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CYCLIC AMP-DEPENDENT AND -INDEPENDENT PROTEIN KINASES OF THE WATER MOLD, *BLASTOCLADIELLA EMERSONII*

MARIA HELENA JULIANI and JOSÉ CARLOS DA COSTA MAIA

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, C.P. 20780, São Paulo 05508 (Brazil)

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Summary

Protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) and cyclic adenosine 3′,5′-monophosphate binding activities have been identified in zoospore extracts of the water mold *Blastocladiella emersonii*. More than 75% of these activities is found in the soluble fraction. Soluble protein kinase activity is resolved in three peaks (I, II and III) by DEAE-cellulose chromatography. Peak I is casein dependent and insensitive to cyclic AMP. Peak II is histone dependent and cyclic AMP independent; this enzyme is inhibited by the heat-stable inhibitor from bovine muscle. Peak III utilizes histone as substrate and is activated by cyclic AMP.

Introduction

During germination of *Blastocladiella emersonii* zoospores, a sequence of biochemical and morphological changes occurs [1]. Most of this sequence does not appear to require either concomitant transcription or concomitant translation [2—4]. Thus, this rapid cellular transition in the life cycle presumably involves control mechanisms not directly related to gene expression. Germination of *B. emersonii* zoospores is an unique, experimentally manipulatable, developmental condition where post-translational events can be temporally dissociated from differential gene expression. The possible role of cyclic nucleotides in regulating this germination process in *B. emersonii* has been studied in this laboratory [5—9]. It has been demonstrated that zoospores contain specific enzymes involved both in the hydrolysis [5,6] and in the synthesis of cyclic AMP [7]. It has also been found that the intracellular concentration of cyclic AMP transiently increases during the germination [7,8]. These results

suggest that cyclic AMP may be an endogenous mediator which couples induction (i.e. interaction of zoospores with their environment) with germination itself [9]. However, the mechanism of action of cyclic AMP at the molecular level in this morphogenetic process is entirely unknown. As a preliminary step in the investigation of the function of protein phosphorylation in the control of germination, we have assessed cyclic AMP-dependent and -independent protein kinase activities (ATP:protein phosphotransferase, EC 2.7.1.37) in zoospores of *B. emersonii*.

Materials and Methods

Growth conditions and zoospore extract preparation. Culture of *B. emersonii* were grown on Standard Cantino peptone-yeast-glucose agar [10]. Freshly released zoospores were harvested by flooding the surfaces of agar cultures with distilled water. Harvested zoospores were immediately filtered through one layer of Nytex cloth (30 μm) to remove sporangia ghosts. The filtered suspensions were washed twice with cold distilled water by centrifugation (500 $\times g$, 5 min) and the washed pellets were frozen at −60°C.

Frozen pellets were thawed in 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 mg/ml of phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 20 000 $\times g$ for 30 min; the pellet was saved and the supernatant was spun down at 105 000 $\times g$ for 60 min. Pellet and supernatant were saved. All operations were carried out at 4°C. Protein was measured as described by Lowry et al. [11] using bovine serum albumin as standard.

Cyclic AMP-binding assays. Binding was performed at 4°C for 45 min in a final volume of 300 μl containing 50 mM acetate buffer (pH 4.5), 1 mM $MgCl_2$, 0.15 mg/ml phenylmethanesulfonyl fluoride, 0.1 μM cyclic [^{3}H]AMP (6.6 Ci/mmol). Assays normally contained about 30 μg protein. Reaction was stopped by filtering the sample through a 0.45 μm Millipore filter, presoaked in acetate buffer. All the values have been corrected for nonspecific retention of cyclic [^{3}H]AMP measured in the presence of non-radioactive 1 mM cyclic AMP.

Binding activity is completely destroyed by heating the extract for 1 min at 100°C. Binding of cyclic AMP reaches a plateau after 10 min at 4°C and the activity is linear with increasing protein concentration up to 200 μg.

