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HEAT TREATMENT OF RAT LIVER ADENOSINE 3′:5′-MONOPHOSPHATE PHOSPHODIESTERASE

KINETIC CHARACTERIZATION OF THE LOW AFFINITY ENZYME

MARINUS M. BEVERS, RIEK A. E. SMITS, JOHANNES VAN RIJN and ROELAND VAN WIJK

Van 't Hoff Laboratorium, Utrecht (The Netherlands)

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SUMMARY

1. Rat liver contains a low and a high K_m cyclic AMP phosphodiesterase activity (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17). The low K_m activity is inactivated irreversibly by heat treatment at a temperature of 45 °C. Column chromatography of heat-treated and untreated samples show the existence of separate proteins which hydrolyse cyclic AMP with a different K_m.

2. The heat-stable high K_m cyclic AMP phosphodiesterase activity shows substrate cooperativity. Cyclic GMP at a concentration of 2 μM activates the enzyme activity and suppresses the substrate cooperativity. Theophylline, final concentration 5 mM, inhibits the enzyme activity and abolishes the activating effects of cyclic AMP and cyclic GMP.

3. The substrate cooperativity and the activation by cyclic GMP are affected by dilution, heat treatment at 60 °C, treatment with sodium dodecylsulfate and gel filtration of the enzyme preparation.

INTRODUCTION

The intracellular concentration of cyclic AMP is determined in part by the activities of one or more cyclic AMP phosphodiesterases. In rat liver extracts low concentrations of cyclic GMP stimulate the hydrolysis of cyclic AMP [1]. Recent studies suggest that cyclic GMP activates the high K_m cyclic AMP phosphodiesterase activity [2]. This paper describes the irreversible inactivation of the low K_m cyclic AMP phosphodiesterase activity without affecting the high K_m enzyme activity. The high K_m cyclic AMP hydrolyzing activity is studied after $(NH_4)_2SO_4$ fractionation of a 100 000 × g supernatant and after subsequent gel filtration on a Sepharose 6-B column, concerning its activation by cyclic AMP and cyclic GMP and its inhibition by theophylline.

Abbreviations: Tris–$MgCl_2$–mercaptoethanol buffer: 40 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 6 mM mercaptoethanol.

MATERIALS AND METHODS

Chemicals

The 3′:5′-cyclic nucleotide monophosphates of adenosine and guanosine were obtained from Boehringer, Mannheim, Germany; 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5) from *Crotalus adamanteus* was supplied by Sigma Chemical Co., St. Louis, Mo.; Bovine serum albumin was from Poviet Producten N.V., Amsterdam, The Netherlands; theophylline was purchased from Schwarz–Mann Inc., Orangeburg, N.Y.; ^{3}H-labeled 3′:5′-cyclic adenosine monophosphate and 3′:5′-cyclic guanosine monophosphate were obtained from the Radiochemical Centre, Amersham, Great Britain. Sepharose 6-B was from Pharmacia, Uppsala, Sweden. Anion-exchange resin Bio-Rad AG1-X2, 200–400 mesh was obtained from Bio-Rad Laboratories, Richmond, Calif.

Before use the resin was washed with 1 M NaOH, 1 M HCl and water to neutrality and finally a few times with absolute ethanol. All other chemicals were of analytical grade.

Preparation of the 50% saturated $(NH_4)_2SO_4$ fractions

Female rats, strain R-Amsterdam, of 250–300 g were killed by decapitation. The livers were quickly removed, rinsed with ice-cold Tris–$MgCl_2$–mercaptoethanol buffer (40 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 6 mM mercaptoethanol) and homogenized in 5 vol. of Tris–$MgCl_2$–mercaptoethanol buffer using an Ultra-Turrax. All steps were carried out at 0–4 °C. Homogenates were centrifuged at 1000 × *g* for 10 min and the supernatants were centrifuged at 105 000 × *g* for 1 h. Neutral saturated $(NH_4)_2SO_4$ solution was added to the 105 000 × *g* supernatant to 50% saturation. The resulting precipitate was collected by centrifugation, resuspended in Tris–$MgCl_2$–mercaptoethanol buffer and dialyzed overnight against 1 mM Tris–HCl buffer (pH 7.5) containing 2 mM $MgCl_2$. This preparation, containing 23 mg protein/ml was stored at −80 °C. Protein was estimated by the method of Lowry et al. [3].

