Identification of a Novel Catalytic Triad with Dual Functions in Enzymatic Cleavage of the P−O Bond

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We report identification of a novel Arg-Asp-His catalytic triad that serves dual functions—phosphate activation and leaving group protonation—to catalyze the P−O bond cleavage for phosphatidylinositol-specific phospholipase C (PI-PLC) from *B. thuringiensis*. PI-PLC cleaves the P−O bond of phosphatidylinositol (PI) to form inositol 1,2-cyclic phosphate (IcP), which is then slowly hydrolyzed to inositol 1-phosphate.[1] X-ray structure[2] along with site-directed mutagenesis[2,3] led to the mechanism in Figure 1A, which involves three elements: His32 as a general base assisted by Asp274, His82 as a general acid, and Arg69, which activates the phosphate group. We recently added two additional aspects to the mechanism: that Arg69 activates the phosphate by hydrogen bonding to the *pro-S* oxygen at the transition state,[4,5] and that Asp33 and His82 function intimately together as a "composite general acid" (Figure 1B).[6,7] We now further show that the functions of Asp33 and Arg69 are also inseparable, which led us to propose the mechanism involving the novel catalytic triad (Figure 1C).

Our conclusions were achieved by rigorously cross-examining the bridging and nonbridging "thio-effects" (k_O/k_S ratios)[6−9] of PI-PLC and its mutants with PI analogues shown in Figure 2. Both types of thio-effects are usually >1 in enzymatic reactions since sulfur weakens potential hydrogen bonding[10] and also introduces possible steric[11] and charge[8] differences. The usefulness of the nonbridging thio-effect lies mainly in the comparison of the enzyme activity toward *R*p- and *S*p-isomers. Bridging thio-effects are more difficult to interpret since they are directly related to the bond being cleaved, and P−S bonds are much weaker than P−O bonds (i.e. k_O/k_S is ≪1 in chemical reactions).[12]

The maximal activities of WT, D33N, and D33A toward substrate analogues are listed in Table 1. While the rates depend on the detergent used to disperse the substrate (see footnote *b*), all data in Table 1 were obtained under the same conditions with dihexanoyl lecithin as a detergent. Furthermore, the analogues having a nonbridging sulfur inhibit the PI cleavage with K_i values close to that of the K_m of PI, and the analogues having the bridging sulfur display the K_m values slightly lower than those of PI.[13] The interpretation of these data is given in the following sections.

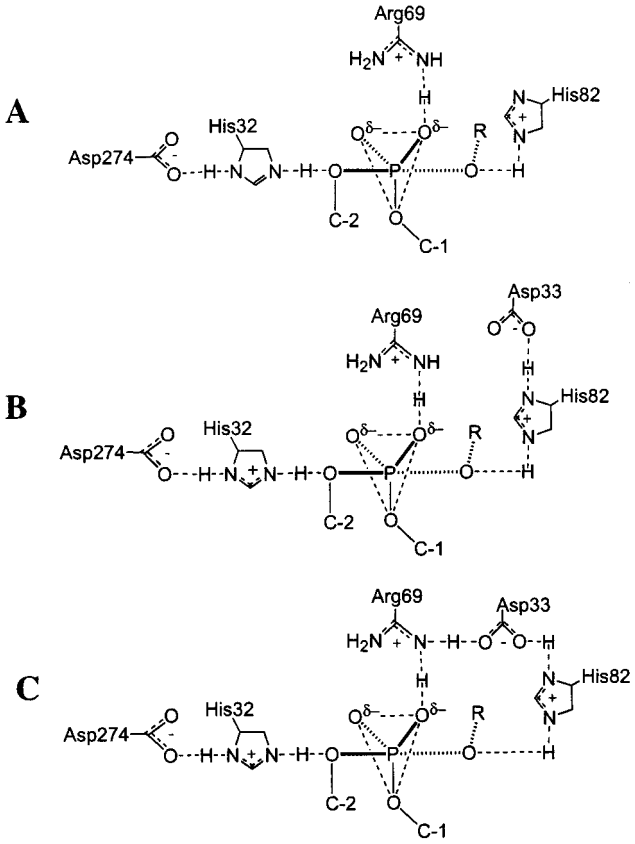

Figure 1. Evolution of understanding of the mechanism of PI-PLC.

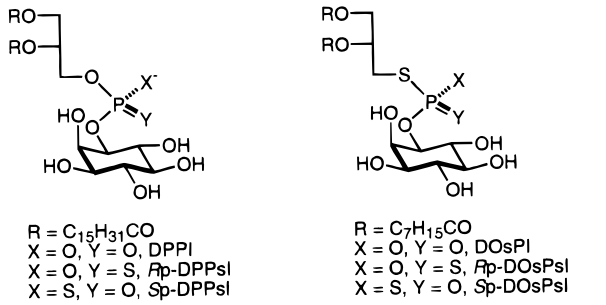

Figure 2. Structures of substrate analogues used in this work. DOsPsI isomers were synthesized analogously as DOsPI,[19] except sulfurization with S_8/CS_2 was used instead of oxidation of the intermediate phosphite.

