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ISOLATION AND PARTIAL CHARACTERIZATION OF A CYCLIC GMP-DEPENDENT CYCLIC GMP-SPECIFIC PHOSPHODIESTERASE FROM *Dictyostelium discoideum*

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The cellular slime mold, *Dictyostelium discoideum*, contains at least two classes of phosphodiesterase activity. One class of enzymes hydrolyses cyclic AMP (cAMP) and cyclic GMP (cGMP) with approximately equal rates. Another enzyme, which is less than 5% of the total activity, specifically hydrolyses cGMP. The cGMP-specific enzyme does not bind to a Con A-Sepharose column, while all the cAMP-hydrolyzing activities are retarded by this column. The cGMP-specific enzyme is activated by low cGMP concentrations (10^{-8} – 10^{-6} M); the enzyme has normal Michaelis-Menten kinetics at high substrate concentrations with a K_m of about 3–6 μ M. The cGMP-binding sites for activation and for catalysis show different cyclic nucleotide specificity, but they are probably located on one protein with a molecular weight of about 70 000. The enzyme is stable only under specific conditions, and the activation property of the enzyme is lost relatively easily. Irreversible modifications occur at temperatures below 0° and above 30°C, and at pH below 6.0. Several other conditions such as high ion concentrations, temperatures just above 0°C and pH above 8.0 lead to reversible modifications of enzyme activity.

Introduction

The amoebae of the cellular slime molds live in the soil where they feed on bacteria. Exhaustion of the food supply induces cell aggregation and the formation of a fruiting body consisting of stalk cells and spores. Vegetative cells are chemotactic to folic acid and pterin [1,2]. Because bacteria excrete these compounds, this chemotactic system has been considered as a food-seeking device [2]. Cell aggregation in *Dictyostelium discoideum* is mediated by chemotaxis [3] to cAMP [4]. Cell aggregation in other species is also mediated by chemotaxis, however, cells are not attracted to cAMP, but to other chemoattractants, called

acrasins. The acrasins of *D. lacteum* [5] and *Polysphondylium violaceum* [6] have been purified and partially characterized.

All these chemoattractants induce a similar transient cGMP accumulation in sensitive cells [7–11]; cGMP levels rise within 2 s, peak after 10 s, and basal levels are recovered at about 30 s after addition of a chemoattractant to a cell suspension. The involvement of cGMP during transduction of chemotactic signals is further suggested by the observation that mutants with altered chemotactic behavior have altered cGMP metabolism [12,13].

cGMP levels rise after chemotactic stimulation due to an activation of guanylate cyclase activity [14]. Not more than 20% of the intracellular cGMP is secreted, which suggests that intracellular cGMP declines mainly by intracellular degradation [15]. Two intracellular phosphodiesterases have been described. One enzyme hydrolyses cAMP about

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Abbreviation: cAMP, cyclic AMP, etc.

3-times faster than cGMP, and is strongly inhibited by dithiothreitol [16,19]. This enzyme is further referred to as nonspecific phosphodiesterase. A second intracellular enzyme is far more specific for cGMP than for cAMP, and is only slightly inhibited by dithiothreitol [9,17]. Mato et al. [9] have shown that the cGMP-specific enzyme possesses positive cooperativity, while Dicou and Brachet [17] observed normal Michaelis-Menten kinetics with a K_m of about 3–5 μ M. Recently, we also observed positive cooperativity at low substrate concentrations (between 10^{-8} and 10^{-6} M), and approximately Michaelis-Menten kinetics at high substrate concentrations (above 10^{-6} M cGMP) with an apparent K_m of about 10^{-5} M [18]. This property was described as activation; low cGMP concentrations activate the enzyme, and complete activation is achieved by micromolar cGMP concentrations.

Activation of the hydrolysis of cGMP by cGMP might be a key element in the signal-transduction pathway. Preliminary results showed that the activation process was very labile; this has prohibited a complete separation of the cGMP-specific enzyme from nonspecific phosphodiesterase. In this report we describe a rapid isolation procedure for a cGMP-specific phosphodiesterase devoid of any cAMP-hydrolyzing activity. The enzyme, and its activation by cGMP, is partially characterized.

