ISOLATION AND PROPERTIES OF A PHOSVITIN KINASE FROM CHICK EMBRYO

L. A. PINNA, V. MORET and N. SILIPRANDI
Institute of Biological Chemistry, University of Padova, Padova, Italy

Received 12 August 1968

Although the presence of phosphoproteins in many organisms and tissues has been reported [1-6], the role of these compounds is still obscure. The turnover of their phosphate groups appears to be regulated by at least two classes of specific enzymes: protein phosphatases which hydrolize the protein phosphate to orthophosphate [7, 8] and protein kinases which transfer phosphate from ATP to phosphoproteins [9-11]. Since it is difficult to isolate and purify endogenous phosphoproteins from tissues, in most instances the activity of these enzymes has been tested by using as substrate foreign phosphoproteins, like casein or phosvitin. However the phosphate transfer to such foreign proteins might be of limited physiological significance.

In order to give an answer to this question it seemed to us of interest to investigate the specificity of the chick embryo enzymes concerned with the phosphate turnover in phosphoproteins, by using as substrate the phosvitin which is available in egg yolk.

Both embryo and yolk, at least up to the 8th day of development, do not exhibit any phosvitin phosphatase activity tested both in the crude homogenate and in the dialysed extract under the most suitable conditions for the test *.

On the other hand, the same extract from embryos on the 7th day of development, actually shows a very significant protein kinase activity catalysing the transfer of ^{32}P from (γ-^{32}P)ATP to added phosvitin (table 1). The yolk, which is lacking in any phosvitin kinase activity, can be phosphorylated, like phosvitin, by embryo extracts.

The embryo phosvitin kinase has been purified about 10-fold, as described in the legend of table 1. The activity of partially purified enzyme has been tested also on other phosphoproteins: casein and protamine, unlike ovalbumin, are phosphorylated, though to a lower extent than phosvitin. The combined addition of phosvitin and protamine results in the formation of an insoluble complex which is phosphorylated to a much lower extent than free phosvitin (see table 1).

The pH dependence of enzyme activity is shown by the curve of fig. 1, while in table 2 the apparent K_m for phosvitin and casein as well as the V_{max}(phosvitin)/V_{max}(casein) ratio are compared with those obtained with rat liver protein kinase. Surprisingly, in spite of the fact that phosvitin is a physiological substrate for the embryo enzyme, the apparent K_m of phosvitin is not higher, but even lower for the liver kinase than for the chick embryo kinase.

This supports the idea that in tissues some phosphoproteins are present which share with phosvitin fundamental similarities of structure.

The reversibility of the phosphate transfer between ATP and phosvitin, catalysed by the partially purified chick embryo enzyme, has been demonstrated by using ^{32}P-labelled phosvitin and unlabelled ADP. Appreciable amounts of (^{32}P)ATP have been detected at the end of incubation (table 3).

In conclusion the presence of a protein kinase catalysing the reversible phosphate transfer between ATP and phosvitin, and the absence of any protein phosphatase activity suggest that the role of phosvitin might be that of supplier of high energy phosphate

* The extract, prepared as described in table 1, was dialysed overnight against 20 mM cysteine pH 6.5 in order to remove the Pi which is known to inhibit protein phosphatase and its phosphatase activity was tested at pH 6.0, in the presence and absence of Mg^{++} and cysteine.

North-Holland Publishing Company – Amsterdam

Table 1

Experiment	mμmoles ^{32}P transferred to phosphoprotein per mg enzyme protein
1. Crude embryo extract	0.82
Mg^{++} omitted	0.06
plus phosvitin (1 mg)	8.52
Dialyzed yolk	0.07
plus phosvitin (1 mg)	0.06
plus crude embryo extract	5.34
2. Partially purified kinase	
plus phosvitin (1 mg)	74.40
plus casein (1 mg)	35.59
plus ovalbumin (1 mg)	0.70
plus ovalbumin (4 mg)	0.50
3. Partially purified kinase	
plus phosvitin (1 mg)	51.60
plus protamine (0.5 mg)	12.67
plus phosvitin plus protamine	18.10

