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Biochemistry

Purified inositol hexakisphosphate kinase is an ATP synthase: Diphosphoinositol pentakisphosphate as a high-energy phosphate donor

(phosphotransferase/pyrophosphate)

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ABSTRACT **Diphosphoinositol pentakisphosphate (PP-IP_5) and bis(diphospho)inositol tetrakisphosphate (bis-PP-IP_4) are recently identified inositol phosphates that possess pyrophosphate bonds. We have purified an inositol hexakisphosphate (IP_6) kinase from rat brain supernatants. The pure protein, a monomer of 54 kDa, displays high affinity (K_m = 0.7 μM) and selectivity for inositol hexakisphosphate as substrate. It can be dissociated from bis(diphospho)inositol tetrakisphosphate synthetic activity. The purified enzyme transfers a phosphate from PP-IP_5 to ADP to form ATP. This ATP synthase activity indicates the high phosphate group transfer potential of PP-IP_5 and may represent a physiological role for PP-IP_5.**

Inositol 1,4,5-trisphosphate (1,4,5-IP_3) is one of the major intracellular messenger molecules in signal transduction and is associated with the regulation of intracellular calcium (1). The importance of 1,4,5-IP_3 in cellular signaling has led to the examination of the numerous other *myo*-inositol phosphates in cells, whose functions are less clear. Of these inositol metabolites, inositol hexakisphosphate (IP_6) is the most abundant, with concentrations in mammalian tissues 10–100 times greater than those of IP_3. The high concentration of IP_6 (15–60 μM) (2, 3) is presumed to be compartmentalized within the cell (4, 5) with at least 80% of IP_6 in releasable cytosolic pools (6).

In plants, IP_6 acts as an antioxidant (7) and phosphate store (8). In humans, dietary IP_6 from cereals and legumes is associated with decreased iron absorption (9) and lowered cancer rates (10). In cells, IP_6 may act as a siderophore (11, 12) and thus block iron-mediated oxidative damage (13). IP_6 also interacts with several proteins that regulate endocytosis (14–18), synaptic vesicle trafficking (19–21), and receptor desensitization (22).

IP_6 was thought to be the metabolic endpoint of *myo*-inositol metabolism, but recently inositol pyrophosphates that have seven and eight phosphates associated with the inositol ring have been identified and are termed diphosphoinositol pentakisphosphate (PP-IP_5) and bis(diphospho)inositol tetrakisphosphate (bis-PP-IP_4) (23–25). In PP-IP_5, the pyrophosphate occurs at the 1 position, and in bis-PP-IP_4, the two pyrophosphates are at the 1 and 2 or 1 and 4 positions (25). The functions of the inositol pyrophosphates have been difficult to demonstrate. We now report purification to homogeneity of an IP_6 kinase and demonstration that this enzyme has an ATP synthase activity, implying that the inositol pyrophosphates function as high-energy phosphate donors.

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MATERIALS AND METHODS

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 DuPont/NEN. [^{32}P]PP-IP_5 and [^{32}P]bis-PP-IP_4 were synthesized as described (26) and supplied by DuPont/NEN. KH_2PO_4, K_2HPO_4, and KCl were 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, 1,4,5-IP_3, and inositol 1,3,4,5-tetrakisphosphate (IP_4) were obtained from LC Services (Woburn, MA). Inositol 1,3,4,5,6-pentakisphosphate (1,3,4,5,6-IP_5) and IP_6 were from Calbiochem. 1,2,4,5,6-IP_5 was a kind gift of James F. Maracek (State University of New York, Stony Brook). IP_6-Affi-Prep resin was prepared as described (27). Polyethyleneimine (PEI)-cellulose TLC plates were obtained from EM Separations (Merck). Hepes buffer was from Research Organics. Nonradioactive nucleotides, nucleotide analogs, and nucleoside-diphosphate kinase (EC 2.7.4.6) were obtained from Boehringer Mannheim. SDS/PAGE gels and molecular mass standards were from NOVEX (San Diego). Protein assay dye reagents were from Pierce. Heparin-agarose, phosphocreatine, creatine kinase (EC 2.7.3.2), and all other reagents were from Sigma.

