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CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN SILKWORM

SEPARATION AND CHARACTERIZATION OF MULTIPLE FORMS OF THE ENZYME

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Summary

The existence of two forms of cyclic AMP phosphodiesterase (3′,5′-cyclic AMP 5′-nucleotidohydrolase, EC 3.1.4.17) was demonstrated in silkworm larvae by kinetic analysis and DEAE-cellulose column chromatography. The two forms of the enzyme (phosphodiesterase II and III) differ apparently in their characteristics from the previously reported cyclic nucleotide phosphodiesterase (phosphodiesterase I) of silkworm. The higher K_m form (phosphodiesterase II) has a molecular weight of approx. 50 000 and optimum pH of 7.8, and requires Mn^{2+} for maximum activity. The lower K_m form (phosphodiesterase III) has a molecular weight of approx. 97 000 and optimum pH of 7.2, and requires Mg^{2+} for maximum activity. Phosphodiesterase II and probably phosphodiesterase III are specific enzymes for the hydrolysis of cyclic AMP.

Introduction

The level of adenosine 3′,5′-monophosphate (cyclic AMP) in any tissue is controlled by the activities of adenyl cyclase and phosphodiesterase. In many mammalian tissues these enzymes have been studied intensively. In insects, however, our knowledge on these enzymes is very meager and little is known about the biochemical role of cyclic AMP, although cyclase [1—3] and phosphodiesterase [4—6] have been identified in some insects. In the previous paper [7], we described the purification and properties of cyclic nucleotide phosphodiesterase in silkworm. The enzyme hydrolyzes both 2′,3′- and 3′,5′-cyclic nucleotides and has an unusually high K_m value (1.3 mM) for 3′,5′-cyclic AMP. It seemed difficult to consider the phosphodiesterase as an enzyme regulating cyclic AMP concentration in vivo, in view of the kinetics and substrate specificity of the enzyme.

Cyclic AMP phosphodiesterase in a number of mammalian tissues exists in multiple forms differing in their K_m values (ref. 8), chromatographic and electrophoretic behavior [9–12] and stability [13]. Some of these enzymes have a sufficiently low K_m value to hydrolyze cyclic AMP at physiological concentrations. These observations led us to identify some other phosphodiesterase in silkworm having a low K_m value as in the case of mammalian tissues.

This paper presents the separation and characterization of two cyclic AMP phosphodiesterases in silkworm larvae. These two enzymes have distinct K_m values of 12 and 0.2 μM, respectively, and differ in their chromatographic behavior, molecular weight, metal requirement, optimum pH and inhibition by various nucleotides. These enzymes have apparently different characteristics from that of previously reported cyclic nucleotide phosphodiesterase [7].

Experimental Procedures

Materials

Silkworm (*Bombyx mori*) used for the experiments was reared on mulberry leaves at 25° C in our laboratory. Nucleotides were obtained from Kyowa Hakko Kogyo Co., Tokyo or Sigma Chemical Co., St. Louis; ^{3}H-labeled 3′,5′-cyclic AMP (7.7 Ci/mmol) and 3′,5′-cyclic GMP (10.2 Ci/mmol) from Daiichi Pure Chemicals Co., Tokyo; snake venom (*Crotalus atrox*), catalase (bovine liver), bovine serum albumin, ovalbumin, alcohol dehydrogenase (yeast), α-chymotrypsinogen and cytochrome *c* from Sigma Chemical Co.; Sephadex G-100 and G-200 from Pharmacia, Uppsala; DEAE-cellulose from Brown Co; aluminium oxide (neutral, activity grade I) from Merck, Darmstadt.

Methods

Preparation of crude enzyme extract. The third-instar larvae of silkworm were homogenized with 3 vol. (v/w) of 0.01 M Tris · HCl, pH 7.5, 5 mM β-mercaptoethanol buffer in a motor-driven Teflon homogenizer at 0° C. The homogenate was centrifuged at 10 000 × *g* for 30 min. The supernatant fluid containing most of the activity was used as the crude extract.

