Biochem. J. (1979) **182**, 547–554
Printed in Great Britain

Changing Pattern of Cyclic AMP-Binding Proteins during Germination of *Mucor racemosus* Sporangiospores

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(*Received* 5 *February* 1979)

Interaction of cyclic AMP with a profoundly changing pattern of specific binding proteins was shown during aerobic germination of sporangiospores from the fungus *Mucor racemosus*. ^{32}P-labelled 8-azido-cyclic AMP, an analogue of cyclic AMP that forms a covalent linkage with the binding proteins under u.v. light, was used as the ligand. Binding proteins carrying this photoaffinity label were separated by polyacrylamide-gel electrophoresis and identified by radioautography. Equilibrium dissociation constants (K_d) and binding-response curves in the presence of competing nucleotides were identical for both 8-azido-cyclic [^{32}P]AMP and cyclic [^{3}H]AMP. A quantitative binding assay with both 8-azido-cyclic [^{32}P]AMP and cyclic [^{3}H]AMP over the time course of sporangiospore germination indicated a parallel relationship between cyclic AMP-binding capacity and the intracellular concentrations of cyclic AMP reported in a previous study [Paznokas & Sypherd (1975) *J. Bacteriol.* **124**, 134–139]. Both of these parameters attained transient high values at a time of development when addition of exogenous cyclic AMP prevents hyphal-germ-tube emergence. The measured K_d values did not change during spore germination.

Mucor racemosus is a phycomycete that exhibits the property of fungal dimorphism. Sporangiospores produced on aerial hyphae develop into budding 'yeast' cells under CO_2 or into hyphae under air. These two vegetative forms are interconvertible on a change of the atmosphere. Cyclic AMP was implicated as a possible regulatory element in the control of *M. racemosus* dimorphism by Larsen & Sypherd (1974). Relatively high concentrations of the nucleotide were found in 'yeasts', whereas low concentrations obtained in hyphae. Exogenous cyclic AMP induced growth in the 'yeast' form under air (Larsen & Sypherd, 1974; Paveto *et al.*, 1975). Paznokas & Sypherd (1975) reported the occurrence of a transient 10-fold rise in the concentration of intracellular cyclic AMP during sporangiospore germination. The peak concentrations occurred during spherical growth (swelling) of the spores and had subsided before the emergence of the first germ tubes or the formation of buds. Orlowski & Sypherd (1976) reported a similar change in the concentrations of cyclic GMP during germination. It has been hypothesized that these cyclic nucleotides may play a regulatory role in sporangiospore germination (Paznokas & Sypherd, 1975; Orlowski & Sypherd, 1976). If this is true, the control is likely to be exerted by interactions of the nucleotides with specific protein molecules (Jost & Rickenberg, 1971; Rickenberg, 1974).

The purpose of the present study was to investigate the binding of cyclic AMP to specific proteins during the germination of *M. racemosus* sporangiospores into aerobic hyphae. This was accomplished by using ^{32}P-labelled 8-azido-cyclic AMP as the ligand. This compound forms a covalent attachment to its binding site on the protein when exposed to u.v. light (Haley, 1975). This facilitates separation and identification of the cyclic AMP-binding proteins by means of gel electrophoresis and radioautography. The present results show that the identity and amounts of cyclic AMP-binding proteins changed considerably during the development. These changes correlated closely with the intracellular cyclic AMP concentrations previously reported by Paznokas & Sypherd (1975).

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and Methods

Organism and cultivation

Mucor racemosus (*M. lusitanicus*) A.T.C.C. 1216B was used in all experiments. The growth medium contained 0.3% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) glucose (adjusted to pH 4.5 with H_2SO_4). Sporangiospores were produced within 3–4 days at 22°C on the above medium solidified with 3% (w/v) agar. The sporangiospores were scraped from the agar (free of any hyphal fragments) into distilled water and inoculated into the liquid growth medium at a final concentration of 5×10^5 spores/ml. Compressed air was bubbled through the culture at a flow rate of approx. 10 vol. of gas/min per vol. of culture fluid.

