Biochimica et Biophysica Acta, 370 (1974) 227–241

BBA 67328

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN SILKWORM: PURIFICATION AND PROPERTIES

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(Received May 1st, 1974)

SUMMARY

A cyclic nucleotide phosphodiesterase (EC 3.1.4.–) has been partially purified from the larvae of silkworm *(Bombyx mori)*. The enzyme catalyzes the hydrolysis of 2′,3′-cyclic nucleotides to 3′-monoesters and 3′,5′-cyclic nucleotides to 5′-monoesters. The 2′,3′-cyclic nucleotide and 3′,5′-cyclic nucleotide phosphodiesterase activities have been purified about 2900- and 2700-fold, respectively. The enzyme preparation is free of phosphomonoesterase, ribonuclease and deoxyribonuclease. Attempts to separate the two activities by a variety of physical and chemical means have been unsuccessful. It is suggested, therefore, that the two activities are associated with the same protein molecule.

The enzyme has a molecular weight of 66 000 and does not require the presence of metal ion for its activity. It shows optimum activity at pH 8.2 with 3′,5′-cyclic AMP as substrate and between pH 7.4 and 8.0 with 2′,3′-cyclic AMP as substrate. Both activities are inhibited by caffeine and theophylline.

INTRODUCTION

Adenosine 3′,5′-monophosphate has been known to be a second messenger in the action of a variety of animal hormones. Recently, it has also been implicated in the action of insect moulting hormone [1–3]. The level of 3′,5′-cyclic AMP in any tissue is a result of a balance between its rate of synthesis by adenyl cyclase and that of hydrolysis by cyclic nucleotide phosphodiesterase. Previous work from this laboratory has described an enzymatic system for the degradation of 3′,5′-cyclic AMP in silkworm and a developmental change of the activity [4]. A similar change of the activity has been observed in the midgut of the tobacco hornworm [5]. So far there are no reports concerning the purification and detailed characterization of cyclic nucleotide phosphodiesterase in insects. It seemed necessary to study the phosphodiesterase activity of insects in more detail in order to obtain further insight into its characteristics and possible biochemical role.

The phosphodiesterase partially purified from silkworm larvae hydrolyzed not only 3′,5′-cyclic nucleotide but also 2′,3′-cyclic nucleotide, unlike 3′,5′-cyclic nucle-

otide phosphodiesterase from animal tissues or bacteria [6–9]. Although enzymes hydrolyzing 2′,3′-cyclic nucleotide have been found in animal [10, 11] and bacterial [12–17] systems, the enzymes do not attack its 3′,5′-isomer. An enzyme which has a similar substrate specificity has been partially purified from some plants, pea seedlings [18] and barley seeds [19]. The silkworm enzyme, however, possessed several properties distinctly different from those reported for the plant enzyme. The most remarkable points were the effect of methylxanthines on the enzyme activity and the hydrolysis product of the enzyme.

This paper presents the procedures for purification of cyclic nucleotide phosphodiesterase from silkworm larvae and properties of the enzyme with respect to its two activities which are probably associated with the same protein molecule.

EXPERIMENTAL PROCEDURES

Materials

Silkworm *(Bombyx mori)* used for enzyme preparation was reared on mulberry leaves at 25 °C in our laboratory. Nucleotides and nucleoside derivatives were obtained from Kyowa Hakko Kogyo Co., Kohjin Co. or Boehringer Mannheim. *p*-Nitrophenyl phosphate, acrylamide, *N,N,N′,N′*-tetramethylenediamine and *N,N′*-methylene bisacrylamide were obtained from Seikagaku Kogyo Co.; snake venom *(Crotalus atrox)*, catalase (bovine liver), hemoglobin (bovine), bovine serum albumin, egg albumin and β-lactoglobulin from Sigma Chemical Co.; alkaline phosphatase (EC 3.1.3.1, calf intestine, grade I) from Boehringer Mannheim; Dowex 1X2 (200–400 mesh) from Dow Chemical Co.; Sephadex G-100 and G-200 from Pharmacia; DEAE-cellulose from Brown Co. Hydroxyapatite was prepared as described by Tiselius et al. [20]. High molecular weight RNA was prepared from commercial yeast by the method of Crestifield et al. [21]. Calf thymus DNA was purified from the commercial product (Sigma Chemical Co.) by the method of Marmur [22].

Methods

Assay of cyclic nucleotide phosphodiesterase. The assay was carried out essentially by the method of Butcher and Sutherland [6]. When 3′,5′-cyclic nucleotide was used as substrate, the incubation mixture (0.50 ml) contained 50 mM Tris–HCl (pH 8.2), 5 mM magnesium acetate, 4 mM substrate, 100 μg of snake venom and the enzyme preparation. When 2′,3′-cyclic nucleotide was used as substrate, Tris–HCl (pH 8.2) and snake venom were replaced by Tris–HCl (pH 7.5) and alkaline phosphatase (0.5 unit), respectively. The reaction mixture was incubated for 30 min at 30 °C. The reaction was terminated by the addition of 0.1 ml of cold 25% $HClO_4$ and the reaction tube was placed in an ice bath for 15 min. The precipitate it formed was removed by centrifugation at 1000 × *g* for 10 min. The resulting supernatant was analyzed for P_i by the method of Fiske and SubbaRow [23]. Color which was fully developed in 10 min was determined in a spectrophotometer at 700 nm. In certain studies the assay was performed in two stages. These consisted of a preliminary incubation of the reaction medium without snake venom or phosphatase for 30 min at 30 °C. After boiling the tube for 90 s, a second incubation was carried out in the presence of snake venom or phosphatase for 30 min at 30 °C. All assays were done in duplicate or triplicate. Under the assay conditions described above, the release of

inorganic phosphate from 2′,3′- or 3′,5′-cyclic nucleotide was proportional to the enzyme concentration, and the assays were linear at least for 60 min.

