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ADENYLATE CYCLASE ACTIVITY IN LUBROL-TREATED MEMBRANES FROM *NEUROSPORA CRASSA*

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SUMMARY

Adenylate cyclase from *Neurospora crassa* membranes was solubilized by treatment with the non-ionic detergent Lubrol. The solubilized enzyme was further purified by DEAE-cellulose column chromatography and sucrose gradient centrifugation. In this last step the enzyme shows a minimal sedimentation constant of 7.1 S.

INTRODUCTION

Previous evidence shows that adenylate cyclase activity in *Neurospora crassa* is associated with membranous structures[1,2]. This property complicates the separation of the enzyme from other membrane-bound proteins. Several attempts were carried out in different laboratories in order to obtain a soluble form of adenylate cyclase. Sutherland *et al.*[3] have obtained a soluble form of the enzyme from skeletal muscle, brain and heart by treatment of partially purified preparations with the non-ionic detergent Triton. With a similar procedure but using Lubrol-PX, a polyoxyethylene ether detergent, Levey[4] obtained a solubilized cyclase preparation. This latter method has been used by other authors[5,6] for the solubilization of $(Na^+–K^+)$-ATPase from guinea pig brain microsomes. In all cases, after lubrol treatment a high proportion of these enzyme activities were extracted from the particulate preparations.

This paper deals with the purification and some properties of *N. crassa* adenylate cyclase activity obtained after treatment of particulate preparations with lubrol.

EXPERIMENTAL PROCEDURE

Cell cultures

Membrane preparations were obtained from the slime mutant of *N. crassa* (strain F_Z:Os-1:N1118-FGSC). This mutant grows as isolated protoplasts surrounded by a plasma membrane; the cellular wall characteristic of the mycelial strain is absent. The organism was grown in the liquid minimal medium of Vogel[7] supple-

mented with 2% sucrose, 0.75% yeast extract and 0.75% nutrient broth. Cultures were grown for 24 h at 32 °C in a rotatory shaker (120 cycles per min).

Enzyme preparation

The cells were collected from the liquid culture by centrifugation at 900 × *g* for 7 min. The supernatant was decanted and the cellular precipitates were resuspended in 1 mM $NaHCO_3$ (one-tenth of the culture volume). The suspension was left in the cold for 30 min and then it was centrifuged for 20 min at 15 000 × *g*. The supernate thus obtained was spun down for 120 min at 105 000 × *g*. After this step more than 80% of the adenylate cyclase activity was recovered in the precipitate ("crude membranes"). This fraction containing about 50 mg protein was homogenized with 20 ml of a cold solution containing 1 mM $NaHCO_3$ and 20 mM Lubrol-PX using a Potter–Elvehjem homogenizer (glass–teflon) and afterwards it was centrifuged for 120 min at 165 000 × *g*. After this step more than 80% of the activity was recovered in the supernatant fluid[2]. An aliquot of this supernatant (about 10 mg protein) was loaded on a DEAE-cellulose column (1.3 cm × 12 cm) equilibrated with 50 mM Tris–HCl buffer, pH 7.4. Elution of the enzyme activity was performed in the absence of lubrol with a linear gradient of KCl (from 0 to 0.75 M) made in the same buffer. The volume of the elution system was 75 ml. Fractions of 2.5 ml were collected at a flow rate of 1 ml per min. Under these conditions, detergent was excluded from the column and the enzyme eluted at a KCl concentration of about 0.36 M (Fig. 1). The

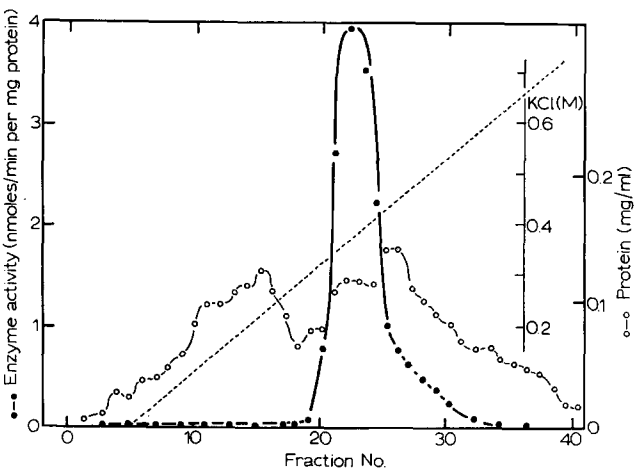


Fig. 1. DEAE-cellulose column chromatography of the solubilized form of adenylate cyclase. ○—○, protein, ●—●, enzyme activity. Conditions were are as described in Experimental Procedure.

fractions obtained from the column having the cyclase activity were dialyzed for 3 h against 25 mM piperazine-*N*,*N*′-bis-ethanesulfonic acid–NaOH buffer, pH 6.35, containing 0.4 mM EDTA and 0.2 mM dithiothreitol. After that, aliquots of this fraction (0.3 ml; 0.05 mg protein) were submitted to centrifugation at 120 000 × *g* for 13 h in a linear sucrose gradient from 20 to 5%. Centrifugation was carried out in an SW 65 rotor of a Spinco Model L preparative ultracentrifuge.

