J|A|C|S
ARTICLES
Published on Web 02/25/2003

Application of Brønsted-Type LFER in the Study of the Phospholipase C Mechanism†

Cornelia Mihai,‡,§ Alexander V. Kravchuk,||,⊥ Ming-Daw Tsai,|| and Karol S. Bruzik*,‡

Contribution from the Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, Illinois 60612, and Department of Chemistry, The Ohio State University, Columbus, Ohio 43210

Received November 15, 2002; E-mail: kbruzik@uic.edu

Abstract: Phosphatidylinositol-specific phospholipase C cleaves the phosphodiester bond of phosphatidylinositol to form inositol 1,2-cyclic phosphate and diacylglycerol. This enzyme also accepts a variety of alkyl and aryl inositol phosphates as substrates, making it a suitable model enzyme for studying mechanism of phosphoryl transfer by probing the linear free-energy relationship (LFER). In this work, we conducted a study of Brønsted-type relationship (log $k = \beta_{lg}\ pK_a + C$) to compare mechanisms of enzymatic and nonenzymatic reactions, confirm the earlier proposed mechanism, and assess further the role of hydrophobicity in the leaving group as a general acid-enabling factor. The observation of the high negative Brønsted coefficients for both nonenzymatic ($\beta_{lg} = -0.65$ to -0.73) and enzymatic cleavage of aryl and nonhydrophobic alkyl inositol phosphates ($\beta_{lg} = -0.58$) indicates that these reactions involve only weak general acid catalysis. In contrast, the enzymatic cleavage of hydrophobic alkyl inositol phosphates showed low negative Brønsted coefficient ($\beta_{lg} = -0.12$), indicating a small amount of the negative charge on the leaving group and efficient general acid catalysis. Overall, our results firmly support the previously postulated mechanism where hydrophobic interactions between the enzyme and remote parts of the leaving group induce an unprecedented negative-charge stabilization on the leaving group in the transition state.

Linear free relationships proved to be valuable tools in studies of reaction mechanisms, in particular in determining the changes in TS state structure due to partial bond formation or scission.[1−5] In our recent papers we have reported on the catalytic mechanism of phosphatidylinositol-specific phospholipase C involving the presence of a new catalytic triad comprised of two protonated amino acid side chains, Arg69 and His82, linked by an Asp33 intermediary.[6−9] In this mechanism, we postulated that the two positively charged residues Arg69 and His82 are responsible for activation of the phosphate group and protonation of the leaving group, respectively, and that the two factors are strongly interrelated.[7−10] Hence, weakening of the interactions of His82 general acid with the leaving group results in significant reduction of interactions of Arg69 with the nonbridging oxygen of the leaving group, and vice versa, reduction of interaction the nonbridging oxygen has a deleterious effect on interactions of His82 with the leaving group. The above conclusions were inferred from the kinetics of reactions of WT and mutant enzymes altered at Arg69, Asp33, and His82 with natural substrate and substrate analogues modified in their bridging and nonbridging oxygen sites by sulfur substitution.[7−10] In addition, we have shown that the catalytic role of His82 general acid and, hence, the whole catalytic triad is strongly affected by the hydrophobic interactions of the diacylglycerol leaving group. We have found that the removal of the hydrophobic fatty acid chains from the leaving diacylglycerol moiety severely impairs general acid catalysis by His82 and Asp33.[9] Hence, reactions of nonhydrophobic substrate analogues should involve the transition state with more negative charge developed on the leaving group oxygen. Furthermore, if interactions of enzyme with bridging and nonbridging oxygens of the substrate are indeed interdependent, the removal of

* To whom correspondence should be addressed. Fax: (312) 996-7107.
† This work was supported by National Institutes of Health Grant GM57568. Abbreviations: AP, alkaline phosphatase; DAST, (diethylamino)-sulfur trifluoride; DCC, dicyclohexylcarbodiimide; diC_6PC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; LFER, linear free energy relationship; MOM, methoxymethylene; NMP, *N*,*N*-methyl-2-pyrrolidone; PI-PLC, phosphatidylinositol-specific phospholipase C; PNP, 4-nitrophenyl; RNase A, ribonuclease A; WT, wild type.
‡ University of Illinois at Chicago.
§ Current address: Department of Chemistry, The University of Iowa, Iowa City, IA 52242.
|| The Ohio State University.
⊥ Current address: Department of Biochemistry, B400 Beckman Center, Stanford University, Stanford, CA 94305.

(1) Aqvist, J.; Warshel, A. *Chem. Biol.* **1999**, *6*, R71−R80.
(2) Warshel, A. In *Computer modeling of chemical reactions in enzyme and solution*; Wiley & Sons: New York, 1991; pp 1−236.
(3) Warshel, A.; Hwang, J. K.; Aqvist, J. *Faraday Discuss.* **1992**, *93*, 225−238.
(4) Warshel, A.; Schweins, T.; Fothergill, M. *J. Am. Chem. Soc.* **1994**, *116*, 8437−8442.
(5) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; Dover: New York, 1987.
(6) Hondal, R. J.; Zhao, Z.; Kravchuk, A. V.; Liao, H.; Riddle, S. R.; Yue, X.; Bruzik, K. S.; Tsai, M.-D. *Biochemistry* **1998**, *37*, 4568−4580.
(7) Kravchuk, A. V.; Zhao, L.; Kubiak, R. J.; Bruzik, K. S.; Tsai, M.-D. *Biochemistry* **2001**, *40*, 5433−5439.
(8) Kubiak, R. J.; Hondal, R. J.; Yue, X.; Tsai, M. -D.; Bruzik, K. S. *J. Am. Chem. Soc.* **1999**, *121*, 488−489.
(9) Kubiak, R. J.; Yue, X.; Hondal, R. J.; Mihai, C.; Tsai, M.-D.; Bruzik, K. S. *Biochemistry* **2001**, *40*, 5422−5432.
(10) Hondal, R. J.; Bruzik, K. S.; Zhao, Z.; Tsai, M.-D. *J. Am. Chem. Soc.* **1997**, *119*, 5477−5478.

