Diphospho-*myo*-inositol phosphates in *Dictyostelium* and *Polysphondylium*: identification of a new bisdiphospho-*myo*-inositol tetrakisphosphate

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Abstract The two major diphospho inositol phosphates from the axenic strain *Dictyostelium discoideum* AX2 were previously investigated and identified as 6-PP-InsP_5 and 5,6-bis-PP-InsP_4. In order to examine whether these findings are representative of Dictyostelids in general, five non-axenic wild-type species of *Dictyostelium* and two of *Polysphondylium* were studied. It was found that all of the *Dictyostelium* species exhibit similar patterns of diphospho inositol phosphates. By contrast, both of the *Polysphondylium* species contain 5-PP-InsP_5 as the predominant isomer. Besides 5,6-bis-PP-InsP_4, a new bis-PP-InsP_4 was detected in *Polysphondylium*. This compound is either 1,5-bis-PP-InsP_4 or its corresponding enantiomer 3,5-bis-PP-InsP_5. The structures were elucidated by two-dimensional ^{1}H-^{1}H and ^{1}H-^{31}P NMR analysis. Additionally, they were confirmed using a specific 6-PP-InsP_5-5-kinase from *D. discoideum* AX2 as an enantio-specific tool and enantiomerically pure reference standards.

Key words: *Myo*-inositol; Kinase; *Dictyostelium*; *Polysphondylium*

1. Introduction

For a long period, InsP_6 has been thought to be the anabolic end point of inositol phosphorylation. However, in recent years it became obvious that InsP_6 can be further phosphorylated yielding a group of highly phosphorylated diphospho inositol phosphates. These compounds seem to be ubiquitous in eukaryotic cells and have been detected in *Dictyostelium* [1–4], free living amoebae [5,6] and yeast [7] as well as in a number of mammalian cell types [8–11].

In *Dictyostelium* the intracellular concentrations of diphospho inositol phosphates range between 10 μM and 300 μM, depending on growth conditions. These amounts are up to 300-fold higher than in mammalian cells [12]. In vitro studies using mammalian cell lines indicated that PP-InsP_5 might be involved in vesicle trafficking. PP-InsP_5 specifically binds to coatomer, a golgi vesicle coat protein complex, and modulates its K^+-channel activity [13]. Additionally, clathrin assembly is blocked by binding of PP-InsP_5 to synapse-specific clathrin assembly protein (AP3) from bovine brain [14]. The high intracellular concentration in *Dictyostelium* contradicts such a regulatory function. It has been speculated that diphospho-*myo*-inositol phosphates may serve as a unimolecular source of chemical energy, phosphate, carbohydrate and metal ions in ancient organisms like amoebae with a dormant stage in their life cycle [1,3].

In mammalian cells, rapid metabolic fluxes between InsP_6, PP-InsP_5 and bis-PP-InsP_4 have been reported which are due to the combined action of ATP-dependent kinases and fluoride sensitive phosphatases [8,9,11]. An ATP-dependent InsP_6-5-kinase has been purified from rat brain, which acts in reverse as an ATP synthase [10]. From *Dictyostelium* cytosolic extracts, a 6-PP-InsP_5-5-kinase has been partially purified which selectively phosphorylates 6-PP-InsP_5 leading to 5,6-bis-PP-InsP_4 [4].

Due to the low amounts of diphospho inositol phosphates in mammalian cells, a purification of sufficient material for NMR analysis has only been possible for the two major diphospho inositol phosphates from the cellular slime mold *Dictyostelium*. PP-InsP_5 has been identified as 6-PP-InsP_5 and bis-PP-InsP_4 has been identified as 5,6-bis-PP-InsP_4 by 2D NMR analysis [3]. Furthermore, stereo-defined synthetic PP-InsP_5 isomers and purified *D. discoideum* 6-PP-InsP_5-5-kinase were used as a diagnostic tool [4]. The structure of 6-PP-InsP_5 in *Dictyostelium* has been recently confirmed by high resolution HPLC and inhibition studies using a hepatic multiple inositol polyphosphate phosphatase (MIPP) for chiral analyses [12]. In that study, smaller amounts (10–25%) of an additional PP-InsP_5 isomer were detected and identified as 5-PP-InsP_5. Structural studies on diphospho inositol phosphates from mammalian cells have also been performed. Extracts from four mammalian cell lines have been analyzed for PP-InsP_5 by high resolution HPLC chromatography. The authors showed that all of them co-eluted exactly with an internal standard of 5-PP-InsP_5 and concluded that 5-PP-InsP_5 is the predominant isomer [12]. Two-dimensional ^{1}H-^{31}P NMR studies of crude extracts from the free living amoebae *Phreatamoeba balamuthi* [5] and *Entamoeba histolytica* [6] indicated that this isomer also exists in these species. However, the structure of bis-PP-InsP_4 found in mammalian cell lines has not been identified yet.

