Phospholipids Chiral at Phosphorus. Stereochemical Mechanism for the Formation of Inositol 1-Phosphate Catalyzed by Phosphatidylinositol-Specific Phospholipase C†

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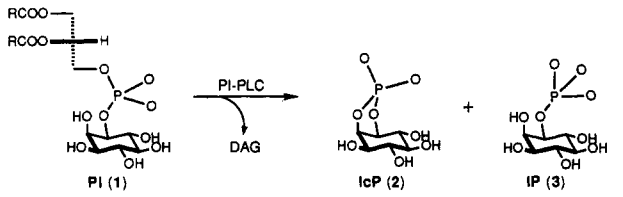
ABSTRACT: The phosphatidylinositol-specific phospholipase C (PI-PLC) from mammalian sources catalyzes the simultaneous formation of both inositol 1,2-cyclic phosphate (IcP) and inositol 1-phosphate (IP). It has not been established whether the two products are formed in sequential or parallel reactions, even though the latter has been favored in previous reports. This problem was investigated by using a stereochemical approach. Diastereomers of 1,2-dipalmitoyl-*sn*-glycero-3-(1*D*-[^{16}O,^{17}O]phosphoinositol) ([^{16}O,^{17}O]DPPI) and 1,2-dipalmitoyl-*sn*-glycero-3-(1*D*-thiophosphoinositol) (DPPsI) were synthesized, the latter with known configuration. Desulfurization of the DPPsI isomers of known configurations in $H_2{}^{18}O$ gave [^{16}O,^{18}O]DPPI with known configurations, which allowed assignment of the configurations of [^{16}O,^{17}O]DPPI on the basis of ^{31}P NMR analyses of silylated [^{16}O,^{18}O]DPPI and [^{16}O,^{17}O]DPPI (the inositol moiety was fully protected in this operation). (R_p)- and (S_p)-[^{16}O,^{17}O]DPPI were then converted into *trans*- and *cis*-[^{16}O,^{17}O]IcP, respectively, by PI-PLC from *Bacillus cereus*, which had been shown to proceed with inversion of configuration at phosphorus [Lin, G., Bennett, F. C., & Tsai, M.-D. (1990) *Biochemistry 29*, 2747–2757]. ^{31}P NMR analysis was again used to differentiate the silylated products of the two isomers of IcP, which then permitted assignments of IcP with unknown configuration derived from transesterification of (R_p)- and (S_p)-[^{16}O,^{17}O]DPPI by bovine brain PI-PLC-β_1. The results indicated inversion of configuration, in agreement with the steric course of the same reaction catalyzed by PI-PLCs from *B. cereus* and guinea pig uterus reported previously. For the steric course of the formation of inositol 1-phosphate catalyzed by PI-PLC, (R_p)- and (S_p)-[^{16}O,^{17}O]DPPI were hydrolyzed in $H_2{}^{18}O$ to afford 1-[^{16}O,^{17}O,^{18}O]IP, which was then converted to IcP chemically and analyzed by ^{31}P NMR. The results indicated that both *B. cereus* PI-PLC and the PI-PLC-β_1 from bovine brain catalyze conversion of DPPI to IP with overall retention of configuration at phosphorus. These results suggest that both bacterial and mammalian PI-PLCs catalyze the formation of IcP and IP by a sequential mechanism. However, the conversion of IcP to IP was detectable by ^{31}P NMR only for the bacterial enzyme. Thus an alternative mechanism in which IcP and IP are formed by totally independent pathways, with formation of IP involving a covalent enzyme–phosphoinositol intermediate, cannot be ruled out for the mammalian enzyme. It was also found that both PI-PLCs displayed lack of stereospecifically toward the 1,2-diacylglycerol moiety, which suggests that the hydrophobic part of phosphatidylinositol is not recognized by PI-PLC.

Phosphatidylinositol-specific phospholipases C (PI-PLC)[1] from mammalian sources are key enzymes in the receptor-mediated metabolism of inositol phospholipids (Rhee et al., 1989, 1992; Rhee, 1991; Berridge, 1987; Hokin, 1985; Shukla, 1982). The function of the corresponding bacterial enzymes is not entirely clear, but they have been implicated in the release of the ectoenzyme activities of some proteins attached to plasma membranes via 6-glycosylated phosphatidylinositol (Ferguson & Williams, 1988; Low & Saltiel, 1988). The two types of enzymes produce different products when acting on analogous phosphatidylinositol substrates **1** (Scheme I): mammalian PI-PLC usually produce a mixture of inositol 1,2-cyclic phosphate (IcP, **2**) and inositol 1-phosphate (IP, **3**) (Kim et al., 1989; Dawson et al., 1971; Lapetina & Michell, 1973; Wilson et al., 1985a,b); bacterial PI-PLC usually produce exclusively IcP **2** (Volwerk et al., 1990; Ferguson et al., 1985). However, formation of both IcP and IP by *Bacillus cereus* PI-PLC has also been reported earlier (Ikezawa et al., 1976), which can now be explained by the recent finding of

Scheme I: Conversion of Phosphatidylinositol to IcP and IP Catalyzed by PI-PLC



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[1] Abbreviations: DPPI, 1,2-dipalmitoyl-*sn*-glycero-3-(1*D*-1-phospho-*myo*-inositol); DPPsI, 1,2-dipalmitoyl-*sn*-glycero-3-(1*D*-1-thiophospho-*myo*-inositol); IP, 1*D*-*myo*-inositol 1-phosphate; IcP, 1*D*-inositol 1,2-cyclic phosphate; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PLA2, phospholipase A_2; DAG, 1,2-diacylglycerol; DPG, 1,2-dipalmitoylglycerol; TFA, trifluoroacetic acid; THF, tetrahydrofuran; EDTA, ethylenediaminetetraacetic acid; DMF, dimethylformamide; Py, pyridine; TLC, thin-layer chromatography.

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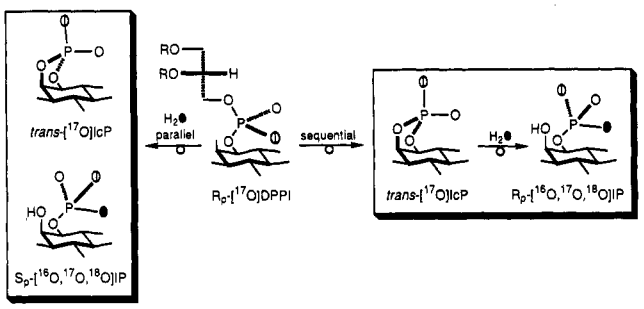
Scheme II: Parallel and Sequential Mechanisms for the Formation of IcP and IP



a low level phosphodiesterase activity of this enzyme converting IcP into IP (Volwerk et al., 1990).

The simultaneous formation of two products by mammalian PI-PLC has been a subject of interest. It is always possible that the mammalian PI-PLC is a bifunctional enzyme with two active sites or that it is a complex of two related proteins (Quinn, 1978). However, there is no available evidence for such possibilities. For a single active site, formation of the two products could follow a parallel or a sequential pathway, as outlined in Scheme II. The parallel mechanism has been favored in previous reports mainly because of lack of evidence for the sequential mechanism (Dawson et al., 1971; Lapetina & Michell, 1973; Quinn, 1978; Wilson et al., 1985a,b; Kim et al., 1989).

Using phosphorothioate analogues of DPPI, we have demonstrated that the formation of IcP proceeds with inversion of configuration, which strongly suggests a single displacement mechanism in the formation of IcP for both *B. cereus* PI-PLC and the isozymes I and II of PI-PLC from guinea pig uterus (Lin & Tsai, 1989; Lin et al., 1990). However, the steric course for the hydrolysis of PI to IP has not been established. As shown in Scheme II, a parallel mechanism predicts that the steric course of the formation of inositol 1-phosphate is also inversion, whereas a sequential mechanism predicts an overall retention for the conversion of phosphatidylinositol to inositol 1-phosphate (Bruzik et al., 1991). In this paper, we present the synthesis of diastereomers of oxygen isotope-labeled P-chiral DPPI and their application toward studying the stereochemical course of the conversion of DPPI to IP catalyzed by *B. cereus* PI-PLC and bovine brain PI-PLC-β_1 [for nomenclature and properties of the different isozymes of mammalian PI-PLCs, see Rhee et al. (1989) and Rhee (1991)].

Materials and Methods

Materials. All reagents were from commercial sources unless otherwise specified. Organic solvents were stored over appropriate desiccants in the stoppered ampoules under vacuum and were distilled into reaction vessels under vacuum prior to the reaction, to avoid atmospheric moisture. 1*D*-2,3-*O*-(*D*-1′,7′,7′-Trimethylbicyclo[2.2.1]hept-2′-ylidene)-4,5,6-*O*-tris(methoxymethylene)-*myo*-inositol (**4**) was obtained as described recently (Bruzik & Tsai, 1992). Unlabeled DPPI was obtained as described previously (Salamonczyk & Bruzik, 1990). (Diphenylphosphoryl)imidazole was obtained according to Abbott et al. (1979). *B. cereus* PI-PLC was from Boehringer and was not further purified. Bovine brain PI-PLC-β_1 was first purified from bovine brain (isozyme I) by Ryu et al. (1987); the enzyme used in this work was purified from the extracts of HeLa cells transfected with psc11 vaccinia virus expression vector which contains the entire coding region of rat PI-PLC-β_1 cDNA (D.-Y. Jhon and S. G. Rhee, unpublished results).

Analytical Methods. NMR spectra were obtained with Bruker AM spectrometers operating at frequencies corresponding to proton 250, 300, and 500 MHz resonance frequency, as stated below. ^{1}H and ^{13}C NMR chemical shifts were indirectly referenced to tetramethylsilane and those of ^{31}P to 85% H_3PO_4. The purity of products was assayed by spectroscopic methods (^{1}H, ^{13}C, ^{31}P NMR) and TLC technique using aluminum foil-based silica gel plates. Phosphomolybdic acid solution (10% in ethanol) was used for visualization of TLC plates.

