PHOSPHORYLATION AND ACTIVATION OF POLY(A)-ENDORIBONUCLEASE FROM CALF THYMUS GLAND

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1. Introduction

mRNA adenylation–deadenylation has been ascribed an important regulatory function in the maturation process of mRNA [1–7]. Polyadenylation–deadenylation of mRNA precursors may play a role in the cleavage of mRNA [8,9] or in mRNA transport [10] to the cytoplasm, or both. A poly(A) stretch in cytoplasmic mRNA may protect the mRNA from degradation [11,12]. Such post-transcriptional modifications could be regulated by the adenylation and/or deadenylation enzymes in nuclei, mitochondria microsomes and cytosol [13]. Poly(A)-polymerase [14], poly(A)-endoribonuclease [14,15] and poly(A)-exoribonuclease [16] have been highly purified and characterized.

The deadenylation enzyme poly(A)-endoribonuclease, and not poly(A)-polymerase, responds under physiological and pathological conditions [17–20]. However, poly(A)-polymerase also undergoes alterations in response to physiological stimuli [21–24] and post-translational modification via phosphorylation–dephosphorylation [25,26].

Protein phosphorylation is a key regulatory event of many cellular processes [27–31]. For this reason and the above discrepancies concerning the responses of adenylation–deadenylation enzymes to physiological and pathological conditions [17–26], we have studied in vitro phosphorylation of soluble calf thymus gland adenylation–deadenylation enzymes in the presence of [γ-^{32}P]ATP and a cAMP-independent soluble protein kinase isolated from the same source. We now report that fractionation of poly(A)-polymerase and poly(A)-endoribonuclease, after phosphorylation in vitro revealed ^{32}P-radioactivity associated with poly(A)-endonuclease and not with the poly(A)-polymerase.

Phosphorylation of poly(A)-endoribonuclease results in a concomitant increase in its catalytic activity.

2. Experimental

2.1. *Materials*

[^{3}H]ATP (30 Ci/mmol) poly([^{3}H]A) (18 Ci/mmol) and [γ-^{32}P]ATP (15–30 Ci/mmol) or 2800–3000 Ci/mmol) were from Amersham, cellulose-phosphate, DEAE-cellulose, GF/C and DE-81 filter discs were a Whatman Reeve Angel product. Poly(A) and oligoriboadenylates were from Boehringer Mannheim. Enzyme grade sucrose, urea, ammonium sulfate and Ampholine carrier ampholytes were from Serva. Acrylamide, methylenebisacrylamide and N_1,N_1,N_1',N_1'-tetramethylethylenediamine were obtaned from Bio-Rad Labs. All other reagents were of the highest grade obtainable.

2.2. *Purification of poly(A)-polymerase and poly(A)-endoribonuclease*

These two enzymes were isolated from the soluble cytosol fraction prepared from calf thymus as in [14,32]. A fraction enriched in poly(A)-polymerase and poly(A)-endonuclease was obtained after chromatography on phosphocellulose, DE-52 cellulose, 70% ammonium sulfate fractionation and a second DE-52 cellulose as in [14].

2.3. *Purification of protein kinase*

The protein kinase which remained associated with poly(A)-polymerase and poly(A)-endonuclease through chromatography on phosphocellulose and DEAE-cellulose could be separated from them by chromatography on Sephacryl S-200 equilibrated with 10 mM

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Tris–HCl (pH 7.5), 2 mM dithioerythritol, 0.1 mM EDTA, 20% glycerol and 1 M KCl.

The active pool obtained from Sephacryl S-200 chromatography was dialyzed against 50 mM K-phosphate buffer (pH 6.8), 4 mM 2-mercaptoethanol, 0.1 mM EDTA, 20% glycerol and applied on a phosphocellulose column equilibrated with the same buffer. Protein kinase activity was eluted with a linear 0–0.3 M KCl gradient in the equilibration buffer. The fractions of the protein kinase peak with spec. act. >13 000 units/mg protein were pooled and stored in 10 mM Tris–HCl (pH 7.5), 2 mM dithioerythritol, 50% glycerol.