10.1021/ja029362s CCC: $25.00

bridging interactions through nonbridging O−S replacement should bring about a similar consequence.

The extent of the negative charge development on the leaving group oxygen in the transition state for phosphate ester hydrolysis can be estimated by employing a Brønsted-type linear free energy relationship (eq 1), by examining rate constants of the cleavage reaction as a function of varying pK_a values of the leaving group.[11−17]

$$\log k = \beta_{lg}\, pK_a + C \qquad (1)$$

where k is the rate constant for P−O bond cleavage.

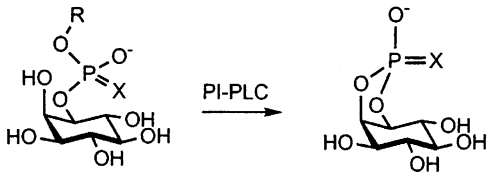
The strong relationship (high negative β_{lg}), indicating a large amount of the negative charge localized on the leaving group, suggests a transition state with negatively charged leaving group (hence resembling the ionized alcohol product), whereas the flat response (low negative β_{lg}) indicates mechanism with such charge absent. It is important to stress, however, that unlike in the nonenzymatic reactions, where the Brønsted coefficient is directly related to the degree of bond formation or bond breaking, enzymatic reactions pose a more complex case. In enzyme reactions, protonation of a leaving group by a general acid function can reduce its negative charge, resulting in a weak response of reaction rates to pK_a change, despite possibly a significant degree of ester bond breaking.[14,15] Before any mechanistic interpretation of the dependence of the rate constants vs leaving group pK_a is attempted, it is also essential to ascertain that the cleavage of the P−O bond is a rate-limiting step. Only in such case, the differences in the stability of transition states (TS) brought upon by the variation in the stability of the leaving group (pK_a) will be manifested in different rate constants for the cyclization reactions. For example, observation of a weak response of rate constants upon pK_a variation can be taken as evidence that very little negative charge is developed on the leaving group in TS. On the other hand, such flat relationship would also be expected if the physical step was rate-determining, since minor changes in the leaving group structure would not have a significant effect on the rates of physical processes such as conformational changes of enzyme, kinetics of binding steps, etc. Once the chemical step rate limitation is established, the comparison of LFER coefficients for a reaction series of different types of substrates should still be a valuable tool in exploring catalytic significance of interactions of the phosphate group with enzyme residues.

One of the requirements for conducting LFER for an enzyme reaction is that the catalytic site accommodate a variety of structures modified such as to alter the pK_a values.[16] This condition is difficult to realize with enzymes displaying strong substrate specificity. Phosphatidylinositol-specific phospholipase C is well suited for this approach, since although it is absolutely specific with respect to the structure of inositol phosphate,[18−21] it has been known to catalyze cleavage of phosphatidylinositol analogues with double or single chain leaving groups and hydrophobic or hydrophilic leaving groups.[22] Additional inspiration to conduct the LFER study on PI-PLC was our earlier finding of strong resistance of alkyl inositol phosphates to chemical cyclization as compared to analogous alkyl ribonucleotides, suggesting different mechanism, as well as the relative paucity of LFER data pertinent to phosphodiester cleavage (other than RNase reactions). Furthermore, application of the Brønsted-type relationship should provide further evidence supporting cooperativity of catalytic functions of Arg69, Asp33, and His82 in their interaction with the nonbridging and leaving group oxygen atoms.

Materials and Methods

Synthesis of Alkyl and Aryl Inositol Phosphates: General Procedure (Scheme 1, Supporting Information). 2,3,4,5,6-Pentakis-(methoxymethylene)-*myo*-inositol[23] (**1**, 1 mmol) was dissolved in anhydrous diisopropylethylamine (2.5 mmol) and a minimum volume of anhydrous chloroform. The solution was cooled to −78 °C, and *O*-methyl phosphorodichloridite (1.25 mmol) was added dropwise via a syringe under nitrogen. The reaction mixture was vigorously stirred and warmed to room temperature over a period of 6 h. The solution of the second alcohol (2 mmol) in dry chloroform (5 mL) was added slowly via a cannula at 0 °C. The reaction mixture was warmed to room temperature and stirred for 12 h. The phosphite triester **2** was oxidized by addding *tert*-butyl hydroperoxide (2 mmol, 5 M solution in decane) via a syringe or sulfurized with elemental sulfur (2 equiv) in anhydrous carbon disulfide (12 h at room temperature). The fully protected phosphate **3a** or phosphorothioate **3b** was treated with anhydrous neat trimethylamine (1 mL) at 50°C for at least 24 h. The progress of the demethylation reaction was monitored by ^{31}P NMR. After the reaction had been completed, trimethylamine was removed under vacuum and the crude product was subjected to final deprotection as follows: Into the phosphate or phosphorothioate diester product from the above trimethylamine reaction was added ethanethiol (1 mL) and boron trifluoride−etherate (100 μL). The reaction mixture was stirred at room temperature for 1 h, and the reaction was monitored by TLC. Ethanethiol was removed under vacuum, and the residue was chromatographed on silica gel using elution with chloroform−methanol−NH_4OH to yield pure deprotected phosphodiesters **4**. This method was applied to all long-chain *O*-alkyl inositol phosphates. Short-chain alkyl phosphodiesters were dissolved in 50% aqueous acetic acid, the obtained solution was heated at 50−60 °C, and the reaction progress was monitored by ^{31}P NMR. The reaction time varied from 30 h to a few days, and the reaction provided a pure product **4** in most cases. Additional purifications were performed by anion-exchange chromatography on Dowex 1×8 200 anion-exchange resin using elution with ammonium formate step gradient. The aryl esters **3a**,**b** were obtained analogously as the alkyl esters, except the phosphites **3** were oxidized with tetrabutylammonium periodate at −20 °C. The aryl esters **3a**,**b** were purified by chromatography on silica gel using hexane−acetone (6:1, v/v) to afford pure products with 60−70% yield as colorless oils. The deprotection of aryl esters **3a**,**b** was performed analogously as described for long-chain aliphatic esters, and the crude products **4** were purified by chromatography on silica gel using chloroform−methanol−acetic acid (6:4:0.1, v/v) as the eluent to yield pure aryl esters **4** as colorless or pale yellow solids. Physical characteristics of each synthesized compound are listed in the Supporting Information.