*Synthesis of (S_p)- and (R_p)-DPPsI (**10a** and **10b**, respectively).* 1*D*-2,3-*O*-(*D*-1′,7′,7′-Trimethylbicyclo[2.2.1]hept-2′-ylidene)-4,5,6-*O*-tris(methoxymethylene)-*myo*-inositol (**4**, 415 mg, 0.9 mmol) was dissolved in chloroform (2 mL) and was treated with diisopropylethylamine (350 μL, 2 mmol) and *N*,*N*-diisopropyl-*O*-methylphosphonamidic chloride (288 mg, 10% excess; Bruzik et al., 1986). The reaction was carried out at 70 °C in a tightly closed flask. After 5 h, the reaction was complete as judged by ^{31}P NMR and by the disappearance of the alcohol **4** on the TLC plate. The solvent and excess amine were removed under vacuum, and the residue was redissolved in the solution of tetrazole (280 mg, 4.5-fold excess) and 1,2-dipalmitoyl-*sn*-glycerol (568 mg, 1 mmol) in THF–acetonitrile (1:1 v/v, 5 mL). After 1 h, the reaction was judged complete by TLC. All solvents were evaporated under vacuum, and the residue was dissolved in benzene, and elemental sulfur (100 mg) was added. The progress of the reaction was monitored by TLC (hexane–acetone, 10:2). Initially, the formation of the less mobile diastereomer of fully protected phosphorothioate **7a** (R_f 0.28) was faster than that of the other one (**7b**, R_f 0.35). After 24 h, the ratio of isomers was close to 1:1. The resulting mixture was diluted with toluene, and the solution was decanted from solid particles and concentrated. The isomers were separated by the chromatography on silica gel (hexane–acetone, 10:2) yielding pure **7a** (374 mg) and **7b** (218 mg): total yield, 58%. **7a** (chromatographically less mobile, slow isomer): ^{31}P NMR ($CDCl_3$) δ 68.4; ^{13}C NMR (C_6D_6) δ 172.5, 172.3 (C=O), 118.0 (C-2′), 97.6, 97.5, 96.3 (CH_2OMe), 76.4, 76.1, 73.9, 69.8, 66.1 (C—O, P-coupled), 80.2, 77.3, 75.2, 62.0 (C—O), 56.0, 55.8, 55.5 (OMe), 54.6 (POMe), 51.9, 48.1, 45.6, 44.3, 34.3, 34.1, 32.3 (camphor), 30.1–29.4 (6 peaks, CH_2), 27.4, 25.2, 23.1, 20.8, 20.4, 14.3, 10.0. **7b** (fast isomer): ^{31}P NMR ($CDCl_3$) δ 67.7.

Diastereomers **7a** and **7b** (327 and 250 mg, respectively) were separately dissolved in trimethylamine (5 mL) below 0 °C and left at ambient temperature for 12 h to give diesters **9**. Diester **9a** (from **7a**) was dissolved in methanol–TFA (1:1 v/v, 8 mL) and heated at 70 °C for 45 min. The progress of the reaction was checked by TLC (methanol–chloroform–water, 20:80:2). The formation of side-products with lower mobility has also been observed (most likely due to competing deacylation). The product was concentrated and chromatographed on a silica gel column using the above solvent for elution to give pure **10a** (280 mg, 100%). Analogously, **9b** was deprotected to give **10b** (148 mg, 78%). **10a**: ^{31}P NMR (CD_3OD) δ 57.2; ^{1}H NMR (250 MHz, CD_3OD, primed numbers refer to inositol ring, unprimed to glycerol protons) δ 5.246 (m, H-2, 1 H), 4.44 (dd, J = 3.3, 12.0 Hz, 1 H), 4.251 (tr, H-2′, J = 2.6 Hz, 1 H), 4.16 (m, 4 H), 3.788 (tr, J = 9.4 Hz, H-6′, 1 H), 3.601 (tr, J = 9.7 Hz, 1 H), 3.394 (dd, J = 2.9, 9.8 Hz, H-3′, 1 H), 3.227 (tr, J = 9.4 Hz, H-5′, 1 H), 2.346, 2.316 (each tr, CH_2CO, 2 H), 1.621 (m, CH_2, 4 H), 1.290 (br s, CH_2), 0.890 (tr, 6 H). **10b**: ^{31}P NMR (CD_3OD) δ 56.6; ^{1}H NMR (250 MHz, CD_3OD) δ 5.26 (m, H-2, 1 H), 4.451 (dd, J = 3.2, 12.0 Hz, 1 H), 4.278 (tr, H-2′, J = 2.7

Hz, 1 H), 4.16 (m, 4 H), 3.791 (tr, J = 9.7 Hz, H-6′, 1 H), 3.638 (tr, J = 9.4 Hz, 1 H), 3.396 (dd, J = 2.9, 9.8 Hz, H-3′, 1 H), 3.225 (tr, J = 9.4 Hz, H-5′, 1 H), 2.351, 2.322 (each tr, CH_2CO, 2 H), 1.613 (m, CH_2, 4 H), 1.296 (br s, CH_2), 0.898 (tr, 6 H).

*Synthesis of 1,2-Dipalmitoyl-sn-glycero-3-(1D-1-[^{16}O,^{17}O]phosphoinositol) ([^{16}O,^{17}O]DPPI, **12a and 12b**).* The phosphitylation of alcohol **4** (1.33 mmol) and further coupling of the resulting phosphoramidite with 1,2-dipalmitoyl-*sn*-glycerol was accomplished essentially as described above. The trivalent phosphorus triester was concentrated under vacuum, and the residue was added with the mixture of pyridine (5 mL), iodine (450 mg), and $H_2{}^{17}O$ (55 μL, 52.8% ^{17}O) at 0 °C until the iodine color persisted. After 30 min, the mixture was diluted with ethyl acetate (25 mL) and washed with aqueous sodium bisulfite and water. The organic layer was concentrated and chromatographed on a silica gel H column (Sigma, 10–40-μm particle size) using hexane–ether (10:3) as the eluting solvent to give two fractions containing isomers of the fully protected [^{17}O]DPPI, **8a** ("fast", 220 mg) and **8b** ("slow", 180 mg), and mixed fraction **8a** + **8b** (130 mg): total yield, 34%. **8a**: high-performance TLC R_f 0.21 (hexane–ether, 10:3); ^{31}P NMR ($CDCl_3$) δ −1.653 (37%), −1.694 (63%). The ratio of [^{16}O] and [^{18}O]phosphate peaks indicates 20% ^{16}O, 32% ^{18}O, and 47% ^{17}O composition of phosphates **8**: ^{1}H NMR ($CDCl_3$) δ 5.237 (br q, H-2, 1 H), 4.793 (m, CH_2OMe, 6 H), 4.54 (m, H-3, 2 H), 4.342 (dd, H-3′, J = 12.0, 4.1 Hz, 1 H), 4.248 (m, H-1′, 1 H), 4.173 (m, 2 H), 4.018 (m, 2 H), 3.876 (tr, H-4′ (6′), J = 7.1 Hz, 1 H), 3.781 (d, OMe, J = 11.3 Hz, 3 H), 3.588 (tr, H-5, J = 7.1 Hz, 1 H), 3.425, 3.419, 3.406 (each s, CH_2O*Me*, 3 H), 2.303 (dtr, CH_2CO, 4 H), 1.959 (m, 2 H), 1.75–1.68 (m, 3 H), 1.60 (m, CH_2, 4 H), 1.447 (d, J = 13.0 Hz, 1 H), 1.26 (br s, 48 H), 0.99, 0.91, 0.86 (each s, C*Me*, 3 H), 0.880 (tr, CH_2*Me*, 6 H); ^{13}C NMR ($CDCl_3$) δ 173.0, 172.6 (C=O), 117.9 (C-2″, camphor), 97.6, 96.1 (CH_2OMe), 79.9, 76.0, 75.4, 69.3, 65.4 (d, C—O, P-coupled), 76.9, 74.7, 73.5, 61.6 (s, C—O), 56.0, 55.9, 55.6 (OMe), 54.5 (POMe), 51.5, 47.8, 45.0, 43.9, 34.1, 33.9 (camphor), 31.8, 29.7–29.0 (8 peaks, CH_2), 26.9, 24.8, 22.6, 20.4, 20.2, 14.0, 13.9, 9.6. **8b**: high-performance TLC R_f 0.18 (hexane–ether, 10:3); ^{31}P NMR ($CDCl_3$) δ −1.728 (37.5%), −1.768 (62.5%); ^{1}H NMR ($CDCl_3$) δ 5.24 (br q, H-2), 4.810 (m, CH_2OMe, 6 H), 4.54 (m, H-3, 2 H), 4.339 (dd, H-3′, J = 12.0, 4.4 Hz, 1 H), 4.193 (m, 3 H), 4.030 (m, 2 H), 3.889 (tr, H-4′ (6′), J = 6.5 Hz, 1 H), 3.794 (d, OMe, J = 11.1 Hz, 3 H), 3.599 (tr, H-5, J = 7.1 Hz, 1 H), 3.423, 3.406, 3.405 (each s, CH_2O*Me*, 3 H), 2.303 (dq, CH_2CO, 4 H), 1.957 (m, 2 H), 1.75–1.68 (m, 3 H), 1.60 (m, CH_2, 4 H), 1.447 (d, J = 13.0 Hz, 1 H), 1.26 (br s, 48 H), 0.98, 0.91, 0.86 (each s, C*Me*, 3 H), 0.880 (tr, CH_2*Me*, 6 H); ^{13}C NMR ($CDCl_3$) δ 172.9, 172.5 (C=O), 117.8 (C-2″), 97.6, 97.5, 96.1 (CH_2OMe), 79.7, 75.9, 75.4, 69.2, 65.3 (d, C—O, P-coupled), 74.6, 73.5, 61.6 (s, C—O), 56.0, 55.9, 55.6 (OMe), 54.3 (POMe), 51.4, 47.7, 45.0, 43.9, 34.0, 33.9 (camphor), 31.8, 29.6–29.0 (7 peaks, CH_2), 26.8, 24.7, 22.5, 20.3, 20.2, 13.9, 9.6.

