Identification and Purification of Diphosphoinositol Pentakisphosphate Kinase, Which Synthesizes the Inositol Pyrophosphate Bis(diphospho)inositol Tetrakisphosphate†

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ABSTRACT: Diphosphoinositol pentakisphosphate (PP-IP_5) and bis(diphospho)inositol tetrakisphosphate (bis-PP-IP_4) were recently identified as inositol phosphates which possess pyrophosphate bonds. The molecular mechanisms that regulate the cellular levels of these compounds are not yet characterized. To pursue this question, we have previously purified an inositol hexakisphosphate (IP_6) kinase from rat brain supernatants [Voglmaier, S. M., et al. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4305−4310]. We now report the identification and purification of another novel kinase, diphosphoinositol pentakisphosphate (PP-IP_5) kinase, which uses PP-IP_5 as a substrate to form bis(diphospho)inositol tetrakisphosphate (bis-PP-IP_4) in soluble fractions of rat forebrain. The purified protein, a monomer of 56 kDa, displays high affinity (K_m = 0.7 μM) and selectivity for PP-IP_5 as a substrate. The purified enzyme also can transfer a phosphate from bis-PP-IP_4 to ADP to form ATP. This ATP synthase activity is an indication of the high phosphoryl group transfer potential of bis-PP-IP_4 and may represent a physiological role for PP-IP_5 and bis-PP-IP_4.

Inositol 1,4,5-trisphosphate (1,4,5-IP_3)[1] is a major second messenger molecule whose signal transduction activities stem from its release of intracellular calcium (*1*). Numerous other inositol polyphosphates have been identified, of which inositol hexakisphosphate (IP_6) is the most abundant with millimolar concentrations in some tissues, substantially greater than IP_3 levels (*2*−*6*). Recently, inositol diphosphates, also referred to as inositol pyrophosphates, in which seven or eight phosphates are linked to the inositol ring have been identified (*7*−*9*). These are termed diphosphoinositol pentakisphosphate (PP-IP_5) and bis(diphospho)inositol tetrakisphosphate (bis-PP-IP_4). The inositol diphosphates turn over quite rapidly (*8*, *10*, *11*) and occur in substantial concentrations in cells, with levels estimated in some tissues to be 0.5−3 μM (*8*−*11*).

To characterize the biosynthesis of the inositol diphosphates, we recently identified and purified an IP_6 kinase (IP_6-K) from rat brain (*12*). We demonstrated that this enzyme can also transfer the terminal phosphate of the diphosphate of PP-IP_5 to ADP to form ATP (*12*). We wondered whether a unique enzyme for converting PP-IP_5 to bis-PP-IP_4 exists. In this study, we demonstrate that purified IP_6-K does not possess this activity. Instead, we have identified and purified a novel enzyme which selectively forms bis-PP-IP_4 from PP-IP_5. We also report the newly identified PP-IP_5 kinase (PP-IP_5-K) can convert bis-PP-IP_4 to PP-IP_5 and synthesizes ATP in the process.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]IP_6, [γ-^{32}P]ATP, [^{3}H]PP-IP_5, PP-IP_5, and bis-PP-IP_4 were supplied by NEN/DuPont. KH_2PO_4, K_2HPO_4, and KCI were obtained from Aldrich. Mono Q HR 10/10 and Superdex 75 chromatography columns and gel filtration standards were from Pharmacia. Leupeptin, pepstatin, aprotinin, chymostatin, and antipain were purchased from Chemicon. Okadaic acid, IP_3, and IP_4 were obtained from LC Services. 1,3,4,5,6-IP_5 and IP_6 were from Calbiochem. 1,2,4,5,6-IP_5 was a kind gift from J. F. Maracek (State University of New York, Stony Brook, NY). PEI-TLC plates were obtained from EM Separations (Merck). HEPES buffer was from Research Organics. Nonradioactive nucleotides, nucleotide analogues, and nucleoside diphosphate kinase (EC 2.7.4.6) were obtained from Boehringer Mannheim. SDS−PAGE gels and molecular weight standards were from Novex. Protein assay dye reagents were from Pierce. Heparin−agarose, phosphocreatine, creatine phosphokinase (EC 2.7.3.2), and all other reagents were obtained from Sigma.

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[1] Abbreviations: 1,4,5-IP_3, inositol 1,4,5-trisphosphate; PP-IP_5, diphosphoinositol pentakisphosphate; bis-PP-IP_4, diphosphoinositol tetrakisphosphate; IP_3, inositol trisphosphate; IP_4, inositol tetrakisphosphate; IP_5, inositol pentakisphosphate; IP_6, inositol hexakisphosphate; IP_6-K, IP_6 kinase; PP-IP_5-K, PP-IP_5 kinase; PEI-TLC, polyethyleneimine−cellulose thin-layer chromatography.

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Synthesis of [β-^{32}P]PP-IP$_5$ and [β-^{32}P]Bis-PP-IP$_4$. These compounds were synthesized essentially as described previously (*8*, *12*, *13*) and supplied by NEN/DuPont. Briefly, inositol polyphosphate kinase activity was isolated from rabbit brain homogenates as described previously (*12*). However, homogenates were centrifuged in an SS 34 rotor for 20−50 min at 16 000 rpm. The resulting supernatant was bulk incubated with heparin−agarose (Sigma) for 1 h on ice. The beads were vacuum filtered and rinsed in homogenizing buffer and finally resuspended in buffer containing 0.6 M KCI. After 1 h, the eluate was collected and the proteins were precipitated by addition of 20% PEG 3300 (Sigma). Following centrifugation, the pellets were resuspended in buffer. The crude kinase fraction was then used to synthesize [β-^{32}P]PP-IP$_5$ and [β-^{32}P]bis-PP-IP$_4$. In general, [γ-^{32}P]ATP was added at the desired specific activity and incubated in 1−2 mL of 75 mM Hepes-tris (pH 7.5), 0.2 M KCI, 4 mM DTT, 10 mM NaF, 10 mM $MgCl_2$, 1 mM EGTA, and $^1/_{10}$ volume of a kinase fraction containing either 0.5 mM IP$_6$ or 50−150 μM PP-IP$_5$. The reaction was terminated by deproteinization, and purification of the products was achieved by ion-exchange chromatography (Figure 1).

