Cyclic nucleotide phosphodiesterase activities in *Neurospora crassa*

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Cyclic nucleotide phosphodiesterase activities in soluble *Neurospora crassa* mycelial extracts were resolved into two peaks, phosphodiesterase I and II, by chromatography on DEAE-cellulose columns. Phosphodiesterase I hydrolysed cyclic AMP and cyclic GMP equally well. Phosphodiesterase II was active on cyclic GMP but scarcely active on cyclic AMP. Phosphodiesterase I was resolved by gel filtration and sucrose-density-gradient centrifugation into three peaks having molecular weights of about 57 000, 126 000 and 225 000. This suggests that this enzyme activity has at least three aggregation forms, tentatively defined as monomeric, dimeric and tetrameric. Similarly, phosphodiesterase II was resolved into two forms, having molecular weights of about 170 000 and 320 000. Evidence on the interconversion between phosphodiesterase I forms was obtained.

Evidence obtained in this and other laboratories indicates that cyclic AMP plays an important role in the control of elongation of hyphae in *Neurospora crassa* (Scott & Solomon, 1973; Torres *et al.*, 1975; Terenzi *et al.*, 1976; Trevillyan & Pall, 1979). Such evidence is supported mainly by the discovery that morphological *cr-1* (crisp) mutants of this fungus are deficient in the synthesis of cyclic AMP (Terenzi *et al.*, 1974; Flawiá *et al.*, 1977).

Studies on *Neurospora* adenylate cyclase, the enzyme system responsible for cyclic AMP synthesis, have also been performed in this laboratory. The enzyme activity is dependent on Mn^{2+} , and it is not activated by fluoride or guanine nucleotides (Flawiá & Torres, 1972*a,b*). In addition, some studies on *Neurospora* soluble cyclic AMP-dependent protein kinase have been reported by this (Judewicz *et al.*, 1981) and another laboratory (Powers & Pall, 1980).

Cyclic nucleotide phosphodiesterases (EC 3.1.4.17) have not been studied in detail in *Neurospora*, though the hydrolysis of cyclic AMP by crude mycelial preparations has been reported (Scott & Solomon, 1973; Terenzi *et al.*, 1976).

The present paper describes the partial purification and characterization of two cyclic nucleotide phosphodiesterase activities obtained from soluble extracts of *Neurospora* mycelia.

Materials and methods

Cultures and enzyme preparations

A wild-type *Neurospora crassa* strain (St. Lawrence 74) was grown in Vogel's (1956) minimal medium supplemented with 2% (w/v) sucrose. Cultures were shaken (100 oscillations/min) at 30°C in 2000 ml Erlenmeyer flasks containing 400 ml of medium in a rotatory air incubator. Mycelia were harvested during the exponential phase of growth, collected by filtration, rinsed with distilled water, frozen at -20°C and freeze-dried. The dried material was ground in a mortar cooled with liquid N_2 and extracted with 20 ml/g of dry mycelium of a cold buffer solution containing 25 mM-Tris/HCl buffer, pH 7.5, and 0.5 mM-EDTA (buffer A). The suspension was further homogenized with an SDT Tissuemizer (Tekmar Co.) provided with a 182 E shaft and generator, and centrifuged at 25 000 *g* for 10 min. The supernatant fluid thus obtained was further centrifuged at 105 000 *g* for 60 min. The latter supernatant was dialysed overnight against a large volume of buffer A.

Further purification was performed by chromatography on DEAE-cellulose. The column (30 cm × 2.8 cm), equilibrated with buffer A, was loaded with 70 ml of the dialysed extract (containing about 6 mg of protein/ml), washed with 250 ml of buffer A and eluted with a 0–0.6 M-NaCl linear gradient in the same buffer solution. The total volume of the gradient was 500 ml. Elution was performed at a rate of 1 ml/min.

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Fractions to be subjected to gel-filtration chromatography, centrifugation in sucrose density gradients or isoelectric focusing were first precipitated by the addition of 3 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. After centrifugation of the mixture at 25 000 g for 10 min, the sediment was resuspended in buffer A (one-tenth of the initial volume) and dialysed overnight against the same buffer solution.