In order to get a more complete picture of the diphospho inositol phosphates in Dictyostelids, five non-axenic wild-type species of *Dictyostelium* and two wild-type species of *Polysphondylium* were analyzed in parallel. *Dictyostelium* and *Polysphondylium* are genera of the subclass Dictyostelidae and are considered to be closely related species [15]. During the present study it is demonstrated that, in contrast to *Dictyo-*

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Abbreviations: PP-InsP_5, diphospho-*myo*-inositol pentakisphosphate; bis-PP-InsP_4, bisdiphospho-*myo*-inositol tetrakisphosphate; HPLC-MDD, HPLC metal dye detection

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stelium, 5-PP-InsP_5 is the major PP-InsP_5 in *Polysphondylium*. Furthermore, an additional bis-PP-InsP_4 isomer was observed in these species besides the previously known 5,6-bis-PP-InsP_4. This compound was identified by 2D NMR analysis as 1,5-bis-PP-InsP_4 or its corresponding enantiomer 3,5-bis-PP-InsP_4. The structural assignments were confirmed using the 6-PP-InsP_5-5-kinase isolated from *D. discoideum*.

2. Materials and methods

2.1. Materials

D-1-PP-InsP_5, D-3-PP-InsP_5 [17], D-4-PP-InsP_5, D-6-PP-InsP_5 [12], 2-PP-InsP_5 and 5-PP-InsP_5 [16] were synthesized as described. ATP, ADP, EDTA, Triton X-100, phosphocreatine, creatine phosphokinase, D/L-dithiothreitol, hexokinase and HEPES buffer were supplied by Sigma. Norit A activated charcoal was from Serva. Nitro-blue tetrazolium, $NADP^+$, diaphorase and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim and glucose, K_2HPO_4, $NaH_2PO_4 \cdot H_2O$, trisodium citrate, $MgSO_4 \cdot 7H_2O$, $(NH4)_2SO_4$ and $MgCl_2$ from Merck. Select Agar was supplied by GibcoBRL. 4-(2-Pyridylazo)resorcinol (PAR) was obtained from Fluka and YCl_3 from Aldrich. Tris buffer was purchased from Biomol. Q-Sepharose fast flow, Mono-Q and resource-15-Q anion exchange material and SP-Sepharose fast flow cation exchange material were from Pharmacia. Hydroxylapatit Biogel HTP was obtained from BioRad. Five μm pore size filters were supplied by Nucleopore.

2.2. Strains and cell culture

The following strains were obtained from the American Type Culture Collection: *D. discoideum* AX2 (ATCC 24397) and NC4 (ATCC 11735), *D. brunneum* (ATCC 48616), *D. giganteum* (ATCC 32961), *D. purpureum* (ATCC 26660), *Polysphondylium pallidum* (ATCC 18421). *D. mucoroides* and *P. violaceum* were wild-type isolates from soil of the environment of Wuppertal, Germany. Cells were grown on bacteria suspension (*Klebsiella aerogenes*, OD_{420} 10) in 40 mM phosphate buffer pH 6.5 until all bacteria were consumed. Cells were harvested by centrifugation for 10 min at $5000 \times g$, washed with 17 mM phosphate buffer pH 6.5 and stored at −80°C. Frozen cells were prepared as described [18] for HPLC-MDD analysis. For preparative isolation of diphospho inositol phosphates, *P. pallidum* was cultured on bacteria lawns (*K. aerogenes*, 40×40 cm plates) on minimal agar medium (K_2HPO_4 4.4 g/l, $NaH_2PO_4 \cdot H_2O$ 5.0 g/l, trisodium citrate 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l, $(NH_4)_2SO_4$ 1.0 g/l, KCl 0.5 g/l, $MgCl_2$ 0.25 g/l, Select Agar 15.0 g/l).

2.3. Analysis of inositol phosphates

Inositol phosphates were analyzed using the HPLC-MDD method described previously [18,19]. The compounds were separated on a 10×1 cm Mono-Q column (Pharmacia) for high resolution chromatography of PP-InsP_5 isomers or on a 1×0.5 cm source-15-Q column (Pharmacia) for enzymatic studies using pH and salt gradients as indicated in the legends of each HPLC-MDD chromatogram. Photometric detection at 546 nm was performed using a modified post-column dye reagent (2 M Tris/HCl (pH 8.5)/200 μM 4-(2-pyridylazo)resorcinol/30 μM YCl_3/10% (v/v) MeOH).

2.4. Preparation of diphospho inositol phosphates from cells

The diphospho inositol phosphates were prepared following standard methods [1–3]. The yield and purity of the isolated compounds were examined by HPLC-MDD. The purity of the bis-PP-InsP_4 isomers was found to be >97% whereas the PP-InsP_5 fraction consisted of two PP-InsP_5 isomers as detailed below.

