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CYCLIC NUCLEOTIDE PHOSPHODIESTERASE OF RETINAL PHOTORECEPTORS.

PARTIAL PURIFICATION AND SOME PROPERTIES OF THE ENZYME

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Summary

1. A cyclic nucleotide phosphodiesterase (EC 3.1.4.16) has been partially purified from bovine rod outer segments. The enzyme preparation obtained has a very high specific activity towards cyclic GMP and is still able to hydrolyze cyclic AMP. Upon polyacrylamide gel electrophoresis, one major and three minor protein bands are seen, the enzyme activity being associated with the major band. The enzyme eluted from the gels still hydrolyzes both cyclic nucleotides. At all substrate concentrations tested, cyclic GMP was hydrolyzed at a faster rate. The enzyme eluted from the gel columns migrated as a single band upon electrophoresis in 0.1% sodium dodecyl sulfate-polyacrylamide gels corresponding to a molecular weight of 105 000.

2. A complex kinetic pattern was observed for cyclic GMP hydrolysis: the plot of velocity vs substrate concentration was hyperbolic at low and sigmoidal at higher concentrations. By contrast, simple kinetics were observed for cyclic AMP hydrolysis yielding an apparent K_m of 0.1 mM. The unusual kinetics may be implicated in the regulation of cyclic GMP levels in rod outer segments.

3. Cyclic AMP stimulated the hydrolysis of cyclic GMP at low and inhibited it at higher concentrations. Addition of Mg^{2+} appeared to be necessary for optimum activity. The activity measured in the absence of exogenous Mg^{2+} was abolished by EDTA.

Abbreviations: Buffer A, 50 mM Tris/acetic acid (pH 6.0)/3.75 mM β-mercaptoethanol; Buffer B, 50 mM Tris/acetic acid (pH 6.0)/3.75 mM β-mercaptoethanol/0.2 M sodium acetate; EGTA, ethyleneglycol-bis-(2-aminoethyl ether) *N,N'*-tetraacetate.

Introduction

Evidence has accumulated recently implicating cyclic nucleotides, in particular cyclic GMP, in photoreceptor function. Rod outer segments, the organelles of vertebrate black and white vision, contain high activities of guanylate cyclase [1—3] and cyclic nucleotide phosphodiesterase [4—6]. High concentrations of cyclic GMP [7] much in excess of the concentration of cyclic AMP have been found in mouse photoreceptor cells [8]. Furthermore, illumination of intact retinae causes a swift and drastic drop of the cyclic GMP levels [9]. The reported light-inhibition of retinal guanylate cyclase [2,3] and/or the light-dependent activation of cyclic nucleotide phosphodiesterase [5,6,10,11] is probably responsible for that effect. Experiments, where the incorporation of labelled precursor into cyclic GMP has been measured in the presence of a potent phosphodiesterase inhibitor, point to the phosphodiesterase as the main site for light-regulation of cyclic GMP metabolism [6].

Although some conditions for the light-activation of retinal phosphodiesterase have been elucidated [5,6,10,11] the mechanism by which this effect is brought about is not understood in molecular terms. As a first step towards a better understanding of the underlying process, we purified cyclic nucleotide phosphodiesterase from bovine rod outer segments and studied some of the properties of the enzyme. Previous studies of the characteristics of rod outer segment phosphodiesterase have been carried out with whole rods [5,11—13] or with soluble activity from whole retina [12].

The interrelationship between cyclic AMP and cyclic GMP hydrolyzing activities which appear to co-exist in all tissues studied is at present not clear (for references, see ref. 14). The highly purified photoreceptor enzyme which still retains activity towards both nucleotides should be useful for studying this question. Since there exists no consistent nomenclature on cyclic nucleotide phosphodiesterases, we will call the activity measured using cyclic GMP as substrate "cyclic GMP phosphodiesterase activity" and the activity measured using cyclic AMP "cyclic AMP phosphodiesterase activity". This does not infer that there are two separate enzymes which catalyze these reactions.

Methods

I. Preparation of cyclic nucleotide phosphodiesterase from rod outer segments

Extraction from rod outer segments. Rod outer segments were prepared from calf eyes as previously described [15] and all of the following steps were carried out at +4 °C. The packed outer segments obtained from 50 retinae were homogenized in 25 ml of 10 mM Tris · HCl (pH 7.2) and the suspension centrifuged for 1 h. at $10^5 \times g$. The clear supernatant was stored at −180 °C for several weeks without loss of activity and served as enzyme source. Till that stage, all operations were done at dim red light. Immediately before use, the buffer extracts obtained from the rods of 120—160 retinae were pooled and concentrated through UM-10 membranes to a volume of 1.3—1.6 ml.

