Adenylate Cyclase Activity in Neurospora crassa

I. GENERAL PROPERTIES*

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SUMMARY

Adenylate cyclase activity in Neurospora crassa is a membrane-bound enzyme. Purified preparations of this enzyme appear to be enriched with a component showing the structure of plasma membrane. The activity requires Mn++ and it is not activated by NaF, adrenocorticotropic hormone, epinephrine, or norepinephrine.

Adenylate cyclase, the enzyme responsible for cyclic adenylate synthesis has been found in membrane fractions of animal cells (1-4). This enzyme system is stimulated by several hormones, showing a wide range of specificity. The occurrence of a similar enzyme activity has been also demonstrated in Escherichia coli and Bacillus liquefaciens (5-7), but in these organisms the mechanism that regulates cyclic adenosine 3',5'-monophosphate synthesis is poorly understood.

Recent pieces of evidence indicate that in Neurospora crassa some of the mechanisms designed for the amplification of environmental signals seem to be well evolved. In fact, glycogen metabolism in this ascomycete fungus resembles that of animal cells (8, 9). Glycogen synthetase (UDP-glucose : glycogen 1,4-glucan : orthophosphate glucosyltransferase, EC 2.4.1.11) and phosphorylase (α-1,4-glucan : orthophosphate glucosyltransferase, EC 2.4.1.1) have two interconvertible forms, and as it also occurs in mammalian tissues, conditions for the activation of one of these enzymes appear to be roughly similar to those required for the inactivation of the other and vice versa. Moreover, conversion of the inactive to the active form of glycogen phosphorylase in mycelial extracts proceeds at a higher rate in the presence of cyclic adenosine 3',5'-monophosphate.

The purpose of this paper is to report evidence indicating that membrane preparations from N. crassa catalyze the synthesis of cyclic AMP. The companion paper presents an account of some properties of this enzymatic system.

† This investigation was supported in part by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) and the University of Buenos Aires.
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* The abbreviation used is: cyclic AMP, cyclic adenosine 3',5'-monophosphate.
Adenylyl Cyclase Assay—Unless otherwise indicated, the reaction mixture contained: 2.5 mM [α-32P]ATP (sodium salt, specific activity 100 to 400 cpm per pmole), 2.5 mM MnCl2, 100 mM piperazine-N,N′-bis-2-ethane-sulfonic acid-NaOH (Pipes buffer), pH 6.35, and enzyme (about 0.2 mg of protein). The total volume was 0.1 ml. Incubations were carried out at 37° usually for 15 to 5 min. The reaction was stopped by the addition of 0.1 ml of a solution containing 40 mM ATP and 12.5 mM cyclic AMP, and the radioactive cyclic adenylyl was isolated and counted following the Krishna’s procedure as described by Rodbell (3) with the following modifications. (a) The Dowex columns were 8 cm long and 0.4 cm internal diameter. After application of the sample (0.5 ml) they were washed with 2.5 ml of water. Percolate and washing liquids were discarded. The radioactive cyclic AMP was eluted with 4 ml of water. The effluent was collected in a test tube (1.2 × 10 cm). (b) To the latter fraction 0.3 ml of 0.3 N Ba(OH)2 solution was added. After mixing in a Vortex, 0.3 ml of a 5% ZnSO4 solution was added and mixed again. The mixture was then centrifuged for 30 min at 2000 rpm and the supernatant was transferred to another test tube. The precipitate and centrifugation were afterwards repeated in the same conditions. (c) After the latter centrifugation, the supernatant was again transferred to another test tube and used for determination of radioactivity and absorbance at 290 nm. This last operation was carried out in order to check recoveries; usually they were about 50 to 60%.

Radioactivity was determined on 3-ml aliquots using a naphthalene dioxane system (16). Blanks of the reaction carried out either in the absence of enzyme or in its presence, but not incubated, gave values between 300 and 700 cpm.

The differences between incubated samples and blanks varied with the experimental conditions, but they were in the range of 1,500 to 15,000 cpm. Differences between duplicate samples were between 5 and 10%. Unless otherwise indicated the activities were expressed as nanomoles of 3′,5′-cyclic AMP formed per min of incubation per mg of protein.

