Interaction of the Catalytic Domain of Inositol 1,4,5-Trisphosphate 3-Kinase A with Inositol Phosphate Analogues

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The levels of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] in the cytoplasm are tightly regulated by two enzymes, Ins(1,4,5)P_3 3-kinase and type I Ins(1,4,5)P_3 5-phosphatase. The catalytic domain of Ins(1,4,5)P_3 3-kinase (isoenzymes A, B and C) is restricted to approximately 275 amino acids at the C-terminal end. We were interested in understanding the catalytic mechanism of this key family of enzymes in order to exploit this in inhibitor design. We expressed the catalytic domain of rat Ins(1,4,5)P_3 3-kinase A in Escherichia coli *as a His- and S-tagged fusion protein. The purified enzyme was used in an Ins(1,4,5)P_3 kinase assay to phosphorylate a series of inositol phosphate analogues with three or four phosphate groups. A synthetic route to* D*-2-deoxy-Ins(1,4,5)P_3 was devised.* D*-2-Deoxy-Ins(1,4,5)P_3 and* D*-3-deoxy-Ins(1,4,6)P_3 were potent inhibitors of the enzyme, with IC_{50} values in the micromolar range. Amongst all analogues tested, only* D*-2-deoxy-Ins(1,4,5)P_3 appears to be a good substrate of the Ins(1,4,5)P_3 3-kinase. Therefore, the axial 2-hydroxy group of Ins(1,4,5)P_3 is not involved in recognition of the substrate nor does it participate in the phosphorylation mechanism of Ins(1,4,5)P_3. In contrast, the equatorial 3-hydroxy function must be present in that configuration for phosphorylation to occur. Our data indicate the importance of the 3-hydroxy function in the mechanism of inositol trisphosphate phosphorylation rather than in substrate binding.*

Introduction

Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] is a second messenger that mobilises intracellular Ca^{2+}.[1] Ins(1,4,5)P_3 can be dephosphorylated by an Ins(1,4,5)P_3 5-phosphatase or phosphorylated to form inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] by an Ins(1,4,5)P_3 3-kinase.[2] The Ins(1,4,5)P_3 3-kinase family consists of three distinct isoforms (A, B and C) containing a relatively conserved C-terminal catalytic domain. (For a review, see ref. [3].) The enzymatic activity of the three isoenzymes is regulated by the Ca^{2+}/CaM complex (CaM = calmodulin).[4–8] By using a series of truncated forms of the rat Ins(1,4,5)P_3 3-kinase A, Takazawa and Erneux showed that the catalytic domain of Ins(1,4,5)P_3 3-kinase A is restricted to approximately 275 amino acids at the C-terminal end.[9]

The aim of our study was to express the Ins(1,4,5)P_3 3-kinase in order to obtain sufficient soluble protein for biochemical and structural studies so that we could identify possible inhibitors and substrates of the enzyme(s). Most biochemical studies related to this enzyme have been complicated by the absence of an expression system that would allow large-scale production of an active and stable enzyme.[4–10] The major problem is that endogenous Ins(1,4,5)P_3 3-kinase is very sensitive to proteolytic degradation due to the presence of polypeptide sequences (so called PEST sequences) enriched in proline, glutamic acid, serine and threonine.[11,12] Full-length recombinant Ins(1,4,5)P_3 3-kinase produced in *Escherichia coli* is also easily degraded.[4,5] Another problem is the production of this enzyme in the form of inclusion bodies. For example, Thomas et al. showed that recombinant nonfusion Ins(1,4,5)P_3 3-kinase B was expressed in the insoluble fraction and incorrectly folded.[10]

Comprehension at the molecular level of the way in which Ins(1,4,5)P_3 is recognised by the catalytic domain is essential for the design of selective and potent inhibitors of the enzyme. Very recently, the crystal structure of inositol 1,4,5-trisphosphate 3-kinase A has been provided by two groups.[13,14] The catalytic core domain consists of three lobes. The N and C lobes bind adenosine triphosphate (ATP), whereas the third lobe binds Ins(1,4,5)P_3 and has a unique four-helix insertion into the C lobe. In this study, we expressed the catalytic domain of rat Ins(1,4,5)P_3 3-kinase A (3KA-cat30) in *E. coli*. This domain of 275 amino acids is less susceptible to degradation by proteases as compared to the full-length protein.[8] Surprisingly, our new construct of the catalytic domain, which was a His- and S-tagged fusion protein, yielded about 10–20% of the total soluble fraction of *E. coli* after induction by isopropyl β-D-thiogalactoside (IPTG). Various inositol phosphates and related polyphosphates were tested as substrates and inhibitors of this construct. One of these molecules, D-2-deoxy-Ins(1,4,5)P_3, appears to be a potent inhibitor and good substrate of the

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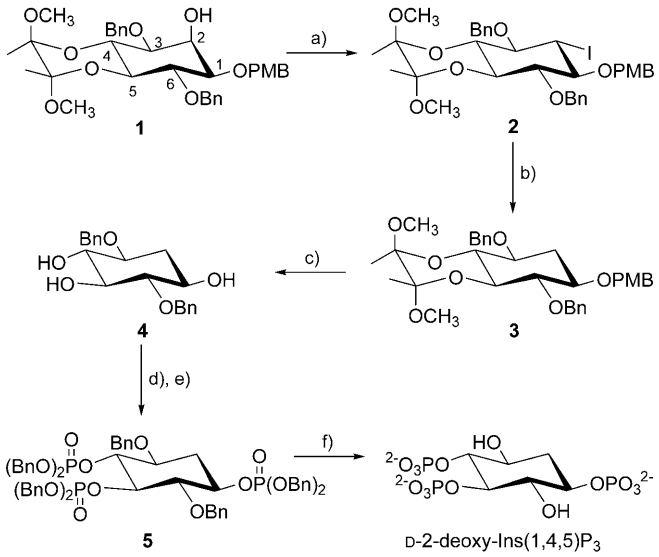
 DOI: 10.1002/cbic.200400443

recombinant enzyme. In contrast, D-Ins(2,4,5)P_3, which was previously claimed to be phosphorylated at the 6-position of the inositol ring by Ins(1,4,5)P_3 3-kinase C,[15] appears to be a very weak inhibitor and poor substrate. We propose that neither the 2-hydroxy nor the 6-hydroxy group is involved in substrate binding but that the 3-hydroxy motif must be equatorial to be part of the catalytic mechanism.

