Phosphatidylinositol-Specific Phospholipase C Cyclic Phosphodiesterase Activity Depends on Solvent Polarity†

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ABSTRACT: Large enhancements (maximum of 82-fold in terms of enzyme efficiency, V_{max}/K_m) of bacterial PI-PLC cyclic phosphodiesterase activity were observed in the presence of organic solvents miscible in water (dimethyl sulfoxide, dimethylformamide, and 2-propanol). In general, organic solvents lowered the K_m for *myo*-inositol 1,2-cyclic phosphate (cIP) and increased V_{max} substantially. This kinetic effect was similar to that obtained with phosphatidylcholine micelles and bilayers in an aqueous assay system for cyclic inositol phosphate hydrolysis [Zhou, C., et al. (1997) *Biochemistry 36*, 347−355]. Solvent properties were examined to determine which ones correlated with the activation of PI-PLC toward cIP in each solvent. Activation correlated best with the solvent polarity as measured by $E_T(30)$; no significant correlation was observed with solution surface tension, the bulk dielectric constant (ϵ), $1/\epsilon$ (a measure of the strength of charge interactions), or the Hildebrand solubility parameter. The sigmoidal curve of the enzyme activity versus solvent polarity was consistent with the solvent promoting a transition in the enzyme from a low-activity to a high-activity form. Possible candidates for this change, including enzyme dimerization, helix B/loop stabilization, and dehydration of the active site, are discussed.

Phosphatidylinositol-specific phospholipase C (EC 3.1.4.11) enzymes have critical roles in cell signaling (Berridge, 1984, 1986; Rhee et al., 1989). Bacterial PI-PLC,[1] specific for nonphosphorylated PI and glycosyl-PI, shares some kinetic properties as well as sequence homology with the mammalian enzymes that prefer phosphorylated PI as a substrate. Hence, it can provide useful insights into the more complex systems (Rhee et al., 1989). Bacterial PI-PLC catalyzes the hydrolysis of phosphatidylinositol (PI) to yield DAG and *myo*-inositol 1,2-cyclic phosphate (cIP) (Figure 1). As shown by Volwerk et al. (1990), this same enzyme can also slowly catalyze the hydrolysis of cIP to D-*myo*-inositol 1-phosphate (I-1-P). Similar to many well-studied phospholipases, PI-PLC shows a preference for aggregated substrate over monomeric PI. With short-chain PI as the substrate, micellization leads to a 5−6 fold-increase in the apparent V_{max} for PI-PLC from *Bacillus thuringiensis* (Lewis et al., 1993). This kinetic phenomenon whereby substrate aggregation leads to enzyme activation is known as "interfacial activation". The same phenomenon for phospholipase A_2 is characterized by a large increase in the apparent V_{max}, and detailed characterization of the interfacial behavior of that enzyme has been reported (Berg et al., 1991; DeHaas et al., 1995; Gelb et al., 1995). Much of the activation observed for that enzyme is caused by anchoring the enzyme to the surface for processive catalysis on its interfacially constrained substrate.

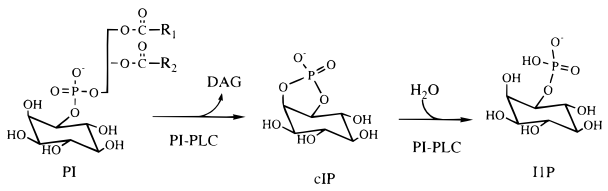

FIGURE 1: Two-step mechanism proposed for PI-PLC-catalyzed hydrolysis of PI.

The activity of PI-PLC toward cIP is unusual in that interfacial activation is observed toward a water-soluble substrate, cIP, that has no tendency to partition to surfaces. We recently reported a 20-fold enhancement in PI-PLC activity (as monitored by V_{max}/K_m) toward cIP in the presence of diC_7PC micelles (Zhou et al., 1997). For activation, the PC had to be organized at an interface, either micellar or bilayer, and the kinetic effect was specific for PC or PE surfaces. Anionic phospholipids such as PS, PA, PG, or PMe led to little significant activation. The kinetic effect of the PC interface was to decrease the cIP K_m 3-fold (from 90 to 29 mM) and to increase V_{max} 6.8-fold (from 20 to 136 μmol min^{-1} mg^{-1}). The mechanism of this interfacial activation appeared to involve a conformational change in the enzyme (Volwerk et al., 1994; Zhou et al., 1997).

Bacterial PI-PLC appears quite stable with respect to organic solvents and has been used with moderate amounts of primary alcohols (0.08−6.0 M) to synthesize inositol phosphodiesters as a competing reaction with respect to cIP hydrolysis (Bruzik et al., 1996). The efficiency of phosphodiester formation from cIP suggested that the added solvent must be localized at the enzyme active site (possibly near the DAG site) where it can compete with water. Secondary alcohols and other solvents which cannot attack bound cIP might be expected to inhibit PI-PLC activity by either blocking the accessibility of water to the substrate or

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[1] Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; PI, phosphatidylinositol; cIP, *myo*-inositol 1,2-cyclic phosphate; I-1-P, *myo*-inositol 1-phosphate; $cICH_2P$, *myo*-inositol 1,2-cyclic 2-methylenephosphonate; PC, phosphatidylcholine; diC_7PC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PMe, phosphatidylmethanol; DAG, diacylglycerol; iPrOH, 2-propanol; DMF, dimethylformamide; DMSO, dimethyl sulfoxide.

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lowering the water activity (Colombo & Bonilla-Rodriguez, 1996; Suzuki & Kanazawa, 1996). Hence, as the percentage of organic solvent is increased, enzyme activity is eventually expected to decrease. With this in mind, we examined several organic solvents that do not compete with the cIP hydrolysis reaction (e.g., DMSO, DMF, and 2-propanol) for their effect on the kinetic parameters for the PI-PLC-catalyzed hydrolysis of cIP. Surprisingly, there was a large enhancement of PI-PLC cyclic phosphodiesterase activity (a maximum 82-fold increase in V_{max}/K_m) at moderately high percentages of organic solvent. In the optimum water/organic solvent mixtures, V_{max} was increased (compared to the value of this parameter in water) and the PI-PLC K_m for cIP was decreased dramatically. Solvent activation efficiency appears to correlate with the polarity of the organic solvent/water mixture (and not with other solvent parameters). Since this solvent activation parallels the PC interfacial activation observed in aqueous systems (Zhou et al., 1997), a similar mechanism is likely to be responsible for both effects.

MATERIALS AND METHODS

Chemicals. iPrOH, DMF, and DMSO were purchased from Aldrich and were used without further purification. $cICH_2P$ was synthesized as described previously (Wu et al., 1997).

