Guanosine Cyclic Monophosphate-Dependent Protein Kinase from Foetal Calf Heart PURIFICATION, GENERAL PROPERTIES AND CATALYTIC SUBUNIT

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Cyclic GMP-dependent protein kinase was purified from foetal calf hearts, and its general properties and subunit structure were studied. The enzyme was purified over 900-fold from the heart extract by pH 5.3-isoelectric precipitation, DEAE-cellulose chromatography, Sephadex G-200 filtration and hydroxyapatite treatment. The purified myocardial enzyme, free from cyclic AMP-dependent protein kinase contamination, exhibited an absolute requirement of stimulatory modulator (or crude modulator containing the stimulatory modulator component) for its cyclic GMP-stimulated activity. Inhibitory modulator (protein inhibitor) of cyclic AMP-dependent protein kinase could not stimulate nor inhibit the cyclic GMP target enzyme. The enzyme had K_s values of 0.013, 0.033 and $3.0\ \mu\text{M}$ for 8-bromo cyclic GMP, cyclic GMP and cyclic AMP respectively. The cyclic GMP-dependent enzyme required Mg^{2+} and Co^{2+} for its activity, with optimal concentrations of about 30 and 0.5 mM respectively. The pH optimum for the enzyme activity ranged from 6 to 9. Histones were generally effective substrate proteins. The enzyme exhibited a greater affinity for histones than did the cyclic AMP-dependent class of protein kinase. The holoenzyme (apparent mol.wt. 150000) of the myocardial cyclic GMP-dependent protein kinase was dissociated into a cyclic GMP-independent catalytic subunit (apparent mol.wt. 60000) by cyclic GMP and histone. The catalytic subunit required the stimulatory modulator for its activity, as in the case of the holoenzyme in the presence of cyclic GMP.

We have reported the involvements of cyclic GMP-dependent protein kinase in certain physiological and pathological processes of the mammalian heart. The concentrations of the enzyme in the heart, as in the lung, of guinea pigs were found to be the highest in the foetus and the lowest in the adult, with intermediate values seen in the newborn (Kuo, 1975). The concentrations of cyclic AMP-dependent protein kinase in both the heart and lung, on the other hand, are conversely the lowest in the foetus and the highest in the adult. The enlarged heart from the spontaneously hypertensive rat was found to contain decreased concentrations of the cyclic GMP-dependent enzyme, which are only about 20–40% of those seen in the heart from the normotensive control rat (J. F. Kuo *et al.*, 1976a). The concentrations of the cyclic AMP-dependent enzyme, in comparison, are unaffected by cardiac hypertrophy.

In view of the potential importance of cyclic GMP in function and metabolism of many tissues (Goldberg *et al.*, 1975), including the heart, and the ubiquitous occurrence of cyclic GMP-dependent protein kinase in mammalian tissues (Kuo, 1974), detailed studies of this cyclic GMP target enzyme from the heart seem to be warranted. The present paper deals with the purification, characterization and subunit structure of the enzyme from the foetal calf heart.

Experimental

Materials

[γ - ^{32}P]ATP was from New England Nuclear, Boston, MA, U.S.A. Various cyclic nucleotides and their analogues were from either ICN (Irvine, CA, U.S.A.), Sigma (St. Louis, MO, U.S.A.), Boehringer (Mannheim) Corp. (New York, NY, U.S.A.) or Plenum Scientific Research (Hackensack, NJ, U.S.A.). The sources of various substrate proteins were indicated in Table 4. Sephadex G-200 was from Pharmacia (Piscataway, NJ, U.S.A.); DEAE-cellulose was from Sigma; hydroxyapatite (Bio-Gel HTP) and 2-mercaptoethanol were from Bio-Rad (Richmond, CA, U.S.A.). Frozen foetal calf hearts were from Pel-Freez Biologicals (Rogers, AR, U.S.A.). Standard proteins used for molecular-weight determinations by means of Sephadex G-200 gel filtration (Fig. 7) were from Sigma.