Preparation of soluble-cell-protein fractions

Cells at the appropriate stage of development were rapidly collected on a filter (Millipore, type AA, 0.8 μm pore size), washed briefly with ice-cold buffer A [10mM-Tris/HCl (pH7.25)/40mM-NaCl/20mM-KCl/2.5mM-$MgCl_2$], quickly placed into a mortar full of liquid N_2 and vigorously ground with a pestle for 2min (dormant spores for 5min). This method was shown to rupture quantitatively all cell morphologies of *Mucor* equally well (Orlowski & Sypherd, 1978*a*,*b*). The broken cells were resuspended in buffer A and centrifuged at 27000*g* for 10min at 4°C. The resulting clear supernatant solution containing the soluble cell proteins was recovered, dialysed against 100 vol. of buffer A and used in all further experiments. In some experiments the 27000*g* pellet was resuspended in buffer A, centrifuged at 2000*g* for 10min to sediment unbroken cells and a rather opaque supernatant fraction (containing membrane and cell-wall fragments) was collected for further experiments.

Cyclic AMP-binding assay

The binding of cyclic AMP to protein was determined essentially by the method of Gilman (1970). Cyclic [8-^{3}H]AMP (13.7Ci/mmol) at various concentrations was incubated for 10min at 0°C with 500 μg of soluble cell protein in buffer A (total volume 500 μl). The assay mixtures were passed over cellulose nitrate filters (Millipore, type HA, 0.45 μm pore size), which were immediately washed with 2ml of ice-cold buffer A. The filters were dried under a heat lamp, placed into Aquasol-2 (New England Nuclear Corp., Boston, MA, U.S.A.) scintillation fluid and assayed for radioactivity in a liquid-scintillation spectrometer. Bound radioactivity was converted into pmol of cyclic AMP by means of a standard curve.

8-Azido-cyclic [^{32}P]AMP labelling of cyclic AMP-binding proteins

The method of Haley (1975) was used to label *M. racemosus* cyclic AMP-binding proteins with 8-azido-cyclic [^{32}P]AMP (74Ci/mmol). An appropriate concentration of the nucleotide (200nM for samples to be electrophoresed) was incubated at 0°C with 125 μg of soluble cell protein in buffer A (total volume 100 μl). The incubation mixture was exposed to a u.v. light source (UVS-11 Mineralight; Ultraviolet Products, San Gabriel, CA, U.S.A.; maximum emission at 254nm) for 60min at a distance of 9cm. The resulting solution was assayed for total cyclic AMP-binding activity by the cellulose nitrate-filter technique (see above) or was used directly as a sample for SDS/polyacrylamide-gel electrophoretic separation of proteins (see below).

SDS/polyacrylamide-gel electrophoresis

The system used for SDS/polyacrylamide-gel electrophoresis was essentially that of Laemmli (1970). The sample was heated for 30min at 100°C in the presence of 2.5% (v/v) β-mercaptoethanol, 1% SDS, 25mM-Tris/HCl, pH7.0, and 10% (w/v) sucrose. This solution (containing 125 μg of protein) was applied to the top of a stacking gel (2mm in width, 2cm in length) containing 4% (w/v) acrylamide, 0.1% methylenebisacrylamide, 160mM-Tris/HCl, pH7.0, and 0.007% SDS. The separating gel (2mm in width, 8cm in length) contained a linear 8–17% acrylamide (0.21–0.45% bisacrylamide) gradient, 0.1% SDS and 0.375M-Tris/HCl, pH8.8. The electrode buffer contained 0.1% SDS and 25mM-Tris/glycine, pH8.2. The electric current was held constant at 15mA while the sample migrated through the stacking gel, and at 25mA until the Bromphenol Blue tracking dye left the separating gel. The temperature was ambient and the run time approx. 4h. The gels were fixed and stained overnight in a solution of 0.5%(w/v) Coomassie Brilliant Blue R in methanol/water/acetic acid (5:4:1, by vol.). The gels were destained in a solution of methanol/acetic acid/water (10:7:83, by vol.).

Radioautography

Polyacrylamide gels, stained as described above, were dried flat on Whatman no. 3 paper by application of heat (60°C) and vacuum. The dried gels were placed in contact with Kodak X-Omat X-ray film in lead-lined holders for several (5–15) days at −60°C. The films were developed at 20°C with Kodak X-ray developer (5min) and fixed in Kodak rapid fixer (5min).

Protein determinations

Protein was measured by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard.