Assay of IP_6 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 dithiothreitol, 6 mM $MgCl_2$, 5 mM Na_2ATP, 10 mM phosphocreatine, 40 units/ml creatine kinase, 5 mM NaF, 5 μM IP_6, and 40–80 nM [^{3}H]IP_6 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 HCl 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 PEI-cellulose TLC (28, 29) 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-cellulose TLC plate, which was developed in 1.1 M KH_2PO_4/0.8 M K_2HPO_4/2.3 M HCl. The lanes were cut into 1-cm strips, and radioactivity was measured 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-cellulose TLC strips were first shaken with 3 ml of concentrated HCl, and radioactivity was measured 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

Abbreviations: IP_6, inositol hexakisphosphate; PP-IP_5, diphosphoinositol pentakisphosphate; bis-PP-IP_4, bis(diphospho)inositol tetrakisphosphate; IP_3, inositol trisphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate; IP_5, inositol pentakisphosphate; PEI, polyethyleneimine.

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comigrated with standard preparations of [^{3}H]IP$_6$, [^{3}H]PP-IP$_5$, and [^{3}H]bis-PP-IP$_4$. No PP-IP$_5$ or 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 kinase (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, phospho*enol*pyruvate, 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.

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 dithiothreitol, 5 mM Na_2ADP, 5 mM $MgCl_2$, and 50 nM [^{32}P]PP-IP$_5$ [20–100 Ci/mmol (1 Ci = 37 GBq)] and 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-cellulose TLC plates developed either as above, or in 1 M KH_2PO_4. In 1 M KH_2PO_4, [^{32}P]PP-IP$_5$ and [^{32}P]ATP migrated with R_f values of approximately 0.05 and 0.55, respectively, and comigrated with standards (see Fig. 3). No [^{32}P]ATP was formed from [^{32}P]PP-IP$_5$ under the following conditions: 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 kinase (40 units/ml) or nucleoside-diphosphate kinase (0.5 mg/ml); with boiled (5 min) brain extract or partially purified enzyme; or in the absence of ADP. No decrease in [^{32}P]PP-IP$_5$ was observed when 5 mM ADP was replaced by 5 mM CDP, GDP, UDP, AMP, creatine, glucose, or 3-phosphoglycerate as phosphate acceptors. Replacing ADP with adenosine 5′-[β-thio]diphosphate (ADP[β-S]) resulted in the formation of a ^{32}P-labeled product of lower mobility than ATP (presumably ATP[β-S]) with 30% efficiency (data not shown). 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 IP$_6$ 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 (20 mM Hepes, pH 6.8/4 mM dithiothreitol/2 mM EGTA/0.75 mM EDTA/5 mM NaF/1.5 mM Na_3VO_4/0.5 mg/liter okadaic acid/4 mg/liter chymostatin/4 mg/liter pepstatin/4 mg/liter antipain/8 mg/liter leupeptin/8 mg/liter aprotinin/200 mg/liter phenylmethylsulfonyl fluoride). All subsequent procedures were performed at 0–4°C. The homogenate was centrifuged at 100,000 × *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% 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propane sulfonate (CHAPS) for 60 min. The agarose was washed with 450 ml of 0.25 M KCl in buffer A with 1 mM $MgCl_2$ minus okadaic acid and was then eluted by agitation 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 into a column, eluted, and then eluted again with an additional 50 ml of the same buffer. The combined heparin column eluate was diluted to 1 liter in buffer B (20 mM Hepes, pH 6.8/1 mM dithiothreitol/1 mM EGTA/2 mg/liter chymostatin/2 mg/liter pepstatin/2 mg/liter antipain/4 mg/liter leupeptin/0.1% CHAPS) and applied to a Mono Q HR 10/10 fast protein liquid chromatography column at 4 ml/min, 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 plus 0.6% CHAPS. All further purification steps used this buffer. The pooled peak of IP$_6$ kinase activity was diluted to 0.05 M KCl and loaded onto a 1 × 8 cm IP$_6$-Affi-Prep column at 0.5 ml/min, and the flow-through was passed over the column again. The column was then washed with 50 ml of 0.15 M KCl and eluted with 16 ml of 25 μM IP$_6$. The peak fractions were diluted to 2 μM IP$_6$ and applied to a 0.5 × 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. Peak fractions from the heparin column were concentrated in a Centriprep-30 concentrator (Amicon) to 100 μl and applied to a Superdex 75 gel filtration column, which was run at 0.2 ml/min. All columns were washed with 2.5 M KCl between runs.

Other Methods. 1,4,5-Trisphosphate 3-kinase activity was assayed as described (29). 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 (30).

