Properties of an Adenosine Cyclic Phosphates Degrading Enzyme in *Nicotiana tabacum* L. var. *Xanthi*

Axel Brennicke and Hans Dieter Frey

Institut für Biologie I der Universität Tübingen

(Z. Naturforsch. **32 c**, 297–300 [1977]; received December 9, 1976/February 14, 1977)

Adenosine Cyclic Phosphate, Inorganic Phosphate, *Nicotiana tabacum*, Ribonuclease II, Theophyllin

This work describes an enzyme which degrades adenosine 3′:5′-cyclic phosphate (3′:5′-cyclic-AMP) and adenosine 2′:3′-cyclic phosphate (2′:3′-cyclic-AMP). The reaction products are 3′-AMP and 5′-AMP in case of 3′:5′-cyclic-AMP, 2′-AMP and 3′-AMP when 2′:3′-cyclic-AMP is given as substrate. Inorganic phosphate acts as a strong inhibitor, whereas theophyllin does not act up to a concentration of 10^{-3} M. Probably the adenosine cyclic phosphates degrading enzyme has no function in a 'second messenger' system with 3′:5′-cyclic-AMP.

Introduction

Adenosine 3′:5′-cyclic phosphate and involved enzymes are suggested by Wood *et al.* [1] in *Catharanthus roseus* and Ames [28] in *Nicotiana tabacum* as a mediator system in the action of phytohormones and supposed to have a central function in the manifestation of crown gall and genetic tumors. Other reports disagree with these results and doubt the existence of a 'second messenger' system in higher plants [2, 3] at all. Three different approaches have been used to prove the function of 3′:5′-cyclic-AMP and related enzymes in higher plants and the following results were found: (1) Some authors [4–6] report effects of exogenous application of 3′:5′-cyclic-AMP and demonstrate responses partly analogous to the effects of phytohormones. But as 3′:5′-cyclic-AMP is usually applied in excess and the responses hardly match those obtained with the plant hormones, they might just as well be explained as being unspecific. (2) The levels of endogenous 3′:5′-cyclic-AMP were determined. The results vary largely from author to author [7–10]; attempts to reproduce results obtained with the same methods failed in other laboratories [11, 12] and left doubts in the occurrance of 3′:5′-cyclic-AMP in higher plants. (3) Attempts to establish the presence of two enzymes which should accompany 3′:5′-cyclic-AMP, adenylate cyclase (E.C. 4.6.1.1) [1, 13, 14] and the 3′:5′-cyclic-AMP phosphodiesterase (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, E.C. 3.14.17) [1, 2, 15–18], did not agree in all details, thus a 'second messenger' function of 3′:5′-cyclic-AMP seems improbable.

Requests for reprints should be sent to Dr. Hansdieter Frey, Institut für Biologie I, Auf der Morgenstelle 1, *D-7400 Tübingen*.

Recent reports strengthen the assumption that the reported – and no doubt existing – 3′:5′-cyclic-AMP degrading activity in higher plants is not specific to 3′:5′-cyclic-AMP, but just as readily attacks 2′:3′-cyclc-AMP [3, 19]. These results led to the conclusion that the function of this activity might primarily be in the process of degrading 2′:3′-cyclic-AMP, which is accumulated during the break-down of RNA.

Concerning the involvement of 3′:5′-cyclic-AMP and the nature and function of the degrading enzyme in higher plants, we isolated an enzyme from leaves of *Nicotiana tabacum* and analysed some of its properties.

Methods

The cyclic mononucleotides 3′:5′-cyclic-AMP and 2′:3′-cyclic-AMP, and the enzymatic 5′-AMP test were obtained from Boehringer Mannheim, the nucleotids 2′-AMP, 3′-AMP, 5′-AMP, the Dowex for column chromatography and Servalyt for isoelectric focusing from Serva Biochemica, Darmstadt. All other chemicals were from Merck, Darmstadt.