Determination of molecular parameters

Sedimentation coefficients were determined by the method of Martin & Ames (1961). Sucrose density gradients (4 ml; 5–20%, w/v) were made in 10 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl (buffer B). Samples (0.25 ml) of the enzyme fractions or solutions of molecular-weight markers were loaded on the top of the gradients. Centrifugations were performed in a Beckman SW-56 rotor at 40 000 rev./min for 18 h at 5°C. Gradients were calibrated with the following molecular-weight markers: liver catalase (0.1 mg/ml; mol.wt. 247 500), muscle lactate dehydrogenase (0.03 mg/ml; mol.wt. 142 000), muscle malate dehydrogenase (0.01 mg/ml; mol.wt. 70 000) and heart cytochrome *c* (2 mg/ml; mol.wt. 13 370).

Stokes radii were determined by gel filtration through agarose columns (Bio-Gel A-1.5 m; 100–200 mesh) by the method of Laurent & Killander (1964). The columns (30 cm × 0.75 cm) were equilibrated and eluted with buffer B at 5°C. Samples (0.3 ml) of the enzyme fractions or solutions of molecular-weight markers (see above) were loaded on top of the columns. Exclusion volumes were determined with Blue Dextran (2 mg/ml). Columns were operated at a rate of 0.15 ml/min. The behaviour of each particular enzyme activity or protein in agarose columns was expressed in terms of K_{el} , defined as the ratio of the elution volume of this activity (or protein) to that of Blue Dextran.

Molecular weights were calculated by the method of Tanford (1961) as described by Clarke (1975), assuming a value for partial specific volumes of 0.74 ml/g. Molecular and hydrodynamic parameters of protein markers have been reported by Haga *et al.* (1977).

Isoelectric focusing

Isoelectric focusing of pooled and concentrated DEAE-cellulose chromatography fractions containing phosphodiesterase I activity was performed on a 110 ml column (LKB Produkter). Ampholine (pH range 3.5–10) concentration in the column was 1% (w/v). The pH gradient was formed at constant power (10 W) for 24 h. After that, 1 ml of enzyme sample was injected into the centre of the column, in accordance with the *LKB 8100 Instrument Manual*. Conditions for enzyme focusing were as follows:

temperature, 5°C; time, 24 h; initial voltage and current, 800 V and 12 mA respectively; final voltage and current, 1600 V and 6 mA respectively. Fractions of volume about 2 ml were collected.

Enzyme assays

Cyclic nucleotide phosphodiesterase activities were determined essentially as described by Thompson & Appleman (1971). Unless otherwise indicated, reaction mixtures contained 40 mM-Tris/HCl buffer, pH 8.0, 5 mM-MgCl₂, 1 μM-cyclic [³H]-AMP or -cyclic [³H]GMP (about 70 000 c.p.m.) and enzyme. The total volume was 0.1 ml. Incubations were performed at 30°C for 5–20 min, and the reactions were stopped by heating for 1 min at 100°C. After the addition of 0.04 ml of a 2 mg/ml solution of king-cobra venom in 10 mM-Tris/HCl buffer, pH 8.0, a second incubation was performed for 30 min at 30°C. Reactions were finally stopped by the addition of 0.02 ml of a solution containing 50 mM-EDTA and 5 mM-adenosine or -guanosine. When cyclic AMP was used as substrate 1 ml of a slurry containing 1 vol. of wet AG 1-X4 (200–400 mesh) resin and 3 vol. of 30% (v/v) ethanol in water was added to each tube (Londesborough, 1976). When cyclic GMP was used, the addition consisted of 0.5 ml of a slurry containing equal volumes of wet AG 3-X4 (200–400 mesh) and water. After centrifugation, samples of the supernatant fluids were counted for radioactivity, with a scintillation mixture containing 0.4% (w/v) Omnifluor (New England Nuclear Corp.) and 30% (v/v) Triton X-100 in toluene.

Under the above-mentioned conditions, reaction rates were linear with time and proportional to enzymic protein concentration. Substrate consumption was not higher than 20% (c.p.m./c.p.m.) of its initial amount.

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and cytochrome *c* by measuring the absorbance of the Soret band at 410 nm. Catalase activity was assayed at room temperature by measuring the disappearance of H₂O₂ by the conversion of iodide into iodine, by the method of Terenzi *et al.* (1971). Lactate dehydrogenase and malate dehydrogenase activities were assayed by measuring NADH consumption by the disappearance of its absorbance at 340 nm as described in the *Worthington Enzyme Manual* (1972, pp. 7–12).

Characterization of phosphodiesterase reaction product was performed as described elsewhere (Flawiá & Torres, 1972a).

