# Mechanism of Phosphatidylinositol−Phospholipase C. 2. Reversal of a Thio Effect by Site-Directed Mutagenesis[1]

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The "thio effect", defined as the ratio $k_O/k_S$ (where O and S stand for a normal oxygen substrate and a sulfur analog, respectively), has been widely used in the mechanistic studies of enzymes and ribozymes. We report the first observation of reversal of a thio effect (from $k_O/k_S > 1$ to $k_O/k_S < 1$) for phosphatidylinositol-specific phospholipase C (PI-PLC) upon single amino acid substitution (D33A, Asp-33 to alanine).

Bacterial PI-PLC hydrolyzes phosphatidylinositol (PI) to 1-inositol phosphate (IP) in two distinct steps:[2] a fast conversion of PI to inositol 1,2-cyclic phosphate (IcP, which is released) and a very slow hydrolysis of IcP to IP (Figure 1). The sulfur analog used for this study is DPsPI [((2*R*)-1,2-((dipalmitoyl)-oxy)propane)thiophospho-1-D-*myo*-inositol], in which the oxygen at the $C_3$ of the diacylglycerol moiety is replaced by sulfur. The thio effect refers only to the first step. The $k_O$ is the specific activity determined by a radioactivity assay at saturating PI, while $k_S$ is $V_{max}$ determined by a continuous assay.[1] The $k_O$ and $k_S$ for wild-type (WT) PI-PLC are 1300 and 53.5 U/mg (1 U = 1 $\mu$mol min$^{-1}$ mg$^{-1}$), respectively, giving a thio effect of 24. The corresponding values for D33A are 1.3 and 13.4 U/mg, respectively, giving a reverse thio effect of 0.1. As a comparison, another mutant, R69K, with a $k_O$ (1.0 U/mg) similar to that of D33A displayed a normal thio effect ($k_S$ = 0.03 U/mg, $k_O/k_S$ = 33).

We further used $^{31}$P NMR to demonstrate the reverse thio effect for D33A. The time courses for the conversion of PI to IcP and IP are shown in Figure 2, where *the amount of D33A used is 500-fold higher than that of WT*. Note that the spectra at time 0 are not shown in Figure 2 because the PI peak is hidden under the large and broad peak of 1,2-diheptanoyl-*sn*-glycero-3-phosphatidylcholine (DHPC), which was used as a detergent.[3] Even at this level of enzyme, the conversion of PI to IcP is slower for D33A, as evidenced by the relative peak intensities between spectrum A (WT, 15 min) and spectrum D (D33A, 15 min). It takes seven times longer for D33A (spectrum E, 105 min) to reach the same extent of conversion as WT in spectrum A. These results indicate that the activity of D33A toward PI is slower than that of WT by up to ca. 3500 times. Similar difference in activity was also observed for the conversion of IcP to IP: IcP is completely converted to IP by WT from A (15 min) to B (105 min), while the conversion is still not fully complete by D33A from E (105 min) to F (525 min). In Figure 3, the time course for DPsPI was compared between *nearly the same amount of WT and D33A*. At the first time point, the extent of conversion of DPsPI to IcP was comparable between