Methods

Purification of cyclic GMP-dependent protein kinase from foetal calf hearts. The procedures are essentially the same as described earlier for the enzyme from foetal guinea-pig lungs (J. F. Kuo *et al.*, 1976b), with a change in the order of the steps used. Eight foetal calf hearts (1150 g), cut into small pieces, were

homogenized for 2 min in 2 vol. of ice-cold 50 mM-potassium phosphate buffer, pH 7.0, with a Waring commercial blender. All buffers used contained 3 mM-mercaptoethanol and all purification procedures were performed at 0–4°C. Crude tissue extract was obtained by centrifuging the homogenate for 40 min at 13 000g. The pH of the extract was then adjusted to 5.3 by slow addition of 0.2 M-acetic acid. The precipitate, which contained most of the cyclic GMP-dependent enzyme activity, was recovered by centrifugation. The supernatant fluid, which contained the cyclic AMP-dependent enzyme activity, was stored at –20°C until further purification.

The pH 5.3 precipitate was dissolved in 80 ml of 50 mM-potassium phosphate buffer, pH 7.0, and was charged onto a DEAE-cellulose column (2 cm × 36 cm) which was previously washed with 1 M-potassium phosphate buffer, pH 7.0, and subsequently equilibrated with 50 mM-phosphate buffer, pH 7.0. After the column was washed with 150 ml of 50 mM-phosphate buffer, pH 7.0, a linear gradient of phosphate buffer, pH 7.0, from 50 to 150 mM (500 ml total volume), was used for the chromatography. The fraction size was 5.5 ml. The fractions from the peak containing the enzyme activity, which appeared at about 110 mM-phosphate buffer, were concentrated to 5 ml by the use of Amicon PM 30 membrane; then the concentrate was loaded onto a Sephadex G-200 column (4 cm × 40 cm), which had been equilibrated with 50 mM-phosphate buffer, pH 7.0. The same buffer was also used for elution. The fraction size was 2.7 ml. The fractions containing the enzyme were pooled (50 ml), and 2 ml of the hydroxyapatite-gel suspension (containing 300 mg of dry matter per ml) was added. The mixture was gently mixed for 5 min in ice. The gel, which quantitatively absorbed the enzyme, was collected by brief centrifugation. The gel was first washed twice with 20 ml portions of 100 mM-potassium phosphate buffer, pH 7.0, by suspending and stirring the gel in the buffer; practically no cyclic GMP-dependent protein kinase activity was detected in the washing solution obtained after brief centrifugation of the mixture. Next, the enzyme was eluted twice from the gel by the use of 17 ml portions of 300 mM-potassium phosphate buffer, pH 7.0. The two eluates, which were obtained after removal of the gel by centrifugation, were combined and were dialysed overnight against 2 liters of 50 mM-potassium phosphate buffer, pH 7.0, with one change of the buffer. The enzyme preparations from either the Sephadex or hydroxyapatite step were found to be suitable for studying the general properties of the enzyme.

Assay for protein kinase activity. The standard assay system (Kuo, 1975; J. F. Kuo *et al.*, 1976b) contained, in a final volume of 0.2 ml; 10 μ M of potassium phosphate buffer, pH 7.0; 0.5 μ M of theophylline; 40 μ g of arginine-rich histone (HA;

Worthington, Freehold, NJ, U.S.A.); 2 μ M of MgCl_2 ; 1 nmol of [γ - ^{32}P]ATP containing 1.1×10^6 – 2.1×10^6 c.p.m.; either crude protein kinase modulator (45–60 μ g protein), the isolated stimulatory modulator (30–45 μ g of protein) or inhibitory modulator (1–6 μ g); 80 pmol of either cyclic GMP or cyclic AMP; appropriate amounts of protein kinase as indicated. A unit of enzyme activity is defined as the amount that transferred 1 pmol of ^{32}P from [γ - ^{32}P]ATP to recovered histone (or other substrate proteins) in 10 min at 30°C under the assay conditions. All experiments were performed in duplicate or triplicate incubations, and the mean values are presented.