The diastereomerically pure esters **8a** (97 mg) and **8b** (55 mg) were separately treated with trimethylamine (3 mL) at the temperature below 0 °C, and the demethylation reactions were carried out in a screw-cap vials at room temperature. The demethylation was followed by TLC (hexane–acetone, 10:3). After the reactions had been complete (48 h), trimethylamine was evaporated and small fractions of the corresponding demethylated diesters **11a** and **11b** were used in ^{31}P NMR experiments as described later. The main batches were dissolved in anhydrous methylene chloride (1 mL) and added with benzenethiol (200 μL) and boron trifluoride etherate (25 μL). After 2.5 h, the deprotection was complete as judged by TLC. No side-products were detected. The deprotection of **11** in methanol–TFA has failed due to the competing deacylation processes. The products were purified without further work-up by column chromatography on silica gel using a gradient of chloroform–methanol-aqueous NH_3 (60:20:0.2) to chloroform–methanol–water (65:35:4) to give pure **12a** (66 mg, from **11a**) and **12b** (42 mg, from **11b**). **12a**: ^{1}H NMR (300 MHz, CD_3OD) δ 5.25 (m, H-2, 1 H), 4.46 (ddd, J = 3.1, 5.6, 12.0 Hz, 1 H), 4.19 (m, 2 H), 4.07 (m, 2 H), 3.90 (ddd, 1 H), 3.78 (tr, J = 9.3 Hz, 1 H), 3.64 (tr, J = 8.5 Hz, 1 H), 3.37 (dd, J = 2.9, 9.8 Hz), 3.21 (tr, J = 9.2 Hz, 1 H), 2.33 (dtr, 4 H), 1.61 (m, 4 H), 1.28 (br s, 52 H), 0.89 (tr, 6 H). Both DPPI samples had identical TLC mobilities and ^{1}H and ^{31}P NMR spectra as those of previously obtained DPPI (Salamonczyk & Bruzik, 1990).

Diastereomeric [^{16}O,^{17}O]DPPI Containing a DL-*1,2-Dipalmitoylglycerol Moiety* (DL-***12a** and* DL-***12b***). These were synthesized and separated in a manner analogous to that of those described above starting from DL-1,2-dipalmitoylglycerol and diasteromerically pure alcohol **4**. The four diastereomers of **8** were separated into only two fractions DL-**8a** and DL-**8b**, since the diastereomers differing in the glycerol configuration were not separable under the conditions used. The intensity ratios in ^{31}P NMR spectra indicated that L:D ratios within separated fractions of triesters DL-**8** were 54:46 for DL-**8a** and 58:42 for DL-**8b**. DL-**8a**: ^{31}P NMR ($CDCl_3$) δ −1.684 [^{16}O], −1.693 [^{16}O], −1.724 [^{18}O], −1.733 [^{18}O]. DL-**8b**: ^{31}P NMR ($CDCl_3$) δ −1.565 [^{16}O], −1.605 [^{18}O], −1.722 [^{16}O], −1.762 [^{18}O]. Deprotection of DL-**8a** and DL-**8b** was carried out analogously as described for **8a** and **8b**. ^{1}H and ^{31}P NMR spectra of DL-**12a** and DL-**12b** were indistinguishable from those of **12a** and **12b**.

*Desulfurization of **9a** and **9b** in $H_2{}^{18}O$.* The diastereomer **9a** (5 mg, 4.3 μmol) was dissolved in dimethoxyethane (0.1 mL) and treated subsequently with $H_2{}^{18}O$ (97% ^{18}O, 6.9 mg, 0.34 mmol), 2,6-lutidine (4.6 mg, 43 μmol), and cyanogen bromide (1.8 mg, 17.2 μmol). The reaction was carried out in a 1-mL silicon rubber-septed screw-cap vial at −15 °C. The reaction was monitored by TLC (chloroform–methanol–aqueous NH_3, 80:10:0.5). Additional cyanogen bromide was needed to complete the reaction. After 48 h, the reaction was essentially complete. The product [^{16}O,^{18}O]**11c** was isolated after chromatography on silica gel (chloroform–methanol–aqueous NH_3, 20:1:0.05). The desulfurization of **9b** was carried out analogously. [^{16}O,^{18}O]**11c**: ^{31}P NMR ($CDCl_3$) δ −2.04. [^{16}O,^{18}O]**11d**: ^{31}P NMR ($CDCl_3$) δ −1.92.

Enzymatic Reactions. The solution of the bovine brain PI-PLC-β_1 in $H_2{}^{16}O$ (100 μg of pure protein in 500 μL) was concentrated using a Centricon-30 molecular filtration device (Amicon) to ca. 150 μL by centrifugation at 2000g for 1 h at 4 °C. The resulting protein solution was diluted with 0.5 mL of $H_2{}^{18}O$ (97%) and further concentrated to 200 μL. This enzyme solution was used for the hydrolysis of **12a** and **12b** in $H_2{}^{18}O$ buffer (buffer A). Buffer solution A was prepared by dissolving sodium deoxycholate (16.6 mg, 40 μmol) and triethylamine (48 μL, 34 μmol) in 97% $H_2{}^{18}O$ (1 mL) and passing carbon dioxide gas through this solution until pH 8 was reached. We found pH 7.0 not suitable due to a buffer gelation. Higher pH also helps increase the output of **3** (relative to **2**) from the reaction. This solution was further added with $CaCl_2$ and NaEDTA to final concentrations 12 mM and 4 mM, respectively. The procedures for individual reactions are described in the following sections.

*Inositol 1,2-Cyclic [^{16}O,^{17}O]Phosphate (**2a** and **2b**) from B. cereus PI-PLC-Catalyzed Transesterification.* [^{16}O,^{17}O]-DPPI (**12a**, 10 μmol) was dispersed in the buffer (0.4 mL, 0.1 M triethylammonium bicarbonate, pH 7.0) containing Triton X-100 (4%) by vortexing at 60 °C, followed by addition of D_2O (0.1 mL) and PI-PLC (*B. cereus*, 2.5 μg). After 10 h, ^{31}P NMR showed solely [^{16}O,^{17}O]IcP (**2a**, δ_{31P} 16.1). The mixture was extracted six times with chloroform–methanol, 9:1 (0.5 mL). The emulsion formed was broken up by centrifugation. The aqueous phase was freeze-dried, and the residue was transferred into a 5-mm NMR tube and rendered anhydrous by the repeated (3×) lyophilization of dioxane dispersions. The resulting sample was used for silylation as described later. Isomer **2b** was obtained in an analogous manner using **12b** as a substrate.

*Inositol 1D-[^{16}O,^{17}O,^{18}O]Phosphate (**3e** and **3f**) from the Hydrolysis of [^{16}O,^{17}O]DPPI by B. cereus PI-PLC.* This preparation was carried out as above except that a greater amount of the enzyme was used (7.5 μg for 20 μmol of **12a**) and the reaction was carried out in $H_2{}^{18}O$ buffer. The progress of the reaction was monitored by means of ^{31}P NMR and TLC (chloroform–methanol–H_2O, 65:35:4). The reaction was complete after 10 days. No side processes (>5%) were detected. The product **3e** was isolated as described later for the bovine enzyme reaction. The other isomer of IcP (**3f**) was obtained analogously starting from **12b**.

*Inositol 1,2-Cyclic [^{16}O,^{17}O]Phosphate (**2c** and **2d**) from Bovine PI-PLC-β_1-Catalyzed Transesterification.* [^{16}O,^{17}O]DPPI (**12a**, 10 μmol) was dispersed in $H_2{}^{16}O$-based triethylammonium bicarbonate buffer (1.0 mL, 0.3 M, pH 7.0) containing sodium deoxycholate (20 mM), $CaCl_2$ (2 mM), and EDTA (1 mM), and PI-PLC was added. After 24 h, the mixture was worked-up as described below. The mixture of products containing 30% of **2c** (70% of **3**) was silylated as described for **2a**. Another isomer (**2d**) was obtained analogously from **12b**.

*Inositol 1D-[^{16}O,^{17}O,^{18}O]Phosphate (**3g** and **3h**) from Bovine PI-PLC Hydrolysis.* The substrate [^{16}O,^{17}O]DPPI (**12a**, 24.3 mg, 30 μmol) was dispersed in the buffer A by vortexing at 60 °C for several minutes. The enzyme solution described above was then added to the resulting slightly opaque dispersion. The appearance of a major signal at 3.1 ppm (IP) and a minor signal at 16.4 ppm (IcP) at the expense of one at −1.5 ppm (DPPI) was observed by ^{31}P NMR. The final product was comprised of 91% IP (**3**) and 9% IcP (**2**). After no more substrate could be detected by ^{31}P NMR and TLC (3 days, end point pH 7.8), the detergent and DPG were removed by the extraction as described above. The aqueous phase was freeze-dried, and the residue was purified by chromatography on QAE-Sephadex column (0.55 × 7 cm) using a gradient of 0.0125–0.5 M triethylammonium bicarbonate buffer, pH 7.4, for elution (12 mL total eluate volume). Fractions (1-mL size) were analyzed by ^1H NMR after lyophilization and redissolving in D_2O. The product was eluted at ca. 0.4 M buffer concentration. The identity of the product was further proven by ^1H NMR. **3g**: ^1H NMR (D_2O, H^+ form) δ 4.13 (tr, H-2, J = 2.8 Hz, 1 H), 3.81 (ddd, H-1, J = 2.8, 8.4, 9.8 Hz, 1 H), 3.66 (tr, H-6, J = 9.5 Hz, 1 H), 3.55 (dd, H-4, J = 9.2, 9.9 Hz, 1 H), 3.47 (dd, H-3, J = 2.7, 9.8 Hz, 1 H), 3.24 (tr, H-5, J = 9.2 Hz). Isomer **3h** was obtained analogously, except that only 9 μmol of **12b** was used.

