Communications to the Editor

Reactivities of Inositol and Ribonucleoside Phosphodiesters toward P−O Bond Cleavage

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Biological cleavage of important phosphodiester molecules, RNA and phosphatidylinositols (PI), occurs with transient formation of five-membered cyclic phosphodiesters.[1,2] The two reactions catalyzed by RNases and phospholipases C (PLC), respectively, involve the intramolecular attack of the β-hydroxy group (2′-hydroxy group of ribonucleoside and 2-hydroxy group of inositol) on phosphorus and departure of the 5′-nucleoside or diacyl glycerol leaving group.[1−3] We and others have shown previously that many analogies exist between catalytic mechanisms and active site structures of RNase A and PLC.[2,4,5] Both enzymes involve a pair of catalytic histidines: His12 and His32, respectively, as general bases, and His 119 and His82, respectively, as general acids.[1,5] In addition, the negative charge of the phosphate group is stabilized by interaction with a positively charged residue, Lys41 and Arg69, respectively.[1,6] Despite these analogies the active site of PLC is much more complex involving additional Asp274 and Asp33 moieties interacting with His32 and His82, respectively.[5,7] Furthermore, Arg69, Asp33, and His82 residues of PI-PLC form a unique catalytic triad involved in both hydrogen bonding to phosphate and leaving group protonation.[8] To understand why a much more complex catalytic mechanism of PI-PLC is necessary to catalyze an analogous reaction, we have investigated nonenzymatic cleavage of inositol phosphodiesters and compared the results to those obtained earlier[9,10] for analogous 3′-ribonucleoside phosphodiesters.

We have found that in sharp contrast to the behavior of 3′-ribonucleoside alkyl phosphoesters,[9−12] 1-inositol alkyl phosphoesters such as 1-glycerol and *n*-butyl inositol phosphates were completely stable to 0.5 M imidazole buffer at pH 6.8 at 80 °C. On the other hand, *p*-nitrophenyl 1-inositol phosphate (NPIP) was cleaved at 25 °C with the rate constant $k = 2.4 \times 10^{-6}$ M^{-1} s^{-1} to afford two products giving rise to ^{31}P NMR signals at 16.1 and 16.8 ppm at the 3:1 ratio (Figure 1A−C). These products were identified as inositol 1,2-cyclic phosphate (1,2-IcP) and inositol 1,6-cyclic phosphate (1,6-IcP) (Scheme 1). The identity of 1,6-IcP is indicated by the low-field chemical shift of ^{31}P NMR signal and the lack of observation of vicinal coupling of H-1 and H-6 protons to phosphorus. These data are consistent with the 1,6-cyclic structure in which both POCH1 and POCH6 dihedral angles are close to 90°. Thus, reactivity of NPIP toward cyclization is 10^3-fold lower than that of *p*-nitrophenyl uridine 3′-phosphate (NPUP).[10] To confirm that the chemical cleavage of NPIP occurs via the same mechanism as the enzymatic