Protein kinase assay. Protein kinase activity was determined by measuring the transfer of ^{32}P from γ-labeled [^{32}P]ATP to histones or casein. Reactions were initiated by adding cell extract aliquots to a mixture composed of either 100 μg histone (type II-A or type VII-S (f2b), Sigma Chemicals) or 200 μg casein, 10 mM $MgCl_2$, 0.15 mg/ml phenylmethanesulfonyl fluoride, 50 mM Tris-HCl (pH 7.5), 1 μM cyclic AMP (when added) and 100 μM [^{32}P]ATP (50–100 cpm/mol) in a total volume of 100 μl. After incubation at 30°C for 10 min, 50-μl samples were pipetted onto squares (2 cm × 2.5 cm) of Whatman 3 M filter paper, placed in cold 15% trichloroacetic acid for 15 min, washed twice in 5% trichloroacetic acid for 10 min, dried, and counted in liquid scintillation fluid. Protein kinase activity was linear for 20 min at 30°C with up to 30 μg of protein/assay.

DEAE-cellulose chromatography. 8.5 ml of the 105 000 $\times g$ supernatant, at 3 mg of protein/ml, were applied onto a column (0.7 cm × 11 cm) of DEAE-

cellulose previously equilibrated with 10 mM Tris-HCl (pH 7.5). After washing with 30 ml of the same buffer, the column was eluted with a linear gradient of 0—0.4 M NaCl in a total volume of 90 ml. Chromatography was carried out at 4°C and 1 ml fractions were collected. Aliquots of 20 μl and 75 μl were used to assay for protein kinase and cyclic AMP-binding activities, respectively.

Materials. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [12] as modified by Walsh et al. [13]. ATP concentrations were determined from absorbance at 260 nm and purity assessed by thin-layer chromatography on polyethyleneiminecellulose (Merck) precoated plates [14], followed by radioautography. Casein was prepared according to Reiman et al. [15]. The heat-stable protein kinase inhibitor protein purified from bovine skeletal muscle was prepared according to Appleman et al. [16].

Cyclic [^{3}H]AMP (37.7 Ci/mmol) was obtained from New England Nuclear. Histones and non-radioactive nucleotides were obtained from Sigma Chemicals. DEAE-cellulose was obtained from Bio Rad Lab. All other chemicals and reagents used were standard reagent grade.

Results

Cyclic AMP binding

Crude zoospore extracts of *B. emersonii* contain components which bind cyclic AMP. As can be seen in Table I, under the conditions used for disrupting zoospores, more than 90% of the total cyclic AMP-binding activity was recovered in the particle-free supernatant fraction.

The specificity of cyclic AMP binding was demonstrated by competition between unlabeled cyclic AMP, dibutyryl cyclic AMP and cyclic GMP for ^{3}H-labeled cyclic AMP-binding activity of the 105 000 × *g* supernatant (Fig. 1A). Unlabeled cyclic AMP readily competed with the labeled cyclic AMP bound as expected from isotope dilution. Dibutyryl cyclic AMP and cyclic GMP were

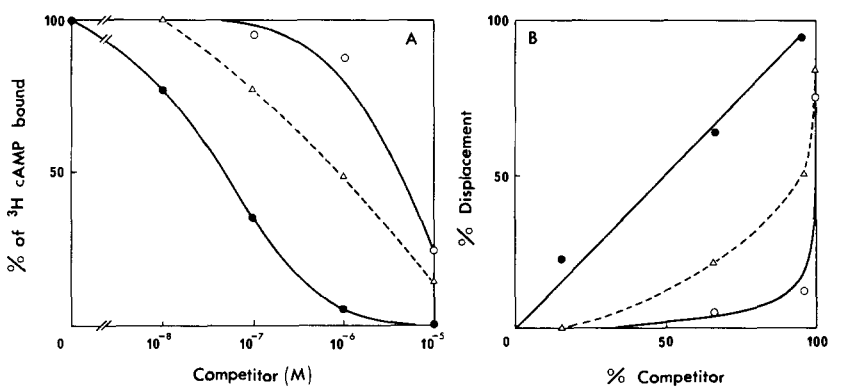

Fig. 1. Competition of ^{3}H-labeled cyclic AMP ($5 \cdot 10^{-8}$ M) binding to $S_{105\,000}$ fraction by unlabeled cyclic AMP (●———●), unlabeled dibutyryl cyclic AMP (△- - - - -△) and unlabeled cyclic GMP (○———○). Data are plotted as percent of labeled cyclic AMP bound vs. competitor added (A) and as percent displacement ([cpm (no competitor) — cpm (with competitor)]/cpm (no competitor)) vs. percent competitor (pmol competitor/pmol competitor + pmol labeled cyclic AMP) (B).