Ion exchange chromatography. A 0.5 × 5 cm column of DEAE-cellulose was equilibrated with Buffer A, the sample applied and eluted stepwise with increasing concentrations of sodium acetate in Buffer A., 3.5-ml fractions being

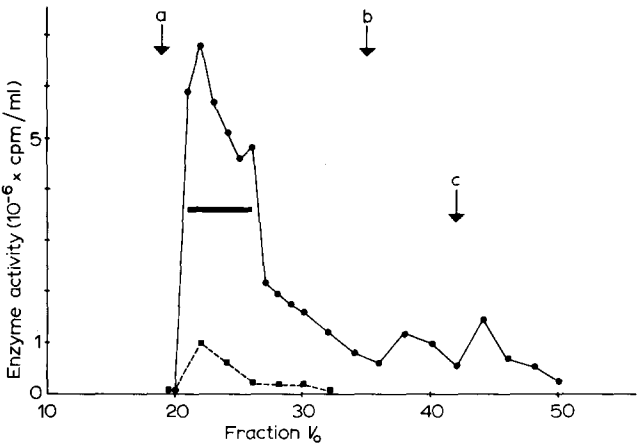

Fig. 1. Ion exchange chromatography of cyclic nucleotide phosphodiesterase from rod outer segments. The concentrated buffer extract (1.6 ml) obtained from rods of 120 retinae was applied to a 0.5 × 5 cm DEAE-cellulose column, equilibrated with Buffer A. Elution was started by applying Buffer A containing 0.1 M sodium acetate. Buffer A with 0.3 M sodium acetate was applied at (a), the sodium acetate concentration was increased to 0.4 and 1.5 M at (b) and (c), respectively. Cyclic nucleotide phosphodiesterase activity was determined with 1 mM cyclic GMP (●———●) or 1 mM cyclic AMP (■- - - - -■) as substrate. The active fractions which were pooled and concentrated are indicated by the horizontal bar.

collected. Phosphodiesterase activity was eluted with 0.3 M sodium acetate. The activity profile is shown in Fig. 1. The most active fractions were pooled and concentrated through UM-10 membranes to a volume of 0.6—0.8 ml.

Gel filtration. The concentrated enzyme fraction from the DEAE-cellulose was applied to a column of Sephadex G-100 (1 × 13.5 cm), which had been equilibrated with Buffer B, and eluted with the same buffer, 1 ml fractions being collected. Enzyme activity was assayed with 1 mM cyclic GMP as substrate (Fig. 2). The peak fractions were pooled, concentrated through UM-10 membranes to a concentration of 0.6—0.9 mg of protein/ml and stored at +4°C.

II. Polyacrylamide gel electrophoresis

Disc gel electrophoresis according to Davis [16] was carried out at pH 8.3 with 2.5% stacking (0.5 × 1.7 cm) and 7.5% running (0.5 × 5.5 cm) gel as described previously [17]. The buffers contained 3.75 mM β-mercaptoethanol. Samples containing 10 to 70 μg of protein in Buffer B containing 15% sucrose were layered onto the gel columns and electrophoresis carried out at 4°C. 0.2 mA per tube were applied till the bromophenol blue marker had penetrated the running gel and 0.5 mA thereafter. The gels were removed when the tracking dye approached the bottom of the gel columns and stained with Coomassie brillant blue. Parallel unstained gels were cut into 2-mm slices, homogenized in Buffer B and the supernatant assayed for phosphodiesterase activity.

For determination of the molecular weight, the enzyme was eluted from polyacrylamide gel as described above and then subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gels according to Weber and Osborn [18]. Bovine serum albumin (monomer, molecular weight 66 000, dimer,

132 000), ovalbumin (molecular weight 46 000) and catalase (monomer, molecular weight 60 000, dimer, 120 000) were used as reference proteins.

III. Assay of phosphodiesterase activity

The assay of cyclic AMP and cyclic GMP phosphodiesterase activities was based on the methods of Thompson and Appleman [19] and Schultz et al. [20]. The incubation mixture contained 80 mM Tris · HCl (pH 8.0); 2 mM $MgSO_4$ and either cyclic [^{3}H]GMP or cyclic [^{3}H] AMP in a final volume of 0.2 ml. Unlabelled cyclic GMP or cyclic AMP was added to give the required concentrations which ranged from $0.1 \cdot 10^{-6}$ to $1 \cdot 10^{-3}$ M. The reaction was initiated by adding suitable amounts of enzyme preparation and left to proceed for 4 min at 30°C. The reaction was terminated by boiling for 3 min. Then, 25 μg of snake venom were added in a volume of 50 μl and the incubation continued for 20 min. 750 μl of 0.1 mM adenosine or guanosine in 20 mM ammonium formate (pH 7.4) were added and the sample applied to a QAE-Sephadex column (0.5 × 3 cm) prepared as previously described [20]. The nucleosides were eluted in 4 ml of 20 mM ammonium formate (pH 7.4) and the radioactivity of the eluate determined by liquid scintillation counting. The values were corrected for recovery by adding [^{3}H]guanosine or [^{3}H]adenosine to samples where the triated cyclic nucleotide had been omitted (recovery for [^{3}H]adenosine and [^{3}H]guanosine 77—87%). Blanks were obtained by using boiled enzyme (3 min at 100°C). For each enzyme preparation, different dilutions were tested to assure that the reaction velocity was proportional to enzyme concentration. All values, where more than 20% of the substrate had been metabolized, were discarded. Enzyme assays were carried out at least in duplicate and results are given as average values.