*Cyclization of Inositol 1D-[^{16}O,^{17}O,^{18}O]Phosphate (**3**).* The solution of inositol 1-phosphate (**3**, 20 μmol) obtained above was passed through the Dowex-H^+ cation-exchange resin, and the acidic eluate was collected and lyophilized. The solid was transferred into a 2-mL vial, dissolved in methanol (0.5 mL), and added with trioctylamine (26 μL). The biphasic mixture was heated at 60 °C until homogenized, and methanol was removed under reduced pressure. The residue was redissolved in anhydrous dioxane and freeze-dried. The operation was repeated twice. (Diphenylphosphoryl)imidazole (12.8 mg) was dissolved in DMF (0.71 mL), and diisopropylethylamine (11 μL) was added. An aliquot of the above solution (0.5 mL) was added to the IP-containing vial, and the reaction was monitored by ^{31}P NMR. After 72 h at room temperature, no more reactant could be detected. The mixture was concentrated under vacuum, and the residue was redissolved in water and extracted 6 times with chloroform–methanol (9:1). ^{31}P NMR showed 75% conversion with the remainder being still unreacted IP. In addition to the main product **2**, up to 25% of unidentified impurities giving rise to ^{31}P NMR signals at 15.7 and 14.9 ppm were formed. These impurities were not separated from **2** since they did not interfere with further analysis. The aqueous phase was lyophilized, and the residue was rendered anhydrous by freeze-drying with dry dioxane. This sample was directly used for the silylation reaction as described below.

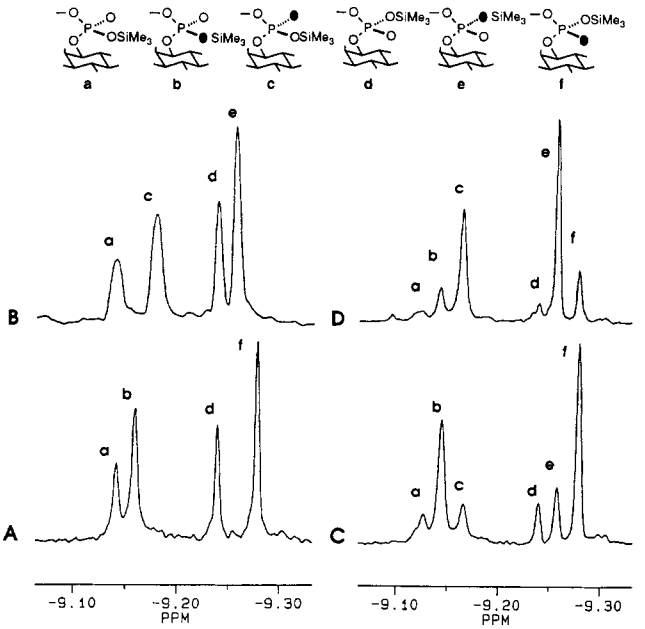
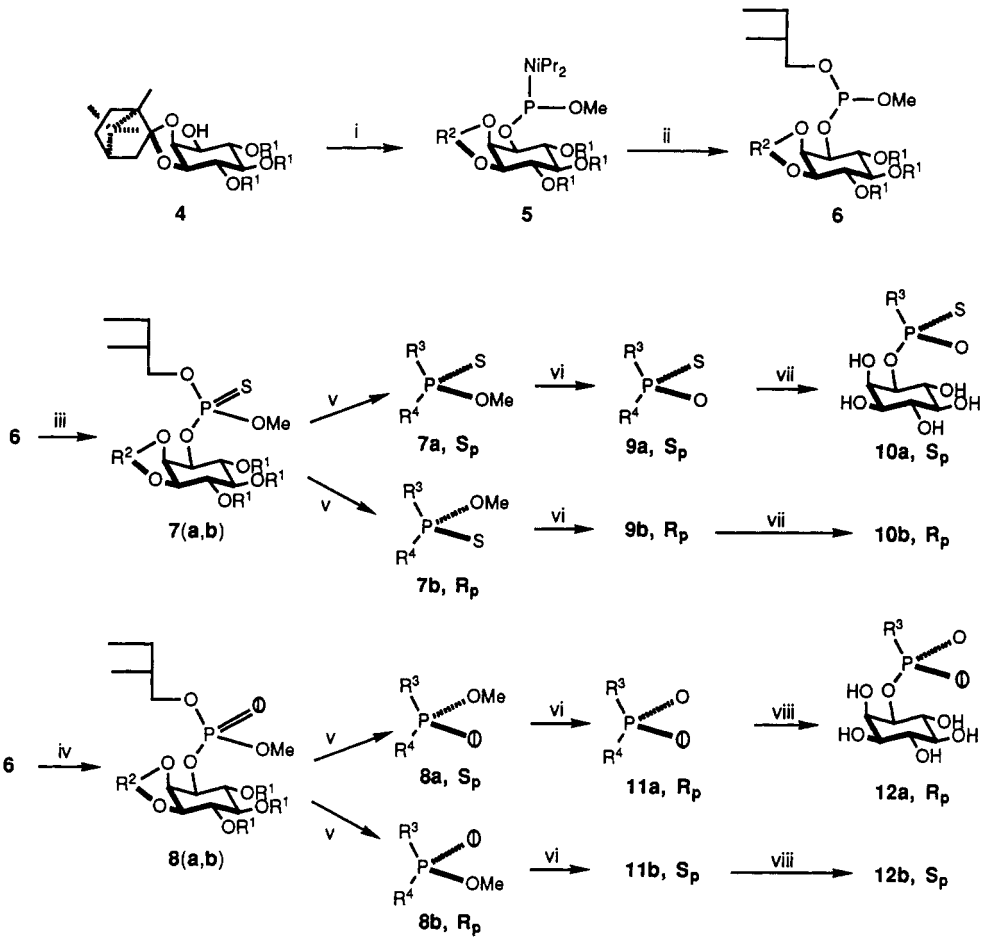


FIGURE 1: The 101.2-MHz (B) and 121.47-MHz (A,C,D) ^{31}P NMR spectra of silylated DPPI precursors: [^{16}O,^{17}O]**11a** (A) and **11b** (B) (from Scheme III, unknown configuration) and [^{16}O,^{18}O]**11c** (C) and **11d** (D) (from Scheme IV, R_p and S_p, respectively). Spectra were apodized by a Gaussian multiplication with LB −1, and GB 0.5 prior to Fourier transformation. The chemical shifts for peaks a–f are, in order, −9.131, −9.149, −9.171, −9.245, −9.263, and −9.284 ppm.

*Analysis of Configurations of Acyclic Diesters **11a–d** and Inositol 1,2-Cyclic Phosphates **2a–h** by ^{31}P NMR.* All silylation reactions were carried out directly in 5-mm NMR tubes. Samples were rendered anhydrous by prolonged evacuation (**11**) or freeze-drying of the dioxane suspensions (**2**). (a) Samples of diesters **11a–d** (17–25 mg) were solubilized in anhydrous $CDCl_3$ (0.5 mL). These samples showed a single peak at −1.92 ppm in ^{31}P NMR. Pyridine (20 μL) and trimethylsilyl chloride (20 μL) were added, and ^{31}P NMR spectra were measured (Figure 1). (b) Samples of anhydrous **2a–h** from cyclization or enzymatic transesterification were suspended in $CDCl_3$ (0.5 mL), pyridine (50 μL) and chlorotrimethylsilane (25–40 μL) were added, and the suspension was heated (above 70 °C) in the tightly closed tube with the heat gun until the solid dissolved. In a few cases, some solid ma-

Scheme III: Synthesis of DPPsI (**10**) and [^{16}O,^{17}O]DPPI (**12**)



terial remained undissolved after treatment with chlorosilane. Such samples were filtered with the aid of the dry syringe and a small cotton swab. ^{31}P NMR spectra of such mixtures were obtained (Figure 2).

