CYCLIC NUCLEOTIDE-INDEPENDENT PROTEIN KINASE FROM PEA SHOOTS[1]

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Received May 14, 1973

Summary: Protein kinase has been isolated from 6-day old etiolated pea shoots. Crude homogenates contain endogenous protein substrates for the kinase. Casein or phosvitin, but not histone, can serve as substrates for assay. DEAE-cellulose columns distinguish several forms of protein kinase activity. Cyclic nucleotides do not modify the activity of these protein kinases in vitro.

I have looked for protein kinase in pea shoots, to inquire into the role of protein phosphorylation as a regulatory mechanism in higher plants.

Many animal hormones operate by raising the intracellular concentration of cyclic AMP (1), which in turn activates protein kinase. Modulation of cellular activity results by phosphorylation of certain critical proteins (2,3). The possible existence and function of cyclic AMP in plants is a controversial area of research. To date, there is no conclusive evidence that cyclic AMP is actually present in higher plant tissues, although a number of reports have attempted to connect cyclic AMP with plant hormone action (4,5,6). This work serves as a probe for possible cyclic AMP-responding systems in plant tissues.

MATERIALS AND METHODS

The enzymes and reagents for ATP-γ-^{32}P synthesis, cyclic nucleotides, phosvitin, calf thymus histone (Sigma type IIA), and bovine serum albumin were obtained from Sigma; casein (vitamin-free), from Calbiochem, was prepared for use as described by Reimann et al. (7).

ATP-γ-^{32}P was synthesised by the method of Penefsky (8) with the following

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1 This investigation was supported by the U.S. Atomic Energy Commision under Contract AT (11-1)-1338.

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TABLE 1

Subcellular distribution of protein kinase activity.

Fraction	Activity pmol/min	Protein mg
1,000 x g pellet	2821	27.1
10,000 x g pellet	1968	39.3
30,000 x g pellet	2920	25.8
105,000 x g pellet	1550	38.6
105,000 x g supernatant	36925	628.0

Activity isolated from 57 g fresh pea shoots.

modifications. Two µmol K_2HPO_4 (2-5 mCi ^{32}P) was included in the reaction mixture and no further carrier ATP was added. The ATP fraction from the Dowex 1 column was desalted by adsorption of the nucleotide onto charcoal (Norit-A, 12 mg) and elution with 50% aqueous EtOH containing 1% saturated NH_4OH. Portions of the lyophilised ATP-γ-^{32}P were diluted for use each day to a specific activity of ca 100 c.p.m./picomol.

Peas (Pisum sativum L. cv Progress #9) were grown on moist vermiculite at 25°C and low humidity in total darkness. Six-day old pea shoots (228 g fresh weight) were homogenised at 2°C in a Waring blendor with an equal volume of 20 mM tris-HCl, 4 mM EDTA, pH 8.0. The filtered homogenate (pH 7.0) was centrifuged at 105,000 x g for 1 hour, and the supernatant was used for the experiments described. Protein was estimated by the method of Lowry (9).

Protein kinase assays were performed at 30°C in a total volume of 0.1 ml containing the following: 0.05 M sodium β-glycerophosphate, pH 6.5, or 0.05 M tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid, pH 8.5; 10 mM NaF; 0.3 mM ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA); 10 mM $Mg(OAc)_2$; 0.05 mM ATP-γ-^{32}P; a protein substrate; and enzyme preparation. A mixture of the ATP and $Mg(OAc)_2$ was the final addition to start the reaction.

TABLE 2.

Activity of pea protein kinase with different protein substrates.

Substrate		Activity with added cyclic nucleotide (pmol/min)			
		None	cyclic AMP	cyclic IMP	cyclic GMP
None added	pH 6.5	4.4	4.4	5.1	4.5
	pH 8.5	5.0	5.8	5.2	5.4
Casein (6mg/ml)	pH 6.5	11.3	11.6	12.0	12.6
	pH 8.5	11.0	12.8	12.7	10.9
Phosvitin (2 mg/ml)	pH 6.5	8.5	-	-	-
	pH 8.5	7.8	-	-	-
Histone (2 mg/ml)	pH 6.5	3.8	4.2	3.2	3.1
	pH 8.5	5.0	5.2	4.8	4.4
Bovine albumin (2 mg/ml)	pH 6.5	5.3	-	-	-
	pH 8.5	6.7	-	-	-

All assays were performed as described in Methods, with incubations of 1 minute duration, each containing 0.02 ml 105,000 x g supernatant (0.19 mg protein); 0.05 mM ATP-γ-^{32}P at 106 c.p.m./pmol; and 2 µM cyclic nucleotides (where added). A blank determination, containing no enzyme, and a control containing enzyme treated at 100°C for 3 minutes contained radioactivity in the precipitated protein equivalent to 0.4 and 0.5 pmol phosphate respectively.

