Phosphatidylinositol-Specific Phospholipase C from *Bacillus cereus* at the Lipid–Water Interface: Interfacial Binding, Catalysis, and Activation†

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ABSTRACT: Binding characteristics of phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* binding to the phospholipid–water interface were determined by spectroscopic methods and correlated with PI-PLC's catalytic properties. Binding of the enzyme to micelles and bilayers of zwitterionic phosphocholines is accompanied by an increase in the fluorescence emission from tryptophan, whereas a decrease in the emission is observed with synthetic anionic lipids containing a phosphomethanol head group. A similar decrease in the tryptophan emission is observed with phosphatidylinositol (PI) analogues containing the phospho-D-1-*myo*-inositol head group, but not with the enantiomeric L-1-*myo*-inositol. In covesicles of PI and phosphatidylcholine (PC), the rate of cleavage of PI is reduced because, as a neutral diluent, PC effectively reduces the surface concentration of PI that the bound enzyme "sees" in the interface. This permits determination of the interfacial Michaelis constant (K_M^*) as 0.26 mol fraction for PI as substrate. On the other hand, ditetradecylglycerophosphomethanol (DTPM) acts as a kinetic competitive inhibitor in the covesicles. The spectroscopic and catalytic activity data taken together show that PI-PLC binds to the interface of aqueous dispersions of phospholipids with an apparent K_d (in terms of the lipid monomers) of about 10–50 μM. However, only lipids with an anionic head group, such as phosphomethanol and phospho-D-1-*myo*-inositol, are able to bind as single molecules into the active site of the enzyme at the interface. Enantiomeric phospho-L-1-*myo*-inositol or the zwitterionic phosphocholine head group has little affinity for the enzyme at the interface. Thus, PI-PLC appears to obey the two-stage, Michaelis–Menten adaptation of interfacial catalysis, according to which the binding of the enzyme to the interface precedes the steps of the catalytic turnover at the interface. Limit estimates suggest that on PI or PI/PC vesicles the catalysis occurs in the "scooting" mode with a moderate processivity. DTPM vesicles also inhibit the activity of PI-PLC toward the synthetic water-soluble substrate *myo*-inositol 1-(4-nitrophenyl phosphate), but the activity is enhanced severalfold in the presence of vesicles of zwitterionic phosphatidylcholine. Several possible explanations of this interfacial activation are considered within the general context of the kinetic scheme for interfacial catalysis. The kinetic results for the action of PI-PLC bound to vesicles are consistent with a model in which the interface acts as an "allosteric" effector of the catalytic rate constant, k_{cat}, without affecting the substrate binding.

Phospholipases C that selectively cleave inositol phosphate from phosphatidylinositol[1] (PI) and its phosphorylated derivatives have been implicated in signal transduction pathways in mammalian cells (Rhee et al., 1989; Rhee & Choi, 1992). PI-PLC's have also been isolated from bacterial sources such as *Bacillus cereus*, *Bacillus thuringiensis*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Ikezawa, 1991). The physiological role of the secreted bacterial PI-PLCs has yet to be defined, but much of the interest in these enzymes derives from their unique ability to cleave the glycosyl-PI residues of membrane protein anchors. It has been suggested that the bacterial PI-PLC may play a role as a virulence factor (Camilli et al., 1991; Mengaud et al., 1991).

Since the naturally occurring substrates of PI-PLCs are normally present in aggregated form, i.e., in the lipid bilayer of biological membranes, the enzyme has probably evolved for interfacial catalysis. According to the minimal adaptation of the Michaelis–Menten formalism for interfacial catalysis (Figure 1), the binding of the enzyme from the aqueous phase precedes the catalytic turnover in the interface. Since this model was originally implicated (Shah & Schulman, 1967; Jain, 1973; Verger et al., 1973; Pieterson et al., 1974) and articulated (Verger & de Haas, 1976), it has been fully developed by emphasizing the processivity of the catalytic turnover in the interface (Jain & Berg, 1989; Berg et al., 1991). This analytical description accounts for virtually all

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[1] Abbreviations: deoxy-LPC, 1-hexadecylpropanediol 3-phosphocholine; DiPC8, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DH-DNS, *N*-dansyl-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine; 2H-GPC, 2-hexadecyl-*sn*-glycero-3-phosphocholine; HPC, hexadecylphosphocholine; HPI-D (or -L), hexadecylphospho-D(or L)-1-*myo*-inositol; HPM, hexadecylphosphomethanol; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; LPI16-D (or -L), 1-hexadecyl-*sn*-glycero-3-phospho-D(or L)-1-*myo*-inositol; LPI18-D (or -L), 1-octadecyl-*sn*-glycero-3-phospho-D(or L)-1-*myo*-inositol; NPIP, *myo*-inositol 1-(4-nitrophenyl phosphate); OPG, octadecylphosphoglycerol; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; PPI, D,L-1-*myo*-inositol 4-(palmitoyloxy)butylphosphonate; X_i, mole fraction of component *i* in the interface; cmc, critical micelle concentration.

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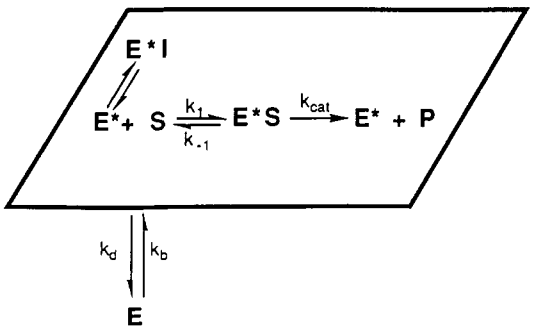

FIGURE 1: Minimal scheme for the action of lipolytic enzymes at the lipid–water interface. The essential feature of this scheme is that an interfacial binding step (E => E*), characterized by a binding rate constant k_b and a desorption rate constant k_d, precedes the classical Michaelis–Menten binding step of a substrate (E* => E*S) or competitive inhibitor (E* => E*I) molecule into the active site. Catalysis and release of products then proceed at the interface (E*S => E* + P). Because the interfacial binding does not depend on the specific recognition of a solitary substrate by the enzyme active site, and interfacial binding and desorption are not necessarily part of the catalytic cycle, the enzyme is able to act in a processive scooting mode where multiple rounds of catalysis can occur without the enzyme leaving the interface. The quantitative kinetic aspects of this scheme have been discussed in detail elsewhere (Berg et al., 1991; Jain & Berg, 1989; Jain et al., 1991a).

kinetic aspects of interfacial catalysis by secreted phospholipases A_2.

When adopted within certain experimental constraints, i.e., with the enzyme acting in a highly processive "scooting" mode, the kinetic scheme has been useful to develop suitable assay protocols (Jain et al., 1986a, d, 1991, 1992b; Jain & Gelb, 1991), to derive the primary rate and equilibrium parameters (Berg et al., 1991; Jain et al., 1991a), to establish substrate specificity (Ghomashchi et al., 1991), to deconvolute the kinetics of inhibition (Jain et al., 1989, 1991d) and activation (Jain et al., 1991c), and to establish that the chemical step is rate-limiting (Jain et al., 1992a). The success of these protocols depends upon the ability to determine experimentally the equilibrium constants of the enzyme–interface (E* to E) and enzyme–ligand complex at the interface (E*L to E* + L) and to distinguish the step for the binding of the enzyme to the interface from the steps involved in the catalytic turnover.

As a minimal scheme, this adaptation of Michaelis–Menten formalism should be useful to describe kinetic features of other lipolytic enzymes. In this article, we examine the interfacial binding, catalysis, and activation of PI-PLC from *B. cereus* by a combination of spectroscopic methods and activity assays without and with competitive inhibitors. It is demonstrated that PI-PLC binds to micelles and bilayers of phosphatidylcholines without binding of these amphiphiles to the active site of the enzyme at the interface, i.e., phosphatidylcholines act as the neutral diluents where E to E* equilibrium (Figure 1) can be monitored without complications from the steps involved in the catalytic turnover cycle. Furthermore, the binding equilibrium of the substrate and substrate analogues bound to the E* form of the enzyme is used to characterize key aspects of interfacial catalysis and activation. The results suggest that the interfacial activation of PI-PLC is due to allosteric activation of the catalytic rate constant, k_{cat}.

EXPERIMENTAL PROCEDURES

Materials. D- and L-phosphoinositols (Filthuth & Eibl, 1992), phosphocholines (Jain et al., 1991a,d), phosphomethanols and OPG (Jain et al., 1986a,b, 1991d), DH-DNS (Jain & Vaz, 1987), PG (Volwerk et al., 1986), PPI (Shashidhar et al., 1990a,b), and NPIP (Shashidhar et al., 1991) were synthesized as described in the literature. DOPC, POPC, and POPG were obtained from Avanti. Radiolabeled PI was from NEN. Phospholipid dispersions were prepared by suspending powdered lipid in water or by rehydrating lipid films dried from organic solvents under high vacuum. Codispersions of phospholipids were prepared by suspending the colyophilized film of the mixture of lipids in water. Diacylphospholipids were dispersed as vesicles by sonication in a bath-type sonicator as described elsewhere (Jain et al., 1986a). Recombinant PI-PLC from *Bacillus cereus* was isolated also as described (Koke et al., 1991). Enzyme concentrations were determined from the absorbance at 280 nm using a calculated $E_{1\%}$ of 18.4 (molar extinction coefficient = 64 000 M^{-1} cm^{-1}) or from the Bradford (1975) method with bovine serum albumin as standard. The latter method overestimates the amount of PI-PLC by 25% compared to the absorbance measurement. For consistency, all PI-PLC concentrations that were determined with the Bradford method have been corrected by multiplying them by a factor of 0.75. This correction factor was obtained by comparing both methods on serial dilutions of PI-PLC and averaging the results (A. Kuppe and J. J. Volwerk, unpublished observations).

