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CALMODULIN-STIMULATED CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM *NEUROSPORA CRASSA*

RUBEN ORTEGA PEREZ [a,*], DIEDERIK VAN TUINEN [a], DIETER MARMÉ [b] and GILBERT TURIAN [a]

[a] *Laboratoire de Microbiologie Générale, Département de Biologie Végétale, Université de Genève, CH-1211 Geneva 4 (Switzerland) and* [b] *Institut für Biologie III, University of Freiburg, D-7800 Freiburg i.Br. (F.R.G.)*

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Cyclic nucleotide phosphodiesterase has been partially purified by calmodulin-Sepharose affinity chromatography from a soluble extract of *Neurospora crassa*. The phosphodiesterase activity remained bound to the affinity column even in the presence of 6 M urea and could only be eluted by calcium chelation. The enzyme exhibits cAMP and cGMP phosphodiesterase activities. Both activities can be enhanced by calmodulin in a Ca^{2+}-dependent manner. Stimulation of cyclic nucleotide phosphodiesterase by calmodulin can be inhibited by calmodulin antagonists such as pimozide, trifluoperazine and chlorpromazine.

Calmodulin was first described by Cheung [1] as an activator of brain cyclic nucleotide phosphodiesterase. It also appears as one of the principal intracellular calcium receptors in eukaryotic cells although some calmodulin-dependent enzymatic activities have been reported in higher plants [2], *Tetrahymena pyriformis* [3] and the slime mold *Dictyostelium discoideum* [4], no calmodulin-dependent enzymes involved in the cAMP metabolism have been described for these organisms or for plants.

We have isolated and characterized calmodulin from *Neurospora crassa* [5,6]. As cAMP has been involved in several physiological responses of this fungus [7–9], it was of particular interest to study the eventual interactions between Ca^{2+} and calmodulin and the enzymes of the cAMP metabolism.

In this paper, we report on a soluble cyclic nucleotide phosphodiesterase activity from *N. crassa* which can be activated by calmodulin in a Ca^{2+}-dependent manner.

The wild-type strain STA_4 262A of *N. crassa* was obtained from the Fungal Genetic Stock Center, Humboldt State University, Arcata, CA, U.S.A.

Growth conditions were as described in Ref. 5. Frozen mycelia were disrupted in a Moulinex blender type 320 for 2 min. The dry powder was resuspended in 2 vols. buffer A: 20 mM Tris-HCl (pH 7.5)/3 mM $MgCl_2$ 3/1 mM 2-mercaptoethanol/1 mM ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA)/1 mM phenylmethylsulfonyl fluoride (PMSF) per g of mycelial wet weight and retreated for 2 min as described above. All further manipulations were carried out at 4°C. The homogenate was filtered through a single layer of nylon cloth and centrifuged at 30 000 × *g* for 30 min. Most of the protein was precipitated at 65% and dialysed against buffer A

* To whom correspondence and reprint requests should be sent at (present address): Institut für Biologie III, University of Freiburg, 7800 Freiburg I.Br., F.R.G.

containing 0.5 M NaCl. 3 mM $CaCl_2$ was added and the solution was centrifuged at 129000 × *g* for 3 h. Endogenous calmodulin was removed by chlorpromazine-Sepharose chromatography [10]. The effluent was loaded on a calmodulin-Sepharose column [11] equilibrated with buffer B (20 mM Tris-HCl (pH 7.5)/3 mM $MgCl_2$/3 mM $CaCl_2$/1 mM EGTA/10 mM 2-mercaptoethanol/0.5 M NaCl) containing 0.3% (w/v) human serum albumin. After loading, both columns were washed with the same buffer. Human serum albumin was removed by washing exhaustively with buffer B. Unspecific protein was removed by washing with buffer B containing 6 M urea. Prior to elution with 5 mM EGTA (in buffer A containing 0.5 M NaCl), urea was removed by washing with buffer B. Protein concentration was determined by the method of Spector [12] using bovine serum albumin as standard. Fractions of the urea and EGTA eluates containing the maximum protein were pooled separately, precipitated at 65% saturation $(NH_4)_2SO_4$ and assayed for cAMP and cGMP phosphodiesterase activity as described by Dieter and Marmé [13].