Protein was measured by the method of Lowry et al. [21] with bovine serum albumin as standard.

IV. Kinetic analysis

For the plot of reaction velocity against substrate concentration $1 \cdot 10^{-6}$—$1 \cdot 10^{-4}$ M cyclic GMP or cyclic AMP were used routinely. Since under our experimental conditions, the fraction of substrate transformed, ranging from 5 to 20%, is not negligible, the mean substrate concentration $S = \frac{1}{2}([S_0] + [S_t])$ was used to compute the velocity substrate-concentration relationship as suggested by Lee and Wilson [22].

Materials

DEAE-cellulose (Servacel, type DEAE 23 SS) was obtained from Serva (Heidelberg G.F.R.), Sephadex-G100 and QAE-Sephadex-A25 from Pharmacia (Uppsala Sweden). Acrylamide and bis-acrylamide were purchased from Eastman Kodak (Rochester U.S.A.). Snake venom (crotalus atrox) and bovine serum albumin were from Sigma (St. Louis U.S.A.), unlabelled nucleotides from Boehringer (Mannheim, G.F.R.). Cyclic[^{3}H]AMP (37.6 Ci/mmol) and cyclic[^{3}H]GMP (2.11 Ci/mmol) were obtained from NEN (Dreieichenhain, G.F.R.). Cyclic[^{3}H]AMP was employed without further purification. Cyclic[^{3}H]GMP was purified by chromatography on QAE-Sephadex-A25 col-

umns. Membranes for ultrafiltration (UM-10 and UM-2) in an Diaflo apparatus were purchased from Amicon (Ivry-sur-Seine, France).

Results

I. Comments on the method of extraction and purification

The cyclic nucleotide phosphodiesterase activity could be extracted from bovine photoreceptor membranes by hyposmotic shock. Over 90% of the recovered activity appeared in the high-speed supernatant (Table I).

A summary of a typical purification is given in Table II. A single hypotonic wash of the rod outer segments was performed, since the enzyme activity was unstable in the highly diluted second wash. The specific activity after gel filtration varied between 1.1 and 7.0 μmol GMP formed/min/mg protein among different preparations. The purified enzyme was sensitive to freezing and thawing and to low osmotic strength. It could however be stored at +4°C for over a month in Buffer B. There was a gradual increase of activity during storage: in the case of the preparation depicted in Table II, the specific activity increased from 3649, if tested immediately after concentration of the Sephadex G-100 eluate, to 7310 in the course of two weeks. For this reason, and because of the concomitant changes of the kinetics (see below) the degree of purification cannot be assessed accurately in terms of enrichment of the specific activity. More indicative is the fact that over 99% of the rod outer segment protein is eliminated during the purification. This figure varied only little among different preparations, despite the fluctuations in enzymatic activity.

II. Polyacrylamide gel electrophoresis: molecular weight

When the purified enzyme preparation was subjected to electrophoresis in 7.5% acrylamide gels, one predominant and three minor bands were observed. Parallel, unstained gels were assayed for cyclic GMP phosphodiesterase activity. A single activity peak was found corresponding to the major band (Fig. 2). The activity profile with 1 mM substrate was superimposable on the one shown in Fig. 2, where 1 μM had been used.

When the enzyme which had been eluted from polyacrylamide gels was

TABLE I

DATA TAKEN FROM A TYPICAL EXTRACTION OF CYCLIC GMP PHOSPHODIESTERASE FROM BOVINE ROD OUTER SEGMENTS

Packed rod outer segments (10 mg of protein) were homogenized in 5 ml of 10 mM Tris · HCl (pH 7.2) and the membranes repelleted (1 h at $10^5 \times g$). The pellet was washed once with 5 ml of the same buffer. 73% of the activity originally present were recovered in the 3 fractions. For calculation of the activities recovered with the three fractions, the total recovered activity was set at 100%. Substrate concentration was 10 μM.

	nmol GMP formed/min/mg protein	Percent recovered activity
Rod outer segments	42.2	—
1st wash	312.4	73.2
2nd wash	226.6	17.1
Remaining pellet	4.0	9.7

TABLE II

DATA TAKEN FROM A TYPICAL PURIFICATION OF CYCLIC GMP PHOSPHODIESTERASE

Cyclic GMP phosphodiesterase activity was determined using 10 μM cyclic GMP as substrate and is expressed as nmol GMP formed/min.