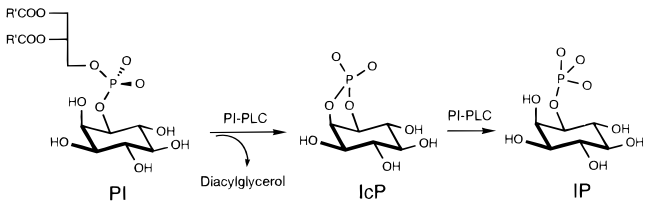


**Figure 1.** The reaction catalyzed by bacterial PI-PLC (R′ = $CH_3$-$(CH_2)_{14}$).

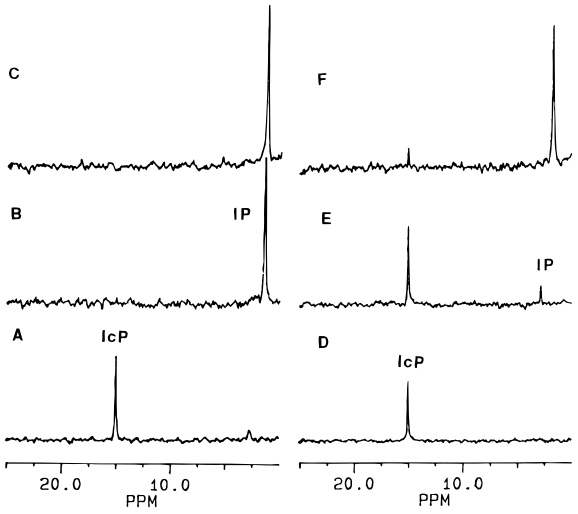


**Figure 2.** $^{31}$P NMR assay of PI/DHPC mixed micelles (where DHPC serves as a detergent[3]), 40 mM sodium borate as buffer, pH 7.5. Only a portion of the spectrum is shown for simplicity. Initially, PI shows a signal hidden underneath the signal of DHPC near −2 ppm: (A) 15 min (midpoint) after the addition of 1.5 $\mu$g of WT, where the peak at 15 ppm is IcP; (B) 105 min (midpoint) post-incubation with WT;, where the large peak near 2 ppm is IP; (C) 525 min (midpoint) post-incubation with WT; (D−F) at the same time points as those in A−C, except 750 $\mu$g of the D33A mutant has been added to the mixed micelles.

WT (A to B) and D33A (E to F), indicating that they have comparable activities toward DPsPI. Upon further incubation IcP was rapidly converted to IP by WT but not by D33A, which is expected since D33A has much lower activity.

The sulfur analog used in this work belongs to phosphorothiolates (sulfur replacing the *bridging* oxygen of a P−O−C group), which are chemically more reactive than normal substrates ($k_O/k_S \ll 1$). The bond energies of the P−S and P−O bonds are 45−50 and 95−100 kcal/mol, respectively,[4] and the chemical reactivity of thiolphosphate esters was shown to be ca. $10^3$ more reactive than the corresponding oxyesters.[5] However, the phosphorothiolates seem to be poorer substrates for enzymes and ribozymes ($k_O/k_S > 1$), possibly because the enzyme−substrate interactions involving the oxygen is unfavorable to sulfur. Such unfavorable interactions have not only "suppressed" the higher intrinsic activity of the S-analog but also further impeded its reactivity.

The catalytic mechanism of PI-PLC has not been established, except that it has been suggested to resemble the mechanism of ribonuclease A.[6] The crystal structure of the *Bacillus cereus* PI-PLC·*myo*-inositol complex has revealed two Asp···His pairs (D274···H32 and D33···H82) at the active site.[7] Our experimental data suggest that Asp-33 is involved, directly or

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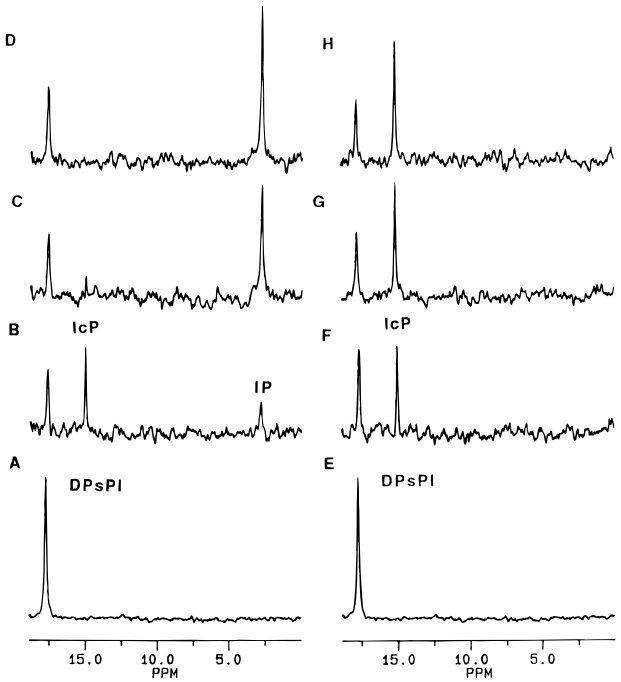


