Characterization of *Neurospora crassa* cyclic AMP phosphodiesterase activated by calmodulin

María T. TÉLLEZ-IÑÓN, Rita M. ULLOA, Gerardo C. GLIKIN* and Héctor N. TORRES

Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET), and Instituto de Investigaciones Bioquímicas, Fundación Campomar, Obligado 2490, 1428 Buenos Aires, Argentina

Activation of cyclic AMP phosphodiesterase I by brain or *Neurospora* calmodulin was studied. The stimulation required micromolar concentrations of Ca^{2+} , and it was observed at cyclic AMP concentrations between 0.1 and 500 μM . Activation was blocked by EDTA and some neuroleptic drugs such as chlorpromazine and fluphenazine. These drugs inhibit the elongation of *N. crassa* wild-type aerial hyphae. These results reinforce the evidence towards the recognition of Ca^{2+} -calmodulin as one of the systems controlling cyclic nucleotide concentrations in *Neurospora*.

INTRODUCTION

Cyclic AMP plays a role in the control of elongation of aerial hyphae in *Neurospora crassa*. This fact is mainly supported by the evidence obtained in the *cr-1* morphological mutant of this fungus, which is deficient in adenylate cyclase activity (Terenzi *et al.*, 1974, 1976; Flawiá *et al.*, 1977; Trevillyan & Pall, 1979).

Some properties of *Neurospora* cyclic nucleotide phosphodiesterase (EC 3.1.4.17) activities have been described by our laboratory. Two different enzyme activities may be resolved from soluble mycelial extracts. One of them (phosphodiesterase I) is active on both cyclic AMP and cyclic GMP, whereas the other (phosphodiesterase II) is more active with cyclic GMP. These two enzyme activities exist in different molecular aggregation forms (Téllez-Iñón *et al.*, 1982).

Calmodulin is a known activator of cyclic nucleotide phosphodiesterase from different sources (Cheung, 1980), including *Neurospora* (Ortega Pérez *et al.*, 1983). In *Neurospora* the modulator seems to play a dual role in the control of cyclic AMP metabolism. Preliminary evidence indicates that calmodulin activates both adenylate cyclase (Reig *et al.*, 1984) and cyclic phosphodiesterase activities (Ortega Pérez *et al.*, 1983). The present paper extends the studies on characterization of the activation of *Neurospora* soluble phosphodiesterase I by homologous and bovine brain calmodulin.

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. Enzyme preparations were obtained by the method described previously (Téllez-Iñón *et al.*, 1982) with the modification described below.

Measurement of cell growth

Cell growth was measured in mycelial cultures grown on either Vogel's minimal liquid or solid medium as described elsewhere (Terenzi *et al.*, 1976). The rate of

mycelial elongation was measured by the method of Ryan *et al.* (1943).

Purification of *N. crassa* phosphodiesterase activity

The 100 000 g supernatant fluid was chromatographed on a DEAE-cellulose column equilibrated with buffer A containing 25 mM-Tris/HCl buffer, pH 7.4, with 0.1 mM-phenylmethanesulphonyl fluoride. The column was eluted with a linear gradient of 0–0.6 M-NaCl in the same buffer, as described elsewhere (Téllez-Iñón *et al.*, 1982).

The phosphodiesterase I activity peak from the first column was rechromatographed on DEAE-cellulose. The column (1 cm $\times$ 15 cm), equilibrated with 25 mM-Tris/acetate buffer (pH 6.5)/2 mM-EDTA (buffer B), was loaded with 47 ml of the enzyme fraction (105 mg of protein) previously dialysed against buffer B. The column was eluted with a 0–0.6 M-NaCl linear gradient (200 ml volume) in buffer B.

The enzyme-activity peak was dialysed against buffer B for 15 h, concentrated in an Amicon PM-10 ultrafilter and stored at -20°C .

