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Water-miscible organic cosolvents enhance phosphatidylinositol-specific phospholipase C phosphotransferase as well as phosphodiesterase activity

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Abstract

Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* catalyzes the hydrolysis of phosphatidylinositol (PI) in a Ca^{2+}-independent two-step mechanism: (i) an intramolecular phosphotransferase reaction to form inositol 1,2-(cyclic)-phosphate (cIP), followed by (ii) a cyclic phosphodiesterase activity that converts cIP to inositol 1-phosphate (I-1-P). Moderate amounts of water-miscible organic solvents have previously been shown to dramatically enhance the cyclic phosphodiesterase activity, that is, hydrolysis of cIP. Cosolvents [isopropanol (iPrOH), dimethylsufoxide (DMSO), and dimethylformamide (DMF)] also enhance the phosphotransferase activity of PI-PLC toward PI initially presented in vesicles, monomers, or micelles. Although these water-miscible organic cosolvents caused large changes in PI particle size and distribution (monitored with pyrene-labeled PI fluorescence, ^{31}P NMR spectroscopy, gel filtration, and electron microscopy) that differed with the activating solvent, the change in PI substrate structure in different cosolvents was not correlated with the enhanced catalytic efficiency of PI-PLC toward its substrates. PI-PLC stability was decreased in water/organic cosolvent mixtures (e.g., the T_m for PI-PLC thermal denaturation decreased linearly with added iPrOH). However, the addition of *myo*-inositol, a water-soluble inhibitor of PI-PLC, helped stabilize the protein. At 30% iPrOH and 4 °C (well below the T_m for PI-PLC in the presence of iPrOH), cosolvent-induced changes in protein secondary structure were minimal. iPrOH and diheptanoylphosphatidylcholine, each of which activates PI-PLC for cIP hydrolysis, exhibited a synergistic effect for cIP hydrolysis that was not observed with PI as substrate. This behavior is consistent with a mechanism for cosolvent activation that involves changes in active site polarity along with small conformational changes involving the barrel rim tryptophan side chains that have little effect on protein secondary structure.

Keywords: Phospholipase C; Organic solvent effects; Phosphatidylinositol; Vesicle solubilization

1. Introduction

Phosphatidylinositol-specific phospholipase C (PI-PLC) catalyzes the hydrolysis of phosphoester bonds of phosphoinositides to produce lipid-soluble diacylglycerol and water-soluble *myo*-inositol phosphates. PI-PLC enzymes are found in a wide range of eukaryotic and prokaryotic cells [1]. In mammalian cells, intracellular PI-PLC is important in signal transduction. Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) catalyzed by PI-PLC produces two second messenger molecules: membrane-localized diacylglycerol, which allosterically activates protein kinase C, and water-soluble D-*myo*-inositol 1,4,5-trisphosphate which is important in intracellular calcium mobilization. In contrast to the mammalian PI-PLC, the bacterial PI-PLC enzymes are secreted and usually show low activity toward PIP_2. They are considerably smaller than mammalian PI-PLCs but their α/β barrel structure is conserved as the catalytic domain in the mammalian enzymes [2–4]. These bacterial PI-PLC enzymes are virulence factors in pathogens like *Listeria monocytogenes* [5,6] and *Staphylococcus aureus* [7].

The *Bacillus thuringiensis* PI-PLC shares some kinetic features with the mammalian PI-PLC enzymes. It exhibits kinetic interfacial activation where aggregated substrates are preferred over monomeric substrates [8], a characteristic of most water-soluble phospholipases including PI-PLCδ1 [1]. However, the enzyme efficiency (k_{cat}/K_m) of the bacterial PI-PLC toward the water-soluble substrate cIP (the cyclic phosphodiesterase step) is also enhanced with the addition of a non-substrate interface (e.g., PC micelles or vesicles)

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[9]; a similar activation was observed toward PI in vesicles that also contained a small amount of PC [10]. A similar interfacial activation is exhibited by PI-PLCδ1 [11] but not by PI-PLCγ [12]. The catalytic efficiencies of the second step for both the bacterial PI-PLC and the PLCδ1 isozyme are also enhanced by addition of water-miscible organic solvents such as isopropanol (iPrOH), dimethylsulfoxide (DMSO), and dimethylformamide (DMF) with maximal activity observed at 30% iPrOH, 40% DMF, and 50% DMSO [11,13].

In this work, the effects of organic solvents on *B. thuringiensis* PI-PLC phosphotransferase activity toward long-chain PI in vesicles and short-chain PI monomers and micelles are examined. Activation of the phosphotransferase step by organic cosolvents parallels that observed for cIP hydrolysis. Although cosolvents alter the distribution and structures adopted by PI, the activation of PI-PLC by cosolvents does not correlate with any substrate aggregate changes. This suggests that PI-PLC activity is enhanced in the presence of organic solvents regardless of the type of substrate presented and regardless of the effect these cosolvents have on the substrate structure. The minimal solvent-induced changes in PI-PLC secondary structure (monitored by CD spectroscopy) are consistent with changes in the local polarity of the active site and conformational changes of side chains as possible causes of the enhanced enzymatic activity.

2. Materials and methods

2.1. Chemicals

L-Phosphatidylinositol and other phospholipids including diheptanoylphosphatidylcholine (diC_7PC) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) were obtained from Avanti and used without further purification. iPrOH, DMF, and DMSO were purchased from Aldrich and were used without further purification. Dibutyroylphosphatidylinositol (diC_4PI) and dioctanoylphosphatidylinositol (diC_8PI) were purchased from Echelon Biochemicals. Other reagents including carboxyfluorescein (CF), D_2O (99.9%), and Sephadex G-100 were purchased from Sigma.

2.2. Purification of PI-PLC

The recombinant *Escherichia coli* cell strain MM294 transfected with *B. thuringiensis* PI-PLC gene [14] was obtained from Dr. Ming-Daw Tsai (Ohio State University). The enzyme was isolated from cell lysates and was purified by chromatography using a 1.6×12 cm column of Q sepharose Fast Flow (Amersham Pharmacia Biotech) and a 1.0×10 cm column of phenyl sepharose (Amersham Pharmacia Biotech). The enzyme from this protocol had less than 5% total impurities as monitored by intensity of the 35-kDa band in Coomassie-stained SDS-polyacrylamide gels compared to other very faint bands. PI-PLC concentration was determined by Bradford assay using bovine serum albumin as a standard. Typically, 20 mg of PI-PLC was obtained from 1 l of culture. The enzyme was stored in 50% glycerol and 20 mM Tris buffer, pH 8.5. The concentrated stock solutions (typically ≥ 0.5 mg/ml) were further diluted with 0.1% bovine serum albumin (Bio-Rad Protein Assay Standard II) in 20 mM Tris, pH 8.5, before enzyme assays. This recombinant PI-PLC had lower (and more reproducible from batch to batch) specific activities toward PI SUVs than PI-PLC secreted into *Bacillus* medium [10], presumably because the secreted protein had lipids or other materials bound which enhanced its activity toward PI vesicles.

2.3. Vesicle preparation

Lipid stock solutions in chloroform were obtained from Avanti and used without further purification. All samples were placed in glass scintillation vials, and the chloroform was removed with a stream of N_2. The lipid film was lyophilized overnight and then rehydrated with 25 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid), pH 7.5, for enzyme assays. SUV and LUV preparations were generated by sonication or extrusion, respectively, as described previously [10]. Mixed PI/PC SUVs were prepared by sonicating a rehydrated PI and PC suspension in 25 mM HEPES using a Branson sonifier cell disrupter and a microtip probe until maximum clarity was achieved (typically 5–10 min). When vesicle preparations were mixed with organic cosolvents, the optical density at 600 nm of solutions was measured as a rough assessment of changes in particle sizes (i.e., increasing OD_{600} indicated larger particles and decreasing OD_{600} indicated a bias toward smaller particles).