2.4. *Assay for poly(A)-polymerase activity*

Poly(A) synthesis was measured essentially as in [14]. Reaction mixtures contained 200 mM Tris–HCl (pH 8.3), 4 mM 2-mercaptoethanol, 20 μM $rA(pA)_3$, 0.5 mM $MnCl_2$, 0.5 mM [^{3}H]ATP (~2000 cpm/nmol) and enzyme. For column assays, 25 μl of a reaction mixture were added to 5 μl every other column fraction and incubated for a fixed time at 35°C. For more precise assays, 100 μl reaction mixture were brought to 110 μl final vol. with enzyme dilution and then several time points were taken over 60 min incubation. All of the aliquots from the reaction mixture were placed on filters discs (GF/C) and batch washed as in [14].

2.5. *Assay for poly(A)-endoribonuclease activity*

Poly(A)-endoribonuclease activity was measured essentially as in [14]. The reaction was carried out at 35°C in 200 mM Tris–HCl (pH 9.0), 0.5 mM $MnCl_2$, 4 mM 2-mercaptoethanol, 0.5 mM poly([^{3}H]A) (~1500 cpm/nmol) and enzyme. Routine enzyme assays on column effluents were done by the addition of 25 μl reaction mixture to 5 μl enzyme solution in Disposo trays and incubated for the times indicated in the text. For more precise assays, 100 μl reaction mixture was brought to 110 μl with enzyme dilution and then several time points were taken for the times indicated in the text. Aliquots from the reaction mixture were placed on separate DE81 discs. The discs were collected in 2 M NaCl, 4 M urea and 1 mM EDTA washed 3 times for 10 min intervals with this solvent, 2 times in 95% cold ethanol, dried and counted [14].

2.6. *Protein kinase assay*

Protein kinase activity was measured by the incorporation of ^{32}P from [γ-^{32}P]ATP into trichloroacetic acid-precipitable material as in [33–34]. The incubation mixtures contained 50 mM Tris–HCl (pH 7.0), 5 mM $MgCl_2$, 1 mM dithioerythritol, 0.2 mM [γ-^{32}P]-ATP (~100 cpm/pmol) in 100 μl reaction mixture. The reaction mixtures were incubated at various times at 35°C and 20 μl aliquot was spotted on Whatman no. 1 discs. The discs were then processed as in [33].

2.7. *Isoelectric focusing on acrylamide gels*

Isoelectricfocusing was done in an apparatus manufactured by Shandon Sci. Co. in (0.5 cm × 7.5 cm) columns of 7.5% acrylamide containing 0.66% ampholine over pH 2–11 as in [35]. Each sample contained 20–50 μg protein. Electrofocusing was done for 3 h at 2.25 mA, 350 V. Enzyme activity and ^{32}P radioactivity were assayed after extraction of the activity from a sliced gel. The gel was frozen and then sliced into 1.5 mM sections. Each slice was placed in a well of a Limbro plate and 50 μl extraction buffer (1 mg BSA/ml in 200 mM Tris–HCl (pH 8.3), 4 mM 2-mercaptoethanol) were added to each well. The gel slices were frozen and thawed 3 times in the extraction buffer and allowed to extract at 4°C overnight. An aliquot of the extract was used to determine activity as described. Other gels were stained with 0.05% Coomassie brilliant blue R-256 after fixation with 14% trichloroacetic acid at 60°C for 1 h and subsequently washed with trichloroacetic acid solutions to remove ampholytes. The gels were destained with methanol–acetic acid–H_2O, 45:9:46 (by vol.). The gels were scanned in a chromoscan gel scanner.

2.8. M_r-*estimation*

The M_r of protein kinase was determined by the ratio of its elution volume (V_e) to the void volume (V_o) of a Sephacryl S-200 column [36] calibrated with standard proteins from Serva. The V_e/V_o ratio of the standard proteins used were (M_r): ovalbumin (45 000) 1.3; chymotrypsinogen (25 000) 1.46; cytochrome *c* (12 400) 1.5. Protein was determined as in [37] unless otherwise specified.

3. Results and discussion

The purification procedure summarized in table 1 yielded a protein kinase preparation with spec. act. 17 380. Analysis of this preparation by isoelectric focusing revealed a predominant protein band at pI 7.5 coinciding with protein kinase activity (fig.1).