Purification of bovine brain cyclic AMP phosphodiesterase activity

Cortex from 650 g of bovine brain was homogenized in 1 litre of 25 mM-Tris/HCl buffer, pH 7.4, containing 0.5 mM-EDTA (buffer C) and 0.5 mM-phenylmethanesulphonyl fluoride with a Teckmar homogenizer with a 182 E shaft, and the homogenate was centrifuged at 20 000 g for 30 min. The supernatant was loaded on a DEAE-cellulose column (2.5 cm $\times$ 40 cm) equilibrated with buffer C and washed with 500 ml of the same solution. Elution was performed with a linear gradient of 0–0.7 M-NaCl (600 ml) in buffer C. Fractions corresponding to the phosphodiesterase activity peak eluted at about 0.3 M-NaCl were pooled and diluted with 9 vol. of buffer C. The diluted fractions were rechromatographed on a DEAE-cellulose column (1.5 cm $\times$ 20 cm). The chromatography was performed as indicated above except for the gradient volume, which was 400 ml. Fractions corresponding to the phosphodiesterase activity

* Present address: Institute de Recherches Scientifiques sur le Cancer, 94800 Villejuif, France.

were pooled, concentrated in an Amicon P-10 ultrafilter and stored at -70°C .

Purification of *N. crassa* calmodulin

Fractions from the DEAE-cellulose column chromatography of the *N. crassa* 100 000 *g* supernatant (see above) having calmodulin activity (assayed with brain phosphodiesterase) and eluted at a concentration of about 0.45 M-NaCl were pooled, heated at 100°C for 10 min, and centrifuged at 10 000 *g* for 10 min. The supernatant was dialysed for 15 h against 50 vol. of buffer A by using a membrane with a cut-off of M_r 8000 (3787-F25; Arthur Thomas) and freeze-dried. The solid material was resuspended in 1.5 ml of 25 mM-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl and filtered through a Bio-Gel P-60 column (1.9 cm $\times$ 95 cm) equilibrated and eluted with the same buffer solution at a rate of 0.2 ml/min; 2 ml fractions were collected.

Calmodulin activity was eluted from this column as a single peak that corresponds to an elution-volume/exclusion-volume ratio of about 1.30. Fractions corresponding to this peak were pooled, dialysed against buffer A and freeze-dried as above.

Calmodulin was further purified by affinity chromatography on 2-chloro-10-(3-aminopropyl)phenothiazine bound to Sepharose by procedures described by Jamieson & Vanaman (1979). The column (1.2 cm $\times$ 20 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 7.4, containing 1 mM- CaCl_2 and 1 mM- β -mercaptoethanol (buffer D) was loaded with 5 ml of the freeze-dried material previously resuspended in water and washed with 50 ml of buffer B. Elution was performed with 20 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-EGTA and 1 mM- β -mercaptoethanol. Fractions containing calmodulin activity were pooled, dialysed and freeze-dried as indicated above.

Purification of bovine brain calmodulin

The modulator was purified by a modification of the method described by Bazari & Clarke (1981). Calmodulin-activity peak fractions from the DEAE-cellulose column chromatography of the brain extract (see above), eluted at about 0.45 M-NaCl, were pooled and heated at 100°C for 10 min. The supernatant was then dialysed as indicated for *N. crassa* calmodulin. The dialysed material (150 ml) was loaded on an hydroxyapatite (Bio-Gel HTP) column (1.8 cm $\times$ 14 cm) equilibrated with 5 mM-sodium phosphate buffer, pH 6.8, and eluted with a sodium phosphate linear gradient (pH 6.8) from 5 to 250 mM (400 ml total volume). Fractions (8 ml) were collected at a rate of 0.5 ml/min. Calmodulin activity was eluted as a single peak at a phosphate concentration of about 0.15 M. Active fractions were pooled, dialysed against water and freeze-dried.

The solid material was resuspended in 1.5 ml of 25 mM-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl, and filtered through a Bio-Gel P-60 column (1.9 cm $\times$ 95 cm) as described above.

Cyclic nucleotide phosphodiesterase assays

Enzyme activities were assayed essentially as described by Thompson & Appleman (1971) with the modification introduced by Londesborough (1976). *N. crassa* phosphodiesterase was assayed in reaction mixtures containing 40 mM-Tris/HCl buffer, pH 7.5, 5 mM- MgCl_2 and 1 μM -cyclic [^3H]AMP (radioactivity 600 c.p.m./pmol)

(Téllez-Iñón *et al.*, 1982). Assay conditions were selected so that cyclic AMP hydrolysis has linear dependence on incubation time and enzyme concentration.