Experimental Section

Synthesis: General synthetic methods were as previously described.[16] Ins(1,4,5)P_3,[17] D- and L-Ins(2,4,5)P_3,[18] Ins(1,3,4,5)P_4,[19] D-2-deoxy-Ins(1,3,4,5)P_4,[20] D-6-deoxy-Ins(1,3,4,5)P_4,[21] adenophostin A,[22] furanophostin,[23] two bicyclic InsP_3 analogues[24] and the epimers of *xylo*-furanophostin[25] were synthesised as previously reported. The synthesis of racemic *epi*-Ins(1,3,6)P_3[26] will be reported elsewhere. D-3-Deoxy-Ins(1,4,6)P_3 was synthesised from D-5-*O*-benzyl-1,4,6-tri-*O*-(*p*-methoxybenzyl)-*myo*-inositol.[27] Briefly, deoxygenation at the 3-position was achieved by stannylene-mediated regioselective tosylation of the O-3 atom followed by treatment with lithium triethylborohydride. Benzylation of the remaining 2-OH group and then cleavage of the three *p*-methoxybenzyl ethers by using trifluoroacetic acid gave D-2,5-di-*O*-benzyl-3-deoxy-*myo*-inositol. Phosphorylation of this triol, followed by deprotection and purification as described below for D-2-deoxy-Ins(1,4,5)P_3 gave D-3-deoxy-Ins(1,4,6)P_3 as the triethylammonium salt. Full synthetic details for D-3-deoxy-Ins(1,4,6)P_3 will be reported elsewhere. All ligands were fully characterised by the usual spectroscopic and analytical methods and gave data that were in accordance with their structure.

Synthesis of D-2-deoxy-*myo*-inositol 1,4,5-trisphosphate: D-2-Deoxy-Ins(1,4,5)P_3 was synthesised in five steps from alcohol **1**[16] (Scheme 1), by using a strategy paralleling the reported synthesis of racemic 2-deoxy-Ins(3,4,5,6)P_4.[28] Thus, iodination of **1** by using



Scheme 1. Synthesis of D-2-deoxy-Ins(1,4,5)P_3. Reagents and conditions: a) I_2, imidazole, Ph_3P, toluene, reflux, 18 h, 93%; b) nBu_3SnH, AIBN, toluene, reflux, 1 h, 74%; c) TFA, CH_2Cl_2, H_2O, 30 min, RT, 85%; d) $(BnO)_2PNiPr_2$, 1*H*-tetrazole, CH_2Cl_2, RT, 3 h; e) *m*CPBA, CH_2Cl_2, −78 °C→RT, 86%; f) H_2, 50 p.s.i., Pd/C, MeOH, H_2O, RT, 20 h, 88%. Bn = benzyl, PMB = *p*-methoxybenzyl, AIBN = azobisisobutyronitrile, TFA = trifluoroacetic acid, *m*CPBA = *meta*-chloroperoxybenzoic acid.

the method of Garegg and Samuelsson[29] proceeded with inversion of configuration at C-2 to give **2.** Free-radical dehalogenation[30] of **2** gave protected deoxy-inositol **3**. The acid-labile butanediacetal (BDA) and PMB protecting groups in **3** were then cleaved by using TFA, thereby exposing the hydroxy groups at positions 1, 4 and 5. Phosphitylation of **4** by using bis(benzyloxy)diisopropylaminophosphine and 1*H*-tetrazole, followed by in situ oxidation with *m*-chloroperoxybenzoic acid gave crystalline **5**. Finally, total deprotection of **5** by hydrogenolysis over palladium on carbon gave D-2-deoxy-Ins(1,4,5)P_3, which was purified by ion-exchange chromatography on Q-Sepharose Fast Flow resin and isolated as the triethylammonium salt.

(2′*S*,3′*S*)-D-3,6-Di-*O*-benzyl-2-deoxy-4,5-*O*-(2′,3′-dimethoxybutane-2′,3′-diyl)-2-iodo-1-*O*-(*p*-methoxybenzyl)-*scyllo*-inositol (2): A solution of (2′*S*,3′*S*)-D-3,6-di-*O*-benzyl-4,5-*O*-(2′,3′-dimethoxybutane-2′,3′-diyl)-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (**1**)[16] (1.19 g, 2.00 mmol), finely powdered triphenylphosphine (2.10 g, 8.00 mmol), imidazole (545 mg, 8.00 mmol) and iodine (1.52 g, 6.00 mmol) in dry toluene (100 mL) was heated at reflux for 18 h. TLC (ether/hexane, 2:1) showed complete conversion of the alcohol (R_f = 0.20) into a less polar product (R_f = 0.60). The solution was allowed to cool and was then stirred with saturated aqueous $NaHCO_3$ (100 mL) for 30 min. At this stage, the aqueous (lower) layer had a brown colour while the organic (upper) layer was colourless. Iodine was added in portions until the organic layer remained purple, and the mixture was then stirred for a further 30 min. Finally, excess iodine was removed by addition of aqueous sodium thiosulfate until two colourless layers were obtained. The organic layer was separated, dried ($MgSO_4$) and concentrated by evaporation under reduced pressure. Purification of the residue by flash chromatography on silica (ether/hexane, 1:2) gave **2** as a colourless oil (1.30 g, 1.85 mmol, 93%); $[\alpha]_D = +43$ (c = 1.1 in $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$): δ = 1.366 (s, 3H; CH_3), 1.374 (s, 3H; CH_3), 3.27 (s, 3H; OCH_3 of BDA), 3.30 (s, 3H; OCH_3 of BDA), 3.55–3.81 (m, 5H; 5×inositol ring CH), 3.80 (s, 3H; OCH_3 of PMB), 3.95 (dd, ^3J(H,H) = 10.4, 10.4 Hz, 1H; H-2), 4.75–4.99 (AB systems, 6H; OCH_2Ar), 6.84–6.92 (m, 2H; *meta*-H of PMB), 7.12–7.40 (m, 10H; Ar*H*), 7.44–7.52 ppm (m, 2H; Ar*H*); ^{13}C NMR (68 MHz, $CDCl_3$): δ = 17.8, 34.0 (C-2), 48.0, 55.3, 70.7, 72.0, 75.6, 75.7, 76.0, 80.5, 81.5, 82.8, 99.3, 99.4, 113.8, 127.7, 127.9, 128.0, 128.3, 128.4, 128.6, 130.0, 130.1, 138.0, 138.5, 159.4 ppm; elemental analysis: calcd (%) for $C_{34}H_{41}IO_8$: C 57.96, H 5.87; found: C 57.8, H 5.93.