Spectroscopy. Unless stated otherwise, all spectroscopic measurements were carried out in 10 mM HEPES and 1 mM EGTA at pH 7.5 and 23 °C. Certain experiments were also carried out with 10 mM Tris buffer at pH 8.0. Except for a small difference in the magnitude of the spectral signal, the spectroscopic behavior in the pH range 7–8 was essentially identical. The PI-PLC concentration for fluorescence measurements was kept at about 1 μM, whereas the concentration was about 15 μM for the absorbance measurements. The steady-state fluorescence measurements were carried out on an SLM 4800S or an AB2 spectrofluorimeter with a xenon light source. The excitation wavelength was 292 nm, with both excitation and emission slit widths set at 4 nm. The fluorescence intensities are expressed as $\delta F (= F/F_0 - 1)$, where F_0 is the intensity of PI-PLC alone, and F is the intensity in the presence of an additive.

Absorbance measurements were carried out on a diode array UV–visible spectrophotometer (Hewlett-Packard 8452A) with a wavelength resolution of 2 nm. Although the software supplied by the manufacturer was adequate for most of the data treatments, satisfactory quantitative scattering corrections for samples containing lipid vesicles were hindered by the intrinsic limitations of the software and hardware. Therefore, only the results with micelles are presented.

Activity Assays. The buffer used in all PI-PLC activity assays was 100 mM Hepes/NaOH (pH 7.0) containing 1 mM EDTA or EGTA. Spectrophotometric assays using the synthetic water-soluble substrate NPIP were performed as reported earlier (Shashidhar et al., 1991; Leigh et al., 1992). Unless stated otherwise, assays were performed at 1 mM NPIP, and the reaction was initiated by the addition of 150 ng of enzyme. Assays with radiolabeled PI in the form of sonicated vesicles alone or mixed with other phospholipid additives were performed essentially as described previously for PI/sodium deoxycholate mixed micelles (Volwerk et al., 1989), except that the reaction volume was reduced 5-fold to 0.1 mL and the reaction was carried out at room temperature. The specific radioactivity of the PI was 125 000 cpm/μmol. Typically, 20 μL of a vesicle suspension containing radiolabeled PI was added to 60 μL of Hepes buffer to give a final bulk concentration in the assay mixture of 2 mM PI, and the reaction was initiated by the addition of 7.5 ng of PI-PLC in 20 μL

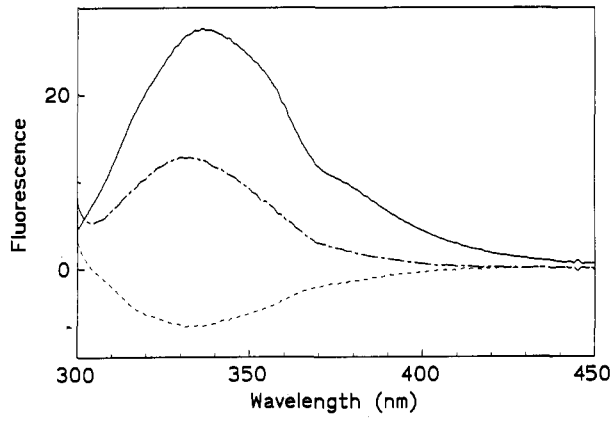

FIGURE 2: Fluorescence emission spectra of (—) 2 μM PI-PLC and difference spectra obtained in the presence of (– - –) 0.35 mM DOPC or (- - -) 0.4 mM DTPM vesicles. The latter were obtained by subtracting the spectrum of PI-PLC from the spectra obtained in the presence of the vesicles. These measurements were carried out in 10 mM Hepes and 1 mM EGTA at pH 7.5 and 23 °C. Excitation was at 292 nm.

of 0.1% bovine serum albumin. Blanks containing no enzyme were always included. After a 10- or 20-min incubation at room temperature, the reaction was terminated by the addition of 0.5 mL of chloroform/methanol/hydrochloride (66:33:1, by vol) with vortex mixing. The phases were separated by brief centrifugation in a microfuge, and 0.1 mL of the upper aqueous phase (0.2 mL total) was counted. Under these conditions, the amount of water-soluble product formed was linear with time up to 20 min. The specific activity of the enzyme acting on sonicated PI vesicles or PI/PC covesicles with $X_{PC} \leq 0.4$ was 300 μmol/min/mg of protein at 2 mM PI, pH 7.0, and room temperature, which corresponds to a turnover number of about 175 s^{-1}.

RESULTS

Fluorescence Emission Properties. PI-PLC has seven tryptophan residues that dominate its fluorescence emission spectrum, with the excitation maximum at 284 nm and the emission maximum at 337 nm. As shown in Figure 2, in the presence of the aqueous dispersion of DOPC, the emission intensity increases and the emission maximum shifts to 331 nm. Such changes were not observed with short-chain phospholipids present as solitary monomers. Thus, the difference spectra of the type shown in Figure 2 are obtained with phospholipids added as vesicles. DOPC increases the fluorescence emission, whereas DMPM decreases the emission intensity with the minimum at 333 nm. As summarized in Table 1, such spectral changes were observed with aqueous dispersions of a variety of amphiphiles; zwitterionic dispersions generally gave an increase in the emission intensity, whereas a decrease was observed with certain anionic phospholipids.

The change in the fluorescence intensity was dependent on the bulk concentration of the phospholipid. As shown in Figure 3, titration of PI-PLC with DOPC vesicles gave a concentration-dependent increase in the fluorescence intensity. Under the same conditions, the dependence on the concentration of DTPM vesicles was biphasic: a small increase at lower concentrations and a decrease at higher concentrations. These results suggest that two separate processes may be involved and that they respond differently to the zwitterionic and anionic amphiphiles. In both cases, an increase in fluorescence is seen below 20 μM. A further increase is seen with the zwitterionic phospholipids which, on the basis of the inhibition results to be described later in this article, do not interact with the catalytic active site. On the other hand, a decrease in the fluorescence emission intensity is seen with the anionic lipids (Table 1). These results suggest that the E to E* change could be a two-step process; a rigorous quantitative analysis of such complex curves was not attempted because of the presence of seven tryptophan residues in PI-PLC and a lack of understanding of the underlying processes. The values of the maximum change in the fluorescence intensity (δF_{max}) and the concentration of phospholipid necessary to obtain a 50% change in the emission intensity were obtained from the titration curves. As summarized in Table 1, zwitterionic amphiphiles induced an increase and anionic amphiphiles induced a decrease in the emission intensity. On the basis of the results to be described later, the increase is due to binding of the enzyme to the interface (E* form), whereas binding of an active-site-directed ligand to the bound enzyme (E*I form) induces a decrease in the emission intensity.

Table 1: Apparent Dissociation Constant (K_d) and the Maximum Change in the Tryptophan Fluorescence Intensity of PI-PLC (δF_{max}) in the Presence of Aqueous Dispersions of Phospholipids[a]

phospholipid	K_d (mM)	δF_{max}
DOPC	0.04	0.33
POPC	0.2	0.33
HPC	0.01	0.43
deoxy-LPC	0.05	0.5
2H-GPC	0.01	0.27
POPG	0.02	–0.14
OPG		–0.25
DTPM		–0.37
HPM		–0.3
PPI		–0.38
HPI-D		–0.13
HPI-L		0.06
HPI16-D		–0.15
LPI16-L		0.05
LPI18-D		–0.22
LPI18-L		0.25

[a] The binding isotherms with anionic lipids were comples; therefore, no attempt was made to obtain the binding parameters by curve fitting. For these amphiphiles, the δF_{max} values were obtained at 0.4 mM.

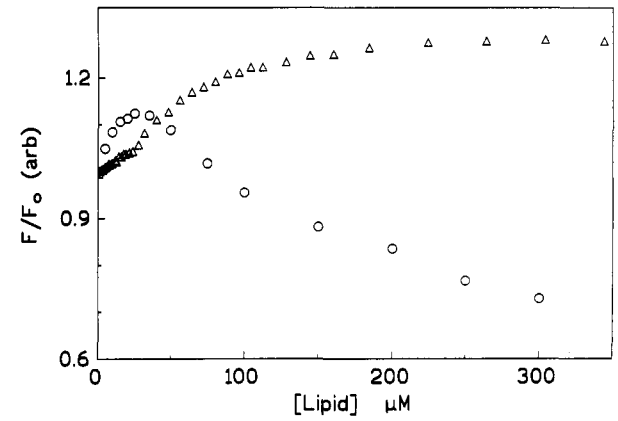

FIGURE 3: Change in the relative fluorescence emission intensity (at 335 nm) of PI-PLC as a function of the concentration of (△) DOPC or (○) DTPM added as small sonicated vesicles. Conditions were as described for Figure 2, except that the buffer was 10 mM Tris/0.1 mM EGTA at pH 7.5.