Assay of phosphodiesterase activity

Activity was measured according to the method of Brooker et al. [4] with slight modifications according to Monard et al. [5]. The hydrolysis of cyclic AMP was studied over a concentration range of 0.2–100 μM. The incubation was performed in Tris–$MgCl_2$–mercaptoethanol buffer at 30 °C and the incubation times allowed measurements of the initial rates of the reaction at any substrate concentration.

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

RESULTS

Kinetics of cyclic AMP hydrolysis

Fig. 1, Curve a, shows a characteristic Lineweaver–Burk plot [6] of the adenosine 3′:5′-monophosphate phosphodiesterase activity as determined in the 50% saturated $(NH_4)_2SO_4$ fraction of 105 000 × *g* rat liver supernatant. Measurements of the cyclic AMP hydrolyzing activity in crude homogenate or 105 000 × *g* supernatant had qualitatively similar results. This plot shows a definite biphasic kinetic behaviour

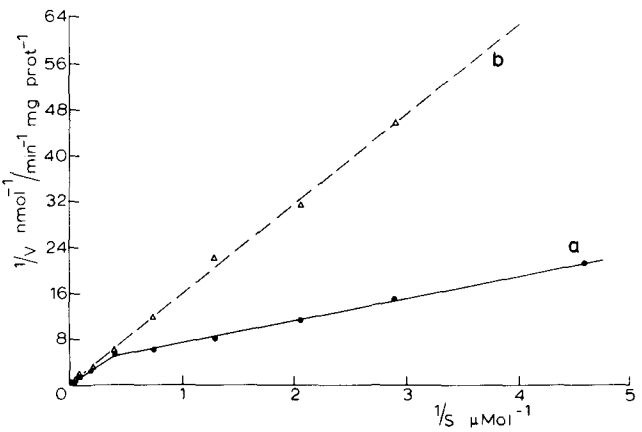

Fig. 1. Lineweaver–Burk plot of the adenosine 3′:5′-monophosphate phosphodiesterase activity of the 50% saturated $(NH_4)_2SO_4$ fraction (1 mg protein/ml Tris–$MgCl_2$–mercaptoethanol buffer) before (Curve a) and after (Curve b) heat treatment of the sample at 45 °C for 1 h. Phosphodiesterase activity was measured at 30 °C as described in Materials and Methods in a concentration range of 0.2–100 μM.

in a substrate concentration range of 0.2–100 μM. At cyclic AMP concentrations below 2.5 μM extrapolation of the linear portion of the curve yielded an apparent K_m of about 1 μM. The apparent K_m at cyclic AMP concentrations above 2.5 μM is discussed later. It must be noted, that this biphasic kinetic behaviour was also observed after the use of other homogenisation media i.e. 10.9% sucrose or 0.15 M KCl. Moreover it is independent of the time of storage at −80 °C for at least 6 months.