(2′*S*,3′*S*)-D-3,6-Di-*O*-benzyl-2-deoxy-4,5-*O*-(2′,3′-dimethoxybutane-2′,3′-diyl)-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (3): A solution of **2** (1.30 g, 1.85 mmol), tributyltin hydride (1.0 mL, 3.7 mmol) and AIBN (60 mg, 0.37 mmol) in dry toluene (100 mL) was heated at reflux under N_2 for 1 h, after which time TLC (ether/hexane, 1:1) showed complete conversion of **2** (R_f = 0.46) into a more polar product (R_f = 0.38). The solution was allowed to cool and was then washed with saturated aqueous $NaHCO_3$ and brine (100 mL of each), dried ($MgSO_4$) and concentrated. Purification of the residue by flash chromatography on silica (ether/hexane, 1:2) gave **3** as a colourless oil (793 mg, 1.37 mmol, 74%); $[\alpha]_D = +61$ (c = 1.1 in $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$): δ = 1.36 (s, 3H; CH_3), 1.38 (s, 3H; CH_3), 1.51 (ddd, ^2J(H,H) = 12.6 Hz, ^3J(H,H) = 11.9, 11.9 Hz, 1H; H-2ax), 2.33 (ddd, ^2J(H,H) = 12.6 Hz, ^3J(H,H) = 4.8, 4.6 Hz, 1H; H-2eq), 3.30 (s, 3H; OCH_3 of BDA), 3.33 (s, 3H; OCH_3 of BDA), 3.34–3.79 (m, 5H; 5×inositol ring CH), 3.79 (s, 3H; OCH_3 of PMB), 4.53–4.95 (AB systems, 6H; OCH_2Ar), 6.82–6.89 (m, 2H; *meta*-H of PMB), 7.22–7.41 ppm (m, 12H; Ar*H*); ^{13}C NMR (68 MHz, $CDCl_3$): δ = 17.8, 34.3 (C-2), 47.9, 55.2, 71.3, 72.6, 73.2, 74.0, 75.5, 77.1, 82.5, 99.1, 99.3, 113.8, 127.4, 127.5, 128.0, 128.2, 128.3, 129.3, 130.6, 138.9, 139.1,

159.2 ppm; elemental analysis: calcd (%) for $C_{34}H_{42}O_8$: C 70.57, H 7.32; found: C 70.6, H 7.35.

D-3,6-Di-*O*-benzyl-2-deoxy-*myo*-inositol (4): A solution of **3** (652 mg, 1.13 mmol) in CH_2Cl_2 (10 mL) and 95% aqueous TFA (10 mL) was stirred at room temperature for 30 min. The solution was concentrated by evaporation under reduced pressure, and the residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH, 30:1) to give **4** as a white solid (332 mg, 0.964 mmol, 85%); m.p. 163–165 °C (from EtOAc/hexane); $[\alpha]_D = +7$ ($c = 1.0$ in MeOH); ^{1}H NMR (270 MHz, $CDCl_3$): $\delta = 1.42$ (ddd, ^2J(H,H) = 12.5 Hz, ^3J(H,H) = 11.9, 11.9 Hz, 1H; H-2ax), 2.36 (ddd, ^2J(H,H) = 12.5 Hz, ^3J(H,H) = 4.4, 4.4 Hz, 1H; H-2eq), 2.45 (d, D_2O exchange, ^3J(H,H) = 2 Hz, 1H; OH), 2.81 (s, D_2O exchange, 1H; OH), 2.91 (s, D_2O exchange, 1H; OH), 3.21–3.40 (m, 2H; 2×inositol ring CH), 3.45–3.57 (m, 3H; 3×inositol ring CH), 4.51 and 4.68 (AB system, ^2J(H,H) = 11.4 Hz, 2H; OC*H*$_2$Ar), 4.73 and 5.01 (AB system, ^2J(H,H) = 11.4 Hz, 2H; OC*H*$_2$Ar), 7.28–7.40 ppm (m, 10H; Ar*H*); ^{13}C NMR (68 MHz, CD_3OD): $\delta = 36.6$ (C-2), 70.2, 73.1, 76.0, 76.2, 78.1, 78.3, 87.2, 128.5, 128.6, 129.0, 129.3, 140.1, 140.5 ppm; elemental analysis: calcd (%) for $C_{20}H_{24}O_5$: C 69.75, H 7.02; found: C 69.6, H 6.94.

D-3,6-Di-*O*-benzyl-2-deoxy-*myo*-inositol 1,4,5-tris(dibenzylphosphate) (5): Bis(benzyloxy)diisopropylaminophosphine (0.3 mL, 0.93 mmol) was added to a suspension of 1*H*-tetrazole (89 mg, 1.3 mmol) and triol **4** (73 mg, 0.21 mmol) in dry CH_2Cl_2 (3 mL) under N_2. The mixture was stirred at room temperature for 3 h and then cooled to −78 °C, before *m*CPBA (57%, 384 mg, 1.3 mmol) was added. The mixture was allowed to warm to room temperature and was then diluted with CH_2Cl_2 (50 mL). The solution was washed with 10% aqueous Na_2SO_3, saturated aqueous $NaHCO_3$ and brine (50 mL of each), dried over $MgSO_4$ and concentrated by evaporation under reduced pressure. The residue was purified by flash chromatography on silica, (EtOAc/hexane, 3:2) to give **5** (204 mg, 0.181 mmol, 86%) as a colourless oil, which slowly crystallised; m.p. 97–99 °C (from EtOH); $[\alpha]_D = -7$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$): $\delta = 1.58$ (ddd, ^2J(H,H) = 12.5 Hz, ^3J(H,H) = 11.9, 11.9 Hz, 1H; H-2ax), 2.58 (ddd, ^2J(H,H) = 12.5 Hz, ^3J(H,H) = 4.4, 4.4 Hz, 1H; H-2eq), 3.48 (ddd, ^3J(H,H) ≈ 12, 9, 4 Hz, 1H; H-3), 3.60 (dd, ^3J(H,H) = 9.0, 9.0 Hz, 1H; H-6), 4.30 (dddd, ^3J(H,H) ≈ 12, 9, 4 Hz, ^3J(H,P) ≈ 8 Hz, 1H; H-1), 4.42–5.06 (m, 18H; H-4, H-5, 8×OC*H*$_2$Ar), 6.98–7.00 (m, 2H; Ar*H*), 7.05–7.35 ppm (m, 38H; Ar*H*); ^{31}P NMR (109 MHz, $CDCl_3$, ^{1}H-decoupled): $\delta = -1.1$ (1P), −0.95 (1P), −0.80 ppm (1P); elemental analysis: calcd (%) for $C_{62}H_{63}O_{14}P_3$: C 66.19, H 5.64; found: C 66.0, H 5.65.

D-2-Deoxy-*myo*-inositol 1,4,5-trisphosphate: Pd/C (10%, 50% water, 200 mg) was added to a solution of **5** (95 mg, 84 μmol) in MeOH (40 mL) and water (5 mL). The mixture was shaken in a Parr hydrogenator under H_2 (50 p.s.i.) for 20 h. The catalyst was removed by filtration through a polytetrafluoroethene (PTFE) syringe filter and 1.0 mol dm^{-3} triethylammonium bicarbonate (TEAB, 1 mL) was added. The solvents were removed by evaporation under reduced pressure and the residue was purified by ion-exchange chromatography on Q-Sepharose Fast Flow resin, with an elution gradient of TEAB (0→1.0 mol dm^{-3}). Fractions containing the target compound were identified by a modification of the Briggs phosphate test.[31] The product eluted at a TEAB concentration of 0.65–0.80 mol dm^{-3}. The combined fractions were concentrated by evaporation in vacuo, and methanol was repeatedly added and evaporated to eventually leave the triethylammonium salt of D-2-deoxy-Ins(1,4,5)P_3 as a colourless glass, which was accurately quantified by a total phosphate assay[31] (74 μmol, 88%); $[\alpha]_D = -14$ ($c = 0.8$ in MeOH); ^{1}H NMR (270 MHz, D_2O): $\delta = 1.43$ (ddd, ^2J(H,H) ≈ 13 Hz, ^3J(H,H) ≈ 12, 12 Hz, 1H; H-2ax), 2.23 (ddd, ^2J(H,H) = 12.7 Hz, ^3J(H,H) = 4.4, 4.4 Hz, 1H; H-2eq), 3.44 (ddd, ^3J(H,H) ≈ 8, 8 Hz, 1H; H-6), 3.52–3.60 (m, 1H; H-3), 3.80–3.90 ppm (m, 3H; H-1, H-4, H-5); ^{31}P NMR (109 MHz, D_2O, triethylamine added, ^{1}H-decoupled): $\delta = 3.37$ (1P), 5.09 (1P), 5.12 ppm (1P); high-resolution FAB-MS (negative ion): calcd for $C_6H_{14}O_{14}P_3^-$: 402.9596; found: 402.9596.