Unit of enzyme activity. One unit of enzyme activity is defined as that amount of enzyme which causes the release of 1 μmole of P_i per 30 min under the assay conditions described above. The specific activity of the enzyme is defined in units/mg of protein.

Other enzyme activities. 3′- or 5′-nucleotidase activity was determined by measuring the release of P_i from 3′- or 5′-AMP. The incubation mixture (0.50 ml) contained 50 mM Tris–HCl (pH 8.0), 5 mM magnesium acetate, 4 mM nucleotide and the enzyme preparation. The reaction was terminated by the addition of 0.1 ml of 25% cold $HClO_4$, and the P_i released was determined as described in the assay of cyclic nucleotide phosphodiesterase. Ribonuclease activity was assayed as described previously [24]. Assay of the deoxyribonuclease activity was essentially the same as that of the ribonuclease activity.

Determination of protein content. Protein was determined unless otherwise noted according to the method of Lowry et al. [25] with crystalline bovine serum albumin as a standard.

Polyacrylamide disc-gel electrophoresis. Electrophoresis was performed according to the method of Ornstein [26] and Davis [27], in 7.5% polyacrylamide gel at 4 °C in Tris–glycine (pH 8.4) with a constant current of 2 mA per tube. After performing electrophoresis, the gel was cut longitudinally into two equal portions. One-half of the gel was stained with Amido Black 10B, and the other half was sliced to 2-mm thickness, each piece being eluted with 1 ml of water for the assay of enzyme activity.

Sucrose density gradient centrifugation. The sample was layered onto a 5–20% sucrose gradient in 0.01 M Tris–HCl (pH 7.5) and centrifuged at 3 °C in RPS-40 rotor of a Hitachi ultracentrifuge at 37 000 rev/min for 20 h. Upon completion of the run, 6-drop fractions were collected from the bottom of the tube using a stainless-steel capillary inserted into the tube.

Dowex ion-exchange chromatography. For the separation of 2′- and 3′-AMP or 3′- and 5′-AMP, a column of Dowex 1X2 (Cl^- form, 200–400 mesh, 0.5 cm × 6 cm) was first calibrated by chromatographing a mixture of authentic 2′- and 3′-isomers or 3′- and 5′-isomers of adenylic acid. The nucleotides were eluted with 2.5 mM HCl. Fractions of 3 ml were collected with a flow rate of 34 ml/h and the absorbance was measured at 260 nm.

RESULTS

Purification of the enzyme

All procedures were carried out in an ice bath or at 4 °C unless otherwise stated.

Step 1: Preparation of crude extract. The fifth-instar larvae of silkworm were treated with chloroform to cause vomiting of the digestive juice. The insects (200 g) were washed with distilled water and homogenized with 450 ml of 0.01 M Tris–HCl (pH 7.5) in a Waring Blender for 2 min. The homogenate was then filtered through a double layer of gauze. The resulting filtrate (506 ml) was centrifuged at 10 000 × g for 30 min and the supernatant fluid was used as the starting material for the purification of the enzyme.

Step 2: pH 5 treatment. The supernatant fluid was adjusted to pH 5.0 with 0.5 M acetic acid. After stirring for 30 min, the precipitate formed was removed by centrifugation at 10 000 × *g* for 20 min. The supernatant fluid was poured through glass wool and adjusted to pH 7.5 with 1 M NH_4OH.

Step 3: $(NH_4)_2SO_4$ fractionation. The supernatant fluid (500 ml) was brought to 40% saturation with $(NH_4)_2SO_4$ by slowly adding 22.6 g of solid $(NH_4)_2SO_4$ per 100 ml of solution with stirring. After stirring for 60 min, the precipitate was removed by centrifugation. The resulting supernatant fluid was adjusted to pH 7.5 with 1 M NH_4OH and then brought to 65% saturation by the addition of 15.3 g of solid $(NH_4)_2SO_4$ per 100 ml of solution. After stirring overnight, the precipitate was collected by centrifugation and dissolved in 40 ml of 0.01 M Tris–HCl (pH 7.5). The resulting solution was dialyzed overnight against 2 l of the same buffer. The precipitate which formed during dialysis was removed by centrifugation.