RESULTS

Purification of IP$_6$ Kinase. IP$_6$ kinase activity was monitored by measuring [^{3}H]PP-IP$_5$ formed from [^{3}H]IP$_6$. ^{3}H-labeled IP$_6$, PP-IP$_5$, and bisPP-IP$_4$ were resolved by PEI-cellulose TLC, with no overlap between the inositol phosphates. In a screen of various tissues, the highest concentrations of enzyme activity are evident in the brain and testis. The thymus possesses about 60% as much activity as brain, whereas enzyme activity in heart, liver, and kidney are 5–25% of brain values. Because of the abundance of phosphatase activity in most tissues, these estimates may not reflect the absolute levels of the IP$_6$ kinase.

In brain extracts, virtually all IP$_6$ kinase activity is recovered in the soluble supernatant fraction after centrifugation at 100,000 × *g*. Enzyme activity adsorbs to a heparin column and is eluted with KCl, providing a 37-fold purification, and purification with the anion exchanger column Mono Q provides a further 4-fold enrichment of enzyme activity. The next greatest purification is obtained by adsorbing the enzyme to an IP$_6$ affinity column, affording a 13-fold increase in specific activity. Adsorption to a second heparin column with a gradient elution provides another 5- to 6-fold purification, and a gel filtration column affords another 3- to 4-fold purification. The final preparation is enriched 40,000-fold in enzyme activity and provides a 2.2% yield (Table 1).

SDS/PAGE of the purified enzyme reveals an apparent molecular mass of 54 kDa (Fig. 1). The elution profile of the enzyme from the gel filtration column is consistent with a

Table 1. Purification of IP$_6$ kinase

Fraction	Protein, μg	Specific activity, nmol/min/mg	Purification, fold	Recovery, %
100,000 × *g* supernatant	1,295,100	0.0365		
Heparin-agarose (batch)	31,000	1.37	37.5	90
Mono Q	4,050	5.48	151	47
IP$_6$-Affi-Prep	73.5	72.9	1,998	11.4
Heparin-agarose	7.26	374.7	10,266	7.9
Superdex S75	0.662	1557.8	42,679	2.2

Enzyme was purified and fractions were assayed as described. Data presented are from a typical purification, which was repeated five times with similar results.

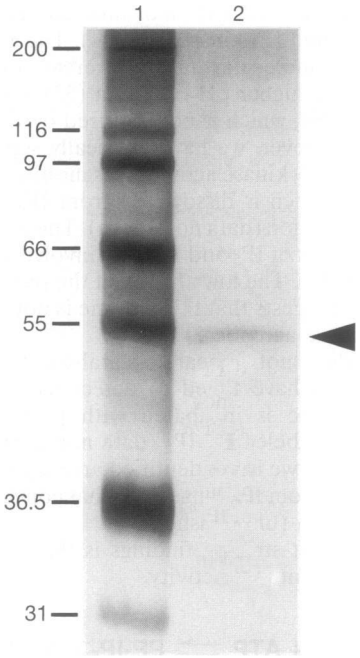

FIG. 1. SDS/PAGE analysis of purified IP_6 kinase. A 12% polyacrylamide gel was silver stained. Lane 1, molecular mass standards: myosin (200 kDa); β-galactosidase (116 kDa); phosphorylase *b* (97 kDa); bovine serum albumin (66 kDa); glutamate dehydrogenase (55 kDa); lactate dehydrogenase (36.5 kDa); carbonic anhydrase (31 kDa). Lane 2, 60 ng of purified IP_6 kinase (arrowhead).

molecular mass of about 60 kDa, which implies that IP_6 kinase is a monomer. The enzyme loses 50% of its activity when stored for 7 days at 4°C. Optimal stability is evident when the enzyme is stored with 20% glycerol at −70°C. Enzyme activity displays a broad pH optimum, with maximal activity at pH 6.8. A variety of buffers can be used, although Hepes is optimal.

The enzyme displays striking selectivity for IP_6 (Fig. 2*A* and Table 2). Unlabeled IP_6 inhibits [^{3}H]PP-IP_5 formation by 50% at about 0.8 μM. The product of the IP_6 kinase, PP-IP_5, is 12% as potent as IP_6 as an inhibitor and is not a substrate for the kinase reaction (data not shown). The two isomers of IP_5 are only about 4% as potent. IP_4 is only 1.5% as potent as IP_6, whereas both 1,3,4-IP_3 and 1,4,5-IP_3 fail to influence enzyme activity at concentrations of 100 μM.

