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A Novel Calcium-Dependent Bacterial Phosphatidylinositol-Specific Phospholipase C Displaying Unprecedented Magnitudes of Thio Effect, Inverse Thio Effect, and Stereoselectivity

Li Zhao,[‡] Yinghui Liu,[§] Karol S. Bruzik,[§] and Ming-Daw Tsai*,[‡]

Departments of Chemistry and Biochemistry and The Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210, and Department of Medical Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, Illinois 60612

Received October 19, 2002; E-mail: tsai.7@osu.edu

Sulfur substitution of a phosphoryl oxygen has been used extensively in mechanistic studies of enzymes and ribozymes that involve phosphoryl transfer reactions.[1−8] The reliability of such applications for the attainment of mechanistic insight has however varied greatly owing to a lack of understanding of what thio effects (k_O/k_S) should be for enzymatic reactions. On one hand, thio effects and R_P/S_P stereoselectivity have been used successfully to elucidate the detailed reaction mechanism of many enzymes. As a prominent example of the contrary, observation of a relatively small thio effect (ca. 3 or less) for the incorporation of dNTPαS into DNA, and the somewhat increased thio effect for mismatches, have been used to conclude that the chemical step is not rate-limiting. This was subsequently interpreted to support that an induced-fit mechanism, where the conformational change is the rate-limiting step, is employed by DNA polymerases.[9−11] On the basis of experimental and theoretical considerations, we have recently suggested that the use of thio effects in this manner has led to major misinterpretation of the catalytic mechanism of DNA polymerases.[12]

Central to the proper interpretation of thio effects is knowledge of the range of their possible magnitudes. In this regard, the magnitude of thio effects in chemical reactions has been taken as a point of reference; these fall between 4 and 11 for phosphodiesters containing sulfur at *non*bridging positions[5] and between 10^{-4} and 10^{-3} for thiolphosphate esters.[13] However, it is not well recognized that the thio effect on an enzymatic reaction is likely to fall in a much greater range, which has been illustrated by data reported in the past two decades for various enzymes. A major goal of this communication is to draw attention to this issue by reporting an even greater span of enzymatic thio effects for a single enzyme.

A thorough examination of thio effects, together with structural and mutagenic information, for *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (btPLC) previously enabled us to uncover a new mechanism of phosphodiester cleavage illustrated in Figure 1A.[6−8,14−16] Herein we report that a novel Ca^{2+}-dependent *Streptomyces antibioticus* PI-PLC (saPLC1) and its mutants display unprecedented magnitudes of thio effects, reverse thio effects, and R_P/S_P stereoselectivity. These findings provide detailed mechanistic insights for this enzyme and further demonstrate that the magnitudes of thio effects in enzymatic reactions cannot be assumed to fall within the narrow range defined by nonenzymatic reactions. Note that all bacterial PI-PLCs known until recently are metal-independent, while their mammalian counterparts require Ca^{2+} for catalysis. On the basis of the sequence homology, saPLC1 has been proposed to utilize the same catalytic mechanism as mammalian PI-PLC.[17]

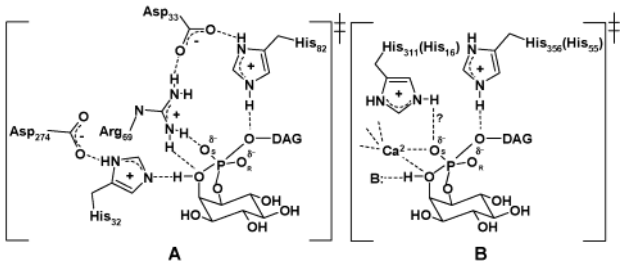

Figure 1. Proposed transition states of btPLC (A) and mammalian PI-PLCδ1 (B). DAG = diacylglycerol. The residues in parentheses are the counterparts in saPLC1.