Step 4: Chromatography on DEAE-cellulose. A column of DEAE-cellulose (3.1 cm × 28 cm) was equilibrated with 0.01 M Tris–HCl (pH 7.5). The dialyzed sample (2.0 g of protein) from step 3 was then applied to the column and washed in 100 ml of the same buffer. The enzyme was eluted with a linear gradient formed with 600 ml each of 0.02 and 0.4 M NaCl in 0.01 M Tris–HCl (pH 7.5) and collected in 11.5-ml fractions at a flow rate of 65 ml/h. The elution profile of enzyme activities (Fig. 1) showed that the two activities toward 2′,3′-cyclic AMP and 3′,5′-cyclic AMP

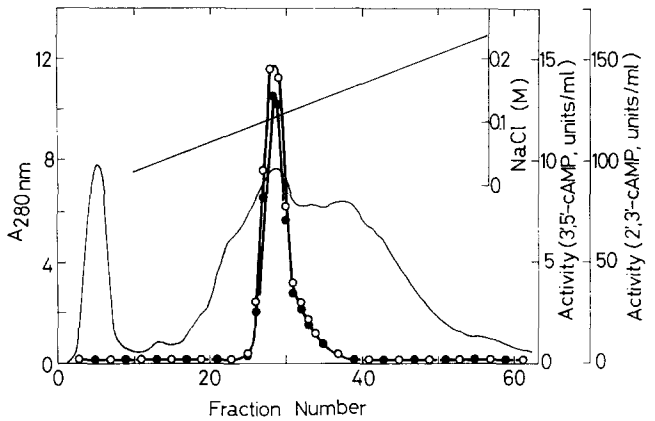

Fig. 1. DEAE-cellulose column chromatography of cyclic nucleotide phosphodiesterase. The enzyme preparation from $(NH_4)_2SO_4$ fractionation was applied to a column (3.1 cm × 28 cm) equilibrated with 0.01 M Tris–HCl (pH 7.5). Elution was carried out with a linear gradient formed with 600 ml each of 0.02 and 0.4 M NaCl in 0.01 M Tris–HCl (pH 7.5). Fractions of 11.5 ml were collected at a flow rate of 65 ml/h. ○—○, enzyme activity toward 2′,3′-cyclic AMP; ●—●, enzyme activity toward 3′,5′-cyclic AMP; ———, absorbance at 280 nm.

were eluted coincidently at about 0.1 M NaCl. Fractions 27–30 (46 ml) were pooled and concentrated to about 2 ml by ultrafiltration, using a Diafilter G-05T (Bio-engineering Co., Japan).

Step 5: Chromatography on Sephadex G-200. A column of Sephadex G-200 (1.7 cm × 94 cm) was equilibrated with 0.01 M Tris-HCl (pH 7.5). The concentrated

sample from Step 4 was then applied to the column. The enzyme was eluted with the same buffer and collected in 4.6-ml fractions at a flow rate of 10 ml/h. Two enzyme activities were also eluted coincidently in a sharp peak. Fractions with high specific activity were pooled (27.6 ml).

Step 6: Chromatography on hydroxyapatite. A column of hydroxyapatite (1.7 cm × 3 cm) was equilibrated with 0.01 M Tris–HCl (pH 7.5) and loaded with the eluate from Sephadex G-200, then washed in 10 ml of the same buffer. Elution was stepwise with 5 mM and 0.1 M sodium phosphate (pH 6.8), and fractions of 2.3 ml were collected at a flow rate of 9 ml/h. The two enzyme activities were appeared in the first peak eluted with 5 mM buffer (Fig. 2). The second peak eluted with 0.1 M buffer contained most of protein loaded on the column but no enzyme activity. Fractions 10–13 (9.2 ml) were pooled and concentrated to about 2 ml by ultrafiltration.

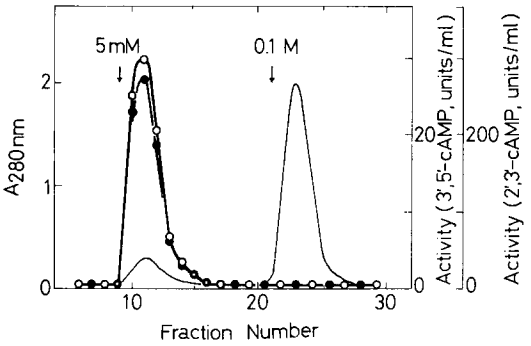

Fig. 2. Hydroxyapatite column chromatography of cyclic nucleotide phosphodiesterase. The enzyme preparation eluted from Sephadex G-200 was applied to a column (1.7 cm × 3 cm) equilibrated with 0.01 M Tris–HCl (pH 7.5). Elution was stepwise with 5 mM and 0.1 M sodium phosphate (pH 6.8). Fractions of 2.3 ml were collected at a flow rate of 9 ml/h. ○—○, enzyme activity toward 2',3'-cyclic AMP; ●—●, enzyme activity toward 3',5'-cyclic AMP; ———, absorbance at 280 nm.

Step 7: Chromatography on Sephadex G-100. The concentrated sample from Step 6 was applied to a column of Sephadex G-100 (1.7 cm × 98 cm) equilibrated with 0.01 M Tris–HCl (pH 7.5). The enzyme was eluted with the same buffer and collected in 3-ml fractions at a flow rate of 11.3 ml/h. The two enzyme activities were also eluted together in one peak followed by a major protein peak which contained no enzyme activity (Fig. 3). Fractions 34–37 (12 ml) were combined, concentrated to 2 ml and stored at −20 °C for further use. At this temperature the enzyme was stable for at least 3 months.

The summary of the purification of cyclic nucleotide phosphodiesterase from silkworm larvae is shown in Table I. In these purification procedures, two enzyme activities toward 2',3'-cyclic AMP and 3',5'-cyclic AMP were not separated and were maintained in a constant ratio. Although the results suggested that the enzymatic hydrolysis of the two cyclic nucleotides might be due to the same protein molecule, further attempts were made to separate the two activities.

