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CALF BRAIN PHOSVITIN KINASE

II. PURIFICATION AND CHARACTERIZATION OF THREE DIFFERENT FRACTIONS OF PHOSVITIN KINASE

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SUMMARY

Three different fractions of calf brain phosvitin kinase have been purified, two of them to a high degree of purity. After purification all three fractions still contained a tightly bound protein which was phosphorylated when the fractions were incubated with [γ-^{32}P]ATP. The [^{32}P]phosphate was bound to serine and threonine residues. Two of the fractions which were resolved by ion exchange chromatography, displayed the same substrate specificity and behaved in an identical fashion on Sephadex chromatography. Furthermore, on polyacrylamide gel electrophoresis in detergent the two purified fractions gave the same protein pattern, characterized by one unlabelled and one ^{32}P-labelled band. The former was attributed to the phosvitin kinase itself or to one a subunit of it (mol. wt about 41 000) and the latter to the phosphoprotein substrate (mol. wt about 26 000).

The third phosvitin kinase fraction was not homogeneous on polyacrylamide gel electrophoresis. Since it was of a lower molecular weight and had a different substrate specificity, it was presumed to be another kind of phosvitin kinase.

In contrast to the cyclic AMP-dependent protein kinases, none of the purified phosvitin kinases catalyzed the phosphorylation of histone to any appreciable extent, nor were they stimulated by cyclic AMP.

INTRODUCTION

The phosvitin kinases belong to the group of enzymes called protein kinases (ATP: protein phosphotransferase, EC 2.7.1.37), which catalyze the transfer of phosphoryl groups from ATP to certain proteins. They are characterized by transferring phosphoryl groups preferentially to phosvitin or casein in a reaction which is generally not stimulated by cyclic AMP[1–3]. The phosvitin kinases should be distinguished from the histone kinases, which preferentially phosphorylate histones, in a cyclic AMP-stimulated reaction[4,5]. The histone kinases are assumed to be involved in hor-

mone action[6], but the intracellular role of the phosvitin kinases is only partly understood.

Calf brain phosvitin kinase has been studied in this laboratory in an attempt to elucidate the physiological function of the enzyme[3]. On purification, three fractions of phosvitin kinase were obtained. Only one of these fractions was further purified and characterized. The purified enzyme was shown to catalyze the phosphorylation of nuclear phosphoprotein[3]. It was tightly bound to a phosphoprotein, which was ^{32}P-labelled when the enzyme fraction was incubated with [γ-^{32}P]ATP. This phosphoprotein was supposed to be a physiological substrate for the kinase. These data are in accordance with those presented by Langan[7], who also suggested that nuclear phosphoprotein was involved in gene activation.

As mentioned above, only one of the three enzyme fractions was further purified and studied in the previous report. This paper deals with the purification of all three fractions by a procedure which is simpler than the previous one and which can be applied to all three enzyme fractions. The substrate specificity, [^{32}P]phosphate incorporation and behaviour on polyacrylamide gel electrophoresis of the three purified fractions have been compared.

EXPERIMENTAL

Materials

Phosphoryl cellulose was a product of Serva (Heidelberg, Germany). DEAE-Sephadex (A-50), Sephadex G-200 and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden). Dithiothreitol (Cleland's reagent) and β-lactoglobulin were from Calbiochem. Phosvitin, histone (Type II-A from calf thymus) and horse albumin were obtained from Sigma. Hammarsten casein was purchased from Merck and pepsin (EC 3.4.4.1) from Boehringer. Human serum albumin was from AB Kabi (Stockholm, Sweden). All reagents were of p.a. grade. [γ-^{32}P]ATP was prepared according to Engström[8] and calcium phosphate gel as described by Tsuboi and Hudson[9].

Analytical methods

Radioactivity was measured as previously described[10]. Protein was assayed according to the method of Lowry *et al.*[11] with human serum albumin as standard. During the chromatographic steps protein was determined by measuring $A_{280\ nm}$ and $A_{260\ nm}$ in a Zeiss spectrophotometer Type PMQ II.