IP_6 Kinase Has ATP Synthase Activity. In the presence of [^{32}P]PP-IP_5 and unlabeled ADP, [^{32}P]ATP is formed (Fig. 3). The reaction is highly selective for ADP as GDP, CDP, and UDP are ineffective (Fig. 3). Also ineffective as PP-IP_5 phosphate acceptors are AMP, creatine, glucose, or 3-phosphoglycerate (data not shown). Unlabeled PP-IP_5 inhibits the ATP synthase activity of the enzyme by 50% at 1.8 μM (Fig. 2*B* and Table 2). IP_6 has a similar IC_{50} value, indicating that end-product inhibition may play a role in the regulation of this activity. 1,3,4,5,6-IP_5 is 14% as potent as PP-IP_5, IP_4 is 5.5% as potent, and 1,4,5-IP_3 is 0.5% as potent at inhibiting enzyme activity.

Kinetic analysis reveals both the forward and reverse reactions to be random bireactant systems (Fig. 4 and Table 3). For the forward reaction, the K_m values for IP_6 and ATP are 0.7 μM and 1.35 mM, respectively. At concentrations of $IP_6 > 50$ μM and ATP > 10 mM, substrate inhibition is observed (data not shown). For the reverse reaction, the K_m values for PP-IP_5 and ADP are 1.97 μM and 1.57 mM, respectively. The V_{max} values for the forward and reverse reactions are 1.41 and 2.64 μmol/min per mg of protein, respectively. Thus, the velocities of the two reactions appear to be similar. However, in the intact organism, the relative concentrations of the substrates are crucial determinants of the direction of an enzyme reaction. The concentration of PP-IP_5 in cells, 0.5–3.0 μM (31), is similar to the observed K_m value, whereas the concentration of IP_6 is considerably higher. Some compartmentalization or complexing of IP_6 is likely (4, 5), so that in cells, the enzyme may be exposed to a smaller effective concentration of IP_6. The physiologic concentrations of ATP and ADP in mammalian brain, 2.7 and 0.7 mM, respectively (32), 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.

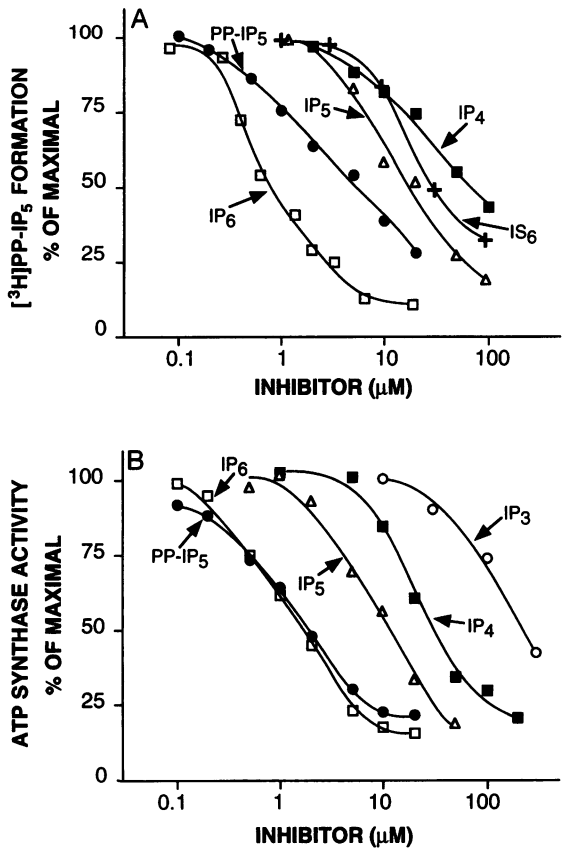

FIG. 2. Inositol phosphate specificity of IP_6 kinase forward and reverse reactions. (*A*) Inhibition of forward reaction ([^{3}H]PP-IP_5 formation), assayed as described with 5 mM ATP and 41 nM [^{3}H]IP_6. IS_6, inositol hexakissulfate. (*B*) Inhibition of reverse reaction (ATP synthase activity), assayed as described with 5 mM ADP and 50 nM [^{32}P]PP-IP_5. Peak fractions from the second heparin column were used (similar results were obtained with purified protein.) Data are means of triplicate determinations of three experiments.