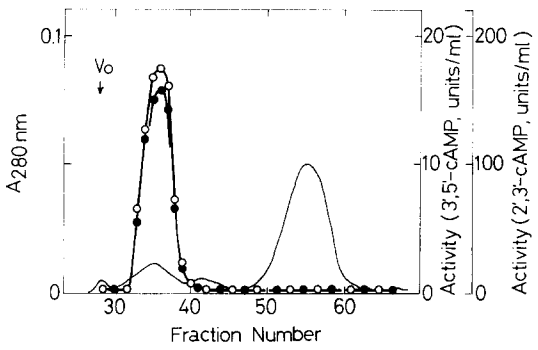

Fig. 3. Sephadex G-100 column chromatography of cyclic nucleotide phosphodiesterase. The enzyme preparation eluted from hydroxyapatite column was concentrated to 2 ml and applied to a column (1.7 cm × 98 cm) equilibrated with 0.01 M Tris–HCl (pH 7.5). Elution was carried out with the same buffer. Fractions of 3 ml were collected at a flow rate of 11.3 ml/h. ○—○, enzyme activity toward 2',3'-cyclic AMP; ●—●, enzyme activity toward 3',5'-cyclic AMP; ———, absorbance at 280 nm.

TABLE I

SUMMARY OF PURIFICATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM SILKWORM LARVAE

Fraction	Total protein (mg)	2',3'-Cyclic AMP as substrate			3',5'-Cyclic AMP as substrate			Ratio of specific activity 2',3'-Cyclic AMP / 3',5'-Cyclic AMP
		Total activity (units)	Specific activity (units/mg)	Yield (%)	Total activity (units)	Specific activity (units/mg)	Yield (%)	
10 000 × *g* supernatant	4710	12 600	2.67	100	1230	0.261	100	10.2
pH 5 supernatant	3350	12 100	3.61	96.0	1070	0.319	87.0	11.3
$(NH_4)_2SO_4$	2000	9 460	4.73	75.1	876	0.438	71.2	10.8
DEAE-cellulose	189	5 470	28.9	43.4	493	2.61	40.1	11.1
Sephadex G-200	27.9	4 830	173	38.3	393	14.1	32.0	12.3
Hydroxyapatite	1.24*	1 850	1490	14.7	171	138	13.9	10.8
Sephadex G-100	0.155*	1 220	7860	9.7	111	716	9.0	11.0

* Protein concentration was determined by measuring the difference in absorbance at 215 and 225 nm [38] using bovine serum albumin as standard.

Attempts to separate two enzyme activities by physical means

The results of sucrose density gradient centrifugation are shown in Fig. 4. Two enzyme activities toward 2',3'-cyclic AMP and 3',5'-cyclic AMP sedimented in an identical pattern with the maximal activity in the same fraction. Compared to bovine liver catalase (11.3 S) and egg albumin (3.66 S), the cyclic nucleotide phosphodiesterase had a sedimentation constant of 4.3 S. The results obtained after polyacrylamide disc-gel electrophoresis indicate that the two enzyme activities have the same mobility (Fig. 5). Furthermore, the area of the gel where the major portion of activity resides coincides with only one demonstrable protein band, and no activity is recognized in the area of the other protein band.

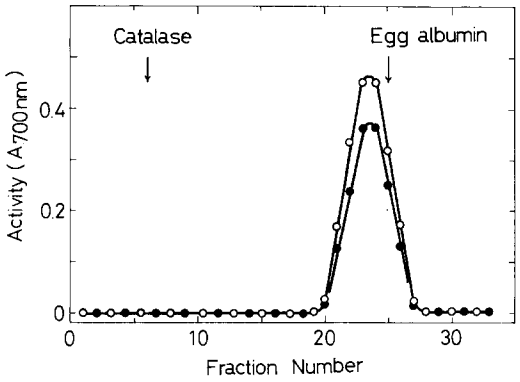

Fig. 4. Sucrose density gradient centrifugation of cyclic nucleotide phosphodiesterase. Purified enzyme preparation containing 1 unit of activity toward 3′,5′-cyclic AMP in 0.2 ml was layered onto a 5 to 20% sucrose gradient in 0.01 M Tris–HCl (pH 7.5) and centrifuged as described in Methods. Bovine liver catalase (11.3 S) and egg albumin (3.66 S) were used as markers, which were indicated by arrows in the figure. After centrifugation 6-drop fractions, total of 34 fractions, were collected. An aliquot was taken for the enzyme assay under the standard conditions. ○—○, enzyme activity toward 2′,3′-cyclic AMP; ●—●, enzyme activity toward 3′,5′-cyclic AMP.

