RESEARCH COMMUNICATION

Diphospho-*myo*-inositol phosphates from *Dictyostelium* identified as D-6-diphospho-*myo*-inositol pentakisphosphate and D-5,6-bisdiphospho-*myo*-inositol tetrakisphosphate

Tim LAUSSMANN*, Komandla Malla REDDY†, K. Kishta REDDY†, J. R. FALCK† and Günter VOGEL*‡

*Fachbereich 9 – Chemie, Bergische Universität GHS Wuppertal, Gausstrasse 20, D-42097 Wuppertal, Federal Republic of Germany, and †Department of Biochemistry, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9038, U.S.A.

Two diphospho-*myo*-inositol phosphates from *Dictyostelium* were recently investigated by two-dimensional $^1\text{H}/^{31}\text{P}$ NMR analysis and assigned to be either D-4-diphospho-*myo*-inositol pentakisphosphate (D-4-*PP*-Ins P_5) and D-4,5-bisdiphospho-*myo*-inositol tetrakisphosphate (D-4,5-bis-*PP*-Ins P_4) or their corresponding enantiomers D-6-*PP*-Ins P_5 and D-5,6-bis-*PP*-Ins P_4 . In the present study the naturally occurring enantiomers were

identified by using defined synthetic *PP*-Ins P_5 isomers as substrates for a partially purified *PP*-Ins P_5 5-kinase from *Dictyostelium*. This enzyme specifically phosphorylates the naturally occurring *PP*-Ins P_5 and the synthetic D-6-*PP*-Ins P_5 , leading to D-5,6-bis-*PP*-Ins P_4 . In contrast, neither D-4-*PP*-Ins P_5 nor D-1-*PP*-Ins P_5 or D-3-*PP*-Ins P_5 are converted by the enzyme.

INTRODUCTION

Dictyostelium cells contain two diphosphorylated *myo*-inositol phosphates at intracellular concentrations over the range 0.02–0.30 mM, depending on cell density and growth conditions. In the original studies, these compounds were preliminarily characterized and recognized as diphosphoinositol pentakisphosphate (*PP*-Ins P_5) and bisdiphosphoinositol tetrakisphosphate (bis-*PP*-Ins P_4) [1,2]. Recently their structures have been elucidated by two-dimensional $^1\text{H}/^{31}\text{P}$ NMR analysis [3]. This investigation showed, that *PP*-Ins P_5 is either D-4-*PP*-Ins P_5 or its enantiomer D-6-*PP*-Ins P_5 and bis-*PP*-Ins P_4 is either D-4,5-bis-*PP*-Ins P_4 or its enantiomer D-5,6-bis-*PP*-Ins P_4 . A differentiation between enantiomers was not possible by the NMR methods used.

Diphosphoinositol phosphates have been detected in a number of mammalian cell types and are currently being intensively investigated with regard to their metabolism and function [4–7].

Up until now, the *in vivo* function of diphosphoinositol phosphates has not been known, but high-affinity binding of *PP*-Ins P_5 to clathrin assembly protein AP3 from bovine brain [8] and Golgi coatmer from ox and yeast [9,10] may indicate that they are involved in the regulation of vesicle trafficking and endocytosis.

The *vicinal* arrangement of the diphospho groups in bis-*PP*-Ins P_4 from *Dictyostelium* probably causes steric and electrostatic constraints and possibly qualifies this compound as a metabolite with high phosphate-group-transfer potential in specialized phosphotransferase reactions. This idea is supported by the recent finding that a purified kinase from rat brain, which specifically phosphorylates Ins P_6 to *PP*-Ins P_5 , can also act in reverse, transferring a phosphate group from *PP*-Ins P_5 to ADP to form ATP [7].

Meanwhile, the total synthesis of *PP*-Ins P_5 isomers has been achieved ([11]; G. W. Mayr, C. Albert, S. T. Safrany, M. E. Bembenek, K. M. Reddy, K. K. Reddy, J. R. Falck and S. B. Shears, unpublished work). In the present study a *PP*-Ins P_5

kinase from *Dictyostelium*, which specifically phosphorylates *PP*-Ins P_5 , leading to bis-*PP*-Ins P_4 , was identified and partially purified. This enzyme was used as a tool to clarify whether D-4-*PP*-Ins P_5 or D-6-*PP*-Ins P_5 is the naturally occurring enantiomer. For that purpose, all available synthetic *PP*-Ins P_5 isomers were employed as substrates in the kinase reaction.