Other methods. Myocardial cyclic AMP-dependent protein kinase from the supernatant fluid obtained after isoelectric precipitation of the cyclic GMP-dependent enzyme was purified to the DEAE-cellulose-chromatography step, as reported earlier (Kuo & Greengard, 1969). Crude protein kinase modulator, stimulatory modulator and inhibitory modulator were prepared from the rat liver or bovine heart by previously described procedures (W.-N. Kuo *et al.*, 1976a,b; Kuo & Kuo, 1976). Conditions for dissociation of cyclic GMP-dependent protein kinase are indicated in the legend to Fig. 7. Measurement of apparent molecular weights of the holoenzyme and catalytic subunit by Sephadex G-200 filtration was performed by the method of Leach & O'Shea (1965) by using several globular proteins as standards as indicated in Fig. 7. Protein was determined by the method of Lowry *et al.* (1951).

Results

Purification of myocardial cyclic GMP-dependent protein kinase

The chromatographic pattern of the pH 5.3 precipitate on a DEAE-cellulose column is illustrated in Fig. 1. The cyclic GMP-dependent enzyme activity was eluted as a single peak. Sephadex G-200 gel filtration of the pooled DEAE-cellulose eluate is shown in Fig. 2. The enzyme activity appeared as a single peak, which was still not completely separated from the contaminating cyclic AMP-dependent protein kinase.

The typical results of the enzyme purification are summarized in Table 1. The enzyme was purified over 900-fold with an activity recovery of 4%. The specific activity of the enzyme from the last (hydroxyapatite) step, based upon the net activity stimulated by 0.4 μ M-cyclic GMP, was about 80 000. This was comparable with that seen in the purified enzyme from foetal guinea-pig lungs (J. F. Kuo *et al.*, 1976b), a tissue shown to have the highest content of this class of protein kinase among all mammalian tissues examined (Kuo, 1975). We have shown (Kuo,

Fig. 1. DEAE-cellulose chromatography of cyclic GMP-dependent protein kinase from foetal calf hearts

Experimental conditions were as described in the text. A sample (0.02 ml) from the fractions was used for assaying the enzymic activity under the standard conditions, in the absence (○) and presence of either cyclic AMP (△) or cyclic GMP (▲), as indicated. —, Phosphate concentration, 50 mM at fraction 40 to 150 mM at fraction 120; ----, A₂₈₀. The bar represents the fractions that were pooled.

1975) that the content of cyclic GMP-dependent protein kinase in the heart, as in the lung, was the highest in the foetus and the lowest in the adult. It would have been more difficult to purify the enzyme had we used the adult bovine heart instead as the starting material. It was found that the enzyme from the adult from the last step of the purification procedure described above had a total activity and a specific activity of only 20 and 40% respectively of the enzyme similarly purified from the same amount of the foetal heart.

Properties of myocardial cyclic GMP-dependent protein kinase

Requirement of stimulatory modulator for the enzyme activity. The crude protein kinase modulator (mixture of the stimulatory and inhibitory modulators) from the rat liver and bovine heart, or the isolated stimulatory modulators from the heart, augmented the myocardial cyclic GMP-dependent protein kinase activity (Table 2). There was no detectable cyclic GMP-stimulated activity in the absence of the added factor. The absolute requirement of the factor for the activity of the highly purified

cyclic GMP-dependent protein kinase from the foetal guinea-pig lung and lobster tail muscle was reported earlier (J. F. Kuo *et al.*, 1976b). The inhibitory modulator was without effect on the enzyme activity.

The isolated inhibitory modulator, on the other hand, decreased the activity of the cyclic AMP-dependent class of protein kinase without affecting the activity of the cyclic GMP-dependent enzyme (Table 2).

Specificity of cyclic nucleotides and analogues. The K_a values of the cyclic GMP-dependent enzyme for 8-bromo cyclic GMP, cyclic GMP and cyclic AMP, determined by the activation curves assayed in the presence of the stimulatory modulator (Fig. 3), were 0.013, 0.033 and $3.0 \mu\text{M}$ respectively. As shown in Table 2, little or no activity stimulated by the above cyclic nucleotides was noted in the absence of the modulator. Lack of cyclic AMP stimulation seen in the absence of the modulator clearly indicated that there was no detectable contamination of cyclic AMP-dependent protein kinase.