Effect of heat treatment on cyclic AMP hydrolysis

The 50% saturated $(NH_4)_2SO_4$ fraction at a concentration of 1 mg protein/ml was heated at a temperature of 45 °C for 1 h. At various times samples were taken and the enzyme activity was measured by incubation at a temperature of 30 °C. Fig. 2 shows the decrease in phosphodiesterase activity to one-third of the activity of a non-treated sample when measured at a cyclic AMP concentration of 0.48 μM which represents mainly the low K_m activity (Fig. 1, Curve a). A decrease of 10% or less was observed at a cyclic AMP concentration of 50 μM which represents mainly the high K_m activity (Fig. 1, Curve a). The low K_m enzyme activity is not seen after heat treatment as shown by the double reciprocal plot in Fig. 1 (Curve b). The low K_m activity was not restored by storage at 4 °C for 2 days. The high K_m fraction could stand the temperature of 45 °C for at least 3 h. The results of these experiments have been interpreted as pointing to the existence of at least two independent enzyme systems with different K_m values. To confirm this hypothesis gel filtration at 4 °C on a Sepharose 6-B column (2.5 cm × 50 cm) of a heat-treated and an untreated sample was performed. Fig. 3A and Fig. 3B show the profiles of phosphodiesterase activity when tested at a low and a high cyclic AMP concentration. Three peaks were observed (as indicated in Fig. 3A): Peak I and Peak III both with a relative high activity at low (0.48 μM) cyclic AMP concentrations and Peak II with a relative high activity at high (50 μM) cyclic AMP concentrations. The heat-treated sample (Fig. 3B) shows only Peak II. The total activity as measured at the high substrate concen-

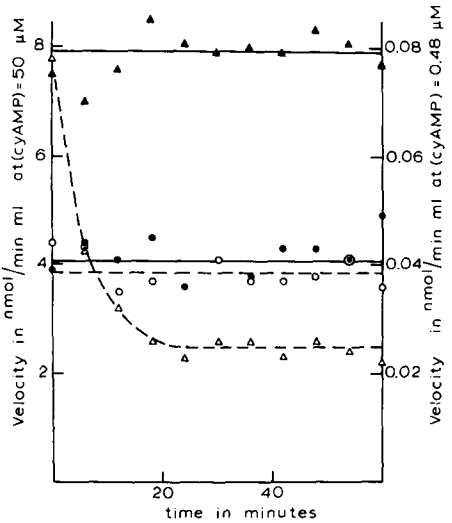

Fig. 2. The effect of heat treatment on adenosine 3′:5′-monophosphate phosphodiesterase activity. A 50% saturated $(NH_4)_2SO_4$ fraction (1 mg protein/ml Tris–$MgCl_2$–mercaptoethanol buffer) was treated for 1 h at 45 °C. At various times a sample was taken and the phosphodiesterase activity was tested at 30 °C at low (0.48 μM), △---△, and at high (50 μM), ○---○, substrate concentrations. The same experiment was done at 30 °C. The activity was tested at cyclic AMP concentrations of 0.48 μM (▲—▲) and of 50 μM (●—●).

