5′-NUCLEOTIDASE REVERSES THE INHIBITORY ACTION OF ACTIN ON PANCREATIC DEOXYRIBONUCLEASE I

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1. Introduction

The interaction of skeletal muscle globular (G-) actin and pancreatic DNase I is now well documented [1–3]. This interaction leads to the formation of a stable stoichiometric 1:1 complex of actin and DNase I, which has been crystallized [4]. After complex formation the biological properties of both proteins are inhibited, i.e., the DNA-degrading activity of DNase I and the ability of G-actin to form high molecular weight polymers [1–3]. So far no method has been found to separate the actin·DNase I complex and recover the biological activities of both constituents. Therefore the biological significance of this complex has been questioned, since in this complex both proteins appear to be almost irreversibly locked to each other due to their high binding constant (5×10^8 M^{-1}, N.G.M. et al., in preparation). However, we have reported the natural occurrence of a secretory actin·DNase I complex in rat pancreatic juice [5]. Furthermore it was found [5] that in bile a protein factor is secreted which is able to reactivate the DNA-degrading activity of either rat secretory or synthetic actin·DNase I complex (the latter being formed from rabbit skeletal muscle actin and bovine pancreatic DNase I). This activating protein has been isolated from rat bile and identified as 5′-nucleotidase (in preparation). In this communication the effect of commercially available snake venom 5′-nucleotidase on synthetic actin·DNase I complex is described.

Abbreviation: Hepes, 2,4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Enzymes: DNase I or deoxyribonuclease I, EC 3.1.4.5.; 5′-nucleotidase, EC 3.1.3.5. (5′-ribonucleotide phosphohydrolase)

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2. Materials and methods

DNase I was a product of Worthington Corp. (cat. no. 2007) and further purified as in [6]. Synthetic actin·DNase I complex was prepared as in [4].

5′-Nucleotidase from *Crotalus adamanteus* venom was obtained from Sigma. In most cases the commercial product was used without further purification, since on SDS–gel electrophoresis it was found to be 70% pure and contaminating enzymatic activities, particularly alkaline phosphatase, were found to be low. In 3 instances commercial 5′-nucleotidase was purified to 99% using the following procedure: The enzyme was taken up in 10 mM Hepes buffer, pH 7.5, 0.1 mM $CaCl_2$ and 0.1 mM NaN_3 (buffer A) and applied to a column of Sepharose-bound 5′-AMP (Pharmacia) of 1 cm diam. and 10 cm ht. This column was developed stepwise: first with 0.5 mM *p*-nitrophenylphosphate to remove any phosphatases and then with 5 mM 5′-AMP. The active fractions were concentrated in a pressure dialysis chamber using an Amicon PM 10 membrane. It was found that the first elution step effectively removed all contaminating phosphatase activity. This procedure resulted in a 1.5-fold increase in specific activity (ranging from 2.47–3.5 μmol AMP hydrolysed/min. mg purified protein). The obtained material exhibited one polypeptide band on SDS–polyacrylamide gels. Its appar-

ent molecular weight from gels with various acrylamide concentrations (w/v) was 5% 68 000, 7.5% 60 000, 10% 53 000 and 12.5% 51 000.

DNase I activity was determined as in [4] and 5'-nucleotidase activity optically [7] in buffer A supplemented with 2 μg/ml adenine deaminase and 0.1 mM 5'-AMP. Enzymatic tests were carried out at 30°C using an Aminco DW 2 spectrophotometer.

Changes in light scattering were followed at 90° and 400 nm with a Perkin Elmer MPF 3 fluorescence spectrometer.

SDS–polyacrylamide gel electrophoresis was as in [8]. Protein concentrations were determined as in [9].

Negatively-stained samples for electron microscopy were prepared on carbon-coated grids [10] and examined in a Zeiss EM 9-S2 electron microscope operated at 60 kV.

Adenosine deaminase (EC 3.5.4.4.) and phalloidin were obtained from Boehringer, *p*-nitrophenylphosphate from Sigma. All other reagents were of analytical grade.