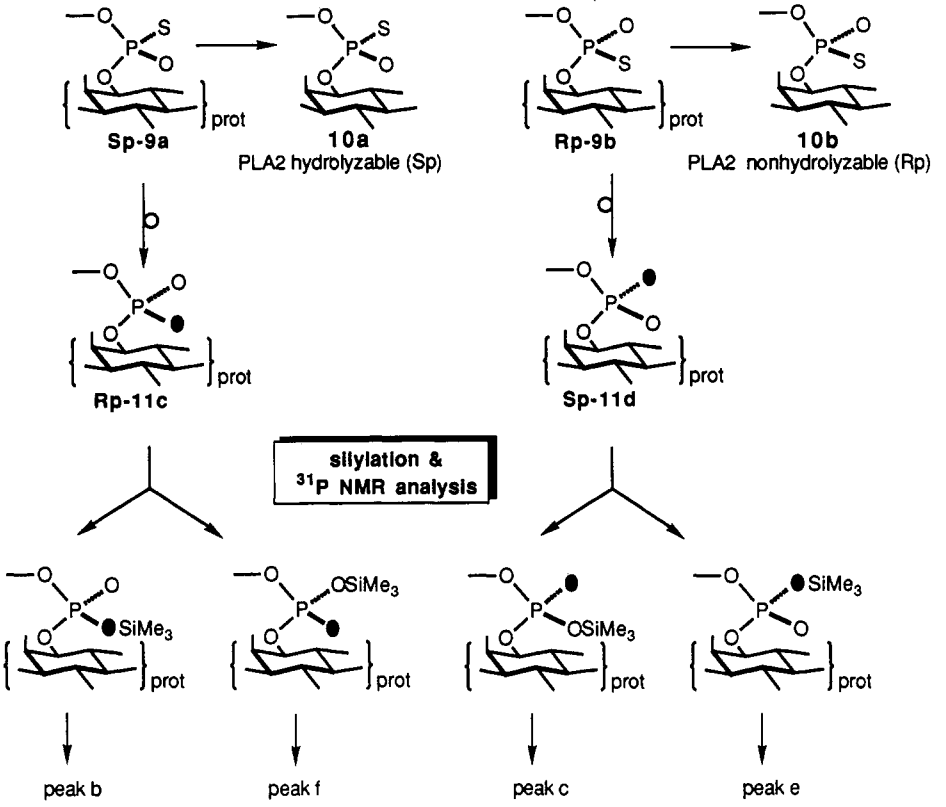
Isolation of DPG from Enzymatic Reactions. [^{16}O,^{17}O]-DPPI (DL-**12a**, 4.0 mg, 4.9 μmol) and sodium deoxycholate (4.7 mg, 10 μmol) were dispersed in 1 mL of water containing 1 mM EDTA. After the pH was adjusted to 7.0, PI-PLC (*B. cereus*, 4 μg) was added to start the reaction. After 25 min the reaction was judged complete by TLC. The entire mixture was lyophilized and the residue was hastily chromatographed on a silica gel minicolumn (hexane–ether, 4:1) to give pure DPG (1 mg, 35%). The whole purification operation was complete within 30 min. TLC of the eluate from column showed no significant isomerization into 1,3-dipalmitoylglycerol.

1,2-Di(palmitoyloxy)propyl 3-(2-Methoxy-2-phenyl-2-(trifluoromethyl)acetate) (DL-**17**). DL-1,2-Dipalmitoylglycerol (19 mg, 33.4 μmol) was dissolved in methylene chloride (0.1 mL) and was treated with triethylamine (5.6 mg, 55.1 μmol) and *S*-(+)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetyl chloride (**16**, 11.1 mg, 43.8 μmol) at room temperature. After 3 h, TLC analysis (hexane–ether, 4:1) showed the absence of DPG. The product was chromatographed in the above solvent to give the pure ester **17**. The synthesis of esters starting from enantiomeric DPG and from DPG obtained from enzymatic processes was accomplished analogously. DL-**17**: ^1H NMR (300 MHz, $CDCl_3$) δ 7.55–7.35 (m, Ph, 5 H), 5.29 (m, H-2, 1 H), 4.586 (dd, H-1, J = 4.0, 11.8 Hz, 1 H), 4.364 (dd, H-1, J = 5.8, 11.8 Hz, 0.5 H), 4.359 (dd, H-1, J = 5.8, 11.8 Hz, 0.5 H), 4.272 (dd, H-3, J = 5.4, 12.0 Hz, 0.5 H), 4.269 (dd, H-3, J = 5.4, 12.0 Hz, 0.5 H), 4.120 (dd, H-3, J = 5.9 Hz, 11.2 Hz, 0.5 H), 4.073 (dd, H-3, J = 5.6, 11.9 Hz, 0.5 H), 3.533 (m, OMe, 3 H), 2.267 (m, CH_2CO, 4 H), 1.569 (m, CH_2, 4 H), 1.255 (br s, CH_2), 0.879 (br tr, Me, 6 H). **17**: ^1H NMR ($CDCl_3$) δ 7.55–7.35 (m, Ph, 5 H), 5.307 (m, H-2, 1 H), 4.591 (dd, H-1, J = 3.9, 11.9 Hz, 1 H), 4.360 (dd, H-1, J = 5.5, 11.9 Hz, 1 H), 4.271 (dd, H-3, J = 5.7, 11.9, 10.5 Hz, 1 H), 4.119 (dd, H-3, J = 6.0, 11.9 Hz, 1 H), 3.538 (m, OMe, 3 H), 2.267 (m, CH_2CO, 4 H), 1.586 (m, CH_2, 4 H), 1.257 (br s, CH_2), 0.879 (br tr, Me, 6 H).

Results

Synthesis of Diastereomers of DPPsI and [^{16}O,^{17}O]DPPI. In order to study the stereochemistry of enzymatic transformations, the diastereomers of DPPsI and ^{17}O- and ^{18}O-labeled DPPI have been synthesized. The synthesis started from diastereomerically pure D-camphor-protected inositol derivative

Scheme IV: Silylation and Configurational Analysis of Inositol-Protected [^{16}O,^{18}O]DPPI



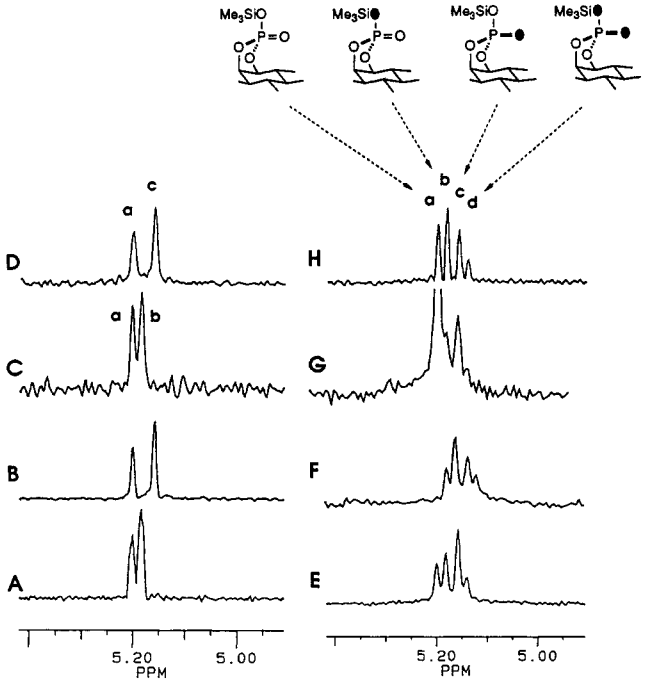


FIGURE 2: The 101.2-MHz ^{31}P NMR spectra of *trans*-silyl esters of IcP isomers **2a–2h** (spectra A–H, in the same order). The sources of samples **2a–2h** are described in the text. The spectral conditions are the same as in Figure 1. The chemical shifts for peaks a–d are, in order, 5.198, 5.180, 5.156, and 5.138 ppm. Due to its small quantity, sample **3g** was mixed with an equimolar amount of unlabeled IP prior to cyclization, which resulted in the intense peak a in spectrum G.

4 (Scheme III), the synthesis of which has been described elsewhere (Bruzik & Tsai, 1992). Treatment of **4** with *N,N*-diisopropyl-*O*-methylphosphonamidic chloride in the presence of diisopropylethylamine afforded the corresponding phosphoramidite **5**. This intermediate was further reacted with 1,2-dipalmitoyl-*sn*-glycerol (DPG) in the presence of tetrazole catalyst to give the trivalent phosphotriester **6**. Treatment of **6** with elemental sulfur afforded the diastereomeric mixture of phosphorothioates **7(a,b)**, while addition of I_2/Py/$H_2{}^{17}O$ afforded the diastereomeric mixture of ^{17}O-labeled triesters **8(a,b)**.

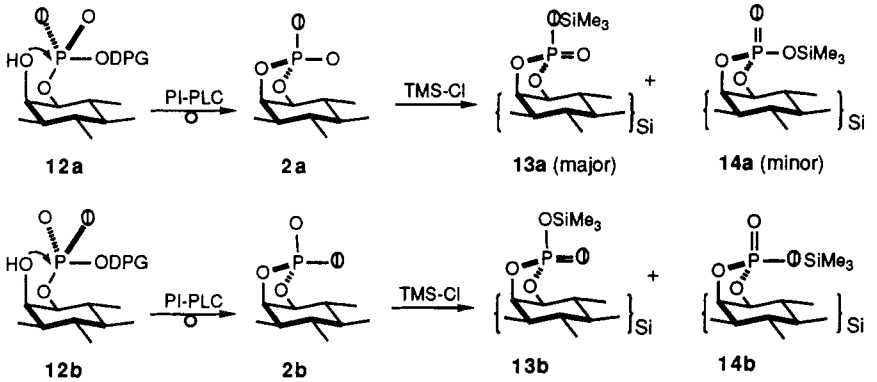
Chromatography of the phosphorothioate mixture **7(a,b)** on silica gel using hexane–ether (10:2) yielded individual isomers **7a** and **7b**. Subsequent demethylation of **7a** and **7b** gave diesters **9a** and **9b**, respectively, and their further deprotection with MeOH–TFA afforded DPPsI (**10a** and **10b**, respectively). On the basis of ^{31}P NMR analysis and the stereospecificity of phospholipase A_2 as described previously (Lin et al., 1990), the configurations of **10a** and **10b** were assigned as S_p and R_p, respectively. Such assignments also led to the conclusion that **7a** and **9a** are S_p while **7b** and **9b** are R_p.

Likewise, separation of the triesters **8(a,b)** using hexane–ether (10:3) gave pure isomers of phosphotriesters **8a** and **8b**, which were demethylated in a manner analogous to that of diesters **11a** and **11b**, respectively. Further deprotection using benzenethiol/BF_3–Et_2O yielded diastereomers of [^{16}O, ^{17}O]-DPPI (**12a** and **12b**, respectively). The assignment of configurations of **12a** and **12b** is explained in the next section.