Materials and Methods

Materials. cGMP, cAMP, cIMP and 8-bromo-cGMP were purchased from Boehringer; snake venom (*Ophiophagus hannah*) and dithiothreitol were obtained from Sigma. Con A-Sepharose was from Pharmacia; [^{3} H]cGMP (0.55 TBq/mmol) and [^{3} H]cAMP (0.9 TBq/mmol) were from Amersham, and Ultragel AcA 34 was from LKB.

Culture conditions. *D. discoideum*, NC-4(H), was grown in association with *Escherichia coli* B/r on a medium containing 3.3 g peptone, 3.3 g glucose, 4.5 g KH_2PO_4 , 1.5 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 15 g agar per l. Cells were harvested in the late-log phase with 10 mM sodium potassium phosphate buffer, pH 6.5, and freed from bacteria by repeated washing and centrifugation at $100 \times g$ for 4 min. Cells were starved by shaking in 10 mM phosphate buffer, pH 6.5, at a density of 10^7 cells

per ml. After 2 h, cells were centrifuged, washed twice with 10 mM phosphate buffer, pH 7.2, and suspended in this buffer at a density of 10^8 cells per ml.

Isolation of cGMP-specific phosphodiesterase. All experiments were done at 0–4°C. Cells were homogenized by sonicating twice for 5 s with a 30 s cooling period in between (Branson B12 with microtip; 50 W). The homogenate was centrifuged at $8000 \times g$ for 1 min, and the supernatant at $48000 \times g$ for 30 min. The supernatant (2 ml) was applied to a Con A-Sepharose column (120×9 mm inner diameter) and the column was eluted with 10 mM phosphate buffer, pH 7.2. The fractions eluting between 6 and 16 ml were combined. The pooled fractions were concentrated on Minicon B15 (Amicon, Oosterhout, The Netherlands) when high protein concentrations were required. The isolation procedure is completed within 2 h.

Phosphodiesterase assay. Unless mentioned otherwise, the incubations at 22°C contained in a total volume of 400 μ l, 10 mM phosphate buffer (pH 7.2), 1 mM MgCl_2 , 10^{-6} or 10^{-8} M [^{3} H]cGMP (approx. 2 kBq per incubation) and 200 μ l phosphodiesterase. The phosphodiesterase was diluted, if necessary, to assure hydrolysis between 5 and 25%. The reaction was terminated after 30 min by boiling the sample for 2 min. After cooling, 100 μ l snake venom (50 μ g) were added and samples were incubated at 36°C during 30 min, followed by the addition of 1 ml anion exchanger (1 part Dowex AG1-X2 and 2 parts water, pH 5.0). Samples were shaken for 2 min, centrifuged at $8000 \times g$ for 1 min, and the radioactivity in 500 μ l of supernatant was determined. Phosphodiesterase was assayed in duplicate. Experiments were performed at least four times; typical experiments are shown. Protein concentrations were determined by the method of Lowry et al. [19]. One unit of phosphodiesterase activity is expressed as 1 μ mol of cGMP or cAMP hydrolysed/min.

Results

The rate of a reaction catalysed by an enzyme with Michaelis-Menten kinetics is given by:

$$v = V_{\max} \frac{[S]}{[S] + K_m} \quad \text{or} \quad \frac{v}{[S]} = \frac{V_{\max}}{[S] + K_m} \quad (1)$$

where [S] is the substrate concentration. This equation shows that $v[S]^{-1}$ is a monotonically decreasing function of [S]. Previously [18], we have observed that $v[S]^{-1}$ of the degradation of cGMP increased between 10^{-8} and 10^{-6} M cGMP. The maximum value of $v[S]^{-1}$ is obtained at 10^{-6} M cGMP; at higher substrate concentrations $v[S]^{-1}$ decreases according to Eqn. 1. These observations show that low cGMP concentrations activate the enzyme, that the enzyme is almost completely activated at 10^{-6} M cGMP, and that at higher cGMP concentrations the activated phosphodiesterase has normal Michaelis-Menten kinetics. The ratio of $v[S]^{-1}$ at 10^{-6} M cGMP and $v[S]^{-1}$ at 10^{-8} M cGMP is called the activation ratio. Values above 1 indicate activation by cGMP.

