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COMPARISON OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN *PHYSARUM FLAVICOMUM*

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ABSTRACT

We have studied both cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase in the myxomycete *Physarum flavicomum*. The cyclic AMP phosphodiesterase preparations were isolated from both the diploid plasmodial stage of the life cycle and the haploid myxamoebal stage. The plasmodial enzyme was prepared from spent medium (extracellular) and also from purified nuclei. The myxamoebal enzyme was prepared from purified nuclei. Cyclic GMP phosphodiesterase activity was studied in purified nuclei isolated from the plasmodium. One unusual feature of all the enzymes from the plasmodium is extreme heat stability; they remain catalytically active even after exposure to a boiling water bath for twenty minutes. The myxamoebae enzyme lost all activity after five minutes in a boiling water bath. All four enzyme preparations gave linear product formation with time and all were inhibited by isobutyl-methyl xanthine, a potent competitive inhibitor of cyclic nucleotide phosphodiesterase.

INTRODUCTION

Cyclic 3 '-5 ' adenosine monophosphate (cyclic AMP) has been shown to be of major importance in regulating cellular events in eukaryotic cells. Cyclic 3 '-5 ' guanosine monophosphate (cyclic GMP), although much less understood, is rapidly gaining attention from many researchers. Both cyclic nucleotides have been studied in many eukaryotic and prokaryotic systems (Pastan *et al.*, 1975).

The regulation of the intracellular (and extracellular) levels of these cyclic nucleotides is coordinated by both synthetic enzymes and degradative enzymes. Cyclic AMP is synthesized by adenylate cyclase and degraded by cyclic AMP phosphodiesterase. Cyclic GMP is synthesized by guanylate cyclase and hydrolyzed by cyclic GMP phosphodiesterase. The work of our laboratory has emphasized the degradative enzymes for both cyclic nucleotides in the myxomycete *Physarum flavicomum*.

Myxomycetes offer a unique model for the study of cellular differentiation and mitotic synchrony (Gray and Alexopoulos, 1968; Aldrich and Daniel, 1982). The life cycle of myxomycetes involves a number of stages that alternate between diploid and haploid structures. The vegetative diploid phase is a macroscopic, single-celled plasmodium. This single cell is multinucleated, with all the nuclei dividing at the same time (mitotic synchrony). Under adverse conditions the plasmodium can differentiate into a dormant sclerotium, which can reform into a plasmodium upon the return of appropriate environmental conditions. A second option available to the plasmodium is the formation of fruiting bodies with the production of haploid spores. These spores can germinate to form the vegetative, single cell haploid phase of the life cycle (myxamoebae and swarm cells). Each of these cells is microscopic and contains one nucleus. These haploid structures can fuse together to form the diploid plasmodium and the life cycle is completed.

Cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase have been studied in the myxomycete *Physarum flavicomum* in our laboratory (Lynch and Farrell, 1984; Lynch and Farrell, 1985; Lynch and Ross, 1985; and Bean *et al.*, 1986). These studies have been aimed at identifying the location of the intracellular (or extracellular) enzymes and characterizing these proteins. We have shown the existence of cyclic AMP phosphodiesterase in the medium of the plasmodium and in several different intracellular locations, including within the nucleus. A cyclic GMP phosphodiesterase from the medium has also been identified in the plasmodium of *P. flavicomum*. We have recently extended our studies to include cyclic AMP phosphodiesterase from the nucleus of the haploid stage of the life cycle (myxamoebae). This paper will compare data from both cyclic AMP and cyclic GMP phosphodiesterase in *P. flavicomum*. Our work here includes three studies on the plasmodium and one on the myxamoeba. The plasmodial investigations confirm the presence of both a nuclear cyclic AMP and cyclic GMP phosphodiesterase as well as an extracellular cyclic AMP phosphodiesterase isolated from the spent medium. Within the myxamoeba we have studied a nuclear cyclic AMP phosphodiesterase. We believe that this is the first such report of a nuclear cyclic AMP phosphodiesterase in the haploid stage of any myxomycete.