TABLE I

SUBCELLULAR DISTRIBUTION OF CYCLIC AMP-BINDING PROTEIN AND PROTEIN KINASE ACTIVITIES IN ZOOSPORE EXTRACTS

Frozen zoospores were thawed in 4.5 ml 0.01 M Tris-HCl (pH 7.5), 0.15 mg/ml phenylmethanesulfonyl fluoride and centrifuged for 30 min at 20 000 × *g*. The resulting pellet was resuspended in 2 ml of buffer and recentrifuged as above. The supernatants were pooled and centrifuged at 105 000 × *g* for 60 min (4°C). The values are the average of two experiments. Endogenous phosphorylation was measured in the absence of histone or casein. cAMP, cyclic AMP; —, —cyclic AMP; +, +cyclic AMP; *R*, ratio of —cyclic AMP to +cyclic AMP.

Subcellular fraction	Protein (mg)	cAMP binding		Protein kinase activity (pmol/mg of protein per min)									
		pmol/mg protein	%	Endogenous activity					Histone II-A activity				
				—	%	+	%	*R*	—	%	+	%	*R*
Homogenate	13.5	9.06		29		37		0.8	122		584		0.2
$P_{20\,000 \times g}$	2.5	3.36	6.7	33	21	30	15	1.1	50	7.6	242	7.7	0.2
$P_{105\,000 \times g}$	3.1	0.77	2.0	11	9	9	6	1.2	17	3.2	175	6.9	0.1
$S_{105\,000 \times g}$	7.9	14.15	91.8	27	55	40	63	0.7	169	81.1	838	84.0	0.2

TABLE I (continued)

Subcellular fraction	Protein (mg)	cAMP binding		Protein kinase activity (pmol/mg of protein per min)									
		pmol/mg protein	%	Histone f2b activity					Casein activity				
				—	%	+	%	*R*	—	%	+	%	*R*
Homogenate	13.5	9.06		296		2141		0.14	952		953		1.0
$P_{20\,000 \times g}$	2.5	3.36	6.7	78	4.9	622	5.4	0.13	538	10.5	521	10.1	1.0
$P_{105\,000 \times g}$	3.1	0.77	2.0	129	10.0	197	2.1	0.65	732	17.7	776	18.7	0.9
$S_{105\,000 \times g}$	7.9	14.15	91.8	440	87.0	3401	93.0	0.13	1230	75.6	1230	75.5	1.0

less effective than cyclic AMP in the competition, the amount of labeled cyclic AMP which remained bound being greater than that expected from isotope dilution (Fig. 1B). The ability of cyclic GMP and dibutyryl cyclic AMP at high concentrations to decrease binding of labeled cyclic AMP suggests that both cyclic nucleotides also bind to cyclic AMP-binding sites, though with lower affinity. 5'-AMP and adenosine were completely ineffective even at concentrations as high as 1 mM and ATP was only slightly effective (20% competition) at 1 mM. The K_d determined for cyclic AMP is about $1 \cdot 10^{-8}$ M.

Protein kinase activity

Table I shows the subcellular distribution of protein kinase activity in the absence of exogenous substrates and in the presence of casein or histone II-A or histone f2b.

Putative phosphorylation of endogenous substrate was measured by the incorporation of $^{32}P_i$ from [^{32}P]ATP to trichloroacetic acid-insoluble material of each subcellular fraction. Specific activities for endogenous phosphorylation ranged from 10 ($P_{105\,000}$) to 33 ($P_{20\,000}$) pmol/min per mg of protein when assayed in the absence of cyclic AMP. No significant stimulation or inhibition by cyclic AMP was observed.

The majority (80—90%) of the histone kinase activity was found in the cytosol fraction. In all fractions the activities were stimulated by cyclic AMP (about 5 fold and 7.5 fold for histone II-A and f2b, respectively).

A cyclic AMP-independent casein kinase activity was also found and 75% of this activity was associated with the post-ribosomal supernatant.

As can be seen in Table II, the analysis of the trichloroacetic acid-soluble ^{32}P-labeled material produced during the kinase reaction revealed that the radioactivity remains trichloroacetic acid-insoluble upon treatment with either