Assay of PP-IP$_5$ Kinase Activity. Enzyme activity during the different purification steps was assayed in 10 μL of reaction mixture containing 20 mM HEPES (pH 6.8), 1 mM DTT, 6 mM $MgCl_2$, 5 mM Na_2ATP, 10 mM phosphocreatine, 40 units/mL creatine phosphokinase, 5 mM NaF, 5 μM PP-IP$_5$, and 10−20 nM [^{3}H]PP-IP$_5$ and incubated at 37 °C for 10−30 min, under zero-order kinetics. Reactions were terminated either by addition of 1 μL of 1 M HCI or by immersion in an ice/water bath. We adapted methods used to separate IP$_6$, IP$_5$, IP$_4$, IP$_3$, ATP, and P$_I$ using polyethyleneimine−cellulose thin-layer chromatography (PEI-TLC) (*14*, *15*) to separate IP$_6$, PP-IP$_5$, bis-PP-IP$_4$, and ATP. The entire reaction mixture was spotted in 2.5 μL aliquots onto a PEI-TLC plate, which was developed in 1.1 M KH_2PO_4, 0.8 M K_2HPO_4, and 2.3 M HC1. The lanes were cut into 1 cm strips, and they were counted with 15 mL of duPont Formula 963 scintillation cocktail. Approximately 25% of the added ^{3}H applied to the plates was recovered by this method. Alternatively, the PEI-TLC strips were first shaken with 3 mL of concentrated HC1 and then counted with 5 mL of H_2O and 10 mL of scintillation cocktail. Up to 80% of the added ^{3}H was recovered by this method. [^{3}H]IP$_6$, [^{3}H]-PP-IP$_5$, and [^{3}H]bis-PP-IP$_4$ migrated with R_f values of approximately 0.75, 0.45, and 0.3, respectively, and comigrated with standard preparations of [^{3}H]IP$_6$, [^{3}H]PP-IP$_5$, and [^{3}H]bis-PP-IP$_4$, respectively. No bis-PP-IP$_4$ was formed under the following conditions: at 0 °C, in zero-time incubations, in the absence of brain extract or partially purified enzyme, with boiled (5 min) brain extract or partially purified enzyme, when partially purified enzyme was replaced with creatine phosphokinase (40 units/mL) or nucleoside diphosphate kinase (0.5 mg/mL), in the presence of 0.1 M HCl or 0.1 M NaOH, in the absence of ATP, or when ATP was replaced by 5 mM CTP, GTP, phosphocreatine, phosphoenolpyruvate, acetyl phosphate, or glucose 6-phosphate (data not shown). For K_m determinations, done under initial rate conditions (<10% product formation), MgATP was used at the indicated concentrations, and 5 mM $MgCl_2$ was also included in the assay.

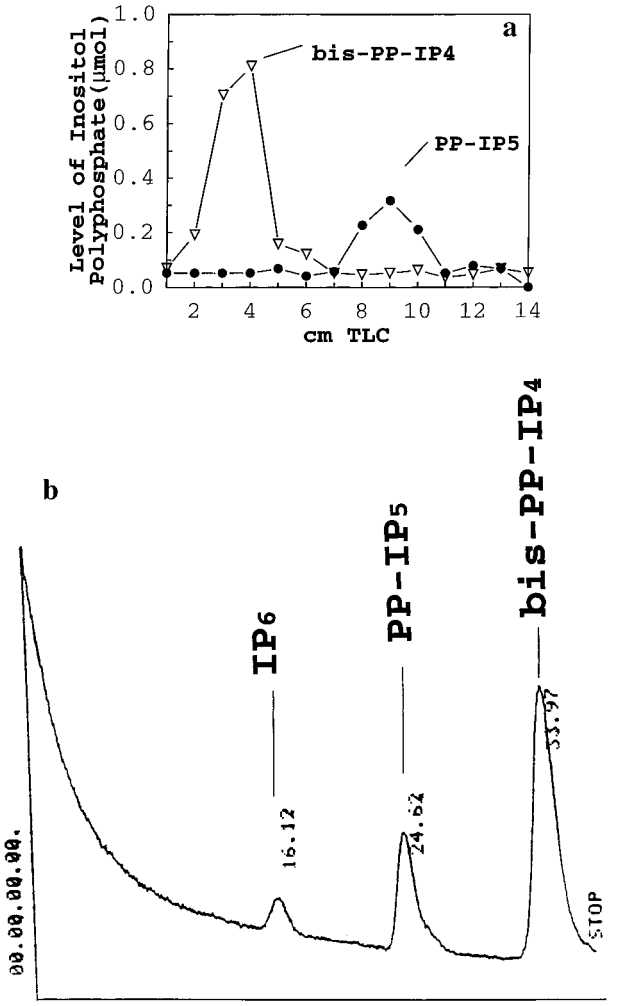

FIGURE 1: Identification of bis-PP-IP$_4$. Enzyme fractions was purified from soluble fractions of rat forebrains on a heparin−agarose column and assayed as described. (a) PEI-TLC analysis of ^{3}H-labeled inositol polyphosphate. Products from the PP-IP$_5$ kinase reaction were spotted onto PEI-TLC plates and developed as described. Plates were visualized and analyzed by phosphorimager or cut into strips and the strips counted with a β-counter in the presence of scintillation cocktail. [^{3}H]PP-IP$_5$ and [^{3}H]bis-PP-IP$_4$ peaks were identified by comparison with known standards. (b) HPLC chromatogram of ^{32}P-labeled inositol polyphosphates. Products from the inositol polyphosphate kinase reaction were fractionated on an ion-exchange column and compared with known standards. Times for elution are 16.12 min for [^{32}P]IP$_6$, 24.62 min for [β-^{32}P]PP-IP$_5$, and 33.97 min for [β-^{32}P]bis-PP-IP$_4$.

