STUDIES OF SUBUNIT STRUCTURE OF PHOSVITIN KINASE FII FROM BOVINE BRAIN

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1. Introduction

Mammalian brain is a rich source of phosphoprotein [1,2]. Such phosphorylation may be caused in part by phosvitin kinase (EC 2.7.1.37) [3–5]. Two kinds of phosvitin kinase, FI and FII, are reported in bovine brain [6], that is, phosvitin kinases FI and FII are eluted with 0.1 M NaCl and 0.5 M NaCl, respectively, on stepwise elution of a DEAE–Sephadex column. Phosvitin kinase FII has been highly purified and is known to have two subunits of M_r 41 000 and 26 000 [6]. The latter is phosphorylated by the enzyme itself.

There are three reports of two types of phosvitin (or casein) kinase, highly purified from tumor [7,8], liver [9,10] and reticulocyte [11]. They are designated types I and II in order of their appearence on DEAE–Sephadex and DEAE-cellulose chromatography [12]. The common characteristics of the type II kinases from these tissues are that they are tetramers of three distinct subunits, and the smallest subunits are autophosphorylated. The occurrence of two subunits in brain phosvitin kinase FII [6] is inconsistent with three kinds of subunit of type II kinases from other tissues.

This paper shows that phosvitin kinase FII from bovine brain is a tetramer of three different subunits like the type II kinases from other tissues [8,9,11], and contrary to the report of two subunits [6]. The enzyme phosphorylated phosvitin, casein and troponin T, but not troponin I.

2. Materials and methods

[γ-^{32}P]ATP, phosvitin–Sepharose 4B, and troponins I and T were prepared by the methods of [13], [14] and [15], respectively. Phosphocellulose, DEAE-cellulose(DE-52), hydroxylapatite and Sephadex G-200 were the products of Brown, Whatman, Seikagaku-kogyo and Pharmacia. Calf thymus histone (type II), salmon sperm protamine, milk α-casein, egg vitellin phosvitin, human γ-globulin, bovine serum albumin, chicken ovalbumin and horse heart cytochrome *c* were purchased from Sigma. Other chemicals were of analytical grade.

2.1. *Polyacrylamide gel electrophoresis*

(i) Two 6% gel electrophoreses of the purified enzyme (10 μg) were performed to estimate the purity of the enzyme preparation [16]. One gel was stained with Coomassie blue and the other was cut into slices (1.7 mm width), followed by extraction of each slice for 48 h at 4°C in 30 mM Tris–HCl, (pH 8.0) and 10 mM 2-mercaptoethanol (200 μl). Phosvitin, α-casein and troponin T phosphorylating activities were each assayed with 50 μl eluate.

(ii) Gel (4.8–8.4%) electrophoreses of the purified enzyme (10 μg) were carried out to determine the molecular weight of the enzyme [17]. Bovine serum albumin solution, stored for 3 months at −20°C, provided standards of M_r 68 000–340 000, because it produced more polymers than the fresh solution.

(iii) The enzyme (16 μg) was preincubated for 60 min at 30°C in 30 μl 30 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (8500 cpm/pmol), followed by sodium dodecyl sulfate gel electrophoresis [18]. The gel stained with Coomassie blue was scanned at 550 nm by a densitometer, and then was sliced into small disks (1.7 mm width). The radioactivity of each slice was

counted by a liquid scintillation spectrophotometer according to [19].

2.2. *Gel filtration*

The purified enzyme (160 μg) was chromatographed on a Sephadex G-200 column (1 × 94 cm) equilibrated with 50 mM Tris–HCl (pH 7.5), 10 mM 2-mercaptoethanol and 0.02% NaN_3 (buffer A) in the presence or absence of 0.4 M NaCl. Fractions (2 ml each) were collected at a flowrate of 7 ml/h.

2.3. *Protein kinase assay*

Enzyme activity was determined by the method in [19].