Chemicals

All radioisotopes were obtained from ICN, Irvine, CA, U.S.A. All other nucleotides were obtained from Sigma Chemical Corp., St. Louis, MO, U.S.A. Acrylamide and other materials for electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. All culture-media components were from Difco, Detroit, MI, U.S.A. All other chemicals were from Sigma, or from Mallinkrodt Chemical Works, St. Louis, MO, U.S.A.

Results

Cyclic AMP-binding activity

Cyclic AMP-binding activity was detectable in extracts of *M. racemosus* made at all stages of develop-

ment. More than 90% of this activity was always associated with the 'high-speed' supernatant cell fraction. Less than 10% of the activity remained with the washed and resuspended particulate fraction (results not shown). Cyclic AMP-binding activity was linear with respect to increasing protein concentration up to 2 mg/ml (results not shown). The linear relationship of binding activity with protein concentration was also true for 8-azido-cyclic AMP (results not shown). Cyclic AMP-binding activity was completely destroyed by boiling the extracts for 3 min. No binding activity was lost on storage at 4°C up to 10 days. Cyclic AMP did not bind non-specifically to proteins, such as bovine serum albumin, in this assay system. The measured kinetics of binding indicated that the binding proteins were saturated in about 2 min at 0°C (Fig. 1). These findings were also true with respect to covalent or reversible binding of 8-azido-cyclic AMP by *M. racemosus* soluble proteins (results not shown).

Cyclic AMP-binding affinity

Equilibrium dissociation constants (K_d) were calculated as a measure of cyclic AMP-binding affinity for the proteins. The Scatchard (1949) plots (Fig. 2) exhibited linearity, interrupted by one extreme change of slope. This observation suggested the presence of two classes of proteins with radically different binding affinities. The first class, with an average K_d of 4.9 (average for three experiments) for spore proteins and 8.3 (average for three experiments) for hyphal proteins, may include protein molecules engaged in an

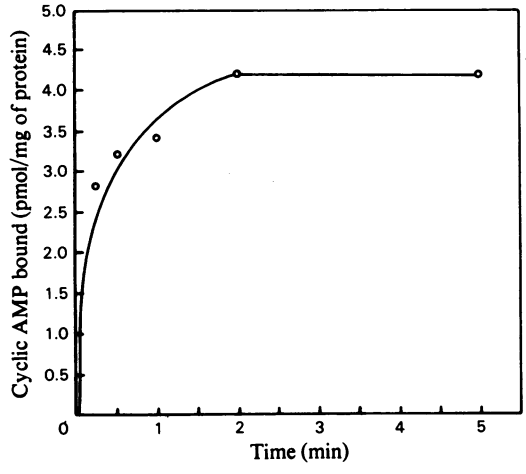

Fig. 1. *Cyclic AMP binding to M. racemosus soluble proteins as a function of time*
Details of the assay are given in the text. The binding reaction was started by the addition of cyclic [^{3}H]-AMP (200 nM final concn.) to dialysed cell-free extract at 0°C and portions of the mixture were withdrawn at intervals for the measurement of bound radioactivity; 12 h-old hyphae were used in the experiment shown. The incubation mixture (500 μl) contained 500 μg of protein.