Calmodulin assays were performed in the presence of 50 μM - CaCl_2 , 1 mg of albumin/ml, 40 μg of brain phosphodiesterase protein/ml and 200 μM -cyclic AMP. Calmodulin was expressed as its capacity to increase brain phosphodiesterase activity according to the definition proposed by Clarke *et al.* (1980); 1 unit of calmodulin activity is equivalent to an increment of 1 nmol of 5'-AMP generated/min.

Analytical methods

Proteins were determined by the method of Lowry *et al.* (1951). Details of the procedures for polyacrylamide-gel electrophoresis of protein samples in the presence of sodium dodecyl sulphate (15% -acrylamide gels) stained with Coomassie Brilliant Blue R-250 were given in a previous paper (Reig *et al.*, 1982).

Reagents

DEAE-cellulose (DE-52) was purchased from Whatman. Bio-Gel P-60 was from Bio-Rad Laboratories. Sepharose 4B (CNBr-activated) was purchased from Pharmacia Fine Chemicals, and 2-chloro-10-(3-aminopropyl)phenothiazine was generously given by Dr. Albert Manian (National Institute of Mental Health, Bethesda, MD, U.S.A.). Chlorpromazine was obtained from Sigma, and fluphenazine was generously given by Dr. J. P. Rossi, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

RESULTS AND DISCUSSION

Fig. 1(a) shows the elution profile of a cyclic AMP phosphodiesterase activity from a DEAE-cellulose column. Enzyme activities were assayed with cyclic AMP in the absence or presence of brain calmodulin. In the absence of the modulator, the elution pattern observed was similar to that previously described (Téllez-Iñón *et al.*, 1982). A variable extent of activation of both phosphodiesterase activity peaks was observed (Fig. 1a) when calmodulin was added to the assay mixtures. Endogenous calmodulin was eluted at about 0.45 M-NaCl, partially overlapping the phosphodiesterase II activity peak. Some modulator activity associated with phosphodiesterase I was also obtained (Fig. 1b).

Since phosphodiesterase I is the major component of *Neurospora* soluble cyclic AMP phosphodiesterase, the effect of calmodulin on this enzyme was studied further. As a first approach, the enzyme fraction was separated from the modulator by rechromatography on DEAE-cellulose (Fig. 2).

N. crassa calmodulin was further purified from fractions obtained in the first DEAE-cellulose column chromatography by heating at 100°C , gel filtration and affinity chromatography on 2-chloro-10-(3-aminopropyl)-phenothiazine-Sepharose. After these purification steps, the modulator shows only one polypeptide band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with an apparent M_r of about 16 000 (Fig. 3), which is identical with that previously reported (Ortega Pérez *et al.*, 1981).

Fig. 4 shows the time course of the reaction catalysed by phosphodiesterase I. The homologous calmodulin is more efficient in the activation of phosphodiesterase I

Fig. 1. Elution pattern of phosphodiesterase and calmodulin activities from a DEAE-cellulose column
(a) Phosphodiesterase activity measured at $1\text{ }\mu\text{M}$ -cyclic AMP in the presence (●) or absence (○) of brain calmodulin; Δ , concentration of protein. (b) Calmodulin activity. Conditions were described in the Materials and methods section. —, [NaCl].

Fig. 2. Rechromatography of phosphodiesterase I activity peak on a DEAE-cellulose column
The enzyme was assayed in the presence (●) or absence (○) of calmodulin. Conditions were described in the Materials and methods section. —, [NaCl]; Δ , concentration of protein.

Fig. 3. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of purified samples of *Neurospora* (b) and brain (a) calmodulin

M_r markers were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor and α -lactalbumin. Conditions were described in the Materials and methods section.

than is the brain modulator. Activations were blocked by EGTA, and sub-micromolar concentrations of free Ca^{2+} were required for maximal enzyme activation (Fig. 5).

