Purification of phosphatidylinositol kinase from bovine brain myelin

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A membrane-bound phosphatidylinositol (PI) kinase (EC 2.7.1.67) was purified by affinity chromatography from bovine brain myelin. This enzyme activity was solubilized with non-ionic detergent and chromatographed on an anion-exchange column. Further purification was achieved by affinity chromatography on PI covalently coupled to epoxy-activated Sepharose, which was eluted with a combination of PI and detergent. The final step in the purification was by gel filtration on an Ultrogel AcA44 column. This procedure afforded greater than 5500-fold purification of the enzyme from whole brain myelin. The resulting activity exhibited a major silver-stained band on SDS/polyacrylamide-gel electrophoresis with an apparent M_r 45000. The identity of this band as PI kinase was corroborated by demonstration of enzyme activity in the gel region corresponding to that of the stained protein. The purified enzyme exhibited a non-linear dependence on PI as substrate, with two apparent kinetic components. The lower-affinity component exhibited a K_m similar to that observed for the phosphorylation of phosphatidylinositol 4-phosphate by the enzyme.

INTRODUCTION

The metabolism of the phosphoinositides is thought to play an important role in the signalling function of biological membranes. Phosphatidylinositol 4,5-bisphosphate (PIP₂) serves as a substrate for a hormonally sensitive phosphodiesterase activity, resulting in the generation of two second messengers, diacylglycerol and inositol trisphosphate (Berridge, 1983). PIP₂ is synthesized by the stepwise phosphorylation of PI, by the activities of the PI and PIP kinases. Although some information is available on the subcellular distribution and substrate specificity (Michell *et al.*, 1967; Too *et al.*, 1969; Harwood & Hawthorne, 1969; Collins & Wells, 1983) of these enzymes, neither has been purified in active form, and consequently their characterization has been limited. Because of their crucial role in the synthesis of the substrate for the generation of diacylglycerol and inositol trisphosphate, the activities of these enzymes may be critical in metabolic regulation by several hormones. In addition, a role for these enzymes in mitogenesis (Taylor *et al.*, 1984), viral transformation (Diringer & Friss, 1977) and phosphorylation of certain brain proteins (Jolles *et al.*, 1980) has been suggested.

PI kinase has been identified in most tissues, and is particularly abundant in myelin (Eichberg & Dawson, 1965; Desmukh *et al.*, 1978), although its role in this specialized membrane is unknown. In this paper, we report the purification and partial characterization of this enzyme from bovine brain myelin.

EXPERIMENTAL

Materials

All chemicals and reagents were from Sigma, except DEAE-cellulose (DE-52) and CM-cellulose (CM-52) (Whatman), Ultrogel AcA-44 (LKB), epoxy-activated

Sepharose (Pharmacia), Extracti-gel (Pierce), silica-gel G plates (Fisher), Triton X-100 and reagents for electrophoresis (Bio-Rad), and [γ -³²P]ATP and silver stain kit (NEN).

Preparation of myelin

Whole brain myelin was isolated by a modification of the method of Norton (1972). Bovine brain (150 g) was minced and homogenized in 10 vol. of 50 mM-Tris/HCl/0.32 M-sucrose, pH 7.4, and centrifuged at 600 g for 10 min. The supernatant was then centrifuged at 27 500 g for 10 min, and the resulting pellet rehomogenized in 600 ml of 50 mM-Tris/HCl/0.8 M-sucrose, pH 7.4. This solution was centrifuged at 27 500 g for 1 h. The supernatant was diluted 1:3 into 50 mM-Tris/HCl, pH 7.4, and centrifuged at 27 500 g for 20 min. The resulting pellet contained whole myelin.

Solubilization of PI kinase

Pellets from the final 27 000 g spin were gently homogenized in 30 ml of 50 mM-Tris/HCl, pH 7.4, containing 75 mM-KCl, 1 mg of poly(ethylene glycol) 20000/ml, 0.1 mM-EDTA, 1.0 mM-dithiothreitol, 0.2 mM-phenylmethanesulphonyl fluoride and 1.0% Triton X-100, at a concentration of 2–3 mg of protein/ml. After a 15 min incubation at 4 °C, the solution was sedimented at 100 000 g for 30 min, and the supernatant was aspirated for further purification.