Materials: DNA polymerase and restriction enzymes were obtained from Boehringer Mannheim. ProBond Nickel-Chelating Resin was purchased from Invitrogen Life Technologies and S-protein Agarose was from Novagen. The HiLoad 16/60 Superdex 75 preparative grade column was obtained from Amersham. Dowex 1-X8 (formate form) and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad Laboratories. Turbo Broth came from AthenaES. Complete tablets (Protease inhibitor cocktail tablets) were from Roche. [^{3}H]-Ins-(1,4,5)P_3 (22 Ci mmol^{-1}) was from Dupont-NEN-PerkinElmer. The Partishere SAX column was purchased from Whatman and Easytide [γ^{32}P]-ATP was from NEN.

Bacterial strains and plasmids: Plasmid constructs were made in *E. coli* strain DH10B (Invitrogen Life Technologies) and protein expression was performed in *E. coli* strain BL21(DE3)pLysS (Novagen). A DNA fragment encoding the rat Ins(1,4,5)P_3 3-kinase A catalytic domain (275 amino acids; Ser185–Arg459) was amplified from the rat Ins(1,4,5)P_3 3-kinase A plasmid[8] by PCR with the 5′-primer, 5′-ggatcctcctgggtgcag-3′, containing a *Bam*H I restriction site (underlined), and with the 3′-primer, 5′-taaagcggccgctcatctctcagccag-3′, containing a *Not* I restriction site (underlined). The PCR fragment digested with *Bam*H I and *Not* I was subcloned in the fusion expression vector pET-30b (Novagen), which carries an N-terminal His-tag/S-tag configuration. The new construct was referred to as the catalytic domain of the rat InsP_3 3-kinase A cloned in the pET-30b vector, namely, plasmid pET30b-3KAcat, which enabled expression of the His-tagged/S-tagged catalytic domain 3KA-cat30.

Expression and purification of recombinant 3KA-cat30: *E. coli* BL21(DE3)pLysS bacteria harbouring the plasmid pET30b-3KAcat were grown overnight in Turbo Broth medium (200 mL) containing kanamycin (10 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}) with shaking at 250 rpm and at 37 °C. This culture was used to inoculate 2 L of Turbo Broth medium containing kanamycin and chloramphenicol. Bacteria were then grown at 37 °C until the absorbance at 600 nm was 0.7; this was then followed by an induction with IPTG (1 mM) for 4 h at 30 °C. The bacteria were harvested by centrifugation at 4000 *g* for 15 min. The pellet was resuspended in extraction/wash buffer (40 mL; 50 mM sodium phosphate (pH 7.8) with 300 mM NaCl in the presence of protease inhibitors). The cell suspension was sonicated for 5 min at 4 °C (pulser 1 s on and 1 s off, amplitude 30%) with a probe sonicator. Unbroken cells and debris were removed by centrifugation at 14000 *g* for 20 min at 4 °C. The soluble fractions containing His-tag/S-tag fusion proteins were incubated overnight with settled ProBond Nickel-Chelating Resin (10 mL) in a batch procedure. The resin was loaded onto a column and washed with 10 volumes of extraction/wash buffer. The fusion protein was eluted with 10 volumes of elution buffer (50 mM sodium phosphate (pH 7.8) with 300 mM NaCl, 250 mM imidazole and 10% glycerol). Further purification by using the S-protein agarose, which specifically retains S-tag fusion proteins, did not improve the purity of the 3KA-cat30 protein sample. Elution was tested with 10 volumes of elution buffer (50 mM trishydroxymethylaminomethane (Tris)/HCl (pH 7.9) with 300 mM NaCl) supplemented with either $MgCl_2$ (3 M) or NaSCN (3 M) but in both cases the same contaminants were found as those observed before purification. So, the active fractions were further purified on a Hiload 16/60 Superdex 75 preparative grade column by using

50 mM Tris (pH 7.8) with 500 mM NaCl and 5% glycerol at a flow rate of 0.25 mL min^{-1}.

Assay of Ins(1,4,5)P_3 3-kinase activity: Ins(1,4,5)P_3 3-kinase activity was measured as previously described[27,32] in 50 μL of medium containing ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA, 0.9 mM), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)/NaOH (84 mM, pH 7.5), bovine serum albumin (BSA, 1 mg mL^{-1}), ATP (1 mM), $MgCl_2$ (20 mM), Triton-X-100 (1%), diluted enzyme and [^{3}H]-Ins(1,4,5)P_3 (1500 cpm per assay, 1 or 10 μM).

HPLC analysis of the phosphorylation of inositol phosphates: The Ins(1,4,5)P_3 3-kinase activity was assayed in a 20 μL volume in the presence of the various derivatives with [γ^{32}P]-ATP (10 μM, 5 μCi), ATP (0.1 mM) and $MgCl_2$ (8 mM) in HEPES (50 mM, pH 7.5). The incubation was for 15 min at 37 °C. The reaction was stopped by addition of buffer A (3 mL, 1 mM ethylenediamine tetraacetate disodium salt (Na_2 EDTA)). The phosphorylation products were analysed by HPLC by using a Partisphere SAX column (25 cm × 4.6 mm). Samples were eluted with a gradient generated by mixing buffer A and buffer B (buffer A plus 1.3 M $(NH_4)_2HPO_4$ (pH 3.35) with H_3PO_4) as described in ref. [33].

Results

Preparation of an active catalytic domain 3KA-cat30

Our aim was to prepare a stable and active recombinant Ins(1,4,5)P_3 3-kinase to identify potential new substrates of the enzyme by using analogues of Ins(1,4,5)P_3 to understand the molecular interactions involved in catalysis. Since the catalytic domain of the rat Ins(1,4,5)P_3 3-kinase A is restricted to approximately 275 amino acids at the C-terminal end,[9] a DNA fragment encoding this domain, starting at Ser185, was amplified by PCR and cloned in the bacterial expression vector pET-30b. This protein, which was His- and S-tagged, proved to be particularly active and stable at 4 °C. We estimate that, after induction, the recombinant enzyme yielded 10–20% of the total soluble protein fraction. The purification of 3KA-cat30 was carried out in two steps. After the first purification step on Pro-Bond Nickel-Chelating Resin, we obtained approximately 40 mg of recombinant catalytic domain per litre of *E. coli* culture. The protein eluted at a protein concentration of 5–15 mg mL^{-1} (Figure 1). These active protein samples were further purified by gel filtration so that approximately 10 mg of purified recombinant protein were obtained after two purification steps. The protein was still active and was shown to be pure and intact at both the N- and C-terminal ends, as demonstrated by mass spectrometry: peptides were found which corresponded to the His-tag, the S-tag and the C terminus of the catalytic 3-kinase domain.

