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Cyclic nucleotide phosphodiesterase activity in *Neurospora crassa*

Purification by immunoaffinity chromatography and characterization

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Monoclonal antibodies to *Neurospora crassa* cyclic nucleotide phosphodiesterase (PDE I) were selected by their capacity to inhibit the enzyme activity. The monoclonal immunoglobulin, coupled to Sepharose 4B, was used for the affinity purification of PDE I activity. After SDS-polyacrylamide gel electrophoresis the affinity purified PDE I fractions showed a single polypeptide band of about 41 kDa. This band reacted in Western blots with the above mentioned monoclonal immunoglobulin.

Monoclonal antibody; Cyclic AMP phosphodiesterase; Immunoaffinity chromatography; (*Neurospora crassa*)

1. INTRODUCTION

Some properties of *Neurospora crassa* cyclic nucleotide phosphodiesterase activities (EC 3.1.4.17) have been described in this laboratory [1]. Two different enzyme activities could be resolved by DEAE-cellulose column chromatography from soluble mycelial extracts. One of them (PDE I) is active on both cyclic AMP and cyclic GMP, whereas the other (PDE II) is more active with cyclic GMP. These two enzyme activities exist in different aggregation forms.

Calmodulin is a known activator of cyclic nucleotide phosphodiesterase from different sources, including *Neurospora* [2,3]. In *Neurospora*, the modulator seems to play a dual role in the control of cyclic AMP metabolism since in the presence of Ca^{2+}, calmodulin activates both adenylate cyclase and PDE I [3,4]. These activations were blocked by EGTA and some neuroleptic drugs such as chlorpromazine and flufenazine. Moreover, these drugs inhibited the elongation of *N. crassa* wild-type aerial hyphae indicating that the Ca^{2+}-calmodulin complex is involved in the control of cyclic nucleotide levels in *Neurospora* [3].

The present paper reports a further purification and characterization of the *N. crassa* cyclic nucleotide phosphodiesterase, PDE I, using monoclonal antibodies with the capacity to inhibit this enzyme's activity.

2. EXPERIMENTAL

2.1. *Materials*

The sources of materials used in this work have been given in previous papers [1,3,5,6]. Immunoglobulins were obtained from Cappel Laboratories, West Chester, PA, USA or from Zymed Laboratories, San Francisco, CA, USA. Cyanogen bromide activated Sepharose 4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

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2.2. *Phosphodiesterase purification*

Cyclic nucleotide phosphodiesterase PDE I was purified by two sequential DEAE-cellulose column chromatographics as previously described [1,3]. Purified PDE I fractions had a specific activity of about 100 pmol/min per mg protein.

2.3. *Preparation of monoclonal antibodies*

The procedure followed has been described elsewhere [5,6]. Clones were selected for the capacity of culture media to inhibit cyclic AMP phosphodiesterase activity. Three hybridoma clones out of 200, showing the highest capacity to inhibit enzyme activity, designated 17, 32, and 37, were subcloned and used for further experiments.

2.4. *Purification and characterization of monoclonal immunoglobulins*

Purification of immunoglobulins from culture media was performed as described by Fundenberg [7] with some modifications. The hybridoma media were precipitated with solid ammonium sulfate to 50% saturation and the sedimented material resuspended and dialyzed against sodium phosphate buffer, pH 8.0. The resuspended fractions (3.6 ml, 2.9 mg/ml of protein for clone 17 and 3 ml, 2.8 mg/ml of protein for clone 32) were loaded onto DEAE-cellulose columns (1 × 6 cm) equilibrated with the same buffer solution, washed with two bed volumes of this buffer solution, and stepwise eluted with two bed volumes of 35, 50, 100, 200, and 300 mM sodium phosphate buffer, pH 8.0. All fractions eluted from the DEAE-cellulose columns were assayed for their capacity to inhibit *Neurospora* phosphodiesterase activity. The protein peak in the flow-through fraction of clone 32 and the fractions eluting at 200 mM sodium phosphate buffer of clones 17 and 37 had the highest inhibitory capacity. These selected fractions were concentrated against solid sucrose and used as the source of monoclonal IgG and IgMs, respectively.

Characterization of monoclonal immunoglobulins was performed by immunoelectrophoresis as described by Williams [8] and by enzyme-linked immunoassays (ELISA) using different goat anti-mouse immunoglobulins coupled to peroxidase (Zymed Laboratories), according to the procedure suggested by the manufacturer. ELISA tests of the culture media were performed on nitrocellulose membranes using as a second layer one of the following immunoglobulins conjugated to peroxidase: rabbit anti-mouse IgD (δ-chain specific), rabbit anti-mouse IgM (μ-chain specific) or goat anti-mouse IgG (heavy and light chain specific). Controls were performed using mouse IgM (positive control) or myeloma cell conditioned medium (negative control). Assays were performed four, five or six weeks after plating the hybrids.

2.5. *Immunoaffinity purification of PDE I*

Purified IgM from clone 17 was covalently coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's protocol and used as an affinity support for PDE I purification. The column (0.6 × 5.5 cm; about 1.35 mg of bound protein/ml) was equilibrated with 25 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl. A PDE I preparation (2 ml, 1.9 mg/ml protein) was loaded onto the column and the sample was allowed to equilibrate for 60 min to promote the antigen-antibody interaction. The column was washed with the same buffer until no absorbance at 280 nm was detected. Bound antigens were eluted with three column volumes of distilled water [9] followed by two column volumes of 0.5 M NaCl. This chromatography was repeated three times using the flow-through fraction of the previous column chromatography, as enzyme source for the second one and in the same form for the third column. Fractions with phosphodiesterase activity were pooled, lyophilized and subjected to polyacrylamide gel electrophoresis under denaturing conditions.

2.6. *Western blot*

Partially purified PDE I (DEAE fraction, 100 μg protein) and protein markers (Pharmacia) were electrophoresed on 0.1% SDS/12.5% polyacrylamide slab gels under denaturing conditions [10]. Transference to nitrocellulose membranes was performed according to the procedure of Towbin et al. [11]. Modifications of this method as well as blockage of the nitrocellulose strips and antibody reactions were as described by Torruella et al. [6]. Proteins were stained with India ink [12].

2.7. *Phosphodiesterase assay*

Enzyme activity was assayed essentially by the method of Thompson and Appleman [13] with the modifications indicated by Londesborough [14] and Téllez-Iñón et al. [1]. Reaction mixtures contained 40 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$ and 1 μM cyclic [^{3}H]AMP (specific radioactivity 600 cpm/pmol). When indicated mixtures were supplemented with 25 μM $CaCl_2$ and 200 ng of *N. crassa* calmodulin. Total volume was 0.1 ml. Assay conditions were selected so that cyclic AMP hydrolysis had a linear dependence on incubation time and enzyme concentration.

To test the inhibitory capacity of the secreting clones, a PDE I preparation was preincubated with variable concentrations of each culture medium or purified IgG or IgM at 4°C for 60, 120 and 180 min. The mixtures were then assayed for phosphodiesterase activity as indicated.

3. RESULTS AND DISCUSSION

N. crassa cyclic AMP phosphodiesterase PDE I activity was purified by DEAE-cellulose column chromatography of soluble mycelial extracts. This PDE I activity preparation, fully dependent on Ca^{2+} and calmodulin, was used to generate monoclonal antibodies.

Three stable lines, designated clones 17, 32, and 37, were selected for their capacity to inhibit enzyme activity. Monoclonal immunoglobulins from these clones were purified and characterized in terms of chain structure and their inhibitory capacity. As shown in table 1, the highest inhibition of PDE