Properties of purified enzyme

Estimation of molecular weight. The molecular weight of the enzyme was estimated as described by Siegel and Monty [28] by means of the following equation: $M = 6\pi\eta N a s/(1-\bar{v}\rho)$, where M = molecular weight, a = Stokes radius, s = sedimentation constant and $\bar{v}$ = partial specific volume. The Stokes radius of the

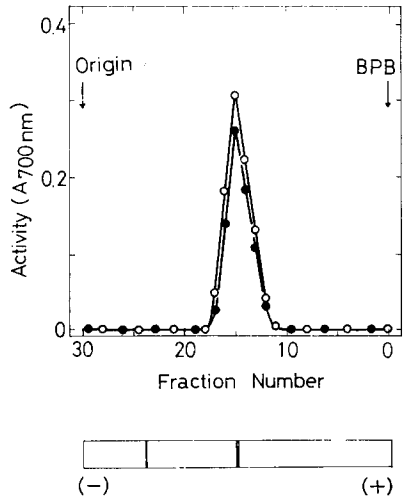

Fig. 5. Polyacrylamide disc-gel electrophoresis of cyclic nucleotide phosphodiesterase. Purified enzyme preparation containing 10 μg of protein was applied to a 7.5% polyacrylamide gel as described in Methods. The gel was cut in half; one half was stained with Amido Black 10B, and the other half was sliced into 2-mm thickness and eluted with 1 ml of water. An aliquot was taken for enzyme assay under the standard conditions. ○—○, enzyme activity toward 2′,3′-cyclic AMP; ●—●, enzyme activity toward 3′,5′-cyclic AMP.

enzyme was calculated to be 36 Å according to the correlation of Porath [29] using K_d values obtained by gel filtration through a Sephadex G-200 column. The column (1.7 cm × 94 cm) used had previously been calibrated with catalase, bovine serum albumin, hemoglobin, β-lactoglobulin and egg albumin. Using the Stokes radius of 36 Å and the sedimentation constant of 4.3 S obtained as described above, together with the assumed $\bar{v}$ of 0.725 cm^3/g (see ref. 30), we calculated the molecular weight of the enzyme to be 66 000.

Substrate specificity. The enzyme showed preferences for 2′, 3′-cyclic nucleotides as shown in Table II. Cyclic GMP was hydrolyzed at a similar rate to cyclic AMP. It should be noted that pyrimidine 3′,5′-cyclic nucleotides, especially 3′,5′-cyclic CMP, was very resistant to hydrolysis by the enzyme, although its 2′,3′-isomer was hydrolyzed at a significant rate. The 2,6-dibutyryl derivative of 3′,5′-cyclic AMP was not hydrolyzed by the enzyme as described for many of the mammalian enzymes [31, 32]. The enzyme preparation had no significant activity toward RNA, native or heat-denatured DNA, 3′- or 5′-AMP or *p*-nitrophenyl phosphate.

TABLE II

SUBSTRATE SPECIFICITY OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

The reaction mixture and conditions were as described in Methods. A value of 100 was assigned to the activity toward 2′,3′-cyclic AMP.

Substrate	Relative activity	Substrate	Relative activity
2′,3′-Cyclic AMP	100	Dibutyryl 3′,5′-cyclic AMP	<0.1
2′,3′-Cyclic GMP	102		
2′,3′-Cyclic UMP	40	*p*-nitrophenyl phosphate	<0.01
2′,3′-Cyclic CMP	24	3′-AMP	<0.01
3′,5′-Cyclic AMP	9.1	5′-AMP	<0.01
3′,5′-Cyclic GMP	9.2	RNA	<0.01
3′,5′-Cyclic UMP	0.11	Native DNA	<0.01
3′,5′-Cyclic CMP	<0.01	Heat-denatured DNA	<0.01

Effect of pH. The effect of pH upon enzyme activity was investigated using sodium acetate (pH 5.0–6.0), Tris–acetate (pH 6.0–8.0) and Tris–HCl (pH 7.5–9.5) buffers. No difference in activity was noted with these buffers. The assay was performed by the two-stage method outlined earlier. In the first stage, the incubation was carried out for 30 min at 30 °C with 0.05 M buffer at pH values ranging from 5.0 to 9.5. After boiling the tube for 90 s, the pH was adjusted to 8.2 with 0.1 M Tris–HCl. Alkaline phosphatase or snake venom was added in a further incubation for 30 min at 30 °C. The enzyme exhibited an optimum pH with a relatively sharp peak at 8.2 with 3′,5′-cyclic AMP and GMP as substrate and an optimum pH range between 7.4 and 8.0 with 2′,3′-cyclic AMP, GMP, CMP and UMP as substrate.

Reaction products. The reaction products formed by the enzymatic hydrolysis of 2′,3′-cyclic AMP and 3′,5′-cyclic AMP were analyzed by anion-exchange chromatography. The chromatograms (Fig. 6) showed that 3′-AMP was formed exclusively from 2′,3′-cyclic AMP, and 5′-AMP from 3′,5′-cyclic AMP.