Assay of enzyme activities

The crude protein fractions were assayed for phosvitin kinase essentially as described previously[3]. The ^{32}P-labelled phosvitin was, however, separated from excess [^{32}P]ATP by Millipore filtration and not by acid precipitation as described before[3]. The inactivated incubation mixtures were applied to Millipore filters (SCWP 2500), and after suction to dryness, the filters were washed three times with 5 ml 10% trichloroacetic acid. They were then placed in aluminium cups and the radioactivity determined as described by Forsberg *et al.*[10]. This method was found to be more rapid than the one used before. The results obtained when assaying the chromatographic fractions with the two methods were in good agreement with each other. However, Millipore filtration was not used in the assay of the crude protein fractions,

since it was difficult to obtain reproducible results. This was ascribed to the large amounts of protein applied to the filters in these cases. One unit of phosvitin kinase is defined as before[3].

When investigating the substrate specificity of the purified kinases, histone and casein were also used as substrates. The assay was performed as described[3].

Determination of [^{32}P]phosphate incorporation into purified enzyme fractions on incubation with [^{32}P]ATP

The purified enzyme fractions were incubated with [^{32}P]ATP as previously described[3]. When the effect of cyclic AMP was investigated cyclic AMP was added to the incubation mixture to a final concentration of 5 μM. Inactivation was performed by adding 0.2 vol. of 50% trichloroacetic acid. A few seconds prior to inactivation 1 mg of bovine serum albumin was added as a carrier. The precipitate obtained after centrifugation was dissolved in 1 ml 0.1 M NaOH. The protein was reprecipitated by the addition of 3 ml ice-cold 10% trichloroacetic acid. After centrifugation the supernatant was sucked off. This procedure was repeated three times. Finally the precipitate was dissolved in 0.1 M NaOH plated in aluminum cups and the radioactivity determined[10]. This method for obtaining protein-bound [^{32}P]phosphate free from [^{32}P]ATP was found to give values which were in good agreement with those obtained by Sephadex chromatography in detergent[3]. The procedure involving washing with trichloroacetic acid was most convenient and was therefore used.

Isolation of [^{32}P]phosphorylserine and [^{32}P]phosphorylthreonine from ^{32}P-labelled enzyme fractions

Each purified enzyme fraction was incubated with [^{32}P]ATP as above. Excess [^{32}P]ATP was removed by repeated washings in trichloroacetic acid. The final protein precipitate was subjected to acid hydrolysis[3], and the amounts of [^{32}P]phosphorylserine and [^{32}P]phosphorylthreonine in the hydrolysate were determined as described previously[3].

Polyacrylamide gel electrophoresis of purified enzyme fractions

The purified enzyme fractions were subjected to polyacrylamide gel electrophoresis as described by Dunker and Rueckert[12]. For the determination of molecular weights, horse albumin, pepsin and β-lactoglobulin with molecular weights of 68 600, 35 500 and 17 500, respectively, were used as reference substances. Before electrophoresis the samples were treated for 30 min at 45 °C with 4 M urea containing 1% (v/v) 2-mercaptoethanol and 1% (w/v) sodium dodecylsulphate. 10% acrylamide gels were prepared in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% sodium dodecylsulphate. Electrophoresis was performed in this buffer for 4 h at 8 mA per gel.

The ^{32}P-labelled purified enzyme fractions were also subjected to polyacrylamide gel electrophoresis. The enzyme fractions were ^{32}P-labelled by incubation with [^{32}P]ATP, essentially as above. After incubation for 30 min at 37 °C, EDTA was added to a final concentration of 0.02 M. After concentration by ultrafiltration, the ^{32}P-labelled fractions were subjected to polyacrylamide gel electrophoresis as above.