Reagents

Bio-Gel A-1.5 m as well as AG 1-X4 and AG

3-X4 resins were from Bio-Rad Laboratories. Blue Dextran, phenylmethanesulphonyl fluoride, cyclic nucleotides, bovine liver catalase, king-cobra venom and bovine heart cytochrome *c* were purchased from Sigma Chemical Co. Rabbit muscle lactate dehydrogenase and malate dehydrogenase were from Boehringer Corp., DEAE-cellulose was from Serva Laboratories and sucrose was from Schwarz-Mann Laboratories. ^3H -labelled cyclic nucleotides were from New England Nuclear Corp.

Results and discussion

Chromatography on DEAE-cellulose

Fig. 1 shows the elution pattern of phosphodiesterase activity from a DEAE-cellulose column. The activity was resolved into two peaks, phosphodiesterase I and phosphodiesterase II. Phosphodiesterase I, eluted at about 0.20 M-NaCl, was active on both cyclic AMP and cyclic GMP. Phosphodiesterase II was eluted at about 0.37 M-NaCl, and was active on cyclic GMP, but showed negligible activity on cyclic AMP.

The presence of the proteinase inhibitor phenylmethanesulphonyl fluoride in homogenization and column buffers did not influence the elution pattern described above. When enzyme activities were assayed at millimolar substrate concentration, the chromatographic profile was similar to that shown in Fig. 1 (results not shown).

Chromatography on paper of phosphodiesterase I incubation mixtures revealed that the enzyme converted cyclic AMP and cyclic GMP into the corresponding nucleoside 5'-monophosphates (results not shown).

Substrate kinetics

Activities of the phosphodiesterase I and phosphodiesterase II peaks were studied at different cyclic AMP and cyclic GMP concentrations. Results of such experiments are shown in Fig. 2. Woolf-Augustinsson-Hofstee plots (Segel, 1975) for the enzyme activities indicated that each of the two fractions analysed had two kinetic components, one of 'high affinity' and the other of 'low affinity' for the cyclic nucleotides. It is important to emphasize that with phosphodiesterase II the low activity detected with cyclic AMP rendered studies on the concentration-dependence for this substrate very difficult. In both phosphodiesterase activity peaks the 'low-affinity' component had higher maximal velocities than did the 'high-affinity' one. Table 1 summarizes some parameters calculated for these enzyme activities.

Molecular properties

Further experiments were performed to determine the number and size of the molecular forms present in the phosphodiesterase I and phosphodiesterase II activity peaks.

The elution pattern of phosphodiesterase I from an agarose column (Fig. 3a) showed three activity peaks, designated PDE I-a, PDE I-b and PDE I-c. Stokes radii for the components of these peaks were 6.1, 4.4 and 3.6 nm respectively. The three components were active on both cyclic AMP and cyclic GMP, but with component PDE I-c activity with the former nucleotide was significantly lower than the corresponding activities found with components PDE I-a and PDE I-b.

Sucrose-density-gradient centrifugation of phos-

Fig. 1. Elution pattern of cyclic nucleotide phosphodiesterase activities from a DEAE-cellulose column. Enzyme activity was assayed with cyclic AMP (○) or cyclic GMP (●) as substrate. ■, Concn. of protein. Conditions were as described in the Materials and methods section.

Fig. 2. Substrate-dependence of phosphodiesterase I (a) and phosphodiesterase II (b) activities

Peak fractions from DEAE-cellulose column chromatography were pooled and assayed for phosphodiesterase activity at various concentrations of cyclic AMP (○) or cyclic GMP (●). Other conditions were as described in the Materials and methods section.

phodiesterase I was also resolved in three activity peaks (Fig. 3b), having sedimentation coefficients of about 8.3, 6.5 and 4.6S respectively. The component of the lightest peak was less active on cyclic AMP than were the other ones.

The results shown in Fig. 3 indicate that three different molecular forms could be resolved from phosphodiesterase I by two different procedures. Assuming that the forms found by gel filtration are identical with those resolved in sucrose density gradients, it may be concluded that phosphodiesterase I has three different molecular forms having molecular weights of about 225 000, 126 000 and 57 000 (Table 2). These values suggest that the enzyme can be in monomeric, dimeric and tetrameric forms.