(1) For extraction of enzyme, leaves of young tobacco plants *Nicotiana tabacum* L. var. *Xanthi* grown under greenhouse conditions were used after surface cleaning. The following processes were done in a cold room (+4 °C); after homogenisation with polyvinylpyrrolidone 1:20 w/w (Polyclar AT) in 50 mM Tris (pH 8.5) and 5 mM $MgSO_4$ in a glass homogenizer (Potter Elvehjem), the obtained homogenate was treated with a Bronson Sonifier B-12 for 5 times 10 sec each at intervals and centrifuged at $6500 \times g$ for 15 min. The supernatant was used as enzyme source.

(2) Disc electrophoresis was carried out according to Ornstein and Davis [20] for 90 min with a constant current of 3 mA per gel. The protein bands were stained with 1% Amidoblack 10B in 7% acetic acid [21]. After isoelectric focusing [22] for 2 h with 2 mA per tube, Coomassie-blue was used for protein detection [21].

(3) Gel chromatography was done with Sephadex G200 in a 45 cm column (ϕ 1 cm) at a flow rate of 6 ml/h and fractions of 3 ml were collected. For the molecular weight determination, catalase, aldolase and hemoglobin were used as standards.

(4) Protein contents were determined according to Lowry [23].

(5) Phosphodiesterase I (oligonucleate 5′-nucleotidohydrolase, E.C. 3.1.4.1) activity was demon-

strated by disc electrophoresis with 2′-deoxythymidin-5′-α-naphtyl phosphate as substrate [24]. After 60 min incubation with diazo red, a dark brown band appears at the site of the phosphodiesterase I.

(6) For localisation of the enzyme after electrophoresis gel slices of 2 mm each (cut at −75 °C) were incubated for 90 min at 30 °C with 5 mg 3′:5′-cyclic-AMP/100 ml in Tris (pH 7). The reaction was stopped by heating.

(7) The pH optimum of the 3′:5′-cyclic-AMP degrading enzyme was determined in a pellet yielded from 30% ammonium sulfate processing in the following buffer system: 0.2 M citric acid-sodium citrate (pH 2.5 – 5.5) 0.2 M Tris malate (pH 4.0 – 8.0), 0.2 M Tris HCl (pH 7.0 – 9.0). Another series of experiments were done by incubating the gel slices containing the enzyme after purification with disc electrophoresis.

(8) The amount of hydrolysed 3′:5′-cyclic-AMP was determined enzymatically by measuring 5′-AMP using a specific test (Boehringer Mannheim, No. 15 980). 10 µl of 15 mM ATP were added to each assay because it is necessary for determination of 5′-AMP in the absence of ADP and ATP when starting the reaction.

(9) Determination of 2′:3′-cyclic-AMP degradation was done analogous to the 3′:5′-cyclic-AMP assay with the purified enzyme after disc electrophoresis.

(10) The hydrolysis products were determined with a Dowex 1 × 2 (200 – 400 mesh, Cl^- form) ion exchange column (0.5 × 10 cm) with 2.5 mM HCl as eluting fluid (flow rate 30 ml/h). The concentration of mononucleotides in the 3 ml fractions was calculated by measuring the optical density at 260 nm [24].

(11) All inhibitor experiments were done with the purified enzyme after disc electrophoresis.

Results

The phosphodiesterase I (E.C. 3.1.4.1) activity could be distinguished from the 3′:5′-cyclic-AMP degrading enzyme in the gel after disc electrophoresis [25] (Fig. 1). The molecular weight found (55 000) is lower than the one previously reported for the enzyme from pea seedlings (350 000) [19], the one from sugar beet leaves (110 000) [26], the one from carrots (100 000) [27] and the one from *Bombyx mori* (66 000) [24].