	Total protein mg	Total activity nmol/min	Specific activity nmol/min/mg protein
1. Rod outer segments	94.5	6560	69.4
2. $10^5 \times g$ supernatant	12.1	2328	233
3. DEAE-Cellulose	0.72	2524	3497
4. G-100 Sephadex chromatography	0.25	912	3649

reelectrophoresed in the presence of 0.1% sodium dodecyl sulfate, a single protein band was observed. From gels calibrated with proteins of known molecular weight, its molecular weight was estimated to be 105 000. Approximately the same molecular weight could be estimated when the G-100 Sephadex column used during the purification procedure was calibrated with proteins of known molecular weight.

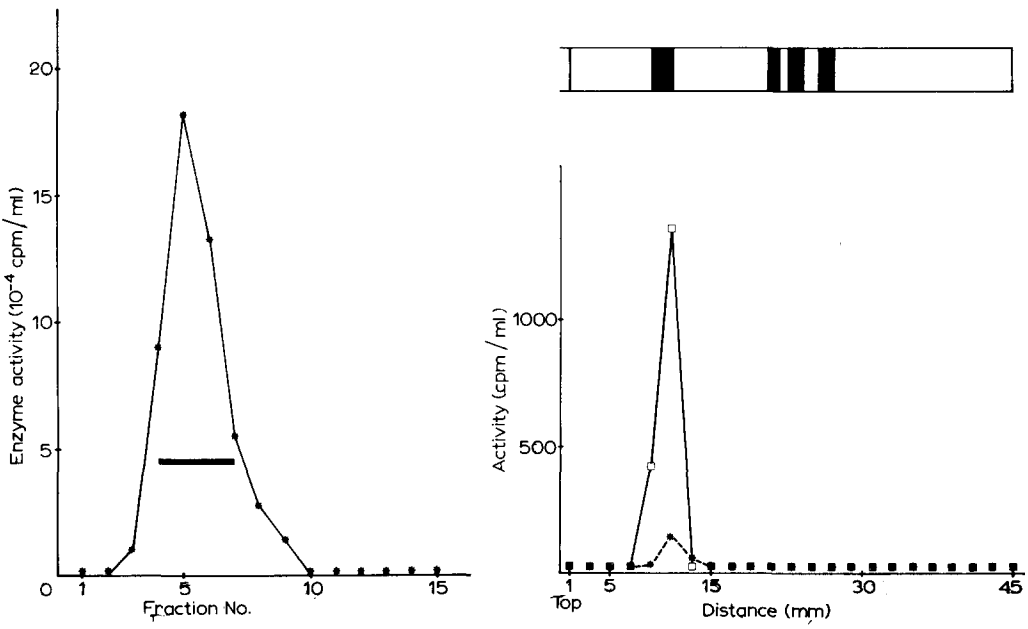

Fig. 2. Gel filtration of cyclic nucleotide phosphodiesterase from rod outer segments. The concentrated enzyme fraction (0.6 ml) from the DEAE-cellulose was applied to a 1 X 13.5 cm Sephadex G-100 column, which had been equilibrated with Buffer B, and eluted with the same buffer, 1 ml fractions being collected. Enzyme activity was assayed with 1 mM cyclic GMP as substrate. The active fractions which were pooled and concentrated are indicated by the horizontal bar.

Fig. 3. Gel electrophoresis of purified phosphodiesterase. Enzyme preparation (12 μg of protein) was applied to the gel columns (2.5% stacking and 7.5% running gels) and subjected to electrophoresis as described in Methods. Some gels were stained with Coomassie brillant blue, a staining profile is shown in the upper part of the figure. Parallel gels were sliced and the cyclic nucleotide phosphodiesterase activity determined in the presence of 1 μM cyclic GMP (□———□) or cyclic AMP (★- - - - -★). The same staining and activity profile has been obtained with two different enzyme preparations.