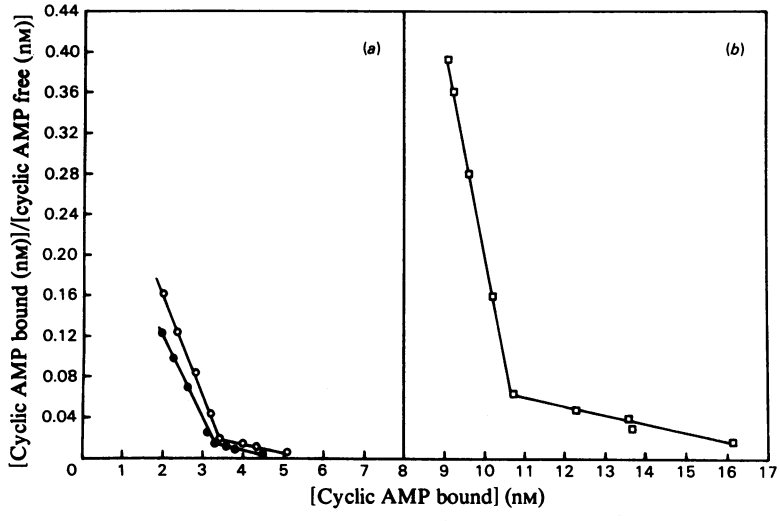

Fig. 2. *Scatchard plots of cyclic [^{3}H]AMP or 8-azido-cyclic [^{32}P]AMP binding by M. racemosus soluble proteins*
The standard assay system described in the text was used. The incubation mixtures (500 μl) contained 500 μg of protein. The amount of cyclic AMP bound was calculated from the amount of radioactivity retained by a cellulose nitrate filter. The amount of free cyclic AMP was calculated from the total amount of cyclic AMP added to the mixture less the amount bound. (*a*) Extracts from 15 h-old hyphae: ○, cyclic [^{3}H]AMP binding; ●, 8-azido-cyclic [^{32}P]AMP binding. (*b*) Extract from dormant sporangiospores: □, cyclic [^{3}H]AMP binding.

avid and highly specific interaction with cyclic AMP. The second class, with an average K_d of 100 (average for three experiments) for spore proteins and 200 (average for three experiments) for hyphal proteins, may represent less avid and perhaps non-specific binding. Alternatively, a strong negative co-operativity within a single class of proteins may be another explanation for the data. 8-Azido-cyclic AMP behaved approximately the same as did cyclic AMP on Scatchard analysis: K_d values of 11.6 and 200 were calculated by using extracts from hyphal cells (Fig. 3).

Cyclic AMP-binding specificity

In an attempt to demonstrate specificity of the binding activity, extracts were treated with a variety of substances that would be expected to alter the structure of the binding proteins and hence affect their interaction with cyclic AMP. These results are presented in Fig. 3. Protein denaturation by boiling or treatment with 6M-urea completely prevented any binding, as did proteolysis by exposure to Pronase. The thiol-group-agents mercurous acetate and *p*-chloromercuribenzoate caused a partial inhibition of cyclic AMP binding, as did the cross-linking agent formaldehyde. The reducing agent β-mercaptoethanol actually enhanced binding, but a similar compound, dithiothreitol, was without effect. The metal chelator EDTA also had no effect. The data indicate that cyclic AMP does not simply bind to protein *per se* in this assay system, but rather to protein molecules of some specific structure.

Nucleotide-binding specificity was investigated in competition-binding experiments (Fig. 4). Unlabelled cyclic AMP competed with equal effectiveness with the labelled form for binding sites. Adenosine 5′-triphosphate and adenosine 5′-monophosphate displayed no competition in the concentration range examined. Cyclic GMP did compete with cyclic AMP for binding sites, although this nucleotide was less effective than cyclic AMP. Identical results were observed when these nucleotides were tested against 8-azido-cyclic AMP. Addition of cyclic nucleotide phosphodiesterase (1 unit/ml; Sigma) to the assay system prevented over 95% of nucleotide binding by *M. racemosus* proteins (results not shown).

Cyclic AMP-binding activity in relation to morphogenesis

A time course of cyclic AMP-binding activity was determined throughout sporangiospore germination and hyphal development (Fig. 5). By using either cyclic AMP or its 8-azido analogue as the ligand, the highest binding activities were observed in germinating spores that had commenced swelling but not yet put out germ tubes (approx. 3 h after introduction of the spores to aerated liquid medium). Very high binding activities (sometimes equalling the maximum values) were also observed in dormant spores. The binding activities dropped 4–5-fold about 3 h before germ-tube emergence (6 h from addition to medium) and remained within relatively low values throughout

