Lipid dependence and activity control of phosphatidylserine synthase from *Escherichia coli*

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Abstract The activity of phosphatidylserine synthase from *Escherichia coli* depends significantly on the nature and level of the lipids in the matrix, at which the enzyme is operating. To elucidate the role of anionic lipids in the regulation of PtdSer synthase, its activity was studied in mixed micelles containing phosphatidylglycerol (one charge) or diphosphatidylglycerol (two charges), the two main anionic membrane lipids in *E. coli*. Membrane association and activity of PtdSer synthase were increased by the two lipids, indicating their essential role in the positive regulation mechanism of the phosphatidylethanolamine level in the *E. coli* membrane.

Keywords: Phosphatidylserine synthase; Mixed micelles; Cooperative Hill kinetics; Lipid activation; *Escherichia coli*

1. Introduction

Many biological processes proceed via membrane proteins and their lipid complexes [1,2]. The lipids in the membrane matrix play an essential and active role in many of these life-sustaining processes, ranging from messenger roles in signal transduction pathways to a role as substrate for many enzymes [3,4]. To keep its cell membrane functioning under all growth conditions, wild-type *E. coli* cells hold the composition of its major lipids nearly constant by a tightly regulated mechanism [5–8]. Two enzymes play essential roles in this regulation [5,9], as reflected by a competition between phosphatidylserine (PtdSer) synthase and phosphatidylglycerophosphate synthase for the substrate 1,2-diacyl-*sn*-glycero-3-cytidinediphosphate (CDP-acyl$_2$Gro) [10,11], providing a certain balance between the zwitterionic lipid phosphatidylethanolamine (PtdEtn) and the anionic lipids phosphatidylglycerol (PtdGro) and diphosphatidylglycerol (Ptd$_2$Gro) [12]. Therefore, any understanding regarding the function of cellular membranes requires detailed information about the synthesis of the necessary lipid components and the regulation of their relative amounts.

PtdSer synthase catalyzes the formation of PtdSer by the condensation of CDP-acyl$_2$Gro and L-serine [13]. PtdSer is then rapidly converted to PtdEtn in *E. coli* [13]. PtdSer synthase belongs to the group of interfacial proteins, which have to bind to a target membrane before enzymatic activity can occur [10,14–16]. This step increases the surface concentration, necessary for hydrophobic interactions to occur, which can facilitate partial penetration of the protein into the bilayer, triggering enzymatic function [17,18]. PtdSer synthase acts on its substrate at the interface between a water phase and a substrate aggregate surface, and the reaction rate is dependent on both the two-dimensional surface concentration as well as the three-dimensional total concentration of amphiphilic substrates, activators, and inhibitors [19–21].

Here, we studied the influence of membrane matrix lipids on the PtdSer synthase activity. Using the two major anionic lipids in *E. coli*, PtdGro and Ptd$_2$Gro, we elucidated the role of membrane association and the nature of participating lipids, information essential to understand the activity and regulation of PtdSer synthase on a molecular level. We explored the role of both lipids for PtdSer synthase activity by monitoring the enzyme kinetics using phospholipid/detergent mixed micelles. By using the non-ionic detergent octaethyleneglycol-*n*-dodecylmonoether ($C_{12}E_8$) with its well-defined chemical structure and low CMC value, we could describe the observed kinetics with a modified surface dilution kinetics (SDK) model, where cooperative kinetic effects were included by using a Hill approach. This way, we could show that membrane association and activity of PtdSer synthase are significantly enhanced by the presence of anionic lipids, where the nature of the lipid plays an essential part in the positive regulation mechanism of the PtdEtn level in the *E. coli* membrane.

2. Materials and methods

2.1. Chemicals

As purchased: L-[3-^{3}H]-serine (Amersham, UK), 1,2-dioleoyl-*sn*-glycero-3-cytidinediphosphate (CDP-oleoyl$_2$Gro) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (PtdGro) from Avanti Polar Lipids (Birmingham, AL), Ptd$_2$Gro isolated from *E. coli* from Sigma (St. Louis, MO), and the non-ionic detergent $C_{12}E_8$ from Nikko Chemicals (Japan).