Fractions from the Bio-Gel column corresponding to PDE I-a, PDE I-b and PDE I-c peaks were pooled, precipitated with $(\text{NH}_4)_2\text{SO}_4$, con-

Fig. 3. Gel filtration (a) and sucrose-density-gradient centrifugation (b) of phosphodiesterase I

Peak fractions from DEAE-cellulose column chromatography were pooled, precipitated with $(\text{NH}_4)_2\text{SO}_4$ and dialysed. Samples of this dialysed preparation (20 mg of protein/ml) were loaded on a column (Bio-Gel A-1.5 m) and on a sucrose density gradient. Conditions and symbols were as in Fig. 1 legend and as described in the Materials and methods section. Key to markers: BD, Blue Dextran; C, catalase; L, lactate dehydrogenase; M, malate dehydrogenase; Cc, cytochrome c.

Table 1. Kinetic parameters of *Neurospora cyclic nucleotide phosphodiesterases*

The data were calculated from the results shown in Fig. 2.

Phosphodiesterase	Substrate	Kinetic component	K_m (μM)	V_{max} (pmol/min)
I	Cyclic AMP	'High affinity'	1.5	65
		'Low affinity'	60.0	820
	Cyclic GMP	'High affinity'	1.0	24
		'Low affinity'	35.0	225
II	Cyclic GMP	'High affinity'	3.5	12
		'Low affinity'	95.0	150

centrated and rechromatographed on agarose. As shown in Fig. 4, each peak generated the other two, resembling the pattern shown in Fig. 3(a). The elution pattern observed in the rechromatography of

Fig. 4. Rechromatography on an agarose column of derived PDE I-a (a), PDE I-b (b) and PDE I-c (c) fractions from phosphodiesterase I

Peak fractions from the column chromatography shown in Fig. 3(a) were pooled, precipitated with $(\text{NH}_4)_2\text{SO}_4$, dialysed and chromatographed on a Bio-Gel A-1.5 m column (43 cm $\times$ 0.75 cm). Conditions and symbols were identical with those described for Fig. 3(a).

component PDE I-c was striking, since the smaller phosphodiesterase form generated a high proportion of the larger one (Fig. 4c). This suggests that the phosphodiesterase I molecular entities are interconvertible. Moreover, on isoelectric focusing phosphodiesterase I shows only one peak at pH 5.0, with hydrolytic activity towards cyclic AMP and cyclic GMP (Fig. 5).

Fig. 6 shows the results obtained with phosphodiesterase II. Gel filtration resolved two activity peaks, designated PDE II-a and PDE II-b (Fig. 6a), the components having Stokes radii of about 7.0 and 5.0 nm respectively. Similarly, sucrose-density-gradient centrifugation resolved two peaks with sedimentation coefficients of about 10.3 and 7.7 S (Fig. 6b). With an approach identical with that used for phosphodiesterase I, two molecular forms may be defined for phosphodiesterase II, having molecular weights of about 320 000 and 170 000.

The evidence reported in the present paper indicates that soluble extracts from *Neurospora* mycelia contain at least two cyclic nucleotide

Table 2. Molecular and hydrodynamic parameters of *Neurospora* phosphodiesterase activities

Phosphodiesterase component	Stokes radius (nm)	$s_{20,w}$ (S)	Mol.wt.
PDE I-a	6.1	8.3	225 000
PDE I-b	4.4	6.5	126 000
PDE I-c	3.6	3.6	57 000
PDE II-a	7.0	10.3	320 000
PDE II-b	5.0	7.7	170 000

Fig. 5. Isoelectric focusing of phosphodiesterase I

Peak fractions from DEAE-cellulose column chromatography were treated as described in Fig. 3 legend. The pH gradient is indicated with triangles (▲); other symbols were as in Fig. 1. Conditions were as described in the Materials and methods section.

Fig. 6. Gel filtration (a) and sucrose-density-gradient centrifugation (b) of phosphodiesterase II. The fraction loaded on a column and on a sucrose density gradient contained 10mg of protein/ml. Other conditions were as indicated in Fig. 3 legend.

phosphodiesterases: one of them is active on both cyclic AMP and cyclic GMP, whereas the other is only active on cyclic GMP. The results also indicate that enzymes exist in different molecular forms.

The existence of cyclic GMP in *Neurospora* mycelia has been reported by Rosenberg & Pall (1980). These authors also showed that mutant strains deficient in cyclic AMP synthesis (Torres *et al.*, 1975; Terenzi *et al.*, 1976) show diminished concentrations of cyclic GMP (Rosenberg & Pall, 1980). The fact that one of the phosphodiesterases found in *Neurospora* soluble extracts is rather specific for cyclic GMP suggests that this nucleotide could play some role in the control of *Neurospora*

metabolism or other cell functions (e.g. growth, differentiation etc.).

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