MATERIALS AND METHODS

Both plasmodia and myxamoebae of *Physarum flavicomum* were grown in liquid shake culture and harvested as previously described (Lynch and Farrell, 1985). The organisms were inoculated into two liter flasks containing one liter of semi-defined medium. Normally 3-6 liters of each batch of cells were grown at one time. Both sets of cells were harvested at late log phase, usually about 5-7 days, depending on the inoculum. It should be emphasized that in liquid cultures both myxamoebae and swarm cells are present. Myxamoebae are the predominate cell type. To facilitate usage, these haploid cells will be referred to as myxamoebae.

Three different preparations were used for the measurement of either cyclic AMP or cyclic GMP phosphodiesterase. Crude spent medium from plasmodial cultures was used to determine the activity of extracellular cyclic AMP phosphodiesterase. This medium was collected from the late log cultures described above. After centrifugation at 2,500xg the medium was decanted and saved for cyclic AMP analysis. The cells were washed several times and used for nuclear isolation. Nuclei were isolated according to Henney and Yee (1979) and Bean *et al.* (1986). The plasmodial nuclear preparations were used for measurements of both cyclic AMP and cyclic GMP phosphodiesterase activity.

Myxamoebae were also collected by centrifugation at 5000 xg for seven minutes. They were washed several times in 20 mM Tris HCl (pH 8.0). The cells were homogenized and the nuclei purified by the procedure described above for the plasmodial nuclei. Myxamoebae nuclei were used to measure cyclic AMP phosphodiesterase activity.

Both cyclic AMP and cyclic GMP phosphodiesterase activity were measured by a two-stage anion-exchange resin procedure as described previously (Lynch and Cheung, 1975). The extracellular cyclic AMP phosphodiesterase and the myxamoebae enzyme were assayed at pH 8.0 (Tris HCl). The cyclic AMP and cyclic GMP phosphodiesterase from the nuclei of the plasmodium were assayed at pH 5.5, where they both exhibited maximum activity. Included in the reaction mixture was 5 mM $MgCl_2$, 0.05 mM $CaCl_2$ and 2 mM ^{3}H-cyclic AMP or ^{3}H-cyclic GMP. The concentration of ^{3}H-cyclic AMP in the measurements of the myxamoeba phosphodiesterase was 0.1 mM. Radioactive cyclic AMP and cyclic GMP were purchased from Amersham. Unlabelled cyclic nucleotides were obtained from Sigma Chemical Co.

The enzyme reaction was initiated with the addition of sample from one of the preparations described above. Incubation was at 30°C for 10-20 minutes, depending on the sample. The enzyme activity of the heat labile preparation was terminated by placing the tubes in a boiling water bath. The heat stable enzyme was terminated with acid and base as described by Lynch and Farrell (1985). Upon equilibration to 30°C, 0.05 mg of snake venom *(Crotalus atrox)* was added as a source of 5 'nucleotidase. This step quantitatively converted all ^{3}H-nucleotides to ^{3}H-nucleosides and inorganic phosphate. The snake venom reaction was stopped by the addition of 1.0 ml of a 33% slurry of AG1-X2 anion exchange resin (Bio-Rad). The tubes were centrifuged and aliquots of the supernatant fraction, which contained either ^{3}H-adenosine or ^{3}H-guanosine, were analyzed for radioactivity in a Packard 300C Liquid Scintillation Counter. When appropriate, enzyme activity was based on protein concentration as determined by the Lowry method (Lowry *et al.*, 1951). Since extracellular (medium) phosphodiesterase activity cannot accurately be expressed on a protein basis, this activity was expressed as product formed per/ml of medium.

RESULTS

One of the characteristics of cyclic nucleotide phosphodiesterase from the plasmodium of *P. flavicomum* is heat stability. We have found that the enzyme from some intracellular locations is extremely heat stable. Table 1 compares the heat stability of phosphodiesterase from different locations in the plasmodium and from the nucleus of the myxamoeba. Heat stability was determined by placing the protein samples in a boiling water bath for five minutes. At the end of five minutes the samples were returned to 30°C and enzyme activity was determined in both the heated sample and in the unheated control sample. Although the specific activities varied, all the plasmodial samples were heat stable for the time indicated. The nuclear enzyme from the haploid stage of the life cycle was denatured during the heating process.

The previous data showed the results of enzyme activity after exposure to a boiling water bath for five minutes. The next set of experiments was designed to determine if the enzymes could be denatured with a longer boiling time. As can be seen in Table 2, the fractions that showed heat stability in Table 1 also retained heat stability when exposed to a boiling water bath for up to twenty minutes. As would be expected from the results of Table 1, the myxamoebae enzyme lost all activity after five minutes.

