# CYCLIC 3',5'-AMP PHOSPHODIESTERASE OF NEUROSPORA crassa

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## SUMMARY

Cyclic 3',5'-AMP (cAMP) phosphodiesterase activity can be demonstrated in extracts of Neurospora crassa. The activity is particulate, has a pH optimum of 7.4, and consists of two forms that have different cAMP binding constants. Methylxanthines, inorganic phosphate, and EDTA are inhibitors of the diesterase as are ATP, ADP, and 8-bromo-cAMP. The enzymatic activity is stimulated by histamine and imidazole. These properties suggest that the Neurospora enzyme is more closely related to the mammalian than to bacterial cAMP phosphodiesterases.

## INTRODUCTION

cAMP has been reported to affect the growth, morphology, and adhesiveness of various types of cells (1-4). Several compounds known to affect either the mammalian adenyl cyclase or cAMP phosphodiesterase have been found to have adverse effects on the growth rate and morphology of wild-type Neurospora (5). To determine whether the action of these agents is the same in Neurospora as in mammalian cells, it is necessary to determine the effects of each compound on the adenyl cyclase and phosphodiesterase in vitro. An adenyl cyclase from Neurospora has been described (6). This report summarizes the properties of a cAMP phosphodiesterase.

## EXPERIMENTAL

Materials: 8-$^{3}$H cAMP (20.4 Ci/mmole) was obtained from

Schwarz/Mann. All other chemicals were Sigma products. Wild-type Neurospora, RL3-8A, was used throughout. Conditions for mass culturing and freeze-drying of mycelia have been described (7).

Partial Purification of cAMP Phosphodiesterase: All steps were performed at 5°. Freeze-dried mycelia were ground in a mortar with an equal weight of sand. The ground cells were extracted for 30 min by stirring with 20 volumes of 0.1 M Tris, pH 7.4, per g dry weight of cells. After centrifugation at 12,000 x g for 10 min, the supernate was further fractionated with ammonium sulfate. The precipitate obtained between 20 and 40% saturation of ammonium sulfate was dissolved in 0.1 M Tris, pH 7.4, (5% of the original volume) and dialyzed overnight against 200 volumes of the same buffer plus 1 mM DTT. Protein was determined by the method of Lowry et al. (8)

cAMP Phosphodiesterase Assay: Assay mixtures contained in a final volume of 100 µl: 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.2 mM cAMP, and 4 p moles of $^3$H-cAMP ($1.5 \times 10^5$ dpm). Reactions were initiated by the addition of enzyme, then incubated for 20 min at 30°, and finally stopped by boiling for 2 min. After centrifugation for 5 min at 5,900 x g, 50 µl of the supernate was removed and mixed with 20 µl of 1 mM adenosine plus 1 mM 5'-AMP which acted as markers for chromatography. Usually 50 µl of this mixture was spotted on Whatman 3 MM paper. Chromatograms were developed by ascending chromatography for 16 hours in a 7:3 mixture of 95% ethanol: 1M ammonium acetate (9). cAMP, adenosine, and 5'-AMP

were visualized with the aid of an ultraviolet lamp. These regions were cut out and radioactivity of each was determined by liquid scintillation counting.

## RESULTS

Since Neurospora extracts contain a large excess of phosphomonoesterase activity, greater than 97% of the radioactive product formed from $^{3}$H-cAMP is recovered as adenosine rather than 5'-AMP. The cAMP phosphodiesterase can be partially separated from the monoesterase on Bio-Gel A-15m columns. Most assays, however, were carried out in the presence of both enzymes. The validity of the phosphodiesterase assay was confirmed by adding snake (Ophiophagus hannah) venom as a source of phosphomonoesterase. No difference was observed in the rate of cAMP conversion to adenosine in the presence or absence of the venom indicating that the phosphodiesterase is the rate limiting step.

Solubility: At least half of the phosphodiesterase activity is particulate. The amount of activity in a 105,000 x g precipitate reaches a constant value after 3 hours of centrifugation and ranges between 65 and 80% of the total activity. Attempts to increase the solubility of the enzyme with 1% digitonin, 1% BRIJ-35, or Lubrol PX were unsuccessful.

Enzymatic Properties: The phosphodiesterase activity in Tris·HCl is approximately two-times that in ADA or maleate buffers at comparable pH values. The pH profile of phosphodiesterase activity in Tris·HCl buffer revealed a narrow peak of activity with a maximum (120 nmoles of adenosine formed/20 min/

mg protein) at pH 7.4. Under these conditions, the amount of adenosine formed from cAMP is directly proportional to the protein concentration up to 3 μg of protein per assay. Kinetics of the enzyme were investigated in an experiment illustrated in Fig. 1.