Assay of PI kinase

Phosphorylation of PI was assayed by a modification of previously described methods (Macara *et al.*, 1984). Enzyme was preincubated for 30 min at 4 °C with 0.1 mM-PI in a total volume of 0.075 ml containing 20 mM-Tris/HCl, 1.0 mM-EDTA and 2.0 mM-dithiothreitol, pH 7.4. Phosphorylation was initiated by

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate.

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addition of [γ - 32 P]ATP and MgCl_2 (final concns. 0.02 and 5.0 mM respectively). After 10 min incubation at 30 °C, reactions were terminated by the addition of 0.66 ml of 1 M-HCl. Polyphosphoinositides were extracted by the sequential addition of 1 ml of chloroform/methanol (2:1, v/v) and 0.66 ml of chloroform. Under these conditions, extraction efficiency was over 90%. After centrifugation at 100 g for 5 min, the upper aqueous phase was washed with an additional 1 ml of chloroform. The lower phases from both steps were pooled, washed with 2 ml of methanol/1 M-HCl (1:1, v/v) and dried under N_2 .

Phosphoinositides were separated on oxalate-pre-treated silica-gel G plates (Rebecchi & Gershengorn, 1983), developed by sequential chromatography in chloroform/methanol/4 M- NH_3 (9:7:2, by vol.) containing diaminocyclohexane-*NNNN'*-tetra-acetic acid ('CDTA'; 0.91 g/90 ml) and chloroform/methanol/acetic acid/water (25:15:4:2 by vol.). ^{32}P incorporation into PIP and PIP_2 was identified by autoradiography on Kodak XAR-5 film, and by comparison with iodine-stained standards. Kinase activity was quantified by scraping the two lipids from the plates and liquid-scintillation counting.

Enzyme activity was proportional to protein concentration, as determined by the method of Lowry *et al.* (1951) after trichloroacetic acid precipitation. Activity of the enzyme was linear with time throughout 15 min. Results are the means of duplicate determinations, in which variability was less than 5%.

SDS/polyacrylamide-gel electrophoresis

Enzyme preparations were solubilized in 62 mM-Tris/HCl/3% SDS/2 mM-dithiothreitol/10% (v/v) glycerol, and run on a 10%-polyacrylamide gel (Laemmli, 1970). For some experiments, 0.5 cm regions of the gel were excised, homogenized in assay buffer, incubated for 16 h at 4 °C, and assayed for PI kinase activity. In parallel lanes, proteins were identified with silver stain, and approximate M_r values were obtained by comparison with standards.

Preparation of PI-Sephacrose

PI (25 mg) was sonicated in 20 ml of 0.1 M- NaHCO_3 , pH 9.5, to prepare vesicles. Then 3 g of preswollen epoxy-activated Sepharose was added, and incubated by mixing at room temperature for 20 h. Then 2 ml of ethanolamine was added to block uncoupled groups, and the gel was washed in water and equilibrated with eluting buffer (50 mM-Tris/HCl/2 mM-dithiothreitol/1 mM-EDTA, pH 7.4). Approx. 1 mg of PI was incorporated per ml of gel.

RESULTS

Purification of PI kinase from bovine brain myelin

The purification of this enzyme is outlined in Table 1. These results are representative of an extraction from 50 g of bovine brain, in which PI kinase was purified 5500-fold with a yield of 13%.

Step 1: solubilization. Whole myelin was solubilized in buffer containing Triton X-100 and KCl as described in the Experimental section. Over 80% of the activity of PI kinase was detected in the 100 000 g supernatant.

