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Purification and Identification of the Fruiting-Inducing Substances in *Coprinus macrorhizus*

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Substances which are effective in inducing fruiting bodies in monokaryotic mycelia of the *fis*$^+$ strain of *Coprinus macrorhizus* were purified and characterized. The active components of fruiting-inducing substances were identified as adenosine-3'-monophosphate, adenosine 3',5'-cyclic monophosphate (cyclic AMP), and a protein which is bound with the cyclic AMP. Cyclic AMP was synthesized from adenine within mycelia of the mutant strains which form monokaryotic fruiting bodies without the addition of fruiting-inducing substances, but not in those of the strains which do not form monokaryotic fruiting bodies. The proteins which bind with cyclic AMP were detected in crude extracts of mycelia of those strains which form monokaryotic fruiting bodies and of the dikaryon, but not in those of the strains which do not form monokaryotic fruiting bodies.

The formation of fruiting bodies in the higher Basidiomycetes is not limited to the dikaryotic mycelium, for it has been shown that the process of monokaryotic fruiting can be initiated by the addition of chemical substances. Fruiting-inducing substances (FIS) which are effective in inducing monokaryotic fruiting were isolated from cell-free extracts of fruiting bodies of the same species or unrelated species of fungi (19, 26, 28). Three types of monokaryotic strains of *Coprinus macrorhizus* were found in our earlier studies (26): *fis*$^-$, which produces no monokaryotic fruiting body irrespective of the presence or absence of FIS; *fis*$^+$, which produces monokaryotic fruiting bodies in the presence of FIS; and *fis*^{c}, which produces monokaryotic fruiting bodies constitutively. Two different fractions of FIS which induced fruiting in the *fis*$^+$ strains were identified after Sephadex G-25 filtration of cell-free extract of fruiting bodies of *C. macrorhizus* or *Lentinus edodes*. One of these contained protein and the other appeared to have a chemical nature similar to nucleotides. The present report demonstrates that the active components of FIS in *C. macrorhizus* are adenosine-3'-monophosphate (3'-AMP) and adenosine 3',5'-cyclic monophosphate (cyclic AMP) which may be made active when bound to a particular protein fraction.

MATERIALS AND METHODS

Organisms. The following strains of *C. macrorhizus* Rea f. *microsporus* Hongo were used: 708-7 *A7B8*, 808-15 *A8B7*, *fis*$^-$, *fis*$^+$, *fis*^{c}, and *ad*$^-$ (T5-M1). The origin and characteristics of these strains have been described elsewhere (26). Double mutants were produced by appropriate crosses of single mutants. Fruiting bodies of *L. edodes* used in this study were obtained at a market.

Media. Fruiting assays, production of mycelia and fruiting bodies, and culture maintenance were carried out on a malt-yeast medium (MY medium) which contained 10 g of malt extract, 4 g of yeast extract, and 4 g of glucose per liter of deionized water. To prepare solid agar medium, 10 g of agar powder was added to 1 liter of MY medium.

Assay for FIS activity. Bioassays for fruiting-inducing activity (FIS activity) were performed as follows. The *fis*$^+$ strain of *C. macrorhizus* was inoculated on a slant of MY medium (10 ml) in a test tube. A 0.2-ml sample of sterilized FIS was added before inoculation of the test strain. As a control test, the same volume of solvent for FIS was added to the test cultures. Slant cultures thus prepared were incubated at 30 C under continuous illumination (1,000 to 6,000 ergs per cm^2 per s). The appearance of fruiting bodies was observed after 5 to 14 days. At least 10 independent cultures were examined to test FIS activity of a sample. Units of FIS activity were defined as follows. An FIS sample was diluted with deionized water to various concentrations, and FIS activity was assayed for each solution as described above. One unit of FIS activity was defined as the

minimal amount with which the probability of induction of fruiting bodies was larger than that in the control test (more than 2 of 10 cultures; 26). Units per milliliter were determined by serial dilution.

Preparation of cell-free extracts. Cell-free extracts of fruiting bodies or mycelia were prepared as follows. Fruiting bodies or mycelia were washed several times with deionized water, pressed dry between filter papers, and macerated for 5 min with approximately the same weight of deionized water in a Waring blender. The suspension of cell fragments was further homogenized with a Braun homogenizer for 3 min. The homogenate was centrifuged at 15,000 $\times$ *g* for 20 min, and the supernatant fraction was designated as crude extract.

Gel filtration. The standard method of gel filtration was employed (2). A gel column (2 by 35 cm) was washed with 0.05 M phosphate buffer or tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer and eluted with the same buffer. Elution positions of Blue Dextran and other known substances were determined by using the same column and are indicated by arrows on the elution profiles.