2.4. Enzymatic synthesis and purification of inositol-(1,2)-(cyclic)-phosphate (cIP)

Crude soybean PI was used for the enzymatic generation of cIP by PI-PLC as described previously [9]. One gram of crude PI was dissolved in 15 ml Tris buffer (pH 7.5) containing 4% Triton X-100. Bacterial PI-PLC (20 μg) was added and the reaction was monitored using ^{31}P NMR spectroscopy. After the PI was hydrolyzed to cIP (typically 4 h at room temperature), further reaction [e.g., hydrolysis of cIP to inositol 1-phosphate (I-1-P)] was quenched by the addition of chloroform. Purification of cIP was carried out as previously described [9].

2.5. ^{31}P NMR assays of PI-PLC activity

The buffer used in all PI-PLC activity assays was 25 mM HEPES, pH 7.5. ^{31}P NMR parameters were based on those previously reported [9,16]. For fixed time point assays using PI as substrate, the amount of enzyme added in each kinetic run was adjusted so that less than 20% hydrolysis of

substrate occurred during the 10-min incubation at room temperature. Since the enzyme was diluted in stocks with 0.1% BSA before kinetics, the final BSA concentration in assay mixtures was $<0.005\%$. After 10 min, the reactions were quenched using chloroform and the aqueous layer was extracted and analyzed for product using ^{31}P NMR spectroscopy (202.3 MHz on a Varian INOVA 500 spectrometer). PI cleavage and cIP hydrolysis rates were measured from the integrated intensity of cIP and I-1-P resonances, respectively, as a function of incubation time. Specific activities are based on assays run at a minimum in duplicate.

2.6. Fluorescence spectroscopy

Two types of fluorescence experiments were performed to characterize the effect of cosolvents on PI vesicles: (i) measurement of 1-myrisotyl-2-pyrenebutanoyl-glycero-3-(D-inositol-1-phosphate), or Pyr-PI (obtained from Dr. Stewart Hendrickson, University of Washington), excimer and monomer band intensities to monitor vesicle solubilization, and (ii) measurement of leakage of entrapped CF by increased fluorescence at 520 nm. Fluorescence studies were done at 25 °C using a Shimadzu RF5000U fluorimeter. The fluorescence spectra of Pyr-PI vesicles (20 μM) in 20 mM HEPES, pH 7.5, were acquired with an excitation wavelength of 350 nm and the emission scanned between 360 and 600 nm. Excitation and emission slit widths were set to 3 nm. The fluorescence intensities of monomer (380–390 nm) and excimer (450 nm) peaks were measured as a function of added organic solvents. CF was trapped inside PI vesicles by sonicating the resuspended PI solution in the presence of 100 mM CF. Free CF in solution was removed by gel filtration through a 0.7×50 cm column of Sephadex G-10-120 obtained from Sigma. CF entrapped in vesicles is self-quenched. However, if vesicles become leaky, the CF is diluted into the solution and the fluorescence at 520 nm (excitation at 490 nm) increased dramatically.

2.7. ^{1}H NMR analyses of PI

^{1}H (500 MHz) spectra (acquired on the same spectrometer mentioned above) were used to compare the conformation and dynamics of naturally occurring PI with synthetic short-chain PI species [17]. NOESY experiments were carried out for 3 mM diC_4PI and 3 mM diC_8PI solubilized in D_2O or iPrOH-d_8/D_2O (30:70, v/v) and 3 mM long-chain PI in iPrOH-d_8/D_2O. Buffers were not included in these solutions (to avoid overlap of buffer peaks with PI protons); however, the pH of the solutions in the absence of cosolvent was typically between pH 6.5 and 7.5. Since no groups in the PI should ionize in this range, the lack of a buffer should not pose a problem. NOESY spectra were acquired with 0.4–0.5 s mixing times. The residual HDO resonance was suppressed using a WET pulse sequence that applies pulse field gradients. Gaussian weighting was used in both F1 and F2 dimensions. ^{1}H spin-lattice relaxation times for the same samples, measured by inversion-recovery, were used to assess PI dynamics in the iPrOH-d_8/D_2O cosolvent system.

2.8. Gel filtration of PI aggregates in water/cosolvent mixtures

PI SUVs (5 mM) in 20 mM HEPES, pH 7.5, were chromatographed on a Sephadex G-100 gel exclusion column (1.5×50 cm equilibrated with 20 mM HEPES, pH 7.5); in the absence of organic solvent, all of the PI eluted in the column void volume. The same column was used to get a rough idea of the particle size distribution for PI vesicles mixed with 10% and 30% iPrOH; for these experiments, the resin was equilibrated with the same concentration iPrOH. cIP (10 mM) was present in all samples as a marker for the elution volume of small molecules. Column fractions were lyophilized, redissolved in 30% iPrOH (additional iPrOH had no effect on the ^{31}P PI intensities) in HEPES, pH 7.5, and quantified by ^{31}P NMR spectroscopy to assess the distribution of PI compared with cIP.

2.9. Characterization of PI/iPrOH aggregates using electron microscopy

The size distribution of PI sonicated vesicles after mixing with different percentages of iPrOH was monitored using a CM10 Phillips Electron Microscope and uranyl acetate (2%) to stain the PI samples. The concentration of PI in SUVs mixed with 0% and 10% iPrOH was 1 mg/ml; 5 mg/ml PI sonicated vesicles were incubated with 20% iPrOH and 10 mg/ml PI SUV preparation was incubated with 30% and 40% iPrOH. Although different SUV preparations were used, the average initial size was similar as monitored by the ^{31}P linewidth. The 2% uranyl acetate (10 μl) was first applied to formvar/carbon-coated 400 mesh grids followed by application of 4 μl of the PI sample of interest. Several pictures of PI vesicles at each percentage of iPrOH were taken and the sizes of the vesicles were measured using catalase crystals (spacing = 87.5 Å) to calibrate sizes at different magnifications. Typically, three to five grids were imaged (5–10 photomicrographs taken of each grid) for each vesicle/iPrOH combination; 20–200 particles, selected randomly, were usually counted from each photomicrograph.

2.10. Circular dichroism

CD spectroscopy was used to monitor changes in secondary structure of PI-PLC induced by iPrOH. CD spectra of 0.2 mg/ml PI-PLC in 10 mM borate buffer, pH 8.0, were obtained between 245 and 190 nm in 1 mm path length cells using an AVIV Circular Dichroism model 202 spectrometer. A baseline scan of the cell filled with buffer (and iPrOH) was subtracted from the spectra obtained with protein. The percentage of secondary structure elements was estimated using the program CDNN [18,19]. Temperature-induced changes in the ellipticity at 222 nm (averaging time of 1

s) were monitored at 1° intervals from 10 to 60 °C for a 0.2–0.3 mg/ml protein solution in a 1-mm-path-length quartz cell. The temperature was maintained to within ± 0.2° with 1 min equilibration time between each increase in temperature. The thermal melting transition, T_m, of the protein under these conditions in the absence and presence of iPrOH and 50 mM *myo*-inositol (a weak inhibitor of the enzyme that does not have any absorbance above 200 nm) was determined by plotting the derivative of the $[\Theta_{222}]$ versus temperature scan; the T_m was taken to be the maximum in the derivative spectrum.