The apparent K_a values of the cyclic GMP-dependent enzyme for various cyclic nucleotides and their analogues, in an order of decreasing affinity,

Fig. 2. *Sephadex G-200 gel filtration of cyclic GMP-dependent protein kinase from foetal calf hearts*
Experimental conditions were as described in the text. A sample (0.02 ml) from the fractions was used for assaying the enzymic activity under the standard conditions, in the absence (○) and presence of either cyclic AMP (Δ) or cyclic GMP (▲), as indicated. ----, A_{280} . The bar represents the fractions that were pooled.

Table 1. *Summary of purification of cyclic GMP-dependent protein kinase from foetal calf heart*
Eight foetal calf hearts (1150 g) were used as the starting material. See the Experimental section for details. The concentration of either cyclic nucleotide was $0.4 \mu\text{M}$.

Fraction	Volume (ml)	Protein (mg)	$10^{-3} \times$ Total activity* (units)	Specific activity of enzyme (units/mg of protein)			Purification* (-fold)	Activity recovery (%)
				Basal	+Cyclic GMP	+Cyclic AMP		
Crude extract	2400	24800	2100	403	488	1130	1	100
pH 5.3 precipitate	79	1400	430	872	1180	1620	4	20
DEAE-cellulose eluate	102	60	153	373	2550	1250	30	7
Sephadex G-200 filtrate	50	9.6	168	964	17500	4190	205	8
Hydroxyapatite eluate	33	1.0	79	9900	79400	20300	935	4

* Calculated on the basis of the net activity stimulated by $0.4 \mu\text{M}$ -cyclic GMP.

are summarized in Table 3. The data were in agreement with those obtained with the enzyme from the lobster muscle (Kuo *et al.*, 1974) and from the foetal guinea-pig lung (J. F. Kuo *et al.*, 1976b). Adenosine 2':3'-monophosphate, guanosine 2':3'-monophos-

phate, 5'-AMP and 5'-GMP were also tested. They were found to be ineffective at all concentrations tested.

Substrate protein and metal ion specificity. Relative ability of various proteins to serve as substrates for

Table 2. Comparative effects of various protein kinase modulator preparations on myocardial cyclic GMP-dependent and cyclic AMP-dependent protein kinases

The cyclic GMP-dependent enzyme (0.3 μg) was from the hydroxyapatite step (Table 1), whereas the cyclic AMP-dependent enzyme (15 μg) was from the DEAE-cellulose step (Kuo & Greengard, 1969). The concentration of either cyclic nucleotide was 0.4 μM.

Source, preparation and amount of modulator	Protein kinase activity (units)			
	Cyclic GMP-dependent		Cyclic AMP-dependent	
	Basal	+Cyclic GMP	Basal	+Cyclic AMP
None	2.6	3.0	2.3	39.6
Rat liver				
Crude modulator (50 μg)	2.4	28.6	1.6	8.2
Bovine heart				
Crude modulator (62 μg)	2.7	19.6	1.5	5.2
Stimulatory modulator (40 μg)	2.6	30.6	2.2	36.8
Inhibitory modulator (6 μg)	2.4	2.8	1.6	8.0

Fig. 3. Stimulation by cyclic GMP, 8-bromo cyclic GMP and cyclic AMP of foetal calf heart cyclic GMP-dependent protein kinase incubated in the absence (a) and presence (b) of protein kinase modulator

The enzyme (0.6 μg) was used from the hydroxyapatite step. When present, the crude modulator (25.5 μg) was prepared from the rat liver. ○, Cyclic AMP; ●, cyclic GMP; △, 8-bromo cyclic GMP.

the myocardial cyclic GMP-dependent enzyme is compared in Table 4. Histones were generally good phosphate acceptors. Casein and phosvitin were poor substrates. Fig. 4 compares the activity of the two classes of myocardial protein kinases as a function of the concentration of arginine-rich histone, lysine-rich

Table 3. Apparent K_a values of foetal calf heart cyclic GMP-dependent protein kinase for various cyclic nucleotides and their analogues

The enzyme (0.6 μg) was from the hydroxyapatite step (Table 1). The concentrations of the compounds used ranged from 0.01 μM to 1 mM.