Isolation of a cGMP-specific phosphodiesterase

A homogenate of *D. discoideum* cells hydrolyzes cAMP and cGMP with approximately equal rates, and the activation ratio is below 1 (Table I). Dithiothreitol is an inhibitor of nonspecific phosphodiesterase in *D. discoideum* [16]. Addition of dithiothreitol to a homogenate results in a strong inhibition of the hydrolysis of cAMP, and in a less strong reduction of the hydrolysis of cGMP; the activation ratio is above 1 (Table I). Thus, addi-

tion of dithiothreitol reveals the presence of a phosphodiesterase which is more specific for cGMP than for cAMP, and which is activated by cGMP. After centrifugation this enzyme is present in the $48\,000 \times g$ supernatant (Table I). Concanavalin A binds to nonspecific phosphodiesterase of *D. discoideum* [20]. Therefore, the $48\,000 \times g$ supernatant was passed through a Con A-Sepharose column (Fig. 1). The eluent hydrolyses only cGMP and not cAMP. Elution of the column thereafter with 0.1 M α -methyl-D-mannoside elutes the nonspecific phosphodiesterase, but no additional cGMP-specific phosphodiesterase is eluted (data not shown). After concentration of fractions 6–16 by Minocon B15 the cGMP-specific phosphodiesterase still has an activation ratio above 1 (Table I). The activity of the isolated phosphodiesterase is linear with incubation time as long as less than 40% of the substrate is hydrolysed (Fig. 2A). Activity is linear with protein concentration at both substrate concentrations, and the activation ratio remains constant upon dilution of the enzyme (Fig. 2B).

We have used this preparation in the subsequent experiments, since further purification of the enzyme failed due to loss of activity and/or activation properties. As an example, the enzyme

TABLE I

ISOLATION OF A cGMP-DEPENDENT cGMP-SPECIFIC PHOSPHODIESTERASE FROM $3 \cdot 10^8$ *D. DISCOIDEUM* CELLS

A homogenate (3 ml) of $3 \cdot 10^8$ cells was centrifuged during 1 h at $48\,000 \times g$. The pellet was washed once with 3 ml phosphate buffer, pH 7.2. The newly obtained pellet was assayed for phosphodiesterase activity. The first supernatant was centrifuged again, and a portion of the newly obtained supernatant was assayed for phosphodiesterase activity. The other part was chromatographed on a Con A-Sepharose column (see Fig. 1) and fractions 6–16 were combined. A portion was assayed for phosphodiesterase activity. The other part was concentrated by Minicon B15 till 1 ml.

Fraction	Protein (mg)	Dithiothreitol	Relative activity ($\text{U} \cdot \text{mg}^{-1} \cdot \text{M}^{-1}$)			Activation ratio
			cAMP (10^{-6} M)	cGMP (10^{-6} M)	cGMP (10^{-8} M)	
Homogenate	18.4	–	98.4	97.8	113.7	0.86
		+	3.7	22.3	12.3	1.81
Pellet	1.0	–	285	296	348	0.85
		+	16.0	10.1	12.2	0.83
Supernatant	15.1	–	124	112	134	0.83
		+	4.1	18.5	10.6	1.71
Con A-Sepharose	11.6	–	< 1 ^a	38.6	15.1	2.54
Amicon B15	9.0	–	< 0.1 ^a	36.1	14.5	2.48

^a Phosphodiesterase activity below the detection level.

Fig. 1. Separation of cGMP phosphodiesterase from non-specific phosphodiesterase (PDE) by Con A-Sepharose column chromatography. 2 ml of the $48000 \times g$ supernatant from $2 \cdot 10^8$ cells were applied to a Con A-Sepharose column (120 $\times$ 9 mm inner diameter). The column was eluted with 10 mM phosphate buffer, pH 7.2, at a flow rate of 25 ml/h and collected in 1-ml fractions. The activity was assayed with 10^{-6} M [³H]cAMP (■), 10^{-8} M [³H]cGMP (○), and with 10^{-6} M [³H]cGMP (●). The activity is expressed as $v[S]^{-1}$ in units $\cdot$ ml⁻¹ $\cdot$ M⁻¹. The activation ratio (▲) is defined as the activity at 10^{-6} M cGMP/activity at 10^{-8} M cGMP (see text).

activity passes unaltered through a Dowex AG1-X2 column in the presence of 0.3 M NaCl. The activity is lost if the enzyme is applied to the column in the absence of NaCl even if the column is immediately eluted with 0.3 or 0.6 M NaCl (data not shown).

A cGMP-dependent cGMP-specific phosphodiesterase with similar properties has also been found in other strains of *D. discoideum* such as AX2 and AX3, and in other species such as *D. lacteum* (data not shown).