3. Results

3.1. Effect of organic solvents on B. thuringiensis PI-PLC activity toward PI vesicles

Phospholipase kinetics with their natural long-chain substrates are complicated because the phospholipids are presented in a two-dimensional matrix and the water-soluble enzyme must interact with the interface as well as an individual substrate molecule. Not only are changes in the protein structure important, but perturbations of the 2D-matrix can affect the observed specific activities. For PI-PLC, cIP serves as a water-soluble substrate that can be used to monitor for effects of nonsubstrate interfaces on enzyme activity. It was observed previously [13] that *B. thuringiensis* PI-PLC activity toward cIP was enhanced in the presence of organic solvents (iPrOH, DMSO, and DMF) (Fig. 1A). As shown in Fig. 1B, the phosphotransferase activity of the enzyme toward small unilamellar PI vesicles was also significantly enhanced by these water-miscible organic solvents, although the mole fraction of each solvent needed for maximum activation was slightly different. Higher mole fractions of cosolvents reduced the activation of PI-PLC, but observed phosphotransferase and phosphodiesterase activities with solvents were still much higher than in the absence of organic solvents. Thus, similar cosolvent effects for both steps of the PI-PLC reaction were observed. PI LUVs are poorer substrates than SUVs for the enzyme, presumably because of the tighter packing of the lipids in the more planar bilayers (Table 1). However, iPrOH increases PI-PLC activity toward both PI bilayer systems to about the same extent. In the case of PI vesicles, the organic solvent can have a pronounced effect on the substrate structure and this could affect the types of interactions PI has with the enzyme.

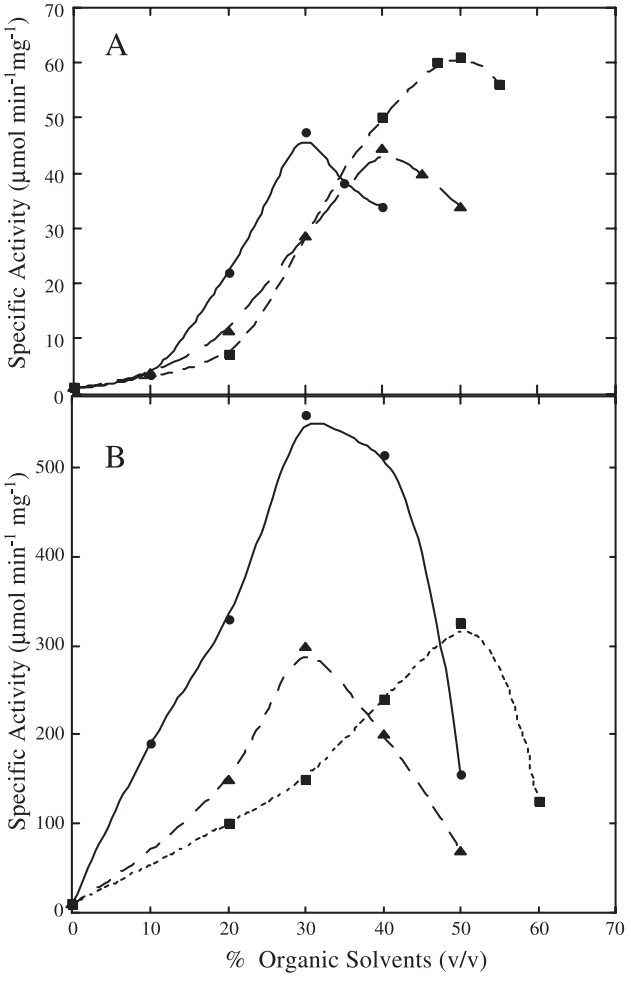

Fig. 1. Effect of iPrOH (●), DMF (▲), and DMSO (■) on the specific activity of PI-PLC toward (A) 8 mM cIP and (B) 6 mM PI SUVs. The data in A are from Ref. [13].

Table 1
PI-PLC phosphotransferase activity toward PI in the absence and presence of 30% iPrOH[a]

Substrate (mM)	Aggregation state	Additive	Specific activity[b] (μmol min^{-1} mg^{-1})	Rate enhancement
diC_4PI (1.5)	monomer	–	0.18	
	monomer	30% iPrOH	12	67
diC_8PI (1.25)	micelle	–	0.32	
	–[c]	30% iPrOH	29	91
PI (8.0)	LUV	–	0.37	
	monomers/small aggregates	30% iPrOH	820	2220
	SUV	–	8.4	
	monomers/small aggregates	30% iPrOH	920	110

[a] Assay conditions include 50 mM Hepes, pH 7.5, and the amount of enzyme added adjusted so that ≤20% PI cleavage occurs in 2 h.
[b] Errors in measuring specific activities were typically ± 15% except for very low specific activities (<1 μmol min^{-1} mg^{-1}) where the error could be as large as 25%.
[c] The addition of the iPrOH increases the CMC so that monomers coexist with micelles.

3.2. Effect of solvents on PI vesicles

To assess the importance of structural perturbations of the long-chain PI substrate to PI-PLC activation by organic

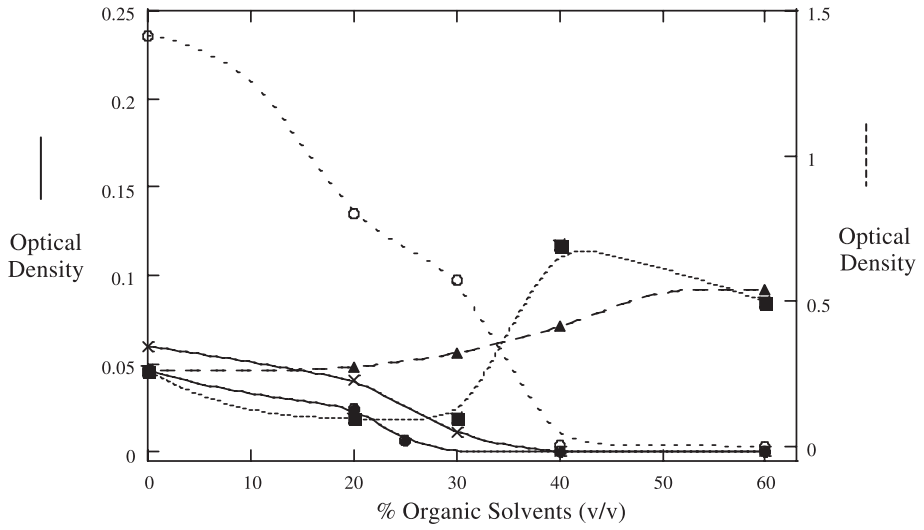

Fig. 2. Optical density (at 600 nm) of PI vesicle solution as a function of percentage of organic cosolvents: PI SUVs mixed with iPrOH (●), DMF (▲), and DMSO (■); PI/PC (1:2) SUVs mixed with iPrOH (×); PI suspensions (formed by hydrating a dry PI film with buffer and shaking) mixed with iPrOH (○). Solid lines are for OD_{600} values shown on the left axis while the dotted lines are for the OD_{600} range shown on the right axis.

solvent, a variety of physical studies of the PI/cosolvent systems were carried out. Both DMF and DMSO at moderate mole fractions caused formation of large particles (larger than 1000–2000 Å LUVs since they sediment by gravity) from PI SUVs (Fig. 2). Centrifugation of the solutions containing PI and >30% DMF or DMSO removed the large particles and produced a supernatant that had virtually no residual PI. These aprotic polar solvents are known to dehydrate protein surfaces [20]. It is likely these solvents also dehydrate the inositol head group, since they promote fusion of SUVs. Since in the absence of organic cosolvents, PI-PLC activity toward SUVs is ~20-fold higher than toward LUVs, the observation of high activity with the addition of DMF and DMSO, where very large vesicles (likely a mixture of multilamellar and large unilamellar species) are formed, is consistent with a direct effect of the cosolvent on the enzyme, since no significant fraction of smaller particles was formed. In contrast to the aprotic cosolvents, iPrOH added to the same PI SUVs decreased the optical density of the solution; MLVs of PI were also clarified by 30% iPrOH (in fact, PI dissolved in 30% iPrOH formed an easy optically clear assay system).