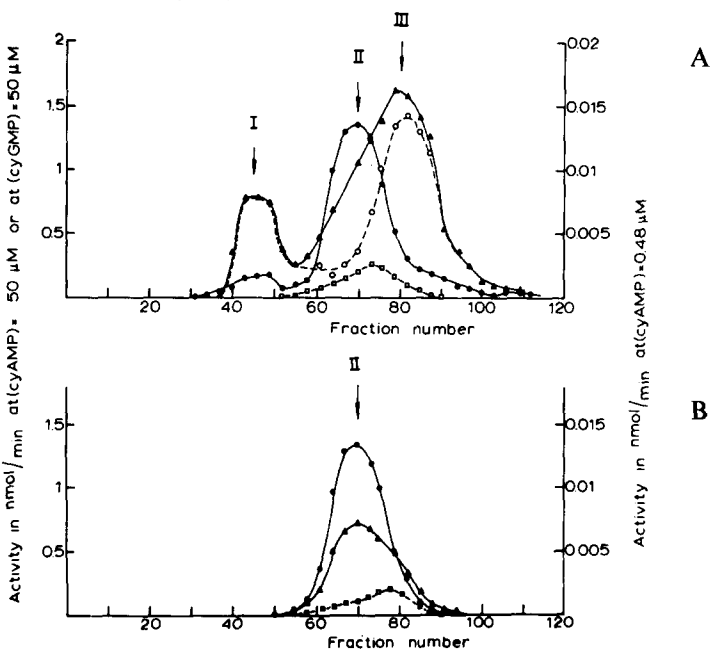

Fig. 3. Gel filtration of a non-heat-treated (Fig. 3A) and a heat-treated (Fig. 3B) 50% saturated $(NH_4)_2SO_4$ fraction (1 mg protein/ml Tris–$MgCl_2$–mercaptoethanol buffer). A volume of 20 ml was applied to a Sepharose 6-B column (50 cm × 2.5 cm). Elution was performed with Tris–$MgCl_2$–mercaptoethanol buffer. Flow rate was 9 ml/h. Fractions of 2 ml were collected in 0.5 ml of Tris–$MgCl_2$–mercaptoethanol buffer containing 5 mg/ml bovine serum albumin. Phosphodiesterase activity was tested at cyclic AMP concentration of 50 μM (●—●) and 0.48 μM (▲—▲). ○---○, the difference between the low substrate activity before and after heat treatment. Cyclic GMP phosphodiesterase activity was tested at a substrate concentration of 50 μM (□---□).

tration is nearly similar after column chromatography of the heat-treated and non-heat-treated sample as was expected from Fig. 2. The profile of the heat unstable activity (Fig. 3A, dotted line) which can be derived from the difference between non-treated (Fig. 3A) and treated (Fig. 3B) enzyme activities, measured at a low substrate level, shows that this activity is only present in Peak I and Peak III.

Effect of heat treatment on cyclic GMP hydrolysis

The hydrolysis of cyclic GMP by a 50% $(NH_4)_2SO_4$ preparation (protein concn 1 mg/ml) shows a linear double reciprocal kinetic plot in a substrate concentration range between 0.2 and 100 μM. Heat treatment of the sample for 1 h at 45 °C results in a decrease of 25% in activity over the whole concentration range. The K_m value before and after heat treatment was about 20 μM and the V 0.6 $\pm$ 0.1 nmole/min per mg protein. The profile of the enzyme activity after gel filtration over Sepharose 6-B as measured at a cyclic GMP concentration of 50 μM is very similar before and after heat treatment of the $(NH_4)_2SO_4$ preparation.

Cyclic AMP hydrolysis by heat stable phosphodiesterase activity: effect of cyclic GMP, theophylline and imidazole

Fig. 4A (Curve a) represents the Lineweaver–Burk plot [6] of the heat-treated 50% saturated $(NH_4)_2SO_4$ fraction in the cyclic AMP range of 3–100 μM. When the best straight line is drawn for data plotted by the 1/S versus 1/v method, an absurd negative value for 1/V is obtained. When the values of the curved part of Curve a (Fig. 4A) are plotted by the $1/S^2$ versus 1/v method, a straight line is obtained. The V calculated by the latter method is 6 $\pm$ 1 nmoles/min per mg protein. This is similar