(11) Davis, A. M.; Hall, A. D.; Williams, A. *J. Am. Chem. Soc.* **1988**, *110*, 5105−5108.
(12) Davis, A. M.; Regan, A. C.; Williams, A. *Biochemistry* **1988**, *27*, 9042−9047.
(13) Hall, A. D.; Williams, A. *Biochemistry* **1986**, *25*, 4784−4790.
(14) Williams, A. *Adv. Phys. Org. Chem.* **1992**, *27*, 1−55.
(15) Hollfelder, F.; Herschlag, D. *Biochemistry* **1995**, *34*, 12255−12264.
(16) Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 7587−7596.
(17) Holtz, K. M.; Catrina, I. E.; Hengge, A.; Kantrowitz, E. R. *Biochemistry* **2000**, *39*, 9451−9458.
(18) Leigh, A. J.; Volverk, J. J.; Griffith, O. H.; Keana, J. F. W. *Biochemistry* **1992**, *31*, 8978−8983.
(19) Bruzik, K. S.; Tsai, M.-D. *Bioorg. Med. Chem.* **1994**, *2*, 49−72.
(20) Lewis, K. A.; Garigapati, V. R.; Zhou, C.; Roberts, M. F. *Biochemistry* **1993**, *32*, 8836−8841.
(21) Bruzik, K. S.; Morocho, A. M.; Jhon, D.−Y.; Rhee, S. G.; Tsai, M.-D. *Biochemistry* **1992**, *31*, 5183−5193.
(22) Bruzik, K. S.; Guan, Z.; Riddle, S.; Tsai, M.-D. *J. Am. Chem. Soc.* **1996**, *118*, 7679−7688.
(23) Kubiak, R. J.; Bruzik, K. S. *J. Org. Chem.* **2002**, *67*, 0000.



pK_a Aryl: 7.1 to 10.2	pK_a Alkyl: 12.4 to 15.9	pK_a Alkyl: 13.3 to 15.9
X = O, S R = Aryl, pK_a = 7.1 - 10.2 Nonhydrophobic	X = O, S R = Alkyl, pK_a = 12.4 - 13.9 Nonhydrophobic	X = O, S R = Alkyl, pK_a = 13.3 - 15.9 Hydrophobic
4-NO_2-Phenyl- 3,5-di-Cl-Phenyl- 2-F-Phenyl- 4-Cl-Phenyl- Phenyl- 4-Me-Phenyl-	CF_3CH_2- CF_2HCH_2- $CFHCH_2$- $CH_3OCH_2CH_2$- CH_3CH_2-	$C_{10}H_{21}CF_2CH_2$-, $C_{10}H_{21}CFHCH_2$-, $C_{10}H_{21}OCH_2CH_2$-, $C_{10}H_{21}OCH_2CH_2CH_2$- $C_{16}H_{33}$-

Figure 1. Structures of substrates used in this work.

^{31}P NMR Assay. Enzymatic Reactions. ^{31}P NMR assays were performed using Bruker DPX-300, DRX-500, and DRX-600 NMR spectrometers equipped with QNP or broad-band probes and temperature control units. The assays were run in 50 mM MOPS buffer, pH 7.5, at 25 °C in the presence of 0.5 mM EDTA. The initial substrate concentration was set at 20−25 mM, which is believed to be saturating concentration for both aryl and long-chain alkyl substrates.[24] In addition, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (diC_6PC) at 80 mM concentration was used to disperse the long-chain alkyl esters. Glucose-1-phosphate (Glu-1-P) and inorganic phosphate were used as internal standards for quantification in the reactions of alkyl and aryl esters, respectively. Samples of amphipathic substrates were prepared in the following manner: The appropriate amounts of the substrate, detergent, and Glu-1-P in an Eppendorf tube were dispersed by vortexing in the buffer solution (300 μL) containing 50 mM MOPS, 5 mM EDTA, pH 7.5, and D_2O (100 μL) and quantitatively transferred into a 5 mm NMR tube. Prior to the assay, the sample was sonicated in the ultrasonic bath to achieve optical transparency. All kinetic runs were performed at 25 °C, and a control spectrum was obtained (t = 0 min) prior to adding the enzyme. The reaction was initiated by adding 50 ng to 5 mg of PI-PLC depending on the enzyme activity with the particular substrate/enzyme. The volume of the enzyme solution added varied from 0.25% to 10% of the entire sample volume. The enzyme solutions were freshly prepared by dissolving the solid protein in dilute buffer, and enzyme concentration was determined spectrophotometrically (ϵ = 18 300 M^{-1} cm^{-1}) at 280 nm. The amount of enzyme used for reaction was sufficient to achieve rates of about 0.05 μmol min^{-1} (1.25% conversion/min); the reactions were typically followed for about 1 h. However, reactions with very low turnover rates were followed for a period of 4−14 days. The typical time course of the cleavage reaction is shown in Figure 1 of the Supporting Information. The rate of the substrate cleavage was calculated by comparing the integral intensity of products with the internal standard Glu-1-P. The initial velocity was obtained by fitting the linear portion of the kinetic curve with KaleidaGraph or SigmaPlot (Jandel Corp.) software.

Nonenzymatic Reaction. Reactions were performed in imidazole buffer (0.5 M, pH 7.0) at 80 °C with the initial concentration of substrate set at 6−8 mM. The time between dissolving the substrate and reaction initiation was kept to a necessary minimum, especially in the case of more reactive substrates. The reaction progress was followed for at least 2 half-lives. Pseudo-first-order rate constants were obtained from nonlinear fits to product concentration vs time plots to the integrated form of the first-order rate law using SigmaPlot.