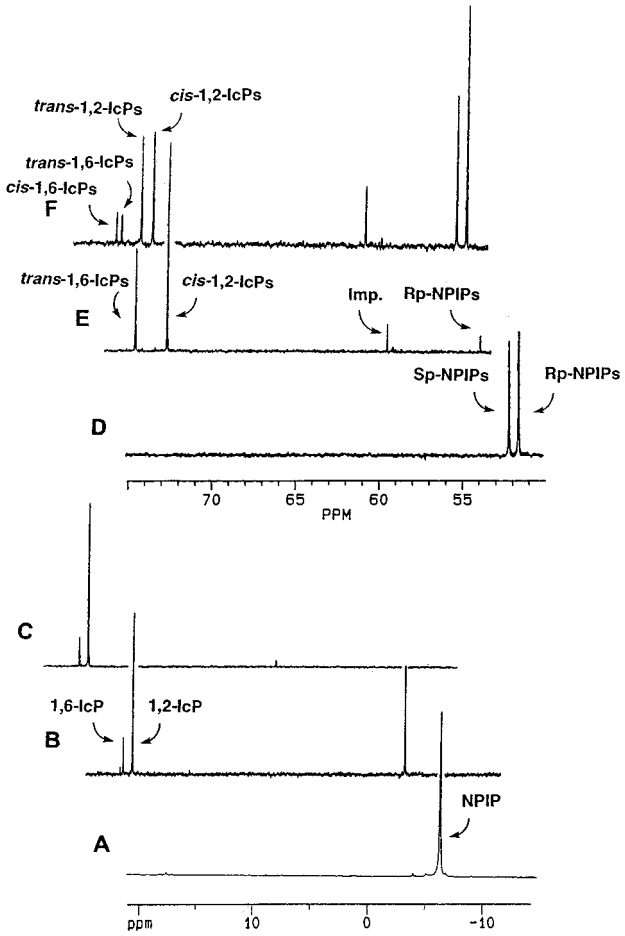
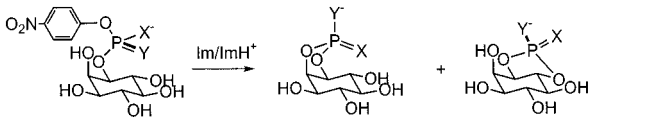


Figure 1. Imidazole buffer-catalyzed cyclization of NPIP (A−C) and NPIPs (D−F). Conditions: concentration of imidazole buffer 0.25 M; pH 6.8; temperature 25 °C. *R*p-NPIPS was obtained by stereoselective PI-PLC-catalyzed cleavage of *S*p-NPIPs in the (*R*p+*S*p)-mixture of diastereomers, followed by chromatographic separation of the intact *R*p-diastereomer. The configurations of *trans*- and *cis*-1,2-IcPs were assigned on the basis of chemical shifts of ^{31}P NMR signals as described earlier.[13]

Scheme 1



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reactions of inositol phospholipids[2] and RNA,[14] we have investigated the steric course of the cleavage of *p*-nitrophenyl inositol phosphorothioate (NPIPs). Cyclization of *R*p-NPIPs in 0.25 M imidazole afforded *cis*-1,2-IcPs (δ 70.35 ppm, Figure 1E), with the second-order rate constant $k = 1.7 \times 10^{-6}$ M^{-1} s^{-1}, and another product giving rise to ^{31}P NMR signals at 72.18 ppm (3:1 ratio). Formation of *cis*-1,2-IcPs from *R*p-NPIPs and the absence of *trans*-1,2-IcPs indicated that analogously to enzymatic cyclization,[2] the chemical reaction proceeds by an in-line mechanism resulting in inversion of configuration at phosphorus and is completely stereospecific. Finally, cyclization of the mixture (*R*p+*S*p)-NPIPs afforded four products giving rise to signals at 70.35 (*cis*-IcPs), 71.05 (*trans*-IcPs), 72.18 (*trans*-1,6-IcPs), and 72.48 ppm (*cis*-1,6-IcPs) (Figure 1F). The intensity of signals arising from the two 1,2-IcPs isomers and two 1,6-IcPs isomers was pairwise identical, indicating that the chemical cyclization proceeds with the same rate constant for either *R*p- and *S*p-diastereomer of NPIPs. Thus, the high *R*p/*S*p stereoselectivities observed in enzymatic cleavage of phosphorothioate analogues of PI[6] are due to enzyme mechanism, and not because of intrinsic differences in isomer reactivities. Unlike in the case of ribosyl aryl phosphates, where cyclization was accompanied by 2′→3′-phosphate migration,[9] we were unable to detect formation of the isomerized inositol *p*-nitrophenyl phosphate. There are two possible reasons for low reactivity of inositol phosphodiesters. First, formation of the five-membered ring in IcP is associated with a greater torsional strain than that in 2′,3′-cyclic ribonucleotide. This is because formation of the five-membered ring in the phosphorane-type transition state, with an almost planar arrangement of O1−C1−C2−O2 fragment, causes distortion of the six-membered inositol ring.[15] The rigidity of the six-membered ring of inositol and an equal distance between 1-oxygen and those in 2- and 6-positions results in no particular kinetic advantage of the nucleophilic attack by the *cis*-2-hydroxyl group as compared to the *trans*-6-hydroxyl group. This fact is manifested in the formation of significant amounts of the 1,6-cyclic phosphate. In contrast, for ribonucleoside 3′-phosphodiester, closer proximity between the 2′- and 3′-oxygens and greater flexibility of the ribose ring as compared to inositol make the 2′-hydroxyl group of the ribose better suited for intramolecular attack.[16] To overcome the greater torsional strain in the inositol system, stronger electrophilic activation of the phosphate group may be necessary. Thus, in PI-PLC-catalyzed reaction, the phosphate group is activated by the dual-function Arg69-Asp33-His82 catalytic triad, responsible for both hydrogen bonding to nonbridging oxygen and leaving group protonation,[8] whereas RNase A uses separate Lys and His residues to accomplish analogous functions.[1] Additional catalytic rate enhancement is due to bridging interactions of the phosphate and the 2-hydroxyl group with Arg69.[17] Second, the calculated pK_a of the inositol 2-hydroxyl group is 14.2 as compared to 12.5[18] for 2′-hydroxyl of uridine. It therefore appears that deprotonation of the inositol 2-hydroxyl requires a stronger general base than that of the ribose 2′-hydroxyl. Consistently, in PI-PLC, activation of the 2-OH nucleophile is achieved by the His32-Asp274 diad,[6] analogously to catalytic triads of serine proteases, whereas a single His12 suffices in RNase A.[1]