Assay of ATP Synthase Activity. The ability of the enzyme to synthesize ATP was assayed in 10 μL of reaction mixture containing 20 mM HEPES (pH 6.8), 1 mM DTT, 5 mM Na_2ADP, 5 mM $MgCl_2$, 5 μM bis-PP-IP$_4$, and 20 nM [β-^{32}P]-bis-PP-IP$_4$ (25−80 Ci/mmol), and the solution was incubated at 37 °C for 10−30 min. All solutions were adjusted to pH 6.8. The reactions were terminated as above and loaded onto PEI-TLC plates developed either as described above or in 1 M KH_2PO_4. In 1 M KH_2PO_4, [β-^{32}P]bis-PP-IP$_4$ and [^{32}P]-ATP migrated with R_f values of approximately 0.03 and 0.55, respectively, and comigrated with standards (data not shown). No [γ^{32}P]ATP was formed from [β-^{32}P]PP-IP$_5$ at 0 °C, in zero-time incubations, in the absence of brain extract or partially purified enzyme, when partially purified enzyme was replaced with creatine phosphokinase (40 units/mL) or nucleosidediphosphate kinase (0.5 mg/mL), with boiled (5

min) brain extract or partially purified enzyme, or in the absence of ADP. No decrease in the [β-^{32}P]bis-PP-IP_4 level was observed when 5 mM ADP was replaced by 5 mM CDP, GDP, UDP, AMP, creatine, glucose, or 3-phosphoglycerate as the phosphate acceptor. For K_m determinations, done under initial rate conditions (<10% product formation), $MgCl_2$ was added in a 1:1 ratio with Na_2ADP.

Purification of the Bis-PP-IP_5 Kinase. Forebrains (70 g wet weight) from 70 adult male (175−300 g) Sprague-Dawley rats were homogenized in 150 mL of ice-cold buffer A containing 20 mM HEPES (pH 6.8), 4 mM DTT, 2 mM EGTA, 0.75 mM EDTA, 5 mM NaF, 1.5 mM Na_3VO_4, 0.5 mg/L okadaic acid, 4 mg/L chymostatin, 4 mg/L pepstatin, 4 mg/L antipain, 8 mg/L leupeptin, 8 mg/L aprotinin, and 200 mg/L phenylmethanesulfonyl fluoride. All subsequent procedures were performed at 0−4 °C. The homogenate was centrifuged at 100000*g* for 90 min. The supernatant was agitated with 70 mL of heparin−agarose in the presence of 1 mM $MgCl_2$ and 0.1% CHAPS for 60 min. The resin was washed with 450 mL of 0.25 M KC1 in buffer A with 1 mM $MgCl_2$ minus okadaic acid, then eluted by agitating for 60 min with 75 mL of 0.5 M KCl at pH 7.4 with 1 mM EDTA in buffer A minus all phosphatase inhibitors, poured onto a column, eluted, and then rinsed with an additional 50 mL of the same buffer. The combined heparin column eluate was diluted to 1 L in buffer B [20 mM HEPES (pH 6.8), 1 mM DTT, 1 mM EGTA, 2 mg/L chymostatin, 2 mg/L pepstatin, 2 mg/L antipain, 4 mg/L leupeptin, and 0.1% CHAPS] and applied to a Mono Q HR 10/10 FPLC column at 4 mL/min; the column was washed with 50 mL of 0.05 M KCl and eluted at 0.5 mL/min with a 60 mL linear KCl gradient from 0.05 to 0.45 M KCl in buffer B and 0.6% CHAPS. This buffer was used in all further purification steps. The pooled peak of bis-PP-IP_5 kinase activity was diluted to 0.05 M KCl and loaded onto a 1 cm × 15 cm ATP−agarose column at 0.5 mL/min and the flow-through passed over the column again. The column was then washed with 50 mL of a 0.05 to 0.15 M KCl gradient and eluted with 20 mL of a 10 to 25 μM IP_6 gradient. The peak fractions were diluted to 2 μM IP_6, applied to a 0.5 cm × 4 cm heparin−agarose column, washed with 20 mL of 0.3 M KCl, and eluted at 0.2 mL/min with a 20 mL linear gradient from 0.3 to 0.6 M KCl. This further purification step resulted in 40% loss of activity, and no apparent increase in enzyme purity was observed. Subsequent purifications were stopped after the ATP−agarose column step.

Other Methods. Inositol 1,4,5-trisphosphate 3-kinase activity was assayed as described previously (*15*). Protein assays were performed according to directions for Pierce Coomassie Blue and Fluoraldehyde protein assay reagents. SDS−PAGE was performed by the method of Laemmli (*16*).