The diastereomers of DPPsI have been synthesized previously (Lin et al., 1990; Salamonczyk & Bruzik, 1990). The present procedure is advantageous in the good accessibility of the starting alcohol **4** (Bruzik & Tsai, 1992). Diastereomers of **7(a,b)** and **8(a,b)** were also more readily separable than the synthetic intermediates reported earlier (Lin et al., 1990), due to the presence of the chiral D-camphor auxiliary in these molecules.

Configurational Assignments of [^{16}O,^{17}O]DPPI Isomers. The configurations of **12a** and **12b** were assigned by a chemical correlation with DPPsI isomers, via desulfurization. The logic of the assignment is shown in Scheme IV. Although it would be simpler to convert **10** to **12** directly by desulfurization, the

Scheme V: Silylation and Configurational Analysis of [^{16}O,^{17}O]IcP



possible participation of the unprotected 2-hydroxyl group of **10** in the reaction would lead to the formation of undesired cyclic products. Such a problem could be overcome by carrying out the conversion when the inositol moiety is still protected, i.e., by converting **9** to **11**. The PS to PO conversions of phosphorothioate diesters in the presence of electrophilic reagents such as *N*-bromosuccinimide, bromine, and cyanogen bromide have been proven to occur with predominant inversion of configuration at the phosphorus atom (Lowe et al., 1982; Connolly et al., 1982; Sammons & Frey, 1982). Thus, reactions of **9a** (S_p) and **9b** (R_p) with cyanogen bromide in the presence of 2,6-lutidine and $H_2{}^{18}O$ afforded **11c** (R_p) and **11d** (S_p), respectively (Scheme IV).

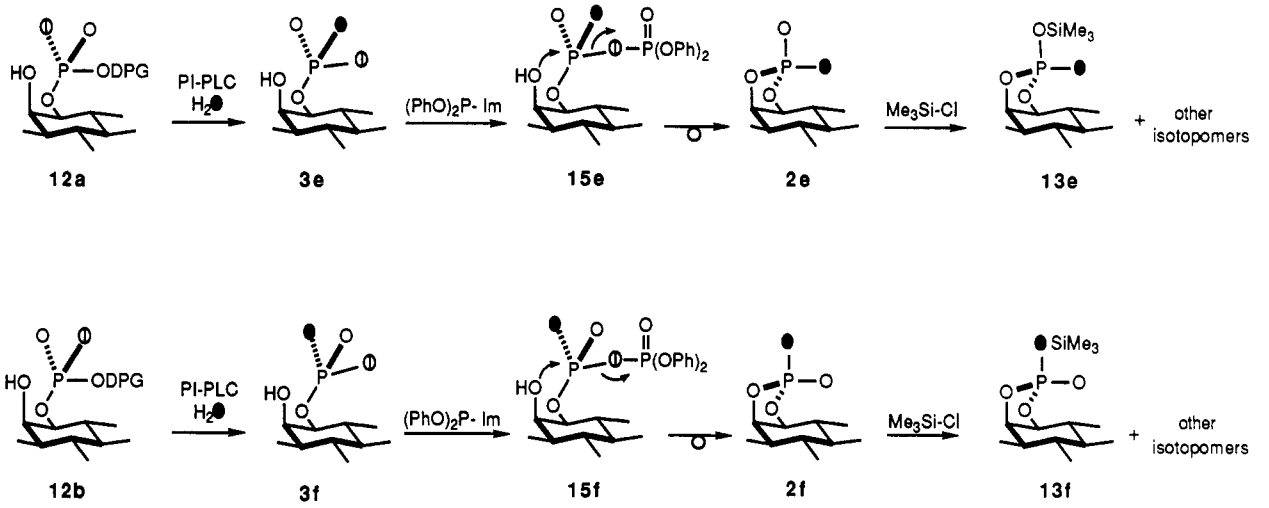
The protected DPPIs **11c** and **11d**, with known configurations, were then used to assign the configurations of **11a** and **11b**. For this purpose, these compounds were subjected to silylation with chlorotrimethylsilane in the presence of pyridine to form the 1:1 mixture of diastereomeric silyl phosphate esters (Bruzik & Tsai, 1984). The ^{31}P NMR spectra of samples derived from **11a**–**11d** are shown in Figure 1 (A–D, respectively). In Figure 1, peaks a and d arise from the two diastereomers of the mono-*O*-silyl phosphotriester, peaks b and e arise from species containing a bridging (P–O–Si) ^{18}O, and peaks c and f arise from species containing a nonbridging ^{18}O (Cohn & Hu, 1980). Spectra C and D indicate that the desulfurization reaction was only 60% stereospecific. Comparison of these spectra allowed us to conclude that [^{16}O,^{17}O]**11a** and [^{16}O,^{18}O]**11c** (and [^{16}O,^{17}O]**11b** and [^{16}O,^{18}O]**11d**) have the same configurations at phosphorus. *The configurations of* ***12a*** *and* ***12b*** *(identical to those of* ***11a*** *and* ***11b****, respectively) can thus be assigned as* R_p *and* S_p*, respectively*. We further infer that the fully protected triesters **8a** and **8b** should be S_p and R_p, respectively. Notice that even though **7a** and **8a** have the same absolute configurations, they have opposite relative configurations as shown in Scheme III.

Preparation and Configurational Analysis of [^{16}O,^{17}O]IcP. As we have demonstrated previously, the transesterification reaction catalyzed by *B. cereus* PI-PLC occurs with inversion of configuration (Lin & Tsai, 1989; Lin et al., 1990). Hence, the reaction of (R_p)-[^{16}O,^{17}O]DPPI (**12a**) afforded *trans*-[^{16}O,^{17}O]IcP (**2a**) exclusively (Scheme V), whereas the reaction of (S_p)-[^{16}O,^{17}O]DPPI (**12b**) gave the corresponding cis-isomer (**2b**). These IcP isomers of known configurations were used as references to develop a method for configurational analysis for other samples with unknown configurations (see next section). To this end, both products and the unlabeled IcP were subjected to the exhaustive silylation reaction with trimethylchlorosilane in $CDCl_3$ in the presence of pyridine (Leavitt & Sherman, 1982). We chose to use silylation instead of methylation due to the ease and the quantitative yield usually obtained in this process. Two diastereomeric esters **13** (major, δ_{31P} 5.2 ppm, 80%) and **14** (minor, δ_{31P} 7.6 ppm, 20%) were produced from each cyclic phosphate as the result of the silylation of one of the two nonbridging oxygen atoms in IcP. As shown in Figure 2A, the major product **13a** (from **2a**) displayed a small ^{18}O-induced shift (0.02 ppm), indicating that silylation occurred at the isotopic oxygen, as shown in Scheme V. The shape of signals arising from the less abundant ester **14** was not clearly defined due to insufficient signal-to-noise ratios and is not shown in Figure 2. The silyl ester **13b** obtained from **2b** displayed a larger isotope shift (0.04 ppm) (Figure 2B), and it thus had ^{18}O-label doubly bonded to phosphorus. We hence conclude that the major product of the silylation of IcP is a *trans*-silyl ester and the minor product is a *cis*-silyl ester. This conclusion is consistent with the expected difference in the reactivity of *trans*- and *cis*-oxygen atoms in IcP due to steric constraints. The trans- and cis-isomers of silyl esters of IcP were not resolved in the previous gas chromatographic study (Leavitt & Sherman, 1982).

Steric Course of Transesterification Catalyzed by Bovine Brain PI-PLC-β_1. Having determined the configurations of both [^{16}O,^{17}O]DPPI (**12a** and **12b**) and [^{16}O,^{17}O]IcP (**2a** and **2b**), we then proceeded to investigate the conversion of DPPI to IcP catalyzed by the PI-PLC-β_1 from bovine brain. The reaction carried out at pH 7.0 produced ca. 30% of [^{16}O,^{17}O]IcP (**2c** and **2d** from **12a** and **12b**, respectively) and 70% of the acyclic phosphate **3**. The amount of the acyclic product formed was greater than that previously reported for the β-isozyme of PI-PLC at this pH (Kim et al., 1989). The mixture of products **2** and **3** was used for stereochemical analysis directly without separation. ^{31}P NMR spectra of silyl esters obtained from the mixture of unlabeled **2** and **3** consisted of three signals: at 5.2 (major, from **13**), 7.6 (minor, from **14**) and −19.0 ppm (major, the bissilyl ester of **3**). The signals corresponding to the *trans*-silyl esters **13** formed from **2c** and **2d** are shown in parts C and D of Figure 2, respectively. The small isotope shift (0.02 ppm) observed for the *trans*-silyl ester **13c** (from **2c**) and large one for **13d** (from **2d**) indicate *inversion of configuration at phosphorus in the transesterification reaction*, which is the same as the result of the *B. cereus* PI-PLC and the PI-PLC (isozymes I and II) from guinea pig (Lin et al., 1990).