Purification of R_p-Phosphorothioates. Cleavage of S_p-components of the mixture of (R_p + S_p)-diastereomers of aryl inositol phosphorothioates with PI-PLC was driven to completion by sample dilution and/or by adding a fresh portion of the WT enzyme as monitored by ^{31}P NMR. Subsequently, samples were lyophilized, resuspended in chloroform−methanol−acetic acid (2:1:0.03, v/v), and chromatographed on silica gel column. R_p-Aryl inositol phosphorothioates were eluted with chloroform−methanol−acetic acid (1:1:0.02, v/v). Organic solvents were removed by evaporation and samples were freeze-dried twice from water to remove the residual acetic acid. Recovery of R_p-phosphorothioates after purification was approximately 70%.

Results and Discussion

The substrate analogues synthesized for the purpose of this study (Figure 1) can be separated into three categories: (i) Aryl inositol phosphate esters with pK_a of the leaving group in the 7.1−10.2 range and the corresponding phosphorothioate analogues with R_p- or S_p-configuration at phosphorus are substrates that are significantly less hydrophobic than the regular phosphatidylinositol substrate. (ii) Short-chain alkyl inositol phosphates and phosphorothioates with pK_a in the 12.4−15.9 range are substrates that contain less than 4 carbon atoms in their leaving group and are also much less hydrophobic than the natural substrate. (iii) The long-chain alkyl inositol phosphates and phosphorothioates with pK_a range 13.3−15.9 are substrates that are only somewhat less hydrophobic than PI and can be considered a close approximation of the natural substrate. Overall, some 43 different substrates have been synthesized and the kinetics of the cleavage by WT PI-PLC was studied. ^{31}P NMR assay enabled determination of rate constants within 5−10% error margin. The derived rate constants are collected in Tables 1 and **2**, and the Brønsted dependencies of log k vs pK_a^{lg} are shown in Figures 2−6.

Chemical Cyclization of Aryl Inositol Phosphodiesters. In several of our recent papers, we have used extensively the magnitude of the thio effect (the ratio of the rate constant for phosphate and phosphorothioate, k_o/k_s) as a probe of enzyme interactions with the substrate. Depending on the site of the substitution in the substrate molecule and the strength of interaction of the modified position with the enzyme/mutant, the values of thio effects ranged from 0.002 to 10^8.[6−10,25,26] Thus

(24) Rukavishnikov, A. V.; Ryan, M.; Griffith, O. H.; Keana, J. F. W. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1239−1242.

(25) Hondal, R. J.; Riddle, S. R.; Kravchuk, A. V.; Zhao, Z.; Liao, H.; Bruzik, K. S.; Tsai, M.-D. *Biochemistry* **1997**, *36*, 6633−6642.

Table 1. Pseudo-First-Order Rate Constants of Imidazole-Catalyzed Cyclization of Aryl Inositol Phosphates at 80 °C[a]

		10^4k, min^{-1}		
leaving group	pK_a	phosphate	S_p-phosphorothioate	R_p-phosphorothioate
4-nitrophenol	7.14	170 ± 6	122 ± 2	152 ± 6
3,5-dichlorophenol	8.18	72 ± 2	61 ± 3	69 ± 3
2-fluorophenol	8.80	nd	8.0 ± 0.2	8.1 ± 0.2
4-chlorophenol	9.38	4.6 ± 0.1	4.62 ± 0.09	4.45 ± 0.07
phenol	9.95	1.85 ± 0.03	1.91 ± 0.03	1.80 ± 0.04
4-methylphenol	10.20	1.35 ± 0.02	1.35 ± 0.03	1.25 ± 0.02

[a] The conditions are the same as those described in Figure 2.

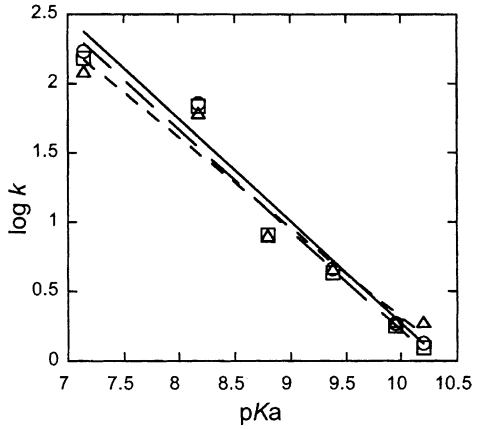


Figure 2. Brønsted dependence of log k vs pK_a for imidazole-catalyzed cyclization of aryl inositol phosphates (□, $\beta_{lg} = -0.74 \pm 0.07$), S_p-phosphorothioates (○, $\beta_{lg} = -0.72 \pm 0.07$), and R_p-phosphorothioates (△, $\beta_{lg} = -0.65 \pm 0.07$). Conditions: 0.5 M imidazole buffer; pH 7.0; 80 °C. The initial substrate concentration was 6−8 mM. The reaction rates were determined by ^{31}P NMR using inorganic phosphate as an internal standard for quantification of the reaction products.

far, we have not been able to compare these values with those of the corresponding base- or acid-catalyzed nonenzymatic reactions since alkyl inositol phosphates are almost completely resistant to chemical cyclization.[27] In this work, the use of the more reactive aryl phosphate esters enabled us to study their chemically catalyzed cleavage as a point of reference for comparison with enzymatic reactions. We have found that the rate constants for the cyclization of aryl inositol phosphates catalyzed by the imidazole buffer at pH 7.0 were close to those of the corresponding R_p- and S_p-phosphorothioates (Table 1). Hence, the thio effect (k_o/k_s) and R_p/S_p-stereoselectivity of chemical cyclization are close to unity. Likewise, the slopes of the dependence of log k vs pK_a (Figure 2) were essentially the same for all three groups of substrates within the experimental error. This result indicates that the previously observed differences between phosphate and phosphorothioate esters and between individual phosphorothioate R_p- and S_p-diastereomers in their enzyme-catalyzed cleavage were entirely due to specific enzyme interactions and were not caused by the intrinsic properties of phosphates vs phosphorothioates.