RESULTS

Identification of PP-IP_5-K Activity Distinct from IP_6-K. In efforts to identify the extent of formation of bis-PP-IP_4, we incubated rat brain supernatant or the supernatant preparation partially purified with a heparin−agarose step with [^{3}H]PP-IP_5 and separated metabolites by PEI-TLC (Figure 1a) and by ion-exchange chromatography (data not shown). With TLC as well as in ion-exchange chromatography, we demonstrate the conversion of [^{3}H]PP-IP_5 to a product that migrates in a manner identical to that of authentic [^{3}H]bis-PP-IP_4. The migratory pattern determined

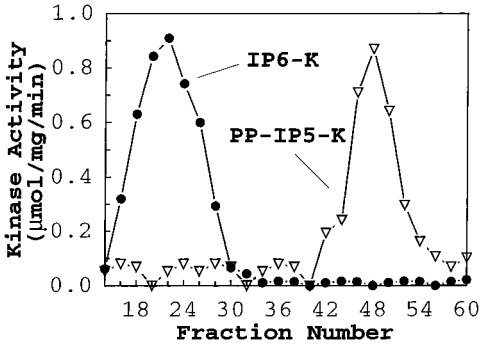

FIGURE 2: Separation of IP_6 kinase and PP-IP_5 kinase by MonoQ chromatography. After the heparin−agarose column step in PP-IP_5-K purification, the preparation was subjected to MonoQ FPLC chromatography. The final eluate was collected as 1 mL fractions. IP_6-K and PP-IP_5-K activities were assayed as described in ref *12*.

Table 1: Purification of PP-IP_5 Kinase[a]

fraction	protein (mg)	purification (-fold)	recovery (%)
rat brain supernatant	1.836	–	–
heparin−agarose (batch)	24	64	83
FPLC Mono-Q	2	520	57
ATP−agarose	0.027	8522	12

[a] Enzyme was purified and fractions were assayed as described. Data presented are from a typical experiment, which was repeated three times with similar results.

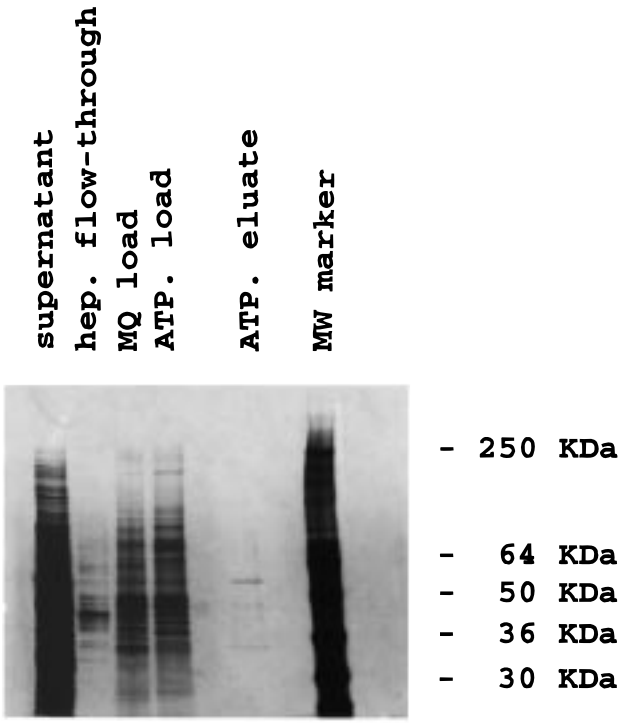

FIGURE 3: SDS−PAGE analysis of purified PP-IP_5 kinase. A 4 to 20% polyacrylamide gradient SDS gel was silver stained: lane 1, 1000*g* brain extract supernatant; lane 2, heparin−agarose column flow-through; lane 3, MonoQ FPLC load; lane 4, ATP−agarose load; lane 6, ATP−agarose eluate; and lane 8, molecular mass standards myosin (250 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), and myoglobin (30 kDa). Lanes 5 and 7 were empty.

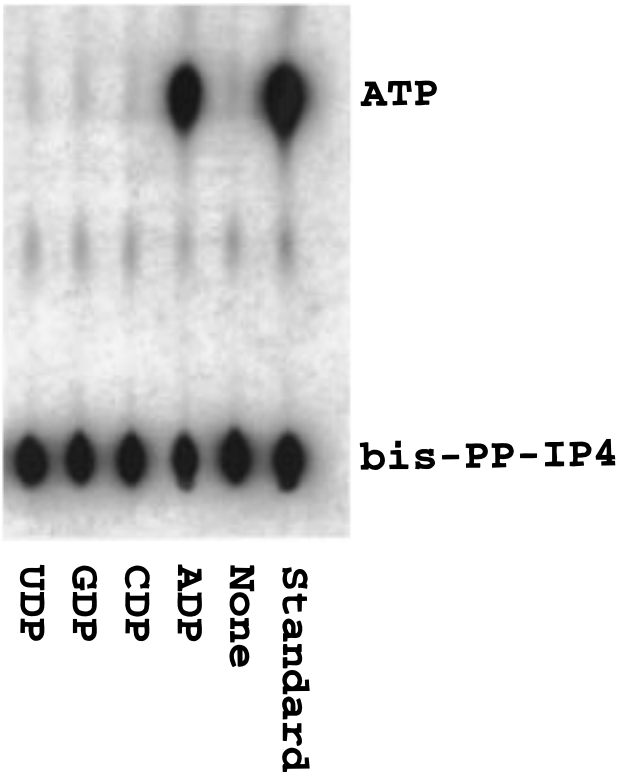

FIGURE 4: Nucleoside diphosphate specificity of the PP-IP_5 kinase reverse reaction (ATP synthase activity). For lanes GDP, CDP, UDP, and ADP, the ATP synthase activity of the purified enzyme was assayed as described with the indicated nucleoside diphosphate at 5 mM. In the lane marked standard, approximately 15 000 cpm each of [γ-^{32}P]ATP and [β-^{32}P]bis-PP-IP_4 were applied at the origin of the PEI-TLC plate. In the lane marked none, no nucleoside diphosphate was added for the ATP synthase activity assay. Tha TLC plate was exposed to a phosphor screen and scanned by a Molecular Dynamics Phosphorimager.