Assay of cyclic AMP phosphodiesterase. The assay of the enzyme activity was based on a modification of the method of Filburn and Karn [14]. The reaction mixture contained 50 mM Tris · HCl, pH 7.2 or 7.8, 5 mM β-mercaptoethanol, 5 mM $MnCl_2$ or 1 mM $MgCl_2$, 100 μg of crystalline bovine serum albumin, cyclic [^{3}H]AMP at concentrations ranging from 0.1 μM to 1 mM (0.15 μCi per reaction mixture) and enzyme in a total volume of 200 μl. Incubations were at 30° C for 15 min. The hydrolysis of substrate was kept at less than 25%. The reaction was terminated by placing the tubes in boiling water for 90 s. After chilling the tubes, 100 μg of snake venom was added in a volume of 20 μl and the incubation continued for 15 min at 30° C. The reaction was terminated by the addition of 50 μl of 0.8 M acetic acid. An aliquot (200 μl) of the mixture was applied to a column (0.7 × 4.2 cm) of aluminium oxide equilibrated with 0.1 M ammonium acetate, pH 4.0, and eluted with the same buffer. First, 0.7 ml of eluate was discarded and next, 1.5 ml of eluate which contained more than 99% of nucleosides applied to the column was collected in a scintillation vial and counted by dissolving in toluene-Triton X-100 scintilla-

tion fluid in a Packard Tri-Carb 3385 liquid scintillation spectrometer. Under the assay conditions described above, the release of [^{3}H] adenosine was proportional to the enzyme concentration until 40% of substrate was hydrolyzed, and the assays were linear for at least 45 min. The enzyme activity was expressed as nmol of cyclic AMP hydrolyzed per 15 min at 30° C. All experiments were carried out in duplicates or triplicates in which the variation in values was usually less than 5%.

Essentially the same assay procedure was used to measure the hydrolysis of cyclic GMP.

Results

Kinetics of cyclic AMP hydrolysis in crude extract

A characteristic Lineweaver-Burk plot of the cyclic AMP phosphodiesterase activity is shown in Fig. 1 as determined in the crude extract of silkworm larvae. The plot shows three distinct straight lines with different slopes in a substrate concentration range of 1—1000 μM. Extrapolation of three linear portions of the curve yielded three apparent K_m values of 10^{-6}, 10^{-5} and 10^{-3} M, respectively. The result suggests a possibility of the existence of three independent enzyme systems in silkworm extract with different K_m values. To confirm this possibility, an attempt was performed to separate these enzyme activities by column chromatography.

Separation of phosphodiesterases by column chromatography

The crude extract was chromatographed on a DEAE-cellulose column. Fig. 2 shows the elution profiles of phosphodiesterase activities when assayed at a low (0.2 μM) and a high (20 μM) cyclic AMP concentration. At a high substrate concentration, two large peaks of I and II were observed; at a low concentration one peak of III. These three forms of the activity in peaks I, II and III were designated phosphodiesterase I, II and III, respectively. Further

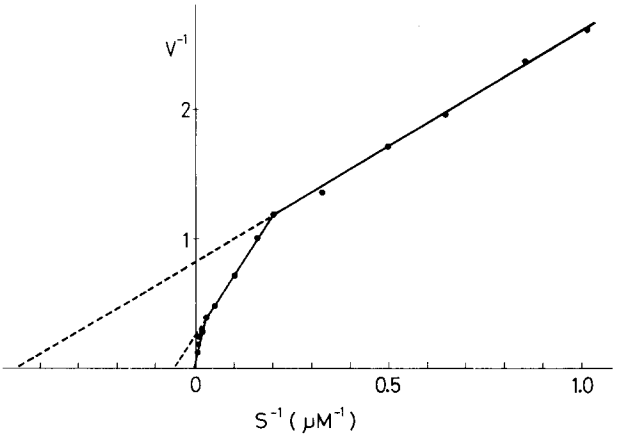

Fig. 1. Lineweaver-Burk plot of cyclic AMP hydrolysis by crude extract. The activity was measured as described in Methods at pH 7.8 with 5 mM $MgCl_2$ in a substrate concentration range of 1—1000 μM. V is expressed as an arbitrary unit.