Fig. 6 shows the dependence of phosphodiesterase I activation on calmodulin concentration. Half-maximal stimulations by *Neurospora* and bovine brain calmodulin were observed at 0.6 and 1.2 $\mu\text{g/ml}$ respectively.

As occurs with cyclic nucleotide phosphodiesterases from a variety of organisms, the *Neurospora* enzyme shows two kinetic components, one of 'high affinity' and the other of 'low affinity' for cyclic AMP (Téllez-Iñón *et al.*, 1982). As shown in Fig. 7, the effect of calmodulin on kinetic properties is rather complex. Increasing concentrations of calmodulin enhance the maximum-velocity values of both kinetic components ($K_m = 2 \mu\text{M}$ and $60 \mu\text{M}$ for high- and low-affinity components respectively). It is also clear that the modulator increased enzyme rates, even at the low cyclic nucleotide concentrations explored. This might indicate that Ca^{2+} -calmodulin influences phosphodiesterase activity at physiological concentrations of the cyclic nucleotide.

The effect of some phenothiazine derivatives on *Neurospora* phosphodiesterase I was studied at the optimal concentration of brain calmodulin. Chlorpromazine and fluphenazine blocked calmodulin stimulation,

Fig. 4. Time course of phosphodiesterase I activity

Reaction mixtures contained 10 μg of *Neurospora* phosphodiesterase protein, 1 μM -cyclic AMP, 25 μM - Ca^{2+} , bovine albumin (1 mg/ml) plus the following additions: ●, *Neurospora* calmodulin (2.0 $\mu\text{g/ml}$); △, brain calmodulin (2.0 $\mu\text{g/ml}$); □, *Neurospora* calmodulin (2.0 $\mu\text{g/ml}$) plus 100 μM -EGTA; ○, no addition. Other conditions were described in the Materials and methods section.

Fig. 5. Phosphodiesterase activity as a function of free Ca^{2+} concentration in the presence (●) or absence (○) of *Neurospora* calmodulin

Reaction mixtures contain 10 μg of phosphodiesterase protein, variable concentrations of Ca^{2+} , and 100 μM -EGTA. Calmodulin concentration was 2 $\mu\text{g/ml}$. Free Ca^{2+} concentrations were calculated as described by Bartfai (1979). Other conditions were as described in the Materials and methods section.

with concentrations of 18 and 35 μM respectively giving half-maximal inhibition (results not shown). Neuroleptic drugs did not affect enzyme activities in the absence of the modulator.

The effect of these neuroleptic drugs on cell growth and rate of mycelial elongation was also studied. Phenothiazine

Fig. 6. Phosphodiesterase activity as a function of *Neurospora* (●) or brain (Δ) calmodulin

Assay mixtures contained 4 μ g of phosphodiesterase protein, 1 μ M-cyclic AMP and the indicated concentration of each calmodulin. Other conditions were described in the Materials and methods section.

Fig. 7. Lineweaver-Burk plots showing the substrate-dependence of phosphodiesterase activity in the absence (○) or presence of brain calmodulin (2 μ g/ml; ●) or *Neurospora* calmodulin (0.5 μ g/ml; Δ)

Reaction mixtures contained 2 μ g of phosphodiesterase protein, 25 μ M- Ca^{2+} and bovine albumin (1 mg/ml). Other conditions were described in the Materials and methods section.

derivatives clearly inhibited aerial growth in standing liquid cultures and the elongation of hyphae in solid medium (results not shown). These effects were specifically quantified by the procedures of Ryan *et al.* (1943). At 30 °C the mycelium elongation rate of a wild-type strain grown in the absence of these drugs was about 0.4 cm/h. In the presence of 100 μ M-chlorpromazine or -fluphenazine this value was decreased to about 0.1 cm/h and reversed

by fresh CaCl_2 -containing Vogel's medium. At this concentration it is possible that phenothiazine derivatives could also have some effects on cell growth, not necessarily related to the inhibition of calmodulin activity.