Inorganic phosphate proved to be a strong inhibitor (50% inhibition at 10^{-3} M Na_2HPO_4), whereas theophyllin up to a concentration of 10^{-3} M produced no definite change in the conversion rate of 3′:5′-cyclic-AMP by the enzyme. Similar results were obtained by other authors [2, 3, 17, 18]. The effect of pH on the degradation of 3′:5′-cyclic-AMP is shown in Fig. 2. There seem to be two pH optima, one at pH 5.5 and one at pH 7.3. Most reports on 3′:5′-cyclic-AMP phosphodiesterase in higher plants agree on the optimal pH 5 – 6 [2, 3, 17–19]; only Wood

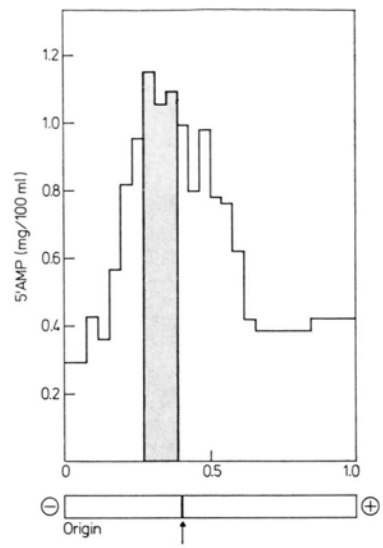

Fig. 1. Localization pattern of 3′:5′-cyclic-AMP degrading activity after disc electrophoresis of enzyme preparations from leaves of *Nicotiana tabacum* L. var. *Xanthi*. The activity is indicated as mg 5′-AMP/100 ml after an incubation of 90 min. The marked gel-slices were taken for further investigations containing an activity of 30 U. The phosphodiesterase I (E.C. 3.1.4.1.) activity is demonstrated in the gel with 2′-deoxythymidin-5′-α-naphthyl phosphate as substrate and incubation with diazo red (arrow).

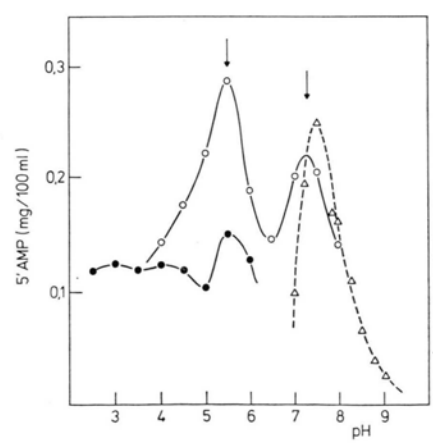

Fig. 2. The pH dependence of 3′:5′-cyclic-AMP degradation with enzyme preparations from leaves of *Nicotiana tabacum* L. var. *Xanthi*. The activity is indicated as mg 5′-AMP/100 ml after an incubation period of 90 min. The assay mixture contained 0.2 M citric acid-sodium citrate (●—●), 0.2 M Tris malate (○—○) or 0.2 M Tris HCl (△—△).

et al.[1] have previously found two pH values giving optimal degradation. The analysis of reaction products with ion exchange chromatography shows 3′-AMP and 5′-AMP as products of 3′:5′-cyclic-AMP hydrolysis (Figs 3 and 4). 2′:3′-cyclic-AMP is broken down by the enzyme to both 2′-AMP and 3′-AMP (Figs 3 and 5). Experiments with enzyme preparations from pea seedlings which degrade adenosine cyclic phosphates[3] showed similar results regarding 3′:5′-cyclic-AMP, but differed in 2′:3′-cyclic-AMP degradation in that only 3′-AMP is produced. In the case of 3′:5′-cyclic-AMP Lin and Varner[3] found as degradation products 3′-AMP and 5′-AMP in a ratio of 7 : 1. Amrhein[2] reports ratios between 8 : 1 and 30 : 1 in *Catharanthus roseus*.

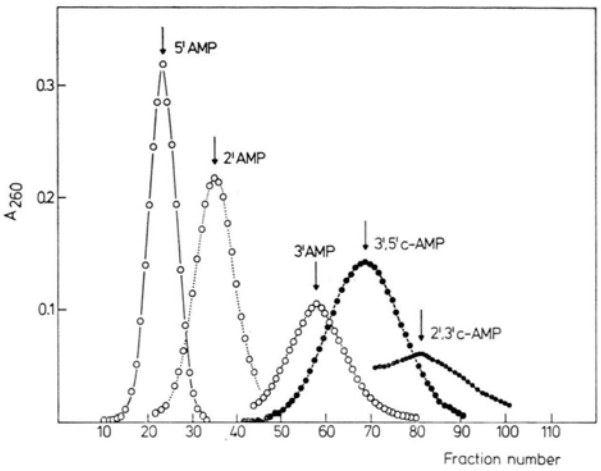

Fig. 3. Separation of the different mononucleotides by ion-exchange chromatography with a Dowex 1×2 (200–400 mesh, Cl^- form) column with 2.5 mM HCl as eluting fluid.