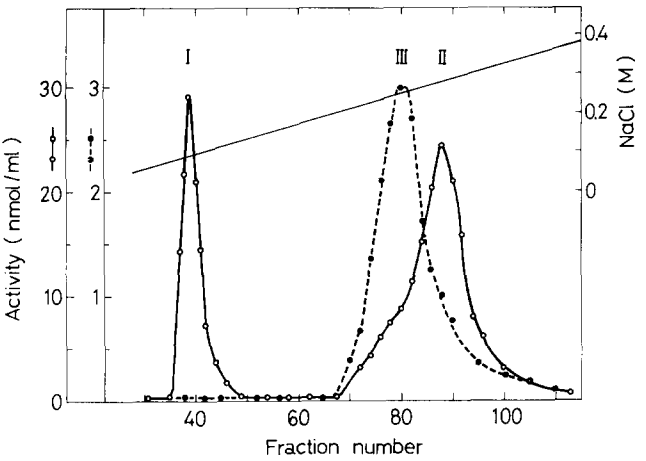

Fig. 2. DEAE-cellulose column chromatography of crude extract. The crude extract (60 ml) was applied to a column (2.9 × 15 cm) equilibrated with 0.01 M Tris · HCl, pH 7.5, 5 mM β-mercaptoethanol and washed in with 50 ml of the same buffer. Elution was carried out with a linear gradient formed with 300 ml each of 0.05 and 0.4 M NaCl in the same buffer. Fractions of 5 ml were collected at a flow rate of 34 ml/h. The activity was assayed at 20 μM cyclic AMP at pH 7.8 with 5 mM $MnCl_2$ (○———○) or at 0.2 μM cyclic AMP at pH 7.2 with 1 mM $MgCl_2$ (●- - - - -●), as described in Methods.

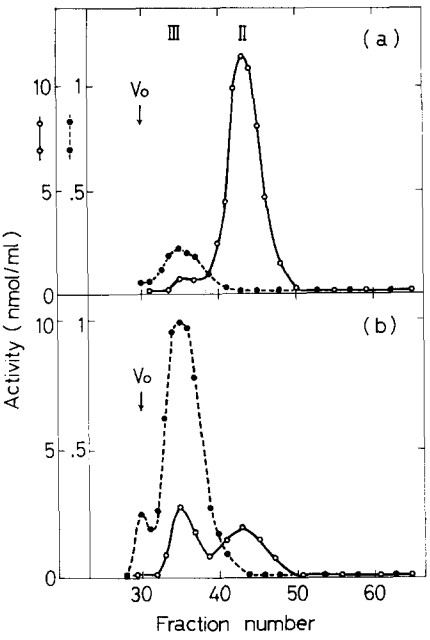

Fig. 3. Gel filtration through Sephadex G-100. The fractions of peak II and III from DEAE-cellulose were concentrated to approx. 1.5 ml, and applied to a column (1.7 × 95 cm) equilibrated with 0.01 M Tris · HCl, pH 7.5, 5 mM β-mercaptoethanol. Elution was carried out with the same buffer. Fractions of 2.7 ml were collected in tubes containing 0.1 ml of 25 mg/ml bovine serum albumin at a flow rate of 11 ml/h. The activity was assayed as described in the legend to Fig. 2. a, Phosphodiesterase II fraction; b, phosphodiesterase III fraction. ○———○, 20 μM cyclic AMP; ●- - - - -●, 0.2 μM cyclic AMP.

purification and characterization of phosphodiesterase I was described previously [7]. This enzyme has an unusually high K_m value of 1.3 mM for cyclic AMP and hydrolyzes both 2',3'- and 3',5'-cyclic nucleotides.