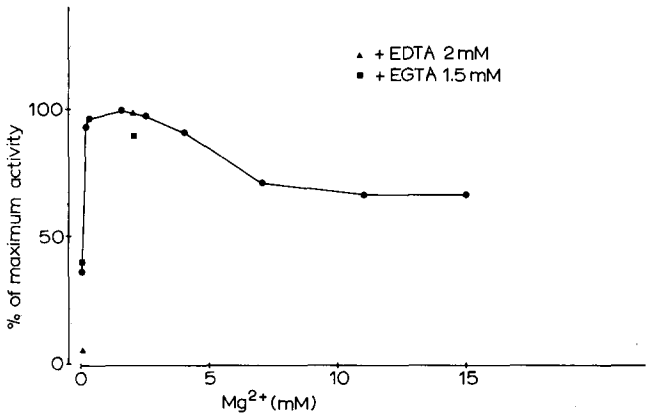

Fig. 4. Effect of Mg^{2+} (●———●), EDTA (▲) and EGTA (■) on cyclic GMP phosphodiesterase activity. Except for the metal ion and the chelating agents, the assay conditions were the same as described in Methods. Substrate concentration was 10 μM. 2 mM EDTA or 1.5 mM EGTA were added either in the absence of added Mg^{2+} or in the presence of 2 mM Mg^{2+}. The activity at 1.5 mM Mg^{2+} (1120 nmol GMP formed/min/mg protein) was taken as 100%. Values presented are means of two experiments which differed less than ±6%.

III. Effect of cations and ATP

The enzyme required Mg^{2+} for full activity. In the absence of added Mg^{2+}, the enzyme showed about one third of its maximum activity (Fig. 4). The residual activity could be nearly completely abolished, when the enzyme was assayed in the presence of 2 mM EDTA without added divalent cations. Addition of 1.5 mM Mg^{2+} gave optimum activity, high concentrations (above 4 mM) being inhibitory. The inhibition by EDTA could be reversed by equimolar concentrations of Mg^{2+} (Fig. 4). EGTA was without appreciable effect on the enzyme activity assayed with or without addition of Mg^{2+} (Fig. 4).

A biphasic effect of ATP on phosphodiesterase activity in whole rod outer segments has been reported [5,13]. No such effect on our purified enzyme preparation could be detected. ATP, added in concentrations from 10^{-10} to 10^{-3} M was without effect on the hydrolysis of 10 μM cyclic GMP.

IV. Kinetic studies

The cyclic nucleotide phosphodiesterase activities from most tissues show non-linear kinetics when a wide range of substrate concentrations is tested. This finding has been explained by the occurrence of different phosphodiesterases (high and low K_m enzyme) [19,23—26] or by a single enzyme showing negative cooperativity [27—29].

When the enzyme purified from rod outer segments was assayed with 1—125 μM cyclic GMP as substrate, the plot of reaction velocity against substrate concentration showed anomalous kinetic behaviour: the curve was hyperbolic at low (<3 μM) and sigmoidal at higher concentrations (Fig. 5). The corresponding Lineweaver-Burk plot is given in Fig. 6. Due to the complex shape of the curve, no estimation of a K_m value was possible.

Upon storage of the enzyme at +4°C, the kinetic behaviour changed gradually, approaching Michaelis-Menten kinetics. After several days, the inter-

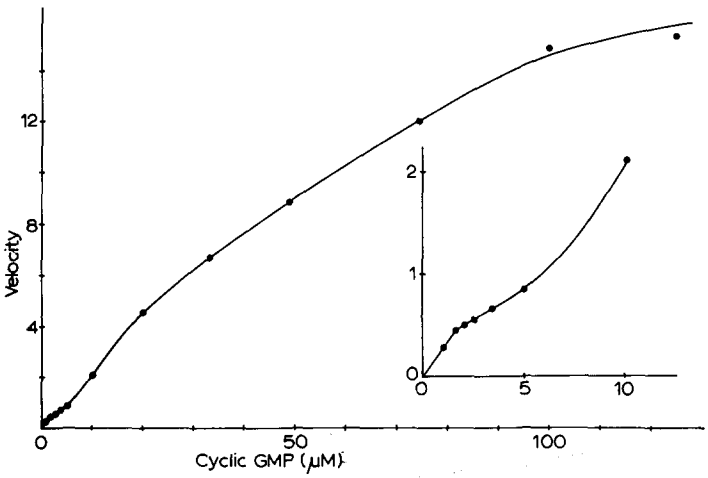

Fig. 5. Saturation plot of cyclic GMP hydrolysis by a freshly purified enzyme preparation. The incubation mixture (0.2 ml) contained 80 mM Tris · HCl (pH 8.0); 2 mM $MgSO_4$ and cyclic[^{3}H]GMP in the concentrations indicated. The reaction (4 min at 30°C) was initiated by addition of 20 μl enzyme in Buffer A containing 75 mM sodium acetate. The unit of velocity is pmol/min/μl enzyme preparation. The mean substrate concentration $\bar{S} = ½ ([S_0] + [S_t])$ was plotted [22]. The smaller panel shows in expanded scale the velocities and substrate levels up to 10 μM. No more than 20% of the cyclic nucleotide initially present was hydrolyzed. The data presented in Figs 5—8 have been obtained on the same enzyme preparation and all points represent mean values of two separate experiments, the duplicates differed less than ±5%. Virtually the same curves as shown in Figs 5—8 were obtained with fresh and aged enzyme from three different preparations despite the fluctuations of the specific activities (1.1 to 7.0 μmol cyclic GMP formed/min/mg protein for 10 μM substrate).