Extracts were prepared by homogenizing 7-days old chick embryos free of yolk in five times their volume of ice-cold 0.1 M phosphate buffer pH 6.8 containing 0.3 M NaCl and 0.1 mM EDTA. After two hours stirring at 2°C the insoluble residue was discarded by centrifugation at 40 000 × *g*. The opalescent supernatant, as well as the yolk, were separately dialysed against 3 changes of 0.05 M Tris-HCl buffer pH 7.0 containing 0.1 mM EDTA. The partially purified enzyme preparation was obtained from the crude embryo extract by following in its general lines the procedure described by Rodnight and Lavin [10] for brain protein kinase, except in that the active fraction used in these experiments was precipitated between 25 and 55% saturation of $(NH_4)_2SO_4$. Protein kinase activity was tested by incubating for 30 min at 37°C 1 ml of a medium containing: 6 μmoles $MgCl_2$; 200 μmoles Tris-HCl buffer pH 7.5; 0.5 μmoles of ATP containing 1-2 μC as (γ-^{32}P)ATP prepared according to Glynn and Chappel [12]; phosphoprotein amounts as indicated. The reaction was started by the addition of the crude extract (1 mg) or of the purified enzyme preparation (0.2 mg) or the dialysed yolk (12 mg protein containing 68 μg of bound alkali-labile phosphate). Incubation was stopped by addition of 0.3 ml of 50% trichloroacetic acid and the precipitate washed 4 times with 5 ml of 10% trichloroacetic acid. Finally the precipitate was transferred to a stainless planchet and counted in a thin window Geiger counter. Phosvitin with 10% alkali-labile phosphate content was prepared according to Mechan and Olcott [13]. 'Hammarsten' casein and ovalbumin were from Merck; salmine was from BDH.

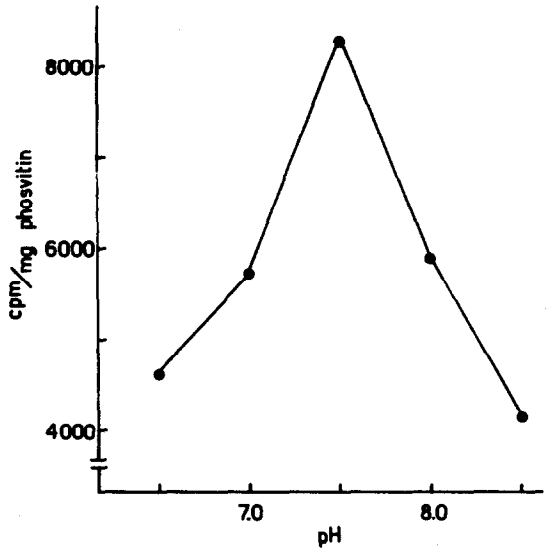

Fig. 1. pH dependence of embryo phosvitin kinase activity. General conditions as described in table 1. 1 mg phosvitin and 0.21 mg of partially purified phosvitin kinase were present. pH 6.5 - 7.5 buffers were Tris-acetate, pH 7.5 - 8.5 buffers were Tris-HCl.

Table 2

Source of protein kinase	Apparent K_m (mg/ml) phosvitin	casein	$\frac{V_{max}(\text{phosvitin})}{V_{max}(\text{casein})}$
Chick embryo	1.2	5.9	1.95
Rat liver (soluble)	0.68	0.66	2.40

General conditions as described in table 1. Incubation time: 15 min. Apparent K_m and V_{max} for the partially purified protein kinase were calculated according to the Lineweaver and Burk 'double reciprocal plot' procedure.

rather than a storage of inorganic phosphate.