Isobutyl-methyl xanthine (IBMX) is generally considered to be a potent competitive inhibitor of cyclic nucleotide phosphodiesterase. Table 3 shows the results of enzyme activity in the presence of various concentrations of IBMX. The extracellular enzyme activity was determined in the presence of only one concentration of IBMX whereas the other samples were measured in the presence of four different IBMX concentrations. All the samples were inhibited by this compound and when used at different concentrations, enzyme inhibition was concentration dependent. Inhibition by IBMX indicates the presence of a cyclic nucleotide phosphodiesterase enzyme.

Another type of experiment that strongly supports the presence of an enzyme is a continuous assay where the amount of product formed is monitored with increasing time. Such a series of experiments is shown in Figure 1. Enzyme activity was measured at each of the time data points shown on the abscissa. At the time intervals indicated, aliquots were removed and the amount of product formed for that time period was determined as described in the Materials and Methods. All four samples showed a linear increase in product formed per time interval.

DISCUSSION

The results presented in this paper compare some of the recent data availble for both cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase in *Physarum flavicomum*. These enzymes have been shown to be present in various intracellular locations of several stages in the life cycle of this organism.

One unusual feature of the plasmodial enzyme studied here is their extreme heat stability. The heat stability of the extracellular phosphodiesterase was first suggested in 1971 when Murray (Murray *et al.*, 1971) commented on the "unusual stability of the excreted enzyme". Extremely stable extracellular enzymes are understandable in that an organism has very little control over its outside environment. The more resistant an enzyme is to the external environment, the greater ability it would have to degrade any compund that might disrupt the organisms normal regulatory mechanisms.

Why a nuclear enzyme would be heat stable is unknown. One obvious suggestion is that the extracellular enzyme and the nuclear enzyme from the plasmodium are identical or similar in structure and that heat stability is an inherent characteristic of the molecule. Reason suggests that the two proteins should be genetically distinct in that any regulatory mechanisms that activate (or inhibit) a nuclear enzyme would presumably be different from those that regulate an enzyme that is released to the outside of the cell.

Table 1. Activity of heated versus unheated enzyme.

	Myxamoebae Nuclear cAMP	Plasmodia Nuclear cAMP	Plasmodia Nuclear cGMP	Plasmodia Medium cAMP
Unheated	5	21	47	25
Heated	0	21	40	24

Plasmodial nuclear activity is in nMoles/min/mg protein. Myxamoebae activity is reported in nMoles/20min/mg protein. Plasmodial extracellular activity is reported in nMoles/min/ml media. Samples were heated for 5 min in a boiling water bath.

Table 2. Variable heating time assay for phosphodiesterase.

Time (min)	Myxamoebae Nuclear cAMP	Plasmodia Nuclear cAMP	Plasmodia Nuclear cGMP	Plasmodia Medium cAMP
0	5	49	45	25
5	0	57	54	24
10	0	47	64	25
15	0	46	63	28
20	0	44	83	25

Nuclear plasmodial activity is in nMoles/1 '/mg protein. Nuclear myxamoebal activity is in nMoles/20 '/mg protein. Extracellular activity is in nMoles/1 '/ml media. Each was heated in a boiling water bath for the times indicated and assayed for phosphodiesterase activity as described in the materials and methods.

Table 3. Inhibition of phosphodiesterase by IBMX.

IBMX (mM)	Myxamoebae Nuclear cAMP	Plasmodia Nuclear cAMP	Plasmodia Nuclear cGMP	Plasmodia Medium cAMP
0.0	100%	100%	100%	100%
0.2	73	80	65	
0.5	60	51	37	44*
0.8	29	20	24	
1.2	25	10	0	

*The concentration of IBMX for this data is 0.6mM. Enzyme activity is expressed as a percentage of each control tube i.e. without added IBMX.