MATERIALS AND METHODS

Materials

D-1-*PP*-Ins P_5 , D-3-*PP*-Ins P_5 , D-4-*PP*-Ins P_5 and D-6-*PP*-Ins P_5 were synthesized as described ([11]; G. W. Mayr, C. Albert, S. T. Safrany, M. E. Bembenek, K. M. Reddy, K. K. Reddy, J. R. Falck and S. B. Shears, unpublished work). *PP*-Ins P_5 was additionally isolated from *Dictyostelium discoideum* (strain AX2, A.T.C.C. 24397) by a procedure described previously [3]. NaF, MgCl $_2$, Na $_2$ ATP, phosphocreatine, creatine kinase and Hepes buffer were supplied by Sigma. 4-(2-Pyridylazo)resorcinol was obtained from Fluka and YCl $_3$ from Aldrich. Tris buffer was purchased from Biomol. Sephacryl S 200 HR gel-chromatography material, Q-Sepharose fast flow and Source-15 Q anion-exchange material and sulphopropyl (SP)-Sephacryl fast flow cation-exchange material were from Pharmacia. Standard proteins for molecular-mass determination were obtained from Boehringer-Mannheim and Sigma. Filters (5 μm pore size) were supplied by Nucleopore. Centriprep 10 concentrators were purchased from Amicon.

Assay of *PP*-Ins P_5 kinase activity

Enzyme activity was assayed in a reaction mixture containing 30 mM Hepes (pH 6.8), 6 mM MgCl $_2$, 5 mM NaF, 5 mM Na $_2$ ATP, 5 mM phosphocreatine, 2 units/ml creatine kinase and 7.5 μM of the particular *PP*-Ins P_5 isomer in a final volume of 0.8 ml. The reaction mixture was incubated at 25 °C and the

Abbreviations used: *PP*-Ins P_5 , diphospho-*myo*-inositol pentakisphosphate; bis-*PP*-Ins P_4 , bisdiphospho-*myo*-inositol tetrakisphosphate; SP-, sulphopropyl-; MDD, metal dye detection.

‡ To whom correspondence should be addressed

reaction was terminated after 2–60 min by diluting the sample with 1.2 ml of ice-cold water. Amounts of $PP\text{-InsP}_5$ and bis- $PP\text{-InsP}_4$ were immediately determined by the metal-dye-detection (MDD)/HPLC method [12,13]. Samples were separated on a high-resolution 1 cm $\times$ 0.5 cm Source-15 Q column with a 30 min linear gradient of 200–425 mM HCl. Detection at 546 nm was performed with a modified MDD post-column dye reagent [2 M Tris/HCl (pH 8.5)/200 μ M 4-(2-pyridylazo)resorcinol/30 μ M YCl_3 /10 % (v/v) methanol]. Amounts of 0.2–10 nmol of $PP\text{-InsP}_5$ are detectable using this method. No enzyme activity was observed in zero-time incubations in the absence, or after boiling, of enzyme solutions.

Purification of $PP\text{-InsP}_5$ kinase

D. discoideum cells (strain AX2, A.T.C.C. 24397) were grown in AX2 medium [14], supplemented with 1.8 % maltose instead of glucose. For a typical purification procedure, about 1×10^{10} cells (about 10 g wet weight) were collected by centrifugation for 10 min at 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 μ m-pore-size filter. All operations were performed at 2–4 °C. Cell debris and membranes were removed by ultracentrifugation (45 min, 4 °C, 100 000 *g*). The supernatant was applied on a Q-Sepharose column (1.5 cm $\times$ 20 cm) equilibrated with 50 mM Hepes, pH 6.8. The $PP\text{-InsP}_5$ kinase activity was not retarded, but was eluted with the starting buffer. The enzyme solution was subsequently loaded on an SP-Sepharose column (1 cm $\times$ 3 cm). The column was washed with starting buffer until non-adsorbed proteins were removed. Enzyme activity was eluted with 100 mM NaCl in the same buffer. Active fractions were combined and concentrated with a Centriprep 10 concentrator to 0.7 ml and placed on a Sephacryl S 200 HR gel-filtration column (2 cm $\times$ 80 cm) equilibrated with 50 mM Hepes (pH 6.8)/100 mM NaCl/1 mM EDTA and eluted at 0.4 ml/min. Active fractions were combined and stored in 20 % glycerol at –80 °C with about 30 % loss of enzyme activity after 1 week. Standard proteins for molecular-mass determination were aldolase (158 kDa), creatin kinase (80 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa) and cytochrome *c* (12.5 kDa) in order to calibrate the column.

