Biochimica et Biophysica Acta, 410 (1975) 310—317

BBA 67666

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN SILKWORM

CHARACTERIZATION OF CYCLIC GMP PHOSPHODIESTERASE

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(Received June 9th, 1975)

Summary

The existence of cyclic GMP phosphodiesterase (EC 3.1.4.-) was demonstrated in silkworm larva by gel filtration of the homogenate. The cyclic GMP phosphodiesterase was separated from cyclic AMP phosphodiesterases by column chromatography on hydroxyapatite and Sephadex G-200. The enzyme has a molecular weight of approx. 260 000, an optimum pH of 8.3 and a *Km* value of 2 μM. The enzyme is activated by 5 mM of Mg^{2+} and 2 mM of Mn^{2+}. The cyclic GMP phosphodiesterase activity was greatly inhibited by low concentrations of cyclic IMP but to a lesser extent by cyclic AMP even at a high concentration. The activity was also inhibited by caffeine and theophylline.

Introduction

Cyclic AMP has been suggested to be implicated in the action of insect hormones [1—5], and adenyl cyclase and cyclic AMP phosphodiesterase have been identified in insects [6—12] as in mammals. There is, however, so far no information available in insects on the function of another cyclic nucleotide, cyclic GMP, and on the enzymes concerning its synthesis and degradation, though a relatively higher level of cyclic GMP has been detected in insect tissues [13,14] compared to that in mammalian tissues.

In the previous papers [15,16], we described multiple forms of cyclic nucleotide phosphodiesterase in silkworm; i.e., phosphodiesterase I which is a non-specific enzyme hydrolyzing both 3′,5′-cyclic nucleotides and 2′,3′-cyclic

Abbreviations: cyclic AMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; Tris-mercaptoethanol buffer, 0.01 M Tris · HCl, pH 7.5, containing 5 mM β-mercaptoethanol.

nucleotides, and two cyclic AMP specific enzymes, phosphodiesterase II and III which are different from each other in their molecular weights, *Km* values, optimum pH values and requirements for metal ions. These enzymes can be separated from each other by column chromatography. In addition to these three phosphodiesterases, the possible existence of a cyclic GMP phosphodiesterase was also indicated [16], and we recently described developmental changes of this activity [17]. This paper represents the separation of cyclic GMP phosphodiesterase (EC 3.1.4.-) from cyclic AMP phosphodiesterases and the characterization of the enzyme.

Experimental procedures

Materials

Silkworms (*Bombyx mori*) used for the experiments were reared on mulberry leaves at 25° C. Hydroxyapatite was prepared as described by Siegelman et al. [18]. Other chemicals and reagents were as described previously [16].

Methods

Preparation of crude enzyme extract. The third-instar larvae of silkworm were homogenized with 3 vol. (v/w) of Tris-mercaptoethanol buffer in a motor-driven Teflon homogenizer at 0° C. The homogenate was centrifuged at 12 000 × *g* for 30 min, and the supernatant fluid was used as the crude extract.

Assay of phosphodiesterase. The two step assay for enzymatic activity was similar to that previously described [16,17]: 5′-[^{3}H] AMP or GMP formed by the phosphodiesterase was converted to corresponding [^{3}H] nucleoside by the action of nucleotidase, unreacted substrate was removed by passage through a small column of aluminium oxide at pH 4.0 [19], and the [^{3}H] nucleoside was counted by liquid scintillation. The reaction medium used for the assay of cyclic GMP phosphodiesterase activity was as follows: 50 mM Tris · HCl, pH 8.3, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 100 μg of crystalline bovine serum albumin, 1.0 μM cyclic [^{3}H] GMP and enzyme in a total volume of 200 μl. The medium for the assay of cyclic AMP phosphodiesterase activity was as previously described [16,17]. The enzyme activity was expressed as pmol or nmol cyclic nucleotide hydrolyzed per 15 min at 30° C. All experiments were carried out in duplicate in which the variation in values was usually less than 5%.