UV Absorbance Properties. Changes in the absorbance of PI-PLC were noted in the presence of the aqueous dispersions of phospholipids. The difference UV spectra with zwitterionic phospholipids were noticeably different from those obtained with anionic phospholipids. For example, the difference spectrum at saturating HPC concentrations shows the max-

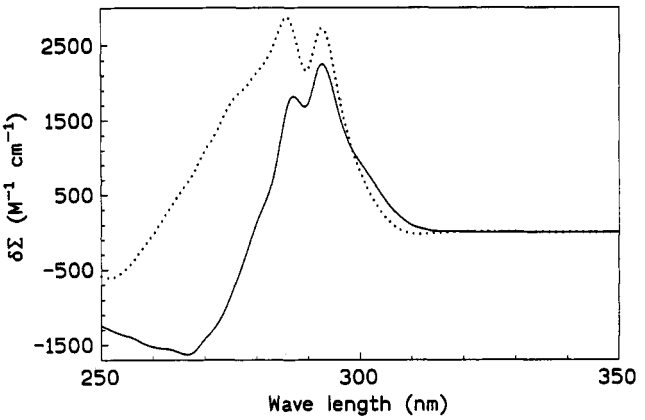

FIGURE 4: Difference absorption spectra of PI-PLC induced by the addition of 0.2 mM (—) HPC (···) HPM micelles. The change in the molar extinction coefficient is expressed on the ordinate. The reaction mixture contained 15 μM PI-PLC in 10 mM Tris and 1 mM EGTA.

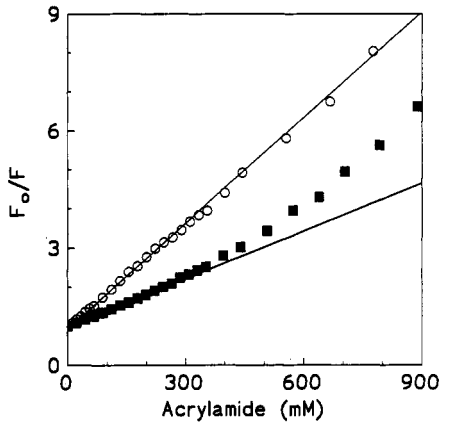

FIGURE 5: Stern–Volmer plot for the quenching of PI-PLC (○) alone or (■) with 0.12 mM DOPC vesicles by acrylamide. Other conditions were as described for Figure 2.

imum change in the absorbance at 294 nm, with $\delta\epsilon$ = 2200 M^{-1} cm^{-1}, and a somewhat smaller peak or shoulder at 286 nm (Figure 4). Such changes result from a red shift, and the shapes are characteristic of those observed upon transfer of a tryptophan residue to a less polar environment (Donovan, 1969). On the basis of the position and magnitude of the peak intensity in the difference spectra, it may be suggested that only one tryptophan residue is perturbed on the binding of PI-PLC to HPC micelles.

As also shown in Figure 4, in the presence of HPM at saturating concentrations, the change in the absorbance at 294 nm has $\delta\epsilon$ = 2600 M^{-1} cm^{-1} and a somewhat larger peak at 286 nm. The most significant difference between the difference spectra of HPC and HPM is that the negative trough at 268 nm observed with HPC is not seen with HPM. The spectral changes with HPC and HPM showed a dependence on the concentration of the amphiphile; however, these changes were only in the overall intensity, rather than a qualitative difference in the spectrum. From the concentration dependence of the change in the absorbance at 294 nm, it was possible to estimate the apparent dissociation constants for the enzyme bound to the interface; these values were generally consistent with those obtained by monitoring the change in the fluorescence intensity (Table 1). The relative change in the absorbance of PI-PLC at 294 nm (about 4%) was, however, considerably lower than the maximum change in the fluorescence emission (about 40%). This suggests that the changes in the fluorescence properties are not primarily due to the changes in the ground-state environment of the indoles, but

Table 2: Concentrations Required for 50% Quenching of PI-PLC

conditions	acrylamide (mM)	succinimide (mM)
buffer	110	208
+0.13 mM HPC	185	538
+0.6 mM HPM	187	516
+0.1 mM DOPC	251	368
+0.05 mM DTPM	287	473

are probably due to a change in the local quenching environment of the fluorophore. Finally, it may also be noted that the spectral changes observed with micellar dispersions were indistinguishable from those observed with lipid vesicles. Only the results with micellar amphiphiles are shown in Figure 4 where the turbidity corrections are not significant.

In summary, qualitatively different spectral changes observed on the binding of PI-PLC to zwitterionic and anionic phospholipid dispersions suggest that the enzyme may be present in two discrete states. Evidence developed below shows that such changes are due to the formation of E* on the zwitterionic interface and the formation of E*L on anionic interfaces of phosphomethanols and phosphoinositols, where these amphiphiles also bind at the catalytic active site. Such spectroscopic changes were not observed in the presence of amphiphiles dispersed as solitary monomers in the aqueous phase.

Quenching of Tryptophan Emission. Binding of PI-PLC to phospholipid dispersions influences quenching by water-soluble quenchers. The fluorescence emission from PI-PLC was quenched by water-soluble agents such as acrylamide or succinimide. As shown in Figure 5, the emission from PI-PLC could be virtually completely quenched by acrylamide, which suggests that most, if not all, of the fluorescent tryptophan residues are accessible to acrylamide. In the presence of DOPC vesicles, the quenching of the tryptophan emission of PI-PLC was observed at a higher concentration of the quencher. The curvature in the plot would be consistent with the conclusion that, in the presence of DOPC, only some of the tryptophan residues are protected from quenching by acrylamide. Essentially similar behavior was observed with succinimide. The concentrations of the quencher required for the 50% decrease are summarized in Table 2. Values obtained under different conditions suggest that at least some of the tryptophan residues in PI-PLC are freely accessible from the aqueous phase, and they are protected in the presence of micelles and vesicles of anionic or zwitterionic phospholipids.

Binding of PI-PLC to the Bilayer Interface. The dansyl fluorophore with the excitation maximum at 346 nm is an efficient acceptor for fluorescence emission from tryptophan, with a resonance energy transfer distance of about 20 Å (Vaz & Schoellman, 1976; Vaz et al., 1977). Therefore, a dansyl group located at the lipid–water interface is useful probe to monitor binding of a protein to the interface. For example, as shown in Figure 6, the fluorescence emission intensity of the dansyl fluorophore at 490 nm in codispersions of DH-DNS and DTPM shows a pronounced increase in the presence of PI-PLC due to resonance energy transfer from the tryptophan(s) of the protein (excitation at 292 nm) to the dansyl groups at the interface. Also, as summarized in Figure 7, the increase in the intensity is proportional to the concentration of PI-PLC added to DH-DNS codispersed with phospholipids. Beyond a certain concentration, addition of more PI-PLC does not cause a further increase in the fluorescence emission. The overall increase in the intensity due to energy transfer is more pronounced with DTPM vesicles than it is with DOPC vesicles. These results show that PI-

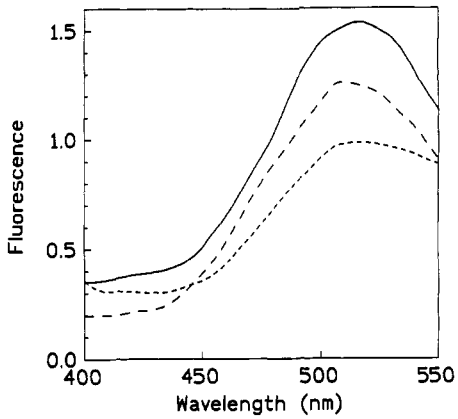

FIGURE 6: Fluorescence emission spectra of codispersed vesicles of DH-DNS + DTPM (1:25 mol ratio and 55 μM DTPM) (- - -) alone or (—) with a saturating amount of PI-PLC (about 15 μM); (– –) spectrum of codispersed vesicles of DH-DNS + DOPC (1:25 mol ratio and 55 μM DOPC) with 15 μM PI-PLC. Excitation was at 292 nm. Other conditions were as described in the legend to Figure 2.

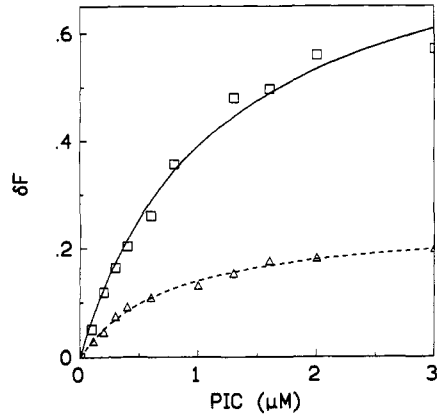

FIGURE 7: Increase in the fluorescence emission intensity (at 490 nm) of (□) DH-DNS + DTPM vesicles (55 μM) or (△) of DH-DNS + DOPC vesicles (55 μM) as a function of PI-PLC concentration. Other conditions were as described in the legend to Figure 2.

PLC binds to the bilayer-water interface. On the basis of the analytical relationships described elsewhere (Jain et al., 1982), from the results shown in Figure 7, by extrapolation of the initial region or by fitting the whole data set (Jain et al., 1982), it is estimated that each PI-PLC molecule binds to about 60 phospholipid molecules at the DTPM interface. Parallel experiments with pig pancreatic phospholipase A_2 showed that about 35 phospholipid molecules at the interface are required for the binding of each enzyme molecule and that the overall increase in the fluorescence was about 2.5-fold [see Jain et al. (1986b); Jain & Vaz, 1987].