Cyclic nucleotide or analogue	Apparent K_a (μM)
8-Bromo cyclic GMP	0.013
Cyclic GMP	0.033
8-Benzylamino cyclic AMP	0.67
8-Bromo cyclic AMP	1.0
8-Bromo cyclic IMP	1.1
8-Benzylamino cyclic GMP	1.7
8-Methylthio cyclic AMP	2.1
<i>N</i> ² -Monobutyl cyclic GMP	2.3
Cyclic AMP	3.0
Cyclic IMP	8.5
8-Benzylamino cyclic IMP	10
<i>N</i> ⁶ -Monobutyl cyclic AMP	13*
2'- <i>O</i> -Monobutyl cyclic GMP	43*
Cyclic UMP	44*
Cyclic CMP	48*
<i>N</i> ² -2'- <i>O</i> -Dibutyl cyclic GMP	68*
2'- <i>O</i> -Monobutyl cyclic AMP	800*
<i>N</i> ⁶ -2'- <i>O</i> -Dibutyl cyclic AMP	800*

* Estimated by assuming these compounds at concentration greater than 1 mM would also stimulate the enzyme to the same maximal value as cyclic GMP and other compounds.

histone and slightly lysine-rich histone. All these histone subfractions exhibited a higher affinity toward the cyclic GMP-dependent enzyme than toward the cyclic AMP-dependent enzyme. Thus the K_m values for the three histone subfractions of the former enzyme were estimated to be about 50, 120 and 50 μg/ml respectively. The corresponding values of the

Table 4. Substrate-protein specificity of cyclic GMP-dependent protein kinase from foetal calf heart

Assay conditions were as described in the Experimental section except for the variation in the kind and amount of substrate proteins used. The enzyme (0.9 μg) used was from the hydroxyapatite step. The concentration of either cyclic nucleotide was 0.4 μM .

Substrate protein and source	Protein kinase activity (units)		
	Basal	+Cyclic GMP	+Cyclic AMP
None	1.2	1.4	1.3
Arginine-rich histone (40 μg)			
Worthington, HA	10.8	97.5	36.9
Sigma, type VIII-S	3.9	22.2	8.7
Schwarz/Mann	11.2	103.8	26.1
Lysine-rich histone (40 μg)			
Worthington, Hf1	18.3	154.2	54.4
Mixed histone (40 μg)			
Sigma, type II	9.8	96.6	22.8
Schwarz/Mann	7.7	81.2	19.3
Casein, Sigma (20–400 μg)	1.0	1.3	1.3
Phosvitin, Sigma (10–250 μg)	1.0	1.5	1.2

Fig. 4. Comparison of ability of various histones to serve as substrates for cyclic GMP-dependent protein kinase (a) and cyclic AMP-dependent protein kinase (b), both from foetal calf hearts

The cyclic GMP-dependent enzyme (0.3 μg), from the hydroxyapatite step (Table 1), was assayed in the presence of 0.4 μM -cyclic GMP, whereas the cyclic AMP-dependent enzyme, from the DEAE-cellulose step (Kuo & Greengard, 1969), was assayed in the presence of 0.4 μM -cyclic AMP. O, Arginine-rich histone; ●, lysine-rich histone; ▲, slightly lysine-rich histone.

cyclic AMP-dependent enzyme were much higher (Fig. 4). The present data were in line with those reported earlier for the enzyme from the arthropod tissue (Kuo & Greengard, 1970, 1974; Kuo *et al.*, 1971) and the foetal guinea-pig lung (J. F. Kuo *et al.*, 1976b).

The dose-dependent activation of the enzyme by

Mg^{2+} and Co^{2+} is presented in Fig. 5. The optimal concentrations of Mg^{2+} and Co^{2+} were found to be about 50 and 0.5 mM respectively. Other ions, such as Mn^{2+} , Fe^{2+} , Pb^{2+} and Zn^{2+} were ineffective. Even though without effect when present alone, Ca^{2+} antagonized the stimulatory effect of Mg^{2+} and Co^{2+} in a dose-related manner (Table 5).

pH optimum. The optimal pH for the enzyme activity was found to be rather broad and ranged from pH 6 to 9 (Fig. 6). It should be pointed out that the basal activity of the enzyme greatly increased at the pH values between 8 and 9. The activity in the presence of cyclic GMP, however, remained unaffected, resulting in a decreased stimulation by cyclic GMP.