For the further separation and the characterization of phosphodiesterase II and III, fractions of peak II and III were concentrated to about 1.5 ml, respectively, by means of ultrafiltration and then gel filtrated through Sephadex G-100. As observed in the elution profiles shown in Figs 3a and 3b, the two phosphodiesterase activities were clearly separated from each other. In Fig. 3b, a small peak of low K_m enzyme activity was observed at the exclusion region. This exclusion peak was usually observed when the concentrated peak III fraction from DEAE-cellulose was gel filtrated. The peak may represent an aggregate of phosphodiesterase III. The peak fraction of phosphodiesterase II showed no activity for cyclic GMP when tested at a cyclic GMP concentration of 0.2 or 20 μM. On the other hand, the phosphodiesterase III fraction hydrolyzed cyclic GMP at a concentration of 0.2 μM at a rate of approx. one-fourth of that of cyclic AMP hydrolysis. The activity for cyclic GMP was, however, more labile than that for cyclic AMP, and the ratio of the two activities was significantly changed under storage at 4° C.

Conditions for optimal activity

Both phosphodiesterase II and III required bivalent cations for activity. The dependence of the activities on Mg^{2+} and Mn^{2+} is shown in Fig. 4. For phosphodiesterase II activity Mn^{2+} was more effective than Mg^{2+}, and the optimal concentration of Mn^{2+} was 3 mM. In contrast, both Mg^{2+} and Mn^{2+} were similarly effective for phosphodiesterase III activity, high concentrations of Mn^{2+}, however, were slightly inhibitory. The optimal concentration of Mg^{2+} was 1 mM. Therefore, 5 mM of Mn^{2+} was employed for the phosphodiesterase II assay and 1 mM of Mg^{2+} for the phosphodiesterase III assay routinely. Other

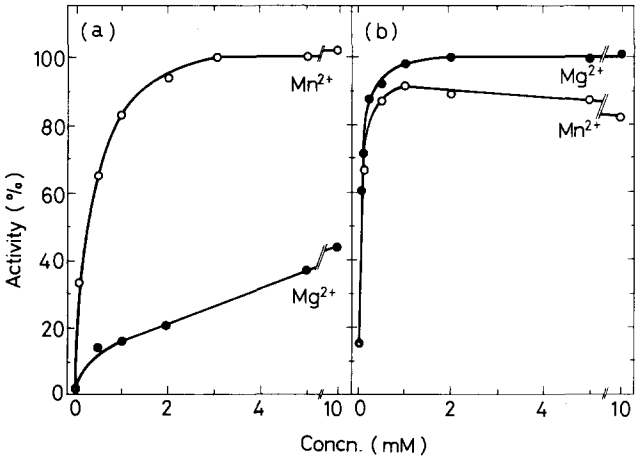

Fig. 4. Effect of Mg^{2+} and Mn^{2+} concentration on the activity of two phosphodiesterases. Phosphodiesterase II activity was assayed at 20 μM cyclic AMP at pH 7.8 (a), and phosphodiesterase III activity at 0.2 μM cyclic AMP at pH 7.2 (b). $MgCl_2$ was added to a final concentration of 10 mM for the nucleotidase reaction.

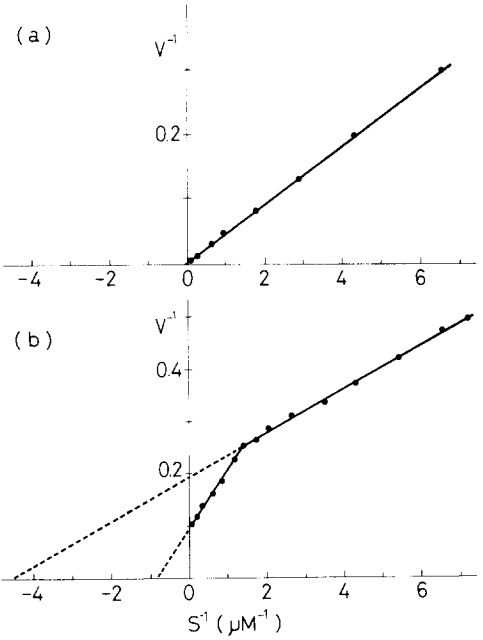

Fig. 5. Lineweaver-Burk plot of cyclic AMP hydrolysis by purified phosphodiesterases. Phosphodiesterase II activity was assayed at pH 7.8 with 5 mM $MnCl_2$ in a cyclic AMP concentration range of 0.1—200 μM (a), and phosphodiesterase III activity at pH 7.2 with 1 mM $MgCl_2$ in the same substrate range as above (b). *V* is expressed as an arbitrary unit.

bivalent cations tested such as Ca^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+} were not effective. EDTA at 10 mM completely inhibited the activities. The pH optima of phosphodiesterase II and III were pH 7.8 and 7.2, respectively, when Tris/acetate was used as buffer. To stabilize the enzymic activities, 5 mM of β-mercaptoethanol and 100 μg/200 μl of bovine serum albumin were routinely included in the assays.