Binding of PI-PLC to Micelles. The change in the fluorescence emission intensity of PI-PLC was also monitored in the presence of aqueous dispersions of several phosphatidylcholine analogues. Results summarized in Figure 8 show that the fluorescence increase is observed only above the critical micelle concentration. For example, with micelles of HPC (with cmc of about 10 μM), the fluorescence emission increases sharply above 5 μM. On the other hand, with diPC8 the increase is observed above 0.1 mM, compared to its cmc of about 0.2 mM (Tausk et al., 1974). The fact that the increase in the emission intensity occurs at concentrations somewhat below the critical micelle concentration suggests that PI-PLC may have a tendency to promote self-aggregation of these amphiphiles.

Effect of Phosphoinositols on the Binding to the Interface. One of the possible interpretations of the differences in the

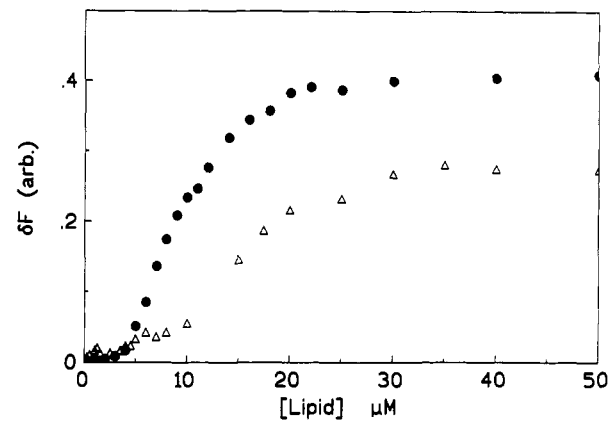

FIGURE 8: Change in the tryptophan fluorescence intensity of PI-PLC (1 μM) as a function of (●) HPC or (△, on a 10× compressed abscissa scale) diPC8. Other conditions are as described in the legend to Figure 2.

fluorescence emission characteristics of PI-PLC in the presence of zwitterionic and certain anionic phospholipids is that anionic phospholipids such as phosphomethanols bind to the active site of PI-PLC at the interface, whereas zwitterionic phospholipids do not. This conclusion is supported by binding studies carried out with several enantiomeric phosphoinositol derivatives. Because PI-PLC has been shown to specifically recognize the D-*myo*-inositol substituent of water-soluble substrates (Volwerk et al., 1990; Leigh et al., 1992), one can predict that only D-phosphoinositols (the naturally occurring enantiomers) produce the spectroscopic signature consistent with binding into the catalytic active site. This is exactly what was observed experimentally. For example, as summarized in Table 1, the tryptophan fluorescence emission intensity of PI-PLC decreases in the presence of several amphiphiles with the D-inositol substituent, whereas an increase was observed with the L enantiomers. The discrimination between these enantiomers was also observed when DOPC vesicles were added to a mixture of PI-PLC and the inositides. By using dansyl-labeled vesicles, it was found that the rate of intervesicle transfer of PI-PLC was less than 10 s. Such a rapid rate was also found under the kinetic conditions that will be described later. These results show that the residence time of PI-PLC on the interface is relatively short (see Discussion).

In order to characterize the absorbance and fluorescence changes induced by the substrate inositides, it was necessary to record the emission spectra within a few seconds after the addition of the dispersions. After longer times there were time-dependent changes in the spectra produced by the D-inositides, but not in those produced by the L-enantiomers. Thin-layer chromatographic analysis of the reaction mixtures incubated for about 30 min under the spectroscopic binding conditions showed that the D-inositides were cleaved, whereas the L-inositides were not. Thus, the time-dependent spectral changes observed with the D-inositides are due to PI-PLC-catalyzed cleavage of these substrates. This conclusion is supported by the UV difference spectra summarized in Figure 9. Again, the changes observed following the addition of HPI-D were time-dependent. The spectrum obtained within 10 s after the addition of HPI-D was similar to that observed with DTPM or HPM (Figure 4); however, within 5 min the spectrum had changed significantly, such that it resembled the spectrum observed immediately after the addition of HPI-L. The spectrum in the presence of HPI-L did not change noticeably with time. It also differed from the spectrum obtained with the zwitterionic micelles (Figure 4), indicating

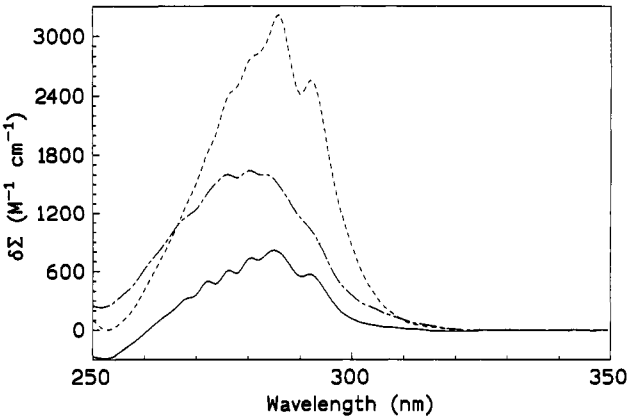

FIGURE 9: UV difference spectra for 15 μM PI-PLC obtained following the addition of (- - -) 0.5 mM HPI-D at time <10 s and (– – –) after 5 min or (—) after the addition of 0.5 mM HPI-L at time < 10 s. Other conditions were as described in the legend to Figure 2.

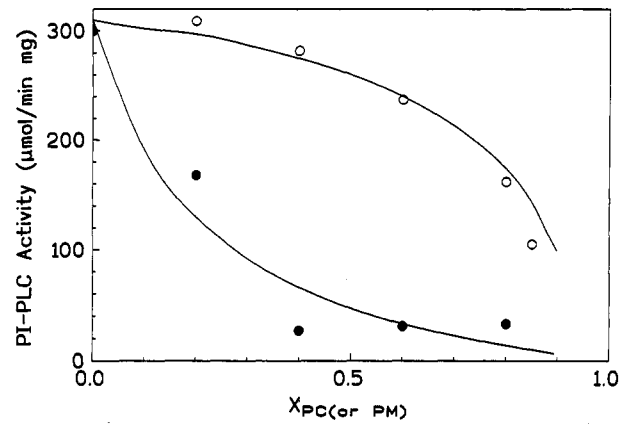

FIGURE 10: Activity of PI-PLC toward sonicated, mixed vesicles of DOPC and PI (○) or DTPM and PI (●) as a function of the mole fraction of the non-PI lipid. The bulk concentration of PI was kept constant at 2 mM in these experiments. The data points represent the mean of two independent experiments. The solid lines were calculated using the appropriate equations with the estimated parameter values as described in the main text. Conditions were as described under Experimental Procedures.

that subtle differences in the modes of binding of the enzyme to these interfaces are reflected in the spectral properties. A UV difference spectrum similar to that observed with HPM and HPI-D, but which remained constant over time, was observed with the noncleavable phosphonate inositide, PPI. Taken together, the spectroscopic results obtained with the enantiomeric inositides lend further support to the notion that the binding of PI-PLC to the lipid–water interface and the binding of a substrate or inhibitor into the active site of E* are distinct.

Catalytic Properties of PI-PLC on Substrate Vesicles. The effect of DTPM or DOPC on the interfacial catalytic properties of PI-PLC acting on codispersions with the substrate, PI, was investigated. Mixed vesicles with varying mole fractions of DOPC or DTPM were prepared, and the initial rates of hydrolysis of radiolabeled PI were measured at a constant bulk concentration of 2 mM PI. Since the apparent K_d values for E* and E*L (Table 1) are in the range of 10 μM, this excess ensures that all of the enzyme is at the interface. As shown in Figure 10, the activity versus mole fraction of PI codispersed with DOPC is typical for surface dilution with a neutral diluent in a situation where the interfacial Michaelis constant, K_M^*, for the enzyme–substrate complex (E*S) is about 0.25 mol fraction unit. The theoretical curve (top) for surface dilution shown in Figure 10 was calculated from the following equation for interfacial catalysis (Berg et al., 1991):

$$v = \frac{k_{cat}X_S}{X_S + K_M^*} \quad (1)$$

where v is the initial rate at mole fraction X_S of the substrate. At $X_S = 1$, the rate is defined as $v_o = k_{cat}(1 + K_M^*)^{-1}$, which is the maximal achievable rate at the maximum possible concentration of the substrate at the interface. K_M^* is the interfacial Michaelis constant, and k_{cat} is the catalytic rate constant or the maximal turnover at saturating substrate mole faction (which may or may not be achievable in practice, depending on the magnitude of K_M^*). According to this relationship, at higher mole factions of the substrate, there is little decrease in the observed rate because the E* to E*S equilibrium (Figure 1) remains in favor of E*S. Only at higher mole fractions of the diluent, where the mole fraction of the substrate approaches K_M^*, does the rate decrease due to surface dilution. This surface dilution tends to lower the effective E*S concentration, as dissociation to E* is favored by the lower surface concentration of the substrate. From the results shown in Figure 10, the calculated value of $k_{cat} = 220\ s^{-1}$ and $K_M^* = 0.26$ mol fraction.

