Effects of Adenosine-3′:5′-monophosphate (cAMP) on the Activity of Soluble Protein Kinases in Maize *(Zea mays)* Coleoptile Homogenates

B. Janistyn

Institut für Pharmazeutische Biologie der Albert-Ludwigs-Universität Freiburg i. Br., Schänzlestraße 1, D-7800 Freiburg i. Br.

Z. Naturforsch. **41c,** 579–584 (1986); received January 3/February 3, 1986

Maize, cAMP, Protein Kinases, Substrate-Inclusion Electrophoresis

Seven protein kinases (PI–PVII) from maize *(Zea mays)* coleoptile homogenates could be detected which phosphorylate a histone-substrate in a polyacrylamide-matrix. The kinases PI, PII and PVI were stimulated by 6 μM cAMP, PIII, PIV and PV were cAMP independent, while PVII was inhibited by 6 μM cAMP.

Introduction

Reversible, post-translational modification of proteins by phosphorylation has been shown to play a key role in the regulation of various cellular functions in animals and men [1]. Experimental evidence indicates that the phosphorylation is mediated by cAMP-dependent and cAMP-independent protein kinases [2] and by the Ca^{2+}-calmodulin-system [3].

In higher plants, protein phosphorylation has been reported for nuclear [4–10], ribosomal [11, 12] and membrane proteins [13, 14]. It has been suggested that phosphorylation of specific proteins is a final step in different pathways for signal transduction by which regulatory agents stimulate cellular activities [15]. Whereas the presence of cAMP and cAMP binding proteins in higher plants have been demonstrated [16], no stimulatory effect of cAMP on higher plant protein phosphorylation could be detected [5, 7, 8, 11, 14, 17]. Recently in the case of *Lemna paucicostata* cAMP was reported to have either stimulatory or inhibitory effects on protein kinases [18, 19] and [20] reported effects of cAMP and cGMP on the autophosphorylation of elongation factor 1 from wheat embryos.

Materials and Methods

Plant material and chemicals

Conditions for aseptic growth and the harvesting of the maize seedlings had been described elsewhere [21].

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0341–0382/86/0500–0579 $ 01.30/0

[γ^{32}P] ATP was obtained from Amersham Buchler, Braunschweig, F.R.G. Coomassie blue R and G 250, bovine serum albumin and histone II-A were purchased from Sigma, München, F.R.G. Acrylamide, N′,N′-methylene-bis-acrylamide (BIS), N′,N′-tetramethylethylendiamine (TEMED), ammoniumperoxodisulfate (PER), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), ethylene diaminetetraacetic acid (EDTA), adenosine-5′-triphosphate (ATP), adenosine-3′:5′-monophosphate (cAMP) and ascorbic acid were from Merck, Darmstadt, F.R.G. Cytidine-3′:5′-monophosphate (cCMP), uridine-3′:5′-monophosphate (cUMP), inosine-3′:5′-monophosphate (cIMP) and guanosine-3′:5′-monophosphate (cGMP) were purchased from Sigma-Chemie, München, F.R.G. Molecular weight calibration kits were obtained from Pharmacia, Freiburg, F.R.G. Kodak X-Omat AR 5 films were from Siemens, Mannheim, F.R.G.

Buffer:

A) 30 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM $MnSO_4$ and 0.1 mM ascorbic acid (pH 6.8).

B) 18.17 g Tris-HCl/100 ml bidist. H_2O (pH 8.8).

C) 6.06 g Tris-HCl/100 ml bidist. H_2O (pH 6.8).

D) 14.4 g glycine and 1.5 g Tris-HCl per 1000 ml bidist. H_2O (pH 8.3).

E) 0.05 M potassium phosphate (pH 7.0).

Preparation of the cell-free maize coleoptile homogenate

40 g five days old etiolated maize seedlings were frozen in liquid nitrogen and stored at −20 °C. The seedlings were homogenized (2 g fresh weight ml^{-1} buffer) in a pre-cooled (0 °C) mortar in buffer A.

After filtration through a nylon cloth, the crude extract was centrifuged for 30 min at 50,000 × *g* at 0 °C. The supernatant (4 mg protein ml^{-1}) was used for the phosphorylation experiments and was stored in 100 µl portions at −20 °C. Protein determinations were carried out by the Coomassie blue G-250 method [22] using bovine serum albumin as standard.