After electrophoresis the gels were immersed in 12.5% trichloroacetic acid over night in order to extract the sodium dodecylsulphate. Staining was performed

with Comassie brilliant blue in 12.5% trichloroacetic acid and destaining with 7.5% acetic acid. By this treatment, [^{32}P]ATP and [^{32}P]orthophosphate were effectively removed from the gels. The destained gels were cut to about 2 mm slices, which were placed in aluminum cups and the radioactivity was determined as described above.

RESULTS

Purification procedure

The results of a typical preparation are given in Table I. The first steps leading to the three different fractions of phosvitin kinase (Fractions I, II_A and II_B) have been described previously[3]. All centrifugations were performed in a Sorwall RC2-B centrifuge unless otherwise stated. Ultrafiltration and dialysis were performed as described previously[3].

TABLE I

PURIFICATION OF CALF BRAIN PHOSVITIN KINASE

2.3 kg of calf brain were used. The initial purification procedure consisting of homogenization, calcium phosphate gel fractionation and $(NH_4)_2SO_4$ precipitation was performed as previously described[3]. The recoveries and purification factors are calculated in relation to the homogenate. For details see under Results.

Purification step	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg)*	*Purification factor*	*Recovery (%)*
First DEAE-Sephadex					
Fraction I (0.1 M NaCl)	25 900	475	55	16	13
Fraction II (0.5 M NaCl)	51 500	510	100	29	25
Further purification of Fraction I:					
Phosphoryl cellulose	12 800	20.3	630	180	6.2
Sephadex G-200	7950	7.3	1100	310	3.8
Further purification of Fraction II:					
Second DEAE-Sephadex					
Fraction II_A (first peak)	13 700	89	150	44	6.6
Fraction II_B (second peak)	26 000	61	420	120	13
Phosphoryl cellulose II_A	9620	1.8	5500	1600	4.6
Phosphoryl cellulose II_B	15 200	2.8	5500	1600	7.3
Sephadex G-200 II_A	6160	0.60	10 300	2900	3.0
Sephadex G-200 II_B	10 800	1.1	9600	2800	5.2

Initial steps (see ref. 3)

2.3 kg of calf or cow brain were frozen at −20 °C immediately after being brought from the slaughterhouse. The material was defrosted overnight at 4 °C before homogenization as described previously[3]. Adsorption onto calcium phosphate gel and $(NH_4)_2SO_4$ precipitation of the phosvitin kinase eluted from the gel was also performed as before[3]. The phosvitin kinase fraction thus obtained was chromatographed on a column of DEAE-Sephadex, which was eluted by stepwise increments of the NaCl concentration. About 30% of the phosvitin kinase activity eluted from the column was displaced by 0.1 M NaCl (Fraction I) and about 60% by 0.5 M NaCl (Fraction II). Initial freezing and defrosting of the brain was found to be necessary to obtain a separation of the protein fractions eluted from the column. The reason for this has not been investigated closely. Fraction II was applied to a new column of DEAE-

Sephadex, which was subjected to gradient elution[3]. Two enzyme peaks, called Fractions II_A and II_B, were obtained. The kinase activity of Fraction II_A generally amounted to 1/2–1/3 of that of Fraction II_B.

Chromatography on phosphoryl cellulose

Fraction I as well as Fractions II_A and II_B were further purified by chromatography on phosphoryl cellulose. Each fraction was first concentrated by ultrafiltration and Fractions II_A and II_B were also dialyzed against 0.01 M triethanolamine-acetic acid buffer (pH 7.4), containing 0.1 M NaCl and $1 \cdot 10^{-4}$ M Cleland's reagent. The fractions were then applied to separate columns of phosphoryl cellulose, which were equilibrated with the above-mentioned dialysis buffer. As shown in the legend of Fig. 1, each column was eluted with a linear gradient of 0.1 M NaCl to 0.8 M NaCl. For all three fractions most of the protein was present in the run-off volume, which contained practically no phosvitin kinase activity. The kinase was bound to the column, and was in all three cases eluted with about 0.5 M NaCl. The enzyme-containing fractions from each chromatography were pooled and concentrated by ultrafiltration to a volume of 3–6 ml.