**Analytical Procedures**—Protein and DNA were assayed as described by Lowry et al. (17) and Burton (18), respectively. Succinate cytochrome c reductase and pyruvate kinase activities were assayed according to Fleischer and Fleischer (19) and Carminatti et al. (20), respectively. Paper chromatography of cyclic AMP was carried out in Whatman No. 3MM paper, using 2-propanol-ammonium-water (7:2:1) (21) as solvent system.

**Cell Cultures**—The slime mutant of N. crassa (strain F2: Os-1: N1118-FGSC) (22) was used throughout this work. This mutant has two advantages. (a) It grows as isolated protoplasts surrounded by a plasma membrane; the cellular wall characteristic of the mycelial strains is absent. (b) The amount of intracellular membranes is very low.

Culture conditions either in solid or liquid medium were similar to those described by Woodward and Woodward (23). Agar slants containing 2% sucrose, 2% soluble starch, 0.75% nutrient broth, 0.75% yeast extract, and 2.5% agar dissolved in Vogel’s minimal medium (24) were replicated weekly and stored at 30°.

In order to obtain an adequate amount of cells, the organism was grown in Vogel’s liquid minimal medium supplemented as indicated above, except that the addition of agar and soluble starch was omitted. Cultures were carried out in 1000-ml Erlenmeyer flasks containing 200 ml of medium. They were started by inoculation of 106 cells from a solid medium culture. The flasks were then incubated for 18 hours at 32° in a rotatory shaker (120 rpm). After this incubation, cell concentration reached a value of about 107 cells per ml.

**Enzyme Preparation**—The cells, obtained from a liquid culture, were harvested by centrifugation for 7 min at 900 × g. The supernatant was decanted and the cellular pellets were resuspended in 1 mM NaCO3 (one-tenth of the culture volume). The suspension was left in the cold for 30 min (“lysate”) and then it was centrifuged for 20 min at 15,000 × g. The supernatant thus obtained was again centrifuged for 120 min at 105,000 × g. The precipitate (“crude membranes”) was resuspended in 1 mM NaCO3 (5 mg of protein per ml) and used as the source of enzyme for most of the experiments.

Further purification of these crude membranes was carried out as follows. After resuspension in cold 1 mM NaCO3 containing 0.25 M sucrose, the preparation was layered on a discontinuous sucrose gradient made from 0.85 to 0.40 M sucrose, over a 1.3 M sucrose cushion. After centrifugation at 105,000 × g for 3 hours (SW 65 rotor, Spinco model L preparative ultracentrifuge), two bands containing a turbid material were observed. The upper band located above the middle of the gradient contained adenylyl cyclase with the greatest specific activity (see below). After dilution with 1 mM NaCO3 the enzyme contained in this band could be sedimented by centrifugation at 105,000 × g for 120 min (“purified membranes”).

**RESULTS**

Structure of “Slime Cells”—Under light or electron microscopic examination (Figs. 1 and 2, respectively) the slime strain appears as isolated cells surrounded by a plasma membrane containing a vacuole and several nuclei. The size of the cells and vacuole varies with the time of culture in liquid medium. In young cultures (18 hours) the cells were about 15 μ in di-
ameter and the vacuole is small. On the contrary, after 40 hours of culture the cell diameter increases up to 30 μ and the vacuole occupies a large portion of the cellular volume. In the electron micrograph shown in Fig. 2, it can be observed that the cytoplasm contains few endoplasmic reticulum membranes and mitochondrial structures.

Morphology Before and After Cell Lysis—Resuspension of the slime cells in hypotonic medium leads to drastic changes in the cellular morphology. First, the cytoplasm swells and the cellular material undergoes constriction between the vacuole and the cellular surface (Fig. 3A). The image is very similar to that of a fat cell. After that, the cell membrane is broken. In these circumstances two types of structures may be observed: (a) amorphous particulate material, composed of membranous aggregates; and (b) spherical sacs of variable size. Some of these sacs are small (about 5 to 10 μ in diameter) and enclose cellular material with an intense brownian movement. The other sacs are larger (about 15 to 30 μ in diameter) and appear as optically empty. These empty sacs arise from the central vacuole.