Substrate inclusion polyacrylamide gel electrophoresis

The method used for separation of proteins from maize coleoptile was as described in [23] with some modifications. The separation gel stock solution contained:

a) 30 g acrylamide and 0.8 g BIS/100 ml bidist. H_2O; b) buffer B 18.17 g Tris-HCl (pH 8.8)/100 ml bidist. H_2O; c) buffer C 6.06 g Tris-HCl (pH 6.8)/100 ml bidist. H_2O; d) 0.1 g PER/1 ml bidist. H_2O; e) TEMED.

The mixing ratio for the separation gel was as follows:

a) 6.7 ml; b) 4 ml; d) 80 µl; e) 80 µl and 5.3 ml H_2O. When 4 mg histone II-A had been dissolved in this mixture, the system was allowed to polymerize under a layer of *n*-butanole saturated with water. This layer was removed and after rinsing the top of the gel with dist. water the stacking gel was poured on the separation gel. The stacking gel had the following composition:

a) 0.75 ml; c) 1.25 ml; d) 15 µl; e) 5µl and 3.0 ml H_2O. The sample-spacer was choosen to allow 100 µl of the sample per slot. For final polymerization the gel was stored at room temperature over night.

After addition of bromophenol blue 100 µl samples of the homogenate containing about 400 µg protein/slot were loaded on the 1 mm thick slab gels and the electrophoresis was performed with the buffer D for 6 h at 3 W gel^{-1} at 4 °C. The electrophoresis was stopped, when the dye-front and a following yellow band (benzoxarines) had left the gel.

Phosphorylation in slab gels containing histone II-A, in presence and absence of cAMP

The gel was removed from the electrophoresis apparatus, washed at 0 °C in 30 ml of buffer A for 5 min with three changes of the buffer and then cut longitudinal in two equal parts. The two parts were transferred in separate boxes filled with 20 ml of buffer A with and without 6×10^{-6} M cAMP.

The buffer in each box contained 20 µCi (740 kBq) of [γ^{32}P] ATP. The incubation was 30 min at 25 °C. Following incubation the gels were washed with buffer A as described above. Thereafter the gels were transferred separately to 200 ml of buffer E at 25 °C and washed with gentle agitation for 3 h. Thereafter the buffer was exchanged and the washing continued for 4 more h with another charge of buffer E after 2 h. The gels were finally rinsed with dist. water, spread on moist Whatman filter paper and covered with cellophane under a Kodak X-Omat film for autoradiography and exposed in closed boxes at 25 °C for 12 h. The developed films were scanned using a laser densitometer (LKB 2202 ultra scan, LKB Produkter AB, Bromma, Sweden) (Figs. 1 and 2).

Thereafter the gels were cut in 2 mm slices with a gel slicer (Hoffer Scientific Instruments) and the ^{32}P activity of the individual slices determined by measuring Cerenkov radiation in a liquid scintillation spectrometer [24] (Figs. 1 and 2). The counting efficiency was 55%. Only little radioactivity could be found on the gels if the histone II-A substrate was omitted from the gels. When the slices of radioactive zones were treated with hot NaOH, the radioactivity was lost from the slices. Thus, fixation of ^{32}P in the gel matrix is dependent upon the presence of substrate and has the known alkali-lability characteristic of phosphoesters.

Protein kinase assay of the proteins isolated from the slab gels

In addition to the *in situ* phosphorylation experiments the kinases were eluted from the gel after electrophoresis and their activity determined in a standard protein kinase assay [25]. The conditions were the same as described above but without addition of histone II-A and [γ^{32}P] ATP. After electrophoresis the gels were cut horizontally in 2 mm slices, weighted and extracted under sonification (10 sec) at 0 °C with the same weight of buffer A. The extracts were centrifuged for 15 min at 50,000 × *g* at 0 °C and the resulting supernatants were used for the kinase assay. Protein determinations were carried out by the method of Spector [22] using bovine serum albumin as a standard.