Kinetics of cyclic AMP hydrolysis by purified enzymes

The Lineweaver-Burk plot of the purified phosphodiesterase II activity (Fig. 5a) shows a straight line yielding a K_m value of 12 μM in a substrate concentration range of 0.1—200 μM. The plot of phosphodiesterase III activity (Fig. 5b), however, shows a biphasic kinetic behavior in the same substrate concentration range as above. At cyclic AMP concentrations below 0.7 μM extrapolation of the linear portion of the curve yielded a K_m value of 0.22 μM. At substrate concentrations above 1 μM an apparent K_m value was approx. 1 μM.

Effect of various nucleotides and methylxanthines

Table I shows the effect of various nucleotides, caffeine and theophylline on the hydrolysis of cyclic AMP by phosphodiesterase II and III at substrate concentration of 20 and 0.2 μM, respectively. Cyclic GMP in concentrations up to 100 μM did not affect the phosphodiesterase II activity. Only at a higher concentration of cyclic GMP (1 mM) was a slight inhibition observed. In contrast, the phosphodiesterase III activity was inhibited by cyclic GMP at concen-

TABLE I

EFFECT OF VARIOUS NUCLEOTIDES AND METHYLXANTHINES ON THE ACTIVITY OF TWO PHOSPHODIESTERASES

The cyclic AMP concentration was 20 μM for the phosphodiesterase II assay or 0.2 μM for the III assay. A value of 100 was assigned to the activity in the absence of any additions.

Compounds	Concentration (mM)	Activity (%) Phosphodiesterase II	Phosphodiesterase III	Compounds	Concentration (mM)	Activity (%) Phosphodiesterase II	Phosphodiesterase III
None		100	100	2′,3′-cyclic AMP	1	57	39
3′,5′-Deoxy cyclic AMP	0.0002		84	2′,3′-cyclic GMP	1	100	28
	0.02	71	17	2′,3′-cyclic UMP	1	100	81
	0.1	38	3	2′,3′-cyclic CMP	1	99	93
Dibutyl 3′,5′-cyclic AMP				5′-AMP	1	81	53
	0.1	95	35	5′-GMP	1	99	59
	1	41	4	5′-IMP	1	100	78
3′,5′-cyclic GMP	0.001	100	85	5′-UMP	1	100	100
	0.01	100	74	5′-CMP	1	100	100
	0.1	100	63	ATP	1	87	59
	1	92	33	Caffeine	0.2	95	63
3′,5′-cyclic IMP	0.1	100	24		1	71	43
	1	95	7	Theophylline	0.2	82	52
3′,5′-cyclic UMP	1	97	69		1	55	25
3′,5′-cyclic CMP	1	99	87				

trations of above 1 μM, although the rate of inhibition was considered to be relatively small comparing the substrate concentration (0.2 μM) with that of the inhibitor. 3′,5′-Deoxy cyclic AMP was the strongest inhibitor for both phosphodiesterases. Dibutyryl 3′,5′-cyclic AMP also inhibited both activities. 3′,5′-Cyclic IMP at 1 mM, however, showed only slight effect on phosphodiesterase II activity in contrast to a great inhibition on phosphodiesterase III activity. 3′,5′-Cyclic CMP and UMP also showed no effect on phosphodiesterase II activity, and the inhibition of phosphodiesterase III activity by these pyrimidine nucleotides was extremely weak compared to that by the purine nucleotides. Similar effects were observed with 2′,3′-cyclic nucleotides and 5′-nucleotides. Thus, the activity of phosphodiesterase II was inhibited only by adenine nucleotides but not by guanine, inosine or pyrimidine nucleotides. The activity of phosphodiesterase III was, in contrast, inhibited significantly by all of the purine nucleotides tested. The pyrimidine nucleotides were only slightly effective. Caffeine and theophylline were also potent inhibitors for the activity of phosphodiesterase II and III as described for many mammalian enzymes [8].

