Mechanism of Phosphatidylinositol-Specific Phospholipase C: Origin of Unusually High Nonbridging Thio Effects†

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ABSTRACT: Phosphatidylinositol-specific phospholipase C (PI-PLC) has been proposed previously to employ a catalytic mechanism highly reminiscent of that of ribonuclease A (RNase A). Both catalytic sites are comprised of two histidine side chains acting as a general base−general acid pair and a phosphate-activating residue: an arginine in the case of PI-PLC and a lysine in RNase A. Despite these structural similarities, the PI-PLC reaction is slowed 10^5-fold upon substitution of one of the phosphate nonbridging oxygen atoms with sulfur, whereas a much smaller effect is observed in the analogous RNase A reaction. Here, we report a systematic study of this property in PI-PLC, conducted by means of site-directed chemical modification of a cysteine residue replacing the arginine at position 69. The results show that mutant enzymes featuring bidentate side chains at this position display significantly higher activity, higher thio effects, and greater stereoselectivity than do those with monodentate side chains. The results suggest that the bidentate nature of Arg69 is the origin of the large thio effects and stereoselectivity in PI-PLC. We propose that in addition to binding the phosphate, the function of arginine 69 is to bring the phosphate group and the 2-OH group of inositol into proximity and to induce proper alignment for nucleophilic attack, and possibly to lower the pK_a of the 2-OH. The results presented here could be important to mechanisms of phosphoryl transfer enzymes in general, suggesting that a major part of thio effects observed in enzymatic phosphoryl transfer reactions can originate from factors other than direct interaction between a side chain and a phosphate group, and caution the use of the absolute magnitude of the thio effect as an indicator of the strength of such interactions.

Phosphorothioate analogues of phosphate, with one of the nonbridging oxygens replaced with a sulfur atom, were among the earliest and still are among the most popular mechanistic probes of enzymatic (*1*−*3*) and ribozymatic (*4*−*6*) phosphoryl transfer reactions. Phosphorothioates have proven to be particularly valuable for elucidation of the steric course of phosphoryl transfer reactions (*7*−*10*). However, mechanistic interpretations of the kinetic effects of sulfur substitutions (nonbridging thio effects) remain controversial, even for the well-studied ribonuclease A (RNase A)[1] (*11*, *12*).

The chemical reactivity of phosphorothioate diesters does not significantly differ from that of the corresponding phosphates; it is only about 2−4-fold lower for intermolecular reactions, and even less so for intramolecular reactions (*4*, *13*). Therefore, thio effects observed in enzymatic reactions ($k_O/k_S \gg 1$) almost entirely result either from disruption of favorable protein−substrate interactions or from newly introduced unfavorable ones. In the former case, the interactions being affected are hydrogen bonding and/or metal complexation, since sulfur and oxygen differ considerably in their ability to form hydrogen bonds and to chelate metal ions (*1*).

Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) displays unusually high nonbridging thio effects, which are associated with the catalytic function of arginine 69 (*14*). While PI-PLC and RNase A display significant similarities in both the topological structures of their respective substrates and the mechanisms employed in catalysis (Figure 1), the thio effects are significantly different between these two enzymes; the thio effects for R_p-isomers are 2 and 40 and those for S_p-isomers 70 and >100000 for RNase A and PI-PLC, respectively (*11*, *14*, *15*). Such a large difference in thio effects suggests fundamentally different interactions between the enzymes and the phosphate groups of their

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[1] Abbreviations: AA, acetylamidine; EA, ethylamine; CAA, chloroacetylamidine; DHPC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; DOsPI, (2*R*)-1,2-dioctanoyloxypropanethio-3-(1-phospho-1*D*-*myo*-inositol); DPPI, dipalmitoylphosphatidylinositol; DPPsI, 1,2-dipalmitoyl-*sn*-glycero-3-(thiophospho-1-*myo*-inositol); DTBA, dithiobis(acetylamidine); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gnd·HCl, guanidinium hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; IcP, inositol 1,2-cyclic phosphate; IcPs, inositol 1,2-cyclic phosphorothioate; PA, propylamine; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; RNase A, ribonuclease A; SDS, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; WT, wild type.