With slight modifications in respect to [25], the assay was performed by mixing the reactants in the following sequence: 20 µl of a solution of histone II-A from a stock solution of 30 mg histone II-A in 1 ml dist. H_2O. 5 µl of an aqueous solution of [γ^{32}P] ATP (5 µCi; 185 kBq) with or without cAMP at a

concentration of 6×10^{-6} M to the final volume of the reaction mixture of 75 µl. The reaction was started by adding 50 µl of the enzyme samples. The following steps were the same as described in [25]. As the enzyme extracts of the gel zones contain some proteins which could be substrates for the kinases it was necessary to correct for this phosphorylation by two controls. One without histone II-A substrate and one without the enzyme extracts. The thus corrected values are shown in Fig. 3 by picomoles Pi incorporated per µg histone II-A substrate per minute.

Results and Discussion

The substrate inclusion technique [23] was modified to determine protein kinases in the following way. The separation gel was polymerized in presence of histone II-A. The enzyme solution was subjected to electrophoresis at 4 °C and incubation with [γ^{32}P] ATP proceeds phosphorylation where protein kinases are present and will be detected by autoradiography. To distinguish between cAMP dependent and cAMP independent protein kinases the electrophoresis and the incubation procedure must be done twice, once with cAMP and once without cAMP in the incubation mixture.

This paper provides evidence for the presence of seven protein kinases in maize coleoptile homogenates in the incubation mixture. Comparing Figs. 1 and 2 it is obvious from the autoradiogrames (bottom of the figures) that there are three major bands with protein kinase activity with (Fig. 1) and without (Fig. 2)

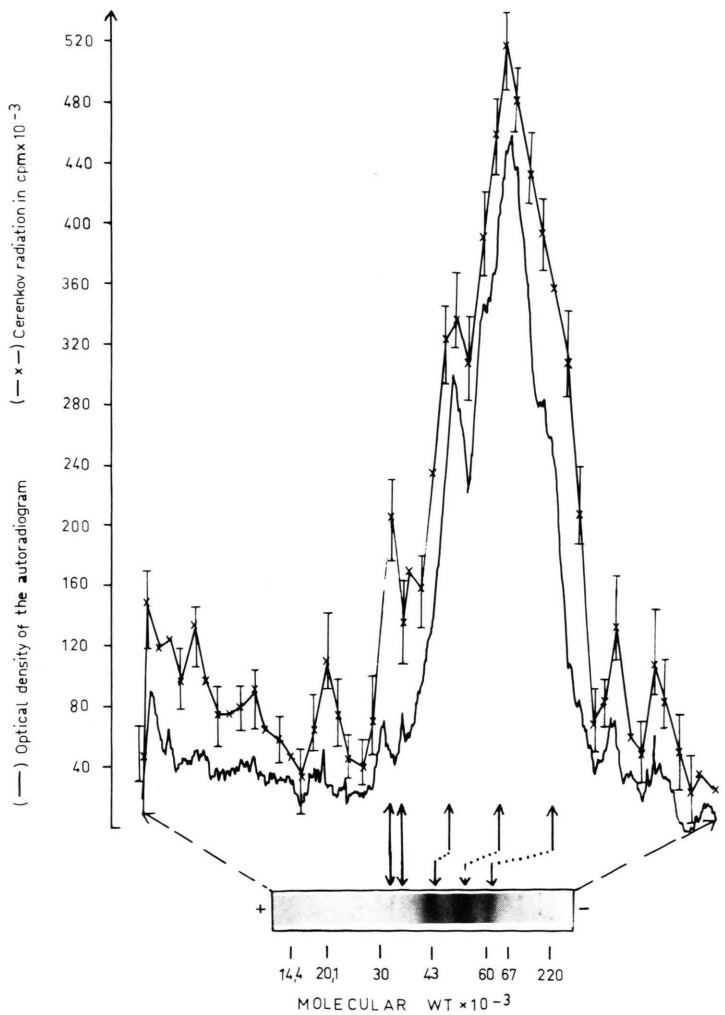

Fig. 1. [γ^{32}P]-ATP phosphorylation pattern of a histone-substrate, in the presence of cAMP (6 µM), in a native polyacrylamide gel after electrophoresis of a maize coleoptile homogenate in the same gel. At the bottom the original autoradiogram of the gel is presented (direct visible zones are indicated by arrows) and in the upper part of the diagram (two times enlarged x-axis) the optical density of the gel (−), and the distribution of the radioactivity measured by extracts from 2 mm slices of the gel by Cerenkov radiation (−x−). I indicates the SE for sixfold samples.