mediary plateau of the saturation plot had disappeared but a small deviation from linearity was still seen in the double-reciprocal plot (Fig. 7). Despite the instability of the kinetic characteristics, the deviation from normal kinetics as shown in Figs. 5 and 6 could be reproduced with three different enzyme preparations.

With cyclic AMP as substrate, fresh or aged preparations displayed normal kinetic behaviour (Fig. 8). From these data an apparent K_m of 0.1 mM can be calculated.

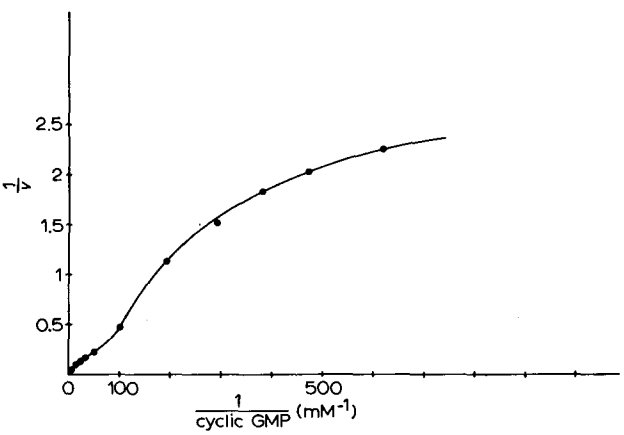

Fig. 6 Lineweaver-Burk plot of cyclic GMP hydrolysis by a freshly prepared enzyme. Experimental conditions and units are the same as described in Fig. 5.

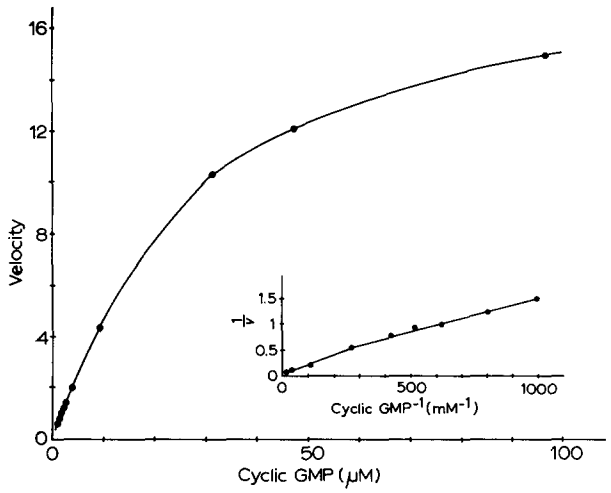

Fig. 7. Saturation plot of cyclic GMP hydrolysis by an aged (1 week at +4°C) enzyme preparation. Experimental conditions and units are as in Fig. 5. The smaller panel shows the corresponding Lineweaver-Burk plot.

V. Interrelationship between cyclic AMP and cyclic GMP hydrolysis

Chromatography on DEAE-cellulose has been used to separate phosphodiesterase activities with different specificities towards the two cyclic nucleotides [24,27]. When the DEAE-cellulose column eluate was assayed with 1 mM cyclic GMP or cyclic AMP, the peak of cyclic AMP hydrolysis was congruent with the peak activity towards cyclic GMP (Fig. 1). A small amount of cyclic GMP hydrolyzing activity could be eluted with increased ionic strength but no activity towards cyclic AMP was detected, most probably because of the much lower activity towards this substrate. Similarly, when the purified preparation was subjected to gel electrophoresis and gel slices were assayed for phosphodi-

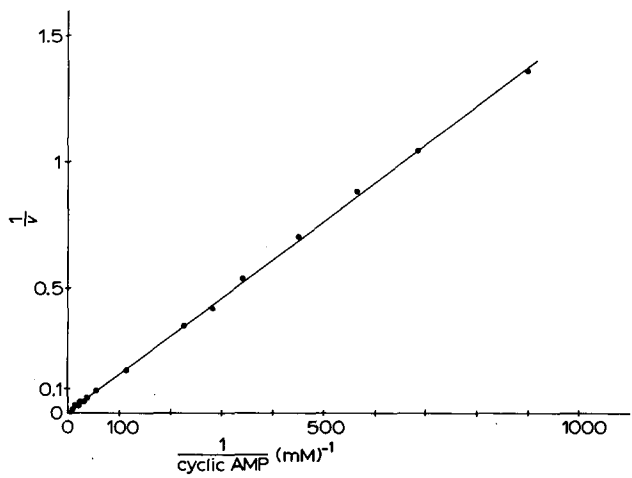

Fig. 8. Lineweaver-Burk plot of cyclic AMP hydrolysis by a freshly purified enzyme preparation. Experimental conditions and units as in Fig. 5, except that cyclic AMP was used as substrate.