2.2. Enzyme preparation

PtdSer synthase (CDP-1,2-diacyl-*sn*-glycerol: L-serine *O*-phosphatidyltransferase, EC 2.7.8.8) was isolated from the *E. coli* strain JA-200/pPS3155-λ [22]. The purification procedure was carried out according to Dowhan [15], which was modified as follows: Triton X-100 was

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Abbreviations: CDP-acyl$_2$Gro, 1,2-diacyl-*sn*-glycero-3-cytidinediphosphate; $C_{12}E_8$, octaethyleneglycol-*n*-dodecylmonoether; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; Ptd$_2$Gro, diphosphatidylglycerol; PtdSer, phosphatidylserine; SDK, surface dilution kinetics

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replaced by 0.6 mM $C_{12}E_8$. In the elution buffer, the NaCl concentration was reduced to 0.7 M to prevent precipitation of the enzyme. The final dialysis step was replaced by gel filtration through Sephadex® G-25M column (Pharmacia, Sweden). Finally, the enzyme was eluted from a pre-equilibrated PD-10 column with 0.1 M potassium phosphate (pH 7.4) containing 0.6 mM $C_{12}E_8$, 25% (w/v) glycerol, 0.5 mM dithiothreitol, and 0.2 M NaCl. Protein concentration and purity were then checked using standard methods.

2.3. Preparation of phospholipid/$C_{12}E_8$ mixed micelles

Stock solutions of phospholipid/$C_{12}E_8$ mixed micelles (for CDP-oleoyl$_2$Gro, PtdGro and Ptd$_2$Gro) were prepared by solubilizing each lipid in a $C_{12}E_8$-solution, followed by sonication at RT until clear solution. Assuming ideal mixing of $C_{12}E_8$ and the phospholipids, the surface concentration of phospholipids was corrected according to:

$$[C_{12}E_8]_{free} = \frac{[C_{12}E_8]_{tot}}{[\text{phospholipid}] + [C_{12}E_8]_{tot}} \cdot \text{CMC} \quad (1)$$

where CMC is the critical micelle concentration of $C_{12}E_8$. The phospholipid fraction was below 15 mol% to prevent any changes in micelle shape [23].

2.4. Enzyme assay

The catalytic activity of PtdSer synthase was measured at 30 °C as described before [15]. The assay mixture included, in addition to the phospholipid/$C_{12}E_8$ mixed micelles, 0.1 M KP_i (pH 7.4), 1.2 mM L-serine, 2.0 μCi/ml L-[3-^{3}H]-serine (26 μCi/nmol) and 1 mg/ml BSA. The ^{3}H-PtdSer product was quantified in a 1214 Rack Beta liquid scintillation counter (LKB-Wallac, Finland). The specific activity of PtdSer synthase is defined as nmoles of PtdSer formed per minute and per mg of enzyme (nmol min^{-1} mg^{-1}). A minimum of 16 replicates was carried out for each molar ratio of CDP-oleoyl$_2$Gro/(PtdGro or Ptd$_2$Gro)/$C_{12}E_8$ used.

2.5. Analysis of kinetic data

A modified SDK model for interfacial enzymes was used where the protein acts according to a two-step mechanism [17]:

$$E + A \underset{k_{-1}}{\overset{k_2}{\rightleftarrows}} EA \quad EA + B \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EAB \xrightarrow{k_3} EA + Q \quad (2)$$

The enzyme, E, associates with the micelle, A, to form an enzyme-mixed micelle complex, EA. Thereafter, the enzyme interacts with the lipid substrate, B, to an EAB-complex, which undergoes a catalytic change, yielding the product Q and regenerating the EA-complex. A is defined as the bulk concentration of the relevant surface ligand (mM), i.e., any molecule that holds the enzyme to the surface, and B is the surface concentration of substrate expressed as mole fraction. The reaction rate is therefore [17]:

$$v = \frac{V_{max} \cdot A \cdot B}{n \cdot K_S^A \cdot K_M^B + x \cdot K_M^B \cdot A + A \cdot B} \quad (3)$$

with

$$x \cdot K_M^B = \frac{(k_{-2} + k_3)}{k_2} \quad \text{and} \quad \frac{n}{x} \cdot K_S^A = \frac{k_{-1}}{k_1}$$

where xK_M^B is the interfacial Michaelis–Menten constant expressed as mole fraction of substrate, nK_S^A/x (mM) is the dissociation constant for the mixed micelle binding site, v is the specific activity (nmol min^{-1} mg^{-1}), and V_{max} is the true maximum specific activity at an infinite mole fraction of substrate. The average surface area per molecule of detergent and substrate is x, and n is the micellar surface area per enzyme binding site.