2.5. Assays of 6-PP-InsP_5-5-kinase activity

HPLC-MDD detection of 6-PP-InsP_5-5-kinase activity: For quantitative studies, enzyme activity was assayed in a reaction mixture containing 20 mM HEPES (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 5 mM phosphocreatine, 2 U/ml creatine phosphokinase, 1 mM D/L-dithiothreitol and 7.5 μM 6-PP-InsP_5 in a final volume of 1.0 ml. The reaction was incubated at 25°C and terminated by freezing the sample in liquid nitrogen. Amounts of PP-InsP_5 and bis-PP-InsP_4 were determined by the HPLC-MDD method [18,19]. Inositol phosphates were separated on a 1×0.5 cm source-15-Q column with a 15 min linear gradient of 200–450 mM HCl.

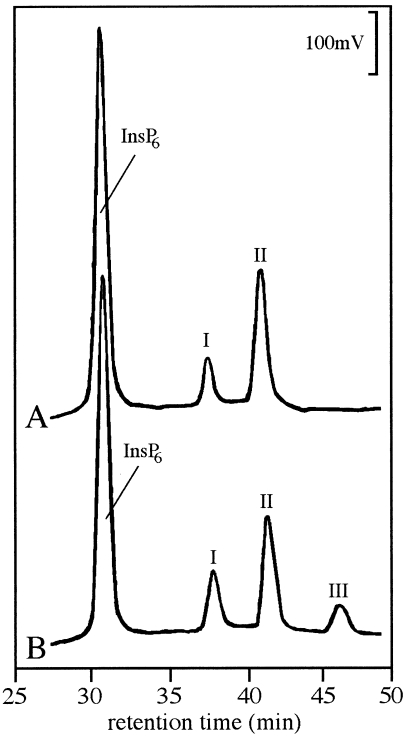

Fig. 1. HPLC-MDD chromatogram of extracts obtained from *D. discoideum* NC4 (A) and *P. pallidum* (B). Gradient: (Mono-Q 1×10 cm) 0 min: 240 mM KCl/40 mM HCl, 30 min: 600 mM KCl/100 mM HCl, 50 min: 600 mM KCl, 100 mM HCl. The peaks I–III represent diphospho inositol phosphates, which were isolated in order to elucidate their structures.

Photometric assay of the ATP synthase activity of the 6-PP-InsP_5-5-kinase: The ATP synthase activity was determined in a coupled enzyme assay using hexokinase, glucose-6-phosphate dehydrogenase and diaphorase.

$$5,6\text{-bis-PP-Ins}P_4 + \text{ADP} \rightarrow \text{6-PP-Ins}P_5 + \text{ATP},$$

$$\text{ATP} + \text{glucose} \rightarrow \text{glucose-6-P} + \text{ADP},$$

$$\text{glucose-6-P} + NADP^+ \rightarrow \text{6-phosphogluconat} + \text{NADPH},$$

$$\text{NADPH} + \text{nitro-blue tetrazolium} \rightarrow NADP^+ + \text{nitro-blue formazane (blue)}.$$

The ATP formed was determined in a microassay using multiwell plates. This assay was used in order to detect active fractions during the preparation of 6-PP-InsP_5-5-kinase. The reaction mixture contained final concentrations of 20 mM HEPES pH 6.8, 5 mM $MgCl_2$, 0.25 mM EDTA, 0.05% Triton X-100, 0.25 mM nitro-blue tetrazolium, 0.5 mM glucose, 40 μM ADP, 40 μM $NADP^+$, 2 U/ml glucose-6-phosphate dehydrogenase, 0.1 U/ml diaphorase, 3 U/ml hexokinase and 15 μM 5,6-bis-PP-InsP_4 isolated from *D. discoideum* AX2 (final volume: 200 μl). Absorbance was measured in a microplate reader (BioRad, model 450) using a 540 nm filter. For kinetic studies this assay was performed in 200 μl cuvettes at 540 nm in a Shimadzu photometer (model UV 240). No reaction was observed in absence of ADP or 5,6-bis-PP-InsP_4 or without 6-PP-InsP_5-5-kinase.