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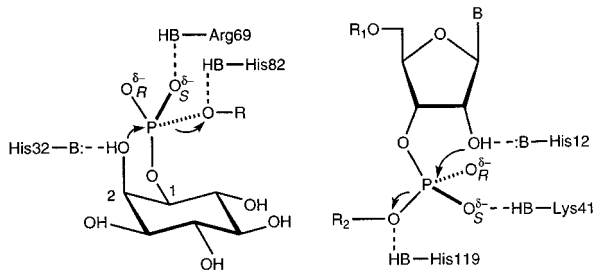

FIGURE 1: Mechanistic analogy between bacterial PI-PLC and RNase A. Shown are structures of both substrates and major components of enzymatic catalytic mechanism: general base (His32 and His12), general acid (His82 and His119), and phosphate activation residues (Arg69 and Lys41).

respective substrates. The only apparent distinction between the modes of phosphate activation in these two enzymes is that for RNase A, activation of the phosphate group is achieved by a lysine 41 residue (*16*, *17*), while in PI-PLC, a similar role was proposed for the arginine 69 side chain (*14*, *18*, *19*). Replacement of arginine 69 with lysine in PI-PLC had a dramatic impact on both activity and thio effects (*14*), but since the structures of these amino acids are different in several respects, it was not clear whether side chain length, the structure of the terminal functional group, or both were responsible for the observed results.

Site-directed chemical modification (sometimes termed "chemical elaboration of cysteine") successfully combines the powers of site-directed mutagenesis and chemical modification of a cysteine side chain to introduce unnatural side chain residues into proteins. Since its first introduction by Smith and Hartman (*20*), this technique has been used in numerous systems: in modulating properties of subtilisin (*21*), as a probe for ion channel properties (*22*), for site-directed incorporation of spin-labels (*23*, *24*), for investigation of membrane-spanning proteins (*25*), and for improving the catalytic power of natural enzymes (*26*). This approach has proven to be particularly valuable for systematic studies of arginines and lysines in enzymatic catalysis and substrate−ligand binding (*16*, *27*−*29*).

Bacterial PI-PLC is a suitable target for site-directed chemical modification since the WT enzyme does not contain any cysteine residues (*30*); thus, cysteine can be introduced as a unique target for chemical site-specific modification. In this paper, we describe application of site-directed mutagenesis and site-directed chemical modification at position 69 of bacterial PI-PLC for delineating the origin of the unusually large nonbridging thio effects and stereoselectivity, as well as the detailed functional role of Arg69.

MATERIALS AND METHODS

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-(thiophospho-1*D*-*myo*-inositol) (DPPsI; mixture of R_p- and S_p-isomers) and enantiomerically pure (2*R*)-1,2-dioctanoyloxypropanethio-3-(1-phospho-1*D*-*myo*-inositol) (DOsPI) were synthesized as described previously (*31*, *32*). 2-Chloroacetamidine hydrochloride was from Lancaster; 2-chloroacetonitrile and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were from Aldrich, and 2-bromoethylamine and 3-bromopropylamine were from Sigma. Dithiobis(acetylamidine) (DTBA) was synthesized using a combination of the two previously published procedures (*33*, *34*). All other reagents were of the highest grade available.

Arg69 → Cys Mutant. The R69C mutant was made via the "QuickChange" (Stratagene) procedure using the following oligonucleotides (mutated nucleotides are in boldface font and the 69th codon is italicized): 5′-CGCATTTTTG-ATATA***T***G***T***GGACGTTTAACAGATG-3′ (sense strand) and 5′-CATCTGTTAAACGTCC***A***C***A***TATATCAAAAAT-GCG-3′ (antisense strand).

To prevent possible metal contamination and sulfhydryl group oxidation, the first purification of the R69C mutant was performed with 10 mM EDTA and 1 mM DTT present in all buffers, with the exception of the final dialysis buffer. After it had become evident (see below) that the cysteine residue is not exposed to solvent, the regular protein purification procedure (*14*, *35*) was used.