Results

Gel filtration of the crude extract on Sephadex G-200

When the crude extract was gel filtrated through Sephadex G-200 in Tris-mercaptoethanol buffer, cyclic GMP phosphodiesterase activity was found in two peaks; a major peak with a molecular weight of approx. 260 000 and a minor peak with a molecular weight of approx. 70 000 (Fig. 1a). The activity in the latter peak was not inhibited by the addition of 10 mM EDTA and showed very low affinity for the substrate. As judged by the characteristics and the molecular weight, the latter peak was considered to be phosphodiesterase I [15]. Addition of 0.4 M NaCl to the buffer employed during gel filtration resulted in a similar profile of cyclic GMP phosphodiesterase activity except the

appearance of a smaller peak at the exclusion volume (Fig. 1b). Cyclic AMP phosphodiesterase III activity assayed at 0.2 μM substrate was found, without NaCl, in a sharp peak at the exclusion volume and in a broad area corresponding to molecular weights of approx. 100 000 to 300 000 (Fig. 1a). Addition of NaCl to the eluting buffer resulted in a different profile of cyclic AMP phosphodiesterase III activity in contrast to that of cyclic GMP phosphodiesterase. Thus, the addition of 0.4 M NaCl led to the near disappearance of the peaks with molecular weight higher than 300 000 and the appearance of a single peak with a molecular weight of approx. 100 000 (Fig. 1b).

The distribution of cyclic AMP phosphodiesterase II activity was also determined at 20 μM substrate. The phosphodiesterase II activity was found in a single peak with a shoulder attributed to phosphodiesterase I; this was not

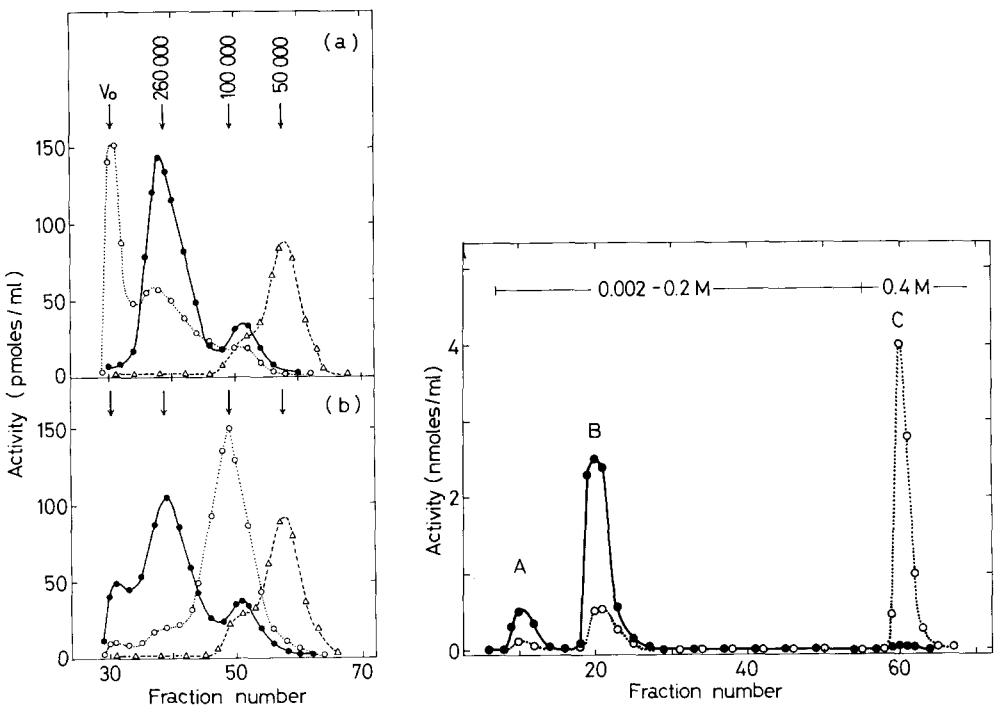


Fig. 1. Gel filtration of crude extract through Sephadex G-200. Two milliliters of the crude extract were applied to a column (1.7 X 100 cm) equilibrated with Tris-mercaptoethanol buffer (a) or the buffer containing 0.4 M NaCl (b). The columns were calibrated with catalase, alcohol dehydrogenase, bovine serum albumin, ovalbumin, α-chymotrypsinogen and cytochrome *c*. Fractions of 2.8 ml were collected at a flow rate of 13 ml/h. Cyclic GMP phosphodiesterase activity (●———●) was assayed as described in Methods; phosphodiesterase II activity (△- - - - -△, X1/50) was assayed at 20 μM cyclic AMP at pH 7.8 with 5 mM $MnCl_2$; phosphodiesterase III activity (○· · · · ·○) at 0.2 μM cyclic AMP at pH 7.2 with 2 mM $MgCl_2$.