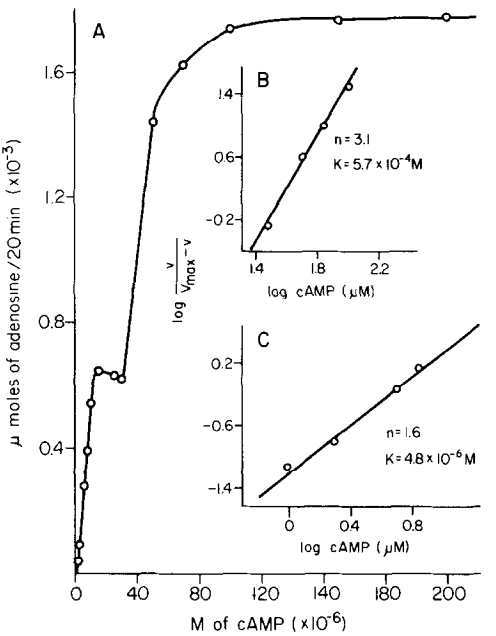


Fig. 1. Kinetics of phosphodiesterase. The velocity versus substrate curve (A) was obtained by varying the nonradioactive cAMP concentration of the standard assay mixture. Other conditions of assay procedure were identical to those described under "Experimental". Curve A. Dependence of the velocity of the phosphodiesterase reaction on the cAMP concentration. Curve B. Hill plot corresponding to the upper portion of Curve A. Curve C. Hill plot of the data in the lower portion of Curve A.

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Two cAMP phosphodiesterases are clearly distinguishable. These data were analyzed by means of a Hill plot (10). A value for n, the Hill coefficient, was calculated for each activity in addition to a value for K. Both activities have apparent values of n

greater than 1.0 and differ by two orders of magnitude in K values.

Effectors: EDTA ($\geq$ 10 mM) inhibits the diesterase activity (see Table 1), however $Mg^{+2}$, $Mn^{+2}$, or $Ca^{+2}$ do not effect the activity up to 10 mM. Inorganic phosphophate is also inhibitory but has no apparent effect on the phosphomonoesterase as described for the yeast system (11). The methylxanthines caffeine, theophylline, and theobromine act as phosphodiesterase inhibitors as does ATP, ADP, and 8-bromo-cAMP. In contrast, both imidazole and histamine are stimulatory.

## DISCUSSION

The Neurospora phosphodiesterase is particulate, inhibited by methylxanthines, and stimulated by imidazole. These properties are similar to those described for several mammalian phosphodiesterases (12-14). Similarly, two forms of the Neurospora enzyme with different affinities for cAMP are found. In contrast to the enzyme from other organisms, both Neurospora activities appear to have Hill coefficients greater than 1.0. This suggests that cAMP interacts in a cooperative manner with these activities, and that there may be some regulation of cAMP levels by the phosphodiesterase.

Experiments to be presented in a subsequent publication indicate that cAMP may influence the morphology of Neurospora. The results presented here demonstrate the existence of a phosphodiesterase-like activity which is a necessary prerequisite for these studies.

TABLE 1

Effect of Various Compounds on cAMP Phosphodiesterase Activity

| Addition | Concentration (mM) | Activity (%) |
|---|---|---|
| None | — | 100 |
| EDTA | 1 | 98 |
| | 10 | 9 |
| $NaH_2PO_4$ | 1 | 82 |
| | 10 | 37 |
| 8-bromo-cAMP | 0.05 | 67 |
| | 0.2 | 38 |
| ATP | 0.01 | 58 |
| | 0.10 | 12 |
| ADP | 0.01 | 88 |
| | 0.10 | 12 |
| Caffeine | 5 | 83 |
| | 10 | 48 |
| Theophylline | 5 | 52 |
| | 10 | 27 |
| Theobromine | 5 | 64 |
| | 9 | 59 |
| Imidazole | 5 | 167 |
| | 10 | 256 |
| Histamine | 5 | 146 |
| | 10 | 179 |

Enzyme activity was estimated by following the formation of $^3$H-adenosine from $^3$H-cAMP. The amount of radioactivity in the area of chromatogram which corresponded to 5'-AMP was also checked at random. This was done to insure that the compound in question did not specifically inhibit or stimulate the mono-esterase. Values represent results from typical experiments. Each determination was carried out at least twice.

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