The isolated enzyme had M_r 42 000 as estimated

Table 1
Purification of cyclic AMP-independent protein kinase from calf thymus cytosol

Fraction	Total protein (mg)	Spec. act. (units/mg)	Total enzyme activity
I First phosphocellulose: 70% $(NH_4)_2SO_4$ concentrate	23.4	2050	47 970[a]
II Sephacryl S-200	6.8	4455	30 300
III Second phosphocellulose	1.1	17 380	19 118

[a] Since separation of phosphoprotein phosphatase from protein kinase is achieved in the next step of purification, a precise estimation of protein kinase activity cannot be obtained to this point

by molecular sieving. The protein kinase catalyzed phosphorylation of α-casein exhibited a wide pH optimum (6.5–7.5). Maximum activity was exhibited at 5 mM Mg^{2+}. Addition of cAMP (1–100 mM) did not stimulate the enzyme activity.

According to these properties the soluble protein kinase, isolated from calf thymus is identified as a casein kinase I, similar to the enzymes isolated from Novikoff ascites tumor cells [36,38] rabbit reticulocytes [39] and calf thymus [40].

An additional feature which relates the soluble protein kinase to known casein kinases is the preference for acidic substrates as shown in table 2, which illustrates the capacity of the protein kinase to phosphorylate various commercial substrates as well as a poly(A)-polymerase- and poly(A)-endoribonuclease-enriched fraction obtained as in section 2.

On the basis of values of pmol ^{32}P incorp./mg pro-

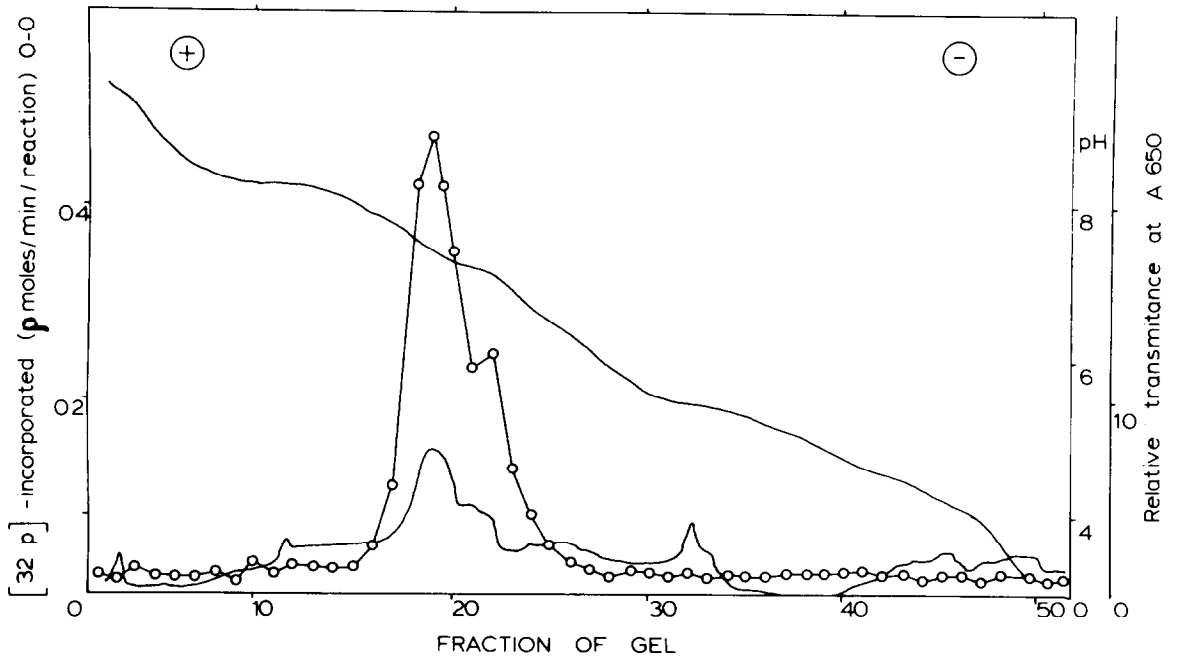

Fig. 1. Isoelectric focussing of calf thymus protein kinase. Electrophoresis conditions, staining and scanning of the gels for protein, extraction of gel slices for enzyme activity and pH determination were described in section 2. Enzyme activity was assayed by incubation of 10 μl gel extract with 50 μl standard R. X mix for 30 min at 37°C; 50 μl were spotted on Whatman no. 1 discs which were processed as in section 2; (——) densitometer tracing of a stained gel; (o——o) enzyme activity obtained from a sliced and extracted gel.