Communication between Arg69, Asp33, and His82. Our previous suggestion that His82 and Asp33 function cooperatively as a composite general acid is based on reversal of the *bridging* thio-effect from >1 in WT to <1 in *both* D33A and H82A mutants (i.e. higher chemical reactivity of the bridging-sulfur analogue is expressed in both mutants).[4,7] We now use the *nonbridging* thio-effect to show that Asp33 and Arg69 also function inseparably. Our previous results showed that PI-PLC displayed a very high stereoselectivity toward the two diastereomers of DPPsI (*R*p/*S*p up to 29 000),[5] and R69K mutation

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(1) Bruzik, K. S.; Tsai, M.-D. *Bioorg. Med. Chem.* **1994**, *2*, 49−72.
(2) Heinz, D. W.; Ryan, M.; Bullock, T. L.; Griffith, O. H. *EMBO J.* **1995**, *14*, 3855−3863.
(3) Gässler, C. S.; Ryan, M.; Liu, T.; Griffith, O. H.; Heinz, D. W. *Biochemistry* **1997**, *36*, 12802−12813.
(4) Hondal, R. J.; Bruzik, K. S.; Tsai, M.-D. *J. Am. Chem. Soc.* **1997**, *119*, 9933−9934.
(5) Hondal, R. J.; Riddle, R. R.; Kravchuk, A. V.; Zhao, Z.; Bruzik, K. S.; Tsai, M.-D. *Biochemistry* **1997**, *36*, 6633−6642.
(6) Hondal, R. J.; Bruzik, K. S.; Zhao, Z.; Tsai, M.-D. *J. Am. Chem. Soc.* **1997**, *119*, 5477−5478.
(7) 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.
(8) Frey, P. A. *Adv. Enzymol.* **1989**, *62*, 119−201.
(9) Herschlag, D. *J. Am. Chem. Soc.* **1994**, *116*, 11631−11635.
(10) Sennikov, P. G. *J. Phys. Chem.* **1994**, *98*, 4973−4981.
(11) Knight, W. B.; Sem, D. S.; Smith, K.; Miziorko, H. M.; Rendina, A. R.; Cleland, W. W. *Biochemistry* **1991**, *30*, 4970−4977.
(12) Bruice, T. C.; Benkovic, S. J. *Bioorganic Mechanisms*; W. A. Benjamin, Inc.; New York, 1966; Vol. 11.

(13) The K_i values for the *R*p- and *S*p-DPPsI determined by DPsPI assay substrate were 2.6 and 0.56 mM, respectively, and the K_m values for DOsPI[19] and DPsPI[5,19] were 0.075 and 0.26 mM, respectively.

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Table 1. Maximal Velocities of the Cleavage of Substrate Analogs by PI-PLC[a,b]

panel	substrate	WT	D33N	D33A
I	DPPI	2000 ± 180	11 ± 0.9	4.6 ± 0.1
	*R*p-DPPsI	53 ± 3.4	2.0 ± 0.1	0.047 ± 0.003
	O/S_{Rp}^{nonbr} [c]	38 ± 6	5.5 ± 0.5	98 ± 9
II	*S*p-DPPsI	0.0065 ± 0.0004	0.07 ± 0.006	0.009 ± 0.001
	O/S_{Sp}^{nonbr} [c]	308000 ± 32000	160 ± 20	510 ± 70
	*R*p/*S*p	8200 ± 1000	29 ± 4	5.2 ± 1
III	DOsPI	58 ± 2		49 ± 2
	O/S^{br} [d]	34 ± 5		0.09 ± 0.1
IV	*R*p-DOsPsI	15 ± 1	0.84 ± 0.06	3.0 ± 0.2
	O/S^{br} [e]	3.5 ± 0.5		0.016 ± 0.002
	O/S_{Rp}^{nonbr} [f]	4 ± 0.3		16 ± 2
V	*S*p-DOsPsI	0.67 ± 0.07		0.1 ± 0.01
	O/S^{br} [e]	0.010 ± 0.002		0.09 ± 0.02
	O/S_{Sp}^{nonbr} [f]	87 ± 10		490 ± 77
	*R*p/*S*p	25 ± 2		32 ± 4.5

[a] All assays were performed by ^{31}P NMR at 25 °C at 121.5 MHz in 50 mM Na-MOPS buffer, 5 mM Na-EDTA, 10% D_2O, pH 7.0. The substrate (10 mM) was dispersed in dihexanoyl lecithin (40 mM) and sonicated in the ultrasonic bath. The activities are expressed in μmol min^{-1} mg^{-1}. The experimental error was obtained individually for each experiment by summation of individual errors for each operation (product and enzyme quantitation and time error). [b] The V_{max} values for the cleavage of DPPI by the WT PI-PLC varied depending on the detergent used, and were 1090 and 60 in the presence of sodium deoxycholate and hexadecylphosphorylcholine, respectively. [c] Ratio of V_{max} values for DPPI and DPPsI. [d] Ratio of the V_{max} values for DPPI and DOsPI. [e] Ratio of V_{max} values for equivalent isomers of DPPsI and DOsPsI. [f] Ratio of the V_{max} values for DOsPI and DOsPsI isomers.