Enzymatic Synthesis and Purification of cIP. Crude soybean PI was used for the enzymatic generation of cIP by PI-PLC. One gram of crude PI was dispersed (by bath sonication) in 15 mL of Tris buffer (pH 7.5) containing 4% Triton X-100. Bacterial PI-PLC (20 μg) was added, and the reaction was monitored by ^{31}P NMR. After the PI was completely hydrolyzed to cIP (about 4 h at room temperature), further reaction (e.g., hydrolysis of cIP to I-1-P) was stopped by the addition of chloroform. DAG and unhydrolyzed lipids were removed by extraction of the aqueous solution with a chloroform/methanol mixture. cIP, in the aqueous phase, was further purified by elution from an AG1-X8 anion exchange column. Five grams of AG1-X8 resin (chloride form, 100−200 mesh), purchased from Bio-Rad, was used for about 100 mg of crude cIP. The resin was converted to its formate form by rinsing with 20 bed volumes of 1 N NaOH, washing with deionized water to neutral pH, and elution of 2 bed volumes of 1 M formic acid, followed by washing with deionized water to pH 7. The crude cIP was applied to the column and eluted with 10 mM formic acid and 40 mM ammonium formate with a linear flow rate of about 0.5 cm/min. About 110 mg of pure cIP was obtained from crude PI in this fashion.

Purification of PI-PLC. PI-PLC, isolated from culture supernatants of *Bacillus subtilis* (BG2320) transfected with the PI-PLC gene from *Bacillus thuringiensis*, was purified by modification of the protocol of Low et al. (1988) (Zhou et al., 1997). The enzyme purity was checked by SDS gel electrophoresis, and its concentration was determined by Bradford assay. The enzyme was stored at −20 °C in 50% glycerol and 20 mM Tris buffer at pH 8.5.

^{31}P NMR Assays of PI-PLC. ^{31}P NMR parameters were based on those previously used by Griffith and co-workers (Volwerk et al., 1990) and Zhou et al. (1997). ^{31}P NMR (202.3 Hz) spectra were acquired using a Varian Unity 500 spectrometer. For all kinetic runs, a control spectrum (t = 0 min) was performed prior to the addition of enzyme. One microgram of PI-PLC [freshly diluted from its concentrated stock solution with 20 mM Tris buffer (pH 8.5) containing 0.1% BSA] was added to initiate hydrolysis, and an arrayed experiment was initiated. cIP hydrolysis rates were measured from the integrated intensity of the I-1-P resonance as a function of incubation time, typically 1−2 h, at 30 °C.

Protein Fluorescence Measurements. The steady-state intrinsic fluorescence of PI-PLC was measured in 50 mM Hepes buffer at pH 7.5 with a Shimadzu RF5000U spectrofluorimeter containing a xenon light source. The temperature was maintained at 10 °C by a RM6 Lauda water circulator. The excitation wavelength was 290 nm with both excitation and emission slit widths set at 3 nm. The excitation slit was shut until right before recording the fluorescence in order to avoid bleaching of the fluorophore. The fluorescence of PI-PLC was reasonably stable with an error estimated to be about 10% from independent measurements. The fluorescence intensity was expressed as $\delta I = (I - I_o)/I_o$, where I_o is the protein fluorescence intensity at 340.8 nm of 0.5−2.0 μM PI-PLC in aqueous solution (in this concentration range, PI-PLC fluorescence intensity was linear with concentration) and I is the fluorescence intensity of the protein at the same concentration in the presence of organic solvents. The shift in the fluorescence emission maximum in the presence of DMSO or iPrOH was less than 1 nm.

Measurement and Calculations of Fundamental Properties of Water/Solvent Mixtures. The surface tension for each solvent mixture used in the enzyme assays was measured with a Fisher model 21 Surface Tensiomat utilizing the du Nouy ring method (Findlay, 1972). Dielectric constants (ϵ) of mixtures of water and organic solvents were obtained from the literature (Åkerlof, 1932; Travers & Douzou, 1974) and corrected to 30 °C using $\log \epsilon = a + bT$, where T is the temperature in degrees Celsius (Travers & Douzou, 1974). The solvent polarity as reflected in experimental $E_T(30)$ values for all the solvent mixtures was obtained from the literature (Ortega et al., 1996). The hydrophobicity values as measured by log(P) for pure solvents were obtained from the literature (Laane et al., 1987). Log(P) values for solvent mixtures were calculated by the following semiempirical equation (Laane et al., 1987):

$$\log P_{mixture} = X_1 \log P_1 + X_2 \log P_2$$

where X_1 and X_2 are the mole fraction of component 1 and 2, respectively. To estimate the Hildebrand parameter, δ_h, for solvent mixtures, the value for each pure solvent was weighted by its mole fraction:

$$\delta_h = X_1\delta_{h1} + X_2\delta_{h2}$$

The Hildebrand solubility parameter of pure solvents was calculated on the basis of the following equation for the density of cohesion energy, in turn proportional to the cavity formation enthalpy of the solvent (Batov & Korolev, 1992):

$$\delta_h^{\,2} = \frac{\Delta\mathrm{Hvap} - RT}{V}$$

where ΔHvap is the vaporization enthalpy of the solvent and V is its molar volume.

RESULTS

Effect of Organic Solvents on PI-PLC Cyclic Phosphodiesterase Activity. In the absence of an interface, cIP is a

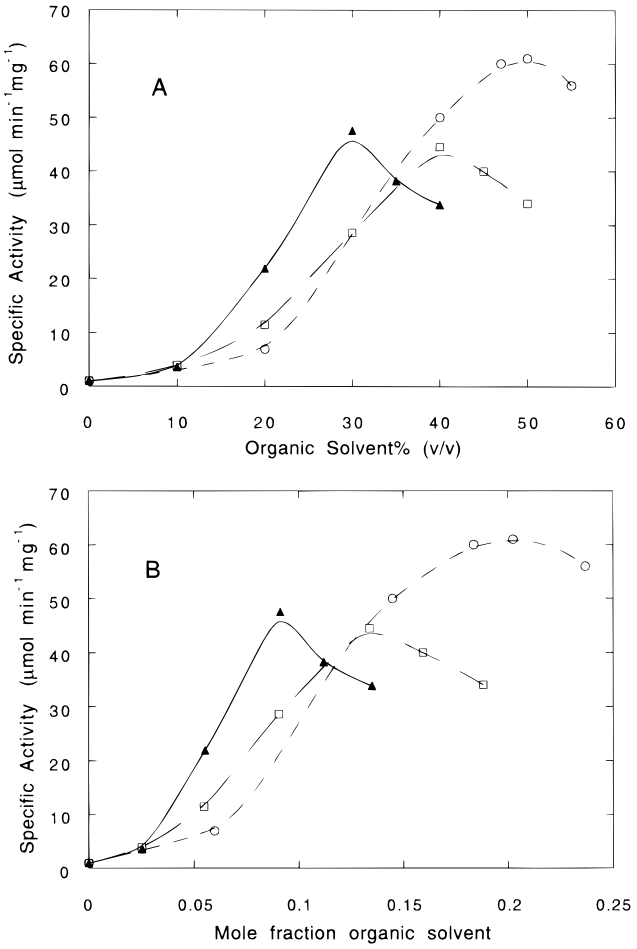

FIGURE 2: PI-PLC cyclic phosphodiesterase activity as a function of (A) percent (v/v) and (B) mole fraction of organic solvent: (▲) iPrOH, (□) DMF, and (○) DMSO. Reaction conditions include 10 mM cIP, 50 mM Hepes buffer (pH 7.5), 1 μg of PI-PLC, and 30 °C.