The effect of increasing the mole fraction of DTPM in the PI interface (Figure 10) is typical of what is expected for a competitive inhibitor, as predicted by the relationship expressed in eq 2 (Berg et al., 1991):

$$\frac{v_o}{v_I} = 1 + \left(\frac{1 + 1/K_I^*}{1 + 1/K_M^*}\right)\left(\frac{X_I}{1 - X_I}\right) \quad (2)$$

where v_o and v_I are the initial rates in the absence and presence of an inhibitor, respectively, X_I is the mole fraction of the inhibitor, and K_I^* is the dissociation constant of E*I in mole fraction units. From the curve based on this equation (Figure 10), the mole fraction of the inhibitor required for 50% inhibition, $X_I(50)$, was estimated as 0.18 mol fraction. From the relationship between $X_I(50)$ and K_M^* (eq 2), the value of K_I^* for DTPM was calculated as 0.045 mol fraction unit. These results show that there is a marked inhibition, even at low mole fractions of DTPM. Thus, DTPM behaves as an active-site-directed ligand competing with the substrate for binding to E*. Inhibition was also observed with HPM and PPI incorporated in covesicles of PI + PC (15:85 mol ratio) as substrate. For example, at 0.15 mol fraction HPM the residual activity was 15%. Similarly, with 0.15 mol fraction PPI, the residual activity was 54% of that observed in its absence. It should be noted that the noncleavable phosphonate analogue, PPI, is racemic; therefore, the effective PPI concentration is 0.075 mol fraction in this experiment. This is because only the D enantiomers are able to bind into the active site and act as substrates or inhibitors. These kinetic results with interfacial substrates and analogues are in agreement with the spectroscopic data and support the conclusion that the binding of a ligand into the catalytic active site of PI-PLC bound to the interface strongly depends on the nature of the phospholipid head group.

For the interpretation of the results in Figure 10, we have discounted the possibility that the observed effects are due to a change in the state of the lipid. This conclusion is based on the following observations: (a) The kinetic and spectral effects similar to those observed with DTPM are also seen with HPM. (b) The inhibitory effect of DTPM is inconsistent with a DTPM-induced phase change. If this was the case, a more pronounced effect would have been seen only at higher mole fractions, rather than a hyperbolic decrease, which is consistent

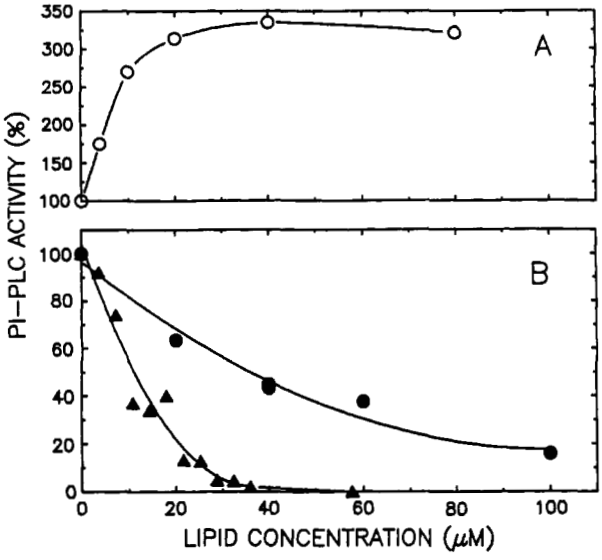

FIGURE 11: Effect of the addition of small sonicated vesicles of (A) DOPC and (B) PG (●), or DTPM (▲) on the PI-PLC-catalyzed cleavage of NPIP. The PG used in this experiment was derived from egg yolk phosphatidylcholine (Volwerk et al., 1986). Conditions were as described under Experimental Procedures. The NPIP concentration was 0.1 mM in A and 1 mM in B. Similar activation by DOPC vesicles was observed at 1 mM NPIP.

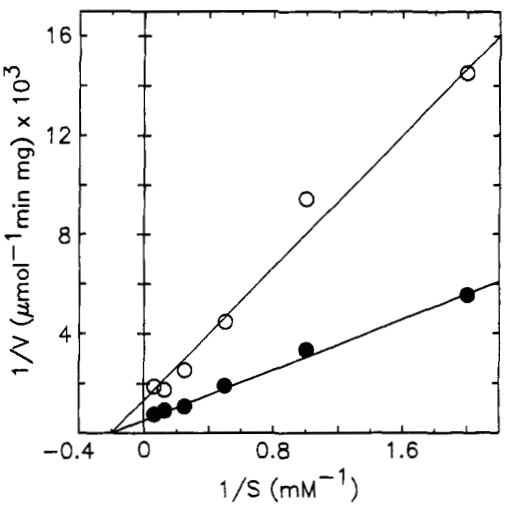

FIGURE 12: Lineweaver–Burk plot of the velocity versus substrate concentration for PI-PLC acting on NPIP in the absence (○) or presence (●) of 80 μM DOPC vesicles. The solid lines were calculated by a linear least-squares procedure. Conditions were as described under Experimental Procedures. See Table 3 for the values of the kinetic parameters obtained by different methods.

with eq 2. (c) The effect of phase separation of lipids on the observed kinetics, if any, would be seen only if the binding of the enzyme (E to E*) to one of the lipids was significantly different from the binding to the other lipid in the covesicles. Virtually all of the interfacial binding studies show that this is not the case, because PI-PLC binds with near-equal affinity to the dispersions of anionic and zwitterionic lipids irrespective of whether they are in the micellar or bilayer form. (d) Inhibitory effects are seen only with the D-inositides, whereas the L enantiomers with comparable physical properties are not inhibitors. (e) The inhibitory effect of DTPM and HPM is also seen under the conditions where catalysis does not occur on the interface, such as upon the hydrolysis of water-soluble substrates. (f) On the basis of the exchange studies, the residence time of the enzyme on the vesicle interface is relatively short. Therefore, on the time scale of reaction progress, the enzyme cannot remain sequestered in any particular "local" lipid environment, as required if phase separation is to induce an inhibitory effect by sequestering the enzyme on the inhibitory lipid away from the substrate. Thus, from the weight of the evidence and the fact that there is no evident need to consider the effect of the phase properties of the lipids on the observed kinetic behavior, it may be concluded that results of the type shown in Figure 10 are a consequence of zwitterionic surfaces on which the E* form is present and that a ligand directed toward the catalytic active site shifts the equilibrium toward the E*L form.

Catalytic Properties of PI-PLC on a Soluble Substrate in the Presence of Interface. The rate of cleavage of the synthetic water-soluble substrate, NPIP, by PI-PLC increases in the presence of DOPC vesicles (Figure 11A), while the rate is reduced in the presence of DTPM or PG vesicles (Figure 11B). Both of these effects depend on the bulk concentration of the phospholipid vesicles, with an apparent K_d in the 10 μM range for both DOPC and DTPM and a somewhat larger value for PG. These values for the binding constants are consistent with the range of values for K_d estimated from the spectroscopic data in Table 1. As expected, the K_d values obtained under the kinetic conditions tend to be smaller than the apparent dissociation constants obtained under the binding conditions. The reason is that, under the binding conditions, the enzyme concentration is considerably higher (2 versus 0.02 μM) than that under the kinetic conditions because under the binding conditions, at lower concentrations of the amphiphile, the condition [total amphiphile] ≈ [free amphiphile] >> [total enzyme] is not satisfied.

On the basis of the spectroscopic and kinetic data summarized thus far, it is clear that the inhibition/activation observed with these lipid vesicles is an interfacial phenomenon and relates to the binding of the enzyme to the vesicle surface. Inhibition of the reaction with the water-soluble substrate, NPIP, by DTPM is expected since, as shown above, this compound is an effective competitive inhibitor of the PI-PLC reaction in covesicles with PI (Figure 10). The unexpected result is that binding of PI-PLC to DOPC vesicles actually stimulates the rate of cleavage of water-soluble NPIP. In order to further examine this interfacial activation observed with a water-soluble substrate, the initial velocity was measured as a function of the NPIP concentration in the absence and presence of a saturating concentration of DOPC vesicles, where all of the enzyme is in the E* form. Figure 12 shows the data as a double-reciprocal Lineweaver–Burk plot of the initial velocity versus the D-NPIP concentration. Since racemic NPIP was used in these studies, the substrate concentration is corrected on the basis of the observation that L-NPIP is neither a substrate nor an inhibitor of PI-PLC (Leigh et al., 1992). The common intersection on the abscissa of the straight lines in Figure 12 suggests that the activation of PI-PLC on binding to DOPC vesicles is primarily due to an effect on V_{max}. There is no significant effect on K_M, which remains 5 mM, as also measured in the absence of the interface. Because of their theoretical significance, these results were also analyzed by other methods. The results summarized in Table 3 show that there is no systematic bias in the data, and the effect of the binding of PI-PLC to the interface is to increase V_{max} by about 2.5-fold without a significant effect on K_M.