Quantification of Free Thiol with DTNB. Typically, 0.5−1 mg of the unmodified or 2−3 mg of the modified R69C mutant was dissolved in 5 M Gnd·HCl (pH 7.5), and DTNB was added to its final concentration of 1 mM. The reaction mixture was incubated at room temperature for 15 min, and the concentration of the released chromogenic product was determined by measuring the absorbance at 412 nm, using a molar extinction coefficient of 13 600 M^{-1} cm^{-1} (*36*).

Chemical Modification Reactions. R69C PI-PLC (∼10 mg) was dissolved in the buffer containing 50 mM Tris (pH 8.0), 3.5 M guanidinum hydrochloride, 5 mM EDTA, and 0.2 M modifying reagent (0.1 M in the case of DTBA). The reaction mixture was flushed with argon and incubated in the dark at room temperature for 1−3 h. Incubation times were determined individually for each reagent in trial experiments. The degree of modification was established by free thiol titration with DTNB. After incubation, the reaction mixture was dialyzed successively against four buffers: (1) 2 M urea, 100 mM HEPES, and 100 mM NaCl (pH 7.0); (2) 1 M urea, 50 mM HEPES, and 100 mM NaCl (pH 7.0); (3) 20 mM HEPES and 100 mM NaCl (pH 7.0); and (4) 10 mM HEPES (pH 7.0). The resulting protein solution was centrifuged and the supernatant concentrated and lyophilized. The recovery of the soluble protein after this refolding procedure varied in the range of 70−90%.

Native PAGE and Isoelectrofocusing. Native PAGE analyses of protein samples were carried out on Phastsystem (Pharmacia) using homogeneous 20% gels according to the manufacturer's recommendations. Isoelectrofocusing (IEF) was conducted on Phastsystem (Pharmacia) in the premanufactured pH gradient (from pH 4 to 6.5). Both native PAGE and IEF gels were silver-stained according to the corresponding manufacturer's protocols.

Mass Spectrometry of Modified Proteins. Electrospray ionization mass spectra of all modified proteins, as well as that of the R69C mutant, were obtained with an API-300 (PE-SCIEX) spectrometer at the biological mass spectrometry facility at The Ohio State University.

Radioactive Assay of PI-PLC with the [^{3}H]PI Substrate. The specific activities of the mutants were measured according to the procedure reported previously (*37*) with a few modifications. [^{3}H]PI (Dupont NEN) was mixed with the unlabeled PI from bovine brain (Avanti Polar Lipids) to obtain the overall PI concentration of 10 mM and the specific radioactivity of $\sim 1.25 \times 10^6$ cpm/μmol. Detergent DHPC was also added to a final concentration of 40 mM. The

reaction mixture contained 20 μL of this substrate solution and 40 μL of 0.1 M HEPES (pH 7.5). An aliquot of 20 μL of PI-PLC was added to the reaction mixture and incubated at 37 °C for 10 min. The reaction was stopped by the addition of 0.5 mL of a $CHCl_3/CH_3OH/HCl$ (66:33:1) mixture. The resulting phases were separated by a brief centrifugation, and the radioactivity of the aqueous phase (50−100 μL) was measured by scintillation counting. Concentrations of WT PI-PLC and mutants were adjusted so that the substrate conversion did not exceed 10−30%. The enzymatic activity was expressed in micromoles per minute per milligram or units per milligram of protein.

^{31}P NMR Assays with DPPsI. ^{31}P NMR assays were performed on Bruker DRX-500 and DRX-600 NMR spectrometers, both equipped with a 5 mm broadband probe and a temperature control unit. All reactions were carried out at 25 °C in 50 mM HEPES buffer (pH 7.5). The mixture of S_p- and R_p-isomers (~6:4 ratio) of DPPsI at a total concentration of 10 mM and a 4-fold excess of DHPC were dispersed as micelles in a bath sonicator. Small aliquots of the prepared substrate (0.5 mL) were used in each assay, and reactions were initiated by adding the appropriate amount of the enzyme diluted in the reaction buffer. In those cases where the enzymatic rates with the two isomers were significantly different, a second aliquot of the protein (usually of significantly higher concentration) was added after the reaction with the preferred (R_p) isomer had been completed. The rates of the reactions were calculated from the linear portions of the plot of IcPs concentration versus time.