poor substrate for PI-PLC (Zhou et al., 1997). At a concentration of 10 mM, cIP is well below its K_m of 90 mM. Since the enzyme exhibits cooperativity with respect to cIP hydrolysis with a Hill coefficient of 2, the PI-PLC activity is quite low under these conditions (Zhou et al., 1997). PI-PLC cyclic phosphodiesterase activity toward 10 mM cIP was assayed with increasing amounts of organic solvents miscible in water (DMSO, DMF, and iPrOH). Dramatic enhancement of activity was observed for all three organic solvents with an eventual decrease in activity at high percent or mole fraction of organic solvent (Figure 2). The maximum activity and the mole fraction of solvent needed to achieve it depended on the identity of the solvent. The cIP molecule is monomeric and is not aggregated by the presence of organic solvents; conformational changes in this constrained cyclic molecule are also minimal. Hence, the sigmoidal curves in Figure 2 suggest a conformational change in the enzyme induced by the presence of the organic solvents. Given the observation that all the organic solvents miscible with water that were examined activated PI-PLC, there may be a common physical parameter for these organic solvent/water mixtures that controls the activity of PI-PLC.

Correlation of PI-PLC Activation with Experimentally Determined Solvent Parameters. PI-PLC is a lipolytic enzyme and needs to bind to an interface for full activation toward cIP in an aqueous assay system (Zhou et al., 1997). The profile for this unusual activation in each organic solvent can be compared to experimental parameters used to characterize solvents. Among these are surface tension (τ), the bulk dielectric constant (ϵ), polarity as measured by the $E_T(30)$ parameter, which is defined as the excitation energy of the $E_T(30)$ dye in the specific solvent, hydrophobicity as measured by log(P) where P is the partition coefficient that describes the water/solvent system with octanol as the reference, and the Hildebrand solubility parameter, a fundamental property of liquids that is related to the cohesive energy density of the solvent (Hildebrand & Scott, 1970; Lin & Nash, 1993; LaPack et al., 1994). If the activation is indicative of a particular solvent property, then the profiles for all three solvents should collapse to a single curve.

For several lipolytic enzymes acting on phospholipid substrates, the surface tension of the assay medium may be an important factor for enzyme activity. Indeed, the activity of several mammalian PI-PLCs, including the β, γ, and δ isoforms, has been reported to be regulated by the surface pressure of the substrate monolayers (Hirasawa et al., 1981; Boguslavsky et al., 1994; James et al., 1997). However, as seen in Figure 3A, there was no correlation between PI-PLC cyclic phosphodiesterase activity and surface tension of the different assay mixtures. While the surface tension could be important for a lipolytic enzyme acting on aggregated substrate because the enzyme must do work to gain access to its substrate (Boguslavsky et al., 1994; James et al., 1997), it is obviously not the case for PI-PLC hydrolysis of the monomeric substrate cIP as shown in Figure 3A.

The bulk solvent dielectric constant is another parameter that might be correlated with PI-PLC activation. The medium dielectric constant, or $1/\epsilon$, could be critical for enzyme activity in aqueous media because virtually all noncovalent interactions (including hydrophobic interactions, salt bridges and H bonds, etc.) in proteins are electrostatic in origin and hence, according to Coulomb's law, are inversely dependent on the dielectric constant of the microenvironment. Dielectric constants for the water/organic solvent mixtures used in assays were obtained from the literature (Åkerlof, 1932; Travers & Douzou, 1974) and plotted versus PI-PLC activity toward cIP (Figure 3B); $1/\epsilon$ for the different solvent mixtures was also plotted versus PI-PLC cyclic phosphodiesterase activity (Figure 3C). There was no obvious correlation between the different solvents. In fact, this parameter enhanced the different efficiencies of the three solvents. Therefore, the bulk solution dielectric constant does not control PI-PLC activity.

Another solvent property that did not correlate with the solvent activation (Figure 3D) was the Hildebrand solubility parameter, δ_h. The square of δ_h is equal to the cohesive energy density, which is directly related to the total molecular interaction energy of liquids. The Hildebrand solubility parameter of a solute is estimated by measuring the solubilities of the solute in solvent mixtures or in a series of solvents that cover the range of interest. The solubility parameter of the solvent or solvent mix that yields the maximum solubility will be equivalent to the solubility parameter of the solute (Lin & Nash, 1993). Hildebrand solubility parameters have been reported to correlate well with membrane permselectivity (Lapack et al., 1994). Because the Hildebrand solubility parameter is directly related to the molecular interaction energy of liquid, it could be important for enzyme solvation and, therefore, regulate enzyme activity. However, we found no correlation between the three different organic solvents in activating PI-PLC hydrolysis of cIP as shown in Figure 3D.