P−O Bond Cleavage as the Rate-Limiting Step in PI-PLC-Catalyzed Cyclization. We have obtained several sets of data indicating that the P−O bond cleavage is indeed a rate-limiting step in the PI-PLC reaction. First, the maximal rate constants

Table 2. Rate Constants for PI-PLC-Catalyzed Cleavage of Aryl and Alkyl Inositol Phosphates and Phosphorothioates

		K_{cat}, s^{-1}		
			phosphorothioate	
leaving group	pK_a	phosphate	preferred isomer	nonpreferred isomer
4-nitrophenol[a]	7.14	1450 ± 8	290 ± 6	(1.4 ± 0.02) × 10^{-1}
3,5-dichlorophenol[a]	8.18	962 ± 5	345 ± 10	(6.3 ± 0.05) × 10^{-2}
2-fluorophenol[a]	8.80	245 ± 3	26 ± 4	(1.1 ± 0.03) × 10^{-2}
4-chlorophenol[a]	9.38	199 ± 4	13.5 ± 0.5	(3.7 ± 0.04) × 10^{-3}
phenol[a]	9.95	68.0 ± 2	2.1 ± 0.2	(6.1 ± 0.07) × 10^{-4}
4-methylphenol[a]	10.20	82.5 ± 2	4.5 ± 0.4	(8.8 ± 0.02) × 10^{-4}
2,2-difluorododecyl[b]	13.3	383 ± 6	18.5 ± 0.5	(3.9 ± 0.05) × 10^{-4}
2-fluorododecyl[b]	14.2	141 ± 3	6.37 ± 0.08	(4.4 ± 0.02) × 10^{-5}
2-decyloxyethyl[b]	14.8	245 ± 5	21.4 ± 0.4	(9.4 ± 0.04) × 10^{-5}
3-decyloxypropyl[b]	15.1	93.0 ± 3	5.7 ± 0.05	(7.5 ± 0.05) × 10^{-5}
hexadecyl[b]	15.9	188 ± 2	2.8 ± 0.02	(1.4 ± 0.06) × 10^{-5}
2,2,2-trifluoroethyl[a]	12.4	1.6 ± 0.1		
2,2-difluoroethyl[a]	13.3	0.25 ± 0.03		
2-fluoroethyl[a]	14.2	0.040 ± 0.005		
2-methoxyethyl[a]	14.8	0.14 ± 0.02		
ethyl[a]	15.9	0.012 ± 0.003		

[a] The conditions are the same as those described in Figure 3. [b] The conditions are the same as those described in Figure 4.

for the cleavage of the micellar phospholipid substrate (such as double-chain phosphatidylinositol) are above 1250 s^{-1}.[9] The cleavage of such aggregated substrates involves, in addition to the typical formation and dissociation of the Michaelis complex, enzyme binding to the water−lipid interface and the lateral diffusion of the substrate within the lipid monolayer to ensure substrate replenishment. We have found that these rate constants are not affected by the micelle makeup, even for the most reactive natural substrate, since similar turnover rates are obtained with detergents so different in their physical properties as structurally rigid, negatively charged deoxycholic acid and conformationally flexible, zwitterionic short-chain phosphatidylcholine.[9] This result suggests that interfacial binding and substrate lateral diffusion are not rate-limiting for PI-PLC. In addition, most of the substrates used in this study are turned over much more slowly than the natural PI, and even those interfacial substrates with the highest k_{cat} are still severalfold less reactive than the natural PI substrate (Table 2). Second, if the chemical step was not rate limiting, minor variations of substrate structure such as the replacement of the nonbridging oxygen with sulfur should have no impact on the reaction rate (small thio effects). Indeed, such behavior has been observed for RNase A, where the substrate desolvation is rate-limiting and where the thio effect is very small.[28,29] For PI-PLC, however, substitution of the *pro-R* oxygen by sulfur results in up to 40-fold rate decrease[7−9] and of the *pro-S* oxygen by a much larger factor.

Furthermore, the data in Figure 3 show a remarkable linear correlation between the rate constants and the pK_a values spanning more than 3 orders of magnitude in the rate constants. Clearly, if the rates of the most reactive substrates were limited by a physical step, those of the slowest substrates would not, and hence, no linearity would be observed. Finally, hydrophobic alkyl R_p-phosphorothioates which react at 30−50 times slower rates display the significantly less negative Brønsted coefficient than the nonhydrophobic esters ($\beta_{lg} = -0.27$, Figure 4), indicating that at least in this case the weak Bronsted relationship

(26) Zhao,L.; Liu, Y.; Bruzik, K. S.; Tsai, M.-D. *J. Am. Chem. Soc.* **2002**, *124*, 0000.
(27) Kubiak, R. J.; Tsai, M.-D.; Bruzik, K. S. *J. Am. Chem. Soc.* **2001**, *123*, 1760−1761.
(28) Herschlag, D. *J. Am. Chem. Soc.* **1994**, *116*, 11631−11635.
(29) Kuteladze, T. G.; Scuster, M. C.; Venegas, F. D.; Messmore, J. M.; Raines, R. T. *Bioorg. Chem.* **1995**, *23*, 471−481.

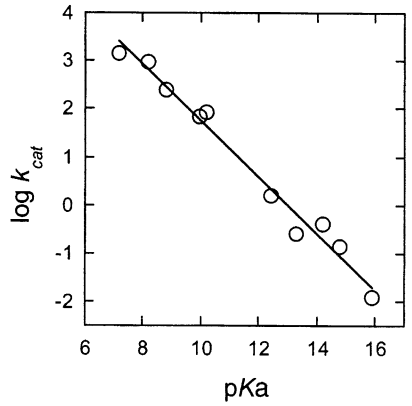


Figure 3. Brønsted dependence of log k_{cat} vs pK_a for PI-PLC-catalyzed cleavage of nonhydrophobic alkyl (pK_a 12.4−15.9) and aryl inositol phosphates (pK_a 7.1−10.2 ($\beta_{lg} = -0.58 \pm 0.06$). Conditions: 50 mM MOPS buffer; pH 7.5; 25 °C. The initial substrate concentration was 20−25 mM. The reaction rates were determined analogously as in Figure 2.