2.6. Preparation of 6-PP-InsP_5-5-kinase

D. discoideum cells (strain AX2, ATCC 24397) were grown in HL5 medium [20]. For a typical purification procedure, about 2×10^{10} cells (ca. 20 g wet weight) were collected by centrifugation for 10 min at

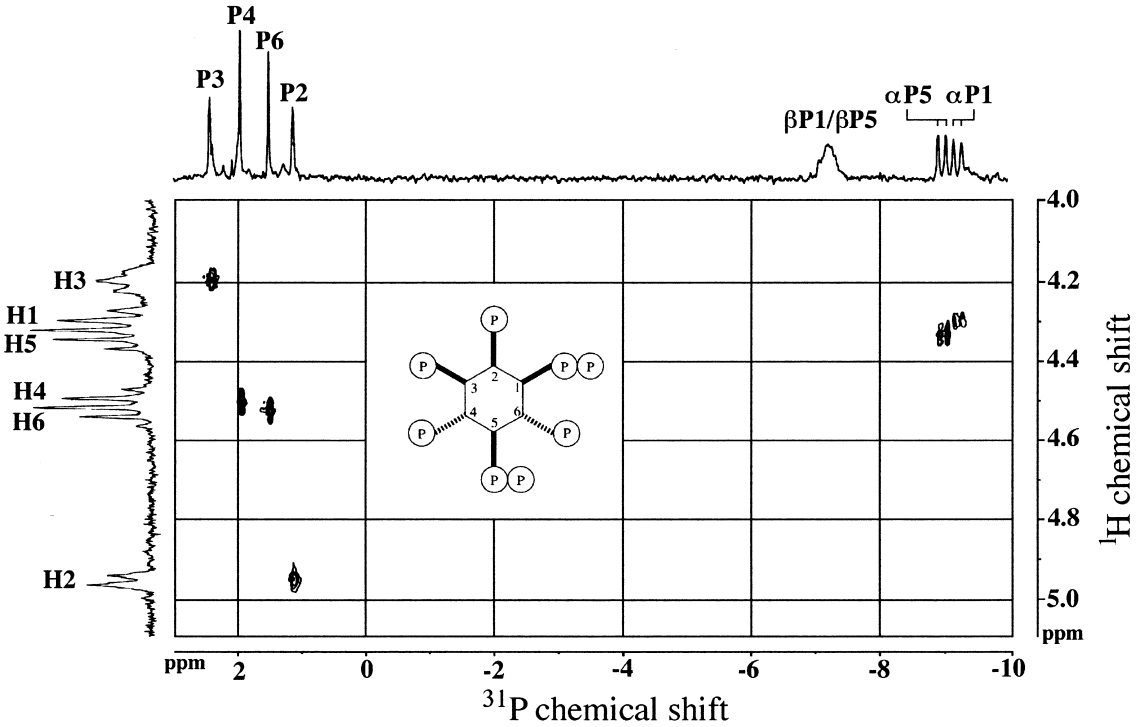

Fig. 2. 2D ^{1}H-^{31}P NMR correlation map (pH 6.0) of 1(3),5-bis-PP-InsP$_4$ isolated from *P. pallidum* (see Fig. 1B, peak III). The proton resonances were assigned by 2D ^{1}H-^{1}H NMR as described previously [3]. The numbers H1–H6 indicate the corresponding position of the proton attached at the inositol backbone. The correlation map demonstrates that the α-phosphates of the two diphospho groups are coupled to H1 and H5, consequently this compound is 1,5-bis-PP-InsP$_4$. Since it is impossible to distinguish between enantiomers by the NMR method used the structure could also be assigned as 3,5-bis-PP-InsP$_4$.

5000×*g*, washed twice with 20 mM HEPES (pH 6.8) and lysed in 50 mM HEPES (pH 6.8) by passing through a 5 μm pore size filter. All operations were performed at 2–4°C. Cell debris and membranes were removed by ultracentrifugation (30 min, 4°C, 150 000×*g*). The supernatant was applied on a Q-Sepharose column (1.5×20 cm) equilibrated with 50 mM HEPES, pH 6.8. The PP-InsP$_5$-kinase activity was eluted with the starting buffer and was bound to SP-Sepharose (1×10 cm column). The column was washed with starting buffer until non-adsorbed proteins were removed. Enzyme activity was eluted with a 60 ml 10–150 mM NaCl linear gradient in the same buffer and detected using the photometric assay for the reverse reaction described above. Active fractions were recovered with 50–70 mM NaCl. The combined active fractions were immediately applied on a 0.5×2 cm hydroxylapatit column and eluted with a linear gradient of 25 ml 10–200 mM NaP$_i$ (pH 6.8). Active fractions were eluted at 80–110 mM NaP$_i$, pooled and stored in 20% glycerol at −80°C. Typical enzyme preparations exhibit specific activities of about 21–28 nmol min^{-1} mg^{-1}. This corresponds to an enrichment of about 2000-fold with a yield of about 30%.

2.7. NMR experiments

2D NMR analysis was performed as described previously [3].