Fig. 2. Hydroxyapatite column chromatography of crude extract. The crude extract (40 ml) was applied to a column (2.7 X 15 cm) equilibrated with 2 mM potassium phosphate, pH 7.0. Elution was carried out with a linear gradient formed with 150 ml each of 2 mM and 0.2 M potassium phosphate, pH 7.0, containing 5 mM β-mercaptoethanol and then with 0.4 M potassium phosphate, pH 7.0, containing 5 mM β-mercaptoethanol. Fractions of 6 ml were collected at a flow rate of 34 ml/h. The activities were assayed as described in the legend to Fig. 1. ●———●, cyclic GMP phosphodiesterase activity; ○· · · · ·○, cyclic AMP phosphodiesterase III activity.

affected by the addition of NaCl (Figs 1a and 1b).

The results indicated the existence of a phosphodiesterase specific for the hydrolysis of cyclic GMP in silkworm extract. To characterize the activity, an attempt was made to separate the cyclic GMP phosphodiesterase from cyclic AMP phosphodiesterases.

Separation of cyclic nucleotide phosphodiesterases by column chromatography

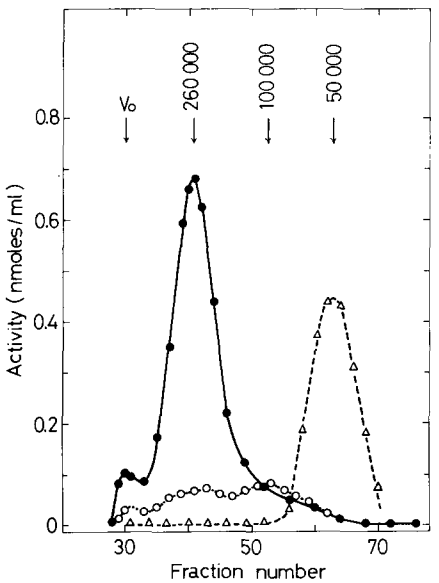
The crude extract of silkworm larva was chromatographed on hydroxyapatite. Three peaks of cyclic nucleotide phosphodiesterase activity designated A, B and C in Fig. 2 were observed when 1 μM cyclic GMP or 0.2 μM cyclic AMP was used as substrate. Peak C was quite specific for cyclic AMP hydrolysis and approx. 51% of the activity in the original crude extract was recovered in this peak. The characterization of the activity showed that the enzyme was phosphodiesterase III. Peak A hydrolyzing both cyclic AMP and cyclic GMP showed very low affinity for the both substrates, and the activity was not affected by 10 mM EDTA. The enzyme was, therefore, considered to be phosphodiesterase I. A large portion of cyclic GMP phosphodiesterase activity (approx. 53% of the activity in the crude extract) appeared in peak B. The peak also contains a minor portion of cyclic AMP phosphodiesterase III activity (Fig. 2) and a large portion of cyclic AMP phosphodiesterase II activity (not shown).

For the further separation of these activities, the peak B fraction was concentrated to approx. 3 ml by means of ultrafiltration, and an aliquot was gel filtrated through Sephadex G-200 in Tris-mercaptoethanol buffer containing 0.4 M NaCl. As shown in Fig. 3, phosphodiesterase II activity was clearly separated from cyclic GMP phosphodiesterase activity. A smaller portion of cyclic AMP phosphodiesterase activity assayed at 0.2 μM substrate was, however, still found in the cyclic GMP phosphodiesterase fraction.

The activity for cyclic AMP could not be separated from that for cyclic GMP by the rechromatography on a hydroxyapatite column. However, the properties such as optimum pH, requirement for metal ions and kinetics of hydrolysis of the cyclic AMP phosphodiesterase activity were the same as that described for phosphodiesterase III [16], but were distinct from that of the cyclic GMP phosphodiesterase. Fractions showing high cyclic GMP phosphodiesterase activity were pooled, concentrated, and stored at 0° C for further use.