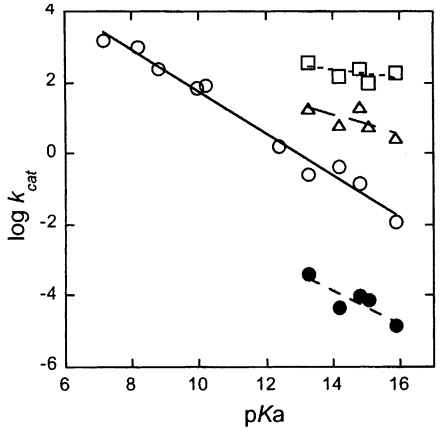


Figure 4. Brønsted dependence of log k_{cat} vs pK_a for PI-PLC-catalyzed cleavage of hydrophobic *O*-alkyl inositol phosphates (□, $\beta_{lg} = -0.12 \pm 0.11$), hydrophobic R_p-*O*-alkyl phosphorothioates (△, $\beta_{lg} = -0.27 \pm 0.15$), and hydrophobic S_p-*O*-alkyl inositol phosphorothioates (●, $\beta_{lg} = -0.46 \pm 0.16$) compared to that of combined short-chain alkyl and aryl esters (○). Conditions: 50 mM MOPS−Na buffer; pH 7.5; 0.5 mM EDTA−Na; 25 °C. The initial substrate concentration was 20 mM, and 1,2-dihexanoyl-*sn*-glycerol-phosphocholine (diC_6PC, 80 mM) was used to disperse the substrate. Glucose-1-phosphate was used as an internal standard for quantification (20 mM).

is not caused by the physical step rate limitation. While the above arguments strongly favor the bond-breaking event as the rate-limiting step, it is possible although not very likely that the physical phenomena partially limit the rate of cleavage, thereby lowering the observed magnitude of Brønsted coefficients in the case of the most reactive substrates.

Chemical vs Enzymatic Cyclization of Nonhydrophobic Substrates. Unlike the enzymatic cyclization of inositol phosphodiesters by PI-PLC which affords inositol 1,2-cyclic phosphates as a sole initial product, we found that the imidazole buffer-catalyzed cyclization produces simultaneously a 4:1 mixture of 1,2- and 1,6-cyclic phosphates.[27] This results indicate that there is no special distinction between reactivity of the axial 2-OH and the equatorial 6-OH group and that regioselectivity of the enzyme reaction is brought about by the regiospecific activation of the 2-OH group by the enzyme. The rate constants reported in Table 1 reflect combined formation of the 1,2- and 1,6-cyclic products as measured by substrate disappearance. The rate constants for the enzymatic cyclization of *O*-aryl and *O*-alkyl inositol phosphates are collected in Table 2 and are graphically expressed in Figure 3. Strikingly, the slopes of the Brønsted correlations in Figures 2 and 3 are rather close, with those for the chemical reactions [$\beta_{lg} = -(0.65-0.74)$] being only slightly more negative than those for the enzymatic reactions ($\beta_{lg} = -0.58$). This result indicates that the enzyme contributes only slightly more to the leaving group stabilization in the transition state than does the imidazole buffer. 4-Nitrophenyl phosphate analogues of RNA and aryl analogues of glycosides have been used previously in conjunction with site-directed mutagenesis for determination of the identity of the general acid residue in RNase A[29] and β-1,4-glycanase,[30] respectively. Recently, we have also used 4-nitrophenyl inositol phosphorothioate for identification of the general acid residue in PI-PLC.[6] The assumption was that, due to high stability of the aryloxide leaving group, the general acid residue of enzyme contributes little to stabilization of the transition state of such substrates. As a result, mutation of the general acid residue had only negligible effect on the rate constants of aryl phosphate esters.[31] Our current results provide experimental confirmation of this assumption by showing that indeed the role of general acid is very limited in the overall catalysis of the cleavage of aryl phosphate esters.

Even more significantly, however, *the enzyme contributes also as little to the leaving group stabilization of the short-chain, nonhydrophobic alkyl esters* (Figures 2 and 3). This finding is important because the latter group of substrates feature leaving groups with pK_a values comparable to that of diacylglycerol of the natural substrate. The poor stabilization of the leaving group in the enzymatic reactions of these *nonhydrophobic* substrates could therefore be the basis of their very slow turnover rates. As we show below, the presence of *hydrophobic* leaving group in the PI-PLC substrate is key to efficient general acid catalysis. Another important conclusion derived from Figure 3 is that the mechanism of the PI-PLC catalyzed cleavage of phosphodiester remains unchanged despite the 10^8-fold increase in the leaving group K_a.

Effect of the Hydrophobic Character of the Leaving Group and Sulfur Substitution. The rate constants for hydrophobic alkyl inositol phosphates and phosphorothioates are listed in Table 2, and the corresponding Brønsted plot is shown in Figure 4. The structures of the leaving groups of these substrates in the immediate vicinity of the phosphate group are very close to those of the short-chain esters considered above; the hydrophobic property is imposed only by the attachment of an additional C10−C14 alkyl chain.

The results indicate that the hydrophobic aliphatic esters behave very differently from the corresponding nonhydrophobic esters, both with regard to the magnitude of the rate constants and the slope of the Brønsted relationship. The hydrophobic esters display much greater rate constants and much weaker dependence on the pK_a as compared to their nonhydrophobic counterparts. This result suggests that introduction of the hydrophobic alkyl group into the substrate induces a quantitative change in the reaction mechanism, affecting enzyme interactions with the leaving group oxygen. The small Brønsted coefficient for the hydrophobic esters indicates a small amount of charge

(30) MacLeod, A. M.; Tull, D.; Rupitz, K.; Warren, A. J.; Withers, S. G. *Biochemistry* **1996**, *35*, 13165−13172.
(31) Thompson, J. E.; Raines, R. T. *J. Am. Chem. Soc.* **1994**, *116*, 5467−5468.

