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 α-1,4-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.

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‡ Career investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).
† The abbreviation used is: cyclic AMP, cyclic adenosine 3',5'-monophosphate.

EXPERIMENTAL PROCEDURE

Materials—Pyruvate kinase (type II), adrenocorticotropic hormone, L-β-3,4-dihydroxyphenylalanine, 5-hydroxytryptamine, histamine, tyramine, epinephrine, norepinephrine, 3-hydroxytryptamine, and ATP were purchased from Sigma. Carrier-free inorganic [32P]phosphate was obtained from the Comisión Nacional de Energía Atómica (Argentina). Isopropyldiene adenosine and trichloroacetoniéride were purchased from Aldrich Chemical Co. Dimethylsulfoxide, acetoniéride and triethylamine were obtained from T. Schwartz, Merck and Matheson, and Coleman & Bell, respectively. These solvents were distilled and stored over calcium hydride. Bio-Rad AG 50W-X8 resin, 100 to 200 mesh, was obtained from Calbiochem and washed as described by Butcher et al. (10). Lubrol-PX was obtained from Dupen-Imperial Chemical Industries (Buenos Aires). Adenylylate kinase was prepared from rabbit skeletal muscle as described by Colowick (11). Specific beef heart cyclic 3',5'-AMP phosphodiesterase was prepared according to Butcher and Sutherland (12).

Preparation of [α-32P]ATP—The radioactive nucleotide was obtained by enzymatic phosphorylation of 32P-labeled 5'-AMP. The latter compound was chemically synthesized by a modification of the method of Symons (13) as follows. A carrier-free inorganic [32P]phosphate solution (20 mCi) was dried in a rotatory evaporator. Then it was dissolved in 2 ml of water and evaporated again. This procedure was repeated twice. The radioative phosphate was then dissolved in 1 ml of water and converted to the triethylammonium salt by passing it through a column (0.4 × 8 cm) of Bio-Rad AG 50W-X8 resin (100 to 200 mesh, H+ form) and adding triethylamine in excess to the percolate. After this operation, the percolate plus the triethylamine was evaporated to dryness in a reaction flask (bulb 3 cm in diameter, neck 10 cm long and 1 cm in diameter). Then 15.3 mg (50 μmoles) of isopropyldiene adenosine were added and the content of the reaction flask was further dried with acetoniéride in a vacuum line as described by Greenless and Symons (14). After that, 0.4 ml of a solution containing 82.5 mm trichloroacetoniéride and 50 mm triethylamine in dimethylsulfoxide was rapidly added to the reaction flask. This was immediately plugged with a glass stopper. After mixing, the components of the reaction were incubated for 30 min at 37°C. Then 1.5 ml of 5 N acetic acid were added and the solution was heated for 90 min at 100°C. The solution was afterwards transferred to a test tube (1.3 × 12.5 cm) and evaporated to dryness on a rota-
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 10^7 cells from a solid medium culture. The flasks were then incubated for 18 hours at 32° in a rotary shaker (120 cpm). After this incubation, cell concentration reached a value of about 10^8 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 NaCO$_3$H (one-tenth of the culture volume). The suspension was left in the cold for 30 min ("lysat") 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 NaCO$_3$H (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 NaCO$_3$H containing 0.25 M sucrose, the preparation was layered on a discontinuous sucrose gradient made from 0.85 to 0.4 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 adenylate cyclase with the greatest specific activity (see below). After dilution with 1 mM NaCO$_3$H the enzyme contained in this band could be sedimented by centrifugation at 105,000 × g for 120 min ("purified membranes").

**RESULTS**

**Structure of "Slim 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-
FIG. 2. Electron micrograph of *Neurospora crassa* slime cells. N, nucleus; M, mitochondria; E. M., endoplasmic membranes. × 10,000.

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. Fig. 3, B and C, shows representative pictures of these structures.

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 × 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 (165,000 x 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 x 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 x g centrifugation yielded the highest specific activity.
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 Teflon) and the extract was then submitted to a differential centrifugation. Other conditions were as indicated under “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Centrifugation</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total DNA</th>
<th>Succinate cytochrome c reductase</th>
<th>Pyruvate kinase</th>
<th>Adenylate cyclase</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (precipitate)</td>
<td>600</td>
<td>5</td>
<td>4.4</td>
<td>0.2</td>
<td>34</td>
<td>170</td>
<td>0.047</td>
</tr>
<tr>
<td>II (precipitate)</td>
<td>600</td>
<td>15</td>
<td>5.0</td>
<td>0.1</td>
<td>39</td>
<td>130</td>
<td>0.057</td>
</tr>
<tr>
<td>III (precipitate)</td>
<td>1,500</td>
<td>20</td>
<td>5.5</td>
<td></td>
<td>7</td>
<td>100</td>
<td>0.050</td>
</tr>
<tr>
<td>IV (precipitate)</td>
<td>15,000</td>
<td>20</td>
<td>3.2</td>
<td></td>
<td>47</td>
<td>190</td>
<td>0.102</td>
</tr>
<tr>
<td>V (precipitate)</td>
<td>105,000</td>
<td>60</td>
<td>12.0</td>
<td></td>
<td>7</td>
<td>100</td>
<td>0.256</td>
</tr>
<tr>
<td>V (supernatant)</td>
<td>105,000</td>
<td>60</td>
<td>22.0</td>
<td></td>
<td>1,800</td>
<td></td>
<td>0.134</td>
</tr>
</tbody>
</table>

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 X g for 2 hours in the presence of 0.25 M sucrose.

<table>
<thead>
<tr>
<th>Centrifugation at 105,000 X g</th>
<th>In the presence of 1.3% Lubrol</th>
<th>In the absence of 1.3% Lubrol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Precipitate</td>
</tr>
<tr>
<td>Lysate</td>
<td>0.041</td>
<td>30.3</td>
</tr>
<tr>
<td>15,000 X g supernate</td>
<td>0.148</td>
<td>34.4</td>
</tr>
<tr>
<td>Crude membranes</td>
<td>0.340</td>
<td>27.2</td>
</tr>
<tr>
<td>Purified membranes</td>
<td>0.950</td>
<td>16.3</td>
</tr>
</tbody>
</table>

* Assayed in the absence of Lubrol before centrifugation at 105,000 X g.
Paper chromatography of the reaction product obtained after incubation of slime membranes with [α-32P]ATP and Mn++.

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 ZnSO₄, as described under "Experimental Procedure." The solution obtained was evaporated under reduced pressure and was 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 MgCl₂, 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.

DISCUSSION

The ascomycete fungus N. crassa has a membrane-bound adenylate cyclase. This was expected from the results obtained in the study of 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.

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REFERENCES

15. PALADINI, A. C., AND LELOIR, L. F. (1952) Biochem. J. 51, 426
18. BURTON, K. (1958) Methods Enzymol. 12, 103
22. EMERSON, S. (1963) Genetics 34, 162
23. WOODWARD, V. W., AND WOODWARD, C. K. (1968) Neurospora Newsletter 13, 18
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