Phosphatidylinositol-Specific Phospholipase C. 3. Elucidation of the Catalytic Mechanism and Comparison with Ribonuclease A[1]

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Substitution of a phosphoryl oxygen with sulfur has been a widely used technique in mechanistic studies of enzymes[2] and ribozymes.[3] We have defined the ratio k_O/k_S for substitution of a nonbridging oxygen and a bridging oxygen as type I and type II thio effects, respectively.[4] We now report the use of thio effects and site-directed mutagenesis to support a mechanism for phosphatidylinositol-specific phospholipase C (PI-PLC), which sheds light on the controversial mechanism of ribonuclease A (RNase A).[5]

PI-PLC catalyzes the conversion of phosphatidylinositol (PI) to 1-inositol phosphate (IP) in two distinct steps, *via* the formation of inositol 1,2-cyclic phosphate (IcP).[6] PI-PLC is similar to RNase A in that both enzymes catalyze rapid conversion of a phosphodiester to a cyclic intermediate *via* intramolecular attack of a neighboring hydroxyl group, followed by slow hydrolysis of the cyclic product to a phosphomonoester. The steric courses of both steps have been shown to be inversion of configuration for both enzymes.[2,6] Moreover, both enzymes contain a pair of histidine residues at the active site (His-32 and -82 for PI-PLC, His-12 and -119 for RNase A) that could function as a general acid−general base (GA−GB) couple.[5,7]

The classical GA−GB mechanism is favored by many for RNase A, although Breslow favors a triester-like mechanism in which a nonbridging phosphoryl oxygen atom is first protonated by His-119.[8] Recent physical organic studies by Kirby indicated that protonation of a nonbridging oxygen atom can activate the phosphodiester for attack by a neighboring OH group by ca. 10^4-10^5-fold.[9] However, Herschlag[10] refuted the triester-like mechanism on the basis of small thio effects observed for the hydrolysis of the phosphorothioate analogs of cUMP and UpA. Raines also refuted the role of His-119 in protonating a nonbridging oxygen of the phosphate,[11] but he subsequently showed that Lys-41 contributes to the catalysis by forming a H-bond with the phosphodiester group.[12]

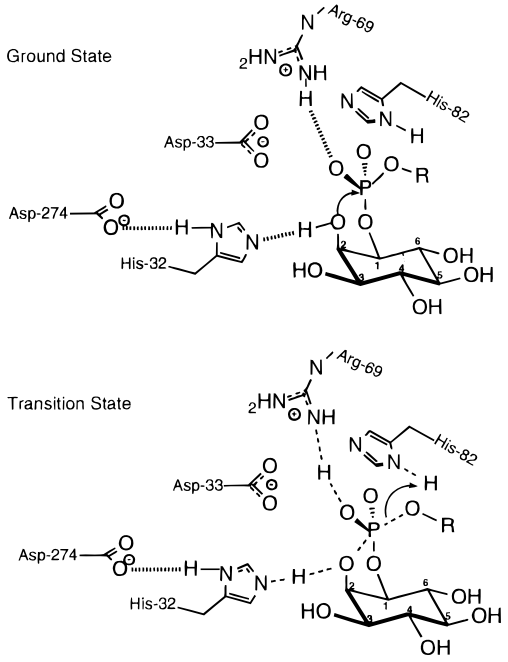

Figure 1. Our proposed mechanism for the phosphatidylinositol cleavage catalyzed by PI-PLC. The spatial relationship between Asp-33 and His-82 remains to be established, but they are both involved in protonating the leaving group. Hydrogen bonds are shown as hash marks. Bonds in the process of being formed or broken in the transition state are shown as dashed lines.

We propose that PI-PLC uses a combination of GA−GB catalysis and arginine···phosphate interaction, as shown in Figure 1. The GA−GB mechanism is based on kinetic analyses of wild type (WT) and mutants and type II thio effects, whereas the arginine···phosphate interaction is based on type I thio effects. The results and rationale supporting our mechanism are elaborated below.

It was predicted from the crystal structure of the PI-PLC−*myo*-inositol complex that His-32, with assistance from Asp-274, acts as the general base for deprotonating the 2-hydroxyl group of inositol and His-82 acts as the general acid protonating the C_3 oxygen of diacylglycerol.[7] We now present results to support and further refine this mechanism: (i) The activities of H32A and H82A toward [^{3}H]PI are reduced to 2×10^{-5} and 1×10^{-5}, respectively, relative to that of wild type. (ii) The activities of D274N and D33N are reduced to 0.3 and 1.6%, respectively, relative to that of wild type. Asp-33 is in proximity to His-82 but not mentioned in the structure paper.[7] The data support that Asp-274 and -33 assist His-32 and -82, respectively. (iii) We recently reported that D33A shows enhanced activity toward the substrate analog with sulfur replacing the C_3 oxygen of diacylglycerol (i.e., a reverse type II thio effect) and concluded that Asp-33 is directly or indirectly donating a proton to the leaving group.[4] We now report that the H82A mutant also shows enhanced activity toward this substrate analog ($k_O/k_S = 0.1$). The results taken together support (independent of the structural evidence) that the Asp-33···His-82 pair functions as the general acid.

For the type I thio effects, we used the stereoisomers of phosphorothioate analogs 1,2-dipalmitoyl-*sn*-glycero-3-(thio-

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phospho)-1-*myo*-inositol (DPPsI) and inositol 1,2-cyclic phosphorothioate (IcPs). The wild-type PI-PLC was known to convert (R_p)-DPPsI to *trans*-IcPs and then further convert *trans*-IcPs to inositol 1-phosphorothioate (IPs),[6] but the thio effects were not measured quantitatively. Using ^{31}P NMR analyses, we have now determined that, for the conversion of DPPI to IcP, $k_O/k_S = 3$ and 10^5 for the R_p and S_p isomers, respectively, of DPPsI. Similar results were obtained for the ring opening of IcPs: the thio effect is 5 for the *trans* isomer while the *cis* isomer was resistant to cleavage even after extended incubation with enzyme in great excess (k_O/k_S is estimated to be $\geq 10^5$).

We recently reported that mutation of Arg-69 to lysine altered the stereoselectivity (R_p/S_p ratio) of the enzyme toward the DPPsI isomers by a factor of 10^4 (from 1.6×10^5 to 16).[13] We now report that this same mutant also shows great relaxation (estimated to be at least 10^3) in stereoselectivity toward the *trans* and *cis* isomers of IcPs in the ring opening reaction. These results taken together establish that the interaction between Arg-69 and the phosphate moiety of substrates plays an important role in PI-PLC catalysis. The very large thio effects and their dependence on phosphorus configuration are consistent with a hydrogen bonding to the *pro-S* oxygen of the phosphate group. Perhaps not coincidentally, the magnitude of thio effect for (S_p)-DPPsI (10^5) and the relaxation of stereoselectivity (10^4) by the R69K mutant are of the same order of magnitude as the rate enhancement achieved by phosphate protonation reported by Kirby.[9]

If one accepts that the two enzymes use the same mechanisms, then the role of Lys-41, suggested to form a H-bond with the phosphodiester group in the most recent work of Raines,[12] should be emphasized. Most likely, RNase A also uses a combination of GA−GB catalysis[4] and lysine···phosphate interaction. The PI-PLC mechanism is better defined than the RNase A one in that the specific oxygen atom involved in the H-bonding has been identified.

If the H-bonding to the phosphate is established, then proton transfer could occur in the transition state. The degree of proton transfer in the transition state could be different between PI-PLC and RNase A and could also depend on the specific substrate (analog) used. If it is a partial proton transfer, then the low-barrier hydrogen bond proposed by Gerlt and Gassman for RNase A[14] is likely. If it is a full proton transfer, then the triester-like mechanism proposed by Breslow[8] is feasible. Thus, neither mechanism should be ruled out for either PI-PLC or RNase A until the transition state structure can be delineated.

In summary, in this short paper, we have elucidated the mechanism of PI-PLC to more certainty than that of RNase A. The similarity between the two enzymes with very different structures and different substrates is impressive. The unequivocal demonstration of the arginine···phosphate interaction in PI-PLC points to the importance of the corresponding lysine···phosphate interaction in the mechanism of RNase A and the possibility of partial or full protonation of the phosphate in the transition state for both enzymes.

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