Table 2
Substrate specificity of the cyclic AMP-independent cytoplasmic non-particulate protein kinase from calf thymus gland (^{32}P radioactivity incorporation)

Phosphate acceptors	μg/reaction	pmol/reaction in 10 min	fmol/μg protein in 10 min
Protamine sulfate	1250	2.10	1.60
α-Casein	1250	11.95	9.56
Phosvitin	1250	10.30	8.24
Calf thymus fraction[a]	140	45.00	264.00

[a] Partially purified fraction enriched in adenylation–deadenylation enzymes or fraction VI [14]

For details of protein kinase reaction see section 2

tein substrate present, the highly purified calf thymus fraction containing both poly(A)-polymerase- and poly(A)-endoribonuclease displayed the highest specific activity of phosphorylation.

Fractionation by isoelectric focusing of an aliquot from such in vitro phosphorylation reaction of poly(A)-polymerase- and poly(A)-endoribonuclease-enriched fraction followed by measurement of their activities and ^{32}P radioactivity revealed that most of the ^{32}P radioactivity on the acrylamide gel focussed with the same pI of ~6.1 as the activity of poly(A)-endoribonuclease (fig.2).

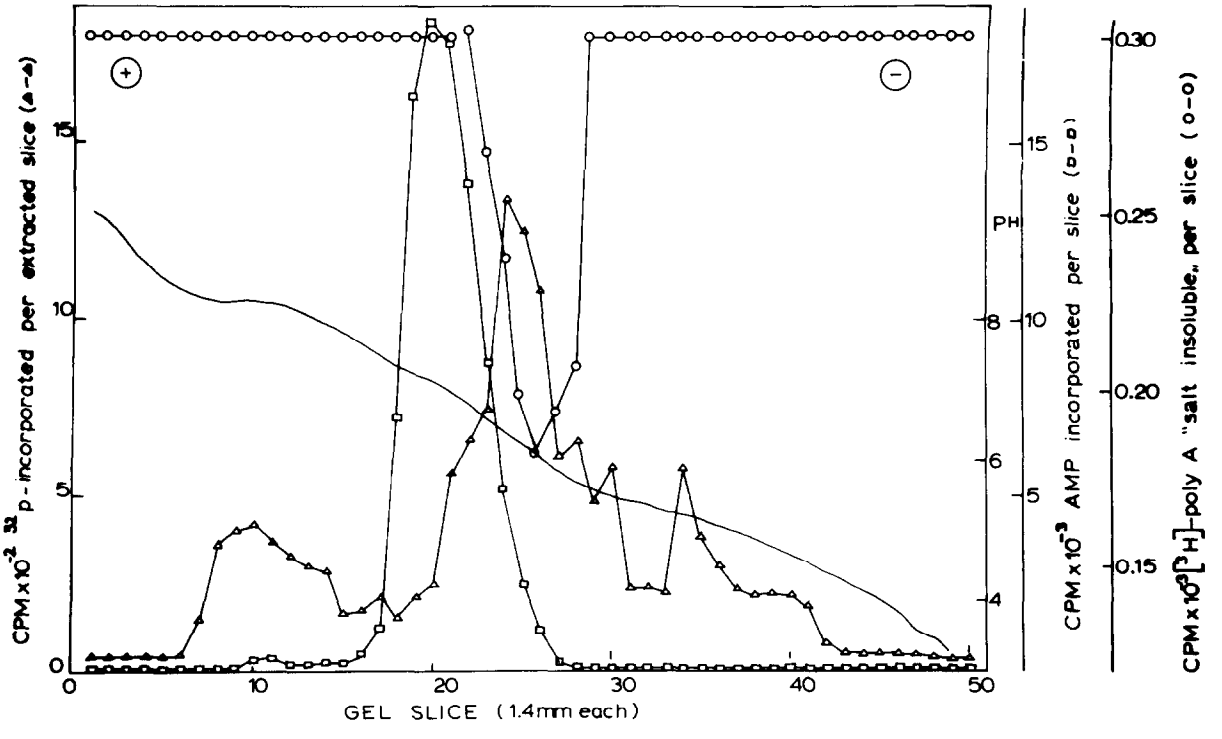

Fig.2. Isoelectric focussing of an in vitro phosphorylated aliquot containing partially purified poly(A)-polymerase and poly(A)-endonuclease. Isoelectric focussing and gel extraction were done as in section 2. Enzyme activities in the gel extracts were determined after 30 min incubation under the same conditions as for column assays see section 2. Aliquots of the gel extracts were used to determine ^{32}P radiation.

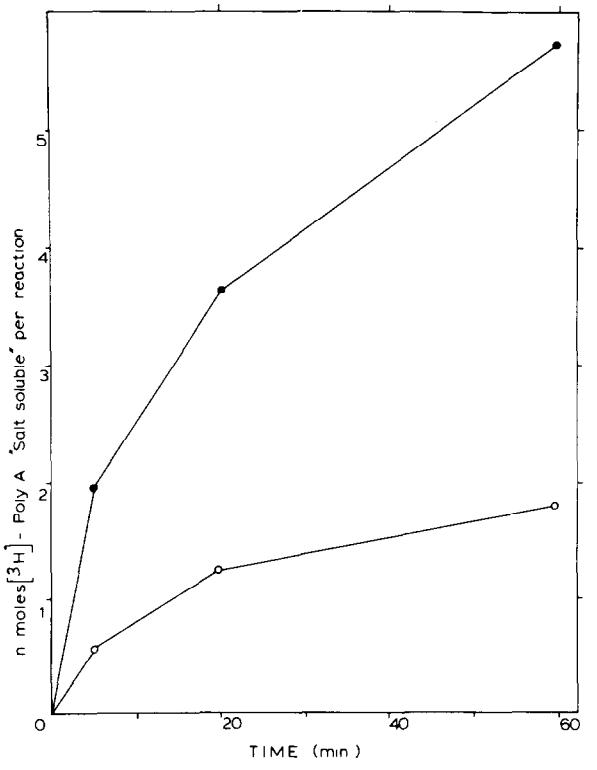