Ion-exchange chromatography. Analytical-grade resin AG50W x4 (hydrogen form) of 200 to 400 mesh was washed twice with 5 volumes of 2 N HCl and then with distilled water, with 2 N NaOH and then with distilled water, and with 2 N HCl and then with distilled water until chloride-free resin was obtained. A sample was placed on the AG50W x4 column (0.9 by 20 cm) and eluted with distilled water. Dowex 1 x2 resin of 200 to 400 mesh was washed with distilled water, then with 5 to 10 volumes of 6 N formic acid containing 1 M ammonium formate, with several volumes of 88% formic acid, and finally with distilled water until the eluate became pH 7.0. A sample was placed on a Dowex 1 formate column (0.9 by 20 cm) and eluted, first with a linear gradient of formic acid (0 to 0.1 N) and then with 0.1 N formic acid. The elution positions of authentic substances were determined by using the same column and are indicated by arrows on the elution profiles.

Paper electrophoresis and paper and thin-layer chromatography. Paper electrophoresis was performed in a cold room at the potential gradient of 40 V/cm for 60 min by using Toyo no. 51A filter paper. Paper chromatography was performed with Toyo no. 51A filter paper. Cellulose thin-layer sheets (Eastman 6065) were used for thin-layer chromatography.

Determination of cyclic AMP-binding activity. The following three different methods originally used for assay of repressor (5) were employed to determine the cyclic AMP-binding activity.

(i) Ammonium sulfate precipitation method: The reaction mixture, containing 0.05 ml of 5×10^{-6} M ^{3}H-cyclic AMP (0.5 μCi) and the protein preparation in 0.15 ml of 0.05 M Tris buffer, pH 7.4, was incubated at 1 C for 5 min in an ice-water bath. The reaction was terminated by the addition of 0.8 ml of a saturated solution of ammonium sulfate and then further incubated at 1 C for 5 min. After removing the supernatant fluid by centrifugation, the precipitate was dissolved in 0.5 ml of distilled water and added to 10 ml of Bray solution. The radioactivity was determined in a liquid scintillation spectrometer. The binding activity is defined as amounts of bound ^{3}H-cyclic AMP per milligram of protein.

(ii) Membrane filter method: The reaction mixture, containing 50 μliters of ^{3}H-cyclic AMP (0.5 μCi), 25 μliters of 0.05 M $MgSO_4$, 25 μliters of 0.05 M Tris buffer, pH 7.4, and 100 μliters of protein preparation, was incubated at 30 C for 30 min. The reaction was terminated by the addition of 1.8 ml of cold 0.01 M Tris buffer, pH 7.4. The reaction mixture was passed through a membrane filter (0.22 μm pore size, Millipore Corp.), and the filter was dissolved in 10 ml of Bray solution. The radioactivity was determined in a liquid scintillation spectrometer.

(iii) Equilibrium dialysis: The equilibrium points were obtained by dialysis of 0.1-ml portions of the respective extract against 10 ml of Tris buffer, pH 7.4, containing both ^{3}H-labeled and nonradioactive cyclic AMP at various concentration. Dialysis was for 10 h in no. 20 dialysis tubing. After equilibrium was attained, the bag was opened and 50 μliters of each sample was counted and compared to an equal volume of the liquid outside the bag. The specific activity was expressed as the percent excess of ^{3}H-cyclic AMP divided by the protein concentration in milligrams per milliliter.

Bray solution. Radioactivity was determined by using the following Bray solution (6): naphthalene, 60 g; 2,5-diphenyloxazole, 4 g; 1,4-di-2-(5-phenyloxazolyl) benzene, 0.2 g; methanol, 100 ml; ethylene glycol, 20 ml; and dioxane to 1 liter.

Protein measurement. Protein concentration was determined by the method of Lowry et al. (20).

Chemicals. Malt extract was a product of Difco, and yeast extract was purchased from Kyokuto. Other special chemicals used were as follows: Dowex 50 and 1 (Dow Chemicals), Sephadex G-25 and G-100, diethylaminoethyl (DEAE)-Sephadex A-50, Sepharose 6B, and Blue Dextran (Pharmacia), AG50W (Calbiochem), cellulose thin-layer sheet (Eastman), ^{3}H-adenine and ^{3}H-cyclic AMP (Radiochemical Centre and New England Nuclear Corp.), alkaline phosphatase (Sigma Chemical Co.), and cyclic AMP, adenosine triphosphate, and other nucleotides (Boehringer Mannheim).