Molecular weight determination

Molecular weights of phosphodiesterase II and III were determined to be approx. 50 000 and 97 000, respectively, by gel filtration through a Sephadex G-200 column (1.7 × 84 cm) equilibrated with 0.01 M Tris · HCl, pH 7.5, 5 mM β-mercaptoethanol containing 0.1 M NaCl, using cytochrome *c*, α-chymotrypsinogen, ovalbumin, bovine serum albumin, alcohol dehydrogenase and

catalase as standard. These values were relatively small compared to the values of 200 000—400 000 reported for mammalian enzymes [10,15].

Discussion

Kinetic studies on the cyclic AMP hydrolyzing activity of silkworm larval extract demonstrated the presence of three apparent K_m values. Chromatographic studies on the extract demonstrate, furthermore, the separation of three forms of phosphodiesterase activity having distinct K_m values for cyclic AMP, which correspond to the apparent K_m values observed with the extract. The characterization of the activity of three forms of phosphodiesterase (I [7], II and III) represents remarkable differences in their properties; i.e. K_m value, optimum pH, dependence on bivalent cations, inhibition by various nucleotides and molecular weight. These results strongly suggest the presence of three separate enzymes in the silkworm extract which hydrolyze cyclic AMP.

As described in the previous paper [7], phosphodiesterase I is a strange enzyme which hydrolyzes 2′,3′-cyclic nucleotides at a rate 10 times greater than that for 3′,5′-cyclic nucleotide hydrolysis. In view of the substrate specificity and unusually high K_m value (1.3 mM) for cyclic AMP, it is unlikely that the enzyme catalyzes the hydrolysis of cyclic AMP in vivo. The other two enzymes, phosphodiesterase II and III, have sufficiently low K_m values comparable to many of mammalian enzymes (ref. 8). A Lineweaver-Burk plot of purified phosphodiesterase II activity shows a straight line yielding a K_m value of 12 μM. The plot of purified phosphodiesterase III activity, however, shows a biphasic kinetic behavior with two apparent K_m values of 0.22 and 1 μM. Principally this phenomenon can be explained by the presence of separate enzymes having distinct K_m values or by a negatively cooperative enzyme as described by Conway and Koshland [16]. In the kinetic experiment in Fig. 5b, the activity was assayed in the presence of 1 mM $MgCl_2$ at pH 7.2. Under these conditions phosphodiesterase II shows only 12% of its maximum activity, while phosphodiesterase III shows its full activity. For the biphasic kinetic plot of phosphodiesterase III to be due to contamination by phosphodiesterase II activity, the phosphodiesterase III fraction would have to contain a great amount of phosphodiesterase II. This is unlikely as judged by the elution pattern in Fig. 3, which shows almost complete separation of the two peaks, the negative cooperative model would therefore seem to be favored. Negative cooperativity of cyclic AMP phosphodiesterase has been recently demonstrated in the case of low K_m enzyme of rat tissues [10,17].

The purified phosphodiesterase II fraction from Sephadex G-100 shows no detectable activity for cyclic GMP, and the activity for cyclic AMP is little or not affected by the addition of a variety of nucleotides other than adenine nucleotides (Table I). These results strongly suggest that the enzyme is very specific for the hydrolysis of cyclic AMP. On the other hand, the purified phosphodiesterase III fraction still contains some detectable activity for cyclic GMP with a K_m value of approx. 1 μM (data not shown). The activity for cyclic GMP is, however, more labile than that for cyclic AMP, and the ratio of the activities for cyclic AMP and cyclic GMP is significantly changed under storage. The result suggests the possibility of the presence of two separate

enzymes in this preparation which catalyze the hydrolysis of cyclic AMP and cyclic GMP independently. This possibility is supported by the fact that inhibition of the activity of cyclic AMP by cyclic GMP is too small to assume that a single enzyme hydrolyzes both nucleotides. Separation and characterization of cyclic GMP hydrolyzing activity are now attempted in our laboratory.

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