INDEPENDENT cAMP AND cGMP PHOSPHODIESTERASES IN *BLASTOCLADIELLA EMERSONII*

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1. Introduction

cAMP+ and cGMP+ are now recognized as key intracellular regulators in several biological functions, including growth and morphogenesis [1,2]. cAMP levels are low during logarithmic growth but rise when contact inhibited cells reach confluency and stop growing [3,4]. The other naturally occurring cyclic nucleotide, cGMP, might also be involved in cell growth regulation, and evidence for an opposing influence of this nucleotide to cAMP, in at least certain stages of growth, has recently been uncovered [5,6].

The intracellular levels of cyclic nucleotides are controlled by synthesis via adenylate or guanylate cyclases, and by degradation via cyclic nucleotide phosphodiesterases. In many cell types there are at least two and perhaps more forms of phosphodiesterases [7]. Chicken embryonic fibroblasts possess cAMP and cGMP phosphodiesterase activities under separate genetic control [8].

The presence of a cAMP phosphodiesterase activity exhibiting a cyclic fluctuation throughout the life cycle has been demonstrated in *Blastocladiella emersonii* [9]. Variations in the size of the intracellular pools of cAMP and cGMP during the life cycle of this fungus have been reported; however, a cGMP phosphodiesterase activity was not found [10]. The present paper shows that *B. emersonii* zoospores contain independent specific enzymes involved in the hydrolysis of cAMP and cGMP.

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+ *Abreviations:* cAMP, cyclic 3′, 5′- adenosine monophosphate; cGMP, cyclic 3′, 5′- guanosine monophosphate.

2. Methods

2.1. *Cells and growth conditions*

Cultures of *B. emersonii* [11] were derived from stocks kindly given by Dr E. Plessmann Camargo.

Large quantities of zoospores were obtained by flooding first generation cultures grown during 16–18 hr at 27°C in Difco–Cantine PYG agar plates. The zoospore suspension was filtered over a 30 μM mesh nylon net to remove vegetative cell debris.

2.2. *Preparation of extracts*

Zoospores were harvested by centrifugation (3 min, 1000 *g*) at 4°C, resuspended in a buffer containing 50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.15 mg/ml of phenylmethane sulphonyl fluoride (PMSF) and 10% glycerol (V/V) (Buffer A), and broken in the French Press cell at 1000 psi. These crude preparations were used directly in most experiments. Where attempts were made to study the activity of cell fractions, the centrifugation steps were performed at 4°C. Protein concentration was determined by the method of Lowry et al. [12] using bovine serum albumin (BSA) as a standard.

2.3. *Assay of the phosphodiesterases*

The phosphodiesterase activities were determined as described [9]. A typical incubation mixture for the cAMP enzyme consisted of 50 mM Tris–HCl buffer, pH 8.0, 1 mM β-mercaptoethanol; 5 mM $MgCl_2$; 40 000

cpm [^{3}H]cAMP (New England Nuclear); 160 μM unlabeled cAMP and 50–100 μg of freshly prepared enzyme source.

The activity of cGMP phosphodiesterase was assayed replacing cAMP by cGMP, at the concentration of 100 μM.

2.4. *Identification of the cGMP phosphodiesterase reaction products*

Reaction mixtures containing crude homogenates yielded as products equal quantities of 5′-GMP and guanosine as determined by descending paper chromatography using 1.0 M ammonium acetate–95% ethanol (2:5, V/V/). The sum of radioactivities found in the 5′-nucleoside and unhydrolysed cyclic nucleotide substrate sections of the chromatogram were equal to the radioactivity of the cGMP substrate added.