Fig.3. Effect of protein kinase on poly(A)-endoribonuclease activity. Protein kinase and poly(A)-endoribonuclease were obtained as in section 2. The reaction was carried out at 35°C in standard (100 μl) reaction mixture 200 mM Tris–HCl (pH 8.7), 0.5 mM $MnCl_2$, 4 mM 2-mercaptoethanol, 1 mM poly([^{3}H]A) (2000 cpm/nmol), 0.8 μg endoribonuclease: (○) in absence; (●) in presence of 3 μg protein kinase. At indicated times 20 μl aliquots from each reaction mixture were placed on DE 81 discs and batch washed and counted for the measurement of poly(A) hydrolysis as in section 2.

The effect of in vitro phosphorylation of poly(A)-endoribonuclease was investigated as in fig.3. Under identical reaction conditions (section 2) the rate of poly(A) hydrolysis by the phosphorylated form of the enzyme up to 1 h of reaction appears to be 4-fold greater than the rate of poly(A) hydrolysis by the unphosphorylated enzyme. Our results on in vitro phosphorylation of poly(A)-endoribonuclease accompanied by a concomitant increase of its ability to hydrolyze poly(A), are in agreement and support the in vivo rapid alterations observed with this enzyme in response to physiological and pathological conditions [17–20].

The in vivo phosphorylation of poly(A)-endonuclease and its possible relationship to the changes of activity reported should be examined. Furthermore, the protein chemistry of the phosphorylated enzyme should be elucidated.

Several functions have been attributed to casein kinases such as transcriptional [41] and translational control [42,43]. The capacity of the cAMP-independent protein kinase from calf thymus to phosphorylate and activate poly(A)-endoribonuclease also suggests a possible role for this class of enzymes in the regulation of post-transcriptional modification of mRNA.

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