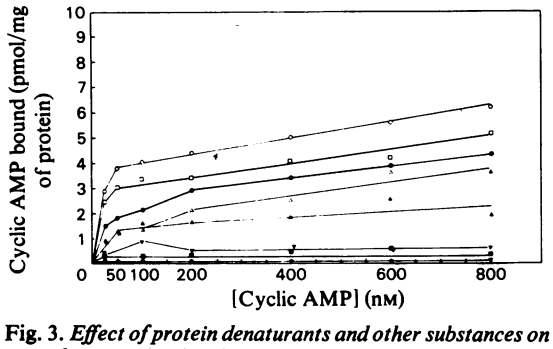

Fig. 3. *Effect of protein denaturants and other substances on cyclic AMP binding by M. racemosus soluble proteins*
The standard binding assay described in the text was used. The compound tested was present at a constant concentration in the presence of increasing amounts of cyclic [^{3}H]AMP. The extracts were from 15 h-old hyphae. □, Control; ○, 1% β-mercaptoethanol; ●, 3.7% formaldehyde; △, 1 mM-mercurous acetate; ▲, 1 mM-*p*-chloromercuribenzoate; ▽, 6M-urea; ■, 1 μg of Pronase B (Calbiochem, San Diego, CA, U.S.A.)/μl; ▼, boiled extract. Binding curves in the presence of 1 mM-dithiothreitol and 1 mM-EDTA were identical with that of the control.

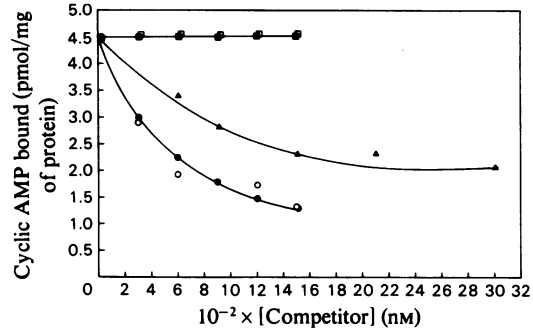

Fig. 4. *Competition of non-radioactive cyclic AMP, cyclic GMP, ATP and 5′-AMP with cyclic [^{3}H]AMP for binding sites on M. racemosus soluble proteins*
The standard binding assay described in the text was used. The nucleotide tested was present at increasing concentrations in the presence of a constant amount of cyclic [^{3}H]AMP (600 nM). The extract used in this experiment was from 15 h-old hyphae. Identical results were obtained by using 8-azido-cyclic [^{32}P]-AMP. Competitors: ○, cyclic AMP; ▲, cyclic GMP; □, ATP; ■, 5′-AMP. ●, Theoretical curve for competition between labelled and unlabelled ligands of equal affinity.

the rest of germ-tube emergence and hyphal growth. The concentrations of intracellular cyclic AMP measured by Paznokas & Sypherd (1975) throughout this developmental sequence are also shown in Fig. 5. This parameter shows a marked correlation with the amounts of cyclic AMP-binding activity reported in the present paper.

Electrophoretic separation of cyclic AMP-binding proteins labelled with the radioactive photoaffinity analogue

Cell-free dialysed extracts were prepared from dormant spores and at intervals throughout germination and hyphal development. These soluble-protein samples were exposed to 8-azido-cyclic [^{32}P]-AMP under u.v. light as described above. A 60 min exposure time was routinely used, although a 10 min period was later shown to be sufficient. The labelled proteins, boiled in the presence of SDS and β-mercaptoethanol, were separated on SDS/polyacrylamide slab gels (see the Materials and Methods section). The gels were stained with Coomassie Blue to show the total pattern of proteins separable by molecular weights. This result is shown in Fig. 6 (*a*). Very few protein bands newly appeared or disappeared or

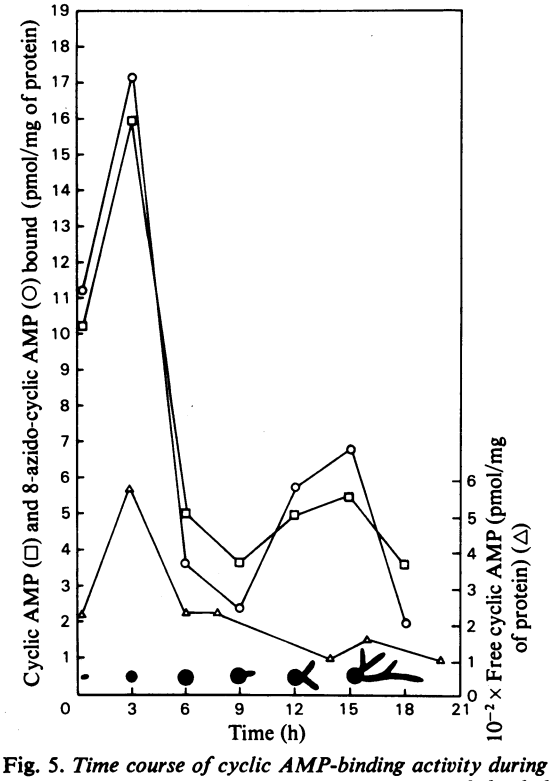

Fig. 5. *Time course of cyclic AMP-binding activity during M. racemosus sporangiospore germination and hyphal development*