Table 2. Specificity of IP_6 kinase forward (IP_6 kinase) and reverse (ATP synthase) reactions

Inhibitor	IC_{50}, μM	
	IP_6 kinase	ATP synthase
IP_6	0.8	1.8
PP-IP_5	6.6	1.8
1,3,4,5,6-IP_5	22	12.8
1,2,4,5,6-IP_5	22*	ND
1,3,4,5-IP_4	61	32.5
1,4,5-IP_3	>100*	253
1,3,4-IP_3	>100*	ND
IS_6	30	ND

Enzyme activity was assayed as described in *Materials and Methods* and Fig. 2. ND, not determined.
*Data not shown.

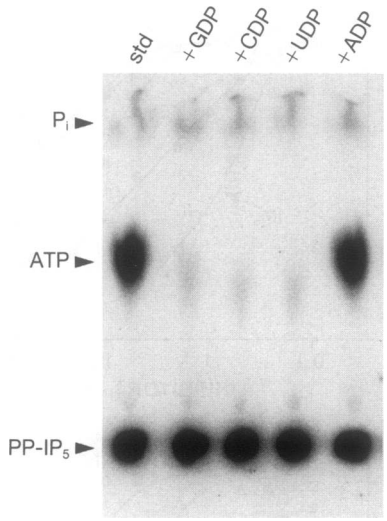

FIG. 3. Nucleoside diphosphate specificity of IP_6 kinase reverse reaction (ATP synthase activity). Lanes: std, approximately 15,000 cpm each of [γ-^{32}P]ATP and [^{32}P]PP-IP_5 were applied at the origin of the PEI-cellulose TLC plate; +GDP, +CDP, +UDP, and +ADP, the ATP synthase activity of the purified enzyme was assayed as described with the indicated nucleoside diphosphate at 5 mM. The TLC plate was exposed to a phosphor screen and scanned by a Molecular Dynamics PhosphorImager.

DISCUSSION

The IP_6 kinase we have purified appears to be the enzyme responsible for the biosynthesis of PP-IP_5, as it displays selectivity and high affinity for IP_6 as a substrate. The low affinity for both 1,4,5-IP_3 and 1,3,4-IP_3 indicates that the enzyme is not identical to purified IP_3 kinases (33, 34). The 1,3,4-IP_3 kinase has a different molecular mass, whereas 1,4,5-IP_3 kinase activity displays a higher pH optimum (33) and is stimulated by calmodulin (29), which is not required by IP_6 kinase (data not shown). Moreover, we have physically separated the IP_6 kinase and 1,4,5-IP_3 kinase activities, as monitored by 1,4,5-IP_3 kinase activity, which is dissociated from IP_6 kinase activity during the purification (data not shown). The exact mechanism for the biosynthesis of IP_5 and IP_6 may involve specific IP_4 and IP_5 kinases (35, 36). The low affinity of the purified IP_6 kinase for IP_4 and IP_5 suggests that this enzyme is not responsible for these other kinase activities.

IP_6 kinase does not appear to catalyze the formation of bis-PP-IP_4, as we have failed to detect its formation when purified IP_6 kinase is incubated with [^{3}H]PP-IP_5 or with [^{32}P]ATP and unlabeled PP-IP_5 (data not shown). In preliminary experiments, we have identified an enzyme fraction that can be separated from IP_6 kinase and that converts [^{3}H]PP-IP_5 into [^{3}H]bis-PP-IP_4 (unpublished).

One of our most striking findings is that the purified IP_6 kinase has ATP synthase activity.

$$\mathbf{IP_6 + ATP \rightleftharpoons PP\text{-}IP_5 + ADP}$$

Structure 1

We have demonstrated that the [^{32}P]ATP formed by IP_6 kinase can phosphorylate protein substrates (data not shown). The enthalpy change (ΔH) of the hydrolysis of a pyrophosphate bond is on the order of -3 kcal/mol (37) (1 kcal = 4.18 kJ). The pyrophosphate of PP-IP_5 formed by an ATP-dependent kinase has been assigned to the C-1 of the inositol ring in