relieved most of the stereoselectivity (*R*p/*S*p = 16). The large stereoselectivity results from an extremely high *pro-S* and a modest *pro-R* nonbridging thio-effect. This was the key evidence supporting the role of Arg69 mentioned above. As shown by the data in Table 1 (panels I−II), *the Rp/Sp rate ratio is also greatly reduced in both D33 mutants*. Thus, the interaction of Asp33 with Arg69 is also important for the catalysis by PI-PLC. The results taken together led us to suggest the new catalytic triad.

Is the Communication Structural or Functional? Before concluding that the communication between the three residues is functionally significant, one needs to rule out the possibility that it is a structural effect, i.e., mutation of one residue significantly alters side-chain positions of the other two residues. We therefore examined the communication between the effects of bridging and nonbridging sulfur using DOsPsI substrates *for the wild-type enzyme*. As shown in panels III−V of Table 1, the *R*p/*S*p ratio of WT is significantly reduced for DOsPsI isomers (relative to DPPsI). This suggests that the role of Arg69 in phosphate activation (reflected by the *R*p/*S*p ratio) is significantly impaired when the role of the general acid is reduced by the presence of the bridging sulfur.

Likewise, while introducing the bridging sulfur into DPPI (DPPI → DOsPI, O/S^{br} in panel III) *reduces* the activity of WT by 34-fold, introduction of bridging sulfur into *S*p-DPPsI (*S*p-DPPsI → *S*p-DOsPsI) *increases* the activity of WT by 91-fold. This suggests that reducing the phosphate activation by introducing a nonbridging sulfur at the *pro-S* position also diminishes the bridging interactions.

This result does not rule out the possibility that the steric effect of the nonbridging sulfur could perturb the exact position of the side chain of Arg69, which in turn perturbs the side chain position of His82 (and *vice versa*). However, it is difficult to account for the very large *R*p/*S*p ratio by the steric effect alone, and it is also difficult to envision how this effect could be transmitted along three residues. The steric effect of the bridging sulfur should be smaller, and it would not explain the greatly diminished stereoselectivity observed with DOsPsI isomers. Finally, the similar K_i values of *R*p- and *S*p-DPPsI suggest that steric interactions of sulfur are not important in the ground state.

Communication between Arg69 and His82 Is Disrupted in D33A. For the D33A mutant, the *R*p/*S*p ratio is comparable between DPPsI (5.2) and DOsPsI (32), and the bridging thio-effect O/S^{br} is identical between DOsPI and *S*p-DOsPsI (0.09). Thus, the communication between the nonbridging thio-effect (reflecting the Arg69 function) and the bridging thio-effect (reflecting the His82 function) is no longer present in D33A. This result strongly supports the interpretations in the previous sections.

Proposed Functional Roles of the Arg-Asp-His Catalytic Triad. The Arg-Asp-His triad is distinct from other catalytic triads[14] in that it is dual-functional. The detailed mechanism, however, remains to be further investigated, particularly regarding the specific nature of the interaction between Arg69 and the phosphate. In our view, our data are most consistent with a proposed dual general acid mechanism: During the initial phase of the reaction, the approach of the negatively charged inositol 2-oxygen toward phosphorus is facilitated by hydrogen bonding or protonation of the nonbridging oxygen by Arg69. The degree of proton transfer would change along the reaction coordinate, since in the later phase of the reaction, protonation of the leaving group (bridging) is necessary and deprotonation of the nonbridging oxygens would be beneficial, as it would help repel the negative charge of the leaving group. This could be accomplished by the movement of the arginine proton away from the nonbridging oxygen with simultaneous movement of the imidazolium proton toward the bridging oxygen, to an extent depending on the reaction progress.

Relevance to Ribonucleases. The reactions catalyzed by PI−PLC are analogous to those of ribonucleases.[15,16] Despite three decades of extensive research, the mechanisms of ribonucleases A and T1 are still under serious debate.[9,17,18] The uncertainties relate mainly to the exact roles of specific active site residues. The experimental approach presented in this work, and the demonstration of communication between active site residues, will likely be useful for the studies of ribonucleases and other phosphoryl transfer enzymes.

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(14) Carter, P.; Wells, J. A. *Nature* **1988**, *332*, 6164.
(15) Raines, R. T. *Chem. Rev.* **1998**, *98*, 1045−1066.
(16) Steyaert, J. *Eur. J. Biochem.* **1997**, *247*, 1−11.
(17) Breslow, R. *Acc. Chem. Res.* **1991**, *24*, 317−324.
(18) Breslow, R.; Chapman, W. H., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 10018−10021.
(19) Mihai, C.; Mataka, J.; Riddle, S.; Tsai, M.-D.; Bruzik, K. S. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1235−1238.