RESULTS

Purification and characterization of the FIS fraction of small molecular weight. It has been indicated previously that FIS was detected in cell-free extracts of fruiting bodies and dikaryotic mycelia of *C. macrorhizus* and fruiting bodies of several other Basidiomycete species (26). Two fractions of FIS, which are now designated as FIS-A and FIS-B, were obtained after Sephadex G-25 filtration. The molecular weight of the active substances in one of these, FIS-B, was estimated to be several hundred, and it contained substances absorbing ultraviolet light (UV) at 260 nm. To characterize further the FIS-B fraction, a significant

amount of this fraction was obtained as follows. Amounts of 300 g of fresh fruiting bodies of *C. macrorhizus* or *L. edodes* were suspended in 700 ml of cold 5% trichloroacetic acid, homogenized with a Waring blender for 5 min, and centrifuged at 1,000 × *g* for 20 min. The supernatant fluid thus obtained was washed three times with 10 volumes of ethyl ether saturated with distilled water to remove trichloroacetic acid. The aqueous phase (pH 5.6), incubated at 90 C for 3 min to remove ethyl ether, was used as the crude FIS-B sample. A 70-ml amount of this sample was applied to a Dowex 1 x2 (formate form) column. As shown in Fig. 1, two peaks of FIS activity were observed: the first small peak designated as FIS-B-1 was eluted at the position of cyclic AMP, and the second large peak designated as FIS-B-2 was eluted at the position of 3'-AMP. Essentially the same elution profiles were obtained for FIS-B samples prepared from *C. macrorhizus* and *L. edodes*. The FIS-B-1 peak showed no detectable UV absorption, and further purification and identification of this peak were impossible. The fractions corresponding to the FIS-B-2 peak were collected, applied to a Dowex 50 x2 (hydrogen form) column, and eluted with distilled water. The fractions absorbing UV were collected, frozen, and dried. The resulting powder was dissolved in 5 ml of distilled water and applied to a Dowex 1 x2 (formate form) column. The column was washed with distilled water, eluted first with a linear gradient of formic acid from 0.02 to 0.1 N, and further eluted with 0.1 N formic acid. All fractions were neutralized and assayed for FIS activity. The fractions showing FIS activity were collected, frozen, and dried. The resulting powder was used as the FIS-B-2 sample for further chemical analysis. The purification procedures of FIS-B-2 described above are summarized in Table 1.

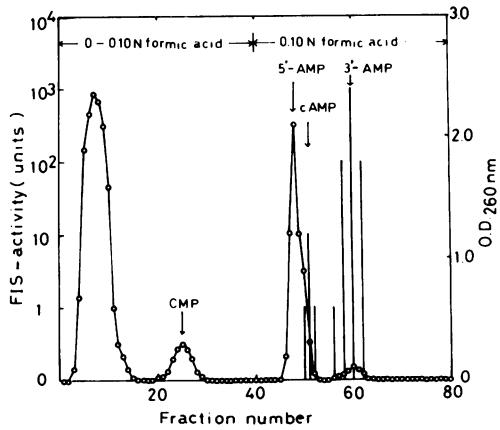

FIG. 1. *Ion-exchange chromatography of FIS-B fraction obtained from fruiting bodies of Coprinus macrorhizus. A 70-ml amount of crude FIS-B sample was applied to a Dowex 1 x2 (formate form) column (0.9 by 20 cm). The column was washed with distilled water and eluted, first with a linear gradient of formic acid (0 to 0.1 N) and then with 0.1 N formic acid, into 6.5-ml fractions. Elution positions of cytosine monophosphate (CMP), 5'-AMP, 3'-AMP, and cyclic AMP (cAMP) are indicated by arrows. Vertical lines, FIS activity; O, optical density at 260 nm.*

The final FIS-B-2 sample was dissolved in distilled water and characterized by several chromatographic and chemical methods. First, the FIS-B-2 sample was applied to Toyo 51A filter paper and to cellulose thin-layer sheets and developed with several kinds of solvent. As shown in Table 2, FIS-B-2 showed R_F values almost identical to those of authentic 3'-AMP. Next, the FIS-B-2 sample was subjected to paper electrophoresis. As shown in Table 2, FIS-B-2 migrated the same distance as authentic 3'-AMP. The UV-absorption spectrum of the FIS-B-2 sample was compared with that of authentic 3'-AMP under acid-neutral, and alkaline conditions; no differences in UV-absorption spectra were found between the two substances. The solutions of FIS-B-2 and 3'-AMP showing the same absorbancy at 260 nm resulted in the same level of FIS activity (Table 3). The FIS-B-2 sample treated with alkaline phosphatase completely lost FIS activity, although it showed the same level of UV absorption at 260 nm (Table 3). The FIS-B-2 sample was frozen and dried, and the infrared spectrum of the resulting powder in a fused KBr pellet was measured. As shown in Fig. 2, the spectrum of this sample completely coincided with that of authentic 3'-AMP, but showed differences from the spectra of 5'-AMP and cyclic AMP. All of these results indicate that the FIS-B-2 sample is identical to 3'-AMP.

TABLE 1. *Purification of FIS-B-2 obtained from Lentinus edodes*

Fraction	OD[a] × ml	Vol (ml)	FIS activity (total units)
Trichloroacetic acid-soluble fraction	37,500	700	3.5×10^7
Fraction treated with ethyl ether	25,600	700	3.5×10^7
First Dowex 1 x2 eluate	35.0	220	1.1×10^7
Dowex 50 x2 eluate	23.5	70	3.5×10^6
Second Dowex 1 x2 eluate	20.5	50	2.5×10^6

[a] Optical density at 260 nm.