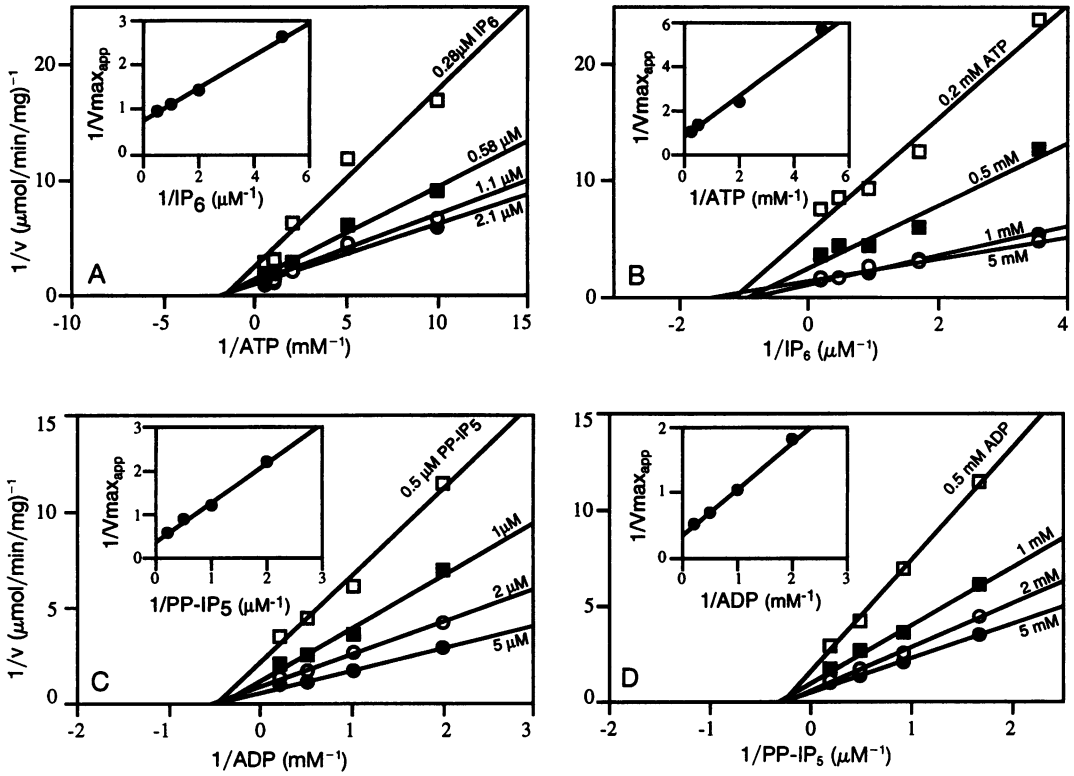

FIG. 4. Determination of IP_6 kinase kinetic constants and reaction mechanism. Enzyme was purified and assayed as described. Dependence of IP_6 kinase activity on the concentration of IP_6 (*A*) and ATP (*B*). Dependence of reverse reaction (ATP synthase activity) on the concentration of PP-IP_5 (*C*) and ADP (*D*). For *A* and *B*, velocity was measured as micromoles of PP-IP_5 formed per minute per milligram of protein. For *C* and *D*, velocity was measured as micromoles of ATP formed per minute per milligram of protein. The insets are plots of the reciprocals of the $1/v$ intercepts ($1/V_{max_{app}}$) vs. the reciprocals of substrate concentration. Data are means of triplicate determinations from a representative experiment, which was repeated twice with similar results.

Table 3. Properties of IP_6 kinase

Kinetic constant	Value
K_m for IP_6	0.70 ± 0.192 μM
K_m for ATP	1.35 ± 0.106 mM
V_{max_f} (forward)	1.41 ± 0.168 μmol/min per mg
K_m for PP-IP_5	1.97 ± 0.312 μM
K_m for ADP	1.57 ± 0.243 mM
V_{max_r} (reverse)	2.64 ± 0.113 μmol/min per mg

Purified enzyme was assayed as described. Values are means (±SD) of three determinations.

mammalian cells (25). Sequential hydrolysis of the phosphate esters of IP_6 has shown that the apparent ΔH of the C-1 monophosphate ester of IP_6, −4 kcal/mol, is higher than that of any of the other positions tested (38). [The C-2 position was not accessible but is thought to possess a higher energy because of charge repulsion (39)]. A pyrophosphate linkage at the C-1 position may possess an even higher ΔH. The bond energy of the P—O—P linkage in PP-IP_5 has been predicted to be −6.6 kcal/mol, similar to ADP (25). The fact that the IP_6 kinase can readily transfer a phosphate group from PP-IP_5 to ADP to form ATP at 37°C suggests that the free energy of hydrolysis of the pyrophosphate bond of PP-IP_5 is similar to that of ATP. Further investigation will be required to determine whether the ATP synthase activity of this enzyme occurs *in vivo*. In liver homogenates incubated with [^{32}P]PP-IP_5, the labeled phosphate group is recovered only as free P_i formed by the action of a specific phosphatase, although PP-IP_5 does accumulate in intact cells (31).