Hydrolytic Reaction of B. cereus PI-PLC. The time course of the hydrolytic reaction of *B. cereus* PI-PLC is shown in Figure 3. The consecutive character of this process is clearly evidenced by the complete disappearance of DPPI before the hydrolysis of **2** into **3** was observed. In order to retain chirality at the phosphorus atom in the hydrolytic product **3**, the hydrolysis was carried out in 97% $H_2{}^{18}O$. Hydrolysis of **12a** and **12b** in $H_2{}^{18}O$ catalyzed by *B. cereus* PI-PLC afforded diastereomers of [^{16}O,^{17}O,^{18}O]IP (**3e** and **3f**, respectively), which were purified by chromatography on QAE-Sephadex. For the

Scheme VI: Configurational Analysis of [^{16}O,^{17}O,^{18}O]IP



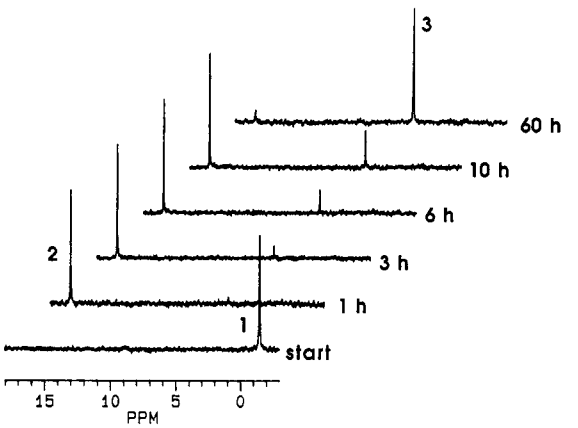


FIGURE 3: Time course of the reaction of DPPI catalyzed by *B. cereus* PI-PLC as monitored by ^{31}P NMR. Sample conditions: DPPI (4 μmol) and PI-PLC (3.3 μg) in 0.1 M triethylammonium bicarbonate buffer, pH 7.0, containing 2% Triton X-100 and 5 mM EDTA. Each spectrum consists of 4000 scans acquired during 1 h. The numbers given to the right of each spectrum correspond to the midpoint of the accumulation period.

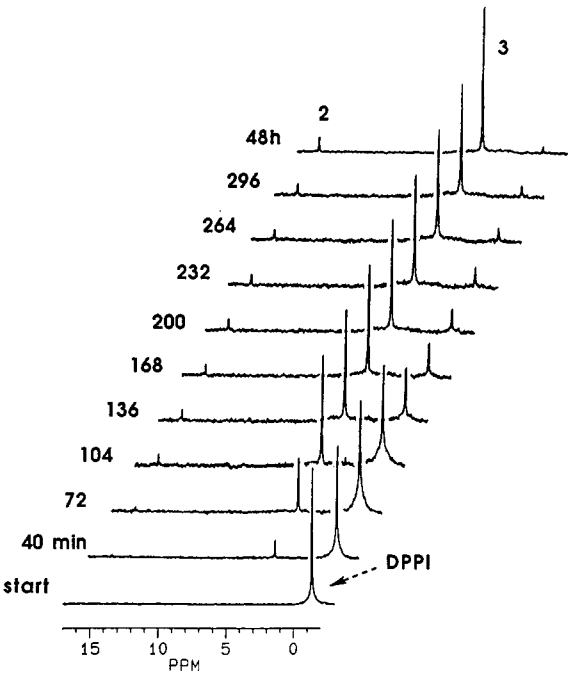


FIGURE 4: Time course of the hydrolysis of [^{16}O,^{17}O]DPPI (**12a**) in $H_2{}^{18}O$ catalyzed by bovine brain PI-PLC-β_1 at pH 8, followed by ^{31}P NMR. Reaction conditions are described in Materials and Methods.

purpose of configurational analysis, **3e** and **3f** were cyclized to give IcP **2e** and **2f**, respectively, after their activation with (diphenylphosphoryl)imidazole in DMF (Scheme VI). The formation of IcP in this process is preceded by the formation of the pyrophosphate intermediate **15** (δ_{31P} −8.2, −20.6 ppm, $^2J_{P\text{-}O\text{-}P}$ = 23 Hz) having one of its three originally peripheral oxygen atoms of IP in the bridging position of the pyrophosphate. The cyclization of phosphate mono(β-hydroxyesters) to cyclic five-membered phosphate diesters is known to proceed with inversion at phosphorus (Abbott et al., 1979). Since the mode of the oxygen phosphorylation of **3** is random, the IcP **2e** and **2f** (from **3e** and **3f**, respectively) are mixtures of isotopomers, in which oxygen atoms at the ^{16}O, ^{17}O, and ^{18}O positions have been removed randomly. The IcP diastereomers **2e** and **2f** were then silylated as described above and the ^{31}P NMR spectra of the corresponding *trans*-silyl esters (**13e** and **13f**, respectively) are shown in Figure 2 (parts E and F, respectively). Only those phosphate species lacking ^{17}O give rise to observable ^{31}P NMR signals (Tsai, 1979). The stereochemical information is contained in the relative intensities of signals b and c of the multiplets resulting from the mixture of isotopomers. The fact that resonance b < c in intensity in spectrum 2E indicates that the major isotopomer of **13e** has a doubly bonded ^{18}O and thus comes from *cis*-[^{18}O]**2e** as shown in Scheme VI. The results, as shown in Scheme VI, indicate that *the conversion of DPPI 12 to IP 3 proceeds with an overall retention of configuration at phosphorus.* This result is consistent with the observation in Figure 3 that the reaction proceeds in two steps, via formation of the IcP intermediate.

Hydrolytic Reaction of Mammalian PI-PLC. The hydrolysis reaction catalyzed by bovine brain PI-PLC-β_1 was performed at pH 8, due to better yields of IP (relative to IcP) obtained at a higher pH (Kim et al., 1989). The time course of the reaction employing (R_p)-[^{16}O,^{17}O]DPPI (**12a**) is shown in Figure 4. The ratio of products was constant throughout the whole reaction time, and the final mixture of products consisted of 90% IP **3** and 10% IcP **2**. The diastereomers of [^{16}O, ^{17}O,^{18}O]IP **3g** and **3h** (formed from **12a** and **12b**, respectively) were purified and cyclized as described in the previous section to afford cyclic esters **2g** and **2h**, respectively. The spectra of silylated derivatives **13** arising from **2g** and **2h** are shown in parts G and H of Figure 2, respectively. These spectra indicate that the configurations of **2g** and **2h** are the same as those of **2e** and **2f**, respectively. Therefore, *the overall steric course of the formation of IP from DPPI mediated by*

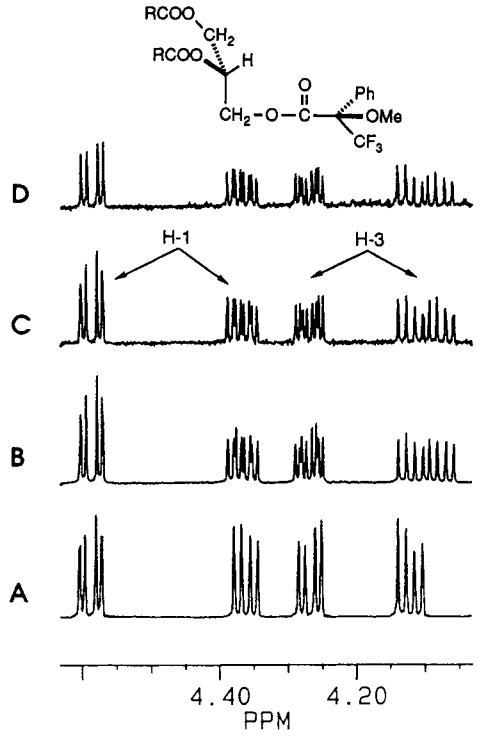
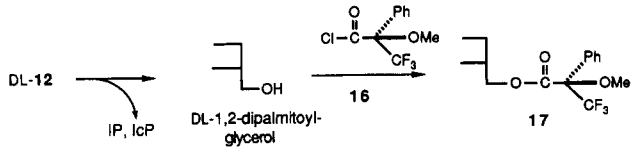


FIGURE 5: The 500-MHz ^{1}H NMR spectra of esters **17** obtained from synthetic and enzymatically generated 1,2-dipalmitoylglycerol: (A) L-**17**; (B) DL-**17**; (C) **17** synthesized from DPG obtained with *B. cereus* PI-PLC; (D) **17** synthesized from DPG obtained with bovine brain PI-PLC-β_1.

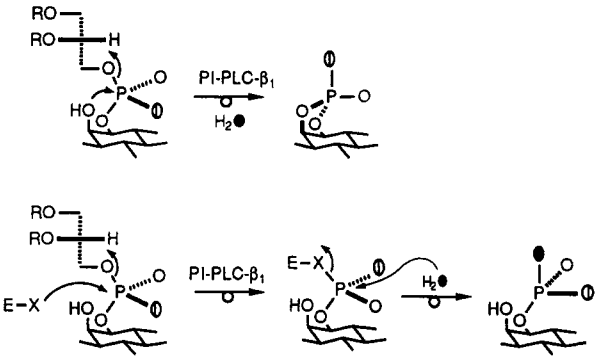
Scheme VII: Derivatization of 1,2-Dipalmitoylglycerol



bovine brain PI-PLC-β_1 is also retention of configuration. This result suggests that the conversion of PI to IP catalyzed by PI-PLC-β_1 also proceeds via a double displacement mechanism. However, since the course of the reaction shown in Figure 4 does not appear to be sequential, the possible mechanisms are elaborated in the Discussion.

PI-PLC Is Nonstereospecific at the C-2 of Diacylglycerol. When the diastereomeric mixture of DPPI synthesized from DL-1,2-dipalmitoylglycerol (with specific 1*D*-configuration in the inositol 1-phosphate moiety) was used as a substrate, both diastereomers were unexpectedly utilized completely by both *B. cereus* PI-PLC and the bovine brain PI-PLC-β. To further demonstrate that both PI-PLC's are nonstereospecific toward C-2 of the glycerol moiety, the product DPG obtained from these reactions was converted into the ester **17** with *S*-(+)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetyl chloride (**16**) (Scheme VII). Such a derivatization is a well-established method to determine enantiomeric excess of chiral alcohols (Dale et al., 1969). As the control experiments, esters of 1,2-dipalmitoyl-*sn*-glycerol and DL-1,2-dipalmitoylglycerol were also obtained. Partial ^{1}H NMR spectra of the methylene protons of the glycerol moiety are shown in Figure 5. The ester from the enantiomerically pure DPG shows only one set of resonances (spectrum A) while that from the racemic mixture shows two sets of resonances for some of the protons due to a mixture of diastereomers (spectrum B). The esters from the products of the two enzymatic reactions are clearly mixtures of diastereomers (spectra C and D).