RESULTS AND DISCUSSION

$PP\text{-InsP}_5$ kinase activity was measured by HPLC/MDD [12,13], which allows the simultaneous determination of $PP\text{-InsP}_5$ decrease and bis- $PP\text{-InsP}_4$ formation. The inositol phosphates were separated on a high-resolution anion-exchange column using a HCl gradient and were detected by a post-column dye reagent.

Almost all $PP\text{-InsP}_5$ kinase activity was recovered in the soluble supernatant fraction of *Dictyostelium* extracts after centrifugation at 100 000 *g*. About 90 % of accompanying

Figure 1 Substrate specificity of $PP\text{-InsP}_5$ kinase from *Dictyostelium*

All samples were incubated for 10 min at 25 °C with pre-purified $PP\text{-InsP}_5$ kinase. Only D-6- $PP\text{-InsP}_5$ and *D. discoideum* (*D. d.*) $PP\text{-InsP}_5$ are almost completely converted into 5,6-bis- $PP\text{-InsP}_4$. It should be noted that D-1- $PP\text{-InsP}_5$ and D-3- $PP\text{-InsP}_5$ have remarkably different retention times in comparison with the natural $PP\text{-InsP}_5$. Contaminating InsP_6 is due to hydrolysis of the particular $PP\text{-InsP}_5$ isomers.

proteins are adsorbed to Q-Sepharose, while the kinase activity is eluted with starting buffer without retardation. However, the enzyme adsorbs to SP-Sepharose and can be eluted with 100 mM NaCl. A gel-filtration column affords another 3-fold purification. The elution profile of the enzyme compared with that of standard proteins indicates a molecular mass of the native enzyme of about 40 kDa. The results of a typical purification procedure, starting with 10 g wet weight of *Dictyostelium* cells are summarized in Table 1 and show that an approx. 170-fold increase in specific activity with 40 % recovery of enzyme activity was achieved.

Subsequently, the specificity of the enzyme was studied by using synthetically derived $PP\text{-InsP}_5$ isomers. The previous NMR

Table 1 Purification of $PP\text{-InsP}_5$ kinase

Fraction	Protein (mg)	Specific activity (nmol/min per mg)	Recovery (%)
Cytosolic extract	356	0.05	—
Q-Sepharose	48	0.4	91
S-Sepharose	9	2.8	83
Sephadex	0.8	8.5	40

Figure 2 Kinetic study of D-5,6-bis-PP-InsP₄ formation (◆)

PP-InsP₅ isolated from *Dictyostelium* (□) and synthetic D-6-PP-InsP₅ (○) were compared as substrates for PP-InsP₅ kinase.

investigation of the diphosphoinositol phosphates from *Dictyostelium* showed that either D-4-PP-InsP₅ or D-6-PP-InsP₅ is the naturally occurring PP-InsP₅ and that bis-PP-InsP₅ is additionally diphosphorylated at the 5-position of the inositol backbone. Therefore D-4-PP-InsP₅ or D-6-PP-InsP₅ must be considered as substrates for a PP-InsP₅ 5-kinase, leading to D-4,5-bis-PP-InsP₄ or D-5,6-bis-PP-InsP₄ respectively. These two compounds, as well as D-1-PP-InsP₅ and D-3-PP-InsP₅, were employed as substrates for the partially purified PP-InsP₅ kinase (Figure 1). Only D-6-PP-InsP₅ was phosphorylated, while its enantiomer, D-4-PP-InsP₅, was not converted. To support this result further, synthetic D-6-PP-InsP₅ and PP-InsP₅ isolated from *Dictyostelium* were compared in a kinetic study. Both compounds show the same kinetics in the kinase reaction (Figure 2). This is strong evidence that D-6-PP-InsP₅ is the natural enantiomer. Since the velocity of the reaction is constant until a substrate concentration below 2 μM is reached, the enzyme obviously has a very high affinity. The K_m value could not be determined exactly by HPLC/MDD because of limited sensitivity, but is significantly lower than 2 μM. This is comparable with the K_m value of 0.7 μM found for the InsP₆ kinase from rat

Figure 3 Structures of diphosphoinositol phosphates from *D. discoideum*

brain [7]. The PP-InsP₅ kinase from *Dictyostelium* is currently being further purified and characterized.

In this investigation it is established that the diphosphoinositol phosphates from *Dictyostelium* are D-6-PP-InsP₅ and D-5,6-bis-PP-InsP₄ (Figure 3). Nevertheless, the structures of the diphosphoinositol phosphates found in other species are still not known with certainty. Therefore it would be of great interest to make a comparative study either by HPLC or by using PP-InsP₅ from other species as substrates for the D-6-PP-InsP₅ 5-kinase from *Dictyostelium*.

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