cAMP in the reaction mixture (45, 50 and 62 kd). In addition there are two peaks with increased radioactivity at the low molecular weight end in the presence of cAMP (36 and 37 kd; Fig. 1). but not in its absence (Fig. 2) as obvious from measurements of radioactivity. At the high molecular weight side there are two small peaks of protein kinase activity in the presence of cAMP (200 and 210 kd; Fig. 1) while in its absence there is only one peak (210 kd; Fig. 2). Whereas the existence in plant tissue of protein kinases stimulated by Ca^{2+} and calmodulin is now well established [14], a stimulation of protein kinases by cAMP so far was only observed by Kato *et al.* [18, 19]. By DEAE-Sephacel chromography three protein kinases could be isolated from cellular extracts of *Lemna paucicostata*. One was partially inhibited, one activated by 10 μM cAMP and the last was cAMP independent. In contrast to Kato *et al.* [18, 19] only little or no cAMP dependent protein kinase activity could be detected, when the maize coleoptile homogenate was fractionated by a DEAE-Sephacel column (data not shown), it could well be that inhibitors which were present could be removed by electrophoresis, or that the protein kinases were stabilized through the polyacrylamide matrix.

Both possibilities require further clearification. To verify the data mentionated above, slab gels without histone II-A substrate where cut in 2 mm slices and the electrophoretically separated proteins extracted. The extracts where measured by standard protein kinase assay with and without cAMP [25] against two blanks. One without substrate and one without the

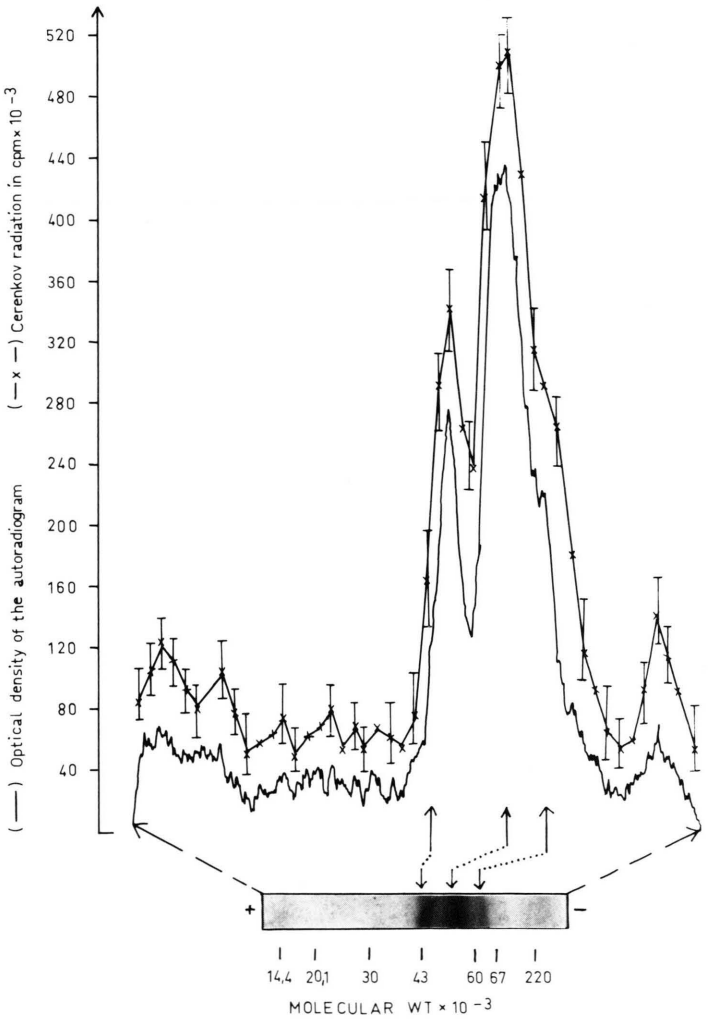

Fig. 2. [γ^{32}P]-ATP phosphorylation pattern of a histone-substrate, in the absence of cAMP, in a native polyacrylamide gel after electrophoresis of a maize coleoptile homogenate in the same gel. At the bottom the original autoradiogram of the gel is presented (direct visible zones are indicated by arrows) and in the upper part of the diagram (two times enlarged x-axis) the optical density of the gel (−), and the distribution of the radioactivity measured by extracts from 2 mm slices of the gel by Cerenkov radiation (−x−). I indicates the SE for sixfold samples.