Step 2: DEAE-cellulose chromatography. The solubilized supernatant was chromatographed directly by adsorption on to a 10 ml column of DEAE-cellulose (DE-52), equilibrated in the solubilizing buffer. Enzyme activity was eluted with a linear gradient (50 ml) of 0–0.25 M-NaCl in the same buffer; 3 ml fractions were assayed for activity (Fig. 1). PI kinase was eluted with approx. 0.05 M-NaCl. Active fractions were pooled and concentrated by precipitation with 60%-satd. $(\text{NH}_4)_2\text{SO}_4$, followed by resuspension of the protein cake in 5 ml of 50 mM-Tris/HCl/2 mM-dithiothreitol/1 mM-EDTA, pH 7.4, and dialysed for 16 h against 400 vol. of the same buffer at 4 °C.

Step 3: affinity chromatography. Detergent was removed from the preparation by twice passing the dialysed solution through a 10 ml column of Extracti-gel, eluted with dialysis buffer. The detergent-free eluate was loaded on a 5 ml column of PI-Sepharose, which was washed with 10 ml of dialysis buffer, followed by elution with 10 ml of 0.5 mg of PI/ml in 0.5% Triton X-100; 2 ml fractions were assayed for kinase activity (Fig. 2). One peak of PI kinase was detected in the PI/Triton X-100 eluate. Removal of the detergent was necessary for adsorption of the enzyme to PI-Sepharose.

Step 4: gel-permeation chromatography. After elution from PI-Sepharose, peak fractions containing the PI kinase activity were pooled, diluted in 62 mM-Tris/HCl/0.2% SDS/2 mM-dithiothreitol/10% glycerol and chromatographed on a 25 ml Ultrogel AcA44 column, eluted in the same buffer (Fig. 3); 1 ml fractions were assayed for activity. PI kinase activity was eluted at approx. 12–13 ml, identical with the elution volume of ovalbumin. Protein could not be detected in any fraction from this column.

Table 1. Purification of PI kinase

Step	Total activity (pmol/min)	Total protein (mg)	Sp. activity (pmol/min mg)	Yield (%)	Purification (fold)
Whole myelin	1057	210.6	5.01	100	—
Triton X-100	898.8	114.0	7.88	84.9	1.6
DEAE-cellulose	948.9	87.0	10.91	89.7	2.2
$(\text{NH}_4)_2\text{SO}_4$	248.1	9.8	25.32	23.4	5.0
PI-Sepharose	224.8	0.063	3568	21.2	710
Ultrogel AcA4	138.8	0.005	27770	13.1	5550

Fig. 1. DEAE-cellulose chromatography of PI kinase

Solubilized myelin (20 ml) was chromatographed on a 10 ml column of DEAE-cellulose (DE-52), equilibrated in solubilizing buffer. The column was eluted with a linear 50 ml gradient of 0–0.25 M-NaCl as indicated. Protein concentration (■) and PI kinase activity (●) were monitored in the eluted 3 ml fractions.

SDS/polyacrylamide-gel electrophoresis

The purified sample from Ultrogel AcA44 was dissolved in Laemmli (1970) sample buffer without boiling, and subjected to SDS/10%-polyacrylamide gel electrophoresis (Fig. 4). The gel was cut into 0.5 cm segments, which were homogenized and assayed for PI kinase. Although the quantity of enzyme activity measured in the gel was approx. 10% of that measured in solution, PI kinase was detected as a single peak which migrated with an apparent M_r of approx. 45000. Silver staining of this preparation electrophoresed in a parallel lane revealed a major band in this region, with another band migrating with an apparent M_r of approx. 36000. Assay of the gel in the M_r -36000 region revealed no enzyme activity.

Kinetic evaluation of the affinity-purified PI kinase

The kinetics of substrate dependence of the affinity-purified PI kinase were examined, and data were

expressed as double-reciprocal plots (Fig. 5). The substrate curve for PI was non-linear, and could be resolved into two kinetic components, with apparent K_m values of approx. $32 \mu\text{M}$ and 0.4 mM. In contrast, evaluation of the dose-dependence of the enzyme on PIP as substrate revealed a linear relationship, with an apparent K_m of approx. 0.5 mM. The K_m for ATP was approx. 0.15 mM (results not shown).