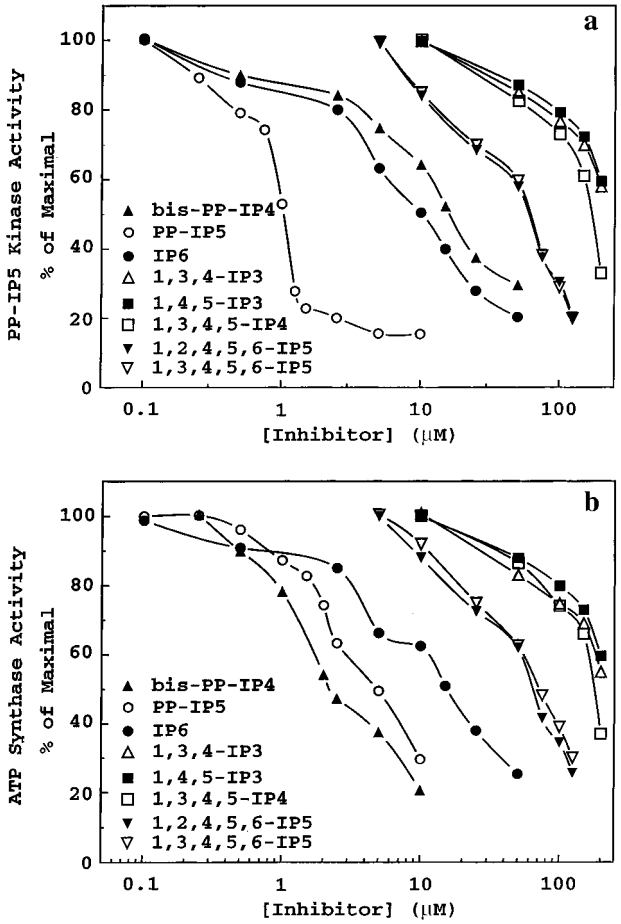

FIGURE 5: Inositol phosphate specificity of the PP-IP_5 kinase forward and reverse reactions: (a) inhibition of the forward reaction (PP-IP_5 kinase activity), assayed with 5 mM ATP and 10 nM [^{3}H]-PP-IP_5, and (b) inhibition of the reverse reaction (ATP synthase activity), assayed with 5 mM ADP and 20 nM [β-^{32}P]bis-PP-IP_4. Peak fractions from the second heparin column were used (similar results were obtained with the purified protein). Data are means of triplicate determinations of three experiments.

by ion-exchange chromatography reveals a greater negative charge for the product, consistent with [^{3}H]bis-PP-IP_4 formation. At the present time, we cannot determine the exact positional arrangement of the two diphosphate groups. Recently, it has been shown that in *Dictyostelium discoideum* the bis(diphospho)inositol tetrakiphosphate exists as 4,5-bis-PP-IP_4 and/or 5,5-bis-PP-IP_4 (*17*, *18*). However, the structure of mammalian bis-PP-IP_4 has not yet been elucidated.

For the synthesis of [β-^{32}P]PP-IP_5 and [β-^{32}P]bis-PP-IP_4, the formation of bis-PP-IP_4 was further established by ion-exchange chromatography (Figure 1b). The TLC and ion exchange chromatography patterns depicted utilize a heparin−agarose-purified preparation, while we obtain essentially the same results with unpurified rat brain supernatant (data not shown).

The heparin−agarose-purified preparation utilized to detect bis-PP-IP_4 formation provides a 37-fold purification of IP_6-K activity from rat brain (*12*). We wondered whether IP_6-K catalyzes the formation of bis-PP-IP_4. Accordingly, we incubated IP_6-K, purified to homogeneity as previously described (*12*), with [^{3}H]PP-IP_5. In multiple experiments with a wide range of temperatures, pH values, and durations of incubation, we do not detect the formation of any bis-PP-IP_4 utilizing the separation techniques depicted in Figure 1.

To ascertain whether a distinct PP-IP_5-K exists in the heparin−agarose-purified preparation, we subjected this preparation to chromatography on a MonoQ column which we previously showed to afford a 16-fold purification of IP_6-K activity over the heparin−agarose-purified preparation (*12*). The MonoQ column provides a complete separation of IP_6-K and PP-IP_5-K enzyme activities, establishing that PP-IP_5-K represents a distinct enzyme (Figure 2).

Table 2: Specificity of PP-IP_5 Kinase[a]

inhibitor	IC_{50} (μM) forward (kinase)	backward (synthase)
bis-PP-IP_4	17.0	2.1
PP-IP_5	1.1	5.0
IP_6	10.0	16.1
1,3,4,5,6-IP_5	59.9	64.9
1,2,4,5,6-IP_5	60.1	70.2
1,3,4,5-IP_4	175.0	160.0
1,4,5-IP_3	>200	>200
1,3,4-IP_3	>200	>200

[a] Enzyme activity was assayed as described.

Purification of PP-IP_5-K. We developed a purification scheme for PP-IP_5-K utilizing some of the steps previously described for purification of IP_6-K (*12*). The heparin−agarose step which yielded a 37-fold purification of IP_6-K provides a 64-fold purification of PP-IP_5 kinase activity with 83% recovery (Table 1). Employing the FPLC MonoQ column, which is used to separate PP-IP_5-K from IP_6-K,