As can be seen, some of these images are very similar to those described for fat cell ghosts (3).

Structure of Membrane—Morphology of the membranes was obtained with the electron microscope. This was carried out with a "purified membrane" preparation. As can be seen in Fig. 4, those fractions with highest adenylate cyclase specific activity (see below) appear to be enriched with a component showing the structure of a plasma membrane; that is, vesiculated structures surrounded by a thick membrane. In some portions the membrane appears diffuse owing to a tangential cutting edge (Fig. 4A), but in others (Fig. 4B) the thickness of the image (100 A) corresponds to plasma membranes as described in animal cells (25).

Adenylate Cyclase Activity in Slime Membrane—In a first approach to identify the cellular fraction responsible for the adenylate cyclase activity in slime cells, the subcellular fractions obtained after homogenization in 0.25 M sucrose were submitted to a differential centrifugation. As can be seen in Table I 50% of the enzyme activity was sedimented at 105,000 X g (Fraction V). DNA and succinate cytochrome c reductase activity sedi-
mented at lower centrifugal forces (Fractions I, II, and IV, respectively). The remaining cyclase activity and glycolytic enzymes were obtained in the last supernatant fluid (Fraction V). This last cyclase activity could also be sedimented at higher speed (105,000 × g for 2 hours). The crude membrane preparation contained in the precipitate of Fraction V was purified by a discontinuous sucrose gradient centrifugation. As can be seen in Fig. 5, the highest cyclase specific activity was recovered in a turbid band located in the upper portion of the gradient (about 0.55 M sucrose). This purified membrane preparation was further purified by resuspension in 0.6 M sucrose and centrifugation for 2 hours at 165,000 × g (SW 65 rotor; Spinco model L preparative ultracentrifuge) over a 1.2 M sucrose cushion. In this condition, the activity was recovered in the fractions above the interphase and the membranes had the structure shown in Fig. 4. In some preparations these fractions showed minor contaminations with microsomal vesicles.

Adenylate Cyclase Activity in Preparations Containing Lubrol-PX—Table II shows that after resuspension of adenylate cyclase preparations at different stages of purification, 15,000 × g
Table I

Distribution of enzyme activities, DNA, and protein in different subcellular fractions of slime cells

Cells from a 200-ml liquid culture were collected, and the cellular precipitate was resuspended in 20 ml of cold 0.25 M sucrose. The cells were broken using a Potter-Elvehjem homogenizer (glass-Tefton) and the extract was then submitted to a differential centrifugation. Other conditions were as indicated under “Experimental Procedure.”

| Fraction          | Centrifugation | Volume | Total protein | Total DNA | Succinate cytochrome c reductase | Pyruvate kinase | Adenylate cyclase |
|-------------------|----------------|--------|--------------|-----------|---------------------------------|-----------------|-------------------|
|                   | × g | min | ml | mg | mg | µmoles/min per mg protein |                   |                   |
| I (precipitate)   | 600 | 5   | 1  | 4.4 | 0.2 | 34                          | 170             | 0.047             |
| II (precipitate)  | 600 | 15  | 1  | 5.0 | 0.1 | 39                          | 130             | 0.057             |
| III (precipitate) | 1,500| 20  | 1  | 5.5 |       | 47                          | 190             | 0.102             |
| IV (precipitate)  | 15,000| 20  | 1  | 3.2 |       | 7                           | 100             | 0.256             |
| V (precipitate)   | 105,000| 60  | 2  | 12.0|       | 7                           | 100             | 0.256             |
| V (supernatant)   | 105,000| 60  | 17 | 22.0|       | 1,800                       |                 | 0.134             |

Supernatant, crude or purified membranes, in 1.3% Lubrol-PX and centrifugation for 2 hours at 105,000 × g, most of the enzyme activity was recovered in the supernatant fluid.