TABLE 2. *Paper chromatography, thin-layer chromatography, and paper electrophoresis of the FIS-B-2 sample obtained from Lentinus edodes in comparison with 3'-AMP, 5'-AMP, adenosine, and cyclic AMP*

Sample	Paper chromatography[a]						Thin-layer chromatography[b]		Paper electrophoresis[c]		
	1	2	3	4	5	6	1	2	1	2	3
FIS-B-2	0.27	0.55	0.29	0.03	0.62	0.23	0.10	0.07	4.8	6.8	7.6
3'-AMP	0.29	0.57	0.27	0.02	0.61	0.25	0.11	0.06	4.8	6.8	7.6
5'-AMP	0.18	0.73	0.18	0.01	0.53	0.20	0.05	0.04	5.0	7.0	7.6
Adenosine	0.63	0.52	0.60	0.17	0.75	0.62	0.39	0.38	—	—	—
Cyclic AMP	0.48	0.59	0.55	0.04	0.61	0.57	0.41	0.27	4.6	5.0	5.4

[a] R_F values developed with the following solvents: (1) ethanol-ammonium acetate buffer (1 M, pH 7.5), 75:30; (2) NH_4HCO_3-water, 16:100 (wt/vol); (3) isopropanol-concentrated ammonia-0.1 M boric acid, 7:1:2; (4) *n*-butanol-water, 86:14; (5) isobutylic acid-1 N NH_4OH-0.1 M EDTA (pH 7.0), 100:60:1.6; (6) isopropanol-concentrated ammonia-water, 7:1:2.

[b] R_F values developed with the following solvents: (1) isopropanol-concentrated ammonia-0.1 M boric acid, 7:1:2; (2) 1 M ammonium acetate-99% ethanol, 30:75.

[c] Distances (centimeters) migrated toward the positive electrode with the following three buffer solutions: (1) 0.02 M sodium citrate buffer (pH 3.5); (2) 0.03 M potassium phosphate buffer (pH 7.0); (3) 0.05 M borate buffer (pH 9.2).

TABLE 3. *Comparison of FIS activity among FIS-B-2 obtained from Lentinus edodes, 3'-AMP, and FIS-B-2 treated with alkaline phosphatase*

FIS sample	OD[a] of FIS sample	No. of cultures producing fruiting bodies[b]
FIS-B-2	1.5×10^{-3}	9
	1.5×10^{-4}	5
	1.5×10^{-5}	1
3'-AMP	1.5×10^{-3}	8
	1.5×10^{-4}	5
	1.5×10^{-5}	1
FIS-B-2 treated with alkaline phosphatase[c]	1.5×10^{-3}	1
	1.5×10^{-4}	1
	1.5×10^{-5}	1
None		1

[a] Optical density at 260 nm.

[b] Ten independent slant cultures of strain *fis*$^{+}$ *A8B7* were made and incubated at 30 C for 15 days under continuous illumination.

[c] FIS-B-2 sample dissolved 0.05 M Tris-hydrochloride buffer, pH 8.0, was incubated with 50 μg of alkaline phosphatase at 37 C for 60 min.

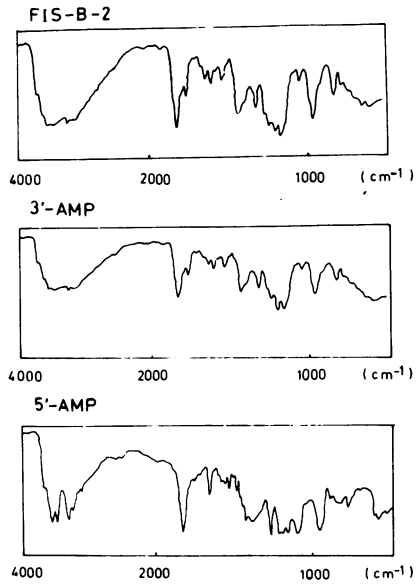

FIG. 2. *Comparison of infrared spectra among FIS-B-2 obtained from Lentinus edodes, 3'-AMP, and 5'-AMP. Ordinate is percent transmission.*

