A Phospholipase with a Novel Catalytic Triad**

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Virtually all chemical reactions in living cells are catalyzed by enzymes. The ability to dramatically enhance the rate of a chemical reaction that would normally not occur under physiological conditions critically depends on the precise spatial arrangement of certain amino acids in the active site of an enzyme. Enzymes are frequently grouped in families depending on their mode of catalysis. The serine proteases, for example, enjoy textbook prominence for being thoroughly investigated and particularly well understood in structural and mechanistic terms. Members of this family, including chymotrypsin, elastase, and subtilisin, cleave peptide bonds in proteins by nucleophilic attack of an activated serine residue.

The serine is part of the famous catalytic triad, a structurally highly conserved feature first detected in the active site of serine proteases. The triad is composed of the pairwise hydrogen-bonded amino acids aspartate, histidine, and serine (Figure 1). Together with other structural elements the triad

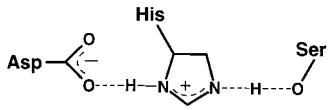

Figure 1. The classical catalytic triad of serine proteases consisting of aspartate, histidine, and serine.

contributes to a rate enhancement of at least nine orders of magnitude relative to the nonenzymatic hydrolysis of amide bonds.[1] In the course of the reaction the serine attacks the carbonyl carbon atom of the substrate's scissile peptide bond. The histidine residue in the center of the triad acts as a general base abstracting the serine proton while the nonsolvated aspartate residue in turn stabilizes the imidazolium ion of the histidine residue through a salt bridge. Subsequently the histidine protonates the amino group in the displaced portion of the peptide substrate leading to a covalent acyl–enzyme intermediate. This is hydrolyzed in the second part of the reaction by a water molecule activated by the same histidine. Equivalent catalytic triads with preserved spatial relationships are observed in other hydrolases and esterases. Deviations from the classical Ser-His-Asp triad are found in a growing number of hydrolytic enzymes, where amino acid variations occur at all three positions, and which probably results in modulations of the catalytic activity.[2]

In a recent paper Kubiak and colleagues describe the identification of a novel and unusual catalytic triad in a phosphatidylinositol-specific phospholipase C (PI-PLC) from the bacterium *Bacillus thuringiensis*.[3] PI-PLCs belong to the important class of phosphodiesterases that include (deoxy)-ribonucleases, cAMP- and cGMP-specific phosphodiesterases, restriction endonucleases, and also certainly catalytic RNAs (ribozymes). The PI-PLC is a ubiquitous enzyme that catalyzes the cleavage of the *sn*-3 phosphodiester bond of phosphatidylinositol (PI) into lipid-soluble diacylglycerol and water-soluble 1,2-cyclic inositolphosphate which can be further hydrolyzed to inositol-1-phosphate.[4] In mammalian cells the enzyme plays a central role in signal transduction cascades while bacterial PI-PLCs are known as virulence factors. The crystal structure of the PI-PLC from *Bacillus cereus* in a complex with its competitive inhibitor *myo*-inositol shows the inhibitor binding to the active site pocket in a substratelike fashion (Figure 2).[5]

Based on the structure and supporting data from site-directed mutagenesis, where single amino acids in the active site were replaced by others,[5–7] a catalytic mechanism was postulated that is highly reminiscent of the general base/acid catalysis of ribonuclease A, another textbook example of a structurally and mechanistically thoroughly investigated enzyme.[8] As in ribonuclease A, two histidine residues in PI-PLC, His32, and His82, respectively, act as a general base and acid (Scheme 1). The first part of the reaction, a transesterification, consists of an in-line S_N2-type displacement reaction at the phosphorus atom, where His32 abstracts the proton from the 2-OH group of the inositol moiety of PI, which facilitates the nucleophilic attack by the O2 atom on the phosphorus atom, in concert with His82 protonating the diacylglycerol leaving group. The collapse of the pentacovalent trigonal bipyrimidal transition state into the stable 1,2-cyclic inositolphosphate intermediate results in the inversion of configuration at the phosphorus atom. The role of His32 as the catalytic base is enhanced by Asp274. In analogy to the serine proteases, Asp274, His32, and the 2-OH group of the substrate (or the *myo*-inositol inhibitor) constitute a "classical" catalytic triad in the active site of PI-PLC (Figure 2).[5, 6] In the second, much slower part of the reaction, the role of both histidine residues is reversed and the 1,2-cyclic phosphate intermediate is hydrolyzed by an activated water