The morphological form of the organism at a given time is represented at the bottom of the Figure. Dialysed 'high-speed' supernatant fractions were prepared at intervals throughout development and assayed for binding activity as described in the text. Both cyclic [^{3}H]AMP and 8-azido-cyclic [^{32}P]AMP were used as the ligand. Data from Paznokas & Sypherd (1975) showing intracellular cyclic AMP concentrations during the same times are also shown (△). □, Cyclic [^{3}H]AMP bound; ○, 8-azido-cyclic [^{32}P]AMP bound; △, free intracellular cyclic AMP concentration.

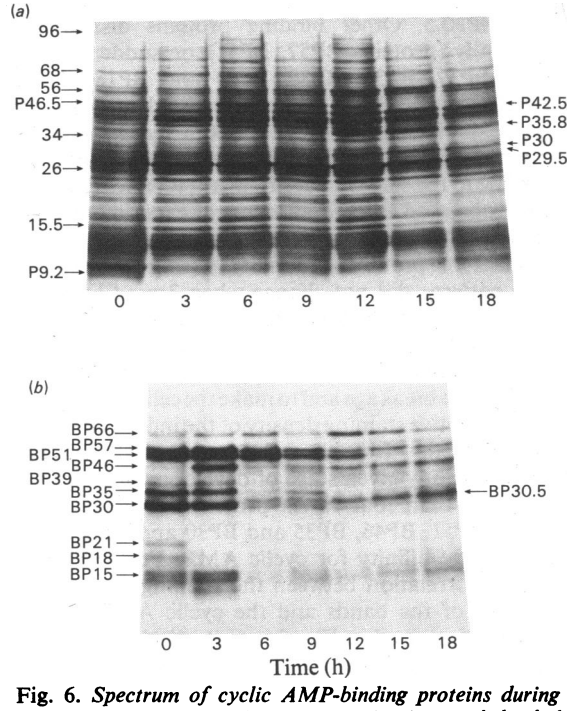

Fig. 6. *Spectrum of cyclic AMP-binding proteins during M. racemosus sporangiospore germination and hyphal development*

(*a*) Total pattern of soluble proteins from *M. racemosus* during development made visible by staining with Coomassie Blue. The time elapsed after introduction of the spores into nutrient medium is indicated below the appropriate track. The morphological form of the organism at the corresponding times was indicated in Fig. 5. The relative mobilities of proteins used as molecular-weight standards are indicated by the molecular weight of the protein multiplied by 10^{-3}. Only those *M. racemosus* proteins whose amounts visibly changed during development are indicated by a 'P' preceding their calculated molecular weight multiplied by 10^{-3}. (*b*) Spectrum of *Mucor* soluble proteins carrying covalently bound 8-azido-cyclic [^{32}P]AMP made visible by radioautography. The gel is the same one shown in (*a*). Those proteins that bound the labelled nucleotide to any significant extent are indicated by 'BP' preceding their calculated molecular weight multiplied by 10^{-3}.

changed density appreciably during the time course. Those bands that did change noticeably are so indicated and identified by their molecular weights, which were calculated from a plot of the mobilities of known protein standards.

The location of proteins on the gels carrying the covalently bound photoaffinity analogue of cyclic AMP was determined by radioautography. A typical radioautogram is shown in Fig. 6. A very limited number of proteins were labelled in any given extract. The labelling pattern changed greatly during morphogenesis. Some binding proteins newly appeared during development, for example proteins BP46 and BP30.5. Other binding proteins disappeared gradually (proteins BP57, BP51) or suddenly (proteins BP39, BP35, BP30, BP21 and BP18) during development. Still others persisted throughout (proteins BP66) or only temporarily disappeared (proteins BP15). Each individual protein pattern is specific to the stage of development; identical patterns were observed in several identical experiments. The changing pattern of binding proteins was not an artefact of proteinase activity during preparation. The patterns did not change when 2mM-EDTA (an inhibitor of metalloproteinases) or 2mM-phenylmethanesulphonyl fluoride (an inhibitor of serine proteinases) was included in the buffer used to wash the cells before breakage and to make the cell-free extracts.