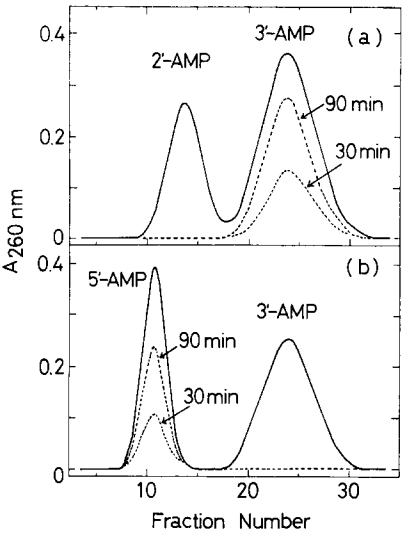

Fig. 6. Ion-exchange chromatography of the hydrolysis products. Purified enzyme preparation was incubated with 4 mM 2',3'-cyclic AMP (a) or 3',5'-cyclic AMP (b), 5 mM magnesium acetate and 50 mM Tris–HCl in a final volume of 1.0 ml for 30 and 90 min at 30 °C. After termination of the reaction by heating in boiling water for 90 s, the whole reaction mixture was streaked on a Whatman 3MM paper. The chromatogram was developed with a solvent system of isopropanol–NH_4OH–water (7:1:2, by vol.) at room temperature. The band containing the product was cut off and eluted with distilled water. The whole eluate was applied to a Dowex 1X2 column as described in Methods. The time (min) for incubation is indicated within the figure. – – –, hydrolysis product; ———, authentic nucleotide.

Effect of bivalent cations on enzyme activity. As shown in Table III, the two activities behaved similarly in response to the presence of various metal ions. A slight increase in both activities was observed in the presence of Mg^{2+} at concentrations of 1–10 mM. Ca^{2+} had no effect up to 10 mM; Mn^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+} inhibited both activities. EDTA had no effect on either activity at a concentration of 10 mM.

Effect of various compounds on enzyme activity. Caffeine and theophylline inhibited the enzyme activities with 3',5'-cyclic AMP and 2',3'-cyclic AMP (Table IV). Theophylline was somewhat more inhibitory than caffeine for both activities. The concentrations of caffeine giving 50% inhibition of the activities toward 2',3'-cyclic AMP and 3',5'-cyclic AMP at 4 mM substrate were 4.2 and 9.2 mM, respectively, and that of theophylline were 2.4 and 7.6 mM, respectively. Imidazole at concentrations of 5–10 mM slightly activated both activities. NaF at 10 mM showed a significant inhibition of 3',5'-cyclic AMP hydrolysis with no apparent effect on 2',3'-cyclic AMP hydrolysis. A similar observation has been reported for cyclic nucleotide phosphodiesterase from pea seedlings [18] and barley seeds [19].

Effect of urea on enzyme activity. Both activities were inhibited in an identical manner by the presence of increasing concentrations of urea (Fig. 7). The concentration of urea giving 50% inhibition of both activities was 2.5 M.

Temperature dependence. The enzyme displayed an optimum temperature at 45 °C with 2',3'-cyclic AMP and 3',5'-cyclic AMP as substrate under the standard

TABLE III

EFFECT OF BIVALENT CATIONS ON THE ACTIVITY OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

The assay was performed by the two-stage method. In the first stage, the reaction mixture containing 50 mM Tris–HCl (pH 7.5 or 8.2), 4 mM substrate, enzyme and bivalent cation or EDTA at the indicated concentration was incubated for 30 min at 30 °C. After termination of the reaction by boiling for 90 s, EDTA (1 or 10 mM), magnesium acetate (20 mM) and alkaline phosphatase or snake venom were added to the tube. The tube was then incubated another 30 min at 30 °C. The P_i released was determined as described in Methods. Under these conditions, the activities of 5′-nucleotidase and alkaline phosphatase were little affected. The activity in the absence of bivalent cation was taken as 100%.

Additions	Concentration (mM)	Relative activity	
		2′,3′-Cyclic AMP as substrate (%)	3′,5′-Cyclic AMP as substrate (%)
None	—	100	100
Mg^{2+}	1	105	106
	10	103	106
Mn^{2+}	1	95	93
	10	76	45
Zn^{2+}	1	32	22
	10	26	21
Ba^{2+}	1	88	93
	10	81	86
Cu^{2+}	1	84	83
	10	30	39
Co^{2+}	1	74	79
	10	51	64
Ca^{2+}	1	100	101
	10	99	100
EDTA	10	98	99

TABLE IV

EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITY OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

The reaction conditions were as described for the standard assay except that the reaction mixture contained the compound to be tested at the indicated concentration.

Compound	Concentration (mM)	Relative activity	
		2′,3′-Cyclic AMP as substrate (%)	3′,5′-Cyclic AMP as substrate (%)
None	—	100	100
Caffeine	2	69	84
	10	29	48
Theophylline	2	53	77
	10	19	44
Imidazole	5	120	118
	10	125	121
NaF	5	100	72
	10	96	54

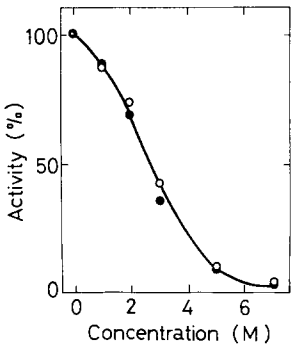

Fig. 7. Effect of urea on the activity of cyclic nucleotide phosphodiesterase. The standard reaction mixtures were incubated with various concentrations of urea. ○—○, 2′,3′cyclic AMP as substrate; ●—●, 3′,5′-cyclic AMP as substrate.

assay conditions. An Arrhenius plot of data taken from the effect of temperature on enzyme activities showed a change in slope at 35 °C (Fig. 8). Using the integrated form of the Arrhenius equation, we calculated the activation energy to be 8.1 kcal/mole for hydrolysis of 2′,3′-cyclic AMP and 7.7 kcal/mole for the hydrolysis of 3′,5′-cyclic AMP between the temperature range of 20 and 35 °C. The values for the hydrolysis of 2′,3′-cyclic AMP and 3′,5′-cyclic AMP are close to those of 8.6 kcal/mole and 7.2 kcal/mole reported by Lin and Varner [18] for pea seedling cyclic nucleotide phosphodiesterase, and the value for the latter is also close to the value of 7.5 kcal/mole reported by Cheung [33] for rat brain 3′,5′-cyclic nucleotide phosphodiesterase.