Scheme VIII: Two-Active Site Mechanism for PI-PLC-β_1



Discussion

Mechanism of Bacterial and Mammalian PI-PLC. In the reactions catalyzed by the *B. cereus* PI-PLC, the acyclic inositol 1-phosphate is clearly a secondary product derived from inositol 1,2-cyclic phosphate. The stereochemical results reported in this paper confirm the sequential nature of the reactions suggested earlier by Volwerk et al. (1990). The rate of formation of IP was estimated to be slower than that of IcP by a factor of 10^3, on the basis of ^{31}P NMR analysis. The sequential mechanism is similar to the mechanism of ribonuclease A (Richard & Wyckoff, 1971); however, the cyclic intermediate is not readily released under normal conditions with natural substrates in the case of ribonuclease A.

Possible Mechanisms of Mammalian PI-PLC. The observation that the steric courses of the formation of IP and IcP catalyzed by mammalian PI-PLC-β_1 (inversion and retention, respectively) are the same as those of the *B. cereus* PI-PLC strongly suggests that the actual mechanism of mammalian PI-PLC is also sequential. On the other hand, the ^{31}P NMR analysis in Figure 4 strongly argues against the idea that IcP is an intermediate in the formation of IP. Scheme VIII shows an alternative mechanism consistent with *both* stereochemistry and ^{31}P NMR results. In this scenario, IcP and IP are formed in parallel and with different mechanisms: IcP via a single displacement mechanism involving a nucleophilic attack by the 2-OH group, and IP via formation of a covalent enzyme–phosphoinositol intermediate.

The parallel mechanism shown in Scheme VIII requires that the enzyme catalyzes two reactions with different mechanisms at a single active site. In our view, such a mechanism is likely only if the mammalian PI-PLC has two active sites or if one of the reactions is catalyzed by a contaminating enzyme. The latter is unlikely since the parallel formation of both IP and IcP has been reported for mammalian PI-PLC from different sources, and the PI-PLC-β_1 used in this study is a recombinant enzyme and thus unlikely to have the same contaminant as the enzyme isolated from natural sources. The possibility of two active sites in a single enzyme (i.e., a bifunctional enzyme) cannot be ruled out, but it is not supported by the recent finding that the 2-deoxy-analogue of phosphatidylinositol is not a substrate for either reaction catalyzed by a mammalian PI-PLC isolated from a human melanoma cell line (Seitz et al., 1992).

The sequential mechanism is attractive in that it implies mechanistic similarity between bacterial and mammalian PI-PLCs. However, the sequential mechanism is peculiar in how the enzymes allow some of the IcP to be released while further hydrolyzing others, and in the total kinetic incompetence of the intermediate IcP. According to Cleland (1990), the total incompetence of an intermediate requires that the ratio $k_3K_{eq}'/K_{ia}K_{eq} \ll 1$, where K_{eq} and K_{eq}' are the equilibrium

constants of substrate to intermediate off and on the enzyme, respectively; K_{ia} is the dissociation constant of the substrate; and k_3 is the rate of the off step of the intermediate. Such a condition is difficult to meet unless k_3 is very small (approaching zero), which is not the case in the present system. However, a possible explanation for the kinetic incompetence of IcP is that IcP cannot reach the active site simply because it is highly hydrophilic and does not mix well with the detergent.

Lack of Stereospecificity at the Glycerol Moiety. The complete lack of stereospecificity of PI-PLC with regard to the configuration of the diacylglycerol moiety also came unexpected. Phospholipases A_2 and C are known for their L-stereospecificity (Kuipers et al., 1990; Snyder, 1987), while the stereospecificity of plant phospholipases D have been shown to be dependent on the source of the enzyme (Heller, 1978, Bugaut, 1985). The stereospecificity of triglyceride lipases also depends on the source of the enzyme: intestinal lipases are generally nonstereospecific, while lipoprotein, plasma, and milk lipases are stereospecific (Paltauf, 1974). Since the cyclic phosphodiesterase activity of *B. cereus* PI-PLC has been shown to be specific to the *D* isomer of IcP (Volwerk et al., 1990), the properties of PI-PLC seem to be similar to those of phosphatidylinositol 4-kinase, which was found to be stereospecific for the 1*D*-inositol 1-phosphate moiety but showed no preference for a particular configuration of diacylglycerol (Young et al., 1990). The lack of stereospecificity of PI-PLC with respect to the configuration of diacylglycerol suggests that the diacylglycerol moiety is probably not involved in recognition or binding by the enzyme.

Comparison between Bacterial and Mammalian PI-PLCs. The major difference between bacterial and mammalian PI-PLCs seems to lie in the relative ratio of the two products. Interestingly, this property also differs among the different isozymes of mammalian PI-PLC, and it depends on pH, Ca^{2+} concentration, and the position and degree of phosphorylation of the substrate (Kim et al., 1989; Rhee et al., 1992). It is possible that the ratio between IcP and IP is subject to metabolic regulations in mammalian PI-PLCs.

As reviewed by Rhee et al. (1989, 1992), the different PI-PLCs also differ in their structures and their substrate specificity. While both types of enzymes use PI as substrates, bacterial PI-PLCs also accept glycosylated PI but not phosphorylated PI, whereas mammalian PI-PLCs accept phosphorylated PI but not glycosylated PI. The two types of enzymes also differ in their calcium dependence (required for mammalian but not *B. cereus* PI-PLC). The molecular weights of mammalian PI-PLCs are substantially larger than those of bacterial PI-PLCs, and different isozymes of mammalian PI-PLCs also differ in size. However, all PI-PLCs share a homologous 70-amino acid segment located in the "X domain". It is therefore highly likely that the active site is located in the homologous segment.

Conclusion. Phosphatidylinositols chirally labeled at phosphorus have been synthesized and used to probe the mechanism of PI-PLC. The results indicate that both *B. cereus* PI-PLC and bovine brain PI-PLC-β_1 catalyze the conversion of phosphatidylinositol to inositol 1,2-cyclic phosphate and inositol 1-phosphate with overall inversion and retention, respectively. The results can be interpreted by a sequential mechanism for both enzymes, with IcP being an intermediate in the formation of IP. However, an alternative two-active site model, with IcP and IP being formed by different mechanisms, cannot be ruled out for the bovine brain PI-PLC-β_1. In addition, the glycerol moiety of the substrate is not the main structural determinant of PI-PLC.

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Different Requirements for Productive Interaction between the Active Site of HIV-1 Proteinase and Substrates Containing -Hydrophobic*Hydrophobic- or -Aromatic*Pro- Cleavage Sites†

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ABSTRACT: The sequence requirements for HIV-1 proteinase catalyzed cleavage of oligopeptides containing two distinct types of junctions (-hydrophobic*hydrophobic- or -aromatic*Pro-) has been investigated. For the first type of junction (-hydrophobic*hydrophobic-) the optimal residues in the P_2 and P_2' positions were found to be Val and Glu, respectively, in accord with recent statistical analysis of natural cleavage sites [Poorman, R. A., Tomasselli, A. G., Heinrikson, R. L., & Kézdy, F. J. (1991) *J. Biol. Chem. 266*, 14554-14561]. For the -aromatic*Pro- type of junction, in the specific sequence context studied here, the value of Glu in the P_2' position was again observed. An explanation for the inefficient cleavage observed for peptides with the sequence -Val-Tyr*Pro- has been provided from molecular modeling of the putative enzyme–substrate complex. A significant effect upon cleavage rates due to the amino acid in the P_5 position has also been documented. While lysine in the P_5 position in one sequence of the -hydrophobic*hydrophobic- type produces a peptide cleaved very efficiently ($k_{cat} > 15$ s^{-1} for Lys-Ala-Arg-Val-Nle*p-nitrophenylalanine-P_2'-Ala-Nle-NH_2, for P_2' = Glu, Gln, Ile, Val, or Ala), for substrates of the -aromatic*Pro- type, the P_5 residue can exert either a positive or negative effect on cleavage rates. These results have again been interpreted in light of molecular modeling. We suggest that interaction of the substrate sequence on the periphery of the active site cleft may influence the match of the enzyme–substrate pair and, hence, control the efficiency of catalysis. Thus, ability of HIV-1 PR to selectively *and* efficiently cleave a variety of totally different sequences may be derived, in part, from extensive interactions at long distances from the actual scissile peptide bond and the inherent flexibility of several key loops of polypeptide structure of the enzyme.

Biochemical characterization of the aspartic proteinase encoded within the genome of the human immunodeficiency virus (HIV)[1] as a dimer of identical subunits (Meek et al., 1989) was rapidly substantiated by crystallographic analysis of native (Navia et al., 1989; Wlodawer et al., 1989) and inhibitor-complexed enzyme (Miller et al., 1989; Swain et al., 1990; Fitzgerald et al., 1990; Erickson et al., 1990; Jaskólski et al., 1991). The proteinase is considered to be responsible for cleavage of (at least) eight sites in the gag and gag-pol

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[1] Abbreviations: HIV, human immunodeficiency virus; HIV PR, HIV proteinase; P_1, P_2, P_1', P_2', S_1, S_2, S_1', S_2', etc., designation of amino acid residues of a substrate or inhibitor and corresponding regions of the enzyme active site involved in a complex according to Schechter and Berger (1967); Nph, *p*-nitrophenylalanine; pepRPC, C_2/C_{18} dual bonded, 100-Å pore size silica-based reversed-phase column; NMR, nuclear magnetic resonance; MHz, megahertz. In all peptides reported here the amino acids are of the L configuration. The cleavage point is indicated in each sequence by an asterisk (*).

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