3. Results

3.1. Activation of PtdSer synthase by anionic lipids

The activity of PtdSer synthase and its regulation depends crucially on the nature and level of the lipids in the surrounding matrix. To reveal any stimulating, or inhibiting, cooperative effects of the involved lipids on the enzyme activity, a concentration of CDP-oleoyl$_2$Gro close to the apparent K_M value (7.5 mol%) was chosen in our initial experiments. In those experiments, we studied the effect of anionic lipids on PtdSer synthase activity (Fig. 1). The function of the non-ionic detergent $C_{12}E_8$ in the experimental system was to serve as a catalytically inert matrix to enable a change in the total lipid concentration as well as its surface concentration. Fig. 1 shows the relative activity of PtdSer synthase as a function of mol% PtdGro or Ptd$_2$Gro in the mixed micelles. Both curves show a similar dependence of activity on surface concentration of the charged lipids and their respective charge. Clearly, the presence of PtdGro and Ptd$_2$Gro has a stimulatory effect on PtdSer synthase, with both lipids promoting binding and activity. The activity response for PtdSer synthase to PtdGro shows a sigmoidal relationship with a threshold concentration of approximately 1 mol%, while Ptd$_2$Gro stimulates PtdSer synthase in a more dose-dependent manner with a faster response. Maximum stimulation was not reached under the conditions used, but in the tested concentration region the stimulation by PtdGro and Ptd$_2$Gro was 2.2-fold and 2.9-fold, respectively. Clearly, there is a strong correlation between the amount of anionic lipids present, their head group charge and enzyme activity.

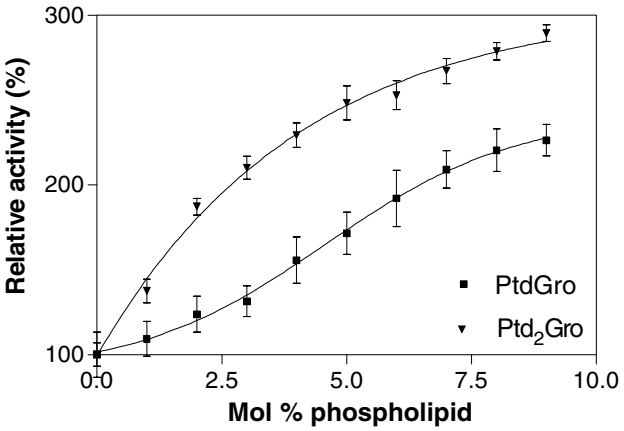

Fig. 1. Relative activity of PtdSer synthase at 30 °C as a function of the mol% of anionic lipids in mixed micelles of $C_{12}E_8$ in the presence of 7.5 mol% CDP-oleoyl$_2$Gro: PtdGro (squares), Ptd$_2$Gro (triangles). Error bars are 95% confidence interval based on a minimum of 18 replicates.

3.2. Regulation of PtdSer synthase kinetics by anionic lipids

To study the influence of anionic lipids and their regulative role on PtdSer synthase, its specific activity was measured as a function of the mol% CDP-oleoyl$_2$Gro at a series of set mol% of PtdGro and Ptd$_2$Gro, respectively (Fig. 2A and B). The fraction of PtdGro and Ptd$_2$Gro in micelles varied between 0 and 6 mol%. The presence of charged lipids increases the specific activity significantly in both cases. However, at low CDP-oleoyl$_2$Gro concentrations, PtdGro has only a minor effect while Ptd$_2$Gro already induces a dramatic increase in activity. This behavior may be explained by the fact that Ptd$_2$Gro has similar head group features as PtdGro, but carries two negative charges. The dependence of the PtdSer synthase activity is cooperative with respect to PtdGro, but almost hyperbolic for Ptd$_2$Gro. To quantify the degree of cooperativity for each lipid, the kinetic data were analyzed according to the Hill equation. This way, Hill coefficients, h, could be determined for each concentration of anionic lipid. They range from 1.8 (only CDP-oleoyl$_2$Gro) to 1.2 and 0.9 for PtdGro and

