation of *myo*-[2^{3}H]Ins into PI by *E. coli* transformant cells

E. coli cells	Triton X-100		Triton X-100+DOC-NA	
	−CDPddg	+CDPdG	−CDPdG	+CDPdG
−PIS	3 4 ± 0 5	3.9 ± 0 3	4.0 ± 0.5	2.9 ± 0.2
+PIS	154.1 ± 4.3	369.0 ± 7.8	44.3 ± 1.7	337.0 ± 10.9

E. coli cells were grown in LB medium. The incorporation of *myo*-[2^{3}H]Ins into PI, performed as described in section 2, is expressed in pmol of Ins/mg of protein/min. The concentrations of Triton X-100 and DOC-Na were 0.26% and 0.2%, respectively The values represent the means of four determinations ± S.D.

Table II

PI synthase activity in *E coli* transformant grown in minimal M9 medium

E coli cells		−CDPdG			+CDPdG		
		−	CMP	CTP	−	CMP	CTP
−PIS	−PI	0.8 ± 0.1	1.8 ± 0.1	0.9 ± 0.2	1.1 ± 0.1	1.7 ± 0.4	1.6 ± 0.3
	+PI	0.9 ± 0.1	1.4 ± 0.6	1.9 ± 0.6	1.2 ± 0.2	1.5 ± 0.6	2.3 ± 0.9
+PIS	−PI	4.0 ± 1.7	4.1 ± 0.2	1.5 ± 0.3	266.3 ± 3.0	252.3 ± 7.5	265.9 ± 12.8
	+PI	41.5 ± 3.8	73.4 ± 3.3	57.4 ± 6.1	245.1 ± 7.8	237.1 ± 10.2	261.8 ± 6.6

The membrane fraction was prepared and used for the enzyme assays as described in section 2. The concentration of CMP and CTP was 4 μM. The concentration of PI was 0.36 mM The activity of PI synthase was expressed as pmol of synthesised labeled PI/mg of protein/min. The values are the means of four determination ± S D

pressed in the *E. coli* cell is quite different to that found in the *Saccharomyces cerevisiae* cell, we showed that the enzyme was still active. It had been shown previously by means of TLC analysis that PI synthase expressed in *E. coli* mediates the synthesis of PI [14]. Moreover, we found that the optimum pH for the forward reaction in our experimental conditions was the same as found for yeast PI synthase isolated by affinity chromatography [16]. The apparent K_m for Ins for this reaction was similar for the differently prepared enzymes: 0.09 mM for yeast PI synthase expressed in *E. coli* and 0.08 mM for purified yeast PI synthase [10]. Consequently, the similar results obtained for the forward reaction with different methods confirm the validity of our model system and of the enzyme activity assays.

It has been shown that purified reconstituted yeast PI synthase did catalyse an exchange reaction only in the presence of CMP [10]. This is in agreement with a general opinion that CMP-dependent and -independent reactions may be catalysed by distinct enzymes, and these processes are referred to as PI synthase and base-ex-

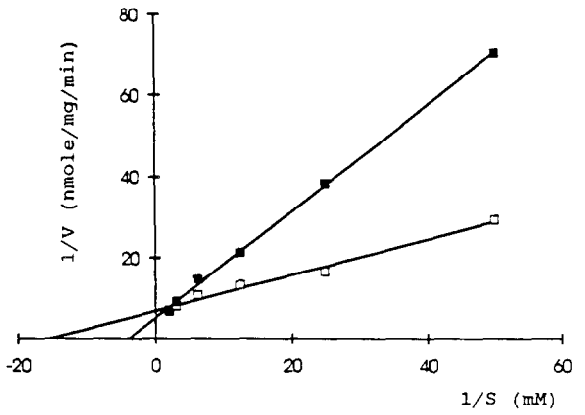

Fig. 1. Ins-dependence of PI synthase exchange activity Enzyme activity was measured in the absence of CDPdG as described in section 2 for 10 min. □, nucleotide-independent reaction (K_m 0.066 mM, V_{max} 0.147 nmol/mg/min); ■, CMP-dependent reaction (K_m 0.277 mM, V_{max} 0.199 nmol/mg/min). Each point is the mean of four determinations.