A minor enzyme peak preceding the main one was often seen. It was of different heights in different preparations and was not investigated further.

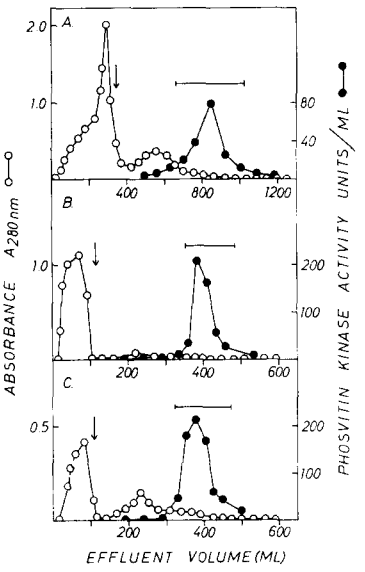

Fig. 1. Chromatography of phosvitin kinase fractions on phosphoryl cellulose. Elution was first performed with 0.1 M NaCl in 0.01 M triethanolamine-acetic acid buffer (pH 7.4) and then with a linear gradient, starting at the arrow, of 0.1 M NaCl to 0.8 M NaCl. All buffers also contained $1 \cdot 10^{-4}$ M Cleland's reagent. A. Fraction I from the first DEAE-Sephadex. Column dimensions: 2 cm × 16 cm. The total volume of the gradient was 1000 ml. Fraction volume, 6–8 ml. B. Fraction II_A from the second DEAE-Sephadex. Column dimensions: 1.5 cm × 14 cm. The total volume of the gradient was 500 ml. Fraction volume, 4–5 ml. C. Fraction II_B from the second DEAE-Sephadex. Column dimensions, volume of the gradient and fraction volume as in B. Fractions were pooled as indicated, concentrated by ultrafiltration and chromatographed on Sephadex G-200.

Chromatography on Sephadex G-200

The concentrated enzyme fractions were then applied to separate columns of Sephadex G-200, which were equilibrated with 0.01 M triethanolamine–acetic acid buffer, pH 7.4, containing 1.0 M NaCl and $1 \cdot 10^{-4}$ M Cleland's reagent. As is shown in Fig. 2, the enzyme activity was eluted as one distinct peak in all three cases. The phosvitin kinase of Fraction I was eluted after 0.64 column-volumes and that of Fraction II_A and II_B after 0.43 column-volumes. Thus, the phosvitin kinase of Fractions II_A and II_B were of the same molecular size, while that of Fraction I was of a smaller size. The enzyme-containing fractions from each chromatography were pooled and concentrated by ultrafiltration to a volume of 3–6 ml and were then stored at −20 °C. The phosvitin kinase of Fraction I had then been purified about 300 times, that of Fraction II_A and II_B 2500–3000 times. The recoveries were about 4, 3 and 5%, respectively. These purified preparations were used for the further studies.

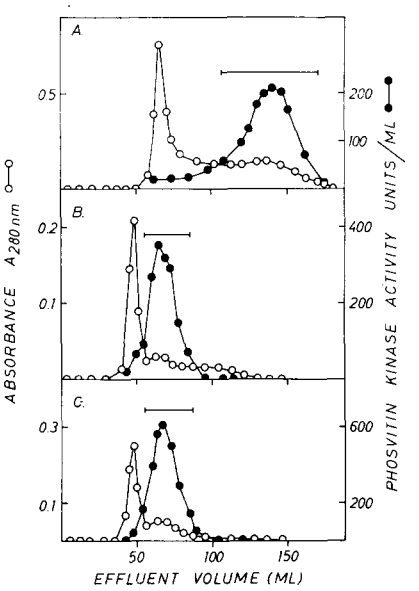

Fig. 2. Chromatography of phosvitin kinase fractions from phosphoryl cellulose on Sephadex G-200. Elution was performed with 0.01 M triethanolamine–acetic acid buffer (pH 7.4) containing 1.0 M NaCl and $1 \cdot 10^{-4}$ M Cleland's reagent. A. Fraction I. Column dimensions: 3 cm × 31 cm. Fraction volume, 3.0 ml. B. Fraction II_A. Column dimensions: 3 cm × 21.5 cm. Fraction volume, 3.0 ml. C. Fraction II_B. Column dimensions and fraction volume as in B.