Dissociation of cyclic GMP-dependent protein kinase to catalytic subunit

When the enzyme was chromatographed on a Sephadex G-200 column, a major enzyme-activity peak (the cyclic GMP-dependent holoenzyme, mol.wt. 150000) and a minor activity peak (the cyclic GMP-independent catalytic subunit, mol.wt. 60000) were obtained (Fig. 7a). If the enzyme was pre-incubated with cyclic GMP (50 μ M) and mixed histone

Fig. 5. Comparative effects of Mg²⁺ and Co²⁺ ions on the activity of cyclic GMP-dependent protein kinase from foetal calf hearts

The enzyme (4.5 μ g) from the Sephadex G-200 step was assayed in the presence of MgCl₂ (●) or CoCl₂ (○), in the presence (—) or absence (----) of 0.4 μ M-cyclic GMP.

Table 5. Antagonism by Ca²⁺ ions of stimulatory effect of Mg²⁺ and Co²⁺ ions on cyclic GMP-dependent protein kinase from foetal calf heart

Incubation conditions were as described in the Experimental section except for the kind and concentration of metal ion used. When present, the concentration of cyclic GMP was 0.4 μ M. The enzyme (0.9 μ g) was from the hydroxyapatite step.

Metal ion and concentration (mm)	Protein kinase activity (units)	
	Basal	+Cyclic GMP
None	0.7	0.6
Mg ²⁺ (30)	6.2	92.5
Co ²⁺ (0.6)	4.6	78.8
Ca ²⁺ (0.5)	0.7	0.9
Ca ²⁺ (2.5)	0.9	1.1
Ca ²⁺ (5)	1.3	1.3
Mg ²⁺ (30)+Ca ²⁺ (0.5)	5.4	81.5
Mg ²⁺ (30)+Ca ²⁺ (5)	4.5	15.8
Co ²⁺ (0.6)+Ca ²⁺ (0.5)	4.0	71.1
Co ²⁺ (0.6)+Ca ²⁺ (2.5)	1.3	18.1

Fig. 6. Effects of pH on the activity of cyclic GMP-dependent protein kinase from foetal calf hearts The enzyme (4.5 μ g) from the Sephadex G-200 step was assayed in the presence (—) and absence (----) of 0.4 μ M-cyclic GMP in 50mM-sodium acetate (○), 25mM-potassium phosphate (●), 50mM-Tris/HCl (Δ) and 50mM-NaHCO₃ (▲). All buffers contained 3mM-mercaptoethanol.

Fig. 7. Dissociation of cyclic GMP-dependent protein kinase from foetal calf heart

Two samples of the enzyme (0.3 ml, 30 μ g) from the hydroxyapatite step (Table 1) were preincubated for 1 h in ice under the following conditions: (a) phosphate buffer (50 mM), pH 7.0, containing 3 mM-mercaptoethanol; (b) same as (a) except containing, in addition, 50 μ M-cyclic GMP and mixed histone (200 μ g/ml). The solutions were charged on to Sephadex G-200 columns (1.5 cm $\times$ 15 cm). The columns were previously equilibrated, and the enzyme was eluted from the columns, with the respective preincubation solutions. The fraction size was 0.56 ml, and 0.02 ml portions were assayed for the enzyme activity in the presence or absence of 0.4 μ M cyclic GMP. Fractions from (b) were individually dialysed against 50 mM-phosphate buffer containing 3 mM-mercaptoethanol to remove cyclic GMP before assay. The mol.wts. of the marker enzymes are as follows: 1, phosphorylase b, 185 000; 2, human γ -globulin, 160 000; 3, creatine kinase, 80 000; 4, horseradish peroxidase, 49 000. RC, holoenzyme; C, catalytic subunit.