Detection of cyclic AMP synthesized in vivo. On the elution profile of Dowex 1 x2 chromatography (Fig. 1), the FIS-B-1 peak corresponds to that of cyclic AMP. However, the FIS-B-1 peak showed no UV absorption, and further direct purification and identification of this peak were impossible. To detect extremely small amounts of cyclic AMP synthesized in cells, ^{3}H-adenine was incorporated into mycelia of *fis*^{c} *ad*$^{-}$ *A8B7* and *fis*$^{-}$ *ad*$^{-}$ *A8B7* strains of *C. macrorhizus*, and the presence of synthesized ^{3}H-cyclic AMP was measured. Adenine-requiring strains were used to raise the efficiency of ^{3}H-adenine incorporation. A mycelial suspension of each of these strains was inoculated into a 100-ml Erlenmeyer flask containing 20 ml of MY medium and incubated for 8 days at 30 C under continuous illumination. A 1-ml amount of a solution containing 1 μmol of carrier adenine and 100 μCi of ^{3}H-adenine, sterilized by filtration, was added to the

flask and incubation was continued for 10 h under the same conditions. The mycelial pad was harvested on a filter paper and washed well with distilled water. The crude FIS-B sample obtained as described above was mixed well with 4 ml of 5% $ZnSO_4$ and 4 ml of 0.3 M $Ba(OH)_2$ solution (pH 7.2 to 7.8). A portion of the supernatant fluid obtained after centrifugation at 1,000 × *g* for 20 min was mixed with carrier cyclic AMP, applied to an AG50W x4 (hydrogen form) column, and eluted with distilled water. The UV absorbancy and ^3H radioactivity were measured for each fraction. As shown in Fig. 3, the FIS-B sample obtained from the *fis*^{c} strain contained a significant amount of radioactivity in the fraction expected for cyclic AMP, but that from the *fis*$^-$ strain showed an extremely small amount of radioactivity in the same fraction. The fractions corresponding to cyclic AMP peak were pooled, frozen, and dried. The resulting powder, dissolved in 50 μliters of distilled water, was applied to a cellulose thin-layer sheet. As shown in Fig. 4a, a significant amount of

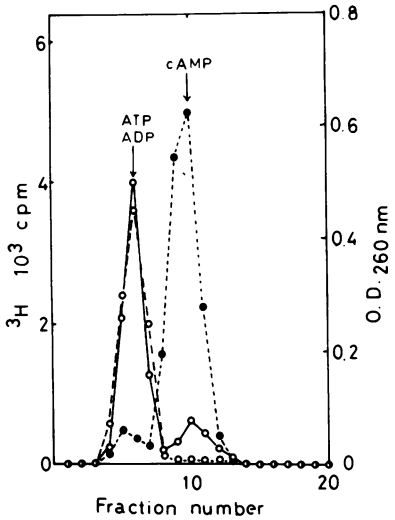

FIG. 3. *Ion-exchange chromatography of FIS-B fraction obtained from mycelia with incorporated ^3H-adenine. The FIS-B fraction was obtained from 8-day-old mycelia of fis*^{c} *ad*$^-$ *and fis*$^-$ *ad*$^-$ *strains grown with ^3H-adenine (100 μCi) for 10 h. A 5-ml amount of FIS-B fraction treated with $ZnSO_4$-$Ba(OH)_2$ containing carrier cyclic AMP (50 μmol) was applied to an AG50W x4 (hydrogen form) column (0.9 by 20 cm) and eluted with distilled water into 4.5-ml fractions. Ultraviolet absorption and radioactivity of each fraction were determined. Elution positions of ATP, ADP, and cyclic AMP (cAMP) are indicated by arrows. Solid lines, fis*^{c}*; dashed lines, fis*$^-$*; ○, ^3H-radioactivity; ●, optical density at 260 nm.*

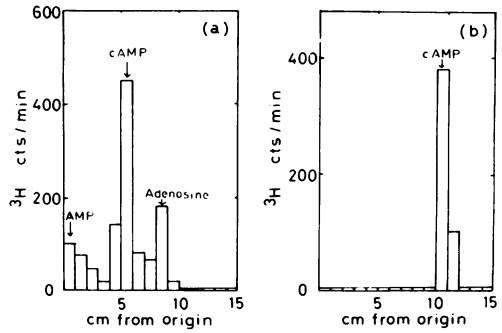

FIG. 4. *Thin-layer chromatography of the cyclic AMP fraction obtained after AG50W x4 chromatography of FIS-B fraction obtained from fis*^{c} *ad*$^-$ *mycelia with incorporated ^3H-adenine. (a) The cyclic AMP fraction obtained after AG50W x4 chromatography (Fig. 3) was chromatographed together with carrier cyclic AMP on cellulose thin-layer sheets (Eastman 6065); the solvent used was 1 M ammonium acetate-99% ethanol (30:75, vol/vol). The thin-layer sheet was separated by 1 cm and the radioactivity was counted. The elution positions of AMP, cyclic AMP (cAMP), and adenosine are indicated by arrows. (b) The cyclic AMP fractions obtained in (a) were rechromatographed by the same method as in (a) except that the following solvent was used: isopropanol-concentrated ammonia-0.1 M boric acid (60:10:30, by volume).*

radioactivity was found at the position expected for cyclic AMP in the case of the sample obtained from the *fis*^{c} strain. The radioactive cyclic AMP fraction obtained from the first thin-layer chromatogram shown in Fig. 4a was rechromatographed. The radioactivity was observed only at the cyclic AMP fraction (Fig. 4b). Table 4 summarizes the purification procedures of ^3H-cyclic AMP. These results clearly indicate that the *fis*^{c} strain produced cyclic AMP in vivo, but that the *fis*$^-$ strain did not.