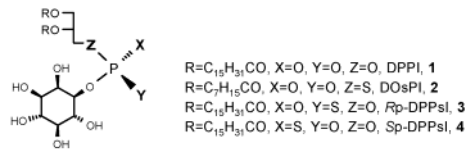

Figure 2. Structures of the substrate and substrate analogues used in this work. DPPI, 1,2-dipalmitoyl-*sn*-glycero-3-(1-phospho-1-D-*myo*-inositol). DOsPI, (2*R*)-1,2-dioctanoyloxypropanethio-3-(1-phospho-1-D-*myo*-inositol). DPPsI, 1,2-dipalmitoyl-*sn*-glycero-3-(1-thiophospho-1-D-*myo*-inositol).

Although the mechanistic details of mammalian PI-PLCs are not as well established as that of btPLC, a mechanism has been proposed for isozyme PLC-δ1 on the basis of crystal structures and limited site-directed mutagenesis studies[18−20] (Figure 1B, where His311 and His356 of PLC-δ1 correspond to His16 and His55 of saPLC1, respectively[17]). His311 is homologous to the general base (GB) His32 in btPLC, but it is not positioned to function as the GB in the crystal structures of PLC-δ1 (the GB residue is not yet established). To dissect the catalytic contributions of both the metal cofactor and the proposed active-site histidines of saPLC1, we employed site-directed mutagenesis in conjunction with thio effects.

First, we tested the bridging thio effect (sulfur substitution of bridging oxygen) by use of DOsPI (**2** in Figure 2) for the wild type (WT) and mutant saPLC1, and evaluated the catalytic contribution of the possible general acid (GA) residues. These results were then compared to those of the WT and mutant btPLC. The WT saPLC1 and most of its mutants demonstrated a very small bridging thio effect ($k_O/k_S = 0.5-2$) (Table 1). *Strikingly, however, H55A mutant displays an inverse thio effect (k_O/k_S) of 0.0019.* To the best of our knowledge, this is the largest inverse thio effect reported for any enzyme-catalyzed phosphoryl transfer reaction. For btPLC, the corresponding k_O/k_S values are 12−24 (WT and most mutants) and 0.1 (D33A and H82A), which led to the conclusion that both Asp33 and His82 are components of the GA.[6,15]

The observation of an inverse bridging thio effect for the H55A mutant of saPLC1 identifies this residue as a part of the GA

[‡] The Ohio State University.
[§] University of Illinois at Chicago.

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Table 1. Summary of Bridging Thio Effects for WT and Mutant saPLC1[a]

enzyme		WT	H16A	H55A
PI [b]	k_O[c]	1122 ± 22	0.0236 ± 0.0005	0.207 ± 0.015
	$K_{m,app}$[e]	58 ± 7	16 ± 3	23 ± 3
DOsPI	k_S[d]	678 ± 28	0.044 ± 0.002	107 ± 8
	$K_{m,app}$[e]	24 ± 5	6 ± 2	36 ± 10
k_O/k_S [f]		1.7	0.54	0.0019

[a] Measured at 37 °C, 0−2.0 mM PI (or DOsPI) and PI (or DOsPI)/Triton X-100 = 5 in 40 mM HEPES, 2 mM $CaCl_2$, 1 mM EDTA, pH 7.0. Activities are expressed in μmol mg^{-1} min^{-1}. [b] Natural phosphatidylinositol, where the chain length of DAG may vary from that of DPPI. The chain length differences between PI and DOsPI might affect the kinetic parameters slightly. However, the mechanistic interpretation was drawn mainly from the comparison between thio effects. Thus, the possible chain length effect was not further pursued. [c] Maximal activity toward PI determined by the radioactivity assay.[7] [d] Maximal activity toward DOsPI obtained by the spectroscopic assay.[7] [e] In μM. [f] Bridging thio effect. The values for N17A, E39A, E39Q, and D41A are 0.94, 0.53, 1.3, and 1.3, respectively.