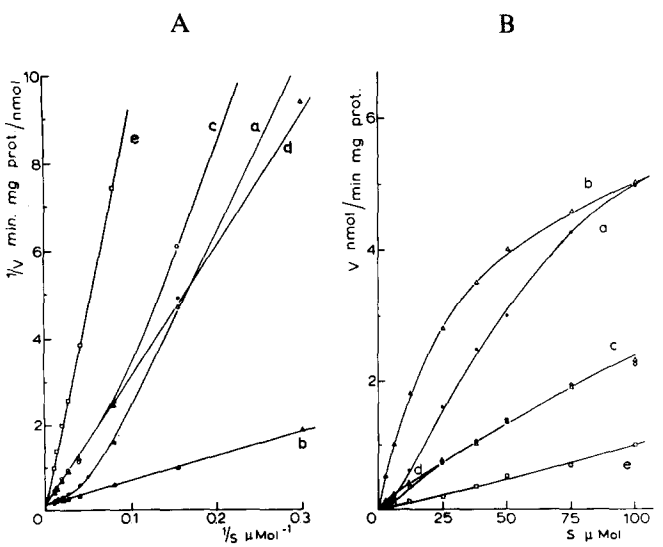

Fig. 4. Lineweaver–Burk plots (Fig. 4A) and substrate saturation plots (Fig. 4B) of cyclic AMP phosphodiesterase activity of a heat-treated 50% saturated $(NH_4)_2SO_4$ fraction (1 mg protein/ml Tris–$MgCl_2$–mercaptoethanol buffer); Curve a (●—●), without additions; Curve b (▲—▲), in the presence of 2 μM cyclic GMP; Curve c (○—○), in the presence of 1 mM theophylline; Curve d (△—△), in the presence of 1 mM theophylline and 2 μM cyclic GMP; Curve e (□—□), in the presence of 5 mM theophylline.

with the V determined by extrapolation of Curve a (Fig. 4A). A non-heated $(NH_4)_2SO_4$ fraction shows identical kinetics at cyclic AMP concentrations of 3–100 μM. Below a concentration of 3 μM the values of the non-heated fraction deviate because of the interference of the low K_m enzyme. The Hill coefficient [7] of the heat-treated $(NH_4)_2SO_4$ fraction was 1.9.

In the presence of 2 μM cyclic GMP the heat-treated 50% $(NH_4)_2SO_4$ fraction shows a straight Lineweaver–Burk curve with a K_m of 25 $\pm$ 5 μM over the cyclic AMP concentration range of 3–100 μM (Fig. 4A, Curve b). When the cyclic GMP concentration was varied between 0 and 2 μM intermediate forms of Curve a and Curve b (Fig. 4) were found. The Hill coefficient in the presence of 2 μM cyclic GMP is 1.0. Substrate velocity plots, which are sigmoidal shaped in the absence of cyclic GMP, obtain the normal hyperbolic shape in the presence of 2 μM cyclic GMP (Fig. 4B, Curve a and Curve b). Theophylline (1 mM) inhibits the cyclic AMP hydrolysis (Fig. 4A and Fig. 4B, Curve c). At a cyclic AMP concentration of 100 μM enzyme activity is inhibited for about 60%. The inhibitor greatly suppresses the activation of the cyclic AMP phosphodiesterase activity by high substrate concentrations (10–100 μM). Moreover 1 mM theophylline greatly abolishes the activating effect of cyclic GMP (Fig. 4A and Fig. 4B, Curve d). Theophylline (5 mM) inhibits the enzyme for about 80% at a cyclic AMP concentration of 100 μM (Fig. 4A and Fig. 4B, Curve e). In the presence of 5 mM theophylline no positive cooperativity of cyclic AMP was detected. The substrate saturation curve has a normal hyperbolic shape. In this case 2 μM cyclic GMP has no effect on the rate of hydrolysis. Imidazole at a final concentration of 40 mM increases the enzyme activity in the absence and presence of 2 μM cyclic GMP by about 50%. The kinetic behaviour was not changed by imidazole at this concentration.

Factors influencing the positive cooperativity and the activation by cyclic GMP of the high K_m cyclic AMP phosphodiesterase

(1) The activity of a heat-treated 50% saturated $(NH_4)_2SO_4$ precipitate (1 mg protein/ml) did not decrease during storage for about 25 h at 4 °C. Moreover the activation by the substrate and by 2 μM cyclic GMP were still the same. A 10-fold dilution of the heat-treated 50% saturated $(NH_4)_2SO_4$ precipitate with Tris–$MgCl_2$–mercaptoethanol buffer resulted in a complete loss of the positive cooperativity by cyclic AMP within 4 h. The phosphodiesterase activity of the 10-fold diluted extract was measured by addition of a sample to the assay medium at various times. The protein concentration in the assay was similar to the undiluted control. The hydrolysis of the substrate showed normal Michaelis–Menten kinetics with a V of 5 $\pm$ 1 nmoles/min per mg protein and a K_m between 100 and 200 μM. The activation by cyclic GMP as measured at a cyclic AMP concentration of 3.3 μM was reduced 5-fold.