3. Results

Figure 1 illustrates the effect of 5'-nucleotidase on the DNA-degrading activity of actin·DNase I complex after 12 h of incubation at room temperature. It can be seen that the sigmoidal time dependence of DNA hydrolysis by actin·DNase I complex is changed into a hyperbolic one resulting in a 20-fold higher activity, i.e., complete reactivation of its DNase activity. Incubation of free DNase I with 5'-nucleotidase under identical conditions did not alter its enzymatic activity. Incubation of actin·DNase I complex with purified 5'-nucleotidase (at a 15:1 molar ratio) at 23°C

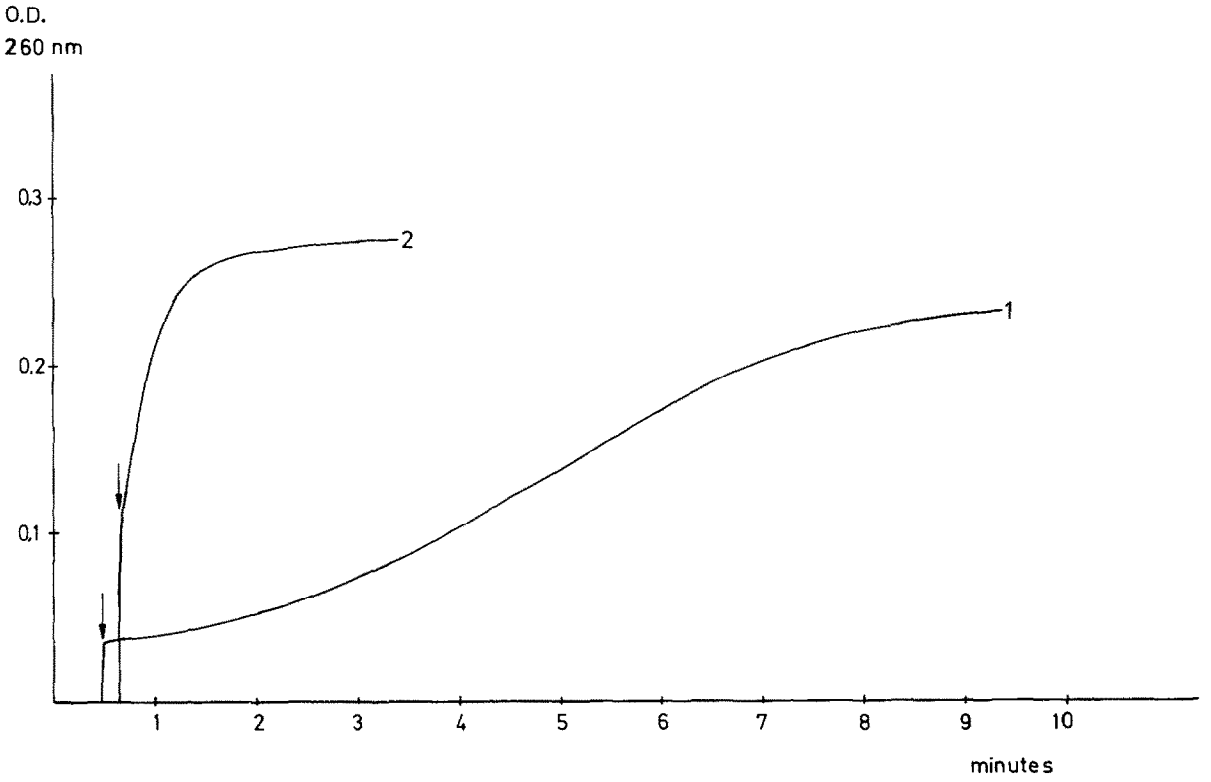

Fig.1. Effect of 5'-nucleotidase on the rate of DNA hydrolysis by actin·DNase I complex. Actin·DNase I complex, 10 μM, and 2 μM 5'-nucleotidase were incubated at 23°C for 14 h. Trace 1: DNase activity of a 5 μl sample just after mixing. Trace 2: 12 h later. Arrows indicate start of enzymatic test.

resulted in 8-fold increase of the DNase activity after 12 h. Under identical conditions the fraction eluted with *p*-nitrophenylphosphate did not affect the DNA-degrading activity of actin-DNase I complex.

In order to obtain information about the mechanism of the reactivation, actin-DNase I complex was incubated with 5′-nucleotidase under actin polymerizing conditions (buffer A supplemented with 0.1 M KCl) and the change in DNase I and 5′-nucleotidase activity, and light scattering was followed with time. Further-