IP_6, or phytate, has long been known to exist in high concentrations in plant seeds. It may be important as an antioxidant during dormancy (7) and may be the storage form for phosphate that can be converted into ATP during germination (8). A kinase from mung bean seeds that transfers a phosphate from IP_6 to GDP (41) was identified, and two kinases that can transfer a phosphate from IP_6 to ADP have been isolated from mung bean (42) and soybean (43) seeds. The phosphotransferase reaction of the soybean 1,3,4,5,6-IP_5 2-kinase was observed in both directions *in vitro* and may proceed in both directions *in vivo* depending on substrate concentrations, including the ATP/ADP ratio of maturing versus germinating seeds (43).

Our results suggest that PP-IP_5 constitutes an energy reservoir for mammalian cells analogous to IP_6 in plants or to phosphocreatine. Because endogenous concentrations of PP-IP_5 are substantially lower than those of phosphocreatine, PP-IP_5 is not likely to be a high-energy phosphate donor for general cellular functions. Instead, PP-IP_5 may have a more specialized role, either by generating ATP or by transferring its energy directly. One role may involve vesicle trafficking, especially in the brain, where IP_6 kinase activity is highest. The purified IP_6 receptor binding protein is identical to the clathrin assembly protein AP-2, which is a component of clathrin-coated pits and vesicles (14, 15). PP-IP_5 and IP_6 also bind with high affinity and specificity to the clathrin assembly protein AP-3 (16, 17), Golgi coatomer (18), and yeast coatomer (26). Although the inositol pyrophosphates have not been directly tested in each case, higher inositol polyphosphates also bind to the synaptic vesicle associated protein synaptotagmin (44), β-arrestin (22), and the cytoskeletal protein band 4.1 (45), indicating a possible role for these inositol compounds at the synapse.

Endocytosis and vesicle recycling are among the most dynamic processes of cellular regulation. Several specific stages of the endocytic process depend on unknown cytosolic factors as well as energy donors. Initiation or nucleation, membrane fission (46), and maintenance of an unassembled pool of coat proteins (47) are all energy-requiring steps of the endocytic process. ATP is required, and other energy sources may be involved as well. Some endocytosis occurs in ATP-depleted cells (48), various methods of ATP depletion differentially affect measures of endocytosis, and neither levels of ATP nor rate of ATP depletion is directly correlated to loss of cell surface receptors (49).

Hydrolysis of the high-energy phosphate bonds of ATP and GTP also drive vesicle uncoating, docking, and fusion events, providing a directional regulatory switch (50). Misassembly of coated pits (51, 52) and functional differences in coat proteins isolated from assembled vs. unassembled pools (53) indicate a regulatory switch in coat assembly (50, 54). IP_6 inhibits AP-2 self-assembly, AP-2 binding to clathrin, and clathrin coat assembly (55). IP_6 and PP-IP_5 also inhibit AP-3 (16, 17) and coatomer-mediated clathrin coat assembly *in vitro* (26), indicating a role for IP_6 or PP-IP_5 in the regulation of coat assembly and disassembly in the cell. Another more specialized regulatory switch or "fusion clamp" (50, 56) may be involved in regulated fusion events like synaptic vesicle exocytosis and recycling or receptor down-regulation, which may involve cross-talk with second messenger systems. Higher inositol phosphates bind to synaptotagmin at the phospholipid binding domain CL2B (44), interfering with β-SNAP/synatotagmin binding (21) and vesicle fusion (19). A regulatory mechanism could involve phosphate transfer or hydrolysis of PP-IP_5 bound at such domains. The high rate of turnover of PP-IP_5 (31) and its regulation by intracellular calcium (57) suggest that interconversion of IP_6 and PP-IP_5 and/or bisPP-IP_4 would be an excellent candidate as an energy source and/or regulatory switch for these processes.

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