Cyclic AMP-binding activity of cell-free extracts. Crude mycelial extracts were prepared from 5- to 14-day-old cultures of four strains of *C. macrorhizus* and assayed for cyclic AMP-binding activity. Crude mycelial extracts of the strains which form fruiting bodies, *fis*^{c}, *fis*$^+$, and the dikaryon, showed cyclic AMP-binding activity, and the activity decreased in the course of fruiting body formation (Fig. 5). Crude mycelial extracts of the *fis*$^-$ strain showed scarcely any cyclic AMP-binding activity.

The partial purification of cyclic AMP-binding activity was performed as follows; 35.2 g of ammonium sulfate was added to 50 ml of the crude extract of *fis*^{c} strain. The solution was allowed to stand at 4 C for 2 h in an ice-water

TABLE 4. *Detection of ^{3}H-cyclic AMP produced by fis^c ad$^-$ A8B7 and fis$^-$ ad$^-$ A8B7 strains grown in a medium containing 100 μCi of ^{3}H-adenine*

Cyclic AMP fraction	*fis^c ad$^-$ A8B7*		*fis$^-$ ad$^-$ A8B7*	
	Total counts/min	Yield (%)	Total counts/min	Yield (%)
Trichloroacetic acid-soluble fraction	3.32×10^6	100	3.62×10^6	100
$ZnSO_4$, $Ba(OH)_2$-treated fraction	2.54×10^6	77	1.12×10^6	31
AG50W x4 chromatogram eluate	3.97×10^5	10	1.02×10^3	0.03
First thin-layer chromatogram eluate	2.08×10^4	0.6	0	0
Second thin-layer chromatogram eluate	1.0×10^4	0.3	0	0

bath, and the supernatant fluid was removed by centrifugation. The precipitate was dissolved in 8 ml of 0.05 M Tris buffer, pH 7.4, and the precipitate was removed by centrifugation. The supernatant fluid was applied to a Sepharose 6 B column. As shown in Fig. 6, the largest peak of cyclic AMP-binding activity agrees with the peak of phosphodiesterase activity which degrades cyclic AMP. The cyclic AMP-binding activity was detected in fractions eluted ahead of the phosphodiesterase peak, but no clear peak was observed in these fractions. A clear peak of cyclic AMP-binding activity found behind that of phosphodiesterase may correspond to the elution position of the FIS fraction of larger molecular weight (FIS-A).

To obtain a partially purified sample of FIS-A, 50 ml of cell-free extract prepared from fruiting bodies of *L. edodes* was fractionated through a Dowex 1 x2 column, and fractions showing FIS activity were pooled, frozen, and dried. The resulting FIS sample was subjected to gel filtration through Sephadex G-25. Fractions corresponding to the first peak, designated as FIS A fraction, were pooled, frozen, and dried. A 3-ml amount of the concentrated FIS-A fraction thus obtained was applied to a DEAE-Sephadex A-50 column (2 by 10 cm) and eluted with 0.05 M phosphate buffer, pH 7.2, into 4-ml fractions. The fractions showing UV absorption at 260 nm were pooled. A 62-ml portion of the pooled solution was precipitated by the addition of 32.9 g of ammonium sulfate, allowed to stand for 12 h at 4 C, and centrifuged. The precipitate obtained was dissolved in 3 ml of 0.05 M phosphate buffer, pH 7.4, applied to a Sephadex G-25 column, and eluted with the same buffer into 6-ml fractions. The fractions corresponding to a clear peak of UV absorption at 260 nm were pooled; 25 ml of this solution was precipitated by addition of 13.1 g of ammonium sulfate, allowed to stand for 12 h at 4 C, and centrifuged. The precipitate was dissolved in 3 ml of the same buffer as described above, applied to a Sephadex G-50 column, and eluted with the same buffer into

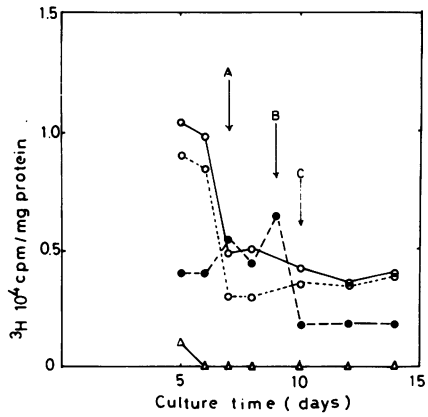

FIG. 5. *Cyclic AMP-binding activity of crude extracts obtained from mycelia (including fruiting bodies) of various ages in four strains of Coprinus macrorhizus. Cyclic AMP-binding activity was determined by the ammonium sulfate precipitation method. The following three stages of fruiting body formation are indicated by arrows: cell aggregation (A), formation of fruiting body primordia (B), and formation of mature fruiting body (C). Symbols: ●----●, dikaryon (A7B8 + A8B7); O——O, fis^c A8B7; O----O, fis$^+$ A8B7; Δ——Δ, fis$^-$ A8B7.*