Kinetic activation of PI SUVs mixed with iPrOH, could reflect contributions from decreasing particle sizes and

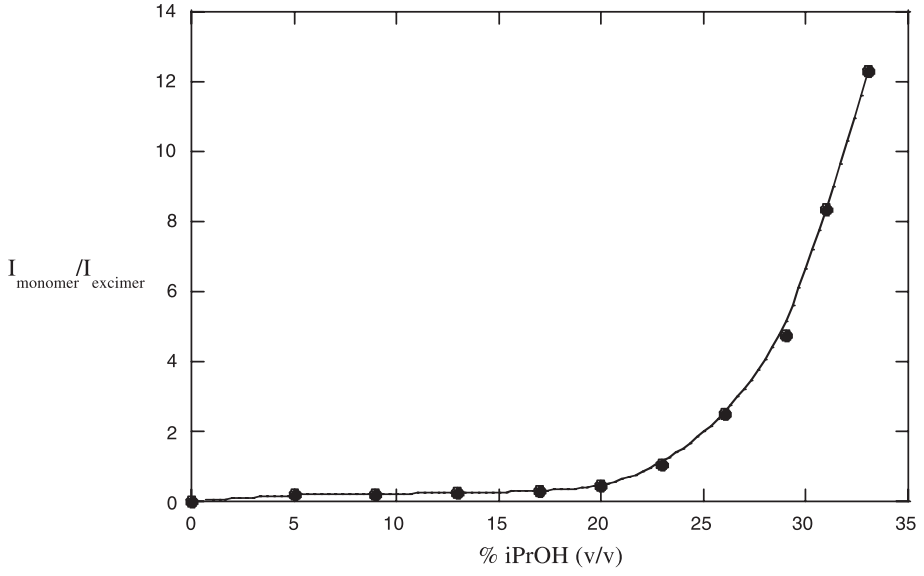

Fig. 3. Ratio of fluorescence intensities of monomer (380–390 nm) and excimer bands (450 nm) of pyrene-labeled PI (20 μM) as a function of percentage (v/v) of iPrOH. The excitation wavelength was 350 nm.

enhanced particle curvature, or isolation of very small aggregates. The generation of smaller particles could account for the fact that the specific activity of PI-PLC toward PI in the presence of iPrOH is relatively higher than that of the reaction in the presence of DMF or DMSO. The fluorescence spectrum of Pyr-PI vesicles (20 μM) is characterized by a strong excimer band at 450 nm and weak monomer bands at 380–390 nm such that the ratio of intensity of monomer at 385 nm compared to excimer is less than 0.05. If the Pyr-PI vesicles are solubilized by iPrOH and monomers are formed, the ratio of fluorescence intensity at 385 nm compared to that at 450 nm should increase. As shown in Fig. 3, an increased proportion of Pyr-PI monomer began to appear with the addition of 20% iPrOH; in 40% iPrOH, the ratio of Pyr-PI fluorescence at 380–390 nm was the same as if the Pyr-PI was solubilized by a large excess of Triton X-100 where Pyr-PI molecules are completely surrounded by detergent and few if any excimers form. Between the two amounts of iPrOH, there must exist some type(s) of aggregated PI to produce the excimer band.

Other techniques were also used to get a sense of changes in average particle sizes. The ^{31}P linewidth of PI decreased and its integrated intensity increased with the addition of iPrOH (data not shown), consistent with the generation of smaller particles. The largest changes in these two parameters occurred between 10% and 20% iPrOH. As little as 5% iPrOH added to PI SUVs or LUVs caused leakage of entrapped CF with very rapid release of CF at 10% iPrOH. For comparison, Anchordoguy et al. [21] showed that 3 M DMSO (~ 20%) added to CF entrapped in egg phosphatidylcholine vesicles did not induce vesicle leakiness.

The distribution of PI in large particles (vesicles) versus small aggregates or monomers in the absence and presence of iPrOH was also assessed with gel filtration chromatography using Sephadex G-100. PI vesicles, regardless of size,

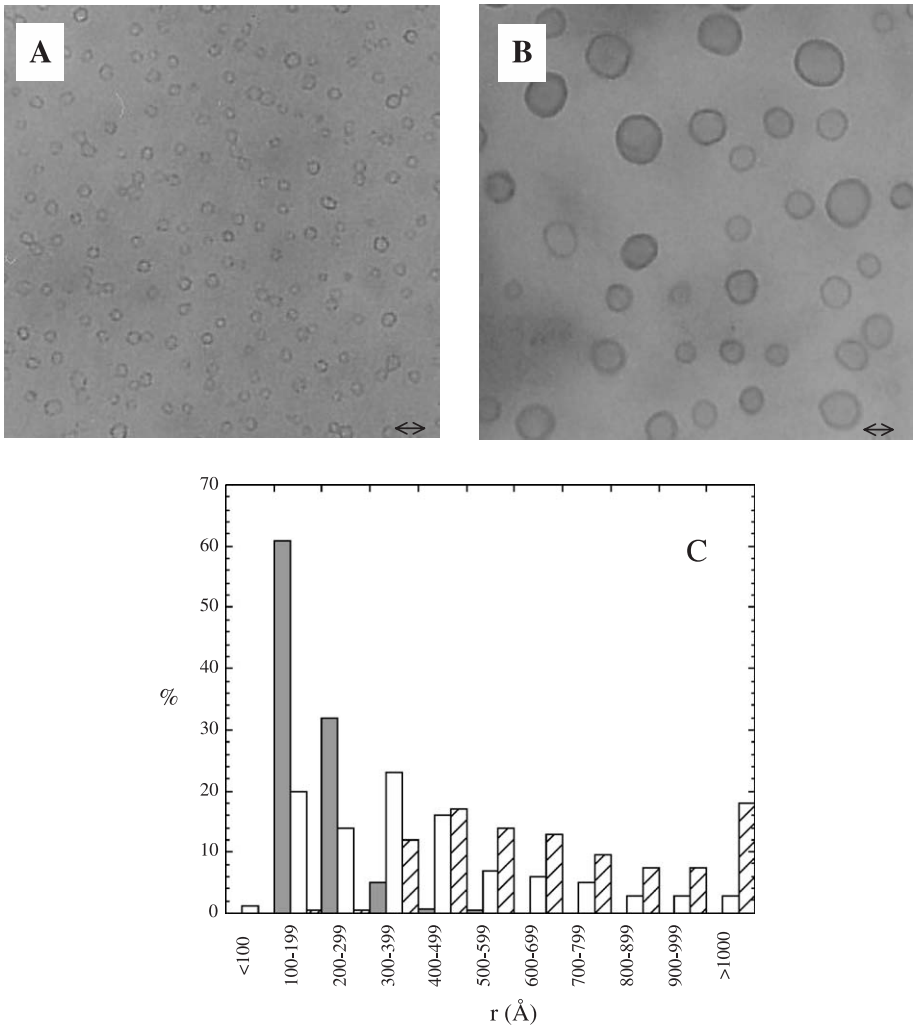

Fig. 4. Electron micrograph showing PI vesicle sizes (A) in buffer alone and (B) with 10% iPrOH. The arrows in both figures correspond to 250 Å. (C) Size distribution of PI vesicles (detected by EM) incubated with different percentages of iPrOH: vesicles of PI (1 mg/ml) in 20 mM Hepes, pH 7.5 (gray bars) and in 10% iPrOH (white bars) and PI (5 mg/ml) in 20% iPrOH (hatched bars) were stained using 2% uranyl acetate.

elute in the void volume of the column. cIP, a molecule that has no tendency to partition into a bilayer [9], is a marker for small molecules and elutes at the column volume. In the elution profile of PI in 10% iPrOH (data not shown), there was a slightly broader distribution of PI sizes with some significantly smaller particles. However, with PI in 30% iPrOH, the range of PI particles was biased toward much smaller particles. Indeed, there was some overlap of fractions containing PI and cIP indicating significant amounts of monomeric PI or very small PI particles. Clearly, iPrOH interacts differently with PI bilayers than the two aprotic cosolvents and leads to mixtures of both smaller and larger particles.