Table 2. Summary of Nonbridging Thio Effects for WT saPLC1 and Mutant H16A in the Presence of Different Metal Ions[a]

enzyme		WT	WT	H16A	H16A
metal Ions		Ca^{2+}	Cd^{2+}	Ca^{2+}	Cd^{2+}
PI	k_O[b]	646	666	0.0041	0.038
R_P-DPPsI	k_{R_P}[b]	13.7	13.3	0.0038	4.8×10^{-6}
	k_O/k_{R_P}[c]	47	50	1.1	7.9×10^3
S_P-DPPsI	k_{S_P}[b]	4.0×10^{-6}	1.6×10^{-3}	ND[f]	ND
	k_O/k_{S_P}[d]	1.6×10^8	4.2×10^5	ND	ND
k_{R_P}/k_{S_P}[e]		6.2×10^6	4.3×10^3	ND	ND

[a] Measured by ^{31}P NMR[7] at 27 °C, in 40 mM HEPES, 1 mM EDTA, optimal metal ion concentrations, saturating substrate concentrations, pH 7.0. Activities are expressed in μmol mg^{-1} min^{-1}. [b] The specific activities should be close to maximal activities, since the substrate concentrations used in the assays are well above the $K_{m,app}$ value for PI and $K_{i,app}$ values (0.161 mM and 0.190 mM for R_P- and S_P-DPPsI, respectively) for DPPsI. [c] R_P-thio effect. [d] S_P-thio effect. [e] R_P/S_P stereoselectivity. [f] ND, nondetectable.

involved in protonation of the DAG leaving group. However, a 50-fold difference in the magnitude of this effect relative to that for His82 mutants of btPLC suggests a significant difference between the catalytic mechanisms of the two enzymes. One of the possible explanations is that His55 of saPLC1 functions relatively "independently" of other active-site residues, in contrast to His82 of btPLC, which functions cooperatively with other residues as shown in Figure 1. Consequently, the strong inverse thio effect of H55A saPLC1 is a reflection of the chemical properties of sulfur versus oxygen as a leaving group. (The bond energies of P−S and P−O bonds are 45−50 and 95−100 kcal/mol, respectively.)[13]

To probe the possible transition-state interaction of the phosphate moiety with the metal cofactor and His16, we also examined the nonbridging thio effect (sulfur substitution at nonbridging oxygen) by use of R_P and S_P isomers of DPPsI (**3** and **4**, respectively, in Figure 2) for saPLC1. We have found (Table 2) that the WT enzyme has an extraordinarily high S_P-thio effect ($O/S_P = 1.6 \times 10^8$) and very high stereoselectivity (6.2×10^6) in the presence of the natural cofactor Ca^{2+}. Both values are 2 orders of magnitude higher than those for btPLC, which are already among the highest of all enzymes. On the other hand, the R_P-thio effect for WT saPLC1 is similar to that found for btPLC but decreases considerably for the H16A mutant ($O/R_P = 1.1$). Hence, the magnitude of nonbridging thio effect for a single enzyme (saPLC1) can vary from 1 to 10^8.

Further analyses of the data in Table 2 indicate several interesting points: (i) Substitution of Ca^{2+} by Cd^{2+} in WT saPLC1 enhances the activity of S_P-DPPsI by a factor of 400 but does not affect the activity with PI or R_P-DPPsI; thus, the S_P-thio effect and the R_P/S_P stereoselectivity are decreased by the same magnitude. (ii) In the presence of Ca^{2+}, the R_P-thio effect of H16A is lowered from that of WT significantly. (iii) Ca^{2+}/Cd^{2+} replacement in H16A dramatically lowers activity toward the R_P-isomer resulting in 10^3-fold increase of the R_P-thio effect. Tentatively, these results suggest that both Ca^{2+} and His16 are involved in the interaction with the *pro*-S_P oxygen of the phosphate moiety, but additional experiments are required to understand the nature of these interactions.