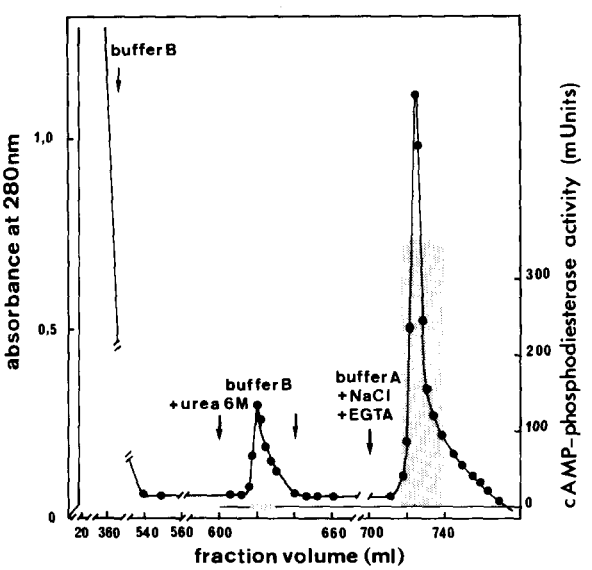

Fig. 1. Calmodulin-Sepharose 4B affinity chromatography profile of the soluble protein fraction from *N. crassa* (●——●). The total cAMP phosphodiesterase activity, expressed in mUnits (shaded area), was measured in the urea and EGTA elution pool. (HSA, human serum albumin)

Bovine brain calmodulin was purified to electrophoretic homogeneity essentially as described in [10].

Drug samples were kindly provided as follows: pimozide by Janssen Pharmaceutica (Baar, Switzerland) and trifluoperazine by Rhône-Poulenc (Paris, France).

The calmodulin-Sepharose affinity chromatography profile of the *Neurospora* soluble protein fraction is shown in Fig. 1. The bulk of proteins was removed by washing with buffer B containing human serum albumin to minimize proteolytic activities. As it has been shown that proteins with specific Ca^{2+}-dependent affinity to calmodulin can form complexes with calmodulin even in the presence of urea [14], the calmodulin-Sepharose column was washed in buffer B containing 6 M urea to remove unspecifically bound proteins (Fig. 1). Urea was then excluded from the column by washing with buffer B. Calcium-dependent calmodulin-binding proteins were eluted with buffer A containing 0.5 M NaCl and 5 mM EGTA. The protein contents of the urea and EGTA eluates were 20 and 80%, respectively, of the total amount retained on the column. Both eluates were tested for cyclic nucleotide phosphodiesterase activity. We could show the presence of a Ca^{2+}-calmodulin-dependent cyclic AMP phosphodiesterase in the EGTA eluate; no activity could be detected in the urea eluate (Fig. 1). The cAMP phosphodiesterase activity could be stimulated by bovine brain calmodulin. In the presence of saturating levels of calmodulin and calcium, the cAMP phosphodiesterase activity could be

TABLE I

Ca^{2+}, CALMODULIN-DEPENDENT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY FROM *NEUROSPORA CRASSA*

The final concentrations in the assay medium were: 20 μg/ml calmodulin, 0.7 mM Ca^{2+}, 0.4 mM EGTA, 2 mM 3',5'-cAMP or 200 μM 3',5'-cGMP.