Conditions for optimal activity

Cyclic GMP phosphodiesterase required bivalent cations for activity. The dependence of the purified enzyme activity on Mg^{2+} and Mn^{2+} is shown in Fig. 4. At lower concentrations, Mn^{2+} was more effective than Mg^{2+}, however higher concentrations of Mn^{2+} were inhibitory, but Mg^{2+} was not. The optimal concentration of Mn^{2+} was 2 mM and that of Mg^{2+} was 5 mM. Therefore, 5 mM of Mg^{2+} was employed for the assay routinely. Other bivalent cations tested such as Ca^{2+}, Cu^{2+}, Zn^{2+} and Co^{2+} at 1 mM were not effective. EDTA at 10 mM completely inhibited the activity. The pH optima was pH 8.3 when Tris-acetate was used as buffer. To stabilize the enzyme activity, 5 mM of β-mercaptoethanol and 100 μg per tube of bovine serum albumin were routinely included in the assays.



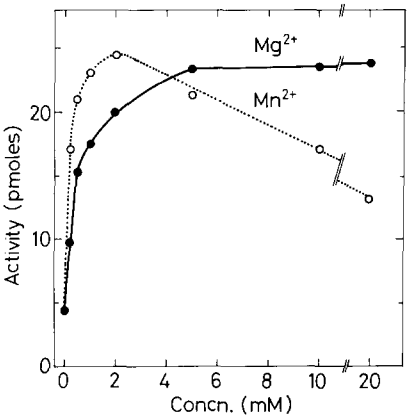


Fig. 3. Gel filtration of hydroxyapatite fraction through Sephadex G-200. The peak B fraction from hydroxyapatite was concentrated and an aliquot (1.5 ml) was applied to the column (1.7 × 100 cm) equilibrated with Tris-mercaptoethanol buffer containing 0.4 M NaCl as described in the legend to Fig. 1. Fractions of 2.5 ml were collected in tubes containing 0.1 ml of 25 mg/ml bovine serum albumin at a flow rate of 13 ml/h. Activities were assayed as described in the legend to Fig. 1. ●———●, cyclic GMP phosphodiesterase activity; △- - - - -△, cyclic AMP phosphodiesterase II activity ×1/30; ○· · · · ·○, cyclic AMP phosphodiesterase III activity.

Fig. 4. Effect of Mg^{2+} and Mn^{2+} concentration on cyclic GMP phosphodiesterase activity. The activity was assayed as described in Methods except the varying concentration of Mg^{2+} or Mn^{2+}.

Kinetics of cyclic GMP hydrolysis

The Lineweaver-Burk plot of the purified cyclic GMP phosphodiesterase activity (Fig. 5) showed a straight line yielding a *K*m value of 2 μM in a substrate concentration range of 0.25—10 μM.

Effect of various nucleotides and methyxanthines

Table I showed the effect of various nucleotides, caffeine and theophylline on the activity of cyclic GMP phosphodiesterase. 3′,5′-Cyclic IMP was the strongest inhibitor among the nucleotides tested; at 0.01 mM of the inhibitor, about 64% of the activity was inhibited. In contrast, 3′,5′-cyclic AMP at 0.1 mM, a concentration 100-times higher than that of the substrate, showed only 20% of inhibition, and at 1 mM about 50% of inhibition. Dibutyl 3′,5′-cyclic AMP, a strong inhibitor for phosphodiesterase II and III [16], showed similar inhibition of the activity as that by 3′,5′-cyclic AMP. At 1 mM 2′,3′-cyclic GMP was a strong inhibitor, but 2′,3′-cyclic AMP had only a slight effect. 5′-Nucleotides had no effect on the activity, though ATP and GTP at 1 mM showed a slight inhibition. The cyclic GMP phosphodiesterase activity was also inhibited by caffeine and theophylline as described for many of cyclic AMP phosphodiesterases. However, the inhibition of the activity by caffeine was slightly greater than that by theophylline, in contrast to the greater inhibition by

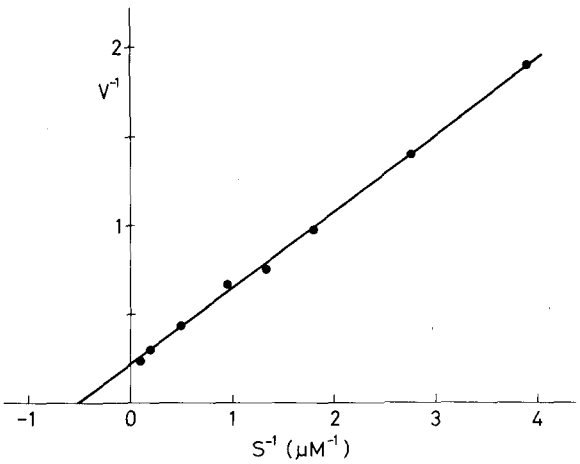


Fig. 5. Lineweaver-Burk plot of cyclic GMP hydrolysis. The activity was assayed as described in Methods except the varying substrate concentrations. *V* is expressed as an arbitrary unit.