DISCUSSION

This paper describes the purification of affinity chromatography of PI kinase from rat brain myelin. A peak of kinase activity was identified by detergent extraction of whole myelin, followed by sequential chromatography on DEAE-cellulose, PI-Sepharose and Ultrogel AcA44. Enzyme activity was detected after electrophoresis on SDS/polyacrylamide gels, apparently associated with a major silver-stained band migrating

Fig. 2. PI-Sepharose chromatography of PI kinase

After chromatography on DEAE-cellulose, active fractions were concentrated with $(\text{NH}_4)_2\text{SO}_4$, dialysed, and detergent was removed as described in the Results section. The detergent-free sample (5 ml) was then chromatographed on a 5 ml column of PI-Sepharose. After application of the sample, the column was washed with an additional 10 ml of buffer. As indicated by the arrow, the column was then further eluted with 10 ml of PI (0.5 mg/ml) in the same buffer containing 0.5% Triton X-100. PI kinase activity (●) and protein concentration (■) were measured in 2 ml fractions.

Fig. 3. Ultrogel AcA44 gel-permeation chromatography

Active samples from the PI-Sepharose column were pooled and diluted 1:1 in the SDS buffer as described in the Results section. The sample was chromatographed on a 25 ml column of Ultrogel AcA44, eluted in the same buffer. PI kinase activity was assayed in 1 ml fractions.

Fig. 4. SDS/polyacrylamide-gel electrophoresis

Purified PI kinase was electrophoresed on 10%-polyacrylamide gel by the method of Laemmli (1970). One lane was cut into 0.5 cm segments and homogenized in PI kinase assay buffer. After a 16 h incubation at 4 °C, the homogenized gel fractions were assayed for PI kinase activity for 1 h. M_r standards were run in a parallel lane.

with an M_r of approx. 45000. However, since only 10% of the enzyme activity was detectable after electrophoresis, the possibility remains that the silver-stained protein migrating at M_r 36000 or other minor proteins contain some enzyme activity, which was not detectable under SDS/polyacrylamide-gel electrophoresis.

Evaluation of the substrate specificity of the affinity-purified enzyme revealed a non-linear substrate curve for PI, with two apparent kinetic components. Interestingly, the lower-affinity component exhibited a K_m for PI (0.4 mM) similar to that observed for the phosphorylation of PIP ($K_m = 0.5$ mM) by this affinity-purified preparation. These results may be explained by distinct binding sites on the enzyme with different preferred substrates, heterogeneity of the enzyme preparation, or negatively co-operative interactions. Alternatively, the conformation of an otherwise homogeneous enzyme may be altered as a consequence of association with a heterogeneous population of detergent/phospholipid micelles. To what extent this observed kinetic behaviour of the purified enzyme mirrors that of the native enzyme in its membrane lipid bilayer will require further study.

The PI kinase activity derived from myelin appears to be an unusually stable enzyme. Activity is retained for 3–4 weeks at 4 °C, and can be assayed after treatment with 0.2% SDS at room temperature. The enzyme appears to be relatively hydrophobic, and is found to be aggregated with other proteins even after affinity chromatography. This finding may explain the previously described detection of PI kinase activity in affinity-purified or immunoprecipitated proteins such as the epidermal-growth-factor receptor (Thompson *et al.*, 1985), the insulin receptor (Machicao & Wieland, 1984),

Fig. 5. Substrate-dependence of the affinity-purified PI kinase

After affinity chromatography, the enzyme preparation was diluted 1:10 in detergent-free buffer to a final concentration of 0.5% Triton X-100. Phosphoinositide phosphorylation was assayed as a function of substrate concentration, with (a) PI or (b) PIP as substrate. Data are expressed as double-reciprocal plots.

and the *v-src* (Sugimoto *et al.*, 1984) and *v-ros* (Macara *et al.*, 1984) gene products.

The relatively high activity of PI kinase in myelin is consistent with previous reports documenting high concentrations of polyphosphoinositides in this fraction, yet the physiological role of these lipids in the structure or function of myelin remains puzzling. Although it is

not clear whether the myelin-derived enzyme is identical with other phosphoinositide kinases related to receptor-activated PI metabolism, the further characterization of this purified PI kinase may be helpful in understanding the molecular mechanisms involved in the modulation of the hormonally sensitive turnover of these phospholipids in other tissues.

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