6-ml fractions. The fractions corresponding to the major part of the peak of UV absorption were collected and used as the partially purified FIS-A sample. The purification procedures of the FIS-A sample are summarized in Table 5. The cyclic AMP-binding activity of the FIS-A sample was measured by the membrane-filter method and the equilibrium dialysis method. The results are shown in Table 6 and Fig. 7. The FIS-A sample (optical density at 280 nm, 0.50) could bind with 20 pmol of cyclic AMP in both assay systems. From the equilibrium dialysis, it was found that the FIS-A sample binds cyclic AMP with an apparent dissociation constant (K_d) of 3.6×10^{-7} M. These results suggest that the FIS-A sample may easily bind with cyclic AMP in vivo.

DISCUSSION

Examination of cell-free extracts obtained from fruiting bodies of *C. macrorhizus* or *L.*

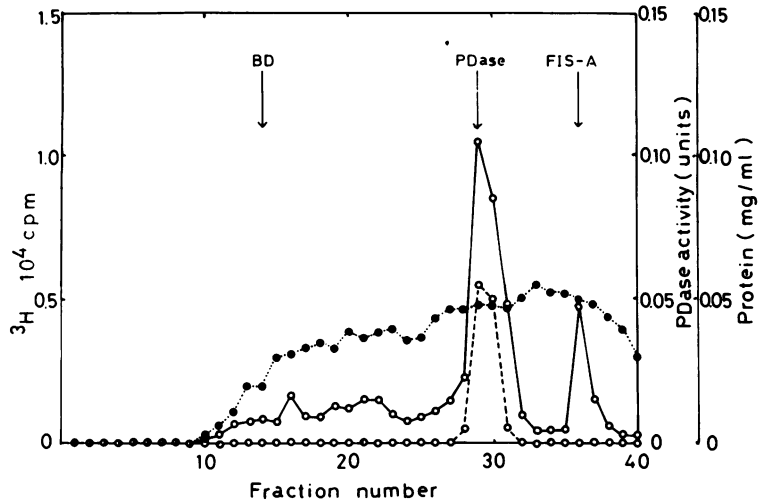

FIG. 6. *Gel filtration of cyclic AMP-binding proteins. A 2-ml amount of the binding protein preparation obtained from 8-day-old mycelia (fis^c A8B7) was put onto a Sepharose 6 B column (2 by 35 cm) and eluted with 0.05 M Tris buffer, pH 7.4, into 4.5-ml fractions. Each fraction was assayed for cyclic AMP-binding activity (by the membrane-filter method), protein concentration, and phosphodiesterase activity assayed as described by Uno and Ishikawa (27). Elution positions of Blue Dextran (BD), phosphodiesterase (PDase), and FIS-A are indicated by arrows. Symbols:* O——O, *cyclic AMP-binding activity;* ●----●, *protein concentration;* O-----O, *PDase activity.*

TABLE 5. *Purification of FIS-A fraction obtained from fruiting bodies of Lentinus edodes*

Fraction	Total OD[a]	Vol (ml)	FIS activity (total units)
First Sephadex G-25 eluate	70	150	7.5×10^5
DEAE-Sephadex A-50 eluate	52	100	5.0×10^5
Second Sephadex G-25 eluate	20	60	3.0×10^5
Sephadex G-50 eluate	15	60	3.0×10^5

[a] Optical density at 280 nm.

TABLE 6. *Cyclic AMP-binding activity of FIS-A protein determined by the membrane-filter method*

Reaction time (min)	Radioactivity on filter (counts/min)[a]	Bound counts/min	Bound cyclic AMP (pmol)
0	310 ± 10	—	—
30	4,053 ± 130	3,742 ± 130	20
60	4,414 ± 115	4,104 ± 135	22

[a] Calculated from four samples.