Effects of analogues on 3KA-cat30 activity

A series of synthetic analogues were tested as inhibitors and substrates of the enzyme (Scheme 2). These molecules could be classified as analogues of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 or adenophostin A. Nalaskowski et al. showed that Ins(2,4,5)P_3 could be phosphorylated at the 6-position of the inositol ring by recombinant Ins(1,4,5)P_3 3-kinase C;[15] we therefore tested D- and L- Ins(2,4,5)P_3 on 3KA-cat30. We previously reported no inhibition by D-Ins(1,2,4,6)P_4, a regioisomer of D-Ins(1,3,4,5)P_4, of Ins(1,4,5)P_3 3-kinase activity (up to 100 μM), although this molecule inhibits type I Ins(1,4,5)P_3 5-phosphatase.[27] Therefore, we did not expect all analogues evaluated here to inhibit the recombinant enzyme.

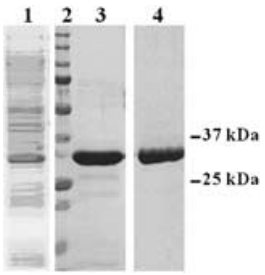
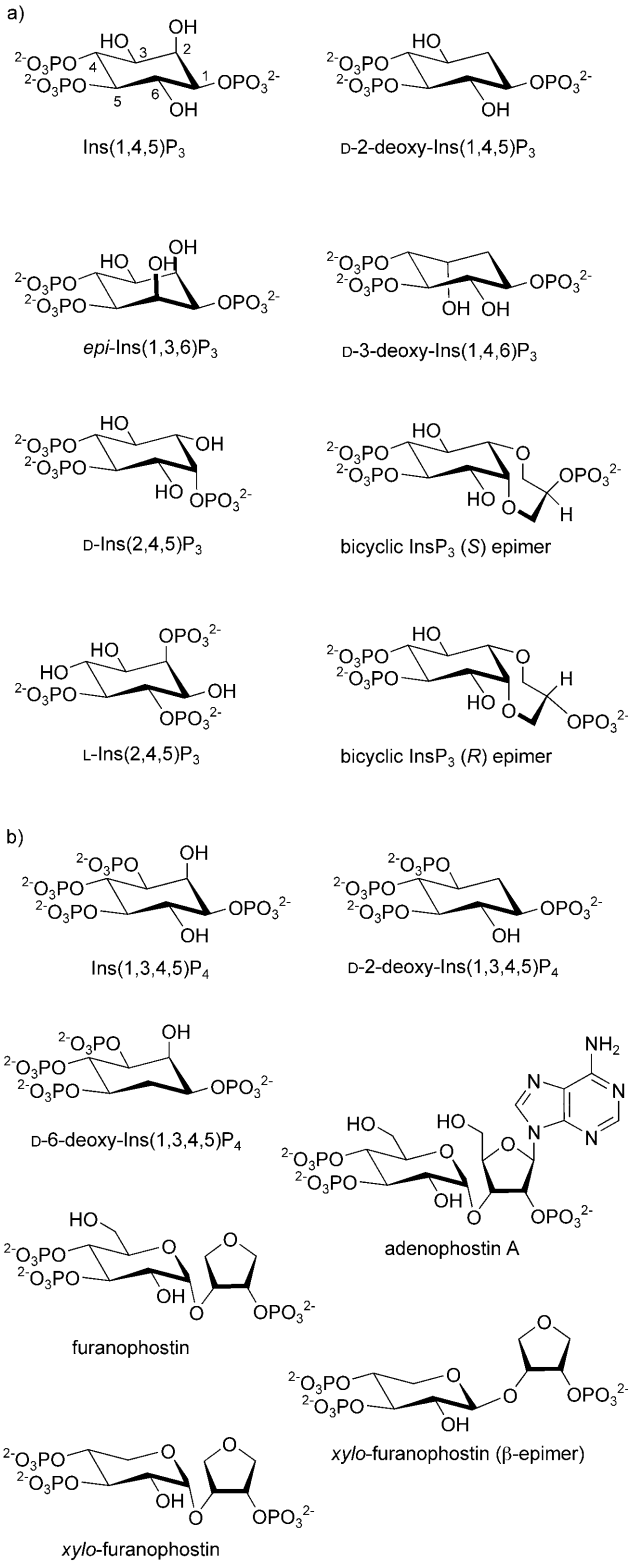


Figure 1. Purification profile of 3KA-cat30. Coomassie blue-stained sodium dodecylsulfate (SDS) PAGE analysis, where each lane represents 10 μL of the 3KA-cat30-containing samples. The Ins(1,4,5)P_3 3-kinase specific activities of the different samples applied to the SDS gel are given in brackets: lane 1: resuspended *E. coli* bacteria BL21(DE3)pLys(pET30–3KAcat) after induction with 1 mM IPTG for 4 h (0.214 μmol min^{-1} mg^{-1}); lane 2: molecular weight marker (Bio-Rad); lane 3: 3KA-cat30 sample after purification on nickel–nitrilotriacetate (Ni-NTA) resin, (3.2 μmol min^{-1} mg^{-1}); lane 4: 3KA-cat30 sample purified on Ni-NTA resin and on the Superdex 75 gel-filtration column, (28 μmol min^{-1} mg^{-1}).

As compared to Ins(1,4,5)P_3 (apparent Michaelis constant, K_m, equals 8 μM), D-2-deoxy-Ins(1,4,5)P_3 and D-3-deoxy-Ins(1,4,6)P_3 (Scheme 2A) were potent inhibitors with IC_{50} values in the low micromolar range (Table 1). Neither D- nor L-Ins(2,4,5)P_3 could be classified as an inhibitor unless very high concentrations were used (higher than 100 μM). Ins(1,3,4,5)P_4 and the deoxygenated analogues D-2-deoxy-Ins(1,3,4,5)P_4 and D-6-deoxy-Ins(1,3,4,5)P_4 (Scheme 2B) were all well recognised by the Ins(1,4,5)P_3 3-kinase with IC_{50} values in the micromolar range. Two synthetic bicyclic Ins(1,4,5)P_3 receptor agonists[24] with unusual positioning of a Ins(1,4,5)P_3 1-phosphate surrogate (Scheme 2A) and the adenophostin analogues[34–36] (Scheme 2B) did not inhibit the recombinant enzyme. Our data are summarised in Figure 2 and Table 1.

