Sulfur substitution of a phosphoryl oxygen has been used extensively in mechanistic studies of enzymes and ribozymes that involve phosphoryl transfer reactions. 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 should be for enzymatic reactions. On one hand, thio effects and Rp/Sp 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 dNTPs 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. As a prominent example of the contrary, observation of a relatively small thio effect (ca. 3 or less) for the incorporation of dNTPs 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.

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. 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<sub>o</sub>/k<sub>s</sub>) should be for enzymatic reactions. On one hand, thio effects and Rp/Sp 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 dNTPs 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.

Figure 1. Proposed transition states of btPLC (A) and mammalian PI-PLC<sub>01</sub> (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, 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-01 on the basis of crystal structures and limited site-directed mutagenesis studies. Figure 1B, where His311 and His356 of PLC-01 correspond to His16 and His55 of saPLC1, respectively). 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-01 (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 (2R)-1,2-dioctanoyloxypropanethio-3-(1-phospho-1-d-myo-inositol), DOSPI, 1,2-dipalmitoyl-sn-glycero-3-(1-thiophospho-1-d-myo-inositol).
Table 1. Summary of Bridging Thio Effects for WT and Mutant saPLC1

<table>
<thead>
<tr>
<th>enzyme</th>
<th>WT</th>
<th>H16A</th>
<th>H55A</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI b</td>
<td>$k_{b}'$</td>
<td>$k_{b}^{d}$</td>
<td>$k_{b}^{d}$</td>
</tr>
<tr>
<td></td>
<td>1122 ± 22</td>
<td>0.0236 ± 0.0005</td>
<td>0.207 ± 0.015</td>
</tr>
<tr>
<td>DOsPI</td>
<td>$k_{b}^{d}$</td>
<td>58 ± 7</td>
<td>16 ± 3</td>
</tr>
<tr>
<td></td>
<td>$k_{b}^{d}$</td>
<td>678 ± 28</td>
<td>0.044 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>$k_{b}^{d}$</td>
<td>24 ± 5</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>$k_{b}^{d}/k_{b}'$</td>
<td>1.7</td>
<td>0.54</td>
<td>0.0019</td>
</tr>
</tbody>
</table>

b 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 CaCl2, 1 mM EDTA, pH 7.0. Activities are expressed in μmol mg⁻¹ min⁻¹. c 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. d Maximal activity toward PI determined by the radioactivity assay. e Maximal activity toward DOsPI obtained by the spectroscopic assay. f In μM. g Bridging thio effect. The values for N17A, E99A, E93Q, 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 d

<table>
<thead>
<tr>
<th>metal ions</th>
<th>WT</th>
<th>PI</th>
<th>DOsPI</th>
<th>R₈-DPPsI</th>
<th>S₂-DPPsI</th>
<th>$k_{b}/k_{b}^{d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺</td>
<td>666</td>
<td>646</td>
<td>13.7</td>
<td>4.0 × 10⁻⁶</td>
<td>4.0 × 10⁻⁶</td>
<td>6.2 × 10⁻³</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>23</td>
<td>71</td>
<td>32</td>
<td>1.1</td>
<td>16</td>
<td>1.6</td>
</tr>
<tr>
<td>Pb²⁺</td>
<td>22</td>
<td>663</td>
<td>664</td>
<td>3.9</td>
<td>3.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

d Measured by ³¹P NMR 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. e 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 $K_{m,app}$ values (0.161 mM and 0.190 mM for R₈- and S₂-DPPsI, respectively) for DPPsIs. f R₈-thio effect. g S₂-thio effect. h $R_{P}/S_{P}$ stereoselectivity. i ND, nondetectable.

The presence of Ca²⁺, the R₈-thio effect of H16A is lowered from that of WT significantly. (iii) Ca²⁺/Cd²⁺ replacement in H16A dramatically lowers activity toward the R₈-isomer resulting in 10³-fold increase of the R₈-thio effect. Tentatively, these results suggest that both Ca²⁺ and His16 are involved in the interaction with the pro-S₉ oxygen of the phosphate moiety, but additional experiments are required to understand the nature of these interactions.

Table summary: The table presents the summary of bridging thio effects for WT and mutant saPLC1, as well as the summary of nonbridging thio effects for WT saPLC1 and Mutant H16A in the presence of different metal ions. The data includes the specific activities (kₐ) and their ratios ($k_{b}/k_{b}^{d}$) for various metal ions and substrates, showing the effect of metal ions on thio effects.

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

References


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