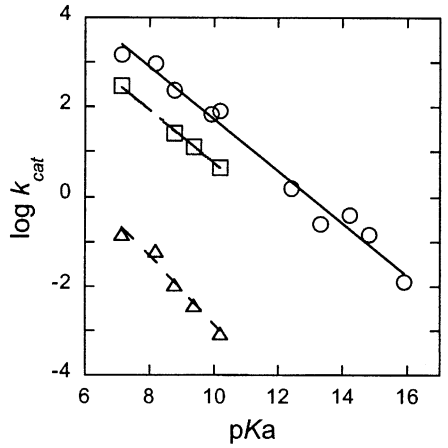


Figure 5. Brønsted dependence of log k_{cat} vs pK_a for the PI-PLC-catalyzed cleavage of aryl inositol phosphates (□, $\beta_{lg} = -0.58 \pm 0.06$), S_p-phosphorothioates (○, $\beta_{lg} = -0.59 \pm 0.05$), and R_p-phosphorothioates (△, $\beta_{lg} = -0.76 \pm 0.07$). The conditions were the same as those described in Figure 3.

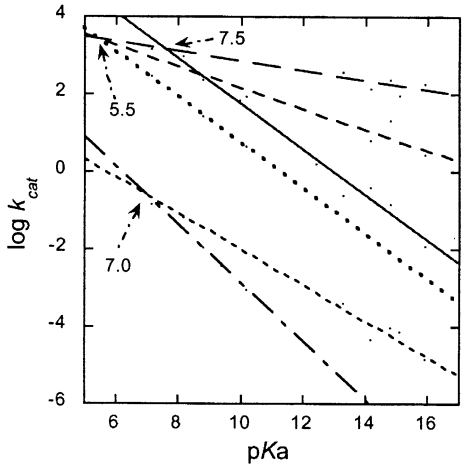


Figure 6. Extrapolation of LFER for groups of hydrophobic and nonhydrophobic substrates showing intersection points at or near neutral pK_a.

on the leaving group oxygen, consistent with the efficient general acid catalysis.[9] It is noteworthy that the rate constant data for alkyl esters show significant scattering, resulting in the apparent large standard deviation of the determination of the Brønsted slope. A closer inspection of the behavior of the long- and short-chain substrates shows, however, that similar modifications of the leaving group structure tend to produce analogous deviations from the linearity. This indicates that although the absolute values of the slopes bear large standard deviation error, the comparison of the values is more precise than what could be suggested by the large error in the determination of the Brønsted coefficients.

In agreement with our expectations, the effect of phosphorothioate modification at the nonbridging position strongly depends on the stereochemical position of the oxygen atom replaced by sulfur. Note that the substitution of the *pro-R* oxygen in alkyl ester and the *pro-S* oxygen in aryl esters by sulfur brings about formation of the analogous stereoisomer despite different R_p/S_p designation. The Brønsted plot for the preferred (R_p) isomers of alkyl phosphorothioates displays only slightly more negative slope as compared to the corresponding phosphates ($\beta_{lg} = -0.27$ vs -0.12, Figure 4). Likewise, sulfur modification has no effect on the Brønsted coefficient in the case of preferred aromatic esters ($\beta_{lg} = -0.59$ vs -0.58, Figure 5). In contrast, the nonpreferred diastereomers of both alkyl (S_p) and aryl esters (R_p) display significantly more negative Brønsted coefficients ($\beta_{lg} = -0.46$ for aliphatic esters and $\beta_{lg} = -0.76$ for aryl esters). Unfortunately, due to very low reactivity, we have not been able to construct the analogous Brønsted correlation for the phosphorothioate analogues of nonhydrophobic alkyl esters.

The above results indicate that alteration of interactions of enzyme with the *pro-S* oxygen in the case of alkyl esters (and stereochemically equivalent *pro-R* oxygen of aryl esters) results in significant impairment of general acid catalysis. Such conclusion is entirely consistent with the mechanism that we postulated earlier,[6,7,9] whereby interactions of enzyme with the nonbridging oxygen and the leaving group oxygen are strongly inter-related.

Hydrophobic Effect of the Leaving Group Minimized at Low pK_a. It is interesting to note that the Brønsted plots of nonhydrophobic and hydrophobic phosphates and the corresponding pairs of nonhydrophobic and hydrophobic R_p-phosphorothioates and S_p-phosphorothioates intersect at the pK_a values near neutral (7.5, 5.5, and 7.0, respectively) (Figure 6).

This result can be interpreted to suggest that the magnitude of the catalysis-enhancing effect of hydrophobic interactions depends on the pK_a of the leaving group, gradually diminishing with more stable leaving groups. Substrates that include leaving groups with pK_a close to neutral no longer require any stabilization of their leaving group by the enzyme by the general acid catalysis, so the hydrophobic activation disappears altogether.

Comparison with Other LFER Studies of Other Phosphoesterases. LFER studies have been used previously to assess mechanisms of enzymatic phosphoryl transfer reactions,[1−5] to interpret differences between chemical and enzymatic mechanisms,[11−13] and to detect changes in the mechanism produced either by enzyme mutation or substrate modification[15−17] In view of an apparent similarity of the nature of the phosphodiester scission by PI-PLC and RNase A (the use of an intramolecular β-hydroxy group as a nucleophile to form a five-membered cyclic phosphate product), and structural resemblance of the active sites of these two enzymes,[32] it is useful to compare and contrast the corresponding LFER data. First, the imidazole-catalyzed cyclization of aryl uridine phosphates displays the value of $\beta_{lg} = -0.59$.[11] Given the fact that a similar value of $\beta_{lg} = -0.54$ was obtained for the hydroxide-catalyzed cleavage of aryl uridine phosphates,[11] the imidazole catalysis does not seem to involve stabilization of the leaving group by protonation and, hence, could be used as a reference system for detection of general acid catalysis. Accordingly, RNase A-catalyzed cleavage of the same class of compounds is characterized by a value of $\beta_{lg} = -0.17$, consistent with strong general-acid catalysis reducing the effective negative charge on the leaving group in the TS.[12] The calculation of the rate constants for the RNase A-catalyzed cleavage of dinucleotide (pK_a 14.8) using the LFER equation obtained for aryl esters[12] gives the value of log $k_{cat}/K_m = 3.3$, in good agreement with the experimental data for UpA.[29] It therefore appears that aryl nucleotides and dinucleoside phosphates are cleaved by RNase A via a similar

(32) Gässler, C. S.; Ryan, M.; Liu, T.; Griffith, O. H.; Heinz, D. W. *Biochemistry* **1997**, *36*, 12802−12813.

mechanism, despite a large difference in the leaving group structure.