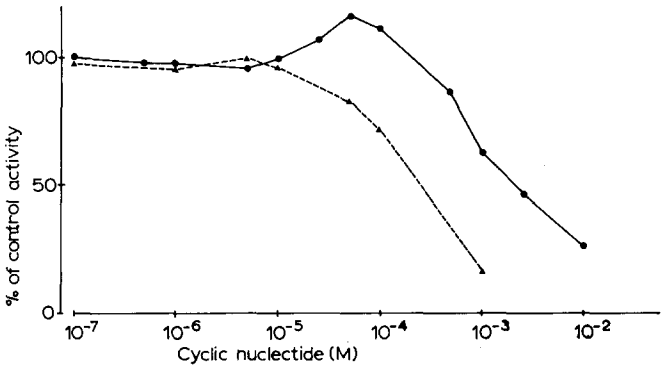

Fig. 9. Effect of increasing concentrations of cyclic AMP on the hydrolysis of 10 μM cyclic GMP (●——●) and of cyclic GMP on the hydrolysis of 10 μM cyclic AMP (▲- - - - -▲). The hydrolysis of 10 μM cyclic[^{3}H]GMP was measured in the presence of the cyclic AMP concentrations indicated. Similarly, enzyme activity with 10 μM cyclic[^{3}H]AMP as substrate was measured in the presence of cyclic GMP. The values obtained in the absence of added unlabelled nucleotide (1350 nmol and 23 nmol AMP formed/min/mg protein) were set at 100%. All points represent mean values of two separate experiments, the duplicates differed less than ±6%.

esterase activity, hydrolysis of cyclic AMP was detectable only in the region of the gel showing activity towards cyclic GMP (Fig. 2).

The unusual kinetics of cyclic GMP hydrolysis and their change with time make it difficult to compare the activities towards cyclic GMP and cyclic AMP, since no reliable estimate of the K_m with cyclic GMP as substrate could be obtained. In the purified enzyme preparation the velocity of cyclic GMP hydrolysis was always considerably greater over the whole range of substrate concentrations tested. In the case depicted in Figs 5 and 8, the ratio of activity towards cyclic GMP or cyclic AMP was 37.3 at 1.1 μM cyclic nucleotide, 22.7 at 5 μM, 33.9 at 25 μM and 22.8 at 90 μM.

Cyclic AMP in 0.1 mM concentration has a small stimulatory effect on the hydrolysis of 10 μM cyclic GMP, concentrations above 0.5 mM were inhibitory (Fig. 9). The highest concentration tested (10 mM) inhibited cyclic GMP hydrolysis 75%. No stimulatory effect of cyclic GMP on the hydrolysis of 10 μM cyclic AMP was observed. Phosphodiesterase activity towards cyclic AMP was inhibited by cyclic GMP concentrations above 50 μM; the inhibition reached 84% at a cyclic GMP concentration of 1 mM.

Discussion

The overwhelming majority of the cyclic nucleotide phosphodiesterase activity associated with rod outer segments appears in the $10^5 \times g$ supernatant after hyposmotic shock and a substantial purification can be obtained by a simple two column procedure. The purified enzyme has a high specific activity (1.1—7.0 μmol cyclic GMP hydrolyzed/min/mg protein at 10 μM substrate), which compares favourably to partially purified preparations from other sources [24,27,30—32] though differences in test conditions and substrate concentrations make an exact comparison difficult.

Rod outer segments can hydrolyze both cyclic GMP and cyclic AMP,

though cyclic GMP appears to be the preferred substrate [4,5,11,12]. The purified phosphodiesterase still hydrolyzed both cyclic nucleotides. At all substrate concentrations tested, the reaction velocity was greater with cyclic GMP than with cyclic AMP. When the enzyme was subjected to polyacrylamide gel electrophoresis, a single peak of activity was detected which could still act on both cyclic nucleotides. In the case of liver, it has been postulated that there are separate phosphodiesterases for cyclic AMP and cyclic GMP, which can aggregate and form a high molecular weight complex with low affinity for both nucleotides [24]. A different situation appears to prevail in rod outer segments: the purified enzyme has a relatively low molecular weight and is highly active with low concentrations of cyclic GMP.