Determination of Bridging Thio Effects. Reactions with the chromogenic substrate, DOsPI, were performed as reported previously (*14*, *31*). Reaction mixtures included 1 mM DOsPI and 5 mM DTNB in 50 mM HEPES (pH 7.5). The initial reaction velocity (V_o) was obtained by monitoring the change in absorbance at 412 nm. The bridging thio effects were obtained by comparing activities of enzymes derived from this assay with those from the radioactive assay.

RESULTS AND DISCUSSION

Chemical Modification of the R69C Mutant and Characterization of the Modified Proteins. Wild-type (WT) PI-PLC does not contain any cysteines, so mutation of arginine 69 to cysteine (R69C) created a unique target for chemical modification. A total of four modifications were made, including two arginine analogues (cysteine acetamidine and cysteine thioacetamidine) and two lysine analogues (cysteine ethylamine and cysteine propylamine) (Figure 2).

Under native conditions, neither the modification reagents nor DTNB was able to react with the thiol group in the pH range of 7.0−9.0. Thus, even though arginine 69 is located in the active site, the cysteine side chain in the R69C mutant appears to be completely shielded from the solvent. The modification reactions were therefore performed under partially denaturing conditions in the presence of 3.5 M guanidinium hydrochloride. The modified proteins were subsequently refolded by a stepwise dialysis against decreasing concentrations of chaotropic agents.

Titration of the modified proteins with DTNB confirmed that reactions were quantitative, with less than 5% of the free thiol remaining in all cases. Analysis of modified proteins by electrospray mass spectrometry confirmed the

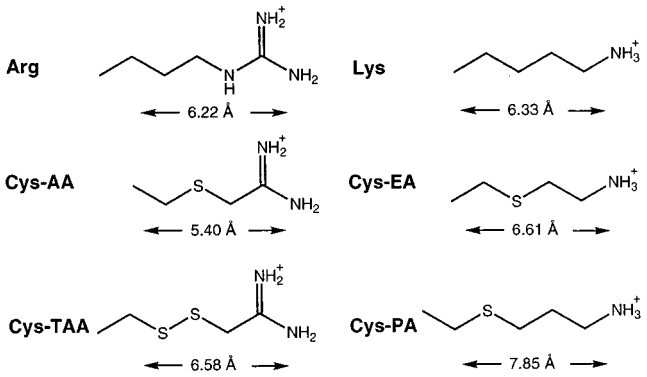

FIGURE 2: Comparison between side chain structures of arginine, lysine, and modified cysteins. The indicated side chain lengths were calculated for the fully extended conformations.

Table 1: Analysis of the PI-PLC R69C Mutant and Its S-Alkylated Analogues by Electrospray Ionization Mass Spectrometry

protein	calculated molecular mass (Da)	found molecular mass (Da)
R69C	34 459	34 461
R69C-EA	34 503	34 510
R69C-PA	34 517	34 522
R69C-AA	34 516	34 521
R69C-TAA	34 548	34 552

1:1 stoichiometry of the modifications (Table 1). The purity of the modified enzymes was determined by native PAGE (not shown), displaying only one band in all cases. Isoelectric focusing demonstrated that modifications of R69C restored its pI to the levels of the WT and R69K mutant.

Bidentate Residues Are Highly Preferred over Monodentate Residues at Position 69. The chemically modified mutants were first used to probe whether the large decrease in activity of R69K (*14*) is caused by a different chain length, or the monodentate nature, of lysine. As shown in Table 2, the activities of the three bidentate enzymes (WT, R69C-AA, and R69C-TAA) are substantially higher that those of the three monodentate enzymes (R69C-PA, R69C-EA, and R69K), even though the side chains of some monodentate analogues are longer than those of some bidentate analogues (Figure 2). These data clearly demonstrate that there is a significant catalytic advantage in having a bidentate functional group (arginine analogue) rather than a monodentate functional group (lysine analogue) at position 69.

The above conclusion was further supported by chemical rescue experiments. The R69A and R69G mutants of PI-PLC were activated by guanidinium hydrochloride to a significant extent (Figure 3). The activation by guanidine appeared to be highly specific, as neither simple amines nor guanidine analogues activated those mutants to any appreciable extent (data not shown). Although the maximal activity was still ~100-fold lower than that of the R69C-TAA modified enzyme, the complexes of R69A and R69G with guanidine had activities comparable to that of R69C-EA, despite the lack of covalent attachment of guanidinium function to the side chain (Table 2 and Figure 3). This fact strongly supports our conclusion about the importance of a bidentate functional group at position 69.