**Figure 3.** $^{31}$P NMR assay of mixed DPsPI/DHPC micelles in 85 mM MOPS buffer, pH 7.5: (A and E) spectra of the mixed micelle before addition of enzyme (time 0); (B) spectra after 15 min (midpoint) with addition of 1.5 $\mu$g of WT enzyme; where the peak at 18 ppm is the substrate DPsPI and the peak at 15 ppm is IcP; (C) 105 min (midpoint) post-incubation with WT, where the peak at 2 ppm is IP; (D) 585 min (midpoint) post-incubation with WT; (F−H) the same time points as B−D except 3.7 $\mu$g of the D33A mutant has been added to the mixed micelles, note that some of the substrate remains unreacted.

indirectly, in protonating the leaving group. Since sulfur is more difficult to protonate than oxygen due to the lower p$K_a$ of the thiol group, this protonation step could be partially responsible for the suppression of the higher intrinsic activity. In the D33A mutant, the lower p$K_a$ of the thiol group (relative to the hydroxyl group) allows the sulfur atom to be unprotonated as it leaves. Thus, the unfavorable protonation step is relieved in the mutant, resulting in higher activity for the S-analog. The mechanistic insight to the catalysis by PI-PLC will be further addressed elsewhere along with the data of other mutants.[8]

It seems useful to put our results into perspective in relation to other reports on the changes of thio effects. For the purpose of discussion, we designate the thio effect of phosphorothiolates (with a bridging sulfur) as type II and the thio effect of phosphorothioates (sulfur replacing a non-bridging oxygen of a phosphate group) as type I. Our results represent a reversal of type II thio effect *by a single mutation.* Change of a type II thio effect by changing metal ions has been demonstrated earlier for the *Tetrahymena* ribozyme.[9] In this case, the unfavorable interaction between $Mg^{2+}$ and sulfur (perhaps along with other factors) has not only suppressed the higher intrinsic activity of the S-analog but also caused a further slowing of the reaction by a factor of 1000 ($k_O/k_S = 1000$). Substitution by $Mn^{2+}$, a more thiophilic metal ion, has "relieved" some of the unfavorable interaction ($k_O/k_S = 3$), but not enough to allow the more chemically reactive S-analog to have higher *enzymatic* activity.

The interpretation of type I thio effects is more straightforward since phosphorothioate analogs are intrinsically less reactive than the regular substrates (i.e., $k_O/k_S > 1$ chemically).[10] Thus, suppression of the intrinsic activity is not an important factor here. Enzymatically, $k_O/k_S$ in the range of 1−1000 have been observed, but most fall within 10−100.[11] The change in the relative thio effect between stereoisomers of ATPαS or ATP$\beta$S upon changes of divalent metal ions can be attributed mainly to the metal ion preference of O versus S.[12] A reversal of thio effect between stereoisomers of ADPαS (i.e., reversal of stereoselectivity) upon single mutation has been achieved for adenylate kinase.[13] However, no absolute reversal of thio effect (i.e., $k_O/k_S < 1$) has ever been achieved for phosphorothioates.

The thio effect has also been used to determine whether the P−O or C−O bond cleavage is rate-limiting in WT and mutant enzymes, such as phospholipase $A_2$ (with a thioester analog, designated as type III)[14] and DNA polymerases (with phosphorothioate analogs).[15,16] An increase in $k_O/k_S$ for a mutant is often used to suggest that the particular step becomes more rate-limiting. One needs to be particularly cautious in this approach for type II and type III thio effects, since a subtle change in the degree of suppression of the intrinsic activity of S-analogs could lead to a large change in the observed thio effect.

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