Kinetics

The hydrolysis of cGMP by the cGMP-specific phosphodiesterase was measured at substrate concentrations between $3 \cdot 10^{-10}$ and 10^{-4} M. The Eadie-Hofstee plot (Fig. 3A) reveals that the enzyme possesses Michaelis-Menten kinetics at substrate concentrations above 10^{-6} M. Between 10^{-8} and 10^{-6} M cGMP, the enzyme is activated about 3-fold by cGMP. Half-maximal activation occurs at approx. 10^{-7} M cGMP. The K_m of the activated enzyme varies between 3 and 6 μ M and the V_{max}

Fig. 2. Linearity of enzyme activity with incubation time and protein concentration. The isolated cGMP phosphodiesterase (PDE) was concentrated by Minicon B15 to 5 ml (1 mg protein/ml). Enzyme activity was assayed at 10^{-8} and 10^{-6} M [³H]cGMP at different protein concentrations, and during different incubation times. (A) Enzyme activity measured at 10^{-6} M [³H]cGMP with different protein concentrations per incubation: 200 (●), 100 (○), 50 (▲), 25 (△), 12.5 (■), 6.25 μ g (□). (B) Enzyme activity measured at 10^{-6} M cGMP (●) and at 10^{-8} M cGMP (○). Hydrolysis was between 5 and 25%. Activation ratio (▲) is the relative activity at 10^{-6} M/relative activity at 10^{-8} M.

Fig. 3. Equilibrium kinetics of the cGMP phosphodiesterase. The cGMP phosphodiesterase was isolated by Con A-Sepharose column chromatography. Enzyme activity was measured at different cGMP concentrations ($3 \cdot 10^{-10}$ – 10^{-4} M). (A) Eadie-Hofstee plot. Arrows and figures indicate the substrate concentrations. Activity (v) expressed as μmol cGMP hydrolysed/min per mg protein. (B) Hill plot of the same data. v in $\log[v/(V-v)]$ is the maximal velocity.

between 350 and 425 pmol/min per mg protein, which corresponds with approx. 60–80 pmol/min per 10^7 cells. The Hill plot (Fig. 3B) reveals a Hill coefficient of 1.03 at cGMP concentrations below 10^{-8} and above 10^{-6} M. The Hill coefficient is 1.32 at concentrations between 10^{-8} and 10^{-6} M cGMP.

The results indicate that the cGMP-specific phosphodiesterase can exist in two conformations; one with low catalytic activity, and one with high catalytic activity. The transition of the low-activity conformation to the high-activity conformation is mediated by cGMP.

Partial characterization

Activation of the enzyme by cGMP might be an artefact if the enzyme degrades during the incubation, and if cGMP would prevent this degradation. This is unlikely, since the enzyme is stable at 22°C during at least 2 h. Furthermore, preincubation of the enzyme with 10^{-6} M cGMP during 2 h, fol-

lowed by removal of cGMP, did not reveal any effect on the enzyme activity measured thereafter at 10^{-8} and 10^{-6} M [^3H]cGMP (data not shown). Thus, activation of the enzyme by cGMP is reversible.

Molecular weight. The isolated cGMP-dependent cGMP-specific phosphodiesterase was chromatographed by gel filtration on Ultragel ACA 34 (Fig. 4). One peak with enzyme activity is eluted from the column, and the activation ratio is constant throughout the peak. The molecular weight is about 70 000. About 50% of the enzyme activity passes through Minicon B₁₂₅ filters which have a molecular weight cutoff of about 125 000. This also suggests a molecular weight of about 60 000–70 000. Approximately the same value has been found previously by Dicou and Brachet [17] for a cGMP phosphodiesterase not activated by cGMP.

Concentration of NaCl. The effect of NaCl concentration on the properties of the cGMP phos-

phodiesterase were investigated during storage of the enzyme, and during the incubation with cGMP (Fig. 5). Storage of the enzyme at different NaCl

Fig. 4. Chromatography of the cGMP phosphodiesterase (PDE) by gel filtration. Fractions 6–16 from the Con A-Sepharose column were concentrated by Minicon B15 to 0.5 ml. This preparation was applied to an Ultragel AcA column (220 × 16 mm inner diameter). The column was eluted with 10 mM phosphate buffer, pH 7.2, at a flow rate of 45 ml/h and collected in 0.5-ml fractions. The enzyme activity was assayed at 10^{-6} M [^{3} H]cGMP (●) and at 10^{-8} M [^{3} H]cGMP (○); (▲) activation ratio. Arrows indicate the elution of ferritin (450 kDa, V_0), bovine serum albumin (68 kDa) and chymotrypsinogen A (25 kDa, V_i).