Authenticity of Reaction Product—In the assay conditions only cyclic AMP was detected as a reaction product. Two criteria were used for the identification of the radioactive compound (Fig. 6A, and B). (a) It co-chromatographed with carrier cyclic 3',5'-AMP; and (b) when the radioactive spot was eluted and the compound was treated with beef heart phosphodiesterase, only one 32P-labeled compound was formed: 5'-AMP.

Requirements for Adenylate Cyclase Activity—As shown in Fig. 7 (A and B) in the assay conditions the production of cyclic AMP was proportional to the incubation time and the enzyme concentration.

The enzyme requires Mn++ specifically. When this divalent cation was replaced by Ca++ or Mg++ the activity was negligible (Fig. 8).

As can be seen in Fig. 8, theophylline did not show any effect, and mercaptoethanol stabilized the enzyme activity. On the other hand, in the presence of an ATP-generating system the production of cyclic AMP was linear with the incubation time up to 15 min.

Fig. 9 shows plots of the enzyme activity versus the pH of the assay mixture. The maximum activity was observed in the range of pH between 5.5 and 6.3. In addition Mg++ could not substitute for Mn++ over all of the pH range tested.

Enzyme Modifiers—Since fluoride is a very well known activator of all animal adenylate cyclases, a similar effect was explored with the slime enzyme; however in all of the conditions assayed this anion did not activate the enzyme.

Searching for an activator of the Neurospora cyclase, several precursors or catabolites of amino acid metabolism and hormones were tested in the reaction mixtures either at low or high concentration of Mn++ATP or Mg++ATP. They included histamine, 5 hydroxytryptamine, 3 hydroxytyramine, epinephrine,

Table II

Effect of Lubrol on sedimentation of N. crassa adenylate cyclase in different stages of purification

Preparation of the different fractions was carried out as described under “Experimental Procedure.” Sedimentation of the enzyme was performed at 105,000 × g for 2 hours in the presence of 0.25 M sucrose.

| Fraction                  | Specific activity | Total activity | In the presence of 1.3% Lubrol | In the absence of 1.3% Lubrol |
|---------------------------|------------------|----------------|-------------------------------|-------------------------------|
|                           | µmoles/min/mg protein | µmoles/min | Supernat | Precipitate | % Non-sedimentable | Supernat | Precipitate | % Non-sedimentable |
| Lysate                    | 0.041            | 30.3          | 37.5  | 8.5       | 82                | 7.4        | 27.2       | 22                |
| 10,000 × g supernate      | 0.148            | 34.4          | 25.0  | 1.9       | 93                | 4.1        | 18.2       | 18                |
| Crude membranes           | 0.340            | 27.2          | 15.0  | 1.2       | 93                | 3.3        | 13.1       | 20                |
| Purified membranes        | 0.950            | 16.3          | 15.0  | 1.2       | 93                | 3.3        | 13.1       | 20                |

* Assayed in the absence of Lubrol before centrifugation at 105,000 × g.
Paper chromatography of the reaction product obtained after incubation of slime membranes with \[^{32}P\]ATP and Mn\(^{2+}\). The enzyme ("crude membranes") was incubated for 10 min at 37° under conditions corresponding to the standard assay for adenylate cyclase activity. The product was purified by Dowex 50 column chromatography and precipitation with Ba(OH)\(_2\) and ZnSO\(_4\) as described under "Experimental Procedure." The solution obtained was evaporated under reduced pressure and then chromatographed on Whatman No. 3MM paper, using 2-propanol-ammonia-water as solvent system and scanned for radioactivity (Packard model 7200 radiochromatogram scanner).

A, scanning of this paper chromatography. B, the radioactive spot with the mobility of cyclic AMP was washed with absolute ethanol and eluted with water. After evaporation under reduced pressure, the sample was incubated for 60 min at 37° in the presence of 5 mM MgCl\(_2\), 50 mM Tris-HCl buffer, pH 7.4, and beef heart phosphodiesterase (60 μg). The total volume was 0.2 ml. The reaction was stopped by the addition of 0.4 ml of methanol and heating for 3 min at 100°. The mixture was then chromatographed and scanned as indicated above.