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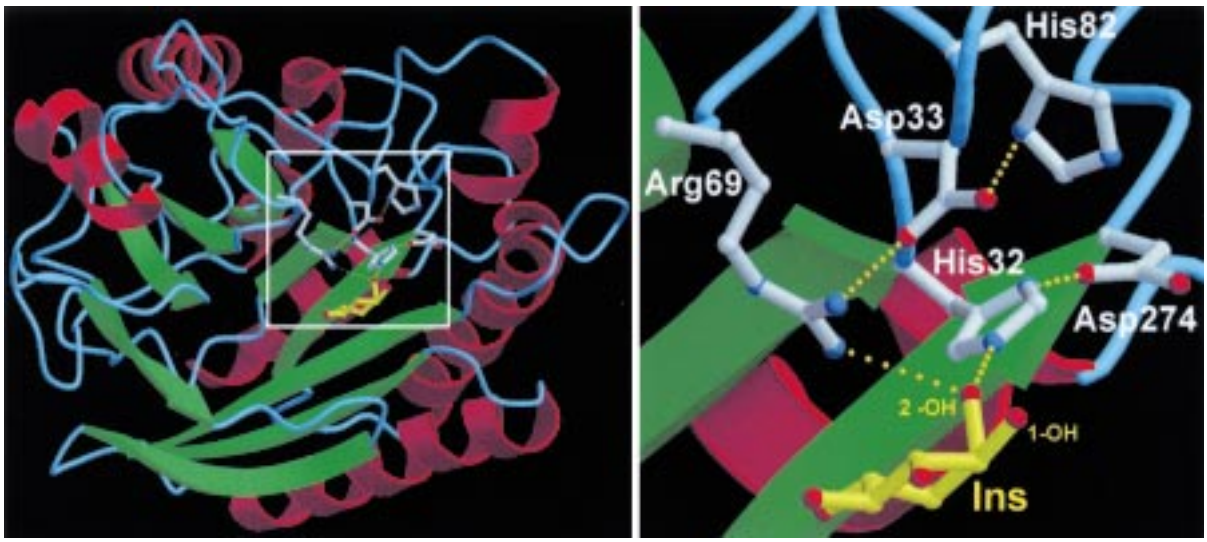
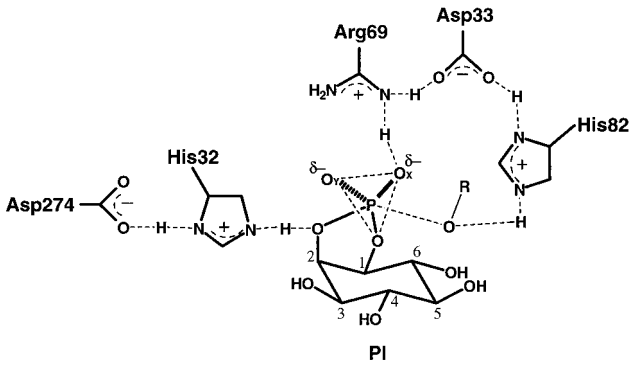


Figure 2. Ribbon diagram of the crystal structure of PI-PLC from *B. cereus* in a complex with *myo*-inositol (left picture). In this picture α-helices are red, β-strands green, and loops light blue. The active site (white square) is magnified in the right picture. The side chains of residues belonging to the two catalytic triads present in the active site of the enzyme as well as the bound inhibitor *myo*-inisotol (Ins) are shown.