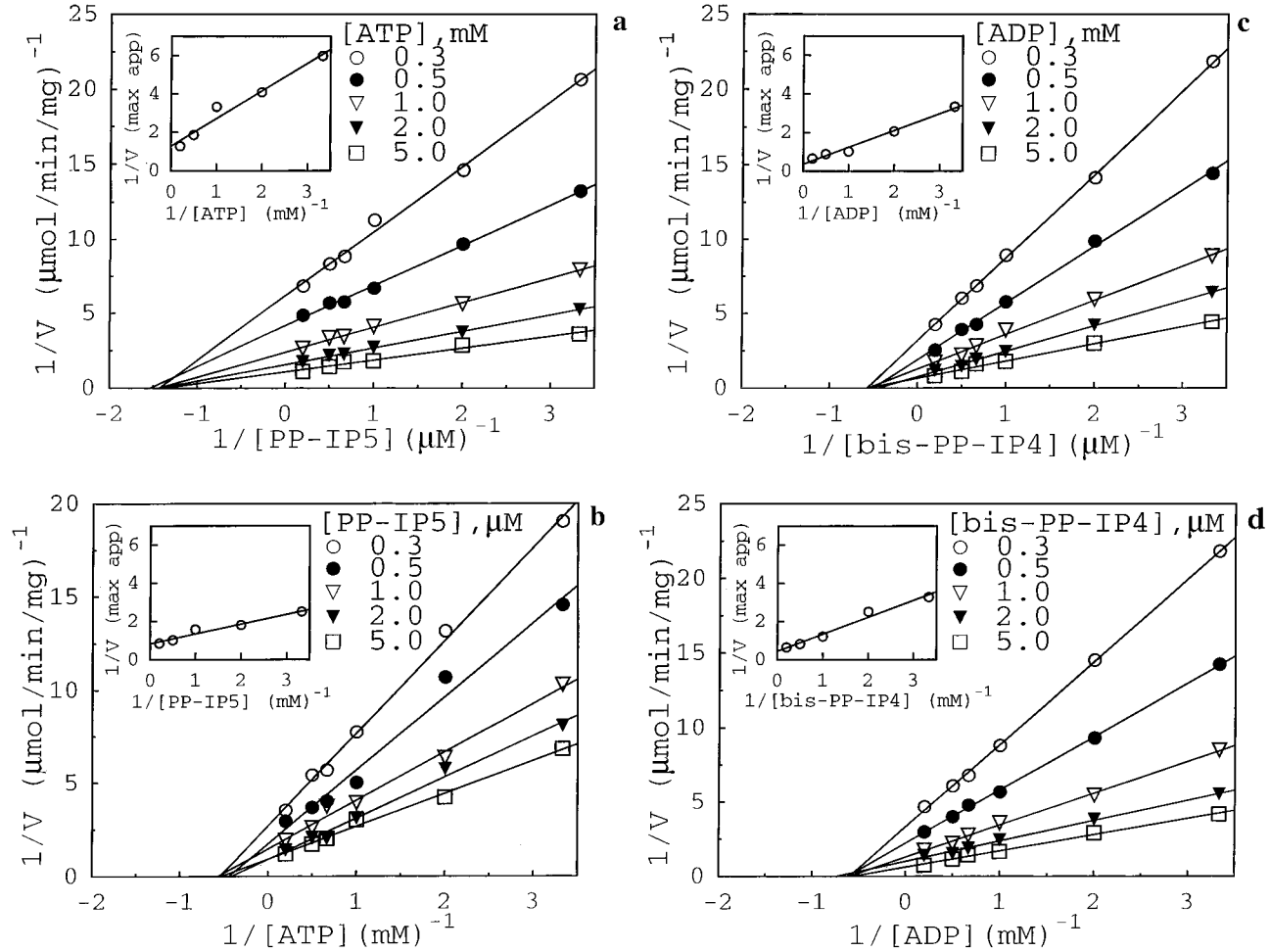

FIGURE 6: Determination of the PP-IP_5 kinase kinetic constants and reaction mechanism. The enzyme was purified and assayed as described. Dependence of PP-IP_5 kinase activity on the concentration of PP-IP_5 (a) and ATP (b). Dependence of the reverse reaction (ATP synthase activity) on the concentration of bis-PP-IP_4 (c) and ADP (d). For panels a and b, the velocity was measured in units of micromoles of bis-PP-IP_4 formed per minute per milligram of protein. For panels c and d, the velocity was measured in units of micromoles of ATP formed per minute per milligram of protein. The insets are plots of the reciprocals of the $1/v$ intercepts ($1/V_{maxapp}$) vs the reciprocals of substrate concentration. Data are means of values obtained from two independent experiments each with triplicate sample setups.

provides a further 8-fold purification of enzyme activity with high recovery. An ATP−agarose column provides a further 15-fold purification with a yield of 12% from the original rat forebrain supernatant.

Purified PP-IP_5-K migrates at about 55 kDa (Figure 3). A few other very faint bands with higher molecular masses are detected. These appear to reflect nonspecific contamination appearing inconsistently in different preparations. To assess whether PP-IP_5-K is a monomer, we examined its migration through a gel filtration column, Superdex S75, and observe its elution with protein markers of about 60 kDa (data not shown). This finding implies that PP-IP_5-K is a monomer.

PP-IP_5-K Acts as an ATP Synthase. We previously found that purified IP_6-K is an ATP synthase, transferring the terminal phosphate from PP-IP_5 to ADP (*12*). To ascertain whether PP-IP_5-K possesses similar activity, we incubated purified PP-IP_5-K in the presence of [β-^{32}P]PP-IP_5 and unlabeled ADP. Under these conditions, [γ-^{32}P]ATP is formed (Figure 4). The reaction is highly selective for ADP, as GDP, CDP, and UDP are ineffective (Figure 4). Also ineffective as PP-IP_5 phosphate acceptors are AMP, creatine, glucose, and 3-phosphoglycerate (data not shown).