Aggregation of phosvitin kinase

When phosvitin kinase was chromatographed on Sephadex G-200 in 0.1 M NaCl instead of 1.0 M NaCl the enzyme displayed a considerable tendency to form high-molecular aggregates. This tendency could be reversed by treatment with 1.0 M NaCl. Since the first DEAE-Sephadex chromatography was preceded by dialysis against 0.1 M NaCl it was desirable to investigate the state of aggregation of Peaks II_A and II_B. Samples of Peaks II_A and II_B from the DEAE-Sephadex chromatography were separately concentrated by ultrafiltration and carefully dialyzed against 0.01 M triethanolamine–acetic acid buffer (pH 7.4) containing 0.1 M NaCl and $1 \cdot 10^{-4}$ M

Cleland's reagent. The two peaks were then separately rechromatographed on DEAE-Sephadex using gradient elution as before. In each case on distinct peak was obtained and there was no evidence that one fraction was an aggregation product of the other.

Substrate specificity of the purified enzymes

Both phosvitin and casein were phosphorylated to a considerable extent by all three fractions of phosvitin kinase (Table II). Casein was the best substrate for Fraction I, while phosvitin was best for Fraction II_A and Fraction II_B. The ratio of the phosphorylation rates with phosvitin and casein was about the same for Fractions II_A and II_B. Histone was not phosphorylated to any appreciable extent by the three enzyme fractions. Neither were they stimulated by cyclic AMP using any of the substrates tested.

TABLE II

SUBSTRATE SPECIFICITY OF PURIFIED PHOSVITIN KINASES

Protein kinase activity was measured as described under Experimental. The concentration of cyclic AMP was 5 μM.

Enzyme fraction	*nmoles of [^{32}P]phosphate transferred per mg of enzyme*					
	Phosvitin		*Casein*		*Histone*	
	−cAMP	*+cAMP*	*−cAMP*	*+cAMP*	*−cAMP*	*+cAMP*
I	820	770	1200	1200	2	5
II_A	6200	6200	3700	3800	20	30
II_B	9200	9200	4700	4500	20	10

[^{32}P]Phosphate incorporated into purified enzyme fractions on incubation with [^{32}P]ATP

The purified enzyme fractions were found to incorporate 0.1–1 nmole of [^{32}P]phosphate per mg of enzyme, under the conditions described above. In accordance with the results in the preceding paragraph, cyclic AMP did not stimulate the phosphorylation of any of the enzyme fractions.

By isolating [^{32}P]phosphorylserine and [^{32}P]phosphorylthreonine after acid hydrolysis of the ^{32}P-labelled enzymes it was concluded that the [^{32}P]phosphate was covalently linked to serine and threonine residues. The isolated phosphoamino acids amounted to 20–25% of the radioactivity of the hydrolysates, which indicates that practically all of the [^{32}P]phosphate was bound to serine and threonine residues before hydrolysis[7]. The ratio of [^{32}P]phosphorylserine to [^{32}P]phosphorylthreonine varied somewhat between different preparations but was generally between 5 and 15 for all three enzyme fractions.

Polyacrylamide gel electrophoresis of the purified enzyme fractions

The purified enzyme fractions were compared with respect to the protein pattern obtained on polyacrylamide gel electrophoresis in sodium dodecylsulphate. Fraction I was found to give several protein bands. Fractions II_A and II_B were found to give identical patterns, consisting of two protein bands (Fig. 3). The identity of the two fractions was tested by mixing the two fractions and subjecting the mixture to electrophoresis. Only two bands were obtained from the mixture (Fig. 3).