3. Results

3.1. *Purification of phosvitin kinase FII*

All operations were performed at 4°C.

3.1.1. The first phosphocellulose chromatography step

Fresh bovine brain (1870 g) was homogenized in 2 vol. 1 mM EDTA in buffer A, followed by centrifugation for 60 min at 10 000 × *g*. After adding 57 g NaCl and 20 g phosphocellulose equilibrated with 0.3 M NaCl in buffer A to the supernatant (3250 ml), the mixture was stirred for 60 min and passed through a filter paper. After washing the cellulose layer with buffer A (1500 ml), it was packed in a column of 3.2 cm diam. A 1600 ml linear gradient of NaCl (0.3–1.0 M) in buffer A was applied. Phosvitin kinase was eluted between 0.50–0.73 M NaCl (620 ml).

3.1.2. The second phosphocellulose chromatography step

After adjusting the sample to pH 6.8 with 1 M phosphoric acid and adding 1240 ml 25 mM KH_2PO_4/KOH (pH 6.8), 10 mM 2-mercaptoethanol and 0.02% NaN_3 (buffer B), the mixture was loaded on a phosphocellulose column (2.2 × 11 cm) equilibrated with buffer B. The column was washed with buffer B (200 ml), followed by a 600 ml linear gradient chromatography of NaCl (0.2–1.3 M) in buffer B. Phosvitin kinase activity was eluted between 0.81–1.16 M NaCl (186 ml).

3.1.3. Affinity chromatography

After adjusting the preparation to pH 7.5 with 1 M Tris base and diluting it with buffer A (600 ml), the mixture was chromatographed on a phosvitin–Sepharose 4B column (1.5 × 9.5 cm) with a 200 ml linear gradient of NaCl (0.25–0.75 M) in buffer A. Phosvitin kinase activity was eluted between 0.40–0.55 M NaCl (69 ml).

3.1.4. DEAE-cellulose chromatography

Buffer A (350 ml) was added to the above fraction, and the mixture was loaded on a DE-52 column (3.5 × 15 cm) equilibrated with buffer A. The chromatography was carried out by a 1400 ml linear gradient of NaCl (0.10–0.35 M). Phosvitin kinase activity was eluted between 0.16–0.26 M NaCl (390 ml). Since the peak of kinase activity was eluted at 0.19 M NaCl, this kinase was equivalent to the phosvitin kinase FII in [6].

3.1.5. Hydroxylapatite chromatography

After adjusting the preparation to pH 6.8 with 1 M phosphoric acid and diluting it with buffer B (390 ml), the mixture was chromatographed on a hydroxylapatite column (2.2 × 5 cm) with a 200 ml linear gradient of KH_2PO_4/KOH (pH 6.8) (0.15–0.35 M), 10 mM 2-mercaptoethanol and 0.02% NaN_3. Phosvitin kinase was eluted between 0.18–0.23 M KH_2PO_4/KOH (62 ml). This fraction was concentrated with Ficoll to 1.6 mg/ml and employed in the subsequent experiments. A summary of the purification is given in table 1. Phosvitin kinase was purified 1746-fold and the final recovery of the enzyme was 6%.

3.2. *Substrate specificity*

Troponin T and acidic proteins like phosvitin and α-casein were phosphorylated, and the relative initial velocities were 2.4, 1.0 and 0.5 for phosphorylation of troponin T, phosvitin and α-casein, respectively. Troponin T phosphorylation reached a plateau level within 20 min on the incubation with the enzyme (80 ng/assay), while phosphorylation of phosvitin and α-casein increased linearly up to 60 min. Histone, protamine and troponin I were not phosphorylated. Cyclic nucleotides and Ca^{2+} had no effect on the protein phosphorylation (not shown).