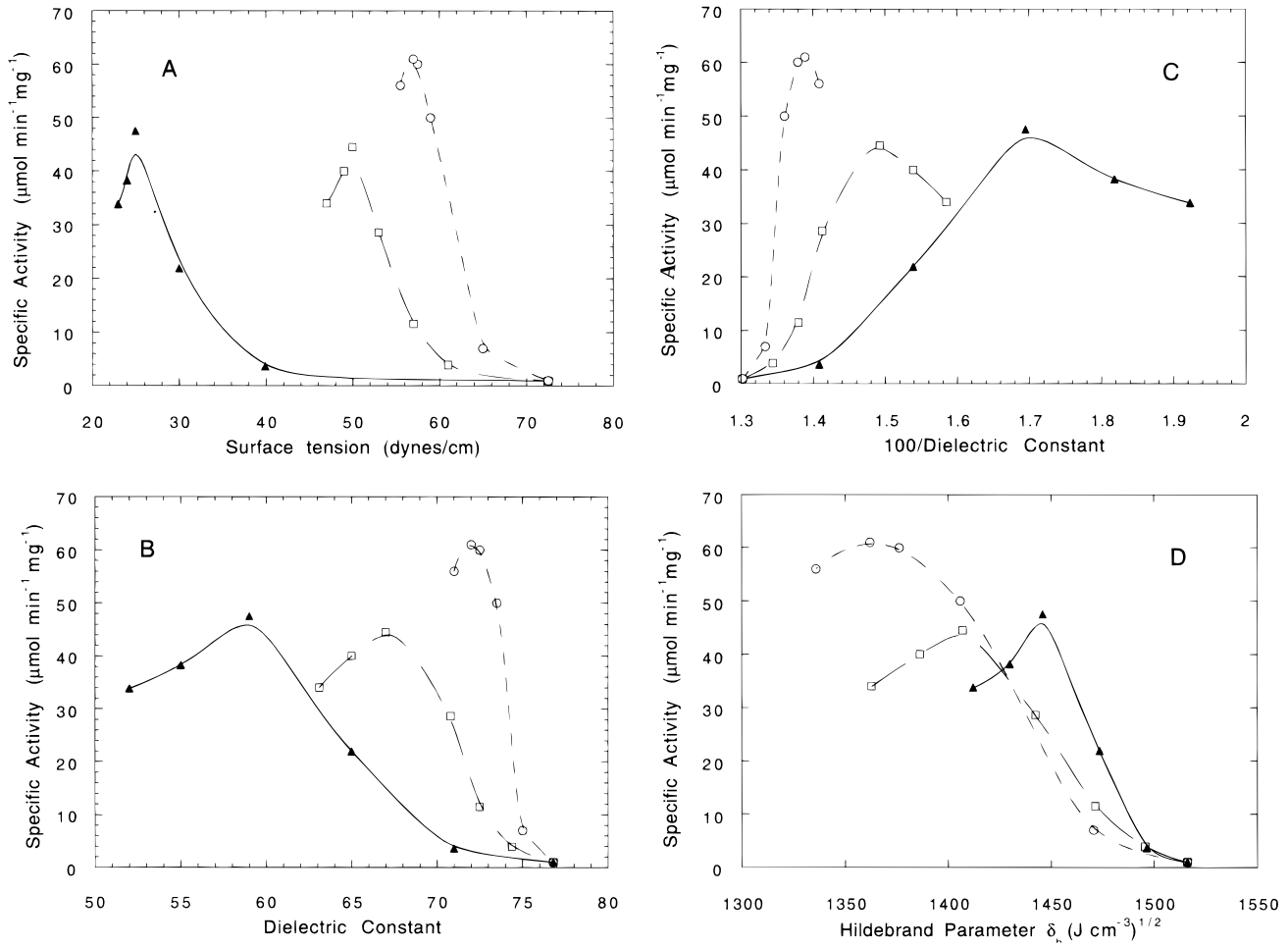

FIGURE 3: PI-PLC cyclic phosphodiesterase activity toward 10 mM cIP as a function of mixed solvent (A) surface tension, (B) dielectric constant ϵ, (C) $1/\epsilon$, and (D) Hildebrand solubility parameter δ_h. Assay conditions are the same as in Figure 2 with iPrOH (▲), DMF (□), or DMSO (○) as the organic solvent.

A critical parameter that collapsed the three solvent profiles to a single curve was the polarity of the water/solvent mixture as measured by $E_T(30)$ values. $E_T(30)$ is defined as the excitation energy of a betaine dye, 2,6-diphenyl-4-(2,4,6-triphenylpyridin-1-io)phenolate [Dimroth et al., 1963; for a recent review see Reichardt (1994)]. The $E_T(30)$ value is a good measure of solvent polarity that can be experimentally determined. Its value in many binary solvent systems has been documented (Ortega et al., 1996; Bosch & Roses, 1996; Acree, et al., 1995; Roses et al., 1995; Mancini et al., 1995). The $E_T(30)$ parameter is a much better polarity indicator than the dielectric constant (ϵ) for solvent effects because solvent/solute interactions take place on a microscopic level within a structured discontinuum consisting of individual solvent molecules capable of mutual solvent/solvent interactions. Therefore, the static dielectric constant approach to medium effects has often failed to correlate with the observed physical parameters (Reichardt, 1994). In contrast, the $E_T(30)$ parameter is an experimentally determined solvent polarity parameter, and its value for a binary mixture has been shown to fit the preferential solvation mode (Ortega et al., 1996). As shown in Figure 4A, all three solvents show an optimum around an $E_T(30)$ value of 56.5 with a half-activation at an $E_T(30)$ of 58.5. The activation is still sigmoidal in shape, suggesting a cooperative process is triggered by the change in solvent polarity.

The other physical parameter that exhibited a good correlation with enzyme activity in water/organic solvent mixtures was the hydrophobicity of the medium as measured by log(P), where P is the partition coefficient for the solvent in an octanol/water two-phase system (Figure 4B). Maximum activation occurs at a log(P) value of -2.61 ± 0.03. The fit is not quite as good as the $E_T(30)$ correlation. A caveat associated with the log(P) values is that we have used linear combinations of pure solvent values to characterize the binary solvent mixtures. If the two solvents mix nonideally, then this correlation may not be as clear-cut. Clearly, the solvent polarity (or hydrophobicity) seems to be a better predictor of PI-PLC activity in mixed media.

Organic Solvents Alter the Kinetic Parameters of PI-PLC. Both V_{max} and K_m for the cyclic phosphodiesterase activity were determined in water/solvent mixtures that corresponded to the maximum activation by each organic solvent. Figure 5 shows the dependence of PI-PLC specific activity on cIP concentration for 30% iPrOH, 40% DMF, and 50% DMSO (these correspond to 0.091, 0.13, and 0.20 mole fraction for iPrOH, DMF, and DMSO, respectively). V_{max} and K_m values are summarized in Table 1. For all the mixed solvent systems, V_{max} increased to a minimum of 4 to a maximum of 9 times the V_{max} for cIP hydrolysis in water. Similarly, K_m for cIP decreased in all solvent systems; this parameter showed a greater range of change (a 3−21-fold decrease). Interestingly, the kinetic parameters of PI-PLC cyclic phosphodiesterase activity in 30% 2-propanol were almost identical to those in an aqueous assay system with diC_7PC present to activate the enzyme (Zhou et al., 1997). 2-Propanol (30%) and diC_7PC (8 mM) both increased V_{max} dramatically and decreased K_m roughly 3-fold. Kinetic

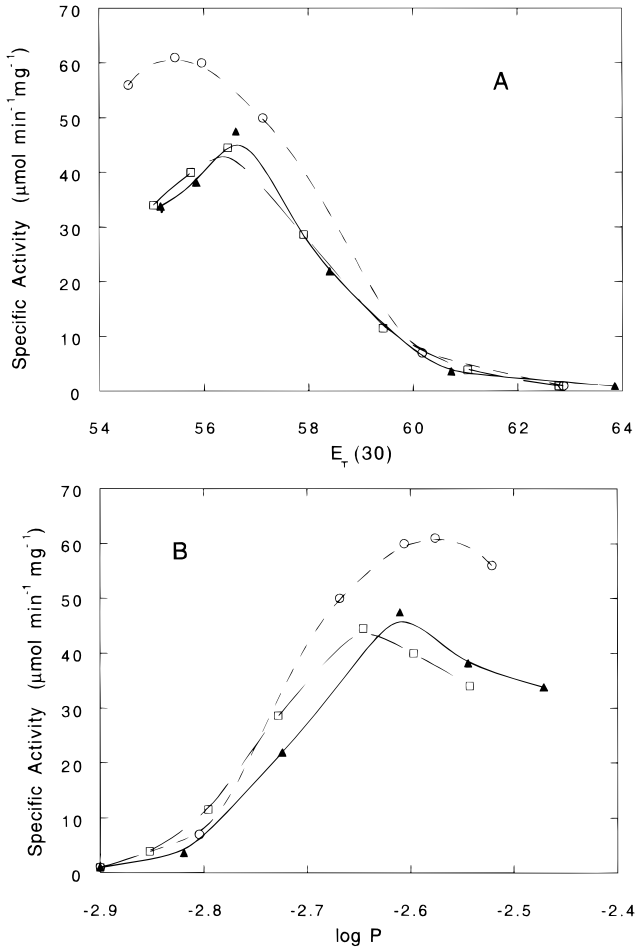

FIGURE 4: PI-PLC cyclic phophodiesterase activity toward 10 mM cIP as a function of mixed solvent (A) polarity as monitored by $E_T(30)$ and (B) hydrophobicity as measured by log(P). Assay conditions are the same as in Figure 2 with iPrOH (▲), DMF (□), or DMSO (○) as the organic solvent.