Properties of Purified PP-IP_5-K. Purified PP-IP_5-K displays a pH optimum of 6.8. However, substantial enzyme activity occurs over a broad pH range with the enzyme activity at pH 5.5 and 8.5 being about $^2/_3$ of the maximal enzyme activity at pH 6.8. The optimal enzyme activity occurs at 37 °C, though at 25 and 40 °C the enzyme activity is 70% of the levels at 37 °C. No enzyme activity is evident in preparations maintained on ice or at 55 °C. Enzyme activity is comparable in Tris, phosphate, and HEPES buffers. Enzyme activity shows virtually no decrease when the purified preparation is stored with 20% glycerol at −70 °C for up to 3 months. However, when the enzyme is stored at −20 °C with glycerol, activity declines 50% in 1 week. At 4 °C, all enzyme activity is lost in 18 h. Enzyme activity is absolutely dependent upon ATP, with no enzyme activity evident with CTP, UTP, or GTP. No activity occurs with boiled tissue or when enzyme is omitted from incubation mixtures. Enzyme activity is not affected by the addition of up to 10 mM Mg^{2+} or Ca^{2+} or 1 mM EDTA.

We examined inositol phosphate specificity by using various inositol phosphates to reduce PP-IP_5-K activity (Figure 5a). PP-IP_5 is most potent, reducing enzyme activity 50% at about 1 μM, while IP_6 is about $^1/_{10}$ as potent and bis-PP-IP_4 about $^1/_{20}$ as potent. The two isomers of IP_5 are less than 2% as potent as PP-IP_5, while 1,3,4,5-IP_4, 1,4,5-IP_3, and 1,3,4-IP_3 are less than 1% as potent as PP-IP_5 (Table

Table 3: Properties of PP-IP_5 Kinase[a]

K_m(PP-IP_5)	0.68 ± 0.04 μM
K_m(ATP)	1.89 ± 0.45 mM
V_{max}(forward)	1.01 ± 0.29 μmol min^{-1} mg^{-1}
K_m(bis-PP-IP_4)	1.94 ± 0.20 μM
K_m(ADP)	1.39 ± 0.54 mM
V_{max}(backward)	2.51 ± 0.33 μmol min^{-1} mg^{-1}

[a] Enzyme activity was assayed as described.

2). Unlabeled bis-PP-IP_4 inhibits the ATP synthase activity of PP-IP_5-K by 50% at 2.1 μM (Figure 5b and Table 2). PP-IP_5 has an IC_{50} value of 5 μM, similar to the bis-PP-IP_4 value, indicating that end product inhibition may regulate this activity. IP_6 is 12% as potent as bis-PP-IP_4; the two isomers of IP_5 are less than 2% as potent as bis-PP-IP_4, while 1,3,4,5-IP_4, 1,4,5-IP_3, and 1,3,4-IP_3 are less than 1% as potent as bis-PP-IP_4 (Table 2).

To determine kinetic constants for enzyme activity, we first monitored PP-IP_5-K activity at varying PP-IP_5 concentrations and observe half-maximal actvity at about 0.7 μM in the presence of a saturating concentration of ATP (5 mM). We then monitored PP-IP_5-K activity while varying both PP-IP_5 and ATP concentrations (Figure 6). The calculated K_m for PP-IP_5 is 0.68 μM, while the K_m for ATP is about 2 mM. The V_{max} is 1.01 μmol min^{-1} mg of $protein^{-1}$. At PP-IP_5 concentrations of >50 μM and ATP concentrations of >10 mM, substrate inhibition is observed (data not shown). For the reverse reaction, the K_m values for bis-PP-IP_4 and ADP are 1.9 μM and 1.4 mM, respectively. The V_{max} value for ATP synthesis is 2.5 μmol min^{-1} mg^{-1} (Figure 6 and Table 3). Thus, the velocities of the two reactions appear to be similar. In the intact organism, the relative concentrations of substrates are crucial determinants of the direction of an enzyme reaction. The concentrations of PP-IP_5 and bis-PP-IP_4 in cells, approximately 0.5−3 μM (*8*−*11*), are similar to the observed K_m value. The physiologic concentrations of ATP and ADP in mammalian brain, 2.7 and 0.7 mM, respectively (*20*), are similar enough to the observed K_m values that both forward and reverse reactions are likely to take place in vivo and the relative extent of the two may be determined by altered physiologic circumstances.

DISCUSSION

Our study establishes that PP-IP_5-K is a distinct enzyme responsible for the formation of bis-PP-IP_4. Purified PP-IP_5-K, like IP_6-K, is a monomer, but in rat brain preparations, PP-IP_5-K appears to be about 5-fold more abundant than IP_6-K.

Strikingly, purified PP-IP_5-K, like IP_6-K, has ATP synthase activity (Figure 4). Since the enthalpy change (ΔH) of the hydrolysis of a diphosphate bond is on the order of −3 kcal/mol (*21*), the bond energy of the P−O−P linkage in bis-PP-IP_4 may be similar to that of ATP (*9*, *21*, *22*). Therefore, PP-IP_5-K can readily transfer a phosphate group from bis-PP-IP_4 to ADP to form ATP at 37 °C. We have demonstrated previously that IP_6-K can produce ATP by transferring a phosphate group from PP-IP_5 to ADP (*12*). It is then reasonable to hypothesize that these inositol diphosphate molecules are physiologic energy sources for specific cellular functions.

Purification of IP_6-K and PP-IP_5-K should facilitate development of antibodies for localizing these enzymes and molecular cloning of their cDNAs. These efforts may provide additional insight into biological functions of the inositol diphosphates. Shears and colleagues (*8*, *10*, *11*) have provided impressive evidence for an extremely rapid turnover of the inositol diphosphates into IP_6. The rapid turnover of these compounds may participate in the recycling of vesicles, especially synaptic vesicles in the brain. Thus, we (*23*) and others (*24*) observed that a high-affinity binding protein for IP_6 is identical to the clathrin-associated protein AP2, which plays a role in vesicle endocytosis (*23*, *24*). Others have demonstrated that IP_6, PP-IP_5, and/or bis-PP-IP_4 can interact with several proteins regulating endocytosis such as synaptotagmin (PP-IP_5 also binds to the coatomer) (*13*, *24*−*33*).