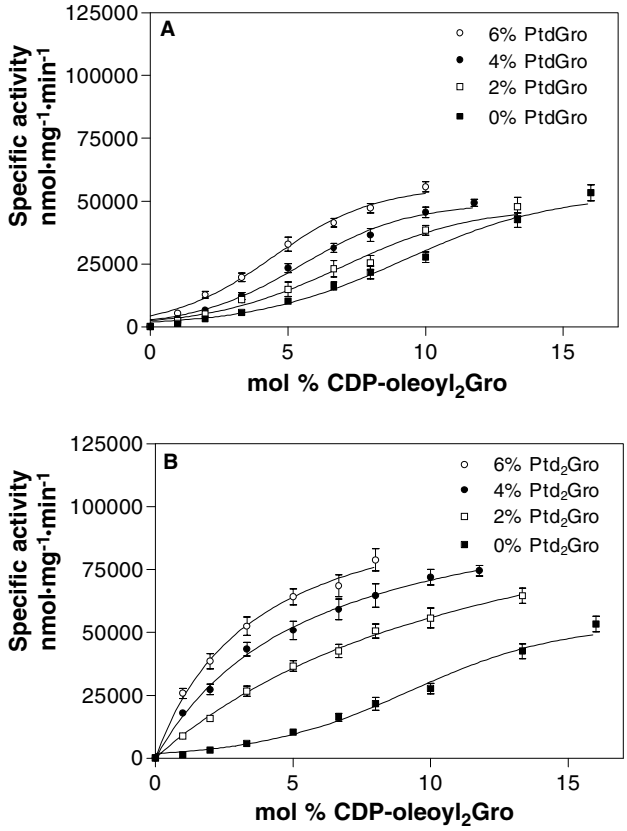

Fig. 2. Specific activity of PtdSer synthase at 30 °C as a function of the mol% of CDP-oleoyl$_2$Gro in mixed micelles of CDP-oleoyl$_2$Gro/$C_{12}E_8$ containing varying molar fractions of anionic lipids as indicated: (A) PtdGro; (B) Ptd$_2$Gro. Error bars as in Fig. 1.

Ptd$_2$Gro, respectively, showing the cooperativity of the enzyme at the presence of PtdGro (Table 1).

Using a combined approach of SDK kinetics and Hill treatment, the kinetics of PtdSer synthase can now be described correctly:

$$v = \frac{V_{max} \cdot A \cdot B^h}{\frac{n}{x} \cdot K_S^A \cdot (x \cdot K_M^B)^h + (x \cdot K_M^B)^h \cdot A + A \cdot B^h} \quad \text{with}$$

$$v = \frac{V_{app} \cdot A}{K_{app} + A} \qquad (4)$$

where V_{app} is the apparent maximum rate and K_{app} is the apparent interfacial Michaelis–Menten constant for each concentration of PtdGro and Ptd$_2$Gro, respectively [20]. This way, each activity curve could be refitted against CDP-oleoyl$_2$Gro

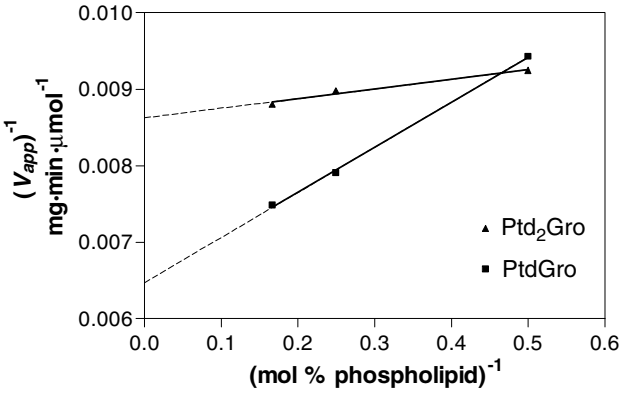

Fig. 3. Relationship between the apparent maximum rate constants (V_{app}) of PtdSer synthase activity in CDP-oleoyl$_2$Gro/$C_{12}E_8$ mixed micelles and the molar fraction of PtdGro (squares) or Ptd$_2$Gro (triangles) present. Values for V_{app} were obtained from the curves in Fig. 2A and B after treatment of the kinetic data with a Hill approach according to equation 4.95% confidence interval of V_{app} is approximately ±10%.

by applying the correct Hill coefficient according to Eq. (4) (data not shown). V_{app} and K_{app} for each concentration of PtdGro and Ptd$_2$Gro were obtained by evaluating the data by nonlinear regression analysis (Table 1). The addition of PtdGro or Ptd$_2$Gro resulted in an increase in V_{app}, but K_{app} remained constant for PtdGro while it decreased for Ptd$_2$Gro. Finally, by replotting $1/V_{app}$ versus 1/(mol% anionic lipid), a true maximum rate (V_{max}) could be calculated from the y-axis intercept (Fig. 3), and the concentration of anionic lipid at half-maximum activation (K_a) could be determined. V_{max} increased for both PtdGro and Ptd$_2$Gro compared to the value for CDP-oleoyl$_2$Gro, with the highest value obtained for PtdGro (Table 1). The K_a values for PtdGro and Ptd$_2$Gro were about eight times and 50 times lower compared to the K_M-value for CDP-oleoyl$_2$Gro alone.