Scheme 1. Schematic representation of the binding of the transition state to the active site of PI-PLC showing the two catalytic triads and a bound PI molecule (R = diacylglycerol for the first part of the reaction, R = H for the second part of the reaction). The nonbridging oxygen atoms are labeled O_X and O_Y according to Scheme 2 (modified from Ref. [3]).

molecule, again by an S_N2 displacement reaction, to yield inositol-1-phosphate with overall retention of configuration of the phosphorus atom. Results from site-directed mutagenesis and enzyme kinetics using natural and synthetic substrates confirmed the importance of residues His32, Asp33, Arg69, His82, and Asp274 for catalysis.[5–7, 9]

By using a combination of site-directed mutagenesis, enzyme kinetic, and stereochemical analysis of sulfur-containing substrate analogues, the groups of Tsai and Bruzik have systematically investigated the catalytic role of certain amino acids in the active site of PI-PLC over the last few years.[7, 9, 10] These efforts culminated in their latest article, in which they propose an additional and novel second catalytic triad in the active site of the enzyme.[3] This triad consists of Arg69, Asp33, and His82 and is proposed to fulfill a dual function by activating the substrate's phosphate group for nucleophilic attack followed by protonation of the leaving group. Although these residues are mutually hydrogen bonded (Figure 2), the crystal structure does not resolve whether this hydrogen bonding is merely a structural feature or whether it is a truly functional prerequisite.

The authors elegantly adressed this problem by a "matched mutation" approach, that is exchange of the amino acids in question as well as substitution of the oxygen by sulfur atoms in the substrate molecules, followed by enzyme kinetic analysis. Substitution of nonbridging or bridging oxygen atoms of phosphodiesters by sulfur has widely been used to investigate the catalytic mechanism of phosphodiesterases.[11] Phosphorothioate diesters are ideal compounds to study the steric course of displacement reactions at the phosphorus center owing to their stereogenic phosphorus atom. As a consequence of the drastically reduced hydrogen-bonding potential of the sulfur atom, relative to oxygen, phosphorothioates in general are chemically less reactive and are cleaved at slower rates by enzymes. In contrast to the phosphorothioates, phosphorothiolates, in which the bridging oxygen is replaced by a sulfur atom, are chemically more reactive than the corresponding phosphate, because of the weaker P–S bond and the lower pK_a value of the thiolate leaving group that does not require protonation by a general base. The "thio-effect" is defined as the ratio k_O/k_S, where O and S denote an oxygen-containing substrate and a sulfur analogue, respectively. The authors designated the thio-effect of phosphorothioates as type I and that of phosphorothiolates as type II.[10] They determined and compared both thio-effects for wild-type and mutant PI-PLCs.

By using a 1:1 mixture of both DPPsI diastereomers (Scheme 2) and ^{31}P NMR spectroscopy to follow the time course of the reaction it was shown that the wild-type enzyme exhibits a large stereoselectivity towards the R_P stereoisomer ($k_{R_p}/k_{S_p} = 10^5$). This remarkable stereoselectivity is largely lost even upon conservatively substituting Arg69 by lysine ($k_{R_p}/k_{S_p} = 16$). This is mainly the result of a 10^4-fold decrease in activity towards the R_p isomer while the rate of conversion of the S_p isomer is essentially maintained for the mutant.[7] The authors conclude that the guanidinium group of Arg69 specifically interacts with the *pro-S* oxygen atom of the phosphate group in the transition state. This leads to an activation of the phosphate group for the nucleophilic attack

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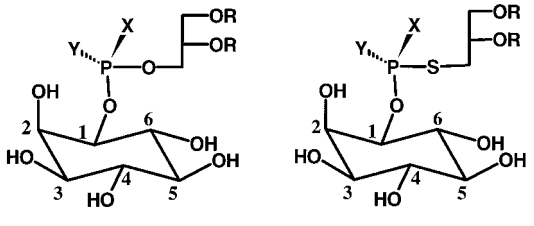

Scheme 2. Structure of thio analogues of PI (modified from Ref. [3]).

by the likewise activated 2-OH group—although the proposed proton transfer from Arg69 to the phosphate group remains to be demonstrated. From the pK_a value of an arginine side chain it is much more likely that Arg69 activates the phosphate group simply by polarization through a hydrogen bond.

The authors furthermore showed that Asp33 and Arg69 function inseparably.[3] Strikingly similar to the effect observed for mutant Arg69 →Lys, the mutation of Asp33 to alanine or asparagine also resulted in a greatly reduced R_P/S_P ratio, which suggests a functionally important interaction between both amino acids.