A more accurate description of the larger particles formed from incubation of PI SUVs with iPrOH was provided by electron microscopy. The distribution of PI SUVs in aqueous solution was used as a standard. In general, the diameters of most PI SUVs in aqueous solution ranged from 100 to 400 Å (Fig. 4). The addition of 10% iPrOH led to a bias with somewhat larger particles while 20% iPrOH promoted fusion to even larger particles (1000–3000 Å diameter). Since 30% and 40% iPrOH yielded mostly small aggregates (which were not detected by electron microscopy), higher concentrations of PI mixtures were applied to the grid. With those amounts of iPrOH, the few vesicles detected had diameters ranging from 2000 to 5000 Å.

3.3. Activity of PI-PLC in the presence of both activators iPrOH and phosphatidylcholine

Phosphatidylcholine activates both the cyclic phosphodiesterase and phosphotransferase activities of PI-PLC [9,10]. It has been shown that a PC monomer binds tightly to PI-PLC [22] and increases the specific activity of PI-PLC toward cIP, although a PC surface is much more effective with PC micelles enhancing the enzymatic activity for cIP cleavage 20- to 40-fold [9]. Incorporation of long-chain PC in PI vesicles was also shown to enhance PI-PLC phosphotransferase activity [10]. Since there appears to be a distinct site for PC binding, an interesting question is what happens to the enzyme when both PC and iPrOH are present simultaneously. The presence of 8 mM diC_7PC and 30% iPrOH separately led to similar values of K_m (29 and 30 mM, respectively). A comparison of PI-PLC specific activities toward 8 mM cIP with 8 mM diC_7PC or 30% iPrOH is shown in Table 2. PI-PLC specific activity was almost 2-fold higher when both activators were present. Multiple repeats of this experiment led to an activation of PI-PLC that ranged from 1.6- to 2.2-fold over iPrOH (or diC_7PC) alone. Given the solubilization of long-chain PI by 30% iPrOH, it is likely that iPrOH increases the CMC of diC_7PC dramatically such that mostly monomeric PC is present in solution under these conditions (and hence one might expect reduced activation by PC). Nonetheless, there was a synergistic effect of both activators on PI-PLC cyclic phosphodiesterase activity.

Table 2
PI-PLC activity toward 8 mM PI and cIP in the presence of activators iPrOH and phosphatidylcholine[a]

Substrate	IPrOH (30%)	diC_7PC	PC[b]	Specific activity (μmol min^{-1} mg^{-1})[c]
cIP	–	+ (8 mM)	–	60
	+	–	–	65
	+	+ (8 mM)	–	100
PI	–	–	+	10
	+	–	–	542
	+	–	+	575
	–	+ (40 mM)	–	240
	+	+ (40 mM)	–	590

[a] Assays were carried out in 50 mM Hepes, pH 7.5; the amounts of enzyme added was adjusted to produce ≤20% hydrolysis in 10 min at room temperature. The enzyme stock used for the kinetics in this table was >6 months old and had a reduced specific activity compared that was ~ 60% that newly purified recombinant PI-PLC toward PI dispersed in 30% iPrOH.

[b] SUVs were prepared for PI or PI/PC in the absence of iPrOH or diC_7PC. The PI/PC (8 mM:16 mM) vesicle sizes were distributed around 300 Å.

[c] Errors in specific activities are ≤15%.

The simultaneous effect of PC in the PI vesicles (1:2 PI/PC) and iPrOH on PI-PLC phosphotransferase activity was also measured (Table 2). As observed previously for this composition of vesicles, there was no activation of PI-PLC compared to the enzyme specific activity toward PI SUVs [10]. In contrast, the addition of iPrOH to SUVs of PI or PI/PC greatly enhanced PI cleavage. With this amphiphilic substrate, a good portion of the enhancement is likely caused by the conversion of PI from a bilayer to the smaller particles (Fig. 2). If PI-PLC phosphotransferase specific activity was compared for PI in 30% iPrOH to PI/PC in the same cosolvent, there was no synergistic effect of the two activator molecules as there was for cIP as the substrate. An alternate assay system is PI dispersed in diC_7PC micelles [9]. Again, PI-PLC phosphotransferase activity for PI/diC_7PC (1:5) with iPrOH is the same as that for iPrOH alone. For both these phosphotransferase assay systems, the increased PI-PLC activity with both activators was comparable to that for iPrOH alone. A synergistic effect of diC_7PC and iPrOH was only observed for cIP hydrolysis. This suggests that acyl chain binding of the substrate is important for optimal PI-PLC activity and that with cIP, the missing substrate acyl chains can be in part replaced by nonsubstrate PC binding to the enzyme.

3.4. PI-PLC activity toward short-chain PI

The phosphotransferase activity of PI-PLC toward the short-chain substrates diC_4PI and diC_8PI was also monitored for any enhancement by iPrOH (Table 1). It was previously observed that PI-PLC activity toward low concentrations (<2 mM) of diC_6PI exhibited a sigmoidal dependence on PI [8]. For 1–1.5 mM monomeric diC_4PI and micellar diC_8PI (1.25 mM), the specific activity was

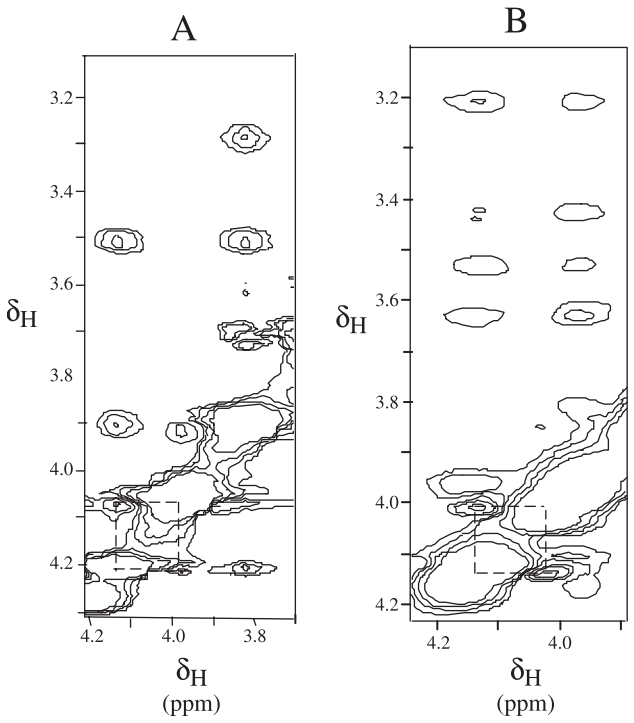

Fig. 5. NOESY contour plots for 3 mM diC$_4$PI (A) and diC$_8$PI (B) dispersed in D_2O. The dashed lines in each plot indicate the crosspeaks between the inositol C(2)–H and one of the glycerol *sn*-3 CH_2 protons.

very low (<0.5 μmol min^{-1} mg^{-1}). However, the addition of 30% iPrOH enhanced PI-PLC activity 70- to 90-fold. With that amount of cosolvent, diC$_4$PI is monomeric and diC$_8$PI will have an increased proportion of monomers (water-miscible alcohols increase the CMC of most phospholipids), so that this activation does not represent the interfacial activation exhibited for a micellar versus a monomeric substrate. The observed increase in specific activity for PI-PLC in the presence of iPrOH toward substrates that form monomers (diC$_4$PI), micelles (diC$_8$PI), and vesicles (long-chain PI) indicates that PI-PLC is activated by organic cosolvents regardless of the type of substrate.