theophylline for the activity of cyclic AMP phosphodiesterases of silkworm [15,16] and mammalian tissues [20,21]. Similar results were described for the cyclic GMP phosphodiesterase of mouse epidermis [22].

Discussion

Gel filtration of the silkworm homogenate shows that the elution profile of phosphodiesterase III activity is greatly altered depending on the presence or absence of 0.4 M NaCl, i.e., in the absence of NaCl most of the activity is eluted in an area corresponding to molecular weights greater than 200 000, but

TABLE I

EFFECT OF VARIOUS NUCLEOTIDES AND METHYLXANTHINES ON THE CYCLIC GMP PHOSPHODIEASTERASE ACTIVITY

The reaction conditions were as described in Methods except that the reaction medium contained the compounds to be tested at the indicated concentration. A value of 100 was assigned to the activity in the absence of any addition.

Compounds	Concentration (mM)	Activity (%)	Compounds	Concentration (mM)	Activity (%)
None		100			
3',5'-Cyclic AMP	0.1	80	5'-AMP	1	94
	1	54	5'-GMP	1	94
Dibutyl 3',5'-cyclic AMP	1	53	5'-IMP	1	96
3',5'-Cyclic IMP	0.01	36	ATP	1	78
	0.1	17	GTP	1	81
3',5'-Cyclic UMP	1	96	Caffeine	0.2	78
2',3'-Cyclic AMP	1	74		1	47
2',3'-Cyclic GMP	1	27	Theophylline	0.2	84
				1	56

in the presence of NaCl it is eluted in a single peak corresponding to a molecular weight of approx. 100 000 (Fig. 1). When the peak fraction with a molecular weight of 100 000 eluted in the presence of NaCl is concentrated and reapplied to Sephadex G-200 in the absence of NaCl, the activity is also eluted in a single peak with a molecular weight of approx. 100 000 (data not shown). Furthermore, when the phosphodiesterase III fraction eluted from DEAE-cellulose with NaCl is gel filtrated with a buffer not containing NaCl, the activity is also eluted in the same region as above corresponding to a molecular weight of approx. 100 000 [16]. The results indicate the 100 000 molecular weight form and higher molecular weight forms of phosphodiesterase III are not interconvertible. The observations are not in accord with the interconvertibility of cyclic AMP phosphodiesterase between high and low molecular weight forms reported for bovine liver enzyme [23]. The phosphodiesterase III may be present in homogenate as a complex formed with the enzyme protein molecule(s) of molecular weight of 100 000 and some other molecule(s). If it is true, once the complex is dissociated under high ionic strength and the components are separated by chromatography from each other, the 100 000 molecular weight component alone could not reform the complex of higher molecular weight.

Cyclic GMP phosphodiesterase is almost completely separated from phosphodiesterase I, II and III by column chromatography on hydroxyapatite and gel filtration. The peaks of phosphodiesterase II and III have no activity for cyclic GMP (Figs 2 and 3), thus there appear to be very specific enzymes for the hydrolysis of cyclic AMP. The partially purified preparation of cyclic GMP phosphodiesterase still contains a small activity for cyclic AMP. However, the properties of cyclic AMP hydrolyzing activity are distinct from that for cyclic GMP phosphodiesterase, but are the same as that for phosphodiesterase III. Furthermore, the inhibition of cyclic GMP phosphodiesterase activity by cyclic AMP is seemed to be too small to assume that one molecule has two discrete binding sites for the two cyclic nucleotides (Table I). The results suggest a possibility that the cyclic GMP phosphodiesterase is very specific for the hydrolysis of cyclic GMP and the cyclic AMP hydrolyzing activity remained in the cyclic GMP phosphodiesterase preparation is due to phosphodiesterase III which would be aggregated to cyclic GMP phosphodiesterase in an unknown manner. The aggregation of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase is proposed in the case of mouse liver [23].

Acknowledgements

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

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