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REFERENCES

1. Berridge, M. J., and Irvine, R. F. (1989) *Nature 341*, 197−205.
2. Szwergold, B. S., Graham, R. A., and Brown, T. R. (1987) *Biochem. Biophys. Res. Commun. 149*, 874−881.
3. Pittet, D., Schlegel, W., Lew, D. P., Monod, A., and Mayr, G. W. (1989) *J. Biol. Chem. 264*, 18489−18493.
4. Irvine, R. F., Moor, R. M., Pollock, W. K., Smith, P. M., and Wreggett, K. A. (1988) *Philos. Trans. R. Soc. London, Ser. B 320*, 281−298.
5. Downes, C. P. (1988) *Trends Neurosci. 11*, 336−338.
6. Stuart, J. A., Anderson, K. L., French, P. J., Kirk, C. J., and Michell, R. H. (1994) *Biochem. J. 303*, 517−525.
7. Mayr, G. W., Radenberg, T., Thiel, U., Vogel, G., and Stephens, L. R. (1992) *Carbohydr. Res. 234*, 247−262.
8. Menniti, F. S., Miller, R. N., Putney, J. W., Jr., and Shears, S. B. (1993) *J. Biol. Chem. 268*, 3850−3856.
9. Stephens, L., Radenberg, T., Thiel, U., Vogel, G., Khoo, K. H., Dell, A., Jackson, T. R., Hawkins, P. T., and Mayr, G. W. (1993) *J. Biol. Chem. 268*, 4009−4015.
10. Glennon, M. C., and Shears, S. B. (1993) *Biochem. J. 293*, 583−590.
11. Shears, S. B., Ali, N., Craxton, A., and Bembenek, M. E. (1995) *J. Biol. Chem. 270*, 10489−10497.
12. Voglmaier, S. M., Bembenek, M. E., Kaplin, A. I., Dorman, G., Olszewski, J. D., and Prestwich, G. D., and Snyder, S. H. (1996*) Proc. Natl. Acad. Sci. U.S.A. 93*, 4305−4310.
13. Ali, N., Duden, R., Bembenek, M. E., and Shears, S. B. (1995) *Biochem. J. 310*, 279−284.
14. Spencer, C. E. L., Stephens, L. R., and Irvine, R. F. (1990) in *Methods in Inositide Research* (Irvine, R. F., Ed.) pp 39−43, Raven Press, Ltd., New York.
15. Ryu, S. H., Lee, S. Y., Lee, K. Y., and Rhee, S. G. (1987) *FASEB J. 1*, 388−393.
16. Laemmli, U. K. (1970) *Nature 227*, 680−685.
17. Laussmann, T., Eujen, R., Weisshuhn, C. M., Thiel, U., and Vogel, G. (1996) *Biochem. J. 315*, 715−720.
18. Laussmann, T., Reddy, K. M., Reddy, K. K., Falck, J. R., and Vogel, G. (1997) *Biochem. J. 322*, 31−33.
19. Albert, C., Safrany, S. T., Bembenek, M. E., Reddy, K. M., Reddy, K., Falck, J., Brocker, M., Shears, S. B., and Mayr, G. W. (1997) *Biochem. J. 327*, 553−560.
20. Veech, R. L., Lawson, J. W., Cornell, N. W., and Krebs, H. A. (1979) *J. Biol. Chem. 254*, 6538−6547.
21. Ging, N. S., and Sturtevant, J. M. (1954) *J. Am. Chem. Soc. 76*, 2087−2091.
22. Raison, J. K., and Evans, W. J. (1968) *Biochim. Biophys. Acta 170*, 448−451.
23. Voglmaier, S. M., Keen, J. H., Murphy, J. E., Ferris, C. D., Prestwich, G. D., Snyder, S. H., and Theibert, A. B. (1992) *Biochem. Biophys. Res. Commun. 187*, 158−163.

24. Timerman, A. P., Mayrleitner, M. M., Lukas, T. J., Chadwick, C. C., Saito, A., Watterson, D. M., Schindler, H., and Fleischer, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8976−8980.
25. Norris, F. A., Ungewickell, E., and Majerus, P. W. (1995) *J. Biol. Chem. 270*, 214−217.
26. Ye, W., Ali, N., Bembenek, M. E., Shears, S. B., and Lafer, E. M. (1995) *J. Biol. Chem. 270*, 1564−1568.
27. Fleischer, B., Xie, J., Mayrleitner, M., Shears, S. B., Palmer, D. J., and Fleischer, S. (1994) *J. Biol. Chem. 269*, 17826−17832.
28. Llinas, R., Sugimori, M., Lang, E. J., Morita, M., Fukuda, M., Niinobe, M., and Mikoshiba, K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12990−12993.
29. Niinobe, M., Yamaguchi, Y., Fukuda, M., and Mikoshiba, K. (1994) *Biochem. Biophys. Res. Commun. 205*, 1036−1042.
30. Schiavo, G., Gmachl, M. J., Stenbeck, G., Sollner, T. H., and Rothman, J. E. (1995) *Nature 378*, 733−736.
31. Palczewski, K., Pulvermuller, A., Buczylko, J., Gutmann, C., and Hofmann, K. P. (1991) *FEBS Lett. 295*, 195−199.
32. Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S., and Mikoshiba, K. (1994*) J. Biol. Chem. 269*, 29206−29211.
33. el Ouggouti, S., Bournier, O., Boivin, P., Bertrand, O., and Dhermy, D. (1992) *Protein Expression Purif. 3*, 488−496.

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