3.5. Conformation of PI in 30% iPrOH

The conformation and dynamics of synthetic short-chain PI in water were assessed by ^{1}H NMR spectroscopy and compared to previous NMR studies of diC$_6$PI and diC$_7$PI species in D_2O [17]. Key observations in the earlier work were an NOE between a glycerol C(3)–H and the inositol C(2)–H and motional constraints on several of the inositol ring protons consistent with intramolecular hydrogen-bonding. An NOE consistent with this interaction is also seen in micellar diC$_8$PI and monomeric diC$_4$PI as well (Fig. 5A and B). This NOE observed for both short-chain PIs had a positive sign (opposite sign of the diagonal). The rest of the NOEs were all positive in the case of diC$_4$PI monomers and mostly negative in the case of micellar diC$_8$PI. This is consistent with the difference in aggregation state of the two different PI species in aqueous solution. The ^{1}H spectrum of long-chain PI solubilized in 30% iPrOH-d_8/D_2O exhibited relatively sharp resonances (Fig. 6A), although they were not as sharp as observed for monomeric diC$_6$PI

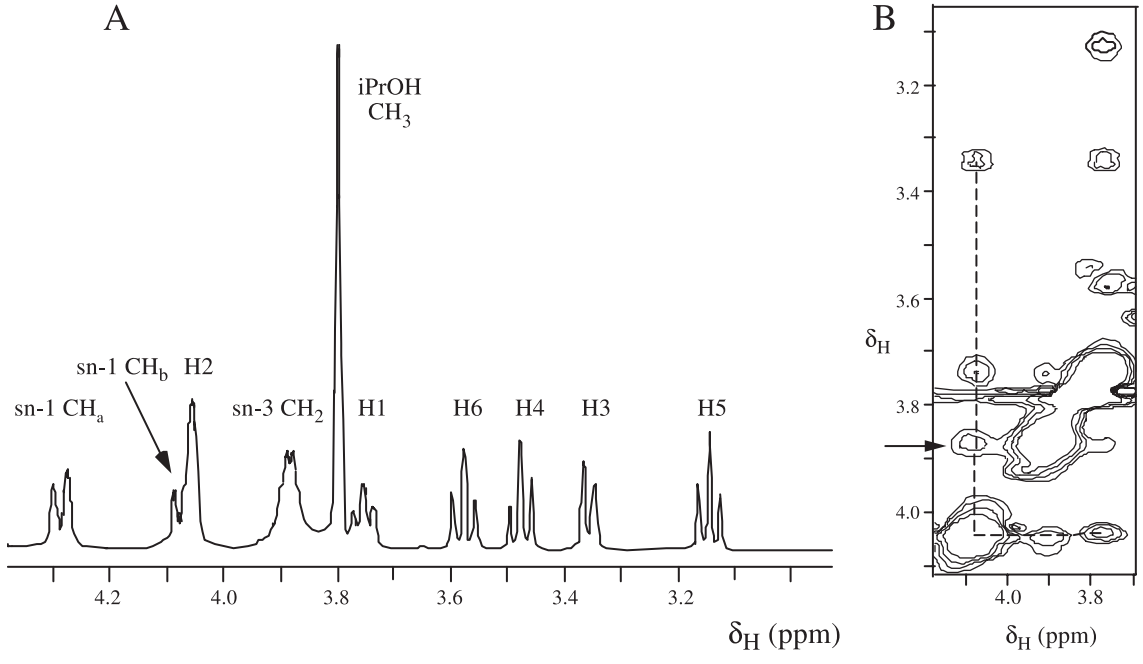

Fig. 6. (A) ^{1}H (500 MHz) spectrum of naturally occurring PI (3 mM) in 30% iPrOH-d_8/D_2O. (B) NOESY contour plot showing crosspeaks of the inositol C(2)–H with the glycerol *sn*-3 CH_2, and inositol C(1)–H and C(3)–H (the dashed line indicates these crosspeaks and the arrow specifically the interaction with the glycerol *sn*-3 CH_2).

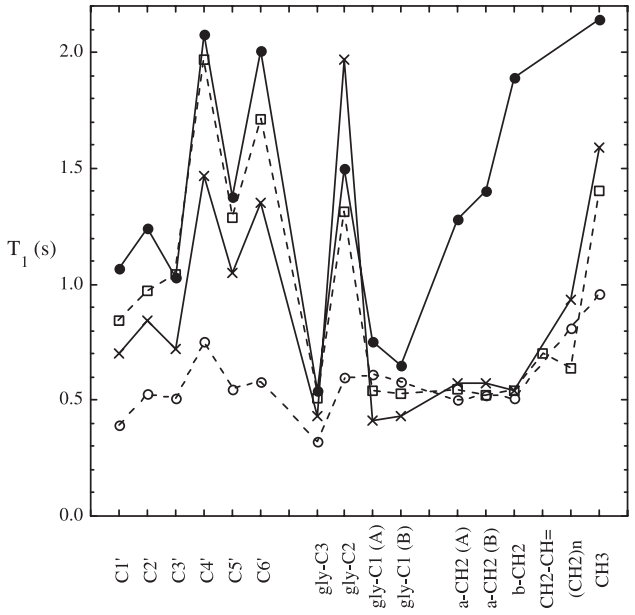

Fig. 7. Comparison of ^{1}H T_1 values for 3 mM long-chain PI (□) and 3 mM diC_8PI (×) in 30% iPrOH-d_8/D_2O, and for comparable concentrations of diC_8PI (○) and diC_4PI (●) dispersed in D_2O. The identification of the protons is indicated on the plot.

in D_2O [17]. This observation is consistent with small aggregates and fast exchange of individual PI molecules between the different species. A NOESY experiment (Fig. 6B) clearly showed inositol ring interactions (as negative NOEs). Crosspeaks reflecting the inositol C(2)–H/C(1)–H and C(2)–H/C(3)–H NOEs were clearly observed. The NOE between a glycerol C(3)–H and inositol C(2)–H was also detected in PI solubilized in the organic solvent/water mixtures. The crosspeak in the NOESY spectrum for the glycerol C(3)–H/inositol C(2)–H interaction in diC_8PI was reduced in 30% iPrOH-d_8/D_2O (data not shown). Under these conditions, the inositol C(2)–H/C(3)–H and C(1)–H crosspeaks were negative while that for the glycerol/inositol interaction was smaller and positive. Similarly, the glycerol C(3)–H/inositol C(2)–H crosspeak in diC_4PI solubilized in the iPrOH/D_2O cosolvent mixture was nearly nonexistent.

^{1}H spin lattice relaxation times of the long-chain PI in iPrOH/D_2O were also measured and compared with T_1 values for diC_8PI in the absence and presence of iPrOH. As shown in Fig. 7, the T_1's for the inositol protons of long-chain PI in 30% iPrOH were similar to those for diC_8PI in iPrOH (as well as the diC_4PI in water) but much longer than for the inositol protons of diC_8PI in water (micelles). Acyl chain T_1's of the terminal CH_3 group were longer in samples with iPrOH, consistent with small aggregates. The more pronounced change in the inositol T_1's was consistent with increased flexibility of this moiety in PI dispersed in iPrOH.

Regardless of the acyl chain length, PI in iPrOH is a flexible molecule with reduced interactions between the inositol and the glycerol backbone. However, this cannot be the sole reason for the enhanced PI-PLC phosphotransferase activity, since diC_4PI in water also has a more flexible inositol ring, but the same low hydrolysis rate as seen for diC_8PI (where the NOE between the glycerol backbone and the inositol C-2 is visible). If changes in the substrate do not correlate with cosolvent activation, the enzyme must be altered in some way—either its conformation, aggregation state, or microenvironment is altered by cosolvent.