3. Results and discussion

3.1. Diphospho inositol phosphates in Dictyostelids

Cells were harvested in the early stationary phase for comparative studies. All of the *Dictyostelium* species investigated contained similar amounts of PP-InsP$_5$ and 5,6-bis-PP-InsP$_4$ (Table 1). Typical amounts of PP-InsP$_5$ were about 20 nmol per 10^9 cells, corresponding to intracellular concentrations of about 100 μM. The amounts of 5,6-bis-PP-InsP$_4$ were approximately three times higher and found to be about 60 nmol per 10^9 cells, corresponding to intracellular concentrations of about 300 μM. Both compounds were also found in similar quantities in the two *Polysphondylium* strains. As will be shown below, the PP-InsP$_5$ fraction in both species is a mixture of 6-PP-InsP$_5$ and 5-PP-InsP$_5$. However, in both of the *Polysphondylium* strains an additional compound was observed by HPLC analysis which was stronger retarded than 5,6-bis-PP-InsP$_4$. This compound was identified as a new bis-

Table 1
Representative amounts (nmol/10^9 cells) of highly phosphorylated inositol phosphates in Dictyostelids

Species	InsP$_6$	PP-InsP$_5$[a]	5,6-bis-PP-InsP$_4$	1(3),5-bis-PP-InsP$_4$
D. brunneum	185	21	74	–
D. discoideum AX2	149	15	48	–
D. discoideum NC4	156	19	47	–
D. giganteum	205	14	39	–
D. mucoroides	171	13	39	–
D. purpureum	176	17	59	–
P. pallidum	157	14	50	12
P. violaceum	190	24	65	17

[a]PP-InsP$_5$ is a mixture of 5-PP-InsP$_5$ and 6-PP-InsP$_5$ (*Dictyostelium* species: about 10% 5-PP-InsP$_5$, 90% 6-PP-InsP$_5$; *Polysphondylium* species: about 70% 5-PP-InsP$_5$, 30% 6-PP-InsP$_5$).

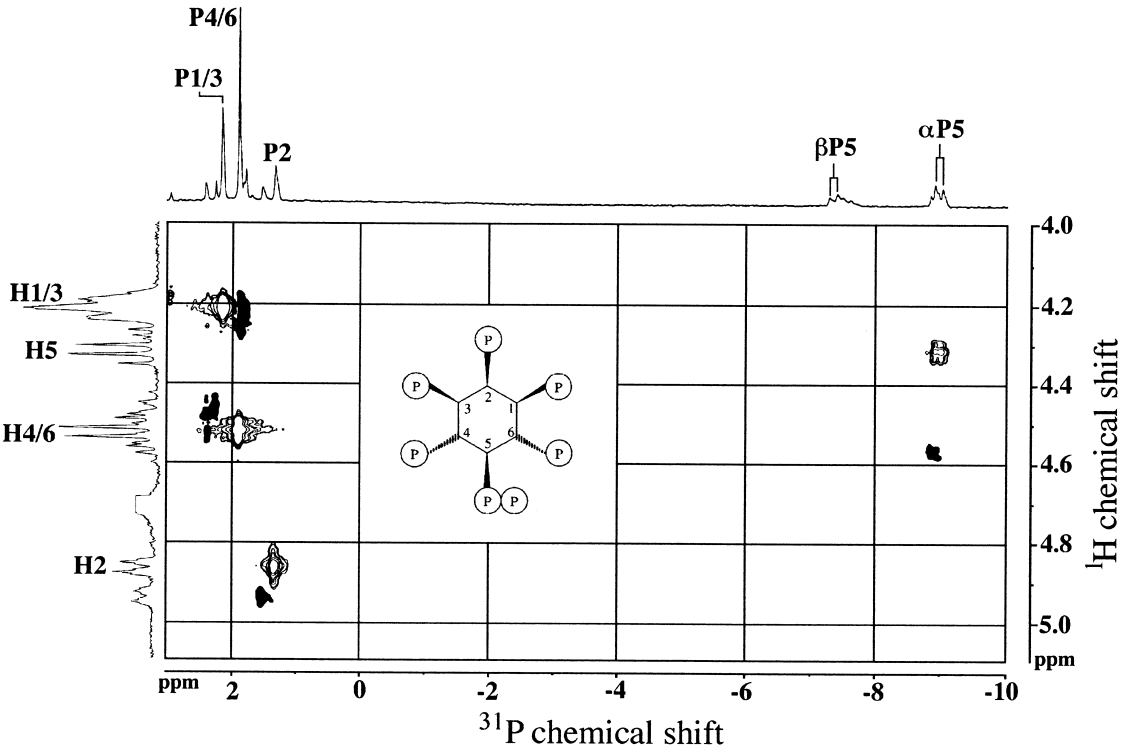

Fig. 3. 2D ^{1}H-^{31}P NMR correlation map (pH 6.0) of PP-InsP$_5$ isolated from *P. pallidum* (see Fig. 1B, peak I). This material is a mixture of two PP-InsP$_5$ isomers, as can be readily seen by the appearance of two H2 signals at 4.95 ppm (smaller signal) and 4.86 ppm (larger signal). The smaller signals are due to 6-PP-InsP$_5$, a compound known from *D. discoideum* [4]. The cross-signals of 6-PP-InsP$_5$ are filled black. The signals of the symmetric 5-PP-InsP$_5$ were assigned as described in Fig. 2. The ratio of 5-PP-InsP$_5$ and 6-PP-InsP$_5$ was determined by the intensity of the P2 resonances and confirmed enzymatically to be about 70:30.