Our results on the influence of cyclic AMP on cyclic GMP hydrolysis and vice versa are in essential agreement with the data obtained by Chader et al. [12] who used the soluble activity from whole retina as enzyme source. Only high concentrations of cyclic AMP (above 0.5 mM) inhibited the hydrolysis of 10 μM cyclic GMP and there was a small stimulatory effect of lower concentrations of cyclic AMP (around 0.1 mM) on cyclic GMP hydrolysis. By contrast, cyclic GMP did not stimulate the hydrolysis of cyclic AMP, but inhibited it already at concentrations above 50 μM.

Freshly prepared enzyme preparations when tested over a wide range of substrate concentrations (1—125 μM) showed a complex kinetic behaviour when cyclic GMP was used as substrate. Chader et al. [12], working with the soluble activity of whole retina reported normal Michaelis-Menten kinetics for cyclic GMP hydrolysis. Interestingly, a similar situation appears to exist in the liver [27], where a partially purified cyclic AMP phosphodiesterase showed anomalous kinetics not seen in the crude homogenate.

Non-linear Lineweaver-Burk plots for cyclic nucleotide hydrolysis have been commonly observed with phosphodiesterase from various sources. Plots of reaction velocity against substrate concentration such as found for cyclic GMP hydrolysis with the purified photoreceptor enzyme have been obtained with cyclic AMP in rat liver [32] and rat adipose tissue [33]. They resemble the curves analyzed by Teipel and Koshland [34]. Following their interpretation, such curves can be obtained if (1) in a mixture of two proteins one shows classical Michaelis-Menten or negatively cooperative and the other one positively cooperative kinetics; (2) in the case of a single protein the substrate induces conformational changes which lead to negative cooperativity at low and positive cooperativity at high concentrations. We tend to favor the second possibility since a single peak of enzymatic activity was obtained upon polyacrylamide gel electrophoresis. A phosphodiesterase showing negative cooperativity at low and positive cooperativity at higher concentrations might be physiologically important for the regulation of photoreceptor cyclic GMP levels: at low concentrations (below around 2 μM), the negatively cooperative kinetics will favor a build-up of cyclic GMP levels, the sigmoidal shape of the velocity vs substrate-concentration plot at higher concentrations will prevent a further rise. It is tempting to speculate that the light-dependent activation of photoreceptor phosphodiesterase [5,6,10,11] is accompanied by a shift to normal kinetics. The "buffering" effect of the unusual kinetic behavior would otherwise prevent the rapid drop of cyclic GMP levels observed [9]. Such a change accompanied

by activation (compare Figs 5 and 7) was seen upon storage of the enzyme preparation. Lineweaver-Burk plots of cyclic GMP phosphodiesterase activity in dark- or light-adapted rod outer segments have been presented [5]. However, Miki et al. [5] do not report activities at concentrations low enough to reveal the unusual kinetic characteristics.

Addition of Mg^{2+} appeared to be essential for optimum enzyme activity. There was appreciable activity without adding Mg^{2+} most likely due to divalent cation tightly bound by the enzyme, since EDTA almost completely abolished this residual activity. A similar dependence on Mg^{2+} has been observed by Chader et al. [12] in whole rods. The inhibition with higher concentrations, however, was seen only with the purified enzyme. ATP appears to be required for the light-stimulation of phosphodiesterase activity in isolated rod outer segments [5,10,11] but not in retinal homogenates [6] and has a biphasic effect on phosphodiesterase activity in illuminated rod outer segments [5,13]. In the purified preparation, ATP had no marked effect.

Rod outer segments contain high activities of cyclic nucleotide phosphodiesterase [2,5,6] which may play a key role in regulating the cyclic nucleotide concentration of these organelles [5,6,10,11]. The studies performed on whole retinae [6], purified rod outer segments [5,10,11,13] or hypotonic extracts derived from them [11] did elucidate some features of the light-regulation of cyclic nucleotide phosphodiesterase. The mechanism, however, by which this effect is brought about remains to be demonstrated. Purified phosphodiesterase showed the properties of a regulatory enzyme which might contribute to this mechanism. Using polyacrylamide gel electrophoresis as additional purification step, an essentially pure enzyme can be obtained (Coquil, J.F. and Goridis, C., unpublished). Work is now in progress to determine the nature of the effector (s) serving as a link between rhodopsin bleaching and changes of phosphodiesterase activity. An understanding of the regulation of the photoreceptor enzyme may lead to more insight into the mechanisms by which phosphodiesterase activity is changed in other systems, where an acute regulation by physiological stimuli has been demonstrated [35,36].

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