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CYCLIC NUCLEOTIDE BINDING PROPERTIES AND MOLECULAR FORMS OF THE CYCLIC-AMP-DEPENDENT PROTEIN KINASE FROM BOVINE EYE LENS

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Summary

The activity of the cyclic-AMP-dependent protein kinase found in the crude extract of bovine eye lens cortical fibers was increased by 10^{-6} M cyclic AMP or 10^{-5} M cyclic GMP to the same extent, However, the interaction between cyclic AMP and cyclic GMP in the course of binding to the cyclic-AMP-dependent protein kinase did not seem to be competitive.

Scatchard analysis of cyclic GMP binding by the crude extract indicated the presence of two types of cyclic GMP binding sites (Kd_1 about $2 \cdot 10^{-7}$ M, Kd_2 about $5 \cdot 10^{-6}$ M).

Different species of cyclic nucleotide binding fractions were separated by Sephadex G-200 gel chromatography of the crude extract. The bulk of the low affinity cyclic GMP binding activity was found in the exclusion volume. The cyclic-AMP-dependent protein kinase eluted in two fraction (apparent molecular weight 300 000 and 150 000) and both protein kinase fractions were accompanied by the high affinity cyclic GMP binding activity. However, the ratios of this activity to the cyclic AMP binding activity were different in the two fractions, suggesting that different molecular weight forms of the holoenzyme had different cyclic nucleotide binding properties.

Introduction

Homogeneus cyclic-AMP-dependent protein kinases from rabbit skeletal muscle, bovine heart and rat liver have been shown to be tetramers consisting of two catalytic subunit monomers (C) and one regulatory subunit dimer (R_2) [1—3]. The molecular weight of the R_2C_2 structure has been reported to vary

between 150 000 and 176 000. However, in several cases, apparently different molecular weight fractions of the holoenzyme have been separated [4—6]. This might have been due to limited proteolysis of the enzyme complex or to some other reason. It is conceivable that different molecular weight forms of the holoenzyme represent different states of aggregation of the enzyme complex and that the ability of the protein kinase to form various aggregates has significance in the regulatory process [7].

As reported, the crude extract of bovine eye lens cortical fibers contains a protein kinase that can be activated 25-fold by cyclic AMP [8]. Sephadex gel chromatography of the crude extract separated distinct fractions of cyclic-AMP-dependent protein kinase. Since the eye lens contains a very low level of proteolytic activity compared to other tissues [9], proteolysis of the enzyme is improbable in the freshly prepared extract. In order to detect functional differences between the distinct molecular forms of the holoenzyme we investigated the cyclic AMP and cyclic GMP binding properties of the eye lens protein kinase.

Earlier studies in this laboratory [10] demonstrated the existence of two types of cyclic GMP binding sites associated with the cyclic-AMP-dependent protein kianse of human lymphocytes. Although cyclic GMP exerted the same effect as cyclic AMP on the catalytic activity of the enzyme [10,11], the kinetic results showed no simple competitive relationship between the two nucleotides in the course of binding to the enzyme. The cyclic GMP binding activity of the lymphocyte protein kinase seemed to be strongly influenced by the state of aggregation of the enzyme complex [10].

The present work shows that different molecular weight fractions of the eye lens protein kinase holoenzyme have different cyclic nucleotide binding properties. Some aspects of this work have been published in a preliminary form [12].

Methods

Chemicals. Cyclic AMP and cyclic GMP were purchased from Sigma Chemical Co. Cyclic-[8-^{3}H]AMP, (27.5 Ci/mmol) and cyclic-[8-^{3}H]GMP (20.7 Ci/mol) were obtained from the Radiochemical Centre, Amersham, U.K. γ-[^{32}P]-ATP (200 Ci/mol) was produced by the photosynthetic method of Avron [13]. Calf thymus H2b histone was purified according to method I of Johns [14].

Preparation of lens cortical extract. Fresh bovine eyes were obtained from the slaughterhouse. The lenses were excised, rinsed in 0.9% NaCl and the lens capsule with the epithelial layer and the nucleus were removed and discarded. The cortical parts from 50 lenses were minced with scissors and homogenized in 5 vols. 5 mM potassium phosphate (pH 7.0), 4 mM EDTA. All procedures were carried out at 4° C. The homogenate was filtered through 4 layers of gauze and centrifuged at 27 000 × g for 30 min. The supernatant was dialyzed against 5 mM potassium phosphate (pH 7.0) and if precipitation appeared during the dialysis it was centrifuged at 27 000 × g for 30 min. The supernatant was the crude extract of eye lens cortical fibers. This crude extract contained about 100 mg/ml protein determined by the method of Lowry et al. [15].