Furthermore it is worth underlining that protamine forms with phosvitin a complex which is less suitable for kinase reaction than phosvitin alone. Such a finding, in agreement with the Langan view concerning nuclear phosphoproteins [15], suggests that phosvitin might also play in chick embryo a concomitant role in 'depressing' nucleic acid synthesis by binding histones.

Table 3

	(^{32}P)ATP formed (counts/min)
Protein kinase omitted	55
ADP omitted	100
Complete system	1125

The complete incubation system contained in a final volume of 1.0 ml: 200 μmoles Tris-HCl buffer pH 6.5; 6 μmoles $MgCl_2$; 5 μmoles ADP and approximately 1 mg of ^{32}P-phosvitin having a specific radioactivity of 30 000 counts/min per mg. Incubation was started by the addition of 0.7 mg of enzyme preparation; a second addition of 0.7 mg enzyme was made after 2 hr. Incubation lasted 5 hr in the presence of a drop of toluene. The reaction was stopped with 0.3 ml 50% trichloroacetic acid. After removal of precipitate by centrifugation the supernatant was stirred at 2°C with 200 mg of Norit and centrifuged. The precipitated Norit, after two washings with cold 5% trichloroacetic acid, was submitted to hydrolysis in 1 M HCl at 100°C for 10 min, and the radioactivity released in the supernatant was determined. Organic and inorganic ^{32}P radioactivity present in the trichloroacetic supernatants after the remotion of Norit was evaluated following a procedure similar to that described by Wadkins and Lehninger [14]. The identification of (^{32}P)ATP has been confirmed by means of the glucose-hexokinase trap and by the identification of glucose-6-^{32}P formed by paper ascending chromatography (solvent: *n*-butanol : *n*-propanol : acetone : 80% formic acid : 30% trichloroacetic acid, 40 : 20 : 25 : 25 : 15). Partially purified phosvitin kinase was prepared as described in table 1. ^{32}P-labelled phosvitin was prepared by incubating phosvitin in the presence of (γ-^{32}P)ATP and rat liver cytosol protein kinase as described in a previous paper [11].

The authors wish to thank Miss Carla Munari for technical assistance.

This work was supported by the Consiglio Nazionale delle Ricerche.

References

[1] M. Friedkin and A. L. Lehninger, J. Biol. Chem. 177 (1949) 775.
[2] K. Ahmed and J. D. Judah, Biochim. Biophys. Acta 57 (1962) 245.
[3] P. J. Heald, Biochem. J. 66 (1957) 659.
[4] S. Sperti, M. Lorini, L. A. Pinna and V. Moret, Biochim. Biophys. Acta 82 (1964) 476.
[5] L. A. Pinna, M. Lorini, V. Moret and N. Siliprandi, Biochim. Biophys. Acta 97 (1965) 398.
[6] D. Rudack and R. A. Wallace, Biochim. Biophys. Acta 155 (1968) 299.
[7] T. A. Sundararajan and P. S. Sarma, Biochem. J. 56 (1954) 125.
[8] S. P. Rose and P. J. Heald, Biochem. J. 81 (1961) 339.
[9] M. Rabinovitz and F. Lipman, J. Biol. Chem. 235 (1960) 1043.
[10] R. Rodnight and B. E. Lavin, Biochem. J. 93 (1964) 84.
[11] L. A. Pinna, V. Moret and N. Siliprandi, Biochim. Biophys. Acta 159 (1968) 563.
[12] J. M. Glynn and J. B. Chappel, Biochem. J. 90 (1964) 147.
[13] D. K. Mechan and H. S. Olcott, J. Am. Chem. Soc. 71 (1949) 3670.
[14] C. L. Wadkins and A. L. Lehninger, in: Methods in Enzymology, vol. 6, eds. S. P. Colowick and N. O. Kaplan (Academic Press, New York, 1963) p. 265.
[15] T. A. Langan, in: Regulation of nucleic acid and protein biosynthesis, eds. V. V. Koningsberger and L. Bosch (Elsevier, Amsterdam, 1967) p.233.