The reaction was stopped by adding 0.02 ml 0.1 M EDTA with 50 mg/ml bovine serum albumin and transfer to 0°C. Protein was precipitated with 3 ml 10% CCl_3COOH-20 mM $Na_4P_2O_7$. Precipitates were kept for 2 hours at 2°C and one hour further at 23°C before collection on Millipore glass-fibre filters (AP-20, 25 mm). The filters were washed 5 times with 3 ml 10% CCl_3COOH-20 mM $Na_4P_2O_7$, and dried at 80°C; the radioactivity was determined in a scintillation counter.

For the isolation of phosphoserine, protein precipitates were collected by centrifugation, washed 3 times with fresh 10% CCl_3COOH-20 mM $Na_4P_2O_7$, and once with diethyl ether, and were taken up in 0.5 ml 2 M HCl containing 0.2 mM O-phospho-L-serine as carrier. Samples were hydrolysed in a sealed tube at 100°C for 4-12 hours, and liberated amino acids were analysed by paper electrophoresis at pH 1.9 (2.5% HCOOH, 8.7% CH_3COOH; 2 hr at 20 V/cm).

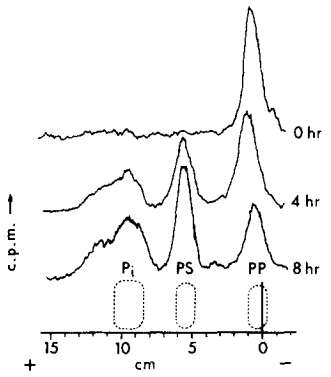

Figure 1. Phosphoserine release during hydrolysis of the ^{32}P-phosphorylated endogenous protein substrate. Samples of the crude supernatant enzyme were incubated for 5 minutes with ATP-γ-^{32}P at 250 c.p.m./pmol, without externally added protein substrate. Samples were hydrolysed for 4 hr or 8 hr and were analysed by paper electrophoresis (see Methods). An unhydrolysed sample (0 hr) was also examined, to show that all radioactivity was associated with the phosphorylated protein (PP) in the kinase reaction product. Hydrolysis released O-phosphoserine (PS) and inorganic phosphate (P_i).

RESULTS

Protein kinase activity was found in a supernatant fraction from pea shoots (Table 1). The enzyme catalysed the transfer of γ-phosphate from ATP to serine residues in casein or phosvitin (data not shown). The supernatant contained an endogenous substrate or substrates for the enzyme, but the activity was enhanced by the addition of casein or phosvitin. Bovine serum albumin was a poor substrate, and calf thymus histone apparently could not serve as substrate at all (Table 2). Cyclic nucleotides did not significantly modify the activity of the protein kinase preparation.

The reaction with endogenous substrate was verified by partial acid hydrolysis (Fig. 1). A phosphorylated protein precipitate, washed free of excess ATP-γ-^{32}P, was redissolved in electrophoresis buffer for comparison with the hydrolysates. Before hydrolysis, all ^{32}P radioactivity was associated with the phosphorylated polypeptide. Acid hydrolysis released progressively phosphoserine and P_i. The time course of P_i appearance was consistent with its origin through hydrolysis, and not by release of non-covalently bound ATP or P_i.

The activity of the crude supernatant enzyme was proportional to the

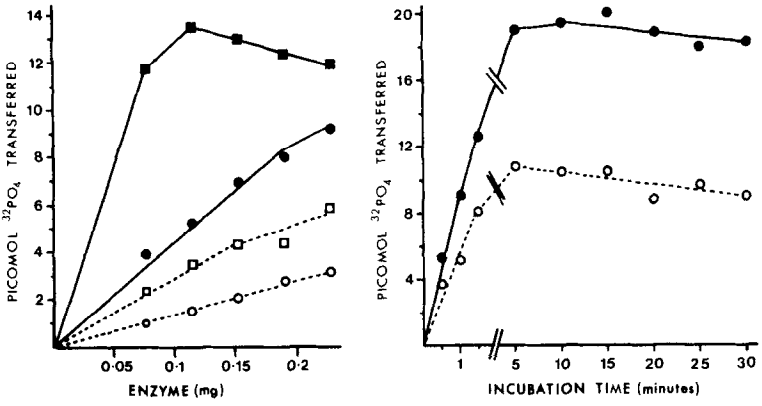

Figure 2. Protein kinase activity of 105,000 x g supernatant: effect of (A) enzyme concentration, and (B) time of incubation.
(A) Incubation with casein (6 mg/ml), for 5 minutes (—■—); for 1 minute (—●—): without added protein substrate, for 5 minutes (··□··) and for 1 minute (··○··).
(B) All incubations with 0.19 mg supernatant enzyme; with casein (—●—), and without added protein substrate (··○··).

amount of enzyme added when incubation time was short (Fig. 2A). The reaction was linear with respect to time for about 90 seconds, and the reaction ceased within 5 minutes (Fig. 2B). Paper electrophoresis of reaction mixtures at various times indicated that 98% of the ATP-γ-^{32}P was destroyed in 5 minutes because of ATPase present in the homogenate. Assays involving the crude kinase preparation were therefore limited to 1 minute. The enzyme was active between pH 6.0 and pH 9.0 without a sharp optimum.