Determination of protein kinase activity. The assay procedure was essentially the same as that described by Kuo and Greengard [16], with slight mofidica-

tions. [17] The standard assay mixture contained 0.05 M potassium phosphate (pH 6.2), 0.01 M NaF, 0.002 M theophylline, 0.01 M $MgCl_2$, 1 nmol γ-[^{32}P]ATP, 1.6 mg histone in 1.6 ml final volume. The reaction mixture was supplemented with the appropriate amount of cyclic nucleotides where indicated. The reaction was started by the addition of the enzyme solution.

Assay of ^{3}H-labeled cyclic nucleotide binding. The incubation mixture contained 0.05 M potassium acetate (pH 4.0) and appropriate amounts of ^{3}H-labeled and unlabeled cyclic nucleotides. The sample of protein solution applied in the incubation mixture contained no more than 0.4 mg protein. The assay was carried out exactly as described by Gilman [18]. Sartorius membrane filters (No. 11307) were used. Binding reactions, incubation for 1 h at 0°C, were started by the addition of protein solution. The equilibrium of the binding reaction was reached after 30 min either with cyclic AMP or with cyclic GMP. Each value presented is the mean of duplicate measurements.

Sephadex G-200 gel chromatography of the lens cortical extracts. Gel chromatography was carried out on Sephadex G-200 column, 2 × 50 cm, equilibrated with 5 mM potassium phosphate (pH 7.0). 0.6 ml crude extact was applied on to the column (3 ml fractions). The protein content of the fractions were determined from absorption at 280 nm. The exclusion volume of the column was detected by the first protein peak containing alfa crystalline; average molecular weight 800 000 [19], and the molecular weights of the fractions were estimated by using the equation of Determann [20]. The protein kinase activity was measured in 0.4 ml samples, while 0.2 ml samples were used for determining the cyclic nucleotide binding activity.

Results

The activation of cyclic-AMP-dependent protein kinase of eye lens cortical fibers

The crude extract of eye lens cortical fibers contained only a cyclic-AMP-dependent protein kinase activity when measured in the presence of H2b histone as a substrate. Neither a specific cyclic-GMP-dependent protein kinase nor a cyclic-nucleotide-independent protein kinase could be detected in the extract of cortical fibers.

However, the cyclic-AMP-dependent protein kinase found in the crude extract was very sensitive. All the applied purification procedures resulted in enzyme preparations with significantly increased basal activity and the rate of activation caused by cyclic AMP decreased from the factor of 25 to 3—10. In order to avoid the partial dissociation of the protein kinase complex and to investigate the behaviour of the native form of the enzyme, the crude extract of cortical fibers was used in the experiments.

The eye lens protein kinase was also activated by cyclic GMP. In four different preparations tested the activation evoked by 10^{-5} M cyclic GMP was identical with that pronounced by 10^{-6} M cyclic AMP, though the kinetics of activation characteristic of the two cyclic nucleotides were different. The double reciprocal plot of cyclic GMP-induced enzyme activity seemed to result in a straight line, while complex kinetics of activation was found with cyclic AMP (Fig. 1). Previously we found different kinetics of cyclic AMP and cyclic