The substrate DPsPI, which contains a sulfur atom in place of the bridging oxygen atom of the leaving group (Scheme 2), was used to investigate the role of His82 and Asp33 in catalysis.[9] The progress of the reaction, that is, formation of the thiolate-containing leaving group, was monitored by a continuous assay by utilizing a subsequent reaction of the free thiolate with 5,5′-dithiobis(2-nitrobenzoic acid). Wild-type PI-PLC cleaved this analogue at slower rates ($k_O/k_S = 24$) relative to PI as a result of the loss or weakening of the stabilization of the leaving group by the general acid His82. In the case of the mutants Asp33 →Ala and His82 →Ala a reverse type II thio-effect ($k_O/k_S = 0.1$) is observed. The higher activity towards the bridging sulfur analogue observed for both mutants is attributed to the difference between the pK_a value of the free thiol of DPsPI (ca. 10), relative to the alkoxy leaving group of diacylglycerol (ca. 16). The lower pK_a value of the thiol partly compensates for the loss of the general acid in both mutants. These results suggest a composite nature of the general acid comprising of the Asp33−His82 diad.

Based on the interdependency of Arg69, Asp33, and His82 it is postulated that these residues act as a new dual-function catalytic triad in PI-PLC (Scheme 1).[3] The critical question of whether the interaction between the three residues is structural or functional was recognized by the authors and addressed by investigating the communication between Arg69 and His82 using diastereomeric DOsPsI (Scheme 2) as a substrate. Interestingly the introduction of the bridging sulfur atom leads to a dramatic reduction in the R_P/S_P ratio relative to DPPsI, mainly as a consequence of an increase in activity towards the S_p-DOsPsI. The R_p/S_p ratio for the mutant Asp33 →Ala is comparable for DPPsI and DOsPsI (namely, a similar type I thio-effect is found) and a nearly identical type II thio-effect is observed for DPsPI and S_P-DOsPsI, which indicates an uncoupling of both effects. These data suggest that Asp33 mediates the "communication" between the type I thio-effect (reflecting the Arg69 function) and the type II thio-effect (reflecting the His82 function). While the data give a clear indication of the proposed effect, cooperative changes in the side-chain conformations of the catalytic residues, induced by the binding of DOsPsI, cannot be ruled out. These steric effects could, for example, lead to a subtle positional displacement of Arg69 causing the observed decrease in stereoselectivity. All PI thio analogues, however, exhibited dissociation constants (as expressed by the Michaelis constant K_M) in a range similar to natural PI, which suggests a highly similar binding mode at least in the ground state.[9] This concern also applies to the frequently asked question as to whether the overall structure of the enzyme is possibly perturbed by the mutations in the active-site pocket. A comparison of the circular dichroism and NMR spectra of wild-type and mutant enzymes did not reveal any notable changes caused by the mutation.[9] This is corroborated by the crystal structures of several active site PI-PLC mutants which show no changes in the tertiary structure except for the site of mutation.[6] It is therefore highly likely that the replacement of single amino acids in the active site of PI-PLC does not perturb the overall structure of the enzyme.

Compared to the classical catalytic triad present in the serine proteases the novel triad has a completely different character. While the former triad plays a role as a highly reactive nucleophile, the novel triad acts as a general acid and donates a proton to the leaving group and possibly to a nonbridging oxygen atom of the phosphate group. The proton position of Arg69 within the triad affects (through Asp33) the acidity of the proton on His82, and vice versa. So far nothing is known about the pk_A value of Asp33 and the geometric requirements of the catalytic triad.

Are these results also of relevance to the mammalian PI-PLCs, which are key players in numerous signal transduction pathways? The catalytically important amino acids are highly conserved among bacterial PI-PLCs. While both catalytic histidines are also conserved between bacterial and mammalian PI-PLCs, the residues equivalent to Arg69 and Asp33 are instead replaced by aspartate and asparagine, respectively.[12] Both residues are ligands to a tightly bound Ca^{2+} ion that superimposes with the guanidinium group of Arg69 in bacterial PI-PLC. In analogy to Arg69 the metal ion is thought to be involved in the stabilization of the transition state.[13] Here two different modes of electrophilic catalysis (H-bond versus metal ion) very likely developed through divergent evolution.