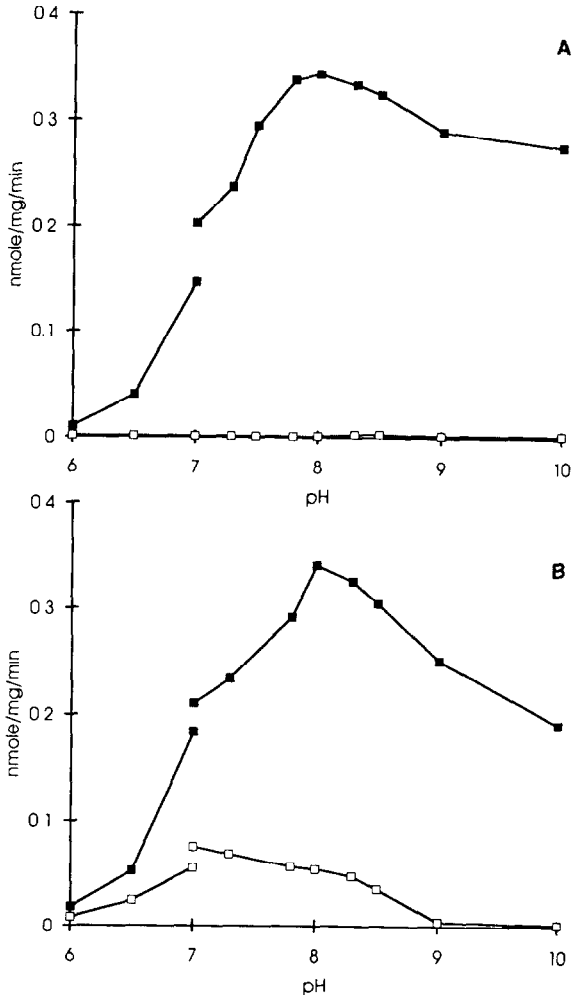

Fig. 2. pH dependence of yeast PI synthase activity The *E coli*–PIS cells were grown in M9 minimal medium The enzyme assays test was performed as described in section 2, in the absence (A) or in the presence (B) of 0.36 mM PI. ■, CDPdG-dependent reaction; □, CDPdG-independent incorporation. PI synthase activity was measured at the indicated pH values with 50 mM MES-HCl (pH 6.0–7.0) or 50 mM Tris-HCl (pH 7.0–10.0). The values are the means of four determinations.

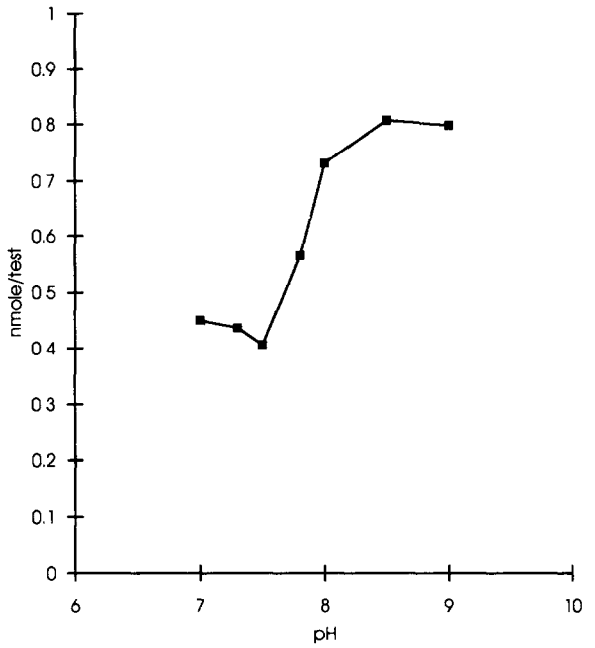

Fig. 3. pH dependence of liberation of *myo*-Ins from *myo*-Ins-2[3]H-labeled PI. The assay test was performed in the absence of Ins. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.0–9.0), 620 μg of protein, 14 nmol of PI (3,500 cpm/nmol), 3 mM CMP, 3 mM $MnCl_2$, 2 mM DTT and 0.26% (w/v) Triton X-100 in a total volume of 0.33 ml. The mixture was incubated for 40 min at 30°C The liberation of free Ins from labeled PI was followed as described in section 2. The values are the means of three determinations.

change reactions, respectively [6,8,10,11]. However, the CMP-dependent exchange reaction of yeast PI synthase had never been further characterised.

The present study determined that *E. coli* cells without the PIS-gene did not accumulate labeled Ins in their membranes (Tables I and II). There was no exchange process between Ins and PI added to the reaction mixture (Table II). In the *E. coli*–PIS cells we found both a CDP-dependent synthesis of PI and two types of CDP-independent incorporation of Ins into PI. So, we conclude that PI synthase is responsible for nucleotide-independent exchange reaction as well as for CMP-stimulated exchange activity.

The enzyme showed different Ins requirements for the two exchange reactions under otherwise indentical experimental conditions. The affinity was higher for the nucleotide-independent reaction (K_m of 0.066 mM) than for CMP-stimulated exchange (K_m of 0.277 mM). This is in agreement with the data reported for turkey erythrocyte membrane: K_m for Ins for CMP-independent and -dependent exchange were estimated as 0.01 mM and 0.3 mM, respectively [11]. When the synthesis of PI was measured in the presence of CDPdG, a higher apparent K_m for Ins was obtained (0.09 mM) as compared to nucleotide-independent exchange reaction (0.066 mM). The same observation was made previously by other investigators [2,5,17,18].

The present study showed that CMP maximally stimulated the exchange activity at a concentration much lower than that found for the reverse reaction: 4 μM and 0.32 mM (K_m of 0.022 mM), respectively. A similar observation was made for rat pituitary cells [19].

In addition, we have found that the kinetics for the CMP-stimulation of exchange and the reversal process were quite different. Stimulation of exchange activity did not follow Michaelis–Menten kinetics, whereas formation of free Ins from PI did.

It has been previously suggested that CMP may act by enhancing the headgroup exchange reaction [20] and this reaction is different from the reversal of PI synthase [19]. In agreement with these workers the present study also showed that CMP-stimulated exchange and reversal of PI synthase are distinct reactions due to their different optima pH and requirements for Ins and CMP.

Consequently, our results indicate that a single enzyme, PI synthase of *Saccharomyces cerevisiae*, is able to catalyse CDPdG-dependent incorporation of Ins into PI, as well as its reversal, and two types of exchange reactions between PI and Ins (nucleotide-independent and -dependent). At this point it is difficult to give clues to the physiological significance of these many activities.

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