Fig. 5. Effect of NaCl concentration on the cGMP phosphodiesterase (PDE). (A) The isolated cGMP phosphodiesterase was concentrated by ultrafiltration to 1 ml. Aliquots (20 μ l) were incubated (22°C) at different NaCl concentrations in a total volume of 40 μ l. After 30 min, the NaCl concentration was adjusted to 0.1 M in a total volume of 0.2 ml, and the enzyme was incubated with [^{3} H]cGMP for 30 min in a total volume of 0.4 ml. (B) The isolated phosphodiesterase was incubated with [^{3} H]cGMP in the presence of different NaCl concentrations for 30 min. At the end of the incubation period, the NaCl concentration was adjusted to 0.25 M, then the reaction was terminated by boiling. (●) Relative phosphodiesterase activity at 10^{-6} M cGMP; (○), relative activity at 10^{-8} M cGMP; (▲) activation ratio.

concentrations during 30 min and detection of the enzyme activity thereafter at 0.05 M NaCl does not reveal any effect on kinetic properties of the enzyme (Fig. 5A). Detection of the enzyme activity in the presence of different NaCl concentrations shows an inhibition of the hydrolysis of cGMP at higher ion concentration (Fig. 5B). The activation ratio slightly increases at higher ion concentrations. This indicates that the catalytic reaction is impaired by NaCl, but that activation of the enzyme is relatively unaltered. Similar results were obtained with other salts such as KCl, KNO₃ and LiCl (data not shown).

Temperature. The enzyme was stored at -16 and $+3^{\circ}\text{C}$, followed by detection of the activity at 22°C (Fig. 6A). The enzyme is relatively stable at $+3$ but not at -16°C . Storage of the enzyme at -16°C does not alter the activity when measured at 10^{-8} M; however, the activity is reduced when assayed at 10^{-6} M cGMP. The activation ratio becomes 1, and the enzyme has approximately Michaelis-Menten kinetics after storage for 1 day at -16°C . Since the activity at 10^{-8} M is unaltered, the enzyme is probably in the low-activity conformation. Apparently, the enzyme is irreversibly modified at -16°C by which cGMP is no longer able to activate the enzyme.

Storage of the enzyme at higher temperatures and detection of the activity at 22°C show other effects (Fig. 6B). The activity at 10^{-6} M cGMP is relatively constant, while that at 10^{-8} M cGMP increases by a preincubation of the enzyme at temperatures up to 40°C . This suggests that a short incubation of the enzyme at higher temperatures leads to an irreversible modification to the high-activity conformation. Preincubation of the enzyme at still higher temperatures leads to the loss of the enzyme activity.

The activity was also assayed at different temperatures (Fig. 6C). Although the enzyme is stable at 4°C , activation by cGMP does not take place at low temperatures. The optimal temperature seems to be about 30°C ; however, the properties of the enzyme change at this temperature (see Fig. 6B).

Proton concentration. Storage of the cGMP-phosphodiesterase at different pH values during 30 min followed by measurement of phosphodiesterase activity at pH 7.2 (Fig. 7A) reveal that the enzyme is stable at pH values above 7.2.

The enzyme is not stable at pH values below 7.2. Activity is lost slightly more at 10^{-6} than at 10^{-8} M cGMP, which suggests that the activation property of the enzyme is also instable at pH below 7.2.

Variation of the pH during the hydrolysis of cGMP shows other effects (Fig. 7B). Activity decreases at lower pH values, but activation remains approximately constant. The optimal pH for the catalytic reaction is about pH 8.0; however, activation by cGMP decreases at higher pH values. The complete kinetics (cf. Fig. 3) at pH 7.2 and 8.0 reveal that the V_{max} and the K_{m} of the activated enzyme are the same at both pH values. Also, the cGMP concentration which induces half-maximal activation is not altered by the pH. The only difference was the catalytic activity at low cGMP concentration, which is increased at higher pH values (data not shown).