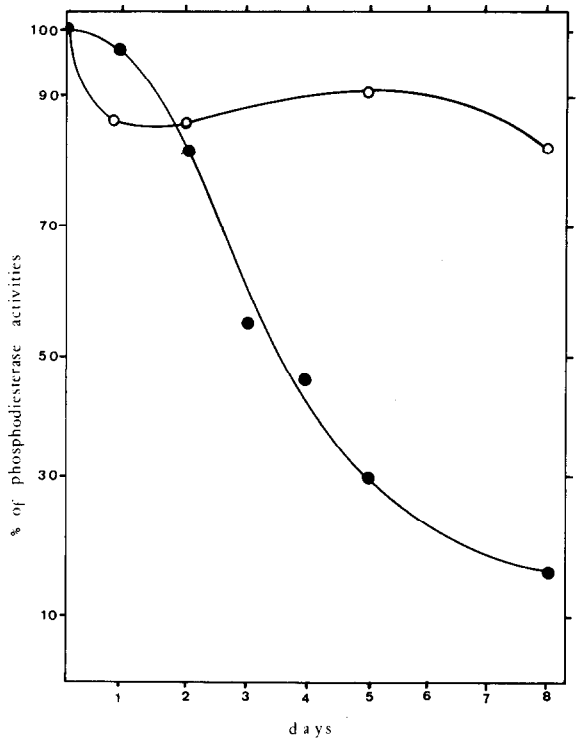

Fig.1. Cyclic nucleotide phosphodiesterase activities as a function of storage time. Samples of a 105 000 *g* supernatant of spores (3.5 mg protein/ml) were stored at 4° C in Buffer A. The enzymatic activities were determined by the standard assay for each nucleotide. (o——o) cAMP phosphodiesterase. (•——•) cGMP phosphodiesterase.

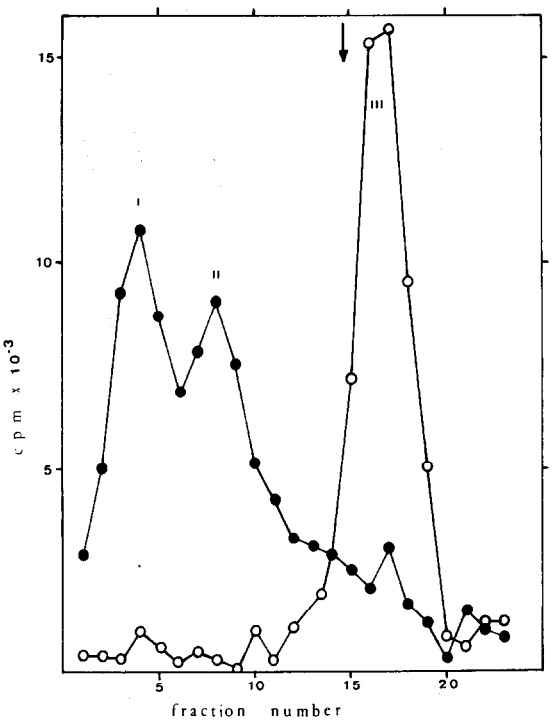

Fig.2. Zone sedimentation of cyclic nucleotide phosphodiesterase activities in a glycerol gradient. 400 μg of a 105 000 *g* supernatant of spores were layered on a 10–30 per cent glycerol gradient and sedimented at 38 000 rev/min for 14 hr. All solutions contained 1 mg/ml BSA and Buffer A. Fractions were collected and analysed for cGMP and cAMP phosphodiesterase activities by the standard assay. A sedimentation standard, *E. coli* alkaline phosphatase, was run at the same time in a duplicate tube. The arrow indicates the peak position of the marker. (o——o) cAMP phosphodiesterase. (•——•) cGMP phosphodiesterase.

Since both 5′-GMP and guanosine were formed, gradient fractions exhibiting cGMP phosphodiesterase activity (fig.2) were used as the enzyme source in standard incubation mixtures from which unlabeled cGMP and snake venom were omitted. After incubation, an aliquot was spotted in Whatman paper No.1 along with 5 μg each of 5′-GMP, 3′-GMP, cGMP and guanosine as standards. The products were separated by ascending bidimensional chromatography using ethanol–1 M ammonium acetate, pH 7.5 (5:2) as the first solvent and saturated aqueous ammonium sulfate–1 M sodium acetate–isopropyl alcohol (80:18:2) as the second solvent. The analysis of the radioactivity on the chromatogram showed that the

only radioactive product in peak I was 5′-GMP. In peak II, 50% of the cpm were located in the 5′-GMP area and 50% in the guanosine area, suggesting that this fraction was still contaminated with a 5′-nucleotidase activity.