In conclusion some key issues remain to be resolved: By what precise mechanism, for example, does the catalytic triad actively facilitate or promote catalysis? Protonation or polarization of a nonbridging oxygen atom by Arg69 will certainly facilitate the nucleophilic attack of the negatively charged O2 atom on the phosphorus atom. Later in the reaction a deprotonation of the nonbridging oxygen would be advantageous to repel the negative charge of the leaving group. The central question is, however, whether Arg69, Asp33, and His82 can indeed be seen as a catalytic triad. In the classical catalytic triad of serine proteases the individual residues function in a concerted manner where the mutation of a single

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member disrupts the entire triad although the rest of the structure remains unchanged.[1] Even though point mutations in the new catalytic triad of PI-PLC lead to a drastic reduction of activity towards PI by a factor of 10^3 (for Asp33 mutations) to 10^6 (for Arg69 or His82 mutations),[6, 7, 9] the mutations could still cause simple steric effects that lead to a minimal shift in the side-chain position of neighboring amino acids and consequently different binding modes of the complex substrate analogues. The crystal structures of PI-PLC in complexes with transition state analogues would certainly help to resolve some of these questions.

Overall the presented results constitute an excellent example of the successful synergy between structural biology, synthetic organic chemistry, and enzymology to find new concepts in enzyme catalysis. While the crystal structure of PI-PLC provided the necessary framework for the identification of catalytically important amino acids and the basis for site-directed mutagenesis experiments, the precise role of some of these amino acids and especially their communication during catalysis remained less clear. The presented "matched mutation" approach using PI-PLC mutants and thio analogues of PI is a powerful technique in addressing these questions and may well be applied to other phosphodiesterases such as the ribonucleases, where the catalytic mechanisms are still vigorously being debated after three decades of research.[14–16]

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[1] P. Carter, J. A. Wells, *Nature* **1988**, *332*, 564 – 568.
[2] G. Dodson, A. Wlodawer, *Trends Biochem. Sci.* **1998**, *23*, 347 – 352.
[3] R. J. Kubiak, R. J. Hondal, X. Yue, M.-D. Tsai, K. S. Bruzik, *J. Am. Chem. Soc.* **1999**, *121*, 488 – 489.
[4] K. Bruzik, M.-D. Tsai, *Bioorg. Med. Chem.* **1994**, *2*, 49 – 72.
[5] D. W. Heinz, M. Ryan, T. L. Bullock, O. H. Griffith, *EMBO J.* **1995**, *14*, 3855 – 3863.
[6] C. S. Gässler, M. Ryan, T. Liu, O. H. Griffith, D. W. Heinz, *Biochemistry* **1997**, *36*, 12 802 – 12 813.
[7] R. J. Hondal, S. R. Riddle, A. V. Kravchuk, Z. Zhao, H. Liao, K. S. Bruzik, M.-D. Tsai, *Biochemistry* **1997**, *36*, 6633 – 6642.
[8] F. M. Richards, H. W. Wyckoff in *The* enzymes (Ed.: P. D. Boyer), Academic Press, New York, **1971**, pp. 647 – 806.
[9] R. J. Hondal, Z. Zhao, A. V. Kravchuk, H. Liao, S. R. Riddle, X. Yue, K. S. Bruzik, M.-D. Tsai, *Biochemistry* **1998**, *37*, 4568 – 4580.
[10] R. J. Hondal, K. S. Bruzik, Z. Zhao, M.-D. Tsai, *J. Am. Chem. Soc.* **1997**, *119*, 5477 – 5478.
[11] P. A. Frey, *Adv. Enzymol.* **1989**, *62*, 119 – 201.
[12] L.-O. Essen, O. Perisic, R. Cheung, M. Katan, R.-L. Williams, *Nature* **1996**, *380*, 595 – 602.
[13] D. W. Heinz, L.-O. Essen, R.-L. Williams, *J. Mol. Biol.* **1998**, *275*, 635 – 650.
[14] D. Herschlag, *J. Am. Chem. Soc.* **1994**, *116*, 11 631 – 11 635.
[15] D. M. Perreault, E. V. Anslyn, *Angew. Chem.* **1997**, *109*, 470 – 490; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 432 – 450.
[16] J. Steyaert, *Eur. J. Biochem.* **1997**, *247*, 1 – 11.

  1433-7851/99/3816-2351 $ 17.50+.50/0