TABLE II

PROPERTIES OF PHOSPHORYLATED PRODUCTS

Experiment 1. Incorporation of ^{32}P was stopped by the addition of 2 mM unlabeled ATP. DNAase (10 μg), RNAase (10 μg) and pronase (20 μg) were added to the appropriate reactions which were incubated along with the control samples at 30°C for 30 min. Reactions were then terminated by spotting an aliquot onto filter paper. Experiment 2. Protein kinase reactions were terminated by the addition of 10% trichloroacetic acid containing 1 mM ATP, 2 mM sodium pyrophosphate and 5 mM sodium phosphate. Samples which were to be treated with hot alkali were centrifuged and the pellet resuspended in 0.5 ml 1 N NaOH. These samples, together with those in 10% trichloroacetic acid were incubated at 100°C for 15 min. 4 ml of 15% trichloroacetic acid were added and acid-insoluble ^{32}P-labeled material was separated from radioactive substrate by centrifugation, dissolved in 0.3 ml 0. 3 N NaOH, reprecipitated with cold 5% trichloroacetic acid and collected on Whatman GFC filters. All the values represent the mean of duplicate determinations.

	^{32}P incorporated (% of control)
Experiment 1	
DNAase	97.2
RNAase	92.1
Pronase	0
Experiment 2	
Hot trichloroacetic acid	95.0
Hot NaOH	0

DNAase or RNAase or hot acid, but is acid soluble after treatment with either pronase or hot alkali. These enzymatic and chemical properties are consistent with the phosphorylated products being proteins containing either phosphoserine or phosphothreonine [17].

DEAE-cellulose chromatography

In order to determine the complexity of protein kinase activity, the $S_{105\,000\times g}$ fraction was chromatographed on DEAE-cellulose as shown in Fig. 2. Under the conditions used, some kinase activity did not bind to the column and appeared in the flow-through fractions (Peak I). This activity was absolutely dependent on added casein and was not affected by cyclic AMP. No cyclic AMP binding was detected in the flow-through fractions. Protein kinase activity eluted with NaCl was measured with histone II-A as substrate and three peaks of phosphorylating activity were observed. The first peak, refered to as peak II, eluted at about 0.07 M NaCl and was completely insensitive to cyclic AMP. In contrast, the other two peaks (IIIa and IIIb), eluting at about 0.16 M and 0.2 M NaCl, respectively, were strongly stimulated by the addition of cyclic AMP. In Fig. 2, peak IIIb has a shoulder, which was not reproducible in other columns.

When cyclic AMP-binding was tested, two components with cyclic AMP-binding activity were detected. The first one coincides with peak IIIa of protein

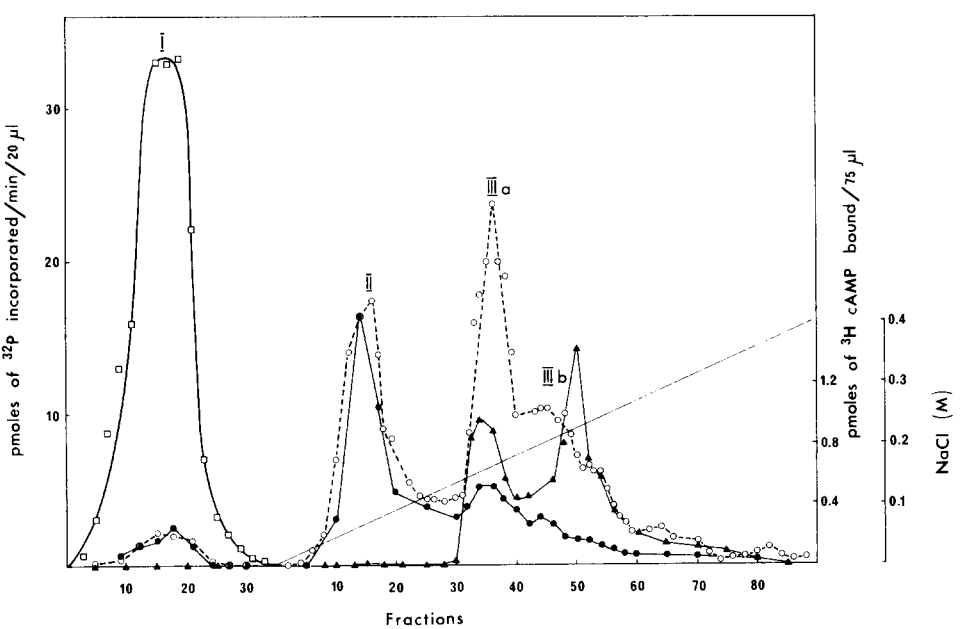

Fig. 2. DEAE-cellulose column chromatography of protein kinases and cyclic AMP-binding protein from 105 000 × *g* supernatant of zoospores. □———□, protein kinase with casein as substrate; ○- - - - -○ and ●———●, protein kinase with histone II-A as substrate in the presence and absence of 1 µM cyclic AMP, respectively; ▲———▲, cyclic AMP binding. Fractions in regions designated IIIa and IIIb were combined and rechromatographed as illustrated in Fig. 3.