PP-InsP$_4$ isomer. A representative HPLC analysis from extracts of *D. discoideum* NC4 and *P. pallidum* is shown in Fig. 1.

3.2. NMR analysis of diphospho inositol phosphates from Polysphondylium

The highly phosphorylated compounds eluting after InsP$_6$ (Fig. 1, peaks I–III) were purified from *P. pallidum* extracts in order to elucidate the structure of the additional substance and to confirm that the compounds eluting in the PP-InsP$_5$ and bis-PP-InsP$_4$ fractions are identical to those found in *Dictyostelium*. Starting from about 2×10^{11} cells, total amounts of about 2 mg of PP-InsP$_5$ (peak I, Fig. 1), 6 mg of bis-PP-InsP$_4$ (peak II, Fig. 1) and 2 mg of the compound eluting as peak III (Fig. 1) were obtained. These substances were investigated by two-dimensional ^{1}H-^{1}H and ^{1}H-^{31}P NMR spectroscopy.

Table 2
Detailed NMR data of 1(3),5-bis-PP-InsP$_4$ (pH 6.0) isolated from *P. pallidum*

Position	δ ^{31}P (ppm)	δ ^{1}H (ppm)	$^2J_{P-P}$ (Hz)
1α	−9.20	4.28	20.0
1β	ca. −7.2	–	20.0
2	1.13	4.95	–
3	2.43	4.19	
4	1.95	4.51	
5α	−8.95	4.33	19.2
5β	ca. −7.2	–	19.2
6	1.51	4.50	–

The ^{31}P-^{1}H NMR of the new compound (peak III, Fig. 1) is shown in Fig. 2. The proton resonances were identified by ^{1}H-^{1}H NMR starting from the only equatorial proton H2, which can be easily assigned as described earlier [3]. The ^{1}H-^{31}P NMR correlation map demonstrates that the α-phosphates of the two diphospho groups are coupled to H1(3) and H5, respectively. Consequently, the structure of this compound is either 1,5-bis-PP-InsP$_4$ or 3,5-bis-PP-InsP$_4$, which is the corresponding enantiomer. Enantiomers can not be distinguished by the NMR methods used. This compound is homogeneous according to NMR and the NMR data are detailed in Table 2.

The compound from *P. pallidum* extracts that elutes as peak II (Fig. 1) was investigated by the same NMR method and found to be 5,6-bis-PP-InsP$_4$ as previously described in *D. discoideum* AX2 (NMR data not shown). Since an achiral NMR method was used, the corresponding enantiomer 4,5-bis-PP-InsP$_4$ could be present as well. This compound was excluded by enzymatic studies, as demonstrated below. Like in *D. discoideum*, this compound is homogeneous according to both NMR and enzymatic experiments.

In contrast, the component of *P. pallidum* extracts that exhibits a similar retention time as 6-PP-InsP$_5$ from *D. discoideum* consists of a mixture of two isomers, namely 6-PP-InsP$_5$ (about 30%) and 5-PP-InsP$_5$ (about 70%) as shown by NMR analysis (Fig. 3). The mass ratio was estimated by the intensity of the P2 signals of both isomers in the ^{31}P NMR spectra. Detailed NMR data of 5-PP-InsP$_5$ are shown in Table 3.