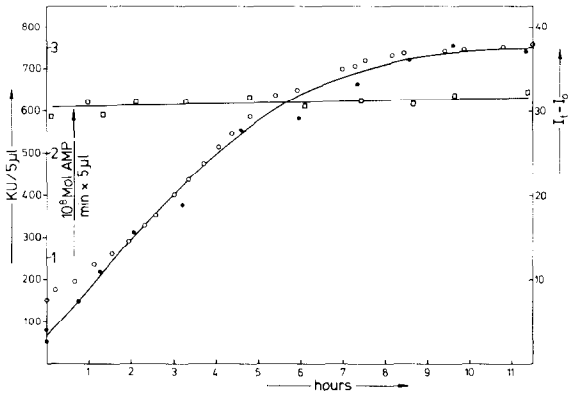

Fig.2. Increase in DNase activity and light scattering during incubation of actin-DNase I complex with 5′-nucleotidase. A final volume of 3 ml containing 17 µM actin-DNase I complex and 5.6 µM 5′-nucleotidase in buffer A supplemented with 0.1 M KCl was placed in a fluorescence cuvette; T = 23°C, (○) intensity of scattered light was recorded continuously ($I_t - I_0$ given in arbitrary units) and at time intervals indicated 5 µl samples were taken to determine (●) DNase and (□) 5′-nucleotidase activity. DNase I activity is expressed in Kunitz units (KU)/5 µl incubation mixture. (1 KU = 0.001 $A_{260\,nm}$ increase/min.).