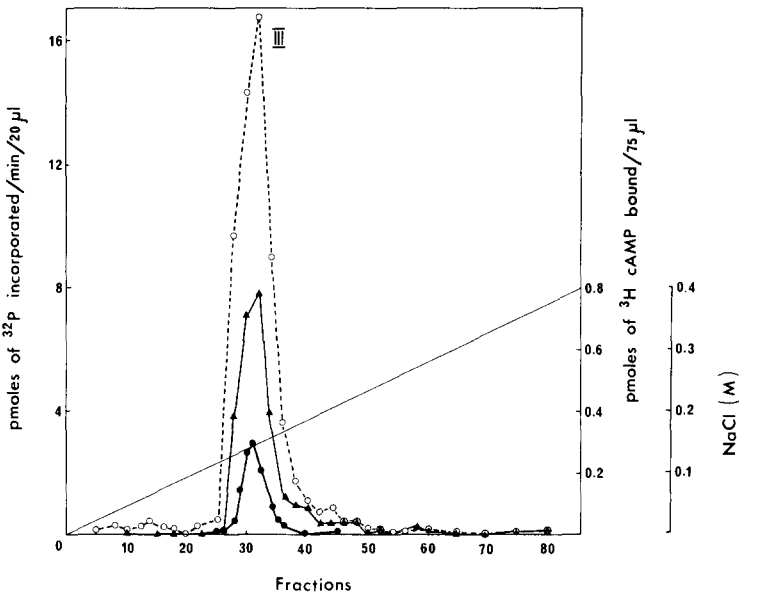

Fig. 3. Rechromatography of fractions combined from regions IIIa and IIIb of Fig. 2. The pooled fractions were dialyzed and then chromatographed on DEAE-cellulose. More than 80% of the total activity was recovered after chromatography ○- - - - -○ and ●———●, protein kinase activity with histone II-A as substrate in the presence or absence of 1 µM cyclic AMP, respectively; ▲———▲, cyclic AMP binding.

kinase activity, whereas the second one is somewhat displaced to the right relative to peak IIIb.

The relative amounts of kinase activities of peaks IIIa and IIIb varied from preparation to preparation. In order to assess whether or not the cyclic AMP-dependent kinase activities represented distinct forms of the enzyme, fractions numbered 30—55 were pooled, dialyzed and rechromatographed on DEAE-cellulose. As illustrated in Fig. 3, the total cyclic AMP-dependent protein kinase activity eluted as a single peak in a position corresponding closely to the position of peak IIIa. the cyclic AMP-binding activity was associated with the cyclic AMP-dependent protein kinase. It is not possible to exclude the possibility that peak IIIb might have been totally converted to form IIIa during the time interval between the two chromatographic runs. Preliminary experiments employing DEAE-cellulose chromatography at different stages of the life cycle, exhibit profiles which demonstrate differential expression of various protein kinases (unpublished data). These studies, as well as further biochemical characterization of these fractions, may shed light on these questions.

Protein substrate specificity and sensitivity to cyclic AMP and cyclic GMP

Since the protein kinase profile depended on the type of protein substrate, each of the fractions were tested for activity with various substrates. As shown in Table III, fraction I was more specific for casein, but exhibited some activity with histone f2b; cyclic AMP had no effect on activity with either casein, histone II-A or histone f2b. In marked contrast, fraction II presented substantial activity with both histones. The relative activities observed were

TABLE III

SPECIFICITY OF THE DIFFERENT FRACTIONS

Fractions I, II, IIIa and IIIb were isolated from zoospore cytosol as described in the legend to Fig. 2. Equal amounts of protein were used to assay for activity of each fraction. cAMP, cyclic AMP; cGMP, cyclic GMP.