Additions	Cyclic nucleotide phosphodiesterase activity (mU/mg protein)	
	cAMP	cGMP
Ca^{2+}	23	12
EGTA + calmodulin	23	12
Ca^{2+} + calmodulin	110	30

enhanced about 5-times as compared to its basal activity (Table I). Ca^{2+} alone or calmodulin in the presence of EGTA do not change the activity of the enzyme. The stimulation of the cAMP phosphodiesterase by calmodulin in the presence of Ca^{2+} is fully reversible. Addition of excess of EGTA during the incubation of the cAMP phosphodiesterase with Ca^{2+} and calmodulin reduces the reaction rate to its basal value (data not shown). It is of interest to point out that this enzyme shows also a cGMP phosphodiesterase activity. However, the maximal stimulation of this activity by calmodulin is lower when cGMP is used as substrate instead of cAMP (Table I). This finding is in accordance with the properties of the bovine heart cyclic nucleotide phosphodiesterase reported by Ho et al. [15].

The cAMP phosphodiesterase activity of *Neurospora* was assayed in the presence of different concentrations of bovine brain calmodulin at saturating calcium concentrations. The maximal phosphodiesterase activity was obtained in the presence of 20 μg/ml of calmodulin. The half-maximal activation of the enzyme was reached in the presence of 2 μg/ml of calmodulin. Compared to the values reported for cyclic nucleotide phosphodiesterase activity from bovine brain, higher concentrations of calmodulin were necessary to obtain half-maximal activation of cAMP phosphodiesterase from *Neurospora* [16]. However, such a high concentration has already been reported by Pizarro et al. [17] for the activation of rat mammary gland cyclic nucleotide phosphodiesterase. We can exclude that the requirement for such high calmodulin concentrations for the activation of the *Neurospora* cyclic nucleotide phosphodiesterase is due to a lower affinity of the enzyme for calmodulin after urea treatment: whether or not the affinity column was washed in the presence of urea, the same concentrations of calmodulin were needed to obtain similar stimulation of the enzyme (data not shown). It is also obvious that the EGTA eluate (Fig. 1) may contain several calmodulin-binding proteins acting as potential competitors for calmodulin.

In order to emphasize the specificity of the interaction between calmodulin and *Neurospora* cyclic nucleotide phosphodiesterase, the calmodulin antagonists chlorpromazine, trifluoperazine and pimozide have been used (Table II). In the presence at saturating concentrations of calmodulin and calcium, the activation of the cyclic nucleotide phosphodiesterase could be inhibited as a function of the drug concentration. The relative inhibitory doses of these drugs which gave 50% inhibition of the calmodulin-stimulated cAMP phosphodiesterase from *N. crassa* are comparable to the values reported for their Ca^{2+}-dependent affinities to calmodulin [18]. However, as compared to these values [18], the absolute concentrations of the calmodulin antagonists needed to obtain 50% inhibition were approx. 4-times higher for *N. crassa*. The apparent discrepancy between the concentrations of calmodulin and its inhibitors, necessary to modify the *Neurospora* cyclic nucleotide phosphodiesterase activity, can be attributed to the competition of calmodulin inhibitors with free calmodulin, phosphodiesterase-linked calmodulin and also with calmodulin complexed by other calmodulin-binding proteins present in the affinity column EGTA eluate.

In contrast to a paper [19] which appeared after this work had been accepted for publication, these data clearly show that a cyclic nucleotide phosphodiesterase of the lower eukaryotic organism *N. crassa* is regulated by calmodulin in a Ca^{2+}-dependent manner. From these data the possibility arises of a concerted regulation of physiological activities by the two second messengers Ca^{2+} and cAMP in

TABLE II

INHIBITION OF THE CALMODULIN-DEPENDENT CYCLIC AMP PHOSPHODIESTERASE FROM *NEUROSPORA CRASSA* BY CALMODULIN ANTAGONISTS

The enzyme activity is expressed in mUnits per mg protein. The tests were performed at saturating concentrations of calmodulin and calcium.

Inhibitors (μM)	cAMP phosphodiesterase activity (mU/mg protein)	Inhibition (%)	
–	110		–
Chlorpromazine	56	96	12.8
	112	62	44.1
Trifluoperazine	24	92	16.7
	48	49	55.9
Pimozide	7	94	15.0
	18	52	52.5

lower eukaryotic organisms.

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