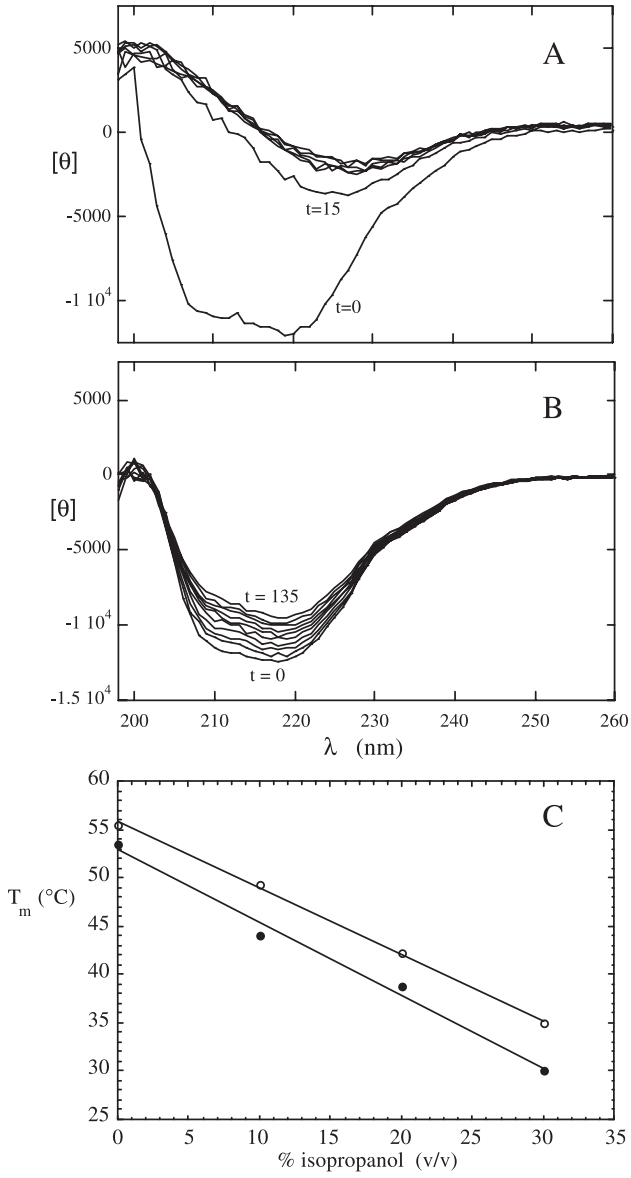

Fig. 8. CD spectra of PI-PLC (0.3 mg/ml) at 25 °C as a function of time incubation in the presence of 30% (v/v) iPrOH in the absence (A) or presence (B) of 50 mM *myo*-inositol. Successive scans are 15 min apart. (C) Dependence of the thermal denaturation temperature (T_m) of PI-PLC as measured by following the ellipticity at 222 nm as a function of temperature on the volume percentage of iPrOH in the absence (●) and presence (○) of 50 mM *myo*-inositol. Experimental conditions included PI-PLC (0.3 mg/ml) in 10 mM borate buffer, pH 8.0, and 1 min incubation times at each temperature.

3.6. Effect of organic cosolvents on PI-PLC secondary structure

Circular dichroism spectroscopy was used to assess the effect of the cosolvent on PI-PLC secondary structure. In general, secondary structure changes of proteins induced by organic solvents cannot be studied because of the strong absorbance by the organic solvents. However, iPrOH does not interfere with the CD spectrum above 200 nm, so that the effect of this cosolvent on PI-PLC secondary structure could be examined. At 25 °C, PI-PLC lost secondary structure in 30% iPrOH (Fig. 8A) as monitored by the loss of negative ellipticity around 220 nm (the increased positive ellipticity around 200 nm is likely the result of unfolded protein aggregation). The loss of structure was biphasic with most of the change occurring within the first 15 min. Loss of secondary structure due to protein denaturation is a common trait of proteins incubated in water/cosolvent mixtures where the amount of organic solvent is between 20% and 70% [23,24]. The hydrophobic interactions between organic solvents and proteins that could destabilize folded structure are known to be favored at higher temperatures [21]. When PI-PLC was incubated with 30% iPrOH at 4 °C, the protein secondary structure was stable for at least 1 week. In parallel, the activities of PI-PLC in 30% iPrOH incubated at 25 and at 4 °C for 30 min and 16 h, respectively, were determined. No activity was observed in the first case and full activity was preserved in the latter case. The thermostability of PI-PLC as monitored by an effective T_m, the midpoint of the thermal denaturation transition as measured by loss of secondary structure, decreased linearly with increasing iPrOH percentage as monitored by CD spectroscopy (Fig. 8C). With 30% iPrOH present, the PI-PLC T_m was decreased from 53.6 to 30 °C. The half-width of the transition was 7–8° regardless of the presence of iPrOH. However, at 25 °C, some unfolding was detected. Since the thermal unfolding of PI-PLC under these conditions was irreversible, the protein incubated at 25 °C would be unfolded over time as was indeed monitored by the CD spectra (Fig. 8A).

This loss of activity of PI-PLC at room temperature when the protein was incubated in 30% iPrOH appears contradictory to ^{31}P NMR assays where there was a linear response for I-1-P production from cIP (or cIP production from PI) during the 2-h time course for samples with 30% iPrOH. A possible explanation is that PI-PLC is stable in organic cosolvents when a molecule occupies the active site. To test this hypothesis, the secondary structure region of the CD spectrum of PI-PLC in 30% iPrOH was monitored in the presence of 50 mM *myo*-inositol. Although *myo*-inositol is not a good inhibitor for this bacterial PI-PLC[K_i ~ 40 mM using PI/Triton X-100 as the substrate (C. Zhou, unpublished results)], this concentration was still able to bind to PI-PLC, retard iPrOH-induced unfolding (Fig. 8B), and preserve enzymatic activity. With *myo*-inositol present, the T_m for PI-PLC in iPrOH is higher than for PI-PLC dispersed in iPrOH alone (Fig. 8C). If *myo*-inositol enhances PI-PLC thermostability in iPrOH, it is likely that cIP (and the amphiphilic substrate PI) and I-1-P do as well.

Since PI-PLC is stable in 30% iPrOH at 4 °C, one can quantify the effect of organic cosolvent on PI-PLC secondary structure. CD spectra of PI-PLC (0.30 mg/ml in 1 mm cuvette) between 190 and 265 nm in the absence and presence of 30% iPrOH were recorded at 4 °C (Fig. 9). The spectra were nearly superimposable with only minor changes. The wavelength dependence of molar ellipticity was analyzed for secondary structure content using the program CDNN 2.1 [18,19]. Clearly, any changes in backbone structure are quite small (at most 0.5–1% change). Therefore, the mechanism by which iPrOH activates PI-PLC is likely to involve small microenvironmental changes

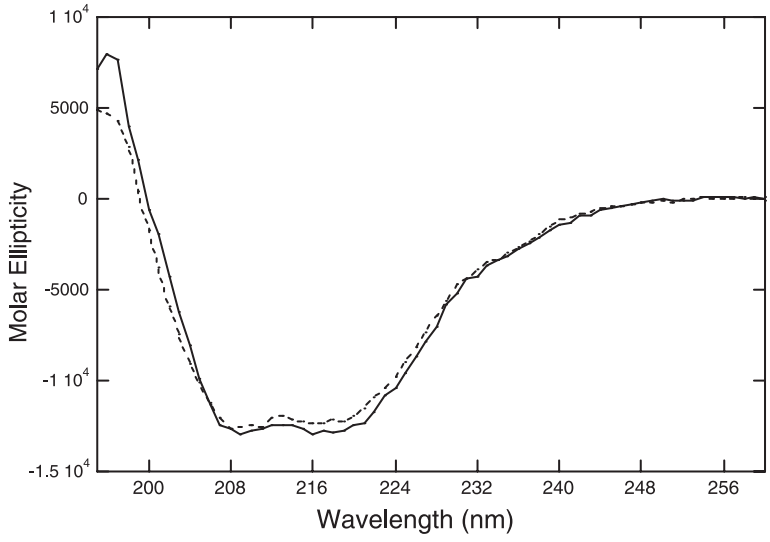

Fig. 9. CD spectra of PI-PLC (0.3 mg/ml in 1 mm cuvette) at 4 °C in the absence and presence of 30% iPrOH (dotted line) is shown.

or orientations of side chains in the active site and not any significant change in protein backbone structure.