3. Results and discussion

cGMP phosphodiesterase activity in spore extracts of *B. emersonii* resides mostly (> 80%) in the 105 000 *g* supernatant fraction, as it was found for the cAMP phosphodiesterase [9] and catalyses the conversion of cGMP to 5′-GMP. The enzyme activity was linear with respect to protein concentration (up to 60 μg) and time of assay (up to 20 min). Maximum activity was observed at pH 8.0, in the presence of 5 mM $MgCl_2$ (2.5 nmol/mg/min).

In contrast to what was found for the cAMP phosphodiesterase, which is three fold stimulated by Mg^{++} or Mn^{++} [9], the cGMP phosphodiesterase activity was stimulated only 34% and 18% by 5 mM $MgCl_2$ and 1.5 mM $MnCl_2$, respectively.

A rapid inactivation of the cGMP phosphodiesterase activity could be demonstrated when cell extracts were maintained at 4°C. Inactivation of the enzyme also occurs, although at a slower rate, in the presence of glycerol and PMSF (fig.1). Under these conditions the two enzymes behaved differently: after 8 days in the cold 80% of the cAMP phosphodiesterase activity could be recovered, as opposed to the remaining 10–20% of the cGMP phosphodiesterase activity. The fact that PMSF slows down the process of inactivation suggests the extreme sensitivity of cGMP phosphodiesterase to proteolytic cleavage. This possibility finds some support in the findings that PMSF (0.15 mg/ml) inhibits 95% of the spore proteolytic activity, as measured by the hydrolysis of azoalbumin at pH 8.0 [13].

Attempts were made to further distinguish the two phosphodiesterase activities. Ammonium sulfate fractionation followed by dialysis and DEAE cellulose column chromatography resulted in extensive losses of the cGMP phosphodiesterase activity. Under the same conditions cAMP phosphodiesterase activity is quite stable, being eluted from the DEAE column in one peak and showing a 20 fold higher specific activity.

The separation of the two activities was accomplish-

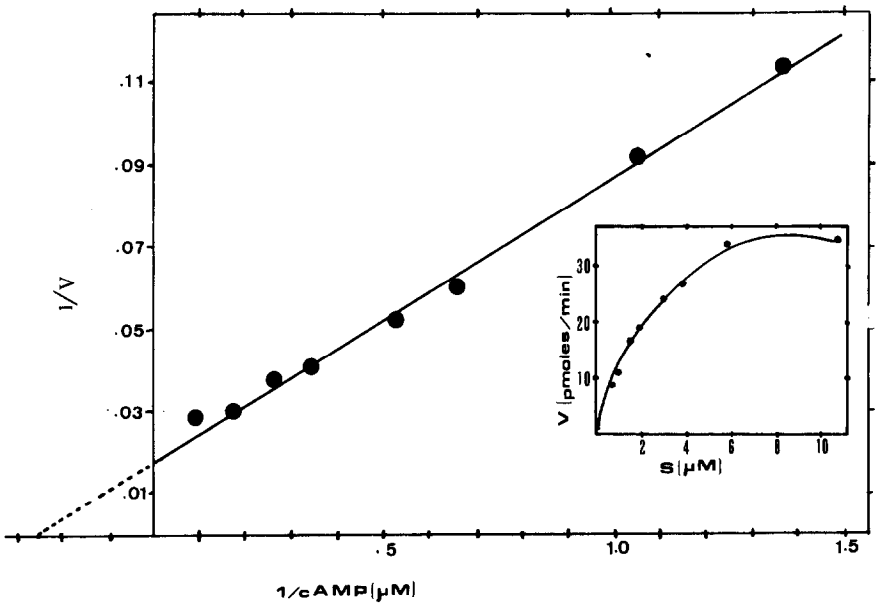

Fig.3. Lineweaver–Burk kinetic plot of the cAMP phosphodiesterase. A 105 000 *g* supernatant of spores was used as the enzyme source.