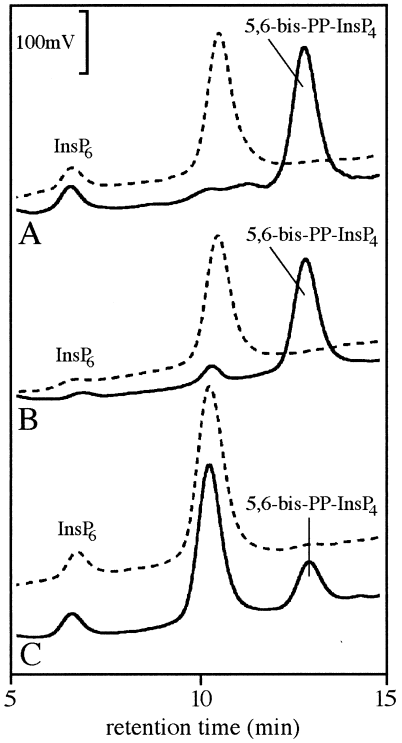

Fig. 4. Enzymatic study of PP-$InsP_5$ isomers using 6-PP-$InsP_5$-5-kinase from *D. discoideum* AX2. Gradient: (source-15-Q, 0.5×2 cm) 0 min: 200 mM HCl, 15 min: 450 mM HCl. Each compound (about 6 μM, 3 nmol injected for HPLC-MDD analysis) was incubated using an ATP regenerating system and 6-PP-$InsP_5$-5-kinase. Dotted lines show zero-time controls. Synthetic 6-PP-$InsP_5$ was completely converted to 5,6-bis-PP-$InsP_4$ after 10 min under these conditions (A). 30% of PP-$InsP_5$ isolated from *P. pallidum* (C) and 90% of PP-$InsP_5$ isolated from *D. discoideum* AX2 (B) were converted after 1 h of incubation. The remaining compound is 5-PP-$InsP_5$, which does not serve as a substrate for 6-PP-$InsP_5$-5-kinase.

3.3. Enzymatic studies using 6-PP-$InsP_5$-5-kinase and $InsP_6$-5-kinase from Dictyostelium

The 6-PP-$InsP_5$-5-kinase has been described in *D. discoideum* [4]. This enzyme is very specific for 6-PP-$InsP_5$, while other PP-$InsP_5$ isomers are not converted to any bis-PP-$InsP_4$. This specificity has been shown for 1-, 3-, 4- and 6-PP-$InsP_5$ previously [4]. Meanwhile, the two remaining meso-isomers 2- and 5-PP-$InsP_5$ have been synthesized [16]. Both meso-isomers are not converted by this enzyme either.

In order to verify the results from the NMR analysis, PP-$InsP_5$ isolated from *P. pallidum* was used as a substrate for the 6-PP-$InsP_5$-5-kinase from *D. discoideum*. While synthetic 6-PP-$InsP_5$ was almost quantitatively converted to 5,6-bis-PP-$InsP_4$, only 28 ± 2% ($n = 3$) of the *P. pallidum* PP-$InsP_5$ fraction was phosphorylated (Fig. 4A, C). This result supports the data obtained by the NMR investigation that PP-$InsP_5$ from *P. pallidum* consists of 6-PP-$InsP_5$ and 5-PP-$InsP_5$ in a ratio of about 30:70. The 5-PP-$InsP_5$ isomer does not modulate the activity of the 6-PP-$InsP_5$-5-kinase since the phosphorylation of synthetic 6-PP-$InsP_5$ is not significantly inhibited in the presence of up to 20 μM synthetic 5-PP-$InsP_5$.

In a recent investigation using high resolution HPLC analysis [12], 5-PP-$InsP_5$ has also been found in small amounts (estimated 10%) in the PP-$InsP_5$ fraction from *Dictyostelium*. However, 6-PP-$InsP_5$ and 5-PP-$InsP_5$ could not be completely resolved. Therefore, PP-$InsP_5$ fractions of *D. discoideum* AX2 cells from three independent preparations were also investigated using 6-PP-$InsP_5$-5-kinase as an analytic tool. The result was that 91 ± 2% ($n = 3$) of these preparations was converted to 5,6-bis-PP-$InsP_4$ (Fig. 4B). Obviously, the remaining 9 ± 2% is 5-PP-$InsP_5$. This result confirms the data obtained by chromatography [12]. It might be speculated whether this compound arises from hydrolysis of 5,6-bis-PP-$InsP_4$ during the isolation procedure using strong acidic conditions. Considering that 5-PP-$InsP_5$ is the predominant isomer in the closely related genus *Polysphondylium*, it seems to be likely that this isomer is a natural metabolite in *Dictyostelium* as well.

The heterogeneity of the PP-$InsP_5$ fraction of Dictyostelids was confirmed by a detailed HPLC analysis which showed