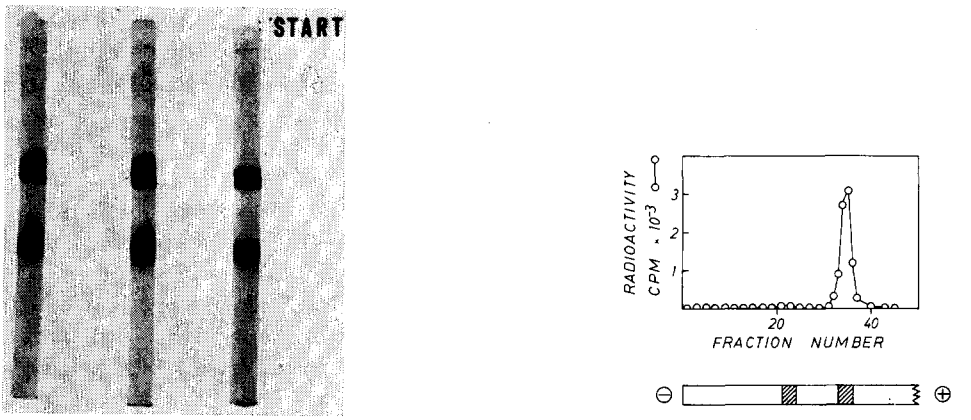

Fig. 3. Polyacrylamide gel electrophoresis of purified unlabelled phosvitin kinases. From right to left: Fraction II_A (50 μg), Fraction II_B (58 μg) and a mixture of the two fractions containing 8 μg of Fraction II_A and 14 μg of Fraction II_B. Electrophoresis was run for 4 h at 8 mA per gel in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% sodium dodecylsulphate. The gels were stained with Coomassie blue as described in the text.

Fig. 4. Polyacrylamide gel electrophoresis of purified ^{32}P-labelled phosvitin kinase of Fraction II_A. The enzyme fraction was ^{32}P-labelled by incubation with [^{32}P]ATP (spec. act. 190 000 cpm/nmole) as described under Experimental. 24 μg of ^{32}P-labelled enzyme were applied to the gel. Electrophoresis was performed as described in the legend of Fig. 3. The gel stained with Coomassie blue is schematically drawn in the lower part of the figure. The radioactivity of the slices obtained after transverse section of the gel is shown in the upper part of the figure.

The ^{32}P-labelled enzyme fractions were also subjected to polyacrylamide gel electrophoresis. Several of the protein bands obtained from Fraction I were ^{32}P-labelled. In the case of Fractions II_A and II_B only the fast-migrating protein band was ^{32}P-labelled, which is shown for Fraction II_A in Fig. 4.

The molecular weights of the two bands obtained from Fractions II_A and II_B, were found to be about 41 000 and 26 000.

DISCUSSION

When a partly purified calf brain homogenate was chromatographed on DEAE-Sephadex, three different fractions of phosvitin kinase were obtained[3]. In this report all three fractions have been further purified by a procedure involving chromatography on phosphoryl cellulose and Sephadex G-200. Chromatography on phosphoryl cellulose afforded a considerable purification (10–40 times). The value of phosphoryl cellulose in the purification of phosvitin kinase was originally described by Baggio *et al.*[13]. It was found that the phosvitin kinase fractions retained on DEAE-Sephadex at pH 7.4 as well as that present in the run-off volume, were all bound by phosphoryl cellulose when chromatographed at this pH. Since these fractions should differ considerably in electric charge there appears to be a specific interaction between the phosvitin kinases and phosphoryl cellulose. After chromatography on Sephadex G-200, the purity of Fraction I had increased about 300 times and that of Fractions IIA and IIB almost 3000 times. The purified fractions were found to incorporate 0.1–1 nmole of [^{32}P]phosphate per mg on incubation with [^{32}P]ATP. Fraction II_B, purified

by a method not involving phosphorylcellulose chromatography, was previously reported to incorporate about 3 nmoles of [^{32}P]phosphate per mg[3]. In view of the results of Takeda *et al.*[2] it seems probable that most of the phosphoprotein was removed from the kinase by chromatography on phosphoryl cellulose.