3.3. *Purity of the enzyme preparation*

Figure 1 shows the result of 6% polyacrylamide gel electrophoreses. A single protein band was seen at 1 cm

Table 1
Purification of brain phosvitin kinase FII

Step	Total protein (mg)	Total act. (units)[a]	Spec. act. (units/mg)	Purification (-fold)
1. Crude extract	57 418	3947	0.07	1
2. Phospho cellulose (1)	124	1697	13.7	198
3. Phospho cellulose (2)	37	1444	39.0	566
4. Phosvitin–Sepharose 4B	5	1095	73.0	1058
5. DE-52-cellulose	10	833	83.3	1207
6. Hydroxylapatite	2	241	120.5	1746

[a] One unit was defined as that amount of enzyme which catalyzed the incorporation of 1 nmol P_i from [γ-^{32}P]ATP into phosvitin/min at 30°C

from the top of the gel (fig.1A). Phosvitin, α-casein (not shown) and troponin T phosphorylating activities were detected in the same protein band (fig.1B). The molecular size of this enzyme protein was estimated to be M_r 320 000 (fig.1C).

3.4. *Disaggregation of the enzyme*

A gel filtration in the absence of NaCl resulted in the elution of phosvitin kinase at the void volume (M_r > 300 000) (fig.2A). In the presence of NaCl, phosvitin kinase was eluted at the position where γ-globulin was eluted (fig.2B).

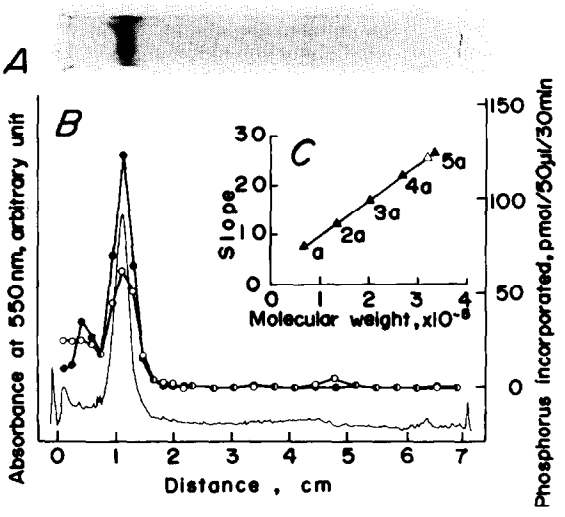

Fig.1. Polyacrylamide gel electrophoresis. Purified enzyme (10 µg) was used in each gel. (A) A 6% gel stained with Coomassie blue. (B) Enzyme activities on a 6% gel. (●,○) Phosvitin and troponin T phosphorylating activities. (——) A_{550}. (C) Estimation of molecular weight of the enzyme. Slope, [100 log(relative mobility × 100)]/% gel, according to [17]. (▲a,2a,3a,4a and 5a) mono-, di-, tri-, tetra- and pentamers, respectively, of bovine serum albumin. (△) The purified enzyme.

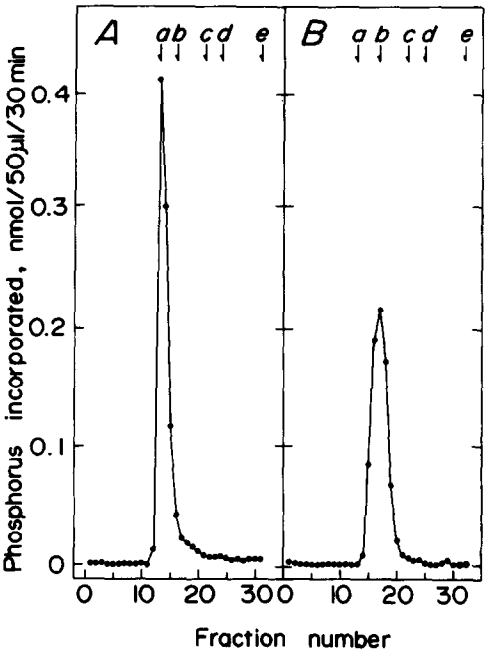

Fig.2. Sephadex G-200 chromatography in the absence (A) and presence (B) of 0.4 M NaCl. The purified enzyme (160 µg) was applied. (●) Phosvitin kinase activity. (a,b,c,d and e) Positions where blue dextran (M_r > 2 000 000), γ-globulin (M_r 160 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000) and cytochrome *c* (M_r 12 400), respectively, were eluted.