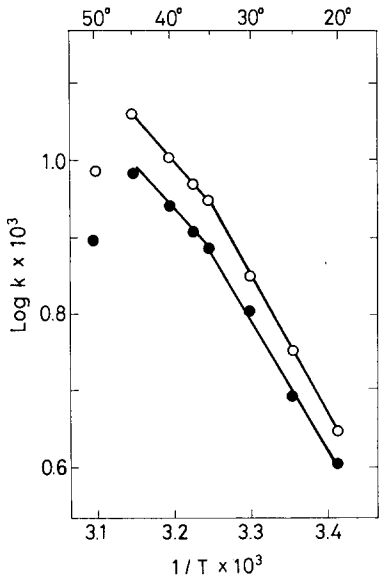

Fig. 8. Arrhenius plots for the activity of cyclic nucleotide phosphodiesterase. The standard reaction mixtures were equilibrated at each temperature for 3 min before the addition of the enzyme. After a 30-min incubation, P_i released was assayed as described in Methods. Substrate used: ○—○, 2′,3′-cyclic AMP; ●—●, 3′,5′-cyclic AMP.

Inhibition by caffeine. The phosphodiesterase activities toward 2′,3′-cyclic AMP and 3′,5′-cyclic AMP were studied at varying substrate concentrations between 0.3 and 4 mM, in the presence or absence of caffeine. Both activities of the enzyme were inhibited by caffeine, the mode of inhibition of the two activities, however, was different. As shown in Fig. 9, inhibition of the activity toward 2′,3′-cyclic AMP appeared to be of a non-competitive type, although inhibition of that toward 3′,5′-cyclic AMP appeared to be of a competitive type. The K_m values were 0.83 mM for 2′,3′-cyclic AMP and 1.3 mM for 3′,5′-cyclic AMP in the absence of inhibitor. The

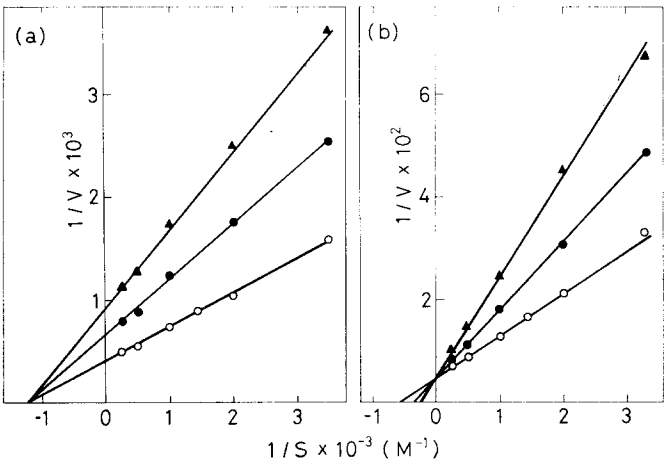

Fig. 9. Inhibition of the activity of cyclic nucleotide phosphodiesterase by caffeine. The reaction medium of 1.0 ml contained the standard components with varying concentrations of substrate and 0, 0.5 or 5 mM of caffeine. The protein concentration was 0.08 μg or 0.8 μg per tube with 2′,3′-cyclic AMP (a) or 3′,5′-cyclic AMP (b) as substrate, respectively. The incubation was reduced to 15 min. *v* is expressed as nmoles cyclic AMP hydrolyzed per min per μg protein. ○—○, no caffeine; ●—●, 2.5 mM caffeine; ▲—▲, 5 mM caffeine.

values are close to those reported for pea enzyme [18], i.e. 0.83 mM for 2′,3′-cyclic AMP and 0.90 mM for 3′,5′-cyclic AMP. The K_i values for caffeine as calculated from Fig. 9 were 4.0 mM with 2′,3′-cyclic AMP and 2.3 mM with 3′,5′-cyclic AMP. With rat brain 3′,5′-cyclic AMP phosphodiesterase [33], the inhibition by caffeine was competitive and the K_i was 3 mM. In the case of human blood platelet [31], it was mixed type and the K_i was 1.5 mM.

DISCUSSION

An enzyme has been partially purified from silkworm larvae which hydrolyzes both 2′,3′-cyclic nucleotide and 3′,5′-cyclic nucleotide. Purification was approximately 2900- and 2700-fold with a recovery of about 9.7 and 9.0% of the total activity with respect to the hydrolysis of 2′,3′-cyclic AMP and 3′,5′-cyclic AMP, respectively. The enzyme preparation is free of non-specific phosphatase, 3′-nucleotidase, 5′-nucleotidase, ribonuclease and deoxyribonuclease activities.