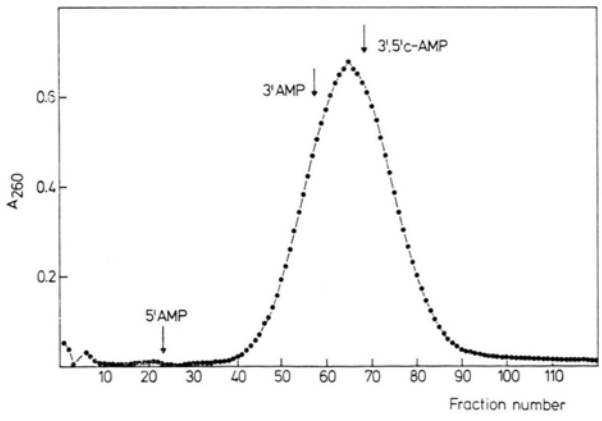

Fig. 4. Degradation of 3′:5′-cyclic-AMP after treatment with the enzyme preparation obtained after disc electrophoresis. The main peak contains the superposition of unhydrolyzed 3′:5′-cyclic-AMP and the reaction product 3′-AMP. The ratio of the reaction products 3′-AMP and 5′-AMP can be calculated as 50:1.

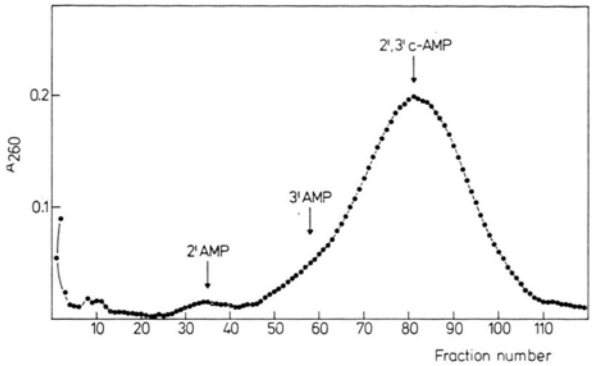

Fig. 5. Elution patterns of 2′:3′-cyclic-AMP and its hydrolysis products after digestion with the enzyme preparation. The ratio of the reaction products 2′-AMP and 3′-AMP can be calculated as 1:1.

Discussion

Our results confirm the existence of an adenosine cyclic phosphates degrading enzyme in *Nicotiana tabacum* L, var. *Xanthi*. Its activity is distinguishable from the phosphodiesterase I (E.C. 3.1.4.1.)[16, 25, 26]. The properties of the enzyme are similar to those obtained by Lin and Varner[3] on pea seedlings and therefore seem to support the opinion that this enzyme plays a role in the reaction chain of RNA degradation. It might be the ribonuclease II (ribonucleate 3′-oligo-nucleotidohydrolase, E.C. 3.1.4.23) with an activity towards 3′:5′-cyclic-AMP. An identification as either nucleoside 2′:3′-cyclic-phosphate 3′-nucleotidohydrolase (E.C. 3.1.4.16) or nucleoside-2′:3′-cyclic-phosphate 2′-nucleotidohydrolase doesn't seem to be correct, as both 2′-AMP and 3′-AMP appear as hydrolysis products. A function of the enzyme in a 3′:5′-cyclic-AMP mediated 'second messenger' system seems very unlikely, as the enzyme is not specific towards 3′:5′-cyclic-AMP, but also degrades 2′:3′-cyclic-AMP. The fact that theophyllin, a characteristic inhibitor in such a 'second messenger' system in animal tissues, does not show a supressing effect on the reaction also supports our hypothesis. The *in vivo* inhibition of 3′:5′-cyclic-AMP degradation with inorganic phosphate has been found in all so far investigated adenosine cyclic phosphates degrading systems in higher plants[2, 3, 6, 8, 16]. A feed-back mechanism via phosphate coming from the 3′:5′-cyclic-AMP-breakdown is hard to imagine, because even if 3′:5′-cyclic-AMP could be detected at all[17],

it is still found in very low intracellular concentrations.