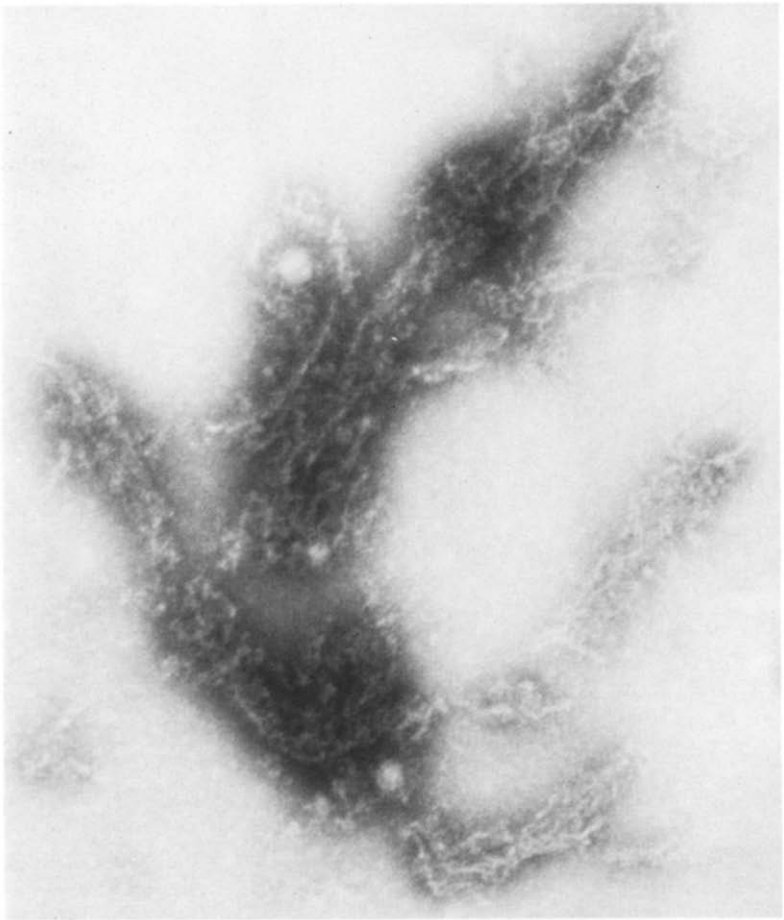

Fig.3a

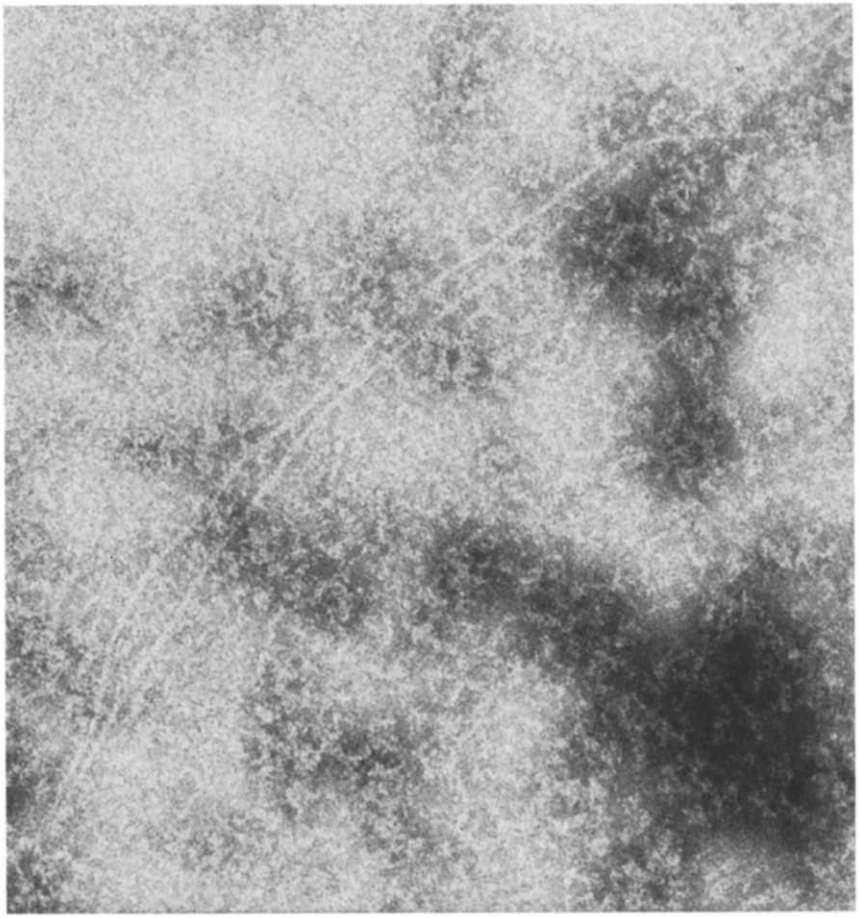

Fig.3b

Fig.3. Electron micrographs of incubation mixtures samples. (a) At 12 h from incubation illustrated in fig.2; (b) at 2 h taken from (▲) from fig.4. (a) 100 000 ×; (b) 62 000 ×.

more samples were taken at different times and examined in the electron microscope. The results are illustrated in fig.2. It can be seen that the increase in DNase I activity is paralleled by an increase in the intensity of scattered light, indicating polymerisation of actin. The activity of 5′-nucleotidase does not alter during the incubation period. Examination of the samples taken for electron microscopy revealed the formation of a filamentous network concomitant to the increase in the intensity of scattered light (fig.3). The increase in the DNA-degrading activity after incubation of actin·DNase I complex with 5′-nucleotidase is strongly dependent on their molar ratio, ionic conditions and temperature. Figure 4 illustrates a similar experiment to the one of fig.2. Here actin·DNase I complex was incubated with 5′-nucleotidase at 37°C at different ionic conditions. It can be seen that the increase in DNase activity is fastest under conditions favouring polymerisation of actin, but is not further accelerated by the addition of a 4 molar excess of phalloidin over actin. Under these conditions full reactivation of the DNase activity by 5′-nucleotidase is reached after 60 min at an actin·DNase I complex: nucleotidase ratio of 3:1. When a sample of the incubation mixture including high salt and phalloidin was examined in the electron microscope, microfilaments could also be detected in addition to the filamentous network (fig.3b).

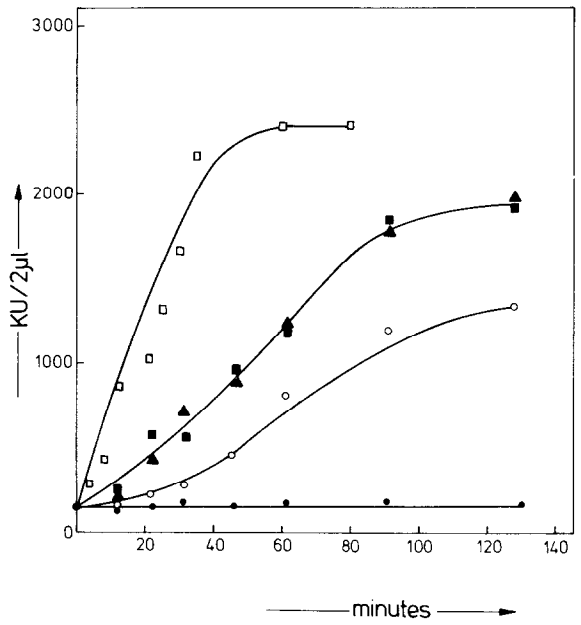

Fig.4. Increase in DNase activity during incubation with 5′-nucleotidase. Incubation mixture contained 30 µM actin·DNase I complex in buffer A. (●) Plus 0.1 M KCl; (○) plus 4 µM 5′-nucleotidase; (■) plus 4 µM 5′-nucleotidase and 0.1 M KCl; (▲) plus 4 µM 5′-nucleotidase, 0,1 M KCl and 125 µM phalloidin; (□) plus 10 µM 5′-nucleotidase and 0.1 M KCl. T = 37°C. DNase I activity in KU/5 µl incubation mixture.