(2) The rate of hydrolysis of cyclic AMP (concentration range of 3.3–100 μM) by fractions of Peak II after gel filtration of a non-heated and heated $(NH_4)_2SO_4$ precipitate (Fig. 3A and Fig. 3B, respectively) showed a Michaelis–Menten substrate saturation plot with a K_m between 100 and 200 μM. No activation at high cyclic AMP concentration could be detected and the activation by cyclic GMP as measured at a substrate concentration of 3.3 μM cyclic AMP was reduced 5-fold. After column chromatography the phosphodiesterase activity has become more labile. Even at −80 °C the activity is reduced to 50% within 6 days.

(3) Treatment of the 50% saturated $(NH_4)_2SO_4$ precipitate for 1 h at 60 °C followed by the assay of the cyclic AMP phosphodiesterase activity under the standard conditions showed that the positive cooperativity of the substrate had disappeared and that the activation by cyclic GMP measured at a concentration of 3.3 μM cyclic AMP was reduced 15-fold. This 60 °C heat treatment resulted in a high loss of activity: after 1 h about 10% of the original activity was left.

(4) Incubation of the $(NH_4)_2SO_4$ fraction in the presence of 0.03% sodium dodecylsulfate resulted in a complete loss of both the positive cooperativity of cyclic AMP and the activation by cyclic GMP. Moreover this treatment showed a 80% loss of activity after a 10-min incubation period.

DISCUSSION

Kinetic studies on the cyclic AMP hydrolyzing activity of rat liver cell extracts demonstrated the presence of two apparent K_m values [1, 2, 8, 9]. Principally this phenomenon can be explained by the presence of separate enzymes acting on the same substrate or by a negatively cooperative enzyme as described by Teipel and Koshland [10]. By DEAE-cellulose chromatography of rat liver homogenate Russell et al. [2] demonstrated the presence of two separate forms of cyclic AMP phosphodiesterase activity with different K_m. The data presented in this paper are also in favour of the former explanation. Thus, the low K_m value is irreversibly eliminated after heat treatment of a 50% saturated $(NH_4)_2SO_4$ fraction. Furthermore, heat treatment results in a difference between the cyclic AMP phosphodiesterase elution patterns on a Sepharose 6-B column. The high K_m activity is associated with one activity peak (Fig. 3, Peak II) while the profile of the heat-unstable enzyme (see dotted line in Fig. 3A) shows two separate peaks. Recently Pichard et al. [11] described elimination of the low K_m activity of cyclic AMP phosphodiesterase in human blood platelets by heating for 30 min at 50 °C. The relationship between Peak I and Peak III which both show low K_m phosphodiesterase activity is not clear. Peak I, the exclusion peak, represents 25% of the total heat-unstable activity. It is possible that Peak I represents an aggregate of the smaller molecular weight enzyme represented by Peak III [12]. On the other hand Peak I may represent an aggregate with other molecules since some evidence exists that the low K_m activity is originally membrane bound [9, 13]. However under our conditions of homogenisation hardly any membrane-bound low K_m cyclic AMP phosphodiesterase activity could be detected.

The data presented in this paper strongly favour the concept [2] that the heat-stable high K_m enzyme is an allosteric protein. The kinetic behaviour agrees with the basic properties of allosteric proteins pointed out by Monod et al. [14]. Thus a strong indication for the existence of multiple interacting sites is the homotropic cooperativity of cyclic AMP, which kinetically is seen by the sigmoid shaped substrate saturation curve. Only in a few cases i.e. involvement of irreversible steps in an enzymic mechanism [15] or when a ternary complex is formed in a random order process with two substrate reactions [16], a non-linear kinetic behaviour is seen which could show a sigmoid shaped s versus v plot without the involvement of an allosteric protein. Cyclic GMP in a final concentration of 2 μM activates the high K_m cyclic AMP phosphodiesterase and changes the Hill coefficient from 1.9 to 1.0. Moreover cyclic GMP changes the s versus v plot from a S-shaped to a normal hyperbolic shaped curve. These properties