The relative staining density of the individual bands is noteworthy. It is clear that most of the binding activity at any given stage of development resided in no more than four to six proteins. Proteins BP66, BP57, BP51, BP46, BP35 and BP30 apparently have the highest affinity for cyclic AMP. Furthermore a strong correlation between the staining density and number of the bands and the cyclic AMP-binding activity described in Fig. 5 is quite obvious. Swollen spores (3h in nutrient medium) possessed the greatest amounts of binding activity; dormant spores possessed nearly as much. Binding activity clearly declined during germ-tube emergence and hyphal growth.

Cycloheximide completely blocks germination of dormant sporangiospores and stops the synthesis of protein (Orlowski & Sypherd, 1978*b*). The spectrum of cyclic AMP-binding proteins was examined in spores exposed to a high concentration of cycloheximide (500μl/ml) in aerated liquid medium for 18h (Fig. 7). Essentially only three species of binding proteins were found in such cells, i.e., proteins BP35, BP30 and BP15. They were all present in much greater quantities than they had been in the dormant spores and all of the higher-molecular-weight species of binding proteins had notably disappeared.

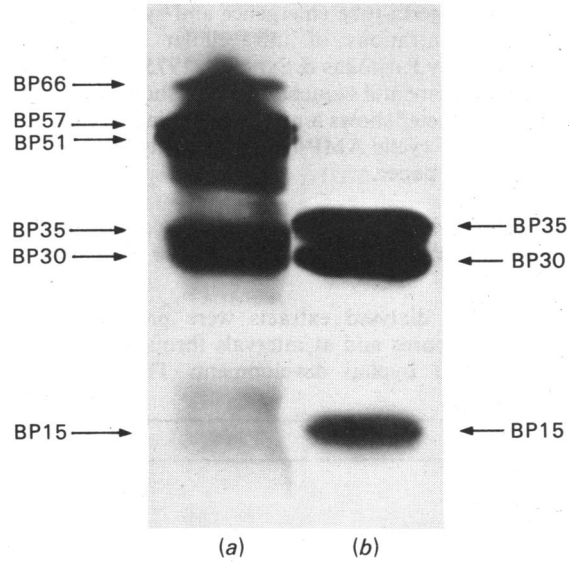

Fig. 7. *Spectrum of cyclic AMP-binding proteins in M. racemosus sporangiospores incubated for* 18*h in nutrient medium containing cycloheximide* (500μg/ml, *final concn.*)
Binding proteins were identified and labelled as in Fig. 6. (*a*) Binding proteins in dormant spores before incubation with cycloheximide; (*b*) binding proteins after 18h incubation in the presence of cycloheximide.

Discussion

The previous work of Larsen & Sypherd (1974) and Paveto *et al.* (1975) established the correlation of *Mucor* morphology with internal cyclic AMP concentrations. In those studies relatively low concentrations of the nucleotide were characteristic of hyphae, whereas high concentrations were characteristic of budding 'yeasts'. Cyclic AMP added to the culture medium prevented growth in the hyphal form: cyclic AMP-treated hyphae became converted into budding 'yeasts'. Paznokas & Sypherd (1975) reported that cyclic AMP concentrations temporarily increased approx. 10-fold during spherical growth (swelling) of the sporangiospore in the process of germination. The concentrations decreased before germ-tube emergence to relatively low concentrations characteristic of hyphal cells. Cyclic AMP added to the culture medium during spherical growth of the sporangiospores prevented the emergence of germ tubes; in *M. racemosus*, spores treated thus continued to swell indefinitely producing giant cells but no buds (A. Larsen, P. Borgia, M. Orlowski & J. Paznokas, unpublished work). The consistent feature among these observations is that high cyclic AMP concentrations seem to be antithetical to hyphal development, although favouring spherical (but not necessarily budding) growth.