'Crisp' is an interesting family of *Neurospora crassa* morphological mutants, characterized by a colonial phenotype showing an impairment in the elongation of aerial hyphae (Perkins, 1959). The 'crisp' phenotype is determined by mutation of any of these different loci: *cr-1*, *cr-2* and *cr-3*. According to results obtained in our laboratory (Terenzi *et al.*, 1974, 1976; Flawia *et al.*, 1977), a deficiency in adenylate cyclase activity produces the 'crisp' phenotype in *cr-1* strains. Consequently, it was interesting to see whether or not a calmodulin deficiency might induce these phenotypes in other 'crisp' strains. Extracts from these 'crisp' strains were analysed for brain phosphodiesterase activation (as described for the wild type in the Materials and Methods section). Values of around 21–26 calmodulin units/mg of protein were obtained for all wild-type and the 'crisp' mutant strains studied.

Evidence presented in this paper and that previously reported (Ortega Pérez *et al.*, 1983; Reig *et al.*, 1984) indicates that the Ca^{2+} -calmodulin system is a major control element of cyclic AMP metabolism in *Neurospora crassa*. Since cyclic AMP plays a crucial role in the control of *Neurospora* morphology (Terenzi *et al.*, 1974, 1976; Torres *et al.*, 1975), it is possible that fluctuations in the intracellular free- Ca^{2+} concentration are involved in the primary events leading to differentiation phenomena such as elongation of aerial hyphae, conidiation and transition towards the sexual cycle. This assumption is in accordance with the observation (Reissig & Kinney, 1983) that the calcium ionophore A23187 induces apical branching in *Neurospora*.

This paper was supported by a grant from CONICET (National Research Council), Argentina.

REFERENCES

- Bartfai, T. (1979) Adv. Cyclic Nucleotide Res. **10**, 219–242
- Bazari, W. L. & Clarke, M. (1981) J. Biol. Chem. **256**, 3598–3603
- Cheung, W. Y. (1980) Science **207**, 19–27
- Clarke, M., Bazari, W. & Kayman, S. G. (1980) J. Bacteriol. **141**, 397–400
- Flawia, M. M., Terenzi, H. F. & Torres, H. N. (1977) Arch. Biochem. Biophys. **180**, 334–342
- Jamieson, G. A. & Vanaman, T. C. (1979) Biochem. Biophys. Res. Commun. **90**, 1048–1055
- Londesborough, J. (1976) Anal. Biochem. **71**, 623–628
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
- Ortega Pérez, R., Van Tuinen, D., Marmé, D., Cox, J. A. & Turian, G. (1981) FEBS Lett. **133**, 204–208
- Ortega Pérez, R., Van Tuinen, D., Marmé, D. & Turian, G. (1983) Biochim. Biophys. Acta **758**, 84–87
- Perkins, D. D. (1959) Genetics **44**, 1185–1208
- Reig, J. A., Kornblihtt, A. R., Flawia, M. M. & Torres, H. N. (1982) Biochem. J. **207**, 43–49
- Reig, J. A., Téllez-Iñón, M. T., Flawia, M. M. & Torres, H. N. (1984) Biochem. J. **221**, 541–543
- Reissig, J. L. & Kinney, S. G. (1983) J. Bacteriol. **154**, 1397–1402
- Ryan, F. J., Beadle, G. W. & Tatum, E. L. (1943) Am. J. Bot. **30**, 784–799

- Téllez-Iñón, M. T., Glikin, G. C. & Torres, H. N. (1982) *Biochem J.* **203**, 611–616
- Terenzi, H. F., Flawiá, M. M. & Torres, H. N. (1974) *Biochem. Biophys. Res. Commun.* **58**, 990–996
- Terenzi, H. F., Flawiá, M. M., Téllez-Iñón, M. T. & Torres, H. N. (1976) *J. Bacteriol.* **126**, 91–99
- Thompson, W. J. & Appleman, M. M. (1971) *Biochemistry* **10**, 311–316
- Torres, H. N., Flawiá, M. M., Terenzi, H. F. & Téllez-Iñón, M. T. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 67–68
- Trevillyan, J. M. & Pall, M. L. (1979) *J. Bacteriol.* **138**, 397–403
- Vogel, H. J. (1956) *Microb. Genet. Bull.* **13**, 4243

Received 20 May 1985/16 July 1985; accepted 25 July 1985