**Fig. 6.** Paper chromatography of the reaction product obtained after incubation of slime membranes with \[^{32}P\]ATP and Mn\(^{2+}\). The enzyme ("crude membranes") was incubated for 10 min at 37° under conditions corresponding to the standard assay for adenylate cyclase activity. The product was purified by Dowex 50 column chromatography and precipitation with Ba(OH)\(_2\) and ZnSO\(_4\) as described under "Experimental Procedure." The solution obtained was evaporated under reduced pressure and then chromatographed on Whatman No. 3MM paper, using 2-propanol-ammonia-water as solvent system and scanned for radioactivity (Packard model 7200 radiochromatogram scanner).

A, scanning of this paper chromatography. B, the radioactive spot with the mobility of cyclic AMP was washed with absolute ethanol and eluted with water. After evaporation under reduced pressure, the sample was incubated for 60 min at 37° in the presence of 5 mM MgCl\(_2\), 50 mM Tris-HCl buffer, pH 7.4, and beef heart phosphodiesterase (60 μg). The total volume was 0.2 ml. The reaction was stopped by the addition of 0.4 ml of methanol and heating for 3 min at 100°. The mixture was then chromatographed and scanned as indicated above.

**Fig. 7.** A, production of cyclic AMP, as a function of the incubation time. The assay mixtures contained the indicated milligrams of protein of a "lysate" preparation. B, dependence on enzyme concentration of the adenylate cyclase activity. Enzymatic activities corresponding to the incubations carried out for 2½ min were plotted as a function of the milligrams of protein in the adenylate cyclase assay. Other conditions were as indicated under "Experimental Procedure."

**Fig. 8.** Time course of the adenylate cyclase-catalyzed reaction by a "lysate" preparation. Conditions were as indicated under "Experimental Procedure." In addition, some mixtures contained 5 mM mercaptoethanol (○), or 10 mM theophylline (△), or 5 mM P-enolpyruvate, 50 mM KCl, and 10 μg of pyruvate kinase (△). In other mixtures MnCl\(_2\) was replaced by MgCl\(_2\) (△) or CaCl\(_2\) (△).

**Fig. 9.** Adenylate cyclase activity as function of the pH. The enzyme ("crude membrane" preparation) was assayed in the presence of 100 mM Tris-maleate (open symbols) or Tris-HCl buffer (closed symbols) of the indicated pH. Concentrations of ATP and divalent cation were 2.5 mM. The incubation time was 2½ min. Other conditions were as indicated under "Experimental Procedure."

Discussion

The ascomycete fungus *N. crassa* has a membrane-bound adenylate cyclase. This was expected from the results obtained in the studies on regulation of glycogen metabolism (8, 9). Under the standard conditions for the assay of this enzyme, the specific activity of a crude membrane preparation is in the range...
between 0.15 and 0.45 nmole per min per mg of protein. This activity is of the same order of magnitude of the maximally stimulated animal adenylate cyclase (26–28).

The availability of a strain devoid of a cellular wall has simplified to a great extent the method of purification of cellular membranes. This strain can be easily maintained in agar slants and adequate amounts of cells may be obtained in liquid cultures. These properties open interesting possibilities to extend the study of membrane-bound enzymes in this simple eucaryotic organism.

Some striking differences were found between the animal cyclases and the enzyme of slime cells. The Neurospora cyclase requires Mn++ specifically and it is not activated by fluoride. On the other hand, several hormones with a known stimulating effect on animal cyclases do not have any action.

Acknowledgments—The authors wish to express their gratitude to Dr. Luis F. Leloir for his continued advice and support, and to Dr. Sara H. Goldenberg and other members of the Instituto de Investigaciones Bioquimicas for helpful discussions and criticisms. We are also indebted to Dr. H. Terenzi for the supply of the slime mutant of N. crassa and advice on the cultures techniques. Special thanks are also due to Dr. E. de Robertis and Dr. J. Russo for their collaboration in the obtention of the electron micrographs. One of the authors (M. M. F.) is grateful to the University of Tucuman for financial support.

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*J. Biol. Chem.* 1972, 247:6873-6879.

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