Phosphorylation of the analogues by the recombinant enzyme

The different analogues were tested as potential substrates of recombinant 3KA-cat30. These experiments were performed in the presence of [γ^{32}P]-ATP (5 μCi) with or without 0.1 mM ATP in the incubation mixture, as described in the Experimental Section. We aimed to compare the results obtained in the presence and absence of cold (nonradioactive) ATP. In the absence of ATP, the concentration of ATP is very far below the K_m value for ATP and therefore the assay conditions could be referred to as forcing conditions. Several molecules which were not phosphorylated in the presence of ATP were very poor substrates under forcing conditions (Table 2). Under forcing conditions, 3KA-cat30 could phosphorylate D-Ins(2,4,5)P_3, D-3-



Scheme 2. a) Structures of Ins(1,4,5)P_3 and analogues. b) Structures of Ins(1,3,4,5)P_4, adenophostin A and analogues.

deoxy-Ins(1,4,6)P_3 and one of the bicyclic Ins(1,4,5)P_3 analogues (Table 2).

D-2-Deoxy-Ins(1,4,5)P_3 (as well as the positive control Ins(1,4,5)P_3) appeared to be largely phosphorylated by the recombinant enzyme both in the presence and absence of cold ATP. In contrast, D-3-deoxy-Ins(1,4,6)P_3, which has an axial hydroxy group in place of the equatorial 3-hydroxy group of D-2-deoxy-Ins(1,4,5)P_3 (Scheme 2A), was not phosphorylated (Figure 3), except under forcing conditions.

Table 1. Inhibition of Ins(1,4,5)P_3 3-kinase activity by analogues of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and adenophostin A.[a]

Analogues	IC_{50} [μM]
Ins(1,3,4,5)P_4	13.08 ± 4.6
D-Ins(2,4,5)P_3	116.65 ± 31
L-Ins(2,4,5)P_3	> 300
D-2-deoxy-Ins(1,3,4,5)P_4	5.4 ± 3.5
D-6-deoxy-Ins(1,3,4,5)P_4	5.1 ± 2.4
D-2-deoxy-Ins(1,4,5)P_3	1.7 ± 1.3
D-3-deoxy-Ins(1,4,6)P_3	1.4 ± 1.3
epi-Ins(1,3,6)P_3	> 300
bicyclic InsP_3 *S* epimer	156
bicyclic InsP_3 *R* epimer	> 300
adenophostin A	> 300
furanophostin	> 300
xylo-furanophostin	> 300
xylo-furanophostin (β epimer)	> 300

[a] Ins(1,4,5)P_3 3-kinase activity was determined at 1 μM Ins(1,4,5)P_3 substrate concentration. The analogues were tested in the 0.1–300 μM range. IC_{50} values are mean values ± the standard deviation of triplicate determinations.

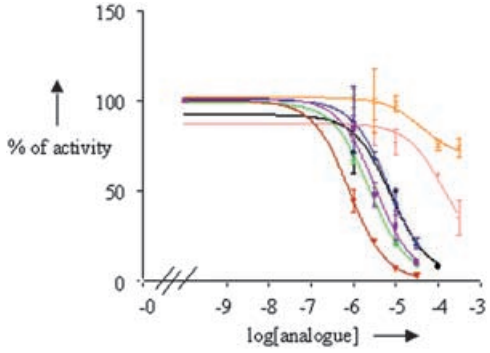


Figure 2. Inhibition of 3KA-cat30 by inositol phosphate analogues. Purple: D-2-deoxy-Ins(1,3,4,5)P_4, blue: D-6-deoxy-Ins(1,3,4,5)P_4, red: D-2-deoxy-Ins(1,4,5)P_3, green: D-3-deoxy-Ins(1,4,6)P_3, black: Ins(1,3,4,5)P_4, orange: L-Ins(2,4,5)P_3, pink: D-Ins(2,4,5)P_3. Ins(1,4,5)P_3 3-kinase activity was determined at 1 μM Ins(1,4,5)P_3 and each analogue was added in increasing concentrations (0–300 μM). IC_{50} values are mean values ± the standard deviation from triplicate determinations.

Discussion

In this study, we aimed to produce the Ins(1,4,5)P_3 3-kinase in order to yield sufficient soluble protein for biochemical and structural studies. Previous constructs coding for the A and B isoforms made in our laboratory led largely to the degradation of the isoforms and to the formation of inclusion bodies which resulted in low amounts of soluble enzymes (see refs. [5,8] and unpublished data). However, when we expressed the catalytic domain as a His- and S-tagged fusion protein, large amounts

Table 2. Analogues tested as substrates of the 3KA-cat30 recombinant enzyme.[a]

Analogue	Kinase substrate with cold ATP (0.1 mM)	Kinase substrate without cold ATP
Ins(1,4,5)P_3	+++	+++
Ins(1,3,4,5)P_4	–	–
D-Ins(2,4,5)P_3	–	+
L-Ins(2,4,5)P_3	–	–
D-2-deoxy-Ins(1,3,4,5)P_4	–	–
D-6-deoxy-Ins(1,3,4,5)P_4	–	–
D-2-deoxy-Ins(1,4,5)P_3	+++	+++
D-3-deoxy-Ins(1,4,6)P_3	–	+
epi-Ins(1,3,6)P_3	–	–
bicyclic InsP_3 *S* epimer	–	+
bicyclic InsP_3 *R* epimer	–	–
adenophostin A	–	–
furanophostin	–	–
xylo-furanophostin	–	–
xylo-furanophostin (β epimer)	–	–

[a] Recombinant enzyme was incubated in the presence of 10 μM of each analogue, [γ^{32}P]-ATP (5 μCi) and with or without 0.1 mM ATP. The incubation time was 15 min.

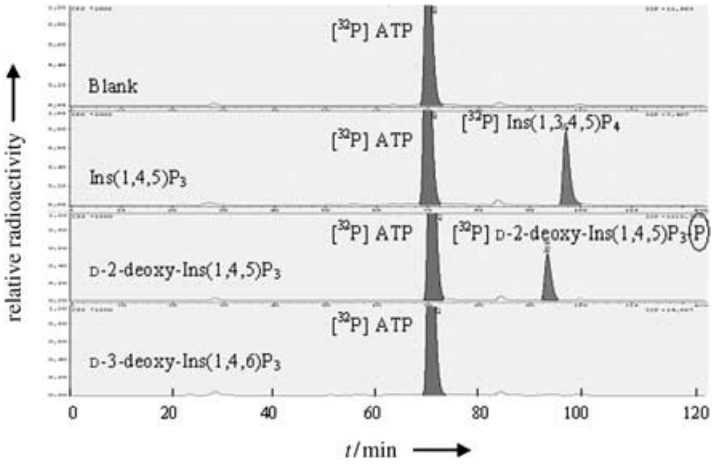


Figure 3. HPLC analysis of the phosphorylation products of D-2-deoxy-Ins(1,4,5)P_3 and D-3-deoxy-Ins(1,4,6)P_3. Recombinant enzyme was incubated in the presence of 10 μM of the analogues in the presence of [γ^{32}P]-ATP (5 μCi) and 0.1 mM ATP. The data are representative of three different experiments.

of the domain could be efficiently produced as soluble protein (10–20% of the total soluble fraction). The recombinant enzyme could be purified in two steps and appears to be very stable at 4°C. It was subsequently used to identify potential new substrates amongst inositol phosphate analogues.