Specificity

Previously, we have shown that the cyclic nucleotide specificity for activation of the enzyme differs from the specificity for binding to the catalytic site [18]. Those experiments were carried out with the $48\,000 \times g$ supernatant in which the non-specific phosphodiesterase was inhibited by dithiothreitol. The more pure preparation used in the foregoing experiments has approximately the same specificity as shown before. 8-bromo-cGMP is a very potent activator of the cGMP hydrolysis, while cAMP and cIMP do not activate the enzyme (Fig. 8A). In Fig. 8B, the enzyme is activated by 10^{-6} M cGMP, and the properties of the cyclic nucleotides for competition with cGMP to occupy the catalytic site are shown. cIMP has a high affinity for the catalytic site, 8-bromo-cGMP binds only at higher concentrations, and cAMP does not bind to the catalytic site. In Fig. 8C, the enzyme is activated by $3 \cdot 10^{-7}$ M 8-bromo-cGMP and the hydrolysis of the cyclic nucleotides at 10^{-5} M was measured by high-performance liquid chromatography. cIMP is hydrolysed at about the same rate as cGMP; 8-bromo-cGMP is hydrolysed more slowly, while cAMP is not hydrolysed by the activated enzyme. Clearly, activation and catalysis occur at different binding sites of the enzyme.

Activation mechanism. Fig. 8 reveals that the enzyme (complex) has at least two different bind-

Fig. 6. Effect of temperature on the cGMP phosphodiesterase. (A) The cGMP phosphodiesterase (PDE) was stored at +3 and -16°C. After different times the enzyme activity was measured (at 22°C) at 10⁻⁶ and 10⁻⁸ M cGMP. (●) Stored at 3°C, measured at 10⁻⁶ M; (○) stored at +3°C, measured at 10⁻⁸ M; (■) stored at -16°C, measured at 10⁻⁶ M; (□) stored at -16°C, measured at 10⁻⁸ M; (▲) activation ratio for enzyme stored at +3°C; (△) activation ratio for enzyme stored at -16°C. (B) The cGMP phosphodiesterase was stored at different temperatures during 30 min, followed by the detection of enzyme activity at 22°C. The hydrolysis was measured at 10⁻⁶ M cGMP (●) and at 10⁻⁸ M cGMP (○). (▲) Activation ratio. (C) The activity of the cGMP phosphodiesterase was measured at different temperatures. The incubation time was 30 min, and the substrate concentration was 10⁻⁶ M cGMP (●) or 10⁻⁸ M cGMP (○). Activation ratio (▲).

Fig. 7. Effect of pH on the cGMP phosphodiesterase (PDE). The isolated cGMP phosphodiesterase was concentrated to 0.2 ml. The concentrate was diluted with 0.1 mM phosphate buffer, pH 7.4, to 10 ml, and the pH was adjusted in 1-ml fractions with KH_2PO_4 or Na_2HPO_4 to the indicated values. Then samples were split in two 0.5-ml fractions. (A) The preparations were stored at 22°C during 30 min. Then the pH was adjusted to pH 7.2 ± 0.05 and homogenates were used in the phosphodiesterase assay. (B) Preparations were used immediately in the phosphodiesterase assay. At the end of the incubation period (30 min) the pH was adjusted to pH 7.2 ± 0.1 and samples were boiled. The ion concentration in the assay varied between 7.5 and 2.5 mM phosphate, and the incubation volume was 0.2 ml. (●) Relative phosphodiesterase activity at 10⁻⁶ M cGMP; (○), relative activity at 10⁻⁸ M cGMP; (▲) activation ratio.

Fig. 8. Cyclic nucleotide specificity of the cGMP phosphodiesterase (PDE). (A) The cGMP phosphodiesterase was incubated at 10^{-8} M [^3H]cGMP with different concentrations cGMP (●), cAMP (○), cIMP (▲) or 8-bromo-cGMP (Δ). The incubation time was 15 and 30 min; relative phosphodiesterase activity is expressed as units cGMP hydrolysed/mg protein per concentration cGMP. (B) The same experiment as in A, except that the enzyme activity was measured at 10^{-6} M [^3H]cGMP. (C) The cGMP phosphodiesterase was concentrated by Minicon B15 to 0.4 ml. The incubation mixture (25 μl) contained 10 mM phosphate buffer, pH 7.2, $3 \cdot 10^{-7}$ M 8-bromo-cGMP, 10^{-5} M cyclic nucleotide and 10 μl enzyme. The reactions were terminated after different times by the addition of 35 μl of 0.01 M phosphoric acid. Samples were centrifuged at $8000 \times g$ during 5 min. The supernatant (40 μl) was analysed by high-performance liquid chromatography on the stationary phase, Lichrosorp 10 RP 18 (Merck), with the mobile phase, 5 mM phosphate buffer, 7.5% methanol, pH 3.0.