After electrofocusing Wood *et al.*[1] obtained two bands with two different pH optima one at pH 4.0 and one pH 7.0, postulating two different enzymes. The possibility of having a mixture of two different enzymes in the preparation with the same electrophoretic qualities and the same molecular weight cannot be excluded. But it seems also possible to explain the described activity as coming from an enzyme complex with two separate active centers suggesting the low molecular weight being the weight of part or monomere of the entire enzyme.

We are grateful to Miss Michelle Poulin for grammatical correction of the manuscript.

[1] H. N. Wood, A. Lin, and A. C. Braun, Proc. Nat. Acad. Sci. U.S. **69,** 403 [1972].
[2] N. Amrhein, Z. Pflanzenphysiol. **72,** 249 [1974].
[3] P. Lin and J. E. Varner, Biochim. Biophys. Acta **276,** 454 [1972].
[4] S. Kamisaka, H. Sano, M. Katsami, and Y. Masuda, Plant and Cell Physiol. **13,** 167 [1972].
[5] P. N. Rustagi and N. Sankhla, Z. Pflanzenphysiol. **73,** 476 [1974].
[6] D. Salomon and J. P. Mascarenhas, Biochem. Biophys. Res. Commun. **47,** 134 [1972].
[7] P. Raymond, A. Narayanan, and A. Pradet, Biochem. Biophys. Res. Commun. **53,** 1115 [1973].
[8] M. Giannattasio and V. Macchia, Plant Sci. Letters **1,** 259 [1973].
[9] B. Janistyn, Z. Naturforsch. **27 b,** 273 [1972].
[10] D. Becker and H. Ziegler, Planta **110,** 85 [1973].
[11] R. A. Bressan, C. W. Ross, and J. Vandepeute, Plant Physiol. **57,** 29 [1976].
[12] N. Amrhein, Planta **118,** 241 [1974].
[13] M. J. Al-Azzawi and J. L. Hall, Plant Sci. Letters **6,** 285 [1976].
[14] M. Giannattasio and G. Sica, Phytochemistry **13,** 2729 [1974].
[15] M. Shimoyama, M. Sakamoto, N. Satoshi, S. Shigehisa, and I. Ueda, Biochem. Biophys. Res. Commun. **47,** 134 [1972].
[16] R. M. Niles and M. S. Mount, Phytochemistry **13,** 2735 [1974].
[17] N. J. Brewin and D. H. Northcote, Biochim. Biophys. Acta **320,** 104 [1973].
[18] M. Giannattasio, E. Mandato, and V. Macchia, Biochem. Biophys. Res. Commun. **57,** 365 [1974].
[19] J. Vandepeute, R. C. Huffaker, and R. Alvarez, Plant Physiol. **52,** 278 [1973].
[20] L. Ornstein and B. J. Davis, Ann N. Y. Sci. **121,** 321 [1964].
[21] R. Maurer, Disk Elektrophorese, Walter de Gruyter & Co., Berlin 1968.
[22] C. W. Wrigley, Meth. Enzym. **22,** 559 [1971].
[23] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265 [1951].
[24] I. Morishima, Biochim. Biophys. Acta **370,** 227 [1974].
[25] W. Heyser, W. Eschrich, A. Hüttermann, R. Evert, R. Burchardt, E. Fritz, and R. Heyser, Z. Pflanzenphysiol. **71,** 413 [1974].
[26] B. Lerch and G. Wolf, Biochim. Biophys. Acta **258,** 206 [1972].
[27] C. Harvey, L. Malsman, and A. L. Nussbaum, Biochemistry **6,** 3689 [1967].
[28] J. H. Ames, Plant and Cell Physiol. **17,** 1059 [1976].