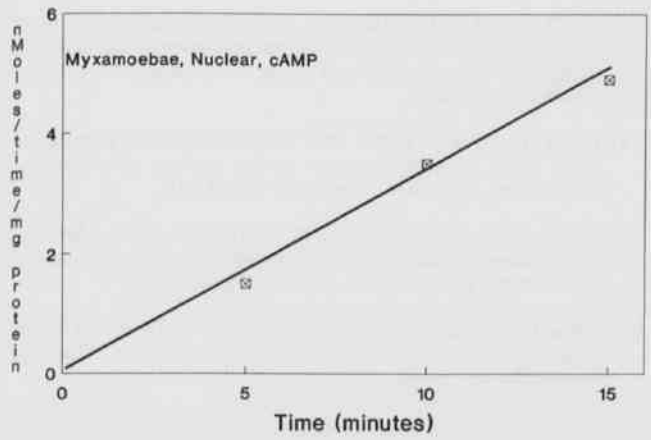

Figure 1. Continuous Assay of Cyclic Nucleotide Phosphodiesterase. Activity of cyclic AMP phosphodiesterase from nuclei of myxamoeba was determined for the times indicated on the abscissa. Enzyme activity is expressed as the amount of product converted for that total time interval.

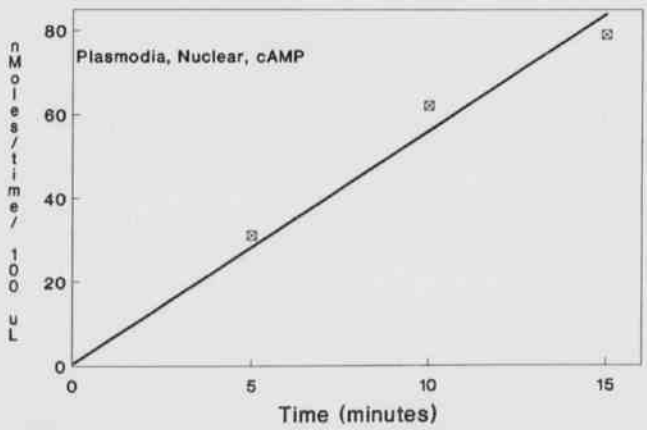

Figure 2. Continuous Assay of Cyclic Nucleotide Phosphodiesterase. Activity of cyclic AMP phosphodiesterase from the nuclei of plasmodia was determined for the times indicated on the abscissa. Enzyme activity is expressed as the amount of product converted for that total time interval.

The regulatory role of the plasmodial cyclic AMP and cyclic GMP phosphodiesterase in the nucleus is unknown at this time. Adenylate cyclase, the enzyme responsible for the synthesis of cyclic AMP, has also been shown to be present in the nucleus of *Physarum* (Atmar *et al.*, 1976). In general it has been noted that in cells growing in culture, factors that decrease cyclic AMP tend to stimulate cell proliferation whereas those that increase cyclic AMP levels tend to inhibit growth rate (Pastan *et al.*, 1975). Knowing the multi-nucleated condition of the plasmodium and the naturally occurring mitotic synchrony, it will be interesting to see if cyclic nucleotides play a role in maintaining this synchronous mitosis.

The nuclear cyclic AMP phosphodiesterase from the myxamoeba does seem to be a separate protein from its counterpart in the plasmodium. This is suggested by the differences in heat stability, with the myxamoeba enzyme being heat labile while that from the plasmodium is heat stable. The myxamoebae are single cells contaiing one nucleus. Even though there is no mitotic synchrony in these cells, it is still possible that cyclic nucleotides may play a role in cell division in the haploid stage of the life cycle.

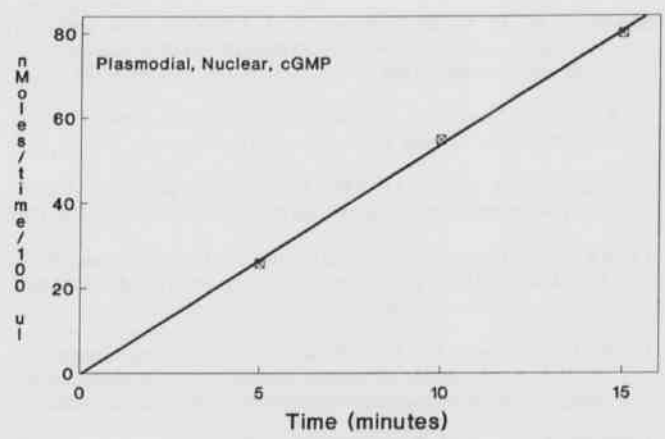

Figure 3. Continuous Assay of Cyclic Nucleotide Phosphodiesterase. Activity of cyclic GMP phosphodiesterase from the nuclei of plasmodia was determined for the times indicated on the abscissa. Enzyme activity is expressed as the amount of product converted for that total time interval.