protein extracts in the assay. The corrected values are shown in Fig. 3 (see text). There are seven different protein kinases, PI to PVII visible. PI, PII and PVI are activated by 6 μM cAMP. PIII, PIV and PV seem to be cAMP independent while PVII is increased in the absence of cAMP. The diagram of the protein kinase assays are in good agreement with the diagrams shown in Figs. 1 and 2. But evidently the activity of the enzymes has been decreased through the extraction procedure. In particular the cAMP dependent protein kinases are mostly decreased in their activity. Therefore it is not astonishing that so far no cAMP dependent protein kinase could be isolated from higher plants in it's substantial form. Recently, S. Ejiri *et al.* [20] reported effects of cAMP and cGMP on the autophosphorylation of elongation factor 1 from wheat embryos. At 10^{-7} M cGMP stimulated and cAMP inhibited the phosphorylation.

By our experiments other cyclic nucleotides as cCMP, cUMP and cIMP showed no significant effects on the phosphorylation activity of the protein kinases. Neither by the substrate inclusion experiments nor by the protein kinase assays with the proteins isolated from the slab gels.

cGMP showed a slow inhibitory effect in the case of the substrate inclusion experiments. This effect will be a part of later investigations in this field.

Acknowledgements

I am obliged to Prof. Dr. E. Wagner for critically reading the manuscript and Mrs. K. Glombik for her help with the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft.

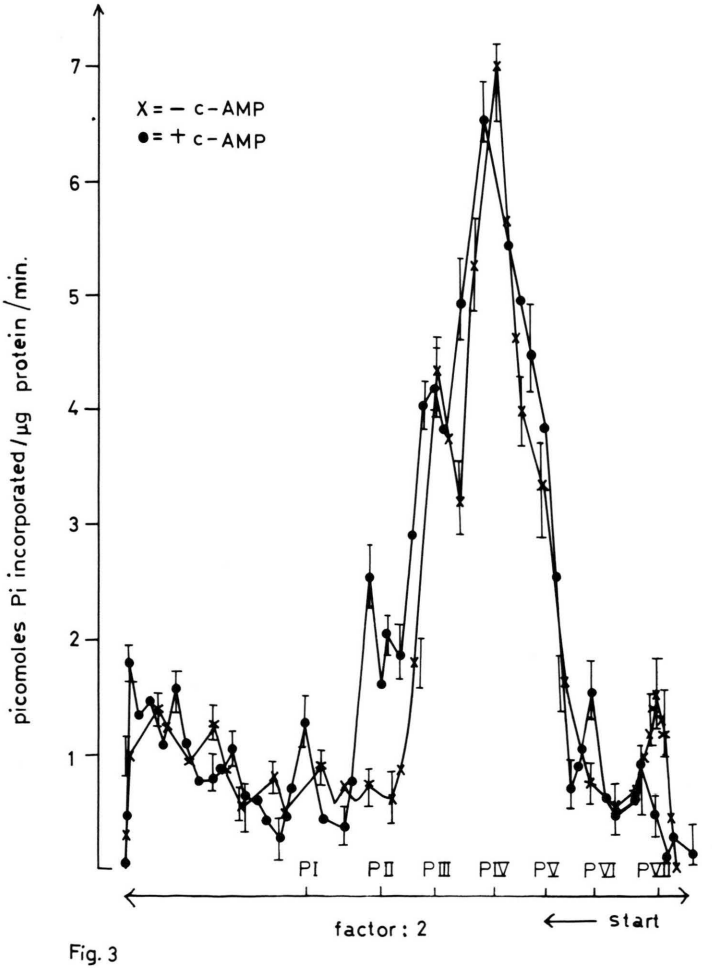

Fig. 3

Fig. 3. Standard protein kinase assay of the proteins isolated from 2 mm slab gel slices without histone-substrate (see text), with cAMP (–●–) and without cAMP (–x–). The x-axis of the diagram is two times enlarged comparison to the original gel. I indicates the SE for sixfold samples.

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