Ins(1,4,5)P_3 analogues have been widely synthesised and used in binding studies and competition experiments with Ins(1,4,5)P_3 3-kinase.[37–45] Safrany et al. reported the interaction of two synthetic analogues, 2-deoxy-2-fluoro-*scyllo*-inositol 1,4,5-trisphosphate (2-F-Ins(1,4,5)P_3) and 2,2-difluoro-2-deoxy-*myo*-inositol 1,4,5-triphosphate (2,2-F_2-Ins(1,4,5)P_3), with 3-kinase activity in rat brain homogenate. Both fluoro analogues were weaker substrates than Ins(1,4,5)P_3 for Ins(1,4,5)P_3 3-kinase but 2,2-F_2-Ins(1,4,5)P_3 was significantly better than 2-F-Ins(1,4,5)P_3, presumably because the axial fluorine atom of the former can act more successfully to mimic the normal electronic environment at the 2-position adjacent to the crucial 3-hydroxy group. The authors concluded that, whilst not an absolute requirement for activity, the 2-hydroxy group is an important element in the recognition of substrates by Ins(1,4,5)P_3 3-kinase.[43] By contrast, Hirata et al. inferred that the hydroxy group of Ins(1,4,5)P_3 at the 2-position was not involved in the recognition by Ins(1,4,5)P_3.[38]

In our study, D-2-deoxy-Ins(1,4,5)P_3 (Scheme 2A) is a potent inhibitor of Ins(1,4,5)P_3 3-kinase (IC_{50} = 1.7 μM) and a substrate of the enzyme. This conclusion was also reached when rat brain cytosol was used as source of Ins(1,4,5)P_3 3-kinase, although the degree of inhibition of the native enzyme was always lower than that for the recombinant enzyme (data not shown). Therefore, the axial 2-hydroxy group appears to be neither involved in binding to the enzyme nor part of the phosphorylation reaction of the inositol ring. The fact that D-2-deoxy-Ins(1,3,4,5)P_4 (Scheme 2B) is a potent inhibitor but not a substrate of the enzyme is consistent with phosphorylation occurring at the 3-position of the inositol ring. In contrast, D-3-deoxy-Ins(1,4,6)P_3 (Scheme 2A) does not appear to be a substrate, although, like Ins(1,4,6)P_3, it could bind in an orientation mimicking Ins(1,4,5)P_3,[40] a fact indicating the importance of an equatorial hydroxy group at the 3-position for acceptance of a phosphate group. D-Ins(1,4,6)P_3 had an IC_{50} value of 30 μM, which suggests that the hydroxy function at the 3-position is influencing the inhibitory potency in comparison to that of D-3-deoxy-Ins(1,4,6)P_3 (data not shown). Finally, D-6-deoxy-Ins(1,3,4,5)P_4 (Scheme 2B) was a potent inhibitor of the kinase, while *epi*-Ins(1,4,5)P_3 (strictly named *epi*-Ins(1,3,6)P_3, Scheme 2A) was not recognised. This suggests that, while the equatorial 6-hydroxy group is not involved in recognition, an axial hydroxy group at this position is not tolerated by the active site of the kinase.

Recent studies provide conflicting results about the phosphorylation of D-Ins(2,4,5)P_3. Bird et al. reported that Ins(2,4,5)P_3 was not phosphorylated by Ins(1,4,5)P_3 3-kinase in lachrymal cells.[42] On the contrary, rat Ins(1,4,5)P_3 3-kinase C was shown to phosphorylate Ins(2,4,5)P_3 at the 6-position.[15] In our hands, D-Ins(2,4,5)P_3 is not phosphorylated by recombinant kinase A unless forcing conditions are used (Table 2). Compared to Ins(1,4,5)P_3 and D-2-deoxy-Ins(1,4,5)P_3, this compound is a very poor substrate requiring assay conditions that will never be seen in intact cells.

The glyconucleotides adenophostin A and B are the most potent known agonists of Ins(1,4,5)P_3 receptors[34–36,46] and have been reported to be resistant to phosphorylation by the 3-kinase activity of rat cerebral cytosol fractions.[46] We questioned whether adenophostins or their analogues lacking the adenine base and other motifs could be recognised by the Ins(1,4,5)P_3 3-kinase. Our experiments showed that this was not the case for adenophostin A and the synthetic analogues examined (Scheme 2B). Therefore, the molecular interactions

involved in Ins(1,4,5)P_3 binding to its receptor and to the inositol 3-kinase are certainly not identical.

Recently, while this paper was in preparation, the crystal structure of the catalytic core of Ins(1,4,5)P_3 3-kinase A was reported by two groups.[13,14] The kinase domain was divided into three subdomains. The N and C lobes and the hinge connecting them are involved primarily in binding the nucleotide and metal cofactor, while the α-helical subdomain is involved in Ins(1,4,5)P_3 binding and has been referred to as the inositol phosphate (IP) binding lobe. Most interestingly, the results from the X-ray diffraction based structure indicate a model of IP binding and a catalytic mechanism where the three phosphate groups interact with positively charged amino acids, while the 2- and 6-hydroxy groups make no direct hydrogen bonds to the enzyme. The equatorial 3-hydroxy group interacts with Lys262 in the rat sequence (264 in the human sequence) from the DxK motif found in several inositol phosphate kinases.[13,14] Mutation of this lysine residue has been shown to abrogate catalytic activity.[47] This residue is therefore essential in the reaction mechanism. In the product complex, Ins(1,3,4,5)P_4 makes the same interactions as Ins(1,4,5)P_3.[13]

From our studies, Ins(1,3,4,5)P_4 is a potent inhibitor; this is in agreement with the interaction of substrate and product in the IP binding lobe, which is comparable for both molecules, and with the in-line mechanism of phosphorylation proposed by the authors.[13] The fact that D-Ins(2,4,5)P_3 is not a potent inhibitor could be explained by the fact that, in place of the equatorial 1-phosphate group of Ins(1,4,5)P_3, it can only provide an axial phosphate group (Scheme 2A), which cannot interact effectively with positively charged amino acids Arg319 and Tyr315. In the crystal structure of the catalytic domain of the human inositol 1,4,5-triphosphate 3-kinase, these residues are hydrogen bonded with the normally equatorial 1-phosphate group and play a crucial role in binding the IP at the active site. Being a bad inhibitor, D-Ins(2,4,5)P_3 can hardly be a substrate. The fact that it can be phosphorylated under forcing conditions is supported by the fact that it can bind (weakly) in such a way as to present the equatorial 6-hydroxy group in a position for phosphorylation. This would account for the product identification of Ins(2,4,5,6)P_4 after phosphorylation of Ins(2,4,5)P_3 by Ins(1,4,5)P_3 3-kinase C isoform in the study of Nalaskowski et al.[15]