Taken together, the observed thio effects for saPLC1 support the transition-state structure shown in Figure 1B. Most importantly, we have observed the strongest bridging and nonbridging thio effects ever recorded, and our results demonstrate that the magnitude of enzymatic thio effects can vary greatly (from 0.002 to 20 for the bridging thio effect and from 1 to 10^8 for the nonbridging thio effect). Likewise, we have shown that the R_P/S_P stereoselectivity of an enzyme may span a large range of values (1 to 10^6). A change in the microenvironment of the active site (such as Arg to Lys, or Ca^{2+} to Cd^{2+}), or in the fine structure of the substrate, is sufficient to perturb these values by several orders of magnitude. Such information is important for proper application of thio effects as a mechanistic probe.

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Supporting Information Available: Experimental details of the enzyme and substrate preparation, kinetic analysis, inhibition study, and 2-D NOESY spectra of WT and mutant enzymes (PDF). This information is available free of charge via the Internet at http://pubs.acs.org

References

(1) Armstrong, V. W.; Yee, D.; Eckstein, F. *Biochemistry* **1979**, *18*, 4120−4123.
(2) Burgers, P. M.; Eckstein, F. *J. Biol. Chem.* **1980**, *255*, 8229−8233.
(3) Eckstein, F.; Romaniuk, P. J.; Connolly, B. A. *Methods Enzymol.* **1982**, *87*, 197−212.
(4) Frey, P. A. *Adv. Enzymol.* **1989**, *62*, 119−201.
(5) Herschlag, D.; Piccirilli, J. A.; Cech, T. R. *Biochemistry* **1991**, *30*, 4844−4854.
(6) Hondal, R. J.; Bruzik, K. S.; Zhao, Z.; Tsai, M.-D. *J. Am. Chem. Soc.* **1997**, *119*, 5477−5478.
(7) Hondal, R. J.; Riddle, S. R.; Kravchuk, A. V.; Zhao, Z.; Liao, H.; Bruzik, K. S.; Tsai, M. D. *Biochemistry* **1997**, *36*, 6633−6642.
(8) Kubiak, R. J.; Yue, X.; Hondal, R. J.; Mihai, C.; Tsai, M. D.; Bruzik, K. S. *Biochemistry* **2001**, *40*, 5422−5432.
(9) Patel, S. S.; Wong, I.; Johnson, K. A. *Biochemistry* **1991**, *30*, 511−525.
(10) Wong, I.; Patel, S. S.; Johnson, K. A. *Biochemistry* **1991**, *30*, 526−537.
(11) Washington, M. T.; Prakash, L.; Prakash, S. *Cell* **2001**, *107*, 917−927.
(12) Showalter, A. K.; Tsai, M.-D. *Biochemistry* **2002**, *41*, 10571−10576.
(13) Milstien, S.; Fife, T. H. *J. Am. Chem. Soc.* **1967**, *80*, 5820−5826.
(14) Hondal, R. J.; Zhao, Z.; Riddle, S. R.; Kravchuk, A. V.; Liao, H.; Bruzik, K. S.; Tsai, M.-D. *J. Am. Chem. Soc.* **1997**, *119*, 9933−9934.
(15) 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.
(16) Kravchuk, A. V.; Zhao, L.; Kubiak, R. J.; Bruzik, K. S.; Tsai, M. D. *Biochemistry* **2001**, *40*, 5433−5439.
(17) Iwasaki, Y.; Tsubouchi, Y.; Ichihashi, A.; Nakano, H.; Kobayashi, T.; Ikezawa, H.; Yamane, T. *Biochim. Biophys. Acta* **1998**, *1391*, 52−66.
(18) Essen, L. O.; Perisic, O.; Cheung, R.; Katan, M.; Williams, R. L. *Nature* **1996**, *380*, 595−602.
(19) Essen, L. O.; Perisic, O.; Katan, M.; Wu, Y.; Roberts, M. F.; Williams, R. L. *Biochemistry* **1997**, *36*, 1704−1718.
(20) Ellis, M. V.; James, S. R.; Perisic, O.; Downes, C. P.; Williams, R. L.; Katan, M. *J. Biol. Chem.* **1998**, *273*, 11650−11659.

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