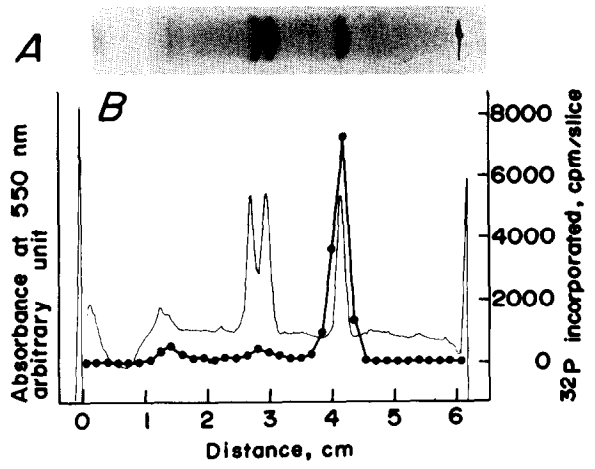

Fig.3. Sodium dodecyl sulfate polyacrylamide gel (8%) electrophoresis. The purified enzyme (16 μg) was employed. (A) Coomassie blue stain. (B) Autophosphorylation of a subunit. (•) radioactivity incorporated in a slice. (——) A_{550}.

3.5. *Subunits and autophosphorylation*

Sodium dodecyl sulfate 8% polyacrylamide gel electrophoresis showed that phosvitin kinase was composed of three subunits of M_r 46 000 (α), 41 000 (α′) and 24 000 (β) (fig.3A). The molar ratios of these subunits were 1.0 (α):1.3 (α′):2.0 (β) by measuring the area of each peak (fig.3B). Moreover, the β-subunit incorporated ^{32}P from [γ-^{32}P]ATP (fig.3B).

4. Discussion

The phosvitin kinase FII as prepared here was composed of three subunits, contrary to the report of only two subunits in the same enzyme [6]. The molar ratios of these subunits (fig.3B) and the enzyme molecular size (M_r 160 000), estimated by the gel filtration in the presence of NaCl (fig.2B), indicate that the most probable structure of the holoenzyme is $\alpha\alpha'\beta_2$. The results of gel filtration without NaCl (fig.2A) and the polyacrylamide gel electrophoreses (fig.1C) show aggregation and dimerization of $\alpha\alpha'\beta_2$, respectively. These findings agree with the behavior of protein kinase KII from Novikoff ascites tumor [8], protein kinase NII from rat liver [9] and casein kinase II from rabbit reticulocyte [11].

The subunits α′ and β in this report may correspond to the subunits of M_r 41 000 and 26 000 of phosvitin kinase FII reported in [6], because of the same molecular size and autophosphorylation, respectively. However, the present authors cannot explain why the phosvitin kinase FII reported in [6] was devoid of the largest subunit α. One possibility is that the subunit α is degraded into the smaller subunit α′ or other fragments by proteolysis during the purification, especially in the defrosting step [6].

The last important point is that the present enzyme catalyzed phosphorylation of troponin T but not troponin I. Although there is no report of the occurrence of troponins in brain so far, muscle troponin T is known to be phosphorylated by phosphorylase *b* kinase [20,21] and troponin T kinase [19,22]. While the former enzyme catalyzes phosphorylation of troponin I and T, the latter phosphorylates troponin T, phosvitin and casein, but not troponin I. Therefore, muscle troponin T kinase may be included in the same class of kinase as phosvitin kinase FII.

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