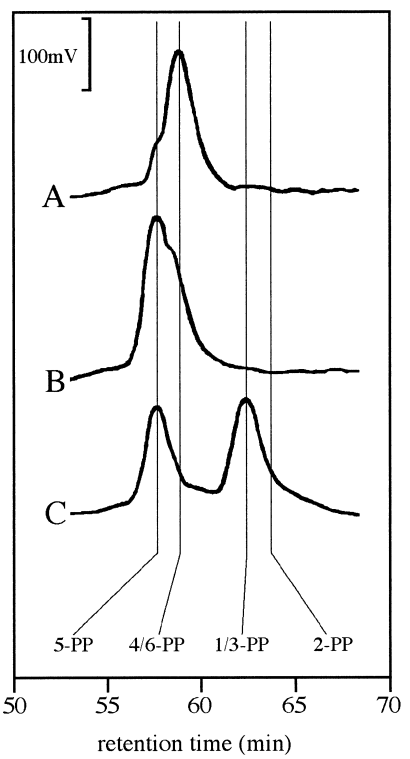

Fig. 5. High resolution HPLC-MDD chromatogram of PP-$InsP_5$ isomers. Gradient: (Mono-Q 1×10 cm) 0 min: 0 mM HCl, 50 min: 500 mM HCl, 70 min: 500 mM HCl. The elution times of the synthetic PP-$InsP_5$ isomers are shown by the vertical lines. A: PP-$InsP_5$ from the wild-type *D. discoideum* NC4 (exhibiting a small leading shoulder of 5-PP-$InsP_5$ followed by 6-PP-$InsP_5$, a similar peak shape was observed in all other *Dictyostelium* species). B: PP-$InsP_5$ from *P. pallidum* (consisting of 70% 5-PP-$InsP_5$, exhibiting a significant following shoulder of 6-PP-$InsP_5$, a similar peak shape was observed in *P. violaceum*). C: Products of spontaneous hydrolysis of 1(3),5-bis-PP-$InsP_4$ (retention time identical to 1(3)- and 5-PP-$InsP_5$).

Table 3
Detailed NMR data of 5-PP-$InsP_5$ (pH 6.0) isolated from *P. pallidum*

Position	δ ^{31}P (ppm)	δ ^{1}H (ppm)	$^{2}J_{P-P}$ (Hz)
1	2.19	4.20	–
2	1.34	4.86	–
3	2.19	4.20	–
4	1.92	4.51	–
5α	−8.93	4.31	19.0
5β	−7.30	–	19.0
6	1.92	4.51	–

that *Dictyostelium* PP-InsP_5 exhibited a leading shoulder of 5-PP-InsP_5 (Fig. 5A, similar as previously described [12]). A similar peak shape of PP-InsP_5 was observed in extracts of all *Dictyostelium* species investigated. In contrast, *Polysphondylium* PP-InsP_5 showed a following shoulder of 6-PP-InsP_5 (Fig. 5B). Additionally, it is demonstrated that during incubation for one day at room temperature at pH 6 about 20% of 1(3),5-bis-PP-InsP_4 is hydrolyzed and 1(3)-PP-InsP_5 and 5-PP-InsP_5 are formed in a 1:1 ratio in amounts corresponding to the hydrolyzed bis-PP-InsP_4 (Fig. 5C).

Since the reverse reaction catalyzed by the 6-PP-InsP_5-5-kinase from *D. discoideum* is very specific for 5,6-bis-PP-InsP_4 as a substrate, the photometric assay can be used to identify bisdiphospho inositol phosphates. The bisdiphospho inositol phosphate from *P. pallidum* eluting as peak II (Fig. 1) was completely converted to 6-PP-InsP_5. This reaction exhibited the same kinetics as observed with 5,6-bis-PP-InsP_4 isolated from *D. discoideum* AX2. Therefore, the occurrence of the enantiomer 4,5-bis-PP-InsP_4 could be excluded. On the other hand, 1(3),5-bis-PP-InsP_4 eluting as peak III, Fig. 1 is not converted by this enzyme.

3.4. Conclusions

Taken together, all five wild-type strains of *Dictyostelium* and the axenically growing mutant *D. discoideum* AX2 contain 6-PP-InsP_5 as the major PP-InsP_5 isomer and additionally lesser amounts of 5-PP-InsP_5. For the axenically growing mutant strain AX2, a similar result has been reported recently [12] using a different approach. In contrast, the two *Polysphondylium* strains investigated contain predominantly 5-PP-InsP_5 and lesser amounts of 6-PP-InsP_5. 5-PP-InsP_5 has been found in a number of phylogenetically different species such as Dictyostelids and mammalian cells.

5,6-bis-PP-InsP_4, previously isolated and identified in *D. discoideum* AX2, is present in similar amounts in all of the *Dictyostelium* wild-type strains. It is also the major bis-PP-InsP_4 isomer in the two *Polysphondylium* strains. An additional bis-PP-InsP_4 (1,5-bis-PP-InsP_4 or its corresponding enantiomer 3,5-bis-PP-InsP_4) is observed in both *Polysphondylium* strains, which is absent in *Dictyostelium*. The ratio of 5,6-bis-PP-InsP_4 to 1(3),5-bis-PP-InsP_4 is about 75:25. It can be excluded that 1(3),5-bis-PP-InsP_4 is formed artificially from 5,6-bis-PP-InsP_4 by phosphate migration, since this was never observed during handling and analysis of isolated 5,6-bis-PP-InsP_4 or in *Dictyostelium* extracts.

The structure of bis-PP-InsP_4 found in mammalian cells is currently unknown and therefore, the two bis-PP-InsP_4 from *Polysphondylium* may serve as standards for comparative HPLC studies. Additionally, the highly specific 6-PP-InsP_5-5-kinase from *Dictyostelium* is a valuable tool to distinguish 6-PP-InsP_5 from other PP-InsP_5 isomers. However, the metabolic network of the various diphospho inositol and the function of these compounds still remains elusive.

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