4. Discussion

PtdGro and Ptd$_2$Gro are the major anionic membrane lipids in *E. coli*. Both lipids significantly stimulate the activity of PtdSer synthase, indicating a major role of this enzyme in the regulation of lipid composition. The ability of anionic lipids to activate the enzyme seems reasonable, since it has several basic amino acid residues accumulated in both the N- and C-termini [25], making electrostatic binding possible. It has also been shown with coupled plasmon-waveguide resonance (CPWR)

Table 1
Kinetic constants for the activity of PtdSer synthase from *E. coli* (18 replicates)

Activator	h	V_{app}[a] (μmol mg^{-1} min^{-1})	K_{app}[a] mol% CDP-oleoyl$_2$Gro	V_{max} (μmol mg^{-1} min^{-1})	xK_a[b] mol% lipid	xK_M[a,c] mol% CDP-oleoyl$_2$Gro
None	1.8	93	12	97[a,c]		7.5
Ptd$_2$Gro, 2 mol%	1.1	109	9.5			
Ptd$_2$Gro, 4 mol%	1.0	111	5.5			
Ptd$_2$Gro, 6 mol%	0.9	114	4.0	116[b]	0.15	
PtdGro, 2 mol%	1.4	107	12			
PtdGro, 4 mol%	1.3	126	14			
PtdGro, 6 mol%	1.2	132	12	154[b]	0.9	

[a] The kinetic constants were estimated using nonlinear regression analysis.
[b] The kinetic constants were estimated using linear regression analysis.
[c] Data from manuscript in preparation [24].

spectroscopy that addition of anionic lipids to a phosphatidylcholine bilayer causes a much stronger binding of PtdSer synthase to the bilayer [18]. Even substrate (CDP-oleoyl$_2$Gro) or product of the enzyme improves membrane association [26].

At low mole fractions, Ptd$_2$Gro has a much more pronounced activating effect than PtdGro (Fig. 2). However, the V_{max} is higher for PtdGro and the V_{app}-values show a steeper increase for PtdGro (Table 1). Thus, the higher surface charge density imparted by Ptd$_2$Gro seems to be the dominating effect at low mole fractions. At higher mole fractions, the ability of the two anionic lipids to perturb the structure of the mixed micelles may be more important. Since Ptd$_2$Gro has a greater tendency than PtdGro to form reversed hexagonal phases [27], the former lipid probably has a stronger tendency to change a globular micelle structure into a more planar-like shape, thus increasing the packing density in the aggregates. This effect decreases the ability of PtdSer synthase to penetrate into the hydrophobic environment of the aggregates and can explain why the V_{max} of PtdSer synthase is lower for Ptd$_2$Gro than for PtdGro. This behavior indicates that interfacial binding of the enzyme is not solely driven by electrostatic interactions, but there is also a hydrophobic component, which has also been inferred from CPWR spectroscopy [18].

PtdSer synthase exhibits positive cooperative kinetics with respect to its lipid substrate CDP-oleoyl$_2$Gro (Table 1) [24]. This feature is retained with PtdGro, although less pronounced, but is on the whole completely lost with Ptd$_2$Gro. Clearly, a sigmoidal binding curve indicates multiple ligand binding sites [28]. The obtained Hill number for CDP-oleoyl$_2$Gro is close to two, indicating that the enzyme contains at least two binding sites, one for the substrate at the active site and one for an effector molecule. Most likely, the activation site is situated at the cationic termini of PtdSer synthase. Binding of PtdGro, Ptd$_2$Gro or another CDP-oleoyl$_2$Gro molecule promotes a conformational change favoring an active form of the enzyme leading to increased activity. The strong activating ability of Ptd$_2$Gro at low mole fractions is reflected by the very low xK_a-value of 0.15 mol% as compared to the xK_M-value of 7.5 mol% for CDP-oleoyl$_2$Gro alone (Table 1).

The membranes of *E. coli* contain 70–80 mol% PtdEtn, 20–25 mol% PtdGro and 5 mol% or less Ptd$_2$Gro. About 5% of the dry weight of *E. coli* is made up of phospholipids [14], which corresponds to 1–1.25 mol% PtdGro and 0–0.25 mol% Ptd$_2$Gro. The obtained xK_a-values are definitely physiologically relevant (Table 1), well related to the requirements on the enzyme in a living *E. coli* cell. Based on the experiments, a model for maintaining a constant polar head group composition has been suggested [18,22,25,29]. The present results give very firm support for this model. PtdSer synthase plays a key role in the model, whose essential feature is that the enzyme molecules are more tightly bound to the membrane when the fraction of anionic lipids increases [18]. Because the enzymatic reaction is carried out when the enzyme is bound to the membrane, the synthesis of PtdEtn (proceeding via PtdSer) will increase under these conditions. The response counteracts the elevated levels of PtdGro and Ptd$_2$Gro, and can result in a balanced composition of polar head groups in *E. coli*.

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