(200 μ g/ml), the holoenzyme peak disappeared completely, accompanied by an increased catalytic subunit peak (Fig. 7b). The low activity recovery in the catalytic subunit was due to the fact that the free subunit was extremely unstable. About 50% of the activity was lost in 6 h at 4°C. We were unable to detect the regulatory (cyclic GMP-binding) subunit of the enzyme in the Sephadex-column eluates. This may be due to an extreme instability of the free regulatory subunit, even though we were able to detect cyclic GMP binding by the holoenzyme (data not shown), as in the case of the cyclic GMP-

Table 6. Requirement of stimulatory modulator for the activity of catalytic subunit of myocardial cyclic GMP-dependent protein kinase

The isolated catalytic subunit (0.4 μ g) from Fig. 7 was incubated in the presence of varying amounts of stimulatory or inhibitory modulator, as indicated. When present, the concentration of cyclic GMP was 0.4 μ M.

Protein kinase modulator	Activity of catalytic subunit (units)	
	-Cyclic GMP	+Cyclic GMP
None	0.3	0.3
Stimulatory modulator (10 μ g)	1.1	1.0
Stimulatory modulator (20 μ g)	1.8	1.8
Stimulatory modulator (30 μ g)	2.6	2.7
Stimulatory modulator (50 μ g)	3.8	3.8
Inhibitory modulator (3 μ g)	0.3	0.2
Inhibitory modulator (6 μ g)*	0.2	0.2
Inhibitory modulator (18 μ g)	0.2	0.2

* Inhibited 20 units of cyclic AMP-dependent protein kinase 70% in the presence of 0.4 μ M-cyclic AMP.

dependent enzyme from foetal guinea-pig lung (J. F. Kuo *et al.*, 1976b). Inclusion of 10 mM-MgCl₂ or bovine serum albumin (1 mg/ml) failed to increase the stability of both catalytic and regulatory subunits in the present studies.

The activity of the isolated catalytic subunit was augmented by the stimulatory modulator (Table 6), as in the case of the cyclic GMP-dependent holoenzyme in the presence of cyclic GMP (Table 2 and Fig. 3). Moreover, the increased activity of the catalytic subunit due to the factor was dose-related. The inhibitory modulator, on the other hand, was without effect.

Discussion

The general properties of cyclic GMP-dependent protein kinase from the calf heart presented in the present paper are similar in many respects to those from the lung (J. F. Kuo *et al.*, 1976b) and the brain (W.-N. Kuo *et al.*, 1976a; J. F. Kuo *et al.*, 1976c) of the guinea pig. These include: an absolute requirement of the stimulatory component of protein kinase modulator for the cyclic GMP-stimulated activity; specificity for substrate proteins, cyclic nucleotides, and metal ions and molecular weight. Many properties of the mammalian enzyme, in turn, are similar to those reported earlier for the cyclic GMP-dependent enzyme from arthropod tissues (Donnelly *et al.*, 1973; Kuo & Greengard, 1974).

Cyclic GMP-dependent protein kinase was also purified from the bovine cerebellum by Takai *et al.* (1975) and from the pig lung by Nakazawa & Sano (1975). One distinct difference between their enzyme

preparations and ours is that their preparations can be stimulated (even though at lower extents) by cyclic GMP in the absence of added modulator. This is analogous to the rather crude enzyme preparations from arthropod tissues, which were stimulated up to about 5-fold by $0.5\ \mu\text{M}$ cyclic GMP alone; the cyclic GMP-stimulated activity, however, was further enhanced by the added modulator (Donnelly *et al.*, 1973). It was suspected that the arthropod enzyme preparations still existed in the form of a complex with the contaminating modulator. An experiment by J. F. Kuo *et al.* (1976b) indicated that the lobster cyclic GMP-dependent enzyme, upon further purification, was no longer stimulated by cyclic GMP unless the factor was added to the incubation system, as in the case of the purified mammalian enzyme. It is therefore likely that their enzyme preparations (Takai *et al.*, 1975; Nakazawa & Sano, 1975) may still significantly be contaminated with the modulator, thus rendering them stimutable by cyclic GMP alone. Further, the specific activity of their enzyme preparations from the final purification steps ranged only from 2000 to 4000. Our preparations from both the lung and the heart, on the other hand, had a value of about 80000, which would have been higher had the enzyme been assayed under the conditions that they have used (higher ATP and substrate protein concentrations, but a shorter incubation time). Whether their enzyme preparations can also be stimulated by the modulator, or their preparations represent a separate subtype of cyclic GMP-dependent protein kinase, is not clear at present.

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