DISCUSSION

Phospholipases C specific for inositol phospholipids (PI-PLC, EC 3.1.4.10), both from eukaryotic and prokaryotic origin, play an important role in processes such as transmembrane signal transduction (Berridge, 1987) and the release

Table 3: Effect of DOPC Vesicles on the Kinetic Parameters (% Standard Deviation) for the Hydrolysis of NPIP by PI-PLC Determined by Different Fitting Routines Used with the Data Shown in Figure 12

	-DOPC		+DOPC	
routine	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
Michaelis hyperbola	4.3 (21)	427 (9)	4.1 (24)	970 (4)
double reciprocal	4.9 (15)	450 (12)	4.3 (18)	973 (9)
Eadie–Hofstee (v vs v/S)	5.1 (11)	458 (18)	4.3 (10)	998 (8)
Hanes–Woolf (S/v vs S)	4.4 (9)	417 (7)	4.2 (7)	866 (12)

of glycosylphosphatidylinositol-linked membrane proteins (Low, 1990). Considerable progress has been made in defining the physiological function and regulation of the family of mammalian PI-PLCs, which plays a key role in phosphoinositide-dependent signaling mechanisms [reviewed by Rhee and Choi (1992)]. Similarly, insights into the catalytic mechanism of both bacterial and mammalian PI-PLCs have been obtained (Volwerk et al., 1990; Bruzik et al., 1992). Resolution of the crystal structure of PI-PLC from *B. cereus* appears imminent (Bullock et al., 1993) and can be expected to provide a structural framework for the design and interpretation of further studies on PI-PLC. Due to the difficulties inherent in crystallizing proteins present at the lipid–water interface, the crystallographic studies alone cannot provide a full understanding of the fundamental functional correlates of interfacial catalysis, which includes determination and evaluation of the relevant equilibrium and rate constants (Berg et al., 1991; Jain et al., 1991a–e).

The purpose of the present work, therefore, was 3-fold: (1) to examine and define experimental conditions for the study of the interfacial binding and catalysis of PI-PLC from *B. cereus*; (2) to evaluate and correlate the enzyme kinetics and binding results within the context of the kinetic scheme for interfacial catalysis shown in Figure 1; and (3) to characterize the features of and to formulate a framework for the interpretation of interfacial activation. With these goals in mind, we developed protocols and obtained binding and kinetic data with aqueous dispersions of PI, PI analogues, and related amphiphiles. The results show that PI-PLC-catalyzed hydrolysis occurs at the interface of phospholipid dispersions, that the binding of PI-PLC to the interface can be effectively dissected from the active-site events, and that the binding of PI-PLC to the interface increases its catalytic efficiency. As elaborated at the end of this section, interfacial activation of PI-PLC is probably the first example of allosteric modulation, where the binding of the enzyme to the interface changes k_{cat}.

Binding of PI-PLC to the interface of phospholipids and analogues present as vesicles or micelles can be monitored by a range of spectroscopic methods. On the basis of the magnitude of the change in the absorbance at 294 nm, it is suggested that at least one, but probably not more than two, tryptophan residues are perturbed upon the binding of PI-PLC to the interface. Specific spectroscopic signatures are observed for the E => E* step and for the subsequent binding of a ligand into the active site, E* => E*L. Formation of E* results in a net increase in the tryptophan fluorescence emission intensity relative to unbound E, probably due to a relief in intermolecular quenching. On the other hand, formation of E*L results in a net decrease of the emission intensity. Formation of E* and E*L gives a characteristic UV difference spectrum with defined features at wavelengths typical for tryptophan perturbation (Figures 4 and 9). The changes observed on the binding of PI-PLC to DTPM and HPM are similar to those observed with inhibitory D-inositides, whereas the changes observed on binding to zwitterionic interfaces are similar to those observed with the enantiomeric inositides. Also, the binding of the enzyme to the zwitterionic interface activates the enzyme, whereas the interface of the active-site-directed ligands inhibits the enzymes. Taken together, these observations suggest that interfaces like those of DOPC bilayers are reasonably good neutral diluents where the E* form of the enzyme predominates.

The spectroscopic and kinetic data indicate values for the apparent dissociation constants (K_d) of the enzyme–phospholipid interface complex (E*) of around 10 μM (in terms of the lipid monomers). The range of apparent K_d values is similar for lipids containing either phosphocholine, phosphomethanol, phosphoglycerol, or phospho-*myo*-inositol head groups, i.e., affinity for the interface does not seem to depend upon the nature of the phospholipid head groups or the net surface charge (negatively charged versus neutral/zwitterionic). This is in sharp contrast to the behavior of secreted phospholipase A_2, where the interfacial binding constant is strongly charge-dependent (Jain et al., 1982, 1986a, 1991b). For phospholipase A_2, interfacial binding is governed by ionic interactions between positively charged surface residues on the protein and negatively charged lipid head groups. It is possible that, for PI-PLC, other forces may play a more significant role, for example, hydrophobic interactions of the type recently described for the pancreatic lipase–procolipase complex (van Tilbeurgh et al., 1993). It is interesting in this respect that a recent monolayer study of a eukaryotic PI-PLC isoform, PLC-δ_1, indicates that a portion of this enzyme is able to insert into the phospholipid monolayer (Rebecchi et al., 1992).

Interfacial binding of the bacterial PI-PLC that is independent of the lipid head group composition is to be expected for an enzyme that has to seek out its substrate, PI or glycosyl-PI, which are normally minor components, in a complex biological membrane. It also implies that the enzyme is capable of acting in the processive scooting mode of interfacial catalysis where the enzyme stays bound for multiple catalytic turnover cycles. From the data presented here, and with some plausible assumptions, it is possible to obtain a limit estimate of the processivity of PI-PLC acting on PI or PI/PC vesicles. From the apparent $K_d < 10$ μM (expressed in terms of the lipid monomers) for interfacial binding, we estimate $K_d^* <$ 400 pM (expressed in terms of the vesicle concentration) because the underlying process involves encounter of the enzyme with a vesicle. K_d^* is obtained by dividing K_d by 25 000, on the basis of the assumption that the number of lipids in a vesicle is 25 000 [for a possible rationale, see Grell (1978); Stankowski, 1984; Jain et al., 1988]. Because of the high vesicle to protein ratio, the excluded area effect is ignored. If it is assumed that the equilibrium constant K_d^* is dominated by the diffusion-limited binding and desorption rate constants, k_b and k_d (Figure 1), k_b having the diffusion limited value of 10^{10} M^{-1} s^{-1} (Grell, 1978; Jain et al., 1988), it follows that k_d is about 4 s^{-1}, that is, the average residence time of a PI-PLC molecule on the surface of a vesicle is about 0.25 s.

With a turnover number of 175 s^{-1} for PI-PLC acting on PI vesicles at pH 7.0 and room temperature, the average processivity is estimated at 40–50 catalytic cycles per binding event. This estimate obviously depends upon the choice of the various input parameters, such as the size of the vesicles and the on-rate constant. If the vesicle size is somewhat smaller the processivity will decrease. On the other hand, the value assumed for k_b is the diffusion-limited on-rate for small molecules, and the corresponding value for the encounter of

vesicle and enzyme would probably be smaller by a factor of 4–10; thus, the processivity number may be correspondingly larger. The estimated residence time for PI-PLC is consistent with the relatively fast intervesicle exchange rate we observed for the enzyme. For example, addition of DTPM vesicles to the reaction mixture containing PI-PLC acting on NPIP in the presence of DOPC vesicles results in immediate inhibition (within the 10-s time resolution of the experiment), and the rate of cleavage thereafter remains constant over time; conversely, inhibition by DTPM is instantaneously overcome by the addition of more DOPC vesicles (data not shown). It may be recalled that, compared to phospholipase A_2 acting on anionic lipids, where the enzyme is effectively irreversibly bound to the interface and cleaves all (several thousand) of the substrate in the outer monolayer in a single visit (Berg et al., 1991), the processivity of PI-PLC is rather modest.

Among phospholipases, bacterial PI-PLC is remarkably specific for phospholipids containing a *myo*-inositol moiety that can be glycosylated but not phosphorylated (Volwerk et al., 1989; Kume et al., 1992). The results presented here show that the enantiomeric substrate specificity of PI-PLC resides entirely in the second binding step, i.e., the formation of the interfacial enzyme–substrate complex, E*S (Figure 1). On the basis of the spectroscopic data supported by the enzyme kinetic results, it is possible to group the phospholipids and amphiphiles into two categories in terms of their binding characteristics toward PI-PLC. The first category consists of lipids that form an interface, to which the PI-PLC readily binds, but that do not bind to the active site. Such amphiphiles are defined as neutral diluents (Jain et al., 1991a). Identification of amphiphiles behaving as neutral diluents is important, in that these agents provide a lipid matrix to which the enzyme can bind without interfering with the interaction of the active site with ligands, as amply demonstrated for phospholipase A_2 (Jain et al., 1991a). Thus, neutral diluents for PI-PLC include phospholipids and analogues with a phosphocholine head group (DOPC, HPC, deoxy-LPC, and 2H-GPC) and those containing a phospho-L-1-*myo*-inositol moiety (HPI-L and LPI16(or 18)-L). The results of Hendrickson et al. (1992) with HPC are consistent with this conclusion.

Some phospholipids were found to be the active-site-directed ligands for PI-PLC, i.e., not only can the enzyme bind to their aqueous dispersions but the amphiphile also has significant affinity for the active site of the bound enzyme. To this group belong DTPM and HPM, as well as naturally occurring inositides and the D enantiomers of synthetic phosphonate inositols such as PPI (Shashidhar et al., 1990a,b). As expected, such amphiphiles are either competitive inhibitors or substrates. The amphiphiles in this category are characterized spectroscopically by inducing a decrease in the tryptophan fluorescence emission intensity of PI-PLC and by a characteristic UV difference spectrum.