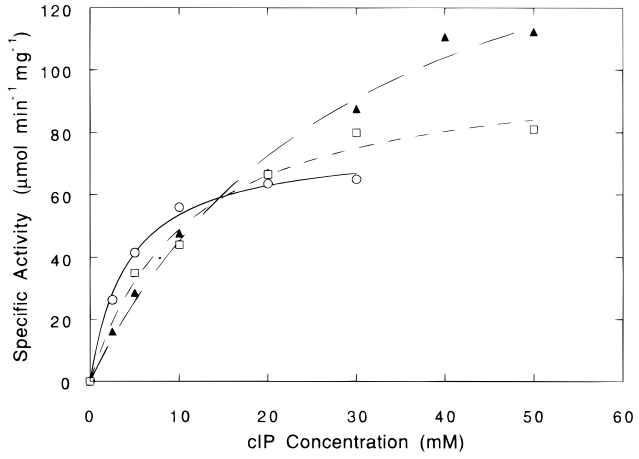

FIGURE 5: PI-PLC cyclic phophodiesterase activity as a function of cIP concentration. Assay conditions include 50 mM Hepes (pH 7.5), 1 μg of PI-PLC, 30 °C, and a fixed concentration of organic solvent: (▲) 30% iPrOH, (□) 40% DMF, and (○) 50% DMSO.

parameters for the two organic solvents with no hydrogen bond donor showed a slightly different pattern. The K_m for cIP in 40% DMF or 50% DMSO was significantly lower than that in the diC_7PC assay system or in 30% iPrOH. This could be related to the active site hydration energy.

cICH_2P is a nonhydrolyzable cIP analog. It has been shown to be a competitive inhibitor of cIP hydrolysis by bacterial PI-PLC with a K_i of 12.3 mM in the presence of 8 mM diC_7PC (Wu et al., 1997). Without the PC interface, it is a very poor inhibitor of the enzyme (K_i > 100 mM). The effectiveness of this inhibitor can also be measured in 50% DMSO. In the water/DMSO medium, cICH_2P exhibits a K_i of 2 mM, roughly half the K_m for cIP in the same assay system. The constant ratio of K_i for cICH_2P to K_m for cIP for both solvent- and interface-activated kinetics again suggests that they share mechanistic similarities.

Table 1: Kinetic Parameters for PI-PLC Cyclic Phosphodiesterase Activity in Different Assay Systems[a]

assay medium	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	V_{max}/K_m
H_2O	20	90	0.22
H_2O/diC_7PC[b]	136	29	4.69
30% 2-propanol	183	30	6.10
40% DMF	102	10.9	9.4
50% DMSO	76.3	4.25	18.0

[a] Reaction conditions include 50 mM Hepes (pH 7.5), 1 μg of PI-PLC, and 30 °C. [b] diC_7PC (8 mM) used to maximally activate PI-PLC.

Is There a Conformational Change Associated with PI-PLC Activation? Whether a lipolytic enzyme changes its conformation by binding to an interface is difficult to assess because of the difficulties in obtaining protein structures in the presence of lipid interfaces. However, previous studies (Volwerk et al., 1994) have suggested that bacterial PI-PLC undergoes a conformational change when binding to an interface. The intrinsic fluorescence of bacterial PI-PLC increased when an interface (notably a short-chain PC or detergent micelle) was added. If this change is critical to enzyme activity, then a similar fluorescence change should occur in response to added organic solvent. The dependence of fluorescence intensity on solvent polarity [as measured by $E_T(30)$] should be sigmoidal and parallel to the dependence of enzyme activity. Interestingly, as shown in Figure 6, the intrinsic protein fluorescence is nearly linear with mole fraction of organic solvent (Figure 6A) as well as linear with log(P) (Figure 6B). However, the intrinsic protein fluorescence is not linear with $E_T(30)$ as shown in Figure 6C. This is consistent with organic solvents inducing a conformational change of the enzyme at a polarity of 58 as measured by the $E_T(30)$ parameter. The linear relationship between the intrinsic protein fluorescence and hydrophobicity as measured by log(P) suggests that tryptophans on the surface relay solvent hydrophobicity changes first and foremost. Any conformational change involving specific protein residues is secondary to the solvent effect on these fluorophores. Alternatively, log(P) is not a good indicator of solvent effects for PI-PLC intrinsic fluorescence, or the calculation of log($P_{mixture}$), which assumes ideal mixing, could be in error. The biphasic behavior of PI-PLC intrinsic fluorescence intensity versus polarity as measured by $E_T(30)$ can only be explained by an enzyme conformational change associated with the medium $E_T(30)$, which is consistent with the enzyme specific activity versus $E_T(30)$ plot. Solvent polarity [as measured by $E_T(30)$] appears to be directly involved in the transition of the protein from a low-activity to a high-activity form.

DISCUSSION

Effect of Organic Solvents on Enzyme Activity. The study of enzyme activity in nonaqueous solvents has been an active research area in recent years because of potential applications in asymmetric organic synthesis (Ke et al., 1996; Almarsson & Klibanov, 1996; Campanella et al., 1994a,b; Ikeda &