ing sites for cGMP, one for activation of the enzyme, and one for hydrolysis of cGMP. The phosphodiesterase may be activated by one of the following mechanisms. (A) The enzyme has two binding sites for cGMP on the same protein; binding of cGMP to the activation site induces a conformational change in the enzyme leading to an increase in the catalytic activity. (B) The enzyme with high activity P^* interacts with an inhibitor I leading to a PI complex with low activity. cGMP induces the dissociation of the PI complex leading to activation of the enzyme. The binding site of cGMP for activation might be located on the enzyme or on the inhibitor. (C) cGMP binds to an activator protein A. This activation complex interacts with the phosphodiesterase with low activity leading to a complex with high activity.

Separation of the isolated cGMP phosphodiesterase by gel filtration revealed the elution of one peak with enzymatic activity. The activation ratio was constant throughout the activity peak (Fig. 4) which suggests a homogeneous enzyme preparation (model A). This is also suggested

by the observation that the activation ratio does not change upon dilution of the enzyme (Fig. 2B). Furthermore, we have not been able to demonstrate activating or inhibiting properties in preparations which do not have enzyme activity (e.g., column fractions and briefly boiled homogenates). Finally, we observed that repeatedly washing of the enzyme by ultrafiltration (molecular weight cutoff 15000 and 125000) does not change the activation ratio. The activation ratio and the recovery of enzyme activity are not changed when cGMP is included in the washing buffer if compared to the washing buffer without cGMP (data not shown). This indicates that cGMP does not alter the molecular weight of the enzyme, and that hydrolysis of cGMP and activation by cGMP probably take place at one protein molecule (model A).

Discussion

Chemoattractants induce a transient accumulation of cGMP levels in sensitive cells of the cellu-

lar slime molds [7–11]. cGMP levels reach a peak at about 10 s after the onset of stimulation, and prestimulated levels are recovered after about 30 s. Evidence is accumulating that cGMP may have a function during chemosensory transduction in the cellular slime molds [7–13].

In this report we presented evidence for an intracellular phosphodiesterase which specifically hydrolyses cGMP and which is activated by low cGMP concentrations (10^{-8} – 10^{-6} M). Such cGMP concentrations are achieved during the chemoattractant mediated cGMP accumulation in vivo [7–11]. Therefore, activation of the enzyme by cGMP might be an essential property of the cGMP-specific phosphodiesterase. The $V_{\max}$ of the enzyme (about 70 pmol/min per 10^7 cells) is sufficiently high to be involved in the degradation of the intracellular cGMP accumulation.

The results show that the cGMP-binding sites for activation and for catalysis are different; they are probably located on one protein of molecular weight about 70 000. The enzyme, and especially the activation process, are rather unstable. Irreversible modifications occur at temperatures below 0°C, at temperatures above 30°C, and at pH below 6.0. Several other conditions modify the enzyme activity; however, these changes are reversible: high ion concentrations, temperatures slightly above 0°C, and pH above 8.0. Activation of the *D. discoideum* enzyme by cGMP has not been observed previously [17], which might be due to the instability of the activation process.

cGMP-dependent phosphodiesterases have been found in other organisms [21–25]. Contrary to the cellular slime molds, these enzymes also hydrolyse cAMP. A cGMP-specific phosphodiesterase has been demonstrated in bovine rod outer segments [26,27]. However, this enzyme is not activated by cGMP.

The function of the cGMP-dependent cGMP phosphodiesterase in the cellular slime molds is most likely the destruction of intracellular cGMP which accumulates after chemotactic stimulation. Activation of the enzyme by cGMP may sharpen the cGMP peak. Therefore, activation might be involved in the dynamics of the transduction of chemotactic signals. Recently, Ross and Newell [13] have described mutants with altered cGMP metabolism. Their cells react to chemotactic sig-

nals for a longer period, and cGMP levels decline to basal levels more slowly. Many biochemical properties of these mutant cells are similar to those of wild-type cells [13], except very low levels of the cGMP-specific phosphodiesterase [28]. This may indicate the function of the cGMP-dependent cGMP-specific phosphodiesterase during chemosensory transduction in the cellular slime molds.

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