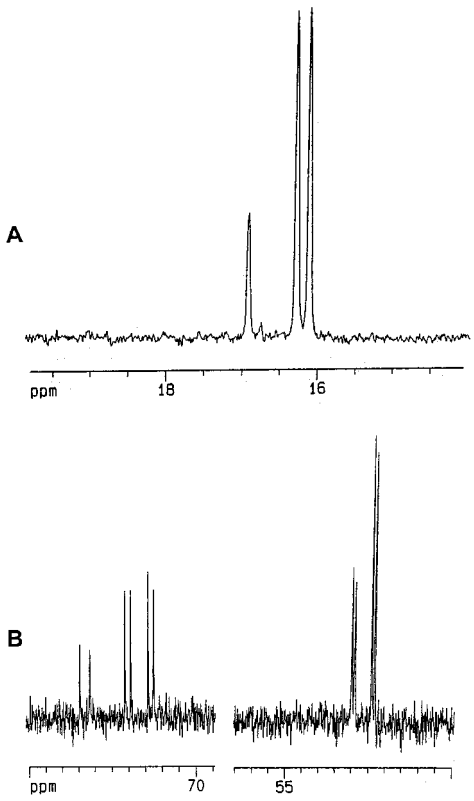

Figure 2. 121.47-MHz ^1H-coupled spectra of the products of NPIP (A) and NPIPs (B) cyclization from Figure 1A and Figure 1F, respectively. The signal of 1,2-IcP at 16.1 ppm shows $^3J_{POCH} = 20$ Hz coupling to inositol H-1 proton and no coupling to H-2, whereas the signal at 16.8 ppm shows no detectable coupling to protons.

In conclusion, since PI-PLC and RNase A attain similar turnover rates (ca. 10^3 s^{-1}), but the chemical reactivity of inositol phosphoesters appears 10^3-fold lower than that of the corresponding ribonucleoside phosphoesters, PI-PLC catalyzes the cleavage of the phosphodiester bond with ca. 10^3-fold greater efficiency.[19,20] This might explain why this enzyme requires a more sophisticated structure of the active site.

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