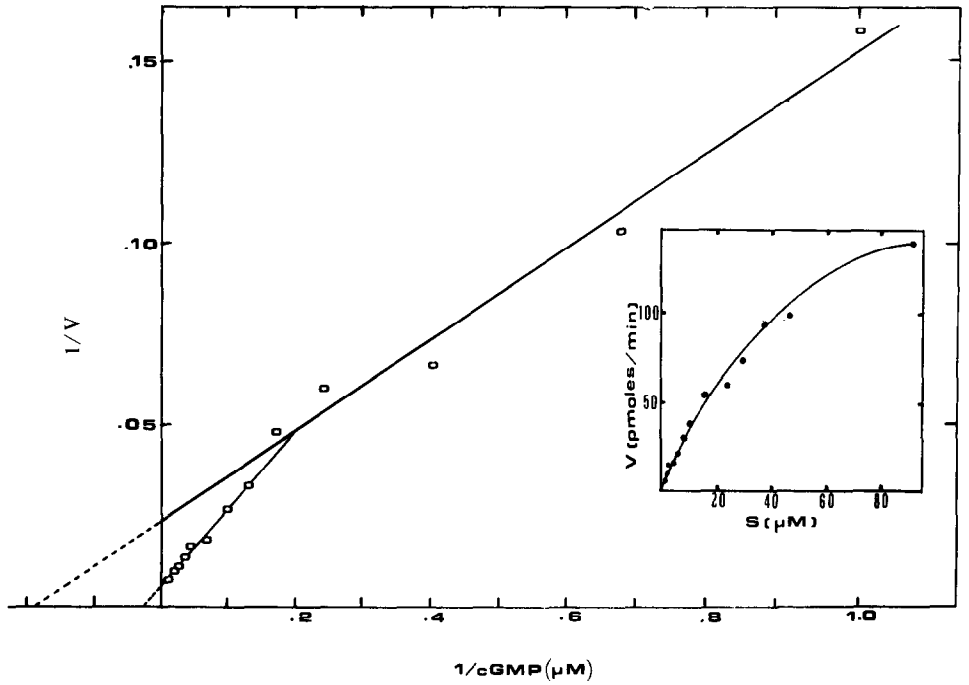

Fig.4. Lineweaver–Burk kinetic plot of the cGMP phosphodiesterase. A 105 000 *g* supernatant of spore was used as the enzyme source.

ed by sedimentation through glycerol gradients, in the presence of BSA (1 mg/ml) and PMSF. Analyses of the 105 000 *g* supernatant of spores by this technique revealed three nucleotide phosphodiesterase fractions, two of them hydrolysing cGMP but not cAMP. The third fraction was specific for cAMP (fig.2). Poor recoveries of the cGMP phosphodiesterase enzyme together with a dispersion of activity throughout the gradient were observed when BSA was omitted. BSA had no effect on the sedimentation behaviour and on the recovery of cAMP phosphodiesterase activity.

At the moment, a definitive explanation for the existence of two distinct peaks of cGMP phosphodiesterase activity can not be offered. However, the possibility of the existence of association equilibrium between sub-units, as demonstrated for other systems [14–16], has to be taken into consideration.

Analysis of the effect of cAMP and cGMP on the kinetic properties of the enzymes has shown that the nucleotides did not interfere with the hydrolysis of each other when a 105 000 *g* supernatant of spores was used. The cAMP phosphodiesterase displays normal Michaelis kinetics with an apparent K_M of 2–4 μM (fig.3). The cGMP phosphodiesterase reveals two Michaelis constants for cGMP hydrolysis (fig.4). These apparent K_M values are 4 μM and 40 μM and may reflect the two forms of enzyme found in the gradient.

Multiple molecular forms of cyclic nucleotide phosphodiesterases have been described to occur in prokaryotes as well as in eukaryotes [7,16–18]. Until now, however, a phosphodiesterase specific for cGMP was described only in rat liver [19].

Our results of metal ion requirements, stability, kinetics and sedimentation analysis strongly suggest that in *B. emersonii* the degradation of both cAMP and cGMP results from the action of different specific enzymes.

Silverman and Epstein have found cGMP in this organism [10]. The failure to find an enzyme active in cGMP hydrolysis reported by these authors can now be understood in the light of the extreme lability of this enzyme.

The determination of the specific activity of this enzyme during the life cycle of *B. emersonii* as well as its possible role in the regulation of cGMP pool sizes is presently under investigation.

Acknowledgements

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