4. Discussion

4.1. Comparison of the effect of organic cosolvents on phosphotransferase and cyclic phosphodiesterase activities

Bacterial PI-PLC has been used to synthesize inositol phosphodiesters by addition of moderate amounts of primary alcohols initiating a reaction that competes with cIP hydrolysis [25]. Thus, it is clear that alcohols can bind to the active site and replace water in the hydrolysis reaction. If the primary alcohols are replaced by a branched chain alcohol (iPrOH) or the aprotic solvents DMSO and DMF, no new inositol phosphodiester is formed, but rather the enzyme is activated for both PI and cIP hydrolysis. The observation that both phosphotransferase and cyclic phosphodiesterase activities of PI-PLC are similarly enhanced by the presence of organic cosolvents might suggest a common mechanism for this kinetic effect. However, the substrates of the two steps differ in structure: cIP is a water-soluble monomer and naturally occurring PI is amphipathic and initially presented in bilayers or detergent-mixed micelles. Cosolvents can have a profound effect on vesicle structure and this could be responsible for the PI 'activation' by cosolvent. The kinetic activation coupled with the effects of cosolvents on PI structure indicate that changes in bilayer structure, curvature, and integrity do not contribute significantly to observed activities.

The activation profiles of PI-PLC toward cIP and PI in the presence of iPrOH show a maximum activation at 30% iPrOH. The bulk of the PI in 30% iPrOH is present in small aggregates with a substantial population of monomers and only a few large vesicles. While PI-PLC activity is enhanced 5- to 10-fold toward PI presented in curved interfaces, it is decreased 4- to 6-fold for monomeric PI compared to micellar PI (the classic 'interfacial activation' [1,8]). Since the enhancement by iPrOH is comparable to that achieved by DMSO or DMF, yet the structure presentation in solution differs radically, the substrate aggregate structure must play only a small role in organic cosolvent activation of PI-PLC. In support of this is the 90-fold enhancement of PI-PLC activity toward diC_8PI by 30% iPrOH which, at the assay concentration used, is predominantly micellar in aqueous solution but will have an increased proportion of monomers in 30% iPrOH. Thus, PI-PLC is activated in organic cosolvents regardless of the type of substrate and regardless of the changes organic solvents introduce to the substrate.

4.2. Synergistic effect of diC_7PC and iPrOH on cIP hydrolysis

The presence of PC and iPrOH along with cIP leads to an enhanced PI-PLC activity toward substrate above what each activator can do alone. However, this synergistic effect is observed for cIP hydrolysis and not PI cleavage. A major difference between PI and cIP is that the first of these is an amphiphile with hydrophobic acyl chains while the second is quite polar. It is possible that acyl chain binding to PI-PLC is crucial for promoting the correct environment for substrate hydrolysis. Organic cosolvent is still effective in activating the enzyme toward PI, but there would be no additive effect with PC binding since acyl chains would already be present with PI (but not cIP) as the substrate. Both iPrOH and diC_7PC activate PI-PLC toward cIP and PI to similar extents. However, the mechanism by which activation is accomplished is different: a number of changes in the orientation of many side chains (in the active site as well as on the surface of the protein) might be expected upon the addition of cosolvent while conformational changes are likely to be more localized with diC_7PC.

4.3. PI-PLC stability in organic cosolvents

Enzymes in neat organic solvents, the area of ''non-aqueous enzymology'', have been widely studied [26,27]. These adopt a tighter structure than the one they adopt in aqueous solution. Although the structure adopted in nearly anhydrous organic solvents is stable, the activity is often low. The decrease in activity is due to the lack of the flexibility in the protein structure making the catalysis less favorable. Proteins in water/organic cosolvent mixtures (typically 20–70% organic solvent) are known to generally unfold. Protein folding is a balance between non-covalent, hydrophobic, and electrostatic interactions. When hydrophobic interactions are disrupted (as will happen with cosolvents), proteins unfold [28]. CD studies showed that PI-PLC in 30% iPrOH at room temperature unfolds over the time. Previous work that monitored an increase in the intrinsic fluorescence of PI-PLC at room temperature as the media polarity was decreased by inclusion of cosolvent (iPrOH or DMSO), is likely to reflect protein unfolding [13]. However, at low temperature (4 °C), the secondary structure and enzymatic activity of PI-PLC in 30% iPrOH were preserved for at least a week. The observation that *myo*-inositol inhibited the cosolvent-induced unfolding indicates that a molecule (even a hydrophilic one) bound in the active site of PI-PLC can stabilize it in $iPrOH/H_2O$ mixtures. A combination of lower temperature and presence of *myo*-inositol (or other substrate and product analogs) could constitute the conditions for future structural studies of PI-PLC in 30% iPrOH.

4.4. Effect of organic cosolvent on the structure of PI-PLC

If cosolvent effects on the substrate do not correlate with the observed rate enhancement for PI-PLC, then the major mechanism for cosolvent activation involves

changes in the enzyme. It has already been observed that a correlation exists between the activation of PI-PLC and two important physical properties, solution polarity and hydrophobicity, of the different cosolvents [13]. Organic cosolvent might (i) change local or global polarity of the enzyme stabilizing the surface binding site for substrate, or (ii) it may remove or change the distribution of water molecules in the active site (particularly critical for the cyclic phosphodiesterase activity) that in turn might affect k_{cat}. There is a large difference in the surface dehydrating ability of the two aprotic solvents DMSO and DMF versus iPrOH. The stronger the dehydrating character, the lower the PI-PLC K_m for cIP. Comparable amounts of DMSO and DMF are more dehydrating than iPrOH, yet all three solvents mixed with water activate the enzyme for both phosphotransferase as well as phosphodiesterase [13] chemistry.

Previous studies showed that PC activation of both cIP hydrolysis and PI cleavage is inhibited when the tryptophans at the rim of the active site, Trp47 in helix B and Trp242 in the 232–244 residue loop, are replaced by alanine [15]. Replacement of these two tryptophans also had a profound effect on PI-PLC binding to PC surfaces. This in turn translates to lower specific activities than the native enzyme. Helix B and the rim loop (residues 237–243) are not themselves part of the catalytic site. They flank the opening of an imperfect αβ-barrel that is fairly open and exposed to solvent. If organic cosolvent merely affected active site polarity, these mutants should show high phosphotransferase activity in iPrOH, yet they showed significant inhibition compared to native protein [15]. In fact, the double mutant (W47A/W242A) showed cleavage rates for PI in iPrOH considerably less that for PI/diC_7PC mixed micelles (and not much higher than cIP hydrolysis with diC_7PC present [15]). Thus, the iPrOH results with the rim tryptophan mutants compared to native protein suggest that either the orientation or dynamics of these segments of the protein are altered by cosolvent and it is this, rather than just the polarity around active site residues, that leads to activation of the enzyme. As observed by CD spectroscopy, addition of iPrOH to PI-PLC at 4 °C (well below the T_m even in the presence of iPrOH) introduces at most a very slight change in secondary structure elements indicating no significant conformational change of the protein backbone. Changes in the orientation of helix B with respect to the 237–243 loop (whose conformation could change significantly without it altering the CD spectrum), in particular in the region involving tryptophans, is likely the target of water-miscible organic cosolvent activation of this bacterial PI-PLC.

Acknowledgement

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