The spectroscopically and kinetically different behavior of the L versus the D enantiomers of the *myo*-inositol-containing lipids dramatically demonstrates the absolute stereoselectivity of the bacterial PI-PLC. Earlier we showed that PI-PLC catalyzes the cleavage of its substrates in two consecutive steps (Volwerk et al., 1990), the first step being a fast intramolecular phospho transfer reaction, producing D-*myo*-inositol 1,2-cyclic monophosphate. At high enzyme concentrations, the cyclic phosphate is slowly hydrolyzed to D-*myo*-inositol 1-monophosphate. Furthermore, we demonstrated that the enzyme is highly stereospecific in both the phosphotransferase (Leigh et al., 1992) and cyclic phosphodiesterase (Volwerk et al., 1990) activities; however, these studies were performed with water-soluble substrates only. The present results show that the stereospecificity of PI-PLC from *B. cereus* is strictly adhered to, also with interfacial inhibitors and substrates, and that the enantiomeric specificity is primarily in the formation of the E*I or E*S complex.

One unexpected observation is the relatively potent inhibition of PI-PLC with lipids containing the phosphomethanol head group. Analysis of the data for DTPM in Figure 10 gives a value for K_I^* of 0.045 mol fraction unit compared to a value for K_M^* of 0.26 mol fraction for PI. Other data support this observation. For example, at equal mole fractions in mixed vesicles, HPM is a more potent inhibitor than PPI, even if one takes into account the fact that only the D enantiomer of racemic PPI is active. Octyl-PM, a short-chain analog of HPM, at submicellar concentrations (up to 5 mM) also inhibits the PI-PLC reaction on NPIP with a K_I of 1.25 mM (data not shown), while the K_M for NPIP is 5 mM (Leigh et al., 1992; Table 3). The putative tight binding of phosphomethanol lipids in the active site of PI-PLC suggests that potent inhibitors for these enzymes may be developed that need not contain the D-*myo*-inositol moiety, which is an advantage from a synthetic point of view. Evidently, the small methyl group of the PM head group can fit in the putative inositol binding pocket in the active site of PI-PLC, but this does not explain the higher affinity of the enzyme for these compounds. The protocols developed here should serve as a guide for future design, characterization, and assay of specific active-site-directed inhibitors for PI-PLCs. Moreover, as elaborated next, the possible presence of an allosteric regulatory site on this enzyme offers an as yet unrecognized locus for pharmacological control.

Kinetic Basis for Interfacial Activation of PI-PLC. A key observation in the present study is the apparent activation of the *B. cereus* PI-PLC acting on a water-soluble substrate in the presence of DOPC vesicles (Figures 11 and 12). According to the suggestion of Roholt and Schlamowitz (1961), the term interfacial activation refers to the enhanced rate of catalysis observed with substrates present at the lipid–water interface compared to that for the same substrates present as solitary monomers in solution. Several other classes of membrane-bound enzymes (Jain & Zakim, 1987), including β-hydroxybutyrate dehydrogenase (Fleischer et al., 1983), show activity when reconstituted at the interface. However, such cases cannot be taken as examples of interfacial activation, because these enzymes may be denatured in the absence of the interface and therefore be catalytically inactive.

The activation of PI-PLC reported here represents a unique situation in that it is observed with a water-soluble substrate lacking a significant hydrophobic moiety and is therefore unlikely to self-aggregate or to partition into a lipid phase. This made it possible to vary the substrate concentration and the concentration of the activating interface independently, as demonstrated in Figures 11 and 12. As a result, the underlying kinetic basis of the interfacial activation of PI-PLC can be deduced as elaborated below.

A general explanation for enhanced catalytic turnover at the interface continues to be the holy grail of research with lipolytic enzymes. In spite of several attempts (Volwerk & de Haas, 1982; Dennis, 1983), no consensus has emerged, largely because a rigorous interpretation of the results in terms of the underlying kinetic and equilibrium parameters has not been possible until recently (Jain & Berg, 1989; Berg et al., 1991; Jain et al., 1992b, 1993). Within the context of the kinetic scheme shown in Figure 1, interfacial activation could

arise from a variety of factors. A short review of the various possibilities may be helpful.

(a) Binding of the enzyme from the aqueous phase to the interface, the E => E* step, is necessary to make a long-chain phospholipid substrate accessible for catalytic turnover by the enzyme. Although this does not constitute interfacial activation in the sense defined above, enhanced binding to the interface increases the observed rates of hydrolysis by phospholipase A_2 by several orders of magnitude (Jain et al., 1982a, 1986a,b; Ghomashchi et al., 1991). This consideration does not apply to the interfacial activation of PI-PLC observed here because the substrate is present in the aqueous phase.

(b) A consideration of the interfacial activation of PI-PLC in terms of a possible difference in the properties of the substrate in the aqueous phase versus the interface can also be ruled out on the basis of the hydrophilic nature of the substrate, NPIP. Other results show that the conformations of the head groups of phospholipid molecules at the interface and in solution are virtually the same as in crystals (Hauser et al., 1981). The energy differences and barriers for the head group conformers of the PI-PLC substrates are probably insignificant compared to the activation energies of the steps explicitly considered in the catalytic turnover.

(c) Considerations related to the partitioning and higher local concentration of the substrate that the bound enzyme "sees" at the interface (Jain et al., 1993) are also of little consequence in the present context. For example, a seemingly obvious explanation of the interfacial activation observed here with PI-PLC would be that, in spite of its hydrophilic nature, the substrate, NPIP, partitions into the bilayer of DOPC vesicles sufficiently for the enzyme at the interface to "see" a higher local substrate concentration than it would in the aqueous phase. The key argument against this possibility derives from the thermodynamic cycle that connects the binding of the enzyme to the interface and the binding of the ligand to the active site. On the basis of the microscopic reversibility of these two steps, it can be argued that a local concentration effect as such will not effectively increase the overall substrate binding to the active site, because the apparent affinity of the substrate for the interface (which would increase the local concentration through the partitioning) will also, to the same extent, reduce the binding of the substrate to the enzyme at the interface.

(d) Having ruled out the physical and substrate-based mechanisms from the present consideration, we seek the basis for interfacial activation in the primary kinetic and equilibrium properties of the enzyme [see Volwerk and de Haas (1982) for some of the early considerations]. According to the formalism of allosteric activation (York, 1992), a K_M (K-) and a k_{cat} (V-) type of activation may be distinguished. On the basis of the results shown in Figure 12, the simplest explanation for the interfacial activation of PI-PLC as observed with water-soluble NPIP is that binding of the enzyme to the interface results in an increase in its k_{cat} from about 420 s^{-1} to about 1000 s^{-1}. The active site of PI-PLC bound to the interface remains accessible to the aqueous substrate with an effective K_M of about 4.5 mM, which is identical to the value observed for the enzyme in the aqueous phase (Leigh et al., 1992). An exchange of water-soluble ligands between the active site of the enzyme and the bulk phase is consistent with the processive mode of action of the PI-PLC on a lipid membrane. The primary products of the PI-PLC-catalyzed reaction are diacylglycerol, which remains in the lipid phase, and water-soluble *myo*-inositol 1,2-cyclic phosphate, which must be released into the aqueous phase.

With interfacial substrates also, at least a part of the activation could be due to an effect of interfacial binding on k_{cat}. The 3-fold increase in the turnover rate of PI-PLC observed here for a water-soluble substrate is similar in magnitude to the interfacial activation observed for PI-PLCs with interfacial substrates. Data published by Hendrickson et al. (1992) for the *B. cereus* PI-PLC using thiophosphate analogues of PI suggest that the effect of incorporating the substrate into the interface increases the rate by less than 10-fold. Similarly, the interfacial activation of the homologous *B. thuringiensis* enzyme observed with short-chain PI analogues above the cmc is about 5-fold (M. F. Roberts, personal communication). Also, for the eukaryotic PLC-δ_1, the increase in the reaction velocity as the concentration of the substrate dioctanoyl-PI is raised above the cmc is only 2–3-fold (Rebecchi et al., 1993). A 3-fold activation has also been observed with a nonspecific phospholipase C (El-Sayed et al., 1985). Although the rate-determining step(s) contributing to k_{cat} in the catalytic cycle of PI-PLC is (are) presently unknown, it is noteworthy that thiophosphate PI analogues are cleaved 10 or more times more slowly by PI-PLC than oxy analogues (Hendrickson et al., 1992). Such an element effect on the rate of hydrolysis suggests that the chemical bond-breaking step(s) may be rate-limiting (Jain et al., 1992a).

Finally, it is of interest to compare the basis of the interfacial activation of PI-PLC with that of pancreatic phospholipase A_2. This enzyme hydrolyzes short-chain phosphatidylcholines dispersed as solitary monomers (below the cmc) at a rate that is considerably lower than that observed with micellar or aggregated substrate (Pieterson et al., 1974). The rate of hydrolysis of these "soluble" substrates does not change significantly in the presence of dispersions of neutral diluents (Jain et al., unpublished observations). On the other hand, the apparent affinity of the active-site-directed ligands is considerably higher for phospholipase A_2 at the interface of the neutral diluent than it is for the enzyme in the aqueous phase (Rogers et al., 1992). Such results suggest that interfacial activation of pig pancreatic phospholipase A_2 is of the K_M type, i.e., the affinity of the enzyme for the active-site-directed ligands is enhanced on the binding of the enzyme to the interface (Jain et al., 1993).