The enzyme could not be purified by methods involving precipitation without modifications causing partial loss of solubility. After $(NH_4)_2SO_4$ treatment to 60% saturation or adjustment to pH 5.3, most of the activity was recovered with precipitated protein, resuspended and dialysed in pH 7 buffer, but a large part of the enzyme was now in an easily sedimented form.

Four major peaks of kinase activity were resolved by DEAE-cellulose chromatography (Fig. 3). The partially purified forms of protein kinase were dependent on added protein substrate for activity, and reaction was linear with respect to time and enzyme concentration. Cyclic nucleotides had no effect on the activity of the different kinases recovered (Table 3). The isolated

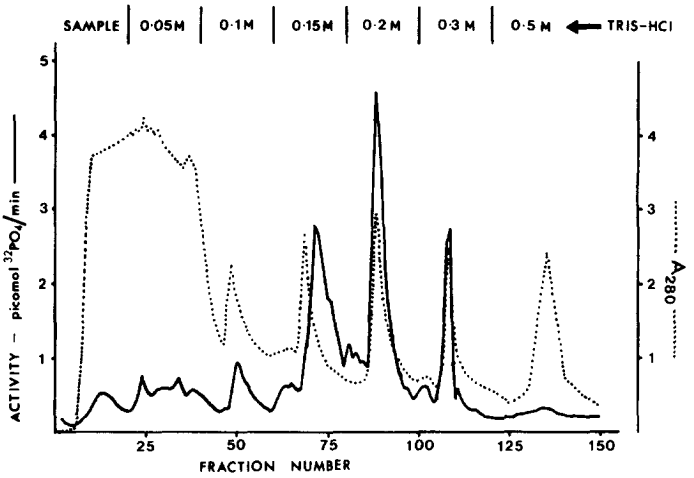

Figure 3. DEAE-cellulose chromatography of pea protein kinase in the 105,000 x g supernatant preparation. The supernatant preparation was loaded onto a column of Whatman DE-52 (4.5 cm high x 5.1 cm diameter), and protein was eluted with tris-HCl, pH 7.5, at the concentrations indicated: these buffers all contained 1 mM EDTA. Aliquots of 0.02 ml from each 12 ml fraction were assayed for protein kinase activity using casein (6 mg/ml) as substrate for 30 minutes of incubation.

TABLE 3.

Pea protein kinase resolved by DEAE-cellulose.

Peak from DE-52 column	Activity recovered (pmol/min)			Protein (mg)	Optimum pH
	No c-NMP	c-AMP	c-GMP		
I	2715	2733	2739	38.7	6.0-9.0
II	9290	9564	8879	78.2	6.0-9.0
III	8023	8264	7957	100.2	7.0
IV	3085	2965	2955	50.9	7.0

All assays were performed as described in Methods, with incubations up to 30 minutes duration and with casein (6 mg/ml) as substrate. Cyclic nucleotides were added at 2 μM concentration as indicated.

forms of pea protein kinase were stable for several weeks when stored at -20°C in 10 mM tris-HCl-2 mM EDTA, pH 7.5.

DISCUSSION

This report presents evidence for a protein phosphorylating system in a

higher plant. Protein kinase activity of 60 picomol/minute/mg total protein was recovered in a pea protein fraction soluble in the presence of 4 mM EDTA: this activity was unchanged in the presence of cyclic AMP. Recovery of ^{32}P-labelled phosphoserine from hydrolysates of proteins extracted from plants incubated in vivo with ^{32}P-phosphate confirms the presence of such enzymes in various plant tissues (author, unpublished data). Homogenates prepared at lower pH, following methods used for the isolation of cyclic AMP-dependent protein kinase from animal tissues (7,10), contained no protein kinase activity with or without added cyclic AMP. If cyclic AMP has a regulatory role in higher plants, the above data suggest that this role may be distinct from its known functions in animal cells.

Although there is no evidence as yet that protein phosphorylation serves as a regulatory mechanism in higher plants, Trewavas has shown phosphorylation in vivo of Lemna ribosomal proteins (11) and more recently, working in collaboration, we have found protein kinase associated with ribosomes isolated from peas and Lemna (12).

Acknowledgments

I wish to thank Dr. N. Amrhein for suggestions on the protein kinase assay procedure, and the Science Research Council, London, for a NATO post-doctoral fellowship.

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