The imidazole-catalyzed cyclization of aryl inositol phosphates displays significantly more negative value of $\beta_{lg} = -0.74$ (this work) than that for aryl uridine phosphates. The difference in β_{lg} between the inositol and nucleoside aryl phosphates arises most likely from significantly lower reactivity of inositol esters,[27] possibly resulting in a more advanced transition state with a larger degree of bond breaking to the leaving group in the latter case. In addition, PI-PLC cleavage of both aryl and short chain alkyl esters displays only slightly lower sensitivity to pK_a changes, $\beta_{lg} = -0.58$, than the imidazole-catalyzed reaction, indicating weak general-acid catalysis for this group of compounds. In stark contrast, PI-PLC-catalyzed cleavage of hydrophobic alkyl esters displays $\beta_{lg} = -0.12$, similar to that of RNase A. These results clearly indicate that there is a significant difference in the way the leaving group structure modulates catalysis in both enzymes. It appears that the structure of the leaving group, and its hydrophobic property in particular, strongly affect the mechanism of PI-PLC, while there is no evidence of an analogous effect in the case of RNase A.

Similarly to our results, a more negative value of β_{lg} was observed for alkaline phosphatase (AP)-catalyzed hydrolysis of aryl phosphorothioates as compared to the corresponding phosphates.[15] In this case, however, it was conclusively shown that while the rates of the reactions of phosphates are limited by the physical steps (resulting in a weak response of rate constants to pK_a changes), those of the phosphorothioates are limited by the chemical step (resulting in a steeper negative slope). This explanation does not hold for PI-PLC as discussed in the earlier section. Although AP-catalyzed hydrolysis of aryl phosphorothioates displayed a less negative Brønsted coefficient than that determined for chemical hydrolysis (-0.77 vs -1.1), this difference was attributed to factors other than the change of transition state structure caused by enzyme interactions.[15] In addition, the mutants of AP altered at the Arg166 residue that interacts with the nonbridging oxygen atom of the phosphomonoester substrate showed largely unaffected Brønsted coefficients as compared to the WT enzyme.[17] The reason for the latter finding could be that Arg166 is involved in stabilization of the negative charge of the *nonbridging* oxygen, and there is no evidence that this residue actually interacts directly or indirectly with the leaving group. Furthermore, R166A mutation causes only a modest 40-fold reduction in k_{cat}, a value probably too small to be reflected in the magnitude of LFER coefficients.

Mechanism of PI-PLC Inferred from This Work. The goal of the current work was to validate the conclusions derived from our earlier, matched enzyme−substrate mutagenesis study of PI-PLC mechanism.[9] Those results indicated the involvement of the novel, dual-function catalytic triad composed of Arg69, Asp33, and His82[8,9] (Figure 7). We have postulated that interactions of this triad with the oxygen atoms of the phosphate group at the nonbridging and the bridging (leaving group) positions are strongly interdependent, i.e., that the weakening of the bridging interactions diminishes or removes the bridging interactions and vice versa. In addition, we postulated that the catalytic roles of Asp33 and His82 are greatly reduced in the case of nonhydrophobic substrates. In this work, we sought to confirm these findings by using Brønsted-type relationship to assess changes in the amount of the negative charge on the leaving group oxygen in response to variations of the substrate hydrophobicity and alteration of nonbridging interactions. The observation of similar highly negative β_{lg} coefficients for the nonenzymatic and enzymatic cleavage of aryl inositol phosphates and enzymatic cleavage of nonhydrophobic esters indicates that the enzyme mechanism employs weak general-acid catalysis, only slightly more efficient than that achieved by the imidazole buffer. In agreement with our earlier postulate,[9] however, the hydrophobic alkyl esters displayed low negative β_{lg} coefficients, consistent with significantly smaller negative charge on the leaving group and stronger general acid catalysis as compared to nonhydrophobic esters.

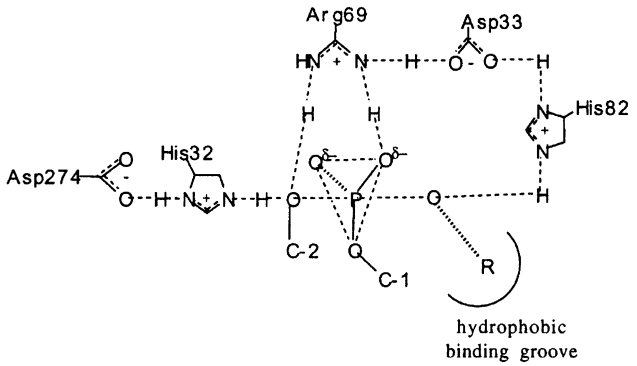


Figure 7. Mechanism of PI-PLC as proposed previously[9] and confirmed by this study. This mechanism is thought to operate in reactions of the hydrophobic substrates, whereas the cleavage of nonhydrophobic esters reduces the degree of cooperation between the three residues in the Arg69−Asp33−His82 triad.

Our results suggest that, in the case of PI-PLC, a major quantitative change in the transition state structure occurs upon a relatively minor alteration of the substrate structure by introducing a remote hydrophobic substituent into the leaving group. This mechanistic change results in effective stabilization of the negative charge on the leaving group. It is possible that analogous binding of remote parts of leaving groups could be important to catalysis by other hydrolytic enzymes.

Supporting Information Available: A description of the synthetic procedures, physical data for PI-PLC substrates, and a listing of Brønsted equations. This material is available free of charge via the Internet at http://pubs.acs.org.

JA029362S