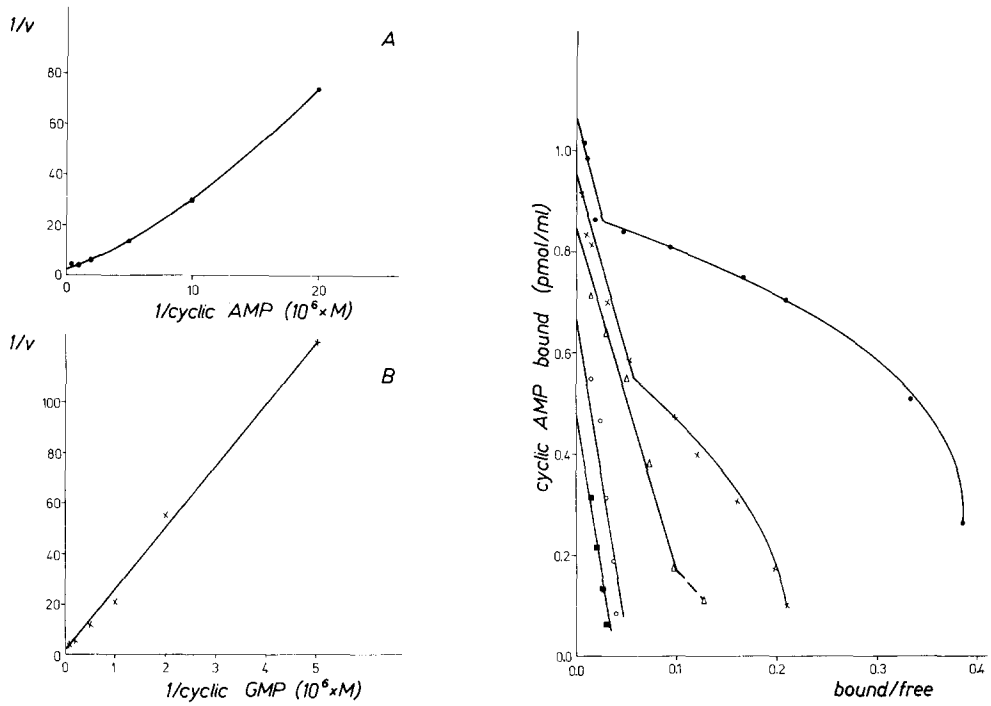

Fig. 1. Double reciprocal plots of activation of the cyclic-AMP-dependent protein kinase by cyclic AMP (A) and by cyclic GMP (B). Reaction velocity (amount of phosphate (pmol) transferred per min per incubation mixture) is expressed as the difference between the values measured in the presence and absence of the cyclic nucleotides. The incubation mixture contained 4 mg protein of the crude extract.

Fig. 2. Scatchard plots of cyclic AMP binding by the lens cortical extract. Cyclic-[^{3}H]AMP binding was measured in the absence (●———●) and in the presence of $5 \cdot 10^{-8}$ M (X———X), 10^{-7} M (△———△), $5 \cdot 10^{-7}$ M (○———○) and 10^{-6} M (■———■) cyclic GMP. The incubation mixture (0.3 ml) contained 0.4 mg protein. The amount of the cyclic AMP bound is expressed as pmol/ml incubation mixture. The values of free cyclic AMP were calculated from the total amount of cyclic AMP and the amount bound.

GMP-induced activation of the cyclic-AMP-dependent protein kinase from human lymphocytes [10], but the activation of the lymphocyte enzyme by the two nucleotides showed reverse kinetic patterns, i.e. in the double reciprocal plot there was a straight line with cyclic AMP and a parabole with cyclic GMP.

Cyclic AMP binding properties of the protein kinase

The cyclic AMP binding of the crude extract was measured at pH 4.0 according to the method of Gilman [18]. Under these experimental conditions the cyclic AMP binding activity obtained at low cyclic AMP concentrations is due exclusively to the regulatory subunit of the cyclic-AMP-dependent protein kinase [3].

Scatchard analysis [21] of cyclic AMP binding by the crude extract indicated a heterogeneity of the cyclic AMP binding sites (Fig. 2). At low saturation of the cyclic AMP binding sites the cyclic AMP binding showed positive cooperativity, while at high saturation a straight line was obtained in the Scatchard plot. The *K*d estimated on the basis of the linear part of the