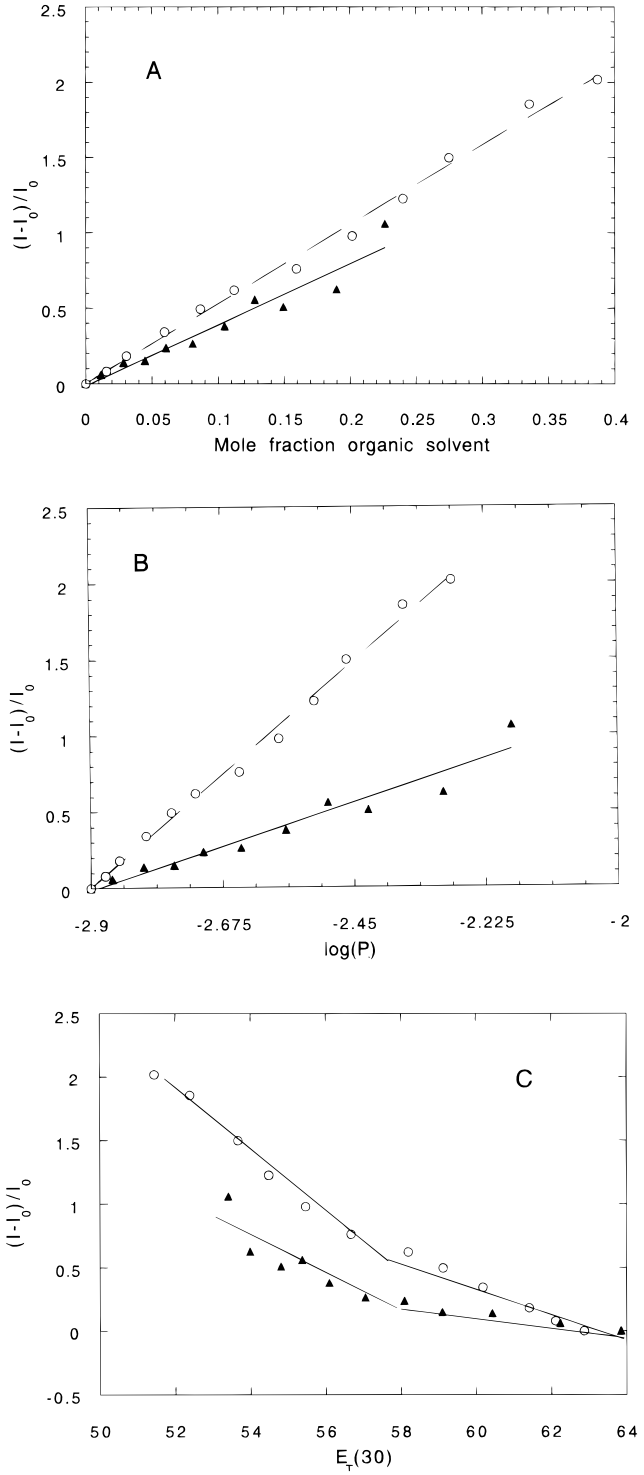

FIGURE 6: Effect of organic solvent on the intrinsic fluorescence of PI-PLC as a function of (A) mole fraction of organic solvent, (B) hydrophobicity expressed as log(*P*), or (C) media polarity as measured by $E_T(30)$. The two solvents examined were iPrOH (▲) and DMSO (○).

Klibanov, 1993; Fitzpatrick et al., 1992; Laane et al., 1987). The enantioselectivity of an enzyme in a given organic solvent can be very different from that in aqueous solution (Ikeda & Klibanov, 1993; Noritomi et al., 1996; Fitzpatrick & Klibanov, 1991; Tawaki & Klibanov, 1992). In general, organic solvents decrease the enzymatic activity, and the addition of DMSO as a "lubricating agent" to increase the enzyme flexibility and, therefore, increase enzymatic activity, has been reported (Almarsson & Klibanov, 1996). In the systems examined carefully, the only physical parameter that appears to be important for enzyme activity in nonaqueous solvents is the solvent hydrophobicity [log(*P*)]. Hydrophobicity and polarity, while not the same parameters, can often lead to similar effects. Both of these parameters correlate with solvent activation of PI-PLC with solvent polarity providing the best normalization of observed activity in different water/solvent mixtures. There appears to be an optimal solvent polarity for cIP hydrolysis. cIP is a water-soluble species that does not partition into amphipathic or hydrophobic aggregates (Zhou et al., 1997); hence, the large activation is not a preferential presentation of the substrate to the enzyme (as it is for lipase, for example). More likely, the solvent polarity controls the enzyme conformation where conformation in a broad sense includes the distribution of solvent molecules in the active site as well as backbone and side chain orientations.

It has also been said (Butler, 1979) that water/organic solvent mixtures may resemble the natural cellular microenvironments more closely than pure aqueous medium. Enhancement of enzyme activity in water/organic solvent mixtures has been reported (Batra & Gupta, 1994; Banno et al., 1988), although the extents of activation have been modest. A 2-fold maximum activation was observed for polyphenol oxidase, peroxidase, acid phosphatase, and trypsin at the optimum percentage of organic solvents (about 10%). Further increases in the percentage of organic solvent generally decreased the enzyme activity. In contrast to these effects, organic solvents have a more dramatic effect on PI-PLC activity. All the solvents examined increased V_{max} for cIP hydrolysis by 3−9-fold with the change comparable to that observed with the best activating PC interfaces. These changes are much larger than that reported for other enzymes whose substrates are monomeric.

Conformational Changes in Lipolytic Enzymes. A number of lipolytic enzymes have been shown to undergo conformational changes when bound to the appropriate interface. These can be quite extensive or relatively subtle depending on the enzyme. X-ray crystallographic studies of triglyceride lipase, a lipolytic enzyme that is very sensitive to interfacial activation, indicate a large conformational change when the enzyme binds to a substrate analog (Derewenda et al., 1992; Brzozowski et al., 1991). A 15-residue "lid" moves away from the active site, making it accessible to the substrate. The lid occupies a new position on the surface which was occupied by 18 water molecules for the inactive enzyme. In this orientation, 15 waters have been expelled. The lid in the active enzyme gains three additional interactions with the main domain of the protein molecule by moving to the new position. There are also secondary structure changes associated with this lid movement. As a result of this change, a hydrophobic area of ca. 800 Å^2 (8% of the total molecule surface) becomes exposed. It is argued that this 800 Å^2 hydrophobic area is the surface binding area, and the conformation with the inhibitor bound is exactly the same as the conformation when the lipase is bound to an interface.

Phospholipase A_2, another lipolytic enzyme sensitive to interfacial activation, shows relatively small conformational changes associated with catalysis at the interface by X-ray crystallographic and NMR investigations (Scott et al., 1990; White et al., 1990; Petters et al., 1992; Van den Berg et al., 1995a,b). Indeed, it is suggested that a nearly isostructural and isoenergetic transfer of phospholipid occurs from the substrate aggregate to the catalytic site through a well-defined, rigid hydrophobic channel (Scott et al., 1990; White

et al., 1990). More recent ^{1}H NMR structural studies have shown small conformational changes that primarily involve decreased flexibility in regions of the protein that contact the substrate (Petters et al., 1992; Van den Berg et al., 1995a,b).

The crystallographic studies of PI-PLC δ1 from rat also showed no large conformational change when the active site was occupied by IP_3 (Essen et al., 1996) or $cICH_2P$ (Essen et al., 1997). However, the authors did observe that there was an unusual hydrophobic area on the rim of the active site, which could be important for surface binding. The binding of inositol to the active site of PI-PLC from *Bacillus cereus* did not induce any notable conformational change in the enzyme as detected in the crystal structures of unliganded and inositol-bound forms (Heinz et al., 1995). As in the mammalian enzyme, the rim of the active site was formed by a hydrophobic, very weakly defined short helix (helix B) as well as by a hydrophobic loop comprising residues 237−243. A reasonable hypothesis explaining both interfacial activation by PC and solvent activation of PI-PLC toward cIP is that this hydrophobic short helix and loop behave like the lid found in triglyceride lipase. The weakly defined electron density map of the helix and the loop means the mobility of this area is extremely high. There could be an equilibrium between active and inactive forms of the enzyme. In the inactive form, the helix B/loop region could partially block the enzyme active site from the accessibility by the substrate. The introduction of an effective interface could stabilize the floppy helix and the loop, as well as form extra interactions with the protein, leaving the active site more accessible to the substrate. Addition of organic solvent could have the same effect as introducing an interface by reducing the solvent polarity and enhancing intramolecular interactions of the protein (the lid in the inhibitor-bound triglyceride lipase has three extra interactions with the main domain of the protein upon moving away from the enzyme active site). The sigmoidal nature of both solvent and interface activation curves indicates that the transition between inactive and active states of PI-PLC is highly cooperative. The biphasic behavior of the intrinsic fluorescence intensity associated with the mixed solvent polarity, $E_T(30)$, also indicates a transition between the inactive and active states of the enzyme. The lack of crystallographic support for a protein conformational change to a high-activity form could arise because inositol is a weak inhibitor and ineffective in stabilizing the more active form of the enzyme. However, with the mammalian PI-PLC, the types of small changes observed with both IP_3 and $cICH_2P$ binding at the active site make a large conformational change of the protein less likely. The lack of such crystallographic support makes this "conformational change" option a less attractive explanation.