	pmol ^{32}P incorporated/min			
	I	II	IIIa	IIIb
Casein				
—cAMP	7.1	1.8	2.5	1.5
+cAMP	7.4	1.8	3.6	1.0
—cAMP/+cAMP	0.96	1.0	0.7	1.5
Histone II-A				
—cAMP	0.3	7.8	2.7	0.9
+cAMP	0.2	9.2	10.8	5.6
—cAMP/+cAMP	1.5	0.85	0.25	0.16
Histone f2b				
—cAMP	4.0	49.3	18.6	5.9
+cAMP	3.7	46.3	81.3	41.7
—cAMP/+cAMP	1.1	1.1	0.23	0.14
Casein				
—	4.1			
+cAMP, 10^{-6} M	4.2			
+cGMP, 10^{-6} M	4.0			
Histone f2b				
—		55.9	12.3	4.6
+cAMP, 10^{-6} M		42.8	64.9	28.7
+cGMP, 10^{-6} M		53.8	23.6	8.3

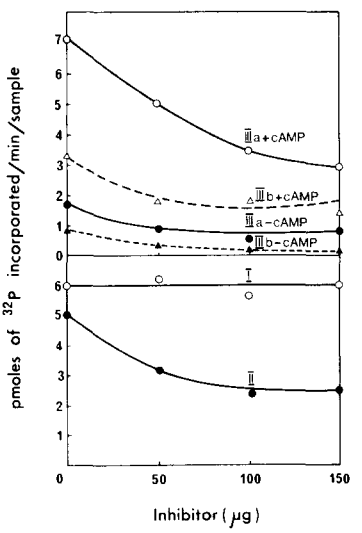

Fig. 4. Inhibition of protein kinase by mammalian heat-stable inhibitor protein. Fractions I, II, IIIa and IIIb were obtained after chromatography on DEAE-cellulose (see Fig. 2) and assayed with casein (fraction I) or histone II-A without and with $1 \cdot 10^{-6}$ M cyclic AMP. Varying concentrations of heat-stable inhibitor protein partially purified from bovine skeletal muscle were added to the standard reaction mixtures. Incubation was carried out for 10 min at 30° C.

100 : 15.8 : 3.6 for histone f2b, histone II-A and casein, respectively. Confirming the results of DEAE-chromatography, this fraction showed no cyclic AMP stimulation when tested with any of these substrates. On the other hand, peaks IIIa and IIIb also had a substantial specificity for histone f2b, but both histone activities were strongly stimulated by cyclic AMP, while the casein activities were not sensitive to this cyclic nucleotide.

Cyclic GMP ($1 \cdot 10^{-6}$ M) was ineffective in stimulating the enzymes present in peak I and II. A 2 fold stimulation was observed in the case of peak III.

Effect of mammalian inhibitor protein

A heat-stable inhibitor protein effective in inhibiting the activity of mammalian cyclic AMP-dependent protein kinase has been described by Walsh et al. [18] and by Ashby and Walsh [19]. The heat-stable inhibitor was shown to inhibit protein kinase activity by directly interacting with the free catalytic subunit, forming and inhibitor-catalytic subunit complex [19,20]. To evaluate the effect of that inhibitor, each of protein kinase activities from zoospores were assayed in the presence of increasing concentrations of inhibitor protein isolated from bovine skeletal muscle (Fig. 4). Histone II-A or casein were used as substrates in the assays. Protein kinase II and III were inhibited. There was no inhibition of the activity of protein kinase I.

Discussion

The data presented above demonstrate that there are three protein kinase activities in the soluble fraction of *B. emersonii* zoospores disrupted by freeze-thawing: (a) a cyclic AMP-independent protein kinase that does not bind to the DEAE-cellulose column; this fraction (I) preferentially phosphorylates casein and is not inhibited by a heat-stable protein kinase inhibitor protein from bovine skeletal muscle; (b) a cyclic AMP-independent protein kinase that elutes at 0.07 M NaCl (peak II); its activity is inhibited by the mammalian inhibitor protein; (c) a cyclic AMP-dependent protein kinase that elutes at 0.16 M NaCl (peak III). The inhibition data suggest that the kinase activity of peak II corresponds to a free catalytic subunit (C), but we do not know whether this activity results from dissociation of the cyclic AMP-dependent protein kinase (peak III). The cyclic AMP-binding protein cochromatographes with peak III.

We were unable to find protein kinase inhibitor activity in zoospore extracts. It is interesting that the mammalian inhibitor is capable of inhibiting 50% of the activity of *B. emersonii* cyclic AMP-dependent protein kinase.

Further studies are needed to evaluate the biological significance of these protein kinase activities present in zoospore extracts, particularly with respect to the post-translational events occurring during early stages of zoospore germination and the triggering of protein synthesis in intermediary stages of germination [2,4]. In this context it may be of particular interest to look for possible specific endogenous substrates.

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