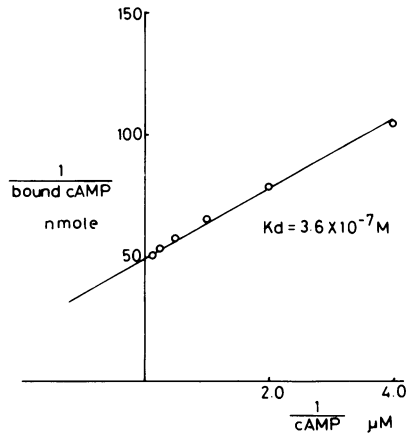

FIG. 7. *Estimation of the dissociation constant for a cyclic AMP and FIS-A complex. The FIS-A sample was dialyzed against various concentrations of ^{3}H-cyclic AMP (cAMP). The reciprocal of the amount of cyclic AMP bound at each external cyclic AMP concentration is plotted against the reciprocal of the cyclic AMP concentration.*

edodes indicated that the substances which are effective in inducing monokaryotic fruiting in the *fis*$^+$ strain of *C. macrorhizus* are 3'-AMP, cyclic AMP, and cyclic AMP-binding protein. The results are compatible with the fact that 3'-AMP and cyclic AMP added to the *fis*$^+$ cultures were effective in inducing monokaryotic fruiting structures (26). 3'-AMP may be produced from cellular ribonucleic acid by the action of ribonucleases or nucleases produced at later stages of growth, and the accumulation of 3'-AMP in old cultures may occasionally be

effective in inducing monokaryotic fruiting bodies in vivo. It has been reported that 3'-AMP can accelerate the morphogenesis of cellular slime mold (13). One of the mechanisms by which 3'-AMP affects the FIS activity may be that addition of 3'-AMP to the cultures results in an increased concentration of cyclic AMP by inhibiting phosphodiesterase, which degrades cyclic AMP in vivo (27).

The present results indicate that the *fis*^{c} strain of *C. macrorhizus* was able to synthesize ^{3}H-cyclic AMP from ^{3}H-adenine, but that the *fis*$^-$ strain failed to synthesize it under the same conditions. The difference between these two strains may indicate that cyclic AMP plays an important role in the induction of monokaryotic fruiting. There are a number of reports indicating that cyclic AMP may play an important role in the expression of hormonal control (12, 24, 25) or in morphogenesis of higher organisms (10, 11). Cyclic AMP is not restricted to higher vertebrates, but has been found in bacteria (8, 21, 31), *Euglena* (22), yeast (29), and cellular slime mold (7, 14). In the cellular slime mold, cyclic AMP has been identified as the acrasin which directs the individual amoebae to aggregate in preparation for the formation of spores. Recently, some reports were published on the study of cyclic AMP in higher plants (3, 23; C. J. Pollard and R. J. Venere, Fed. Proc. **29**:670, 1970). To our knowledge this report is the first to confirm the presence of cyclic AMP in a basidiomycete.

A protein fraction, FIS-A, which was active in inducing monokaryotic fruiting, was partially purified and characterized by its high cyclic AMP-binding activity (an apparent dissociation constant of 3.7×10^{-7} M). This result suggests that the FIS-A protein binds to cyclic AMP in vivo when demonstrating FIS activity. There is the possibility that FIS-A protein added to the *fis*$^+$ culture is not incorporated into mycelial cells but is digested by the action of proteases, thus liberating the bound cyclic AMP.

The existence of cyclic AMP-binding proteins was demonstrated in the mycelial extracts of those strains which form fruiting bodies, *fis*$^+$ and *fis*^{c}, but not in those of the *fis*$^-$ strain. Several kinds of cyclic AMP-binding protein may exist; the most important one may be a particular nonenzymatic protein which binds cyclic AMP, and others may be enzymes which metabolize cyclic AMP, such as phosphodiesterase and adenyl cyclase. Two fractions obtained from crude extracts of *fis*^{c} showed strong cyclic AMP-binding activity: one corresponding to phosphodiesterase, which degrades cyclic AMP, and the other corresponding to the FIS-A fraction. Lack of cyclic AMP-binding proteins in mycelial extract of *fis*$^-$ agrees with the finding that monokaryotic mycelia of *fis*$^-$ contained no FIS effective in inducing monokaryotic fruiting (26) and no enzymes which metabolize cyclic AMP (27). From these results, it may be suggested that the FIS-A fraction which is active in inducing fruiting bodies in the *fis*$^+$ strain is one of the cyclic AMP-binding proteins active in vivo. A number of reports describing cyclic AMP-binding proteins have been published. One of the well-known cyclic AMP-binding proteins is cyclic AMP receptor protein or catabolite gene activator protein which plays a role in the synthesis of inducible enzymes (1, 9, 32). Another cyclic AMP-binding protein may be the transfer factor G (18) or a guanosine triphosphate-dependent cyclic AMP-binding component (4). The existence of cyclic AMP-dependent protein kinases has been described in a number of systems related to the expression of hormonal control (12, 15, 17, 30), and it has been postulated that all of the wide variety of effects elicited by cyclic AMP may be mediated through stimulation of protein kinase (16).

All of the results described in this report strongly suggest that the most important fruiting-inducing substance may be cyclic AMP and that the complex formed between cyclic AMP and a particular protein may play a role in the initiation of fruiting body formation. The manner in which the fruiting body is formed remains to be elucidated.

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