Another type of protein conformational change that could result in enzymatic activation is protein dimerization. At the moment, there is no evidence for functional discrete aggregates of the bacterial PI-PLC (or mammalian PI-PLC δ1). However, looser transient dimers or multimers could be formed and be responsible for enhanced hydrolysis of cIP. Whatever the detailed atomic details of the switch from a low-activity to a high-activity state of PI-PLC, this change affects both V_{max} and K_m and abolishes the cooperativity in cIP hydrolysis in an aqueous system.

Hydration Energy of the PI-PLC Active Site. Triglyceride lipase does not exhibit the phenomenon of interfacial activation in organic solvent that it does in an aqueous solution (Louwrier et al., 1996). The activated state in organic solvent is ascribed to the hydrophobic effect. This observation supports the argument that organic solvent/water mixtures can effectively mimic the microenvironment of an interface. With regard to PI-PLC, addition of organic solvent could have at least two different effects: (i) it could stabilize the surface binding site (helix B and the loop) by providing the polarity of an effective interface microenvironment (as discussed above), or (ii) it could remove some hydration water from the active site, making the active site more accessible for productive binding of the substrate. A PC interface could also stabilize enzyme/substrate interactions by removing tightly bound water at the active site. When the protein-bound water is removed, the less polar active site might allow the hydrophilic cIP to bind in a productive fashion. The three organic solvents studied in detail in this paper all convert the enzyme to the high-activity form. However, there are differences in the kinetic parameters that reflect the behavior of each solvent, in particular their differing affinity for water. 2-Propanol can only form a regular hydrogen bond with water (the strength of a H bond between a 2-propanol and a water molecule should not be much different from that between two water molecules). Hence, 2-propanol is not powerful enough to dehydrate the active site. In contrast, the carbonyl oxygen of DMSO or DMF can form a stronger H bond with a water molecule. These solvents, therefore, have a slightly higher affinity for water and would be expected to remove some water molecules from the active site. Among the three, DMSO has the highest H bond energy with water and should remove the most water molecules from the enzyme active site (Zheng & Ornstein, 1996). In DMSO, cIP has the highest affinity for PI-PLC as measured by K_m. Removing water from the active site will also lower the local concentration of this reactant in the hydrolysis reaction and might also decrease V_{max}. If one compares the V_{max} for the cIP hydrolysis reaction in 2-propanol and in water with diC_7PC with that in DMSO or DMF, this is in fact observed.

$cICH_2P$, a Potent Inhibitor of PI-PLC Cyclic Phosphodiesterase Activity in 50% DMSO but Not in 100% H_2O. $cICH_2P$ is a competitive inhibitor of cIP hydrolysis by bacterial PI-PLC with a K_i of 12.3 mM in the presence of 8 mM diC_7PC (Wu et al., 1997). When the effectiveness of this inhibitor was examined in 50% DMSO, a K_i of 2 mM was obtained. This K_i is lower than the K_m for cIP in the same assay system, consistent with tighter binding of $cICH_2P$ to the PI-PLC active site. The inhibitor has an oxygen replaced by a CH_2 group, making it more hydrophobic. The tighter binding of $cICH_2P$ in water compared to cIP and in the DMSO/water mixture compared to water alone suggests that what hinders cIP binding is water already in the active site or accompanying the substrate. Removal of this solvent is necessary for good binding of soluble substrates to PI-PLC. Since many water molecules are involved, the expulsion of active site water could also have a cooperative profile and thus give rise to the sigmoidal activity versus solvent polarity curve [as well as the biphasic protein fluorescence versus $E_T(30)$]. Replacement of this active site water by organic solvent can also explain the transesterification reactions catalyzed by PI-PLC (Bruzik et al., 1996).

How does this insight into cIP hydrolysis by PI-PLC translate to understanding activity toward a lipophilic substrate (PI)? PC interfaces can also activate PI-PLC

toward PI, although the extent is not as great as with the soluble substrate (Zhou et al., 1997). The conformation of PI in aggregates (and as a monomer) is characterized by an NOE between the inositol C-2 proton and one of the glycerol *sn*-3 protons (Roberts et al., 1996). This constrains the inositol group with respect to the glycerol backbone of the lipid and may shield part of the inositol ring from extensive hydration. Thus, the basic PI structure would have less water associated with it and as such might be expected to bind more tightly to the enzyme.

Summary. Similar to the activation by PC interfaces, the addition of iPrOH, DMF, and DMSO can promote the transition of PI-PLC from a low-activity to a high-activity form better able to catalyze the hydrolysis of the water-soluble substrate cIP. We have demonstrated that media polarity as measured by $E_T(30)$ is a good predictor of the effectiveness of different solvents in activating the enzyme, as well as a good indicator of PI-PLC conformational change. Partial dehydration of the PI-PLC active site could promote better binding of cIP to the enzyme (and decrease K_m). However, the increase in k_{cat} must reflect a change in the enzyme conformation induced by solvent or the PC interface.

REFERENCES

Acree, W. E., Jr., Powell, J. R., & Tucker, S. A. (1995) *J. Chem. Soc., Perkin Trans. 2*, 529−531.

Åkerlof, G. H. (1932) *J. Am. Chem. Soc. 54*, 4125−4139.

Almarsson, O., & Klibanov, A. M. (1996) *Biotechnol. Bioeng. 49*, 87−92.

Banno, Y., Yada, Y., & Nozawa, Y. (1988) *J. Biol. Chem. 263*, 11459−11465.

Batov, D. V., & Korolev, V. P. (1992) *J. Gen. Chem. USSR 62*, 2005−2008.

Batra, R., & Gupta, M. N. (1994) *Biotechnol. Lett. 16*, 1059−1064.

Berg, O. G., Yu, B. Z., Rogers, J., & Jain, M. K. (1991) *Biochemistry 30*, 7283−7297.

Berridge, M. J. (1984) *Biochem. J. 220*, 345−360.