are quite understandable if cyclic GMP acts as an allosteric activator since an allosteric activator tends to abolish the cooperativity of the substrate [14]. The allosteric character of the high K_m cyclic AMP phosphodiesterase was already suggested by Beavo et al. [1] and was supported by the kinetic studies of Russell et al. [2]. Further arguments in favour of the allosteric character of the high K_m cyclic AMP phosphodiesterase and in favour of the action of cyclic GMP as an allosteric activator can be found in the results observed after the different treatments of the enzyme i.e. dilution of the enzyme, gel filtration over Sepharose 6-B, treatment at 60 °C or addition of 0.03% sodium dodecylsulfate. These treatments may alter the homotropic and heterotropic interactions simultaneously according to the stated properties of allosteric proteins [14]. Thus, after changing the enzyme concentration, a different kinetic behaviour of the glutamate dehydrogenase system suggesting association–dissociation phenomena is described by Tomkins et al. [17]. Okazaki and Kornberg [18] and Jensen et al. [19] reported a different kinetic behaviour after column chromatography of the allosteric proteins deoxythymidine kinase and 3-deoxy-D-arabino heptulosonate 7-phosphate synthetase, respectively.

The loss of allosteric control by 0.03% sodium dodecylsulfate can be compared with the effect of Triton X-100 and Lubrol PX on cyclic AMP phosphodiesterase as reported by Beavo et al. [1]. Recently an effect of sodium dodecylsulfate on the allosteric control of rat liver tryptophan hydroxylase was reported by Sullivan et al. [20]. An effect of temperature on substrate cooperativity has also been reported for tryptophan hydroxylase [20] and by Gerhart and Pardee [21] for aspartate transcarbamylase. However we like to state that partially purified enzyme fractions could give only limited information about the allosteric control of a protein and further purification will be necessary to determine the number and kind of subunits and the properties of enzyme–ligand complexes. The hydrolysis of 100 μM cyclic AMP by the 50% saturated $(NH_4)_2SO_4$ fraction in the presence of theophylline is equally inhibited before and after dilution or gel filtration of the fraction. Our observations are in favour of competitive interaction of theophylline with the substrate site(s) [14, 21]. Moreover theophylline (5 mM) was found to abolish the positive cooperativity of cyclic AMP and the activating effect of cyclic GMP on the high K_m cyclic AMP phosphodiesterase activity. It may be hypothesized that occupation of the substrate site(s) by theophylline may prevent conformation changes and consequently activation by cyclic GMP although our data do not exclude a simultaneous interaction with the allosteric activator site.

Competitive inhibition of the high K_m cyclic AMP phosphodiesterase by theophylline is also reported by Pichard et al. [11] for the phosphodiesterase in human blood platelets and by Schröder and Plagemann [22] for the high K_m phosphodiesterase in cultured Novikoff rat hepatomas, human HeLa and mouse L cells.

Many authors reported the hydrolysis of cyclic GMP by partially purified fractions of the high K_m cyclic AMP phosphodiesterase [2, 23, 24]. Schröder and Rickenberg [25] obtained some evidence that the cyclic GMP and the cyclic AMP phosphodiesterase activities are distinct proteins although an aggregate with both activities may occur. Our data support the existence of distinct proteins: at first a difference in heat stability was observed. The high K_m activity decreases about 10% after heat treatment at 45 °C, mainly due to low K_m activity, whereas the cyclic GMP phosphodiesterase activity decreases by about 25%. Secondly the ratio of the cyclic

AMP hydrolysis and the cyclic GMP hydrolysis in the fraction of the Sepharose 6-B gel filtration varies significantly throughout the profile. These properties differ from the characteristics of the cyclic GMP phosphodiesterase activity reported by Russell et al. [2]. In our opinion further separation techniques will be needed to elucidate these discrepancies.

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