Enzyme assay

The standard incubation mixture for the assay of adenylate cyclase contained 100 mM piperazine-*N*,*N*'-bis-2-ethanesulfonic acid–NaOH, buffer, pH 6.35, 2.5 mM $MnCl_2$, 2.5 mM [α-^{32}P]ATP (50 μCi) and enzyme. The total volume was 0.1 ml. Incubations were carried out at 37 °C for 5 min. Reactions were stopped as described by Rodbell[8] and the cyclic adenylate was isolated by the procedure of Krishna *et al.*[9].

Analytical procedures

Sucrose gradient centrifugations were carried out following the method of Martin and Ames[10]. The gradients were calibrated with *Escherichia coli* RNA polymerase (holoenzyme), rabbit muscle phosphorylase *b*, liver catalase and *E. coli* alkaline phosphatase.

Polyacrylamide gel electrophoresis was performed according to the method of Davis[11] using 5% acrylamide gels made in 100 mM sodium phosphate buffer, pH 7.5. Protein was stained with coomassie brilliant blue[12].

Protein was assayed in the enzyme samples following the procedure of Lowry *et al.*[13].

TABLE I

PURIFICATION OF *Neurospora* ADENYLATE CYCLASE

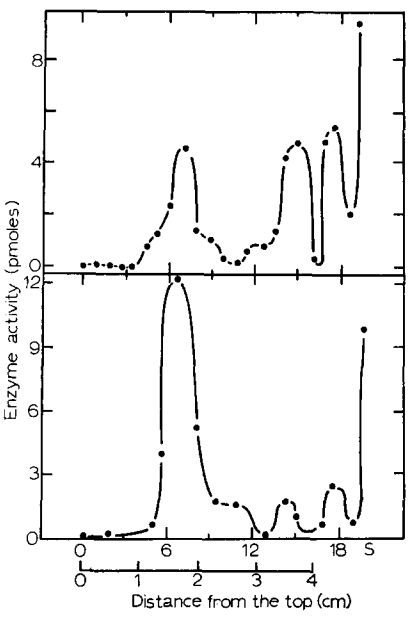
Preparation of the different fractions was carried out as described in Experimental Procedure.

Fraction	*Spec. act (nmoles/min per mg protein)*	*Total activity (nmoles/min)*
Cell lysate	0.03	30
15 000 × *g* supernate	0.13	35
Crude membranes	0.32	25
165 000 × *g* Lubrol supernate	0.60	15
DEAE fraction	4.00	5

RESULTS AND DISCUSSION

Table I shows a summary of the specific and total activities of the fractions corresponding to each purification step of the *Neurospora* cyclase. The enzyme eluted from the DEAE-cellulose with a maximum specific activity of about 4 nmoles per min per mg protein. This value is 100 times higher than the one corresponding to the cell lysate. After this step the enzyme activity was found to be very unstable; most of the cyclase activity was lost by storage at 0 °C for two days.

After sucrose gradient centrifugation, several activity peaks were obtained with a mobility of 7.1, 14.5, and 17.5 S; a variable proportion of the activity also sedimented in the bottom of the tube. This result suggests that after lubrol treatment the enzyme forms multiple molecular aggregates of variable size. Fig. 2 shows the pattern for the distribution of cyclase activity in two gradients carried out on different preparations. The peak with the minimal mobility in the sucrose gradient gave a sedimentation constant of 7.1 S, which corresponds to a molecular weight between 120 000 and 150 000 for a protein with a globular shape. This value is of the same order of magnitude as that reported by Levey[4] for the enzyme from cat myocardium. The existence



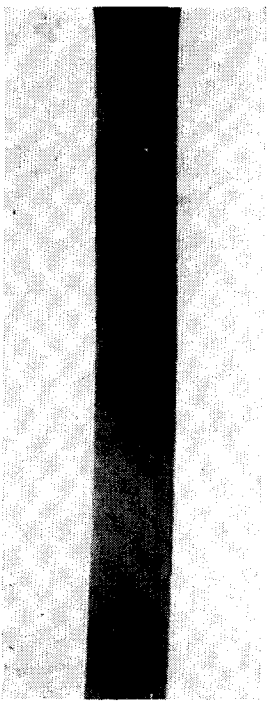


Fig. 2. Sucrose gradient centrifugation of the solubilized form of adenylate cyclase. The centrifugations were carried out with two different preparations performed according to the procedure described in the text.

Fig. 3. Polyacrylamide-gel electrophoresis of adenylate cyclase. The active fractions corresponding to the 7.1-S peak in the sucrose gradient centrifugation were pooled, dialyzed for 4 h against water and concentrated under an N_2 stream. The sample was submitted to a polyacrylamide gel electrophoresis as described in Experimental Procedure.

of multiple forms of variable size is not an unusual observation, since a similar phenomenon has been reported for the brain microsomal ATPase after lubrol treatment[5,6].

On the other hand, polyacrylamide gel electrophoresis of the 7.1-S peak gave only one band of protein (Fig. 3).

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