The results of the present study suggest that the activities toward 2′,3′-cyclic nucleotide and 3′,5′-cyclic nucleotide reside in the same protein molecule. Evidence

for this conclusion is as follows: (1) The two activities show the same behavior on DEAE-cellulose and hydroxyapatite column chromatographies and on gel filtration. (2) The two activities are maintained in a constant ratio throughout the purification procedures. (3) The two activities do not separate by means of sucrose density gradient centrifugation and gel electrophoresis. (4) Studies on the properties of the enzyme show that both activities behave quite similarly under a variety of experimental conditions; response to metal ions, inhibition by methylxanthines, optimum temperature and denaturation by urea.

Most of 3′,5′-cyclic nucleotide phosphodiesterases so far characterized from animal tissues or bacteria show no activity toward 2′,3′-cyclic nucleotide [6–9]. Recently, two enzymes have been partially purified from pea seedlings [18] and barley seeds [19], respectively, which hydrolyze both 2′,3′-cyclic nucleotide and 3′,5′-cyclic nucleotide with velocities of the same order of magnitude. The plant enzymes have optimal activity between pH 5.0 and 6.0, and the pea enzyme shows no requirement for metal ion. The silkworm phosphodiesterase has an optimum pH at 8.2 with 3′,5′-cyclic AMP as substrate and at pH 7.4–8.0 with 2′,3′-cyclic AMP as substrate. The former value is somewhat lower than that reported for the 3′,5′-cyclic nucleotide phosphodiesterase activities of other insects; i.e. pH 8.8 for tobacco hornworm enzyme [5] and pH 8.5 for Madagascar cockroach enzyme [34]. The silkworm enzyme does not require metal ions for its activity in contrast to the enzymes from other animal tissues or bacteria.

The silkworm enzyme catalyzes the formation of 5′-AMP from 3′,5′-cyclic AMP, and 3′-AMP from 2′,3′-cyclic AMP while the plant enzymes hydrolyze 3′,5′-cyclic AMP to a mixture of 3′-AMP and 5′-AMP, and 2′,3′-cyclic AMP exclusively to 3′-AMP. 2′,3′-Cyclic nucleotide phosphodiesterase so far characterized from various microorganisms [14, 15, 17] catalyzes the hydrolysis of 2′,3′-cyclic nucleotides to 3′-monoesters. In contrast, a similar enzyme (2′,3′-cyclic nucleotide-3′-phosphohydrolase) of various animal tissues [10, 11, 35] catalyzes the hydrolysis of the nucleotides to 2′-monoesters.

Caffeine and theophylline, known to be characteristic inhibitors of 3′,5′-cyclic nucleotide phosphodiesterase, also inhibit the silkworm enzyme activities toward both cyclic nucleotides, although the plant enzyme activities are not affected by these compounds. The kinetic data show that the mode of inhibition of the two activities of silkworm enzyme by caffeine was different, i.e. the inhibition with 3′,5′-cyclic AMP as substrate appeared to be competitive whereas that with 2′,3′-cyclic AMP as substrate appeared to be non-competitive. The data in Table IV show great differences in the rate of inhibition by the methylxanthines between the two activities. This is due to the difference in the mode of inhibition; i.e. the degree of inhibition with 2′,3′-cyclic AMP as substrate is independent of the substrate concentration, although that with 3′,5′-cyclic AMP is dependent on the concentration. At a relatively high concentration (4 mM) of substrates used, the apparent inhibition is, therefore, greater in the hydrolysis of 2′,3′-cyclic AMP than in that of 3′,5′-cyclic AMP. The difference in the mode of inhibition by caffeine may suggest that the enzyme possesses two separate sites, with one involved in the hydrolysis of 2′,3′-cyclic nucleotide and the second involved in the hydrolysis of 3′,5′-cyclic nucleotide. The activity of a bis-(*p*-nitrophenyl)phosphate phosphodiesterase of an insect, European corn borer, has been reported to be inhibited by caffeine and theophylline [36]. It is interesting to

note that the methylxanthines inhibit the activities of insect phosphodiesterases which are probably a different type of enzymes from the 3′,5′-cyclic nucleotide phosphodiesterase of mammals.

The silkworm enzyme exhibits unusual substrate specificity, in that the enzyme shows no activity toward pyrimidine 3′,5′-cyclic nucleotides, although the enzyme hydrolyzes its 2′,3′-isomers at a significant rate. The unusual substrate specificity may be explained by the idea that the enzyme has two separate sites.

The 3′,5′-cyclic AMP phosphodiesterase activity of silkworm is greatly changed with its development [4]. At the present time, it is difficult to explain the developmental change of the activity and the biological significance of the enzyme, since there is not much data on the role of 3′,5′-cyclic nucleotide in the insect and since the K_m value for 3′,5′-cyclic AMP of the silkworm enzyme is very high ($1.3 \cdot 10^{-3}$ M) compared to many of mammalian enzymes (10^{-6}–10^{-5} M). Uda et al. [37] have described the isolation of 2′,3′-cyclic nucleotides from the larvae of *Drosophila melanogaster*, and they postulated the cyclic nucleotides as the degradation products of RNA. Although little is known about the biochemical pathway of RNA degradation in insects, the silkworm cyclic nucleotide phosphodiesterase may play an important role in the degradation of RNA.

ACKNOWLEDGEMENTS

This work was in part supported by a Scientific Research Foundation of the Ministry of Education of Japan. The author thanks Professor Dr T. Itoh and Dr S. Hirano for their encouragement in the course of this work.

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