D-2-Deoxy-Ins(1,4,5)P_3 is a potent inhibitor of the kinase and the substrate as well, therefore indicating that the 2-hydroxy function is not required in terms of hydrogen bonding, with the only limitation being space. Thus, removal of the 2-hydroxy group does not interfere with binding to the active site or with the mechanism of phosphoryl transfer (Figure 4 A and B). The structure of D-3-deoxy-Ins(1,4,6)P_3 is almost identical to that of D-2-deoxy-Ins(1,4,5)P_3 except that the orientation of a single hydroxy group is changed from equatorial to axial. This slight change in structure does not affect binding; D-3-deoxy-Ins(1,4,6)P_3 remains a potent inhibitor of the kinase. It does, however, prevent D-3-deoxy-Ins(1,4,6)P_3 from behaving as a substrate, except under forcing conditions. The fact that D-3-deoxy-Ins(1,4,6)P_3 is a potent inhibitor but not a substrate suggests that the hydroxy group presented to the active site for phosphorylation must be equatorial. This can be explained in terms of the crystal structure of the catalytic domain of the human inositol 1,4,5-triphosphate 3-kinase, by noting that the normal interaction of this hydroxy group with Lys262(264) cannot exist in the axial configuration (Figure 4C) and that the required spatial arrangement of Lys262(264), the target OH group and the γ-phosphoryl group of ATP required for in-line phosphoryl transfer will be lost. Therefore, we suggest that Lys262(264) plays an essential role in the mechanism of IP phosphorylation and not in binding of the substrate; for example, this residue may facilitate nucleophilic attack by neutralising the negative charge developed in the transition state, as

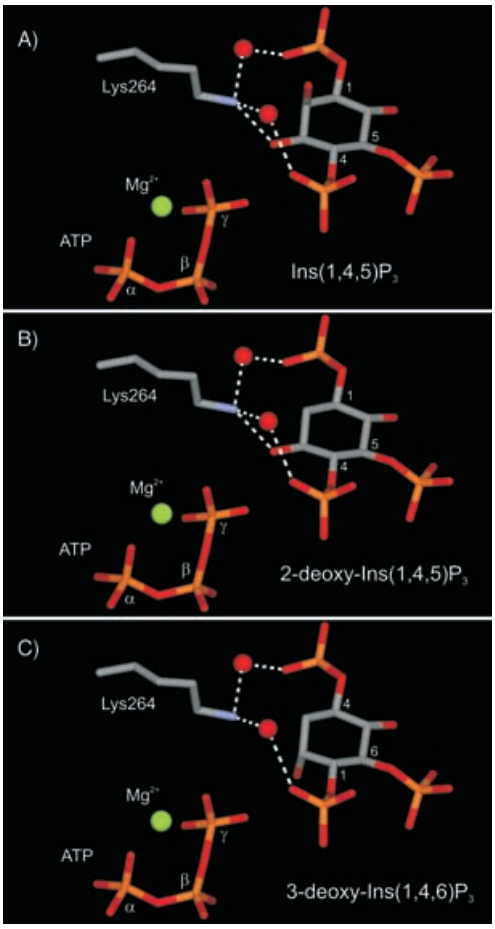


Figure 4. Proposed interactions of D-2-deoxy-Ins(1,4,5)P_3 and D-3-deoxy-Ins(1,4,6)P_3 with human 3-kinase A. A) Lys264 interacts with the 3-hydroxy group of Ins(1,4,5)P_3 in the substrate complex and is likely to be involved in the mechanism of phosphoryl transfer (see text). Two water molecules (red spheres) which mediate indirect interactions of Lys264 with the 1- and 4-phosphate groups of Ins(1,4,5)P_3 are also shown. The 2-hydroxy group of Ins(1,4,5)P_3 is not involved in binding or in the mechanism of phosphoryl transfer (adapted from ref. [13]). B) In the predicted binding mode, D-2-deoxy-Ins(1,4,5)P_3 can reproduce all the important features of Ins(1,4,5)P_3 and is therefore a potent inhibitor and substrate of the kinase. C) In the most likely binding mode for D-3-deoxy-Ins(1,4,6)P_3, the three phosphate groups can effectively mimic those of Ins(1,4,5)P_3 and D-2-deoxy-Ins(1,4,5)P_3, thereby making it a potent inhibitor. However, the interactions with Lys264 that are necessary for phosphoryl transfer are disrupted in this mode, and D-3-deoxy-Ins(1,4,6)P_3 is therefore not a substrate for the kinase, except under forcing conditions.

indicated by Gonzales et al.,[13] and by orientating the transferred phosphate. This also suggests that the presence of phosphate groups in positions 1, 4, and 5 is required for inhibition of the kinase rather than the hydroxy functions on C-2 and C-6. The data we obtained with the Ins(1,3,4,5)P_4 analogues actually confirm that the 2-hydroxy and 6-hydroxy groups are not required in hydrogen bonding during the binding process since the corresponding deoxy analogues were potent inhibitors. This is in agreement with the proximity of the 2-hydroxy and 6-hydroxy functions to the hydrophobic Met316 and Met288 residues, respectively, in the human inositol 1,4,5-triphosphate 3-kinase structure.[13] Finally, we can also suggest why the adenophostins and adenophostin analogues are not recognised. It seems that they cannot mimic the very important 1-phosphate group of the IP substrate of the kinase. This contrasts with the Ins(1,4,5)P_3 receptor, in which the 2′-phosphate group in the adenophostins can still interact well with residues at the site. Similar factors may underlie the poor recognition of the bicyclic Ins(1,4,5)P_3 analogues, in which the position of the 1-phosphate group is altered.

In conclusion, the two hydroxy functions at positions 2 and 6 of Ins(1,4,5)P_3 are not involved in IP binding to the kinase; our data actually indicate that a potent inhibitor could be obtained by removing the hydroxy functions at positions 2 and 6. In contrast, the equatorial 3-hydroxy function requires that configuration for phosphoryl transfer to occur. Our data clearly stress the importance of the 3-hydroxy function in the mechanism of phosphorylation, rather than in IP binding.

Acknowledgements

We thank Dr. V. Dewaste (IRIBHM, ULB Bruxelles, Belgium) for criticism of the manuscript and Dr. G. Horne for some synthetic work. A.P. has been supported by a Marie Curie Fellowship of the European Community Programme "Improving the Human Research Potential and the Socio-Economic Knowledge Base" under contract number HPMF-CT-2002–01683. This work was supported by grants from Fonds de la Recherche Scientifique Médicale, Action de Recherche Concertée of the Communauté Française de Belgique, the Belgium Programme on Interuniversity Poles of Attraction initiated by the Belgium State Prime Minister's Office (Project IUAP P5/05), and by a Wellcome Trust Programme Grant (Grant 060554 to B.V.L.P.).

Keywords: inhibitors · inositol phosphates · kinases · phosphoryl transfer · structure–activity relationships

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Received: December 13, 2004