Bidentate Ligands at Residue 69 Are the Origin of Large Thio Effects and Stereoselectivity. As described in our previous publications (*14*, *15*, *35*, *38*), WT PI-PLC displays

Table 2: Specific Activities, Thio Effects, and Stereoselectivities of PI-PLC with Different Side Chains at Position 69 toward Phosphate and Phosphorothioate Substrates

	PI[a]	(R_p)-DPPsI[b]		(S_p)-DPPsI[b]		stereoselectivity	DOsPI[c]	
enzyme	V_{max}[e]	V_{max}[e]	k_O/k_{R_p}	V_{max}[e]	k_O/k_{S_p}	R_p/S_p	V_{max}[e]	k_O/k_S[d]
WT	2200	53	42	0.007	3.1×10^5	7600	91	24
R69C-AA	160	4.0	40	0.001	1.6×10^5	4000	3.5	46
R69C-TAA	50	1.3	38	0.0004	1.2×10^5	3000	ND[f]	
R69C-PA	1.5	0.043	35	0.0038	3.9×10^2	11	0.031	48
R69C-EA	0.50	0.014	36	0.0046	1.1×10^2	3.0	ND	
R69K	0.10	0.0029	34	0.003	2.7×10	1	ND	

[a] Radioactive assay. [b] NMR assay. [c] Spectrophotometric assay. [d] Bridging thio effect. [e] In micromoles per minute per milligram. [f] ND, not determined.

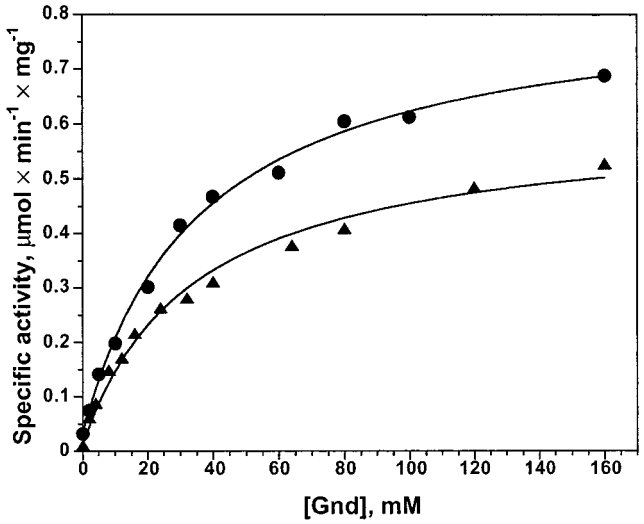

FIGURE 3: Activation of R69A (●) and R69G (▲) mutants by guanidinium hydrochloride. Solid lines represent a nonlinear fit of each data set to the single-site binding equation with the following parameters: $V_{max} = 0.83 \pm 0.03$ μmol min^{-1} mg^{-1} and $K_d = 36 \pm 3$ mM for R69A, and $V_{max} = 0.61 \pm 0.02$ μmol min^{-1} mg^{-1} and $K_d = 34 \pm 4$ mM for R69G. Basal activities of R69A and R69G were 0.03 and 0.01 μmol min^{-1} mg^{-1}, respectively.

an unusually large thio effect toward (S_p)-DPPsI and, consequently, unusually large stereoselectivity (R_p/S_p ratio). The large stereoselectivity was almost completely lost in the R69K mutant. Such results indicated that the side chain of Arg69 interacts specifically with the *pro*-S oxygen of the phosphate group. However, it remained unclear why the S_p thio effect and the R_p/S_p ratio of PI-PLC are larger than the corresponding parameters of most other enzymes, particularly ribonuclease A. To understand the origin of the large thio effect, we determined the nonbridging thio effects and the stereoselectivities for the chemically modified mutants (Table 2).

The results in Table 2 show the following important points. (i) The activities toward the (S_p)-DPPsI isomer are very low and fall within a narrow range. Consequently, all three bidentate enzymes show very large thio effects toward (S_p)-DPPsI ($k_O/k_{S_p} = 1.2-3.1 \times 10^5$), and very large stereoselectivities ($R_p/S_p = 3000-7600$). The corresponding values for all three monodentate enzymes are smaller by 3 orders of magnitude. These results indicate that *the bidentate nature of Arg69 is responsible for the very large k_O/k_{S_p} thio effect and the very high stereoselectivity of WT PI-PLC*. (ii) In contrast, the activities toward (R_p)-DPPsI closely parallel those toward DPPI, and as a result, thio effects with the R_p-isomer (k_O/k_{R_p}) for all analyzed proteins are essentially the same (~40). These results suggest that the R_p thio effects are not caused by phosphate interactions (or their alteration) with the side chain at position 69, but originate from an unfavorable interaction of the sulfur atom with another part of the active site. These results taken together *not only support our previous conclusion that the side chain of Arg69 interacts specifically with the pro-S oxygen of the phosphate group but also further suggest that it does not interact directly with the pro-R oxygen*.

Three Possible Models for the Arg69−Phosphate Interaction. The conclusions from the preceding paper (*15*) and the additional results described above allow us to propose possible models for detailed interactions between Arg69 and the phosphate group of the PI substrate. These models should include the following elements. (i) The Arg69 side chain must form hydrogen bonds with Asp33 (as part of the Arg69−Asp33−His82 catalytic triad) (*38*, *39*). (ii) It must interact exclusively with the pro-*S* oxygen of the phosphate group, not the pro-*R* oxygen. (iii) It must utilize the bidentate nature of the side chain. On the basis of these requirements, we propose three possible models shown in Figure 4A−C.

Model A is proposed because it is not unusual for the nonbridging oxygens of phosphate groups to receive more than one hydrogen bond, and such an interaction is expected to be favorable with positively charged species such as a guanidinium group (*40*, *41*). It is not clear, however, how this second hydrogen bond could contribute to catalysis, particularly if one takes into account that reactions of phosphodiesters proceed through SN2-like transition states (*42*−*44*) with very little additional negative charge developing on the nonbridging oxygens. Model B is proposed because it is straightforward; the two functional groups of the guanidinium group are involved in the two separate functions: activation of phosphate and interaction with the His82−Asp33 general acid diad. Model C is proposed because in the crystal structure of the bacterial PI-PLC complex with *myo*-inositol, Arg69 was shown to be hydrogen-bonded to the 2-OH of inositol (*18*). Although the X-ray structure shows only a static complex with a substrate analogue, the same interaction could occur at the transition state of the reaction. The proposed interaction of arginine 69 with 2-OH of inositol would lower the pK_a of the incoming nucleophile, thereby facilitating proton abstraction by His32, and would help *bring the hydroxyl and the phosphate into proximity and a proper orientation for catalysis*. Studies with different classes of intramolecular reactions have shown that fixing reacting groups in the orientation most favorable for the reaction, by forming the near-attack conformation prior to entering the transition state

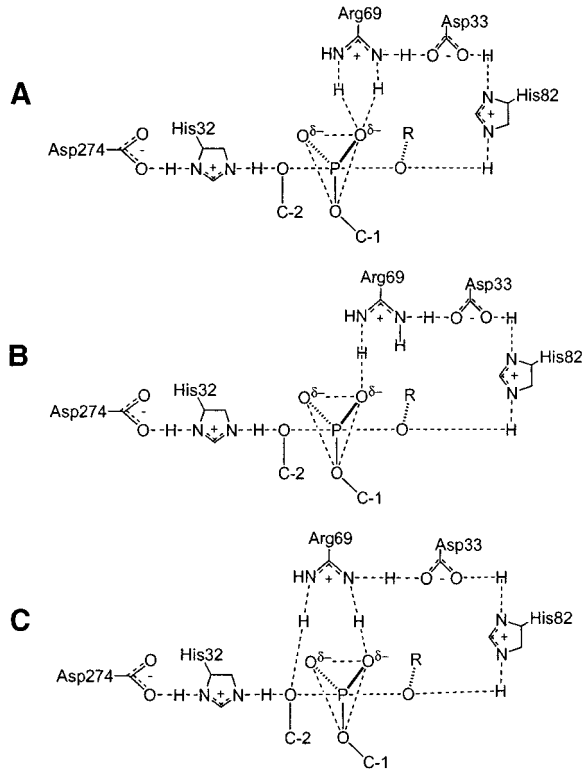

FIGURE 4: Possible arrangements for the bidentate interaction of Arg69 with the substrate and other components of the active site during catalysis. (A) Both η-nitrogens are hydrogen-bonded to the pro-*S* oxygen of the phosphate. (B) Bridging interaction with the pro-*S* oxygen and the general acid dyad (Asp33−His82). (C) Bridging interaction with the pro-*S* oxygen and 2-OH of inositol.

(*45*, *46*), could result in rate increases of up to 10^3-fold.

Model C Is Best Supported by Thio Effects. As the length of the monodentate side chains increases from lysine to aminopropylcysteine, the activities toward PI and (R_p)-DPPsI also increase, as does the stereoselectivity (Table 2). *This suggests that in the monodentate ligand, the amino group still interacts with the pro-S oxygen, albeit weakly*. This result predicts that, if model B were the correct structure, the interaction of the monodentate ligand with the Asp−His diad would be impaired and the general acid function should be seriously perturbed. To determine whether the general acid function is perturbed in the modified proteins, we studied the bridging thio effects. As shown previously (*35*, *47*), mutants of PI-PLC with disrupted general acid function display a "reversed" bridging thio effect; i.e., $k_O/k_S < 1$. This phenomenon is due to the fact that the thiolate has a lower pK_a than an alkoxide and is a better leaving group, thus requiring less assistance from the general acid. The comparison of activities of WT, R69C-AA, and R69C-PA toward PI and the phosphorothiolate analogue, DOsPI, gave "normal" bridging thio effects of 24, 46, and 48, respectively (Table 2, last column). In contrast, the removal of the ω-amino group as in the R69A mutant resulted in a 10-fold reduction in the nonbridging thio effect (*15*). The similarity of the bridging thio effects for both arginine and lysine analogues suggests that the function of the general acid is not perturbed in the lysine analogues. This result does not support model B.

Since R69K is completely nonstereoselective (Table 2), the lysine side chain most likely has little interaction with the phosphate. This conclusion is supported by the fact that the activity of the R69A mutant is only ~20-fold lower than that of R69K (this work and ref *15*). This result supports the cooperative relationship between the functions of the two amino groups of the arginine residue as in model C, as opposed to the additive one predicted by model A. Furthermore, the bridging interaction featured in model C could explain the unusually high thio effects toward (S_p)-DPPsI observed with enzymes containing bidentate groups at position 69. In this model, introduction of sulfur into the pro-*S* position not only would disrupt the interaction of arginine with the phosphate but also would likely reduce its ability to properly align the phosphate group with the nucleophile, even if its interaction with the 2-OH was retained. Supporting this hypothesis is the fact that ribonuclease T1, where Arg77 replaces Lys41 of RNase A, also displays a high thio effect toward (S_p)-phosphorothioate (*48*). Several other instances of enzymes in which the arginine side chain interacts with two functional groups of the substrate have been reported previously (*49*, *50*).

In summary, even though other alternatives cannot be ruled out completely, the bridging interaction with both the phosphate and the 2-OH group of inositol is most likely responsible for the superior catalysis by the bidentate side chains, as compared to monodentate ones, having support from structural and functional data.

Comparison with Mammalian PI-PLC. The crystal structure of mammalian PI-PLC-δ1 was determined in a complex with 1,4,5-*myo*-inositol trisphosphate (*51*), which gives a somewhat clearer picture of the interactions of the 1-phosphate group with the active site residues. There are two major differences relative to the active site of the bacterial enzyme; arginine 69 is replaced with aspartate 343 and a calcium ion, and His311 (corresponding to bacterial His32) is moved from the 2-OH toward the nonbridging oxygen of the phosphate. Consistent with the bridging function of Arg69, the calcium ion is coordinated by both the 2-OH group of inositol and the oxygen atom of the phosphate. Thus, it appears that in mammalian PI-PLC the overall function of arginine is replaced by the combined effort of the calcium ion and His311, where the calcium ion takes over the bridging interaction between the 2-OH and the phosphate (and, most likely, also lowers the pK_a of the former), and His311 contributes to the stabilization of the negative charge on the phosphate.

Comparison with RNase A. Extensive similarities have been found between catalytic mechanisms of RNase A and bacterial PI-PLC (*14*, *19*, *35*). Whereas substrates for both enzymes are topologically similar, inositol phosphodiesters are ~10^3 times more stable to chemical cyclization in imidazole buffers than are the corresponding ribose phosphodiesters (*52*). This fact might explain why PI-PLC must employ a more elaborate mechanism to achieve similar turnover rates. In RNase A, lysine 41 was assigned the function of phosphate activation (*16*, *53*). In the work similar to this one, RNase A was shown to prefer lysine and its analogues at position 41 over arginine-type residues (*16*). However, the difference between "lysine" and "arginine" enzymes was not as dramatic (~100-fold) as in the work presented here (~10^4-fold), and could be attributed to steric

effects resulting from the replacement of the ω-amino group with the much bulkier guanidine group.

Mechanistic Interpretation of Thio Effects. Enzymatic reactions of phosphodiesters are probably the best systems for employing and interpreting nonbridging thio effects since, unlike phosphomonoesters (*3*) and phosphotriesters (*54*), phosphorothioate diesters do not differ in reactivity from their phosphate counterparts (*3*, *55*). Generally, three major factors need to be considered when one attempts to interpret thio effects: (i) the lesser ability of sulfur to accept hydrogen bonds (*56*), (ii) the differences in metal ion chelating specificities between sulfur and oxygen, and (iii) a difference in size between sulfur and oxygen atoms and a bond length to phosphorus (i.e., there is a potential steric hindrance). Since bacterial PI-PLC is a metal-independent enzyme (*6*, *57*, *58*), only factors i and iii need be considered here. The most straightforward interpretation of a thio effect is to indicate a strength of a direct interaction between the replaced oxygen atom and an enzymic residue, with the presumption that with the corresponding phosphorothioate substrate such an interaction is missing or severely limited. The observed kinetic effect would be a direct result of the decrease in transition state stabilization due to the loss or decrease of a single interaction. The results presented here suggest that a part of the observed thio effects could be due to interactions other than that with the phosphate group. Consequently, the strength of the latter could be much lower than that suggested by the thio effect. In the case of PI-PLC, both terminal imino groups of arginine 69 make catalytically important interactions. The replacement of the pro-*S* oxygen with sulfur either disrupts both of these interactions or makes the interaction with the 2-OH ineffective, resulting in the very high overall thio effect. Although the second point of interaction cannot be identified with certainty from the available data, the most likely candidate seems to be the 2-OH group, the catalytic nucleophile.

At least two pieces of experimental evidence suggest that steric effects do not contribute significantly to the thio effect in bacterial PI-PLC. First, the binding affinity of both isomers of the phosphorothioate analogues is similar to that of the phosphate substrate (*15*). Second, enzymatic activities with the (S_p)-phosphorothioate do not show any significant dependence on either the length of the side chain at position 69 or the bulkiness of the functional group it is carrying (guanidine or amidine vs amine, Table 2). In addition, the crystal structure (*18*, *19*) and molecular modeling (K. S. Bruzik and P.-G. Nyholm, unpublished results) indicate that the side chain of arginine 69 is the only part of the active site in proximity to the phosphate group of the substrate; therefore, it is likely that the loss of the hydrogen bond between the pro-*S* oxygen atom and the side chain at position 69 is an important factor (although not the only one) contributing to the observed S_p thio effects and stereoselectivity.

In conclusion, site-directed chemical modification along with the use of phosphorothioate substrate analogues allowed us to gain further insight into the catalytic mechanism of bacterial PI-PLC and to propose a dual function for the catalytic arginine. The work presented here has also demonstrated that a significant portion of nonbridging thio effects in an enzymatic phosphoryl transfer reaction could be attributed to factors other than a direct interaction between a phosphate group and an enzyme.

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