In order to prove the dissociation of DNase I from actin by the interaction with 5′-nucleotidase, the actin·DNase complex was incubated at various ratios with 5′-nucleotidase for 12 h at room temperature. After this time a 6–20-fold increase in DNase I activity was observed. Centrifugation of the incubation mixture for 3 h at 100 000 × g produced a transparent pellet similar to F-actin. Controls of actin·DNase I complex and 5′-nucleotidase were centrifuged separately at concentrations identical to the incubation mixture. No sedimentation of actin·DNase I complex was observed, but 25–32% of the 5′-nucleotidase activity was pelleted. Analyzing the enzymatic activities of the incubation mixture before and the supernatant after centrifugation, it was found that very little DNase I but considerable amounts of 5′-nucleotidase activity exceeding the appropriate control values had sedimented (table 1). SDS–polyacrylamide gel electrophoresis of samples from the incubation mixture before and after centrifugation gave the pattern shown in fig.5. The gels (taken from expt. 2 of table 1) indicate a reduction in the amount of actin in the supernatant and the accumulation of actin but little DNase I in the pellet after centrifugation.

Table 1
Distribution of enzymatic activities before and after centrifugation

No.	Conc. (µM)		Enzymatic activities				
			Before centrifugation		Supernatant after centrifugation		Free 5′-nuc. supernatant
Expt.	Actin·DNase	5′-nuc.	DNase	5′-nuc.	DNase	5′-nuc.	
1	28.5	0.57	600	0.2	540 (90)	0.06 (76)	(98)
2	16	1.4	384	1.4	390 (100)	0.75 (50)	(75)
3	17	4	240	4.9	200 (87)	2.2 (45)	n.d.
4	10	10	400	3.6	360 (100)	1.1 (32)	(70)
5	31	12	240	9.8	219 (91)	3.6 (35)	(68)

n.d., not determined

Experiment 1 was performed with purified 5′-nucleotidase. DNase activity is expressed in KU/µl incubation mixture. 5′-Nucleotidase activity in 10^{-8} mol AMP hydrolysed/min. µl incubation mixture. Numbers in parentheses indicate % enzymatic activity remaining in supernatant. Molarity of 5′-nucleotidase was calculated using mol. wt 70 000

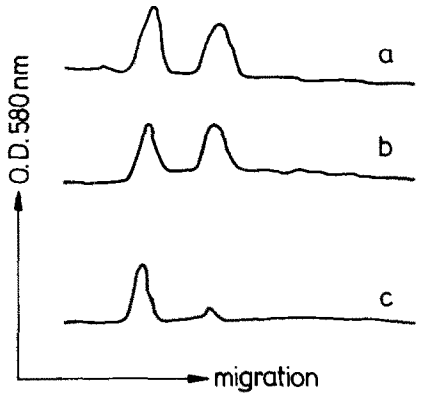

Fig.5. Densitometric traces of gels of incubation of actin·DNase I complex with 5′-nucleotidase taken from expt. 2 of table 1. (a) Incubation mixture before centrifugation; (b) supernatant; (c) pellet after centrifugation. SDS–polyacrylamide gels (10%).

4. Discussion

Evidence has been presented that 5′-nucleotidase is able to reverse the inhibitory action of actin on DNase I. From the results presented it appears that 5′-nucleotidase interacts with actin leading to the formation of a filamentous network and the release of DNase I. The filamentous network resembles in its appearance F-actin after incubation with cytochalasin B [11]. Only if phalloidin is also present filaments resembling natural microfilaments simultaneously appeared. The assumption that 5′-nucleotidase interacts with actin is strengthened by the observation that this enzyme also accelerates the rate of actin polymerisation (to be published). At present, however, we cannot decide whether 5′-nucleotidase directly interacts with actin·DNase I complex or only shifts the equilibrium towards the filamentous form of actin illustrated in fig.3.

So far the effect of 5′-nucleotidase on actin·DNase I complex is the only reported biological mechanism by which this complex is separated into its components. The appearance of actin·DNase I complex [5] and 5′-nucleotidase [12] as secretory products of different organs does not exclude the possibility that the reported interactions of these proteins also occur intracellularly. 5′-Nucleotidase is a widely distributed plasma membrane constituent of eukaryotic cells and therefore might act as an anchorage protein for intracellular filamentous forms of actin modifying its availability for cellular motile responses.

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