According to Paveto *et al.* (1975) the internal cyclic AMP concentrations in *Mucor rouxii* may be adjusted through regulation of the cyclic AMP phosphodiesterase activity. Adenylate cyclase was reported to

remain at constant specific activities in all morphological forms of *M. rouxii*, whereas the phosphodiesterase activity was 6-fold higher in hyphae than in 'yeasts'. The mechanism of phosphodiesterase regulation is not yet understood in *M. rouxii*.

In the present paper we have demonstrated that cyclic AMP interacts specifically with a limited number of soluble proteins whose pattern changes in a characteristic way during the time course of hyphal development from sporangiospores of *M. racemosus*. This was done by using the new technique of labelling the binding proteins with a photoaffinity analogue of cyclic AMP (Haley, 1975). The overall capacity of soluble cell protein for cyclic AMP binding shows the same pattern of flux during development as do free intracellular cyclic AMP concentrations (Fig. 5). Cyclic AMP-binding activity is highest during spherical growth of the spore, when internal cyclic AMP concentrations are also their highest. Both binding activity and internal cyclic AMP concentrations decreased greatly before germ-tube emergence and stayed at relatively low values throughout hyphal growth. The binding activity of individual protein species also changed profoundly during development (Fig. 6). Most of these proteins showed the same behaviour as gross binding activity, that is, their binding activity (as judged by band density on the radioautograms) was highest in the dormant and swelling spores and decreased or disappeared as development progressed. Some notable exceptions were mentioned in the Results section. It seems plausible that the fluctuating concentrations of cyclic AMP may be expressing the regulatory role of this molecule in morphogenesis in interaction with the specific binding proteins, which undergo a parallel change in intracellular concentration.

Since the equilibrium dissociation constants for cyclic AMP binding by *M. racemosus* soluble proteins did not change significantly during the development (Fig. 2), it is likely that the observed changes in binding activity represent adjustments in the number of binding-protein molecules rather than changes in their affinity for cyclic AMP.

Moreno *et al.* (1977) examined cyclic AMP-binding activity in unfractionated hyphal extracts of *M. rouxii*. In contrast with the Scatchard-plot analysis presented in Fig. 2, they reported a single binding site with a K_d of 40nM. Saturation of binding proteins with cyclic AMP took considerably longer in their system (60min) than it did in the present study (2min). In addition, the effectiveness of cyclic GMP in competing with cyclic AMP for binding sites was somewhat less in their system than in the present case. These differences may be a consequence of the different assay conditions or species of *Mucor* used in each study.

The fact that cyclic GMP competes with cyclic AMP for binding sites suggests that perhaps some binding proteins filled by cyclic AMP in the standard assay system may be specific for cyclic GMP in the living cell. Since internal cyclic GMP concentrations fluctuate so dramatically in germinating sporangiospores of *M. racemosus* (Orlowski & Sypherd, 1976), the possibility of specific cyclic GMP-binding proteins certainly bears investigation.

The distribution of cyclic AMP-binding proteins after exposure of sporangiospores to cycloheximide is significant. Only three binding proteins were observed, namely proteins BP35, BP30 and BP15 (Fig. 7). Since new proteins cannot be synthesized in the presence of cycloheximide (Orlowski & Sypherd, 1978*a*,*b*) and since no net loss of cell protein occurs under these conditions (M. Orlowski, unpublished work), it seems likely that the accumulated protein-BP35, -BP30 and -BP15 molecules were degradation products *in vivo* of the higher-molecular-weight species. This is not to say that they are necessarily non-functional in the cell. They may represent activated cleavage products, inactivated degradation products, or perhaps proteins with altered functions. The data suggest that proteins BP66, BP57, BP51, BP46 and BP39 may require constant synthesis to maintain the concentrations observed in control cultures. Since control cultures never accumulated proteins BP35, BP30 and BP15, and in fact preferentially eliminated them, it seems possible that the development of a specific mechanism for their removal or degradation was inhibited by cycloheximide. Alternatively the cycloheximide does not permit the completion of some transient event in which they play an integral function.

The cellular functions of the cyclic AMP-binding proteins described in the present paper are still not known. In other fungi, protein kinases have been reported to co-chromatograph with cyclic AMP-binding activity on ion-exchange columns (Sampson, 1977; Silverman, 1978; Moreno *et al.*, 1977). The possibility that at least part of the binding activity has protein kinase activity during cell morphogenesis of *M. racemosus* requires investigation.

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