The elution volumes of the phosvitin kinase of Fractions II_A and II_B when chromatographed on Sephadex G-200 were similar, indicating that the two kinases have about the same molecular weight. Furthermore the two enzyme fractions gave identical patterns on polyacrylamide gel electrophoresis (Fig. 3) and displayed about the same substrate specificity (Table II). Since phosvitin kinase was shown to form aggregates at concentrations of NaCl as high as 0.1 M NaCl, it was conceivable that Fractions II_A and II_B represented different aggregates of the same phosvitin kinase. The formation of such aggregates has recently been reported for rat liver phosvitin kinase[14]. However, when each of the two fractions was carefully dialyzed against 0.1 M NaCl and then rechromatographed on DEAE-Sephadex no interconversion could be seen. The different behaviour of the two fractions on DEAE-Sephadex might instead depend on some minor difference which does not affect the migration rate on polyacrylamide gel electrophoresis or the elution volume during Sephadex chromatography.

The purified ^{32}P-labelled Fractions II_A and II_B also displayed identical patterns on polyacrylamide gel electrophoresis. In both cases one unlabelled and one ^{32}P-labelled protein band were obtained. The molecular weight of the unlabelled protein was about 41 000, that of the ^{32}P-labelled one about 26 000. The unlabelled band might represent the phosvitin kinase subunit, which had been separated from the phosphoprotein moiety. This could not be verified since phosvitin kinase was not active in 0.1% sodium dodecylsulphate.

On chromatography on Sephadex G-200, the phosvitin kinase from Fraction II was eluted at a position corresponding to a molecular weight in the range of 100 000 to 500 000. It can thus be presumed that native phosvitin kinase is composed of several catalytic subunits (mol. wt about 41 000) with a tightly bound phosphoprotein substrate (mol. wt about 26 000). The phosvitin kinase of Fraction I differed in several respects from that of Fraction II. It was eluted much later during chromatography on Sephadex G-200 (Fig. 2), the substrate specificity was different and it behaved differently on ion chromatography.

The intracellular localization of the purified phosvitin kinases is not known. It is, however, of interest in this context that rat liver contains two phosvitin kinases similar to Fractions I and II from calf brain[2]. Experiments in this laboratory indicate that rat liver cell sap contains mainly the phosvitin kinase corresponding to Fraction II whereas a salt extract of microsomes contains mainly the enzyme corresponding to Fraction I (O. Wålinder, manuscript in preparation).

None of the three fractions of phosvitin kinase showed any appreciable activity when incubated with histone (Table II). Cyclic AMP did not stimulate the activity of any of the fractions. On the other hand, the bovine brain protein kinases purified by Miyamoto *et al.*,[15] Weller and Rodnight[16], and Goodman *et al.*[17] were all active towards histone and were also stimulated by cyclic AMP. The phosvitin kinases described above thus differ from the cyclic AMP dependant protein kinases. However, the latter have been shown to consist of catalytic and cyclic AMP binding subunits[18]. These two types of subunits can be dissociated from each other, and the protein ki-

nases are then no longer stimulated by cyclic AMP[18]. It is of interest in this context that the molecular weight of the catalytic subunit of the bovine brain histone kinase of Miyamoto *et al.*[19], was 40 000. This is about the same as that obtained for the catalytic subunit of Fraction II of calf brain phosvitin kinase. Thus, the relation between the phosvitin kinases described above and the cyclic AMP dependant protein kinases requires further attention.

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