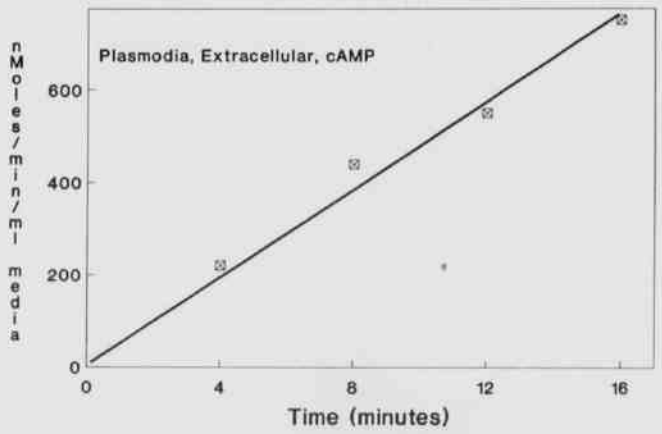

Figure 4. Continuous Assay of Cyclic Nucleotide Phosphodiesterase. Activity of extracellular cyclic AMP phosphodiesterase from the medium of plasmodia was determined for the times indicated on the abscissa. Enzyme activity is expressed as the amount of product converted for that total time interval.

A question that remains to be answered is whether the cyclic AMP and the cyclic GMP phosphodiesterase from the plasmodial nucleus are the same protein or are separate, distinct proteins. Our preliminary data (not shown in this paper) suggest that they may indeed by one and the same protein. This question cannot be answered with certainty however until the enzymes are purified to homogeneity.

LITERATURE CITED

ALDRICH, H. C., and J. W. DANIEL. (Eds.). 1982. Cell Biology of *Physarum* and *Didymium*. 2 Vols. Academic Press, New York, NY.

ATMAR, V. J., J. A. WESTLAND, G. GARCIA, and G. D. KUEHN. 1976. Adenylate cyclases in *Physarum polycephalum:* Inhibition of a nuclear enzyme by polyamines. Biochem. and Biophys. Res. Comm. 68:561-568.

BEAN, J. A., M. G. KLEVE, and T. J. LYNCH. 1986. Isolation of nuclei from *P. flavicomum:* Demonstration of a nuclear cyclic AMP phosphodiesterase. Proc. Ark. Acad. Sci. 40:17-19.

GRAY, W. D., and C. J. ALEXOPOULOS. 1968. Biology of the Myxomycetes. Ronald press, New York, NY.

HENNEY, H. H. JR. and M. YEE. 1979. Nuclear actin and histones from the myxomycete *Physarum flavicomum.* Cytobios 24:103-116.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurements with the folin phenol reagent. J. Biol. Chem. 193:265-275.

LYNCH, T. J. and W. Y. CHEUNG. 1975. Underestimation of cyclic 3 ′-5 ′ nucleotide phosphodiesterase by a radioisotopic assay using an anion-exchange resin. Anal. Biochem. 67:130-138.

LYNCH, T. J. and M. E. FARRELL. 1984. Comparison of cAMP phosphodiesterase from the haploid and diploid phase of the myxomycete *Physarum flavicomum.* Cytobios 39:71-79.

LYNCH, T. J. and M. E. FARRELL. 1985. Extracellular cyclic AMP phosphodiesterase from plasmodia and myxamoebae of *Physarum flavicomum:* Demonstration of a heat stable plasmodial enzyme. Cytobios 44:251-252.

LYNCH, T. J. and M. S. ROSS. 1985. Demonstration of a Heat-Stable cyclic GMP phosphodiesterase in the medium of *Physarum flavicomum.* Proc. Ark. Acad. Sci. 39:139-141.

MURRAY, A., M. SPISZMAN, and D. ATKINSON. 1971. Adenosine 3 ′,5 ′-monophosphate phosphodiesterase in the growth medium of *Physarum polycephalum.* Science 171:496-498.

PASTAN, I. H., G. S. JOHNSON, and W. B. ANDERSON, 1975. Role of cyclic nucleotides in growth control. Ann. Rev. Biochem. 44:491-522.