To recapitulate, the kinetic and binding behavior of PI-PLC is entirely consistent with the kinetic scheme in Figure1. Similar behavior has been observed with several secreted phospholipases A_2. The fact that interfacial activation can also be accommodated within this framework suggests that the primary rate and equilibrium parameters thus obtained could provide a meaningful basis for the structure–function correlation of lipolytic enzymes and insights into the mechanism of interfacial activation. Thus, PI-PLC and phospholipase A_2 may represent examples of lipolytic enzymes for which interfacial activation is brought about by allosteric modulation of these enzymes by the lipid–water interface, either at the substrate binding step (phospholipase A_2) or at a subsequent step (PI-PLC). The kinetic, thermodynamic, and structural consequences of this suggestion are being explored and will be discussed elsewhere.

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REFERENCES

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

Berridge, M. J. (1987) *Annu. Rev. Biochem. 56*, 159–193.

Bradford, M. M. (1975) *Anal. Biochem. 72*, 248–254.

Bruzik, K. S., Morocho, A. M., Jhon, D.-Y., Rhee, S. G., & Tsai, M. D. (1992) *Biochemistry 31*, 5183–5193.

Bullock, T. L., Ryan, M., Kim, S. L., Remington, S. J., & Griffith, O. H. (1993) *Biophys. J. 64*, 784–791.

Camilli, A., Goldfine H., & Portnoy, D. A. (1991) *J. Exp. Med. 173*, 751–754.

Dennis, E. (1983) *Enzymes 16*, 307–353.

Donovan, S. J. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) Part A, pp 101–170, Academic Press, New York.

El-Sayed, M. Y., DeBose, C. D., Coury, L. A., & Roberts, M. F. (1985) *Biochim. Biophys. Acta 837*, 325–335.

Filthuth, E., & Eibl, H. (1992) *Chem. Phys. Lipids 60*, 253–261.

Fleischer, S., McIntyre, J. O., Churchill, P., Fleer, E., & Maurer, A. (1983) in *Structure and Function of Membrane Proteins* (Quagliarielli, E., & Palmieri, E., Eds.) pp 283–300, Elsevier, Amsterdam.

Ghomashchi, F., Yu, B.-Z., Berg, O. G., Jain, M. K., & Gelb, M. H. (1991) *Biochemistry 30*, 7318–7329.

Grell, E. (1978) in *Membrane Spectroscopy* (Grell, E., Ed.) pp 333–376, Springer-Verlag, Berlin.

Hauser, H., Pascher, I., Pearson, R. H., & Sundell, S. (1981) *Biochim. Biophys. Acta 650*, 21–51.

Hendrickson, H. S., Hendrickson, E. K., Johnson, J. L., Khan, T. H., & Chial, H. J. (1992) *Biochemistry 31*, 12169–12172.

Ikezawa, H. (1991) *Cell Biol. Intl. Rep. 15*, 1115–1131.

Jain, M. K. (1973) *Curr. Top. Membr. Transp. 4*, 174–254.

Jain, M. K., & Vaz, W. L. C. (1987) *Biochim. Biophys. Acta 905*, 1–8.

Jain, M. K., & Zakim, D. (1987) *Biochim. Biophys. Acta 906*, 33–68.

Jain, M. K., & Berg, O. G. (1989) *Biochim. Biophys. Acta 1002*, 127–156.

Jain, M. K., & Rogers, J. (1989) *Biochim. Biophys. Acta 1003*, 91–97.

Jain, M. K., & Gelb, M. H. (1991) *Methods Enzymol. 197*, 112–125.

Jain, M. K., Egmond, M. R., Verheij, H. M., Apitz-Castro, R. J., Dijkman, R., & de Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 341–348.

Jain, M. K., Rogers, J., Jahagirdar, D. V., Marecek, J. F., & Ramirez, F. (1986a) *Biochim. Biophys. Acta 860*, 435–447.

Jain, M. K., Rogers, J., Marecek, J. F., Ramirez, F., & Eibl, H. (1986b) *Biochim. Biophys. Acta 860*, 462–474.

Jain, M. K., Rogers, J., & de Haas, G. H. (1988) *Biochim. Biophys. Acta 940*, 51–62.

Jain, M. K., Yuan, W., & Gelb, G. H. (1989) *Biochemistry 28*, 4135–4139.

Jain, M. K., Yu, B.-Z., Rogers, J., Ranadive, G. N., & Berg, O. G. (1991a) *Biochemistry 30*, 7306–7317.

Jain, M. K., Ranadive, G. N., Yu, B.-Z., & Verheij, H. M. (1991b) *Biochemistry 30*, 7330–7340.

Jain, M. K., Rogers, J., Berg, O. G., & Gelb, M. H. (1991c) *Biochemistry 30*, 7340–7348.

Jain, M. K., Tao, W., Rogers, J., Arenson, C., Eibl, H., & Yu, B.-Z. (1991d) *Biochemistry 30*, 10256–10268.

Jain, M. K., Yu, B.-Z., Rogers, J., Gelb, M. H., Tsai, M. D., Hendrickson, E. K., & Hendrickson, H. S. (1992a) *Biochemistry 31*, 7841–7847.

Jain, M. K., Yu, B.-Z., Gelb, M. H., & Berg, O. G. (1992b) *Mediators Inflammation 1*, 85–100.

Jain, M. K., Yu, B.-Z., & Berg, O. G. (1993) *Biochemistry 32*, 11319–11329.

Koke, J. A., Yang, M., Henner, D. J., Volwerk J. J., & Griffith, O. H. (1991) *Protein Expression Purif. 2*, 51–58.

Kume, T., Taguchi, R., Tomita, M., Tokuyama, S., Morizawa, K., Nakachi, O., Hirano, J., & Ikezawa, H. (1992) *Chem. Pharm. Bull. 40*, 2133–2137.

Leigh, A. J., Volwerk, J. J., Griffith, O. H., & Keana, J. F. W. (1992) *Biochemistry 31*, 8978–8983.

Low, M. G. (1990) in *Molecular and Cell Biology of Membrane Proteins: Glycolipid Anchors of Cell-Surface Proteins* (Turner, A. J., Ed.) pp 35–54, Ellis Horwood, New York.

Mengaud, J., Braun-Breton, C., & Cossart, P. (1991) *Mol. Microbiol. 5*, 367–372.

Pieterson, W. A., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13*, 1455–1459.

Rebecchi, M. J., Boguslavsky, V., Boguslavsky, L., & McLaughlin, S. (1992) *Biochemistry 31*, 12748–12753.

Rebecchi, M. J., Eberhardt, R., Delaney, T., Ali, S., & Bittman, R. (1993) *J. Biol. Chem. 268*, 1735–1741.

Rhee, S. G., & Choi, K. D. (1992) *J. Biol. Chem. 267*, 12393–12396.

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

Rogers, J., Yu, B.-Z., & Jain, M. K. (1992) *Biochemistry 31*, 6056–6062.

Roholt, O. A., & Schlamowitz, M. (1961) *Arch. Biochem. Biophys. 94*, 364–379.

Shah, D. O., & Schulman, J. H. (1967) *J. Colloid Interface Sci. 25*, 107–118.

Shashidhar, M. S., Keana, J. F. W., Volwerk, J. J., & Griffith, O. H. (1990a) *Chem. Phys. Lipids 53*, 103–113.

Shashidhar, M. S., Volwerk, J. J., Keana, J. F. W., & Griffith, O. H. (1990b) *Biochim. Biophys. Acta 1042*, 410–412.

Shashidhar, M. S., Volwerk, J. J., Griffith, O. H., & Keana, J. F. W. (1991) *Chem. Phys. Lipids 60*, 101–110.

Stankowski, S. (1984) *Biochim. Biophys. Acta 777*, 167–182.

Tausk, R. J. M., Karmiggelt, J., Oudshoorn, C., & Overbeek, J. T. G. (1974) *Biophys. Chem. 1*, 175–183.

van Tilbeurgh, H., Egloff, M.-P., Martinez, C., Rugani, N., Verger, R., & Cambillau, C. (1993) *Nature 362*, 814–820.

Vaz, W. L. C., & Schoellman, G. (1976) *Biochim. Biophys. Acta 439*, 206–218.

Vaz, W. L. C., Kaufman, K., & Nicksch, A. (1977) *Anal. Biochem. 83*, 385–393.

Verger, R., & de Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77–117.

Verger, R., Van Dam-Mieras, M. C. E., & de Haas, G. H. (1973) *J. Biol. Chem. 248*, 4023–4034.

Volwerk, J. J., & de Haas, G. H. (1982) in *Lipid-Protein Interactions* (Jost, P. C., & Griffith, O. H., Eds.) Vol. I, pp 69–149, Wiley Interscience, New York.

Volwerk, J. J., Jost, P. C., de Haas, G. H., & Griffith, O. H. (1986) *Biochemistry 25*, 1726–1733.

Volwerk, J. J., Wetherwax, P. B., Evans, L. M., Kuppe, A., & Griffith, O. H. (1989) *J. Cell. Biochem. 39*, 315–325.

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

York, J. L. (1992) in *Textbook of Biochemistry* (Devlin, T. M., Ed.) pp 135–193, Wiley, New York.