Berridge, M. J. (1986) in *Receptor Biochemistry and Methodology* (Venter, J. C., & Harrison, L. C., Eds.) Vol. 7, pp 25−45, Alan Liss, New York.

Boguslavsky, V., Rebecchi, M., Morris, A. J., Jhon, D.-Y., Rhee, S. G., & McLaughlin, S. (1994) *Biochemistry 33*, 3032−3037.

Bosch, E., & Roses, M. (1996) *J. Phys. Org. Chem. 9*, 403−410.

Bruzik, K. S., Guan, Z., Riddle, S., & Tsai, M.-D. (1996) *J. Am. Chem. Soc. 118*, 7679−7688.

Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Bjorkling, F., Huge-Jensen, B., Patkar, S. S., & Thim, L. (1991) *Nature 351*, 491−494.

Butler, L. G. (1979) *Enzyme Microb. Technol. 1*, 253−259.

Campanella, L., Favero, G., Sammartino, M. P., & Tomassetti, M. (1994a) *Talanta 41*, 1015−1023.

Campanella, L., Fortuney, A., Sammartino, M. P., & Tomassetti, M. (1994b) *Talanta 41*, 1397−1404.

Colombo, M. F., & Bonilla-Rodriguez, G. O. (1996) *J. Biol. Chem. 271*, 4895−4899.

DeHaas, G. H., Dijkman, R., Boots, J.-W. P., & Verheij, H. M. (1995) *Biochim. Biophys. Acta 1257*, 87−95.

Derewenda, U., Brzozowski, A. M., Lawson, D. M., & Derewenda, Z. S. (1992) *Biochemistry 31*, 1532−1541.

Dimroth, K., Reichardt, C., Siepmann, T., & Bohlmann, F. (1963) *Liebigs Ann. Chem. 661*, 1−37.

Essen, L.-O., Perisic, O., Cheung, R., Katan, M., & Williams, R. L. (1996) *Nature 380*, 595−602.

Essen, L.-O., Perisic, O., Katan, M., Wu, Y., Roberts, M. F., & Williams, R. L. (1997) *Biochemistry 36*, 1704−1718.

Findlay, A. (1972) *Findlay's Practical Physical Chemistry*, 9th ed., John Wiley and Sons, New York.

Fitzpatrick, P. A., & Klibanov, A. M. (1991) *J. Am. Chem. Soc. 113*, 3166−3171.

Fitzpatrick, P. A., Ringe, D., & Klibanov, A. M. (1992) *Biotechnol. Bioeng. 40*, 735−742.

Gelb, M. H., Jain, M. K., Hanel, A. M., & Berg, O. G. (1995) *Annu. Rev. Biochem. 64*, 653−688.

Heinz, D. W., Ryan, M., Bullock, T. L., & Griffth, O. H. (1995) *EMBO J. 14*, 3855−3863.

Hildebrand, J. H., & Scott, R. L. (1950) *The Solubility of Nonelectrolytes*, 3rd ed., Reinhold, New York.

Hirasawa, K., Irvine, R. F., & Dawson, M. C. (1981) *Biochem. J. 193*, 607−614.

Ikeda, I., & Klibanov, A. M. (1993) *Biotechnol. Bioeng. 42*, 788−791.

James, S. R., Paterson, A., Harden, T. K., Demel, R. A., & Downes, C. P. (1997) *Biochemistry 36*, 848−855.

Ke, T., Wescott, C. R., & Klibanov, A. M. (1996) *J. Am. Chem. Soc. 118*, 3366−3374.

Laane, C., Boeren, S., Vos, K., & Veeger, C. (1987) *Biotechnol. Bioeng. 30*, 81−87.

LaPack, M. A., Tou, J. C., McGuffin, V. L., & Enke, C. G. (1994) *J. Membr. Sci. 86*, 263−280.

Lewis, K., Garigapati, V., Zhou, C., & Roberts, M. F. (1993) *Biochemistry 32*, 8836−8841.

Lin, H.-M., & Nash, R. A. (1993) *J. Pharm. Sci. 82*, 1018−1026.

Louwrier, A., Drtina, G. J., & Klibanov, A. M. (1996) *Biotechnol. Bioeng. 50*, 1−5.

Low, M. G., Stiernberg, J., Waneck, G., Flavell, R., & Kincade, P. (1988) *J. Immunol. Methods 113*, 101−111.

Mancini, P. M. E., Terenzan, A., Gasparri, M. G., & Vottero, L. R. (1995) *J. Phys. Org. Chem. 8*, 617−625.

Noritomi, H., Almarsson, O., Barletta, G. L., & Klibanov, A. M. (1996) *Biotechnol. Bioeng. 51*, 95−99.

Ortega, J., Rafols, C., Bosch, E., & Roses, M. (1996) *J. Chem. Soc., Perkin Trans. 2*, 1497−1503.

Petters, A. R., Dekker, N., van den Berg, L., Boelens, R., Kaptein, R., Slotboom, A. J., & de Haas, G. H. (1992) *Biochemistry 31*, 10024−10030.

Reichart, C. (1994) *Chem. Rev. 94*, 2319−2358.

Rhee, S. G., Suh, P.-G., Ryu, S.-H., & Lee, S. Y. (1989) *Science 244*, 546−550.

Roberts, M. F., Wu, Y., Zhou, C., Geng, D., & Tan, C. (1996) *Adv. Enzyme Regul. 36*, 57−71.

Roses, M., Ortega, J., & Bosch, E. (1995) *J. Solution Chem. 24*, 51−63.

Scott, D. L., Otwinowski, Z., Gelb, M. H., & Sigler, P. B. (1990) *Science 250*, 1563−1566.

Suzuki, H., & Kanazawa, T. (1996) *J. Biol. Chem. 271*, 5481−5486.

Tawaki, S., & Klibanov, A. M. (1992) *J. Am. Chem. Soc. 114*, 1882−1884.

Travers, F., & Douzou, P. (1974) *Biochimie 56*, 509−514.

Van den Berg, B., Tessari, M., Boelens, R., Dijkman, R., Kaptein, R., & de Haas, G. H. (1995a) *J. Biomol. NMR 5*, 110−121.

Van den Berg, B., Tessari, M., de Haas, G. H., Verheij, H. M., Boelens, R., & Kaptein, R. (1995b) *EMBO J. 14*, 4123−4131.

Volwerk, J. J., Shashidhar, M. S., Kuppe, A., & Griffith, O. H. (1990) *Biochemistry 29*, 8056−8062.

Volwerk, J. J., Filthuth, E., Griffith, O. H., & Jain, M. K. (1994) *Biochemistry 33*, 3464−3474.

White, S. P., Scott, D. L., Otwinowski, Z., Gelb, M. H., & Sigler, P. B. (1990) *Science 250*, 1560−1563.

Wu, Y., Zhou, C., & Roberts, M. F. (1997) *Biochemistry 36*, 356−363.

Zheng, Y.-J., & Ornstein, R. L. (1996) *J. Am. Chem. Soc. 118*, 4175−4180.

Zhou, C., Wu, Y., & Roberts, M. F. (1997) *Biochemistry 36*, 347−355.

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