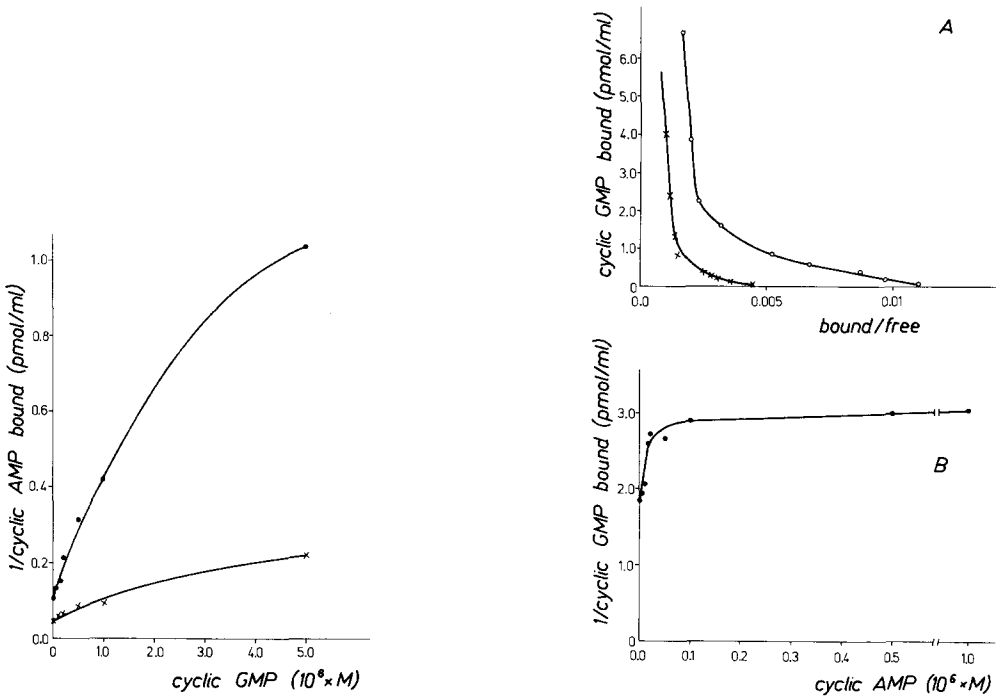

Fig. 3. Reciprocal plots of the amount of cyclic AMP bound vs. cyclic GMP concentration. The concentrations of cyclic-[^{3}H]AMP were $2 \cdot 10^{-9}$ M (●———●) and $5 \cdot 10^{-8}$ M (X———X). The incubation mixture contained 0.4 mg protein.

Fig. 4. The effect of cyclic AMP on the cyclic GMP binding by lens cortical extract. A; Scatchard plots of cyclic GMP binding. Cyclic-[^{3}H]GMP binding was measured in the absence (○———○) and in the presemce of $2 \cdot 10^{-7}$ M cyclic AMP (X———X). The incubation mixture (0.3 ml) contained 0.4 mg protein. The amount of cyclic GMP bound is expressed as pmol/ml of incubation mixture. B; Reciprocal plots of the amount of bound cyclic GMP vs. cyclic AMP concentration. The concentration of cyclic-[^{3}H]GMP was $2 \cdot 10^{-7}$ M. The incubation mixture (0.3 ml) contained 0.4 mg protein.

saturation curve was about 10^{-8} M. In the presence of cyclic GMP the cooperative character of the cyclic AMP binding curve disappeared and the amount of the cyclic AMP binding sites decreased. At high cyclic GMP concentrations the Scatchard plots of cyclic AMP binding showed parallel straight lines (Kd about 10^{-8} M). The inhibition of cyclic AMP binding was not complete even in the presence of cyclic GMP at high concentration, as it was indicated also by the Dixon plot [22] (Fig. 3).

Cyclic GMP binding properties of the extract of cortical fibers

In lymphocytes two types of cyclic GMP binding sites associated with the cyclic-AMP-dependent protein kinase were found and cyclic AMP inhibited selectively the high affinity cyclic GMP binding by reducing the amount of the high affinity cyclic GMP binding sites [10]. The Scatchard analysis of cyclic GMP binding by the extract of cortical fibers showed also two types of cyclic GMP binding sites (Kd$_1$ $2 \cdot 10^{-7}$ M and Kd$_2$ $5 \cdot 10^{-6}$ M, approximately). The amount of the high affinity cyclic GMP binding site decreased in the presence of cyclic AMP, while the low affinity cyclic GMP binding was not influenced

even at high cyclic AMP concentration (Fig. 4A and B). The total concentration of the low affinity cyclic GMP binding sites in eye lens extract, as calculated by an extrapolation of the Scatchard plot, was 5—6 times higher than that of the cyclic AMP binding sites.

Sephadex G-200 gel chromatography of the extract of cortical fibers

Sephadex chromatography of the extract of cortical fibers separated different cyclic-AMP-dependent protein kinase fractions (Fig. 5A and B).

Since the molecular weight of the cyclic-AMP-dependent protein kinase holoenzyme has been reported to be hard to estimate on the basis of gel chromatography [2], the molecular weight data given in this paper refer only to the positions of the cyclic-AMP-dependent protein kinase fractions. The protein kinase fractions of bovine eye lens had apparent molecular weights of about 300 000 and 150 000. The initial high activation rate of the enzyme found in the crude extract decreased to an about 10-fold activation and 5-fold activation of the 300 000 and 150 000 dalton fractions, respectively.

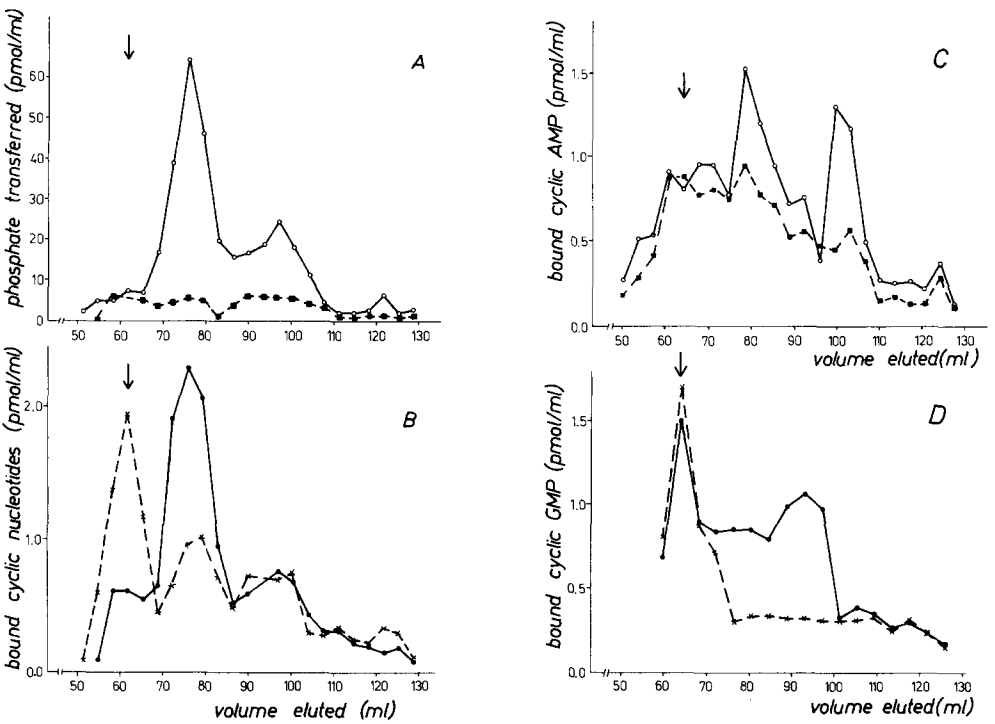

Fig. 5. Sephadex G-200 elution profiles of the protein kinase and cyclic nucleotide binding activities of the eye lens extract. A and B present results obtained from the same run, while the data of C and D pertain to two other experiments. A, Protein kinase activity measured in the absence (■———■) and presence of 10^{-6} M cyclic AMP (○———○). B, Cyclic AMP (●———●) and cyclic GMP (X———X) binding activities measured in the presence of $5 \cdot 10^{-8}$ M cylic-[^{3}H]AMP and $2 \cdot 10^{-7}$ M cyclic-[^{3}H]GMP, respectively. C, Cyclic AMP binding activity measured at $5 \cdot 10^{-8}$ M cyclic-[^{3}H]AMP in the absence (○———○) and in the presence of $2 \cdot 10^{-7}$ M cyclic GMP (■———■). Part D, Cyclic GMP binding activity measured at $2 \cdot 10^{-7}$ M cyclic-[^{3}H]GMP in the absence (●———●) and presence of 10^{-6} M cyclic AMP (X———X). The arrow indicates the exclusion volume of the column.

Both fractions were accompanied by cyclic GMP binding activity in the presence of $2 \cdot 10^{-7}$ M cyclic GMP. This cyclic GMP binding activity was due to the high affinity cyclic GMP binding site because it was inhibited by cyclic AMP (Part D, Fig. 5). The cyclic AMP binding activity of the enzyme fractions was measured at saturating cyclic AMP concentration ($5 \cdot 10^{-8}$ M) and it was also inhibited by $2 \cdot 10^{-7}$ M cyclic GMP. (Fig. 5B and C). The ratios of cyclic GMP binding activity to cyclic AMP binding activity were significantly different in the two fractions, the relative cyclic GMP binding activity being higher in the second enzyme peak.

The bulk of the low affinity cyclic GMP binding activity was found in the exclusion volume of the Sephadex G-200 column. Cyclic GMP binding by this fraction was not inhibited by cyclic AMP, though cyclic AMP binding activity was also detected in the exclusion volume. (Fig. 5B and D). The binding activity measured at $2 \cdot 10^{-7}$ M cyclic GMP represented a small portion of the total cyclic GMP binding capacity of this fraction, since its *K*d was $5 \cdot 10^{-6}$ M (data not shown). The cyclic GMP binding measured at $2 \cdot 10^{-7}$ M cyclic GMP was inhibited by 30% in the presence of 10^{-5} M GDP or GMP.

Discussion

Two cyclic-AMP-dependent protein kinase fractions were separated by Sephadex G-200 gel chromatography of the crude extract of bovine eye lens cortical fibers. Though in some tissues different molecular weight fractions of the protein kinase may arise from a limited proteolysis of the native enzyme [3], in this case such an effect of proteolysis is improbable because in the eye lens proteolytic activity is low [9].

The two protein kinase fractions (apparent molecular weights 300 000 and 150 000) represented different molecular forms of the enzyme. Similar results have been reported on the basis of gel chromatography of the partially purified bovine heart enzyme [4].

None of the protein kinase fractions separated by the Sephadex gel chromatography seemed to be identical with the native form of the enzyme found in the crude extract, since the initial high rate of cyclic AMP-induced activation of the enzyme was strongly decreased after gel chromatography.

Both cyclic-AMP-dependent protein kinase fractions were accompanied by a cyclic GMP binding activity that was inhibited in the presence of cyclic AMP. The kinetic results presented in this paper show two types of cyclic GMP binding sites in the extract of eye lens. Only the high affinity cyclic GMP binding (approx. *K*d $2 \cdot 10^{-7}$ M) was inhibited by cyclic AMP. The measured cyclic GMP binding activity of the cyclic-AMP-dependent protein kinase fractions was due to the high affinity cyclic GMP binding site.

Cyclic AMP binding capacity of the fractions was measured at the saturating cyclic AMP concentration ($5 \cdot 10^{-8}$ M) and cyclic GMP binding activity was measured at $2 \cdot 10^{-7}$ M cyclic GMP concentration. Presuming that the *K*d value of the high affinity cyclic GMP binding site found in the crude extract (approx. $2 \cdot 10^{-7}$ M) was valid also for the fractions isolated by Sephadex G-200, the total cyclic GMP binding capacity of the fractions was 100% higher than the measured cyclic GMP binding activity. On the basis of this rough estimation the

amounts of the cyclic AMP and the high affinity cyclic GMP binding sites in the high molecular weight fraction (apparent mol. wt. 300 000) were approximately equal, while in the low molecular weight fraction (apparent mol. wt. 150 000) the amount of the cyclic GMP binding sites was two times higher than that of the cyclic AMP binding sites. If the affinities of the high and low molecular weight fractions towards cyclic GMP are different, our estimation is not correct. Nevertheless the data show that the cyclic nucleotide binding properties of the two enzyme fractions were different.

The bulk of the low affinity cyclic GMP binding activity, accompanied by a small cyclic AMP binding activity without any protein kinase activity was found in the exclusion volume of the Sephadex G-200 column. The nature of this fraction is obscure. We have no direct evidence showing that this fraction is in any way related to the cyclic-AMP-dependent protein kinase, because the low affinity cyclic GMP binding was not inhibited by cyclic AMP. However, non-cyclic guanine-nucleotides at nearly two orders of magnitude higher concentration than cyclic GMP caused negligible inibition of cyclic GMP binding by this fraction which therefore does not seem to be an unspecific guanine-nucleotide binding protein. We presume that this low affinity cyclic GMP binding fraction is made up from inactive aggregates of the cyclic-AMP-dependent protein kinase of eye lens and it is a characteristic product of the ageing of eye lens proteins. The ageing of eye lens proteins is known to occur by irreversible aggregation of protein molecules [9].

On the basis of the kinetic data presented the interaction between cyclic AMP and cyclic GMP in the course of binding to the cyclic-AMP-dependent protein kinase does not seem to be a 'classical competition'. The results suggest that the cyclic AMP and cyclic GMP binding sites associated with the cyclic-AMP-dependent protein kinase are not completely identical. The cyclic-AMP-dependent protein kinase has probably no specific allosteric binding site for cyclic GMP, but the binding sites of the two nucleotides are overlapping. In spite of the fact that cyclic GMP causes the same effect on the catalytic activity of the enzyme as cyclic AMP, the conformation changes of the regulatory subunit induced by cyclic AMP or by cyclic GMP may not be identical. The complex interaction between cyclic AMP and cyclic GMP is presumably due to cooperative effects of the regulatory subunits, and the functional state of the cyclic nucleotide binding sites may be influenced by the state of aggregation of the protein kinase holoenzyme.

A comparative study of cyclic AMP and cyclic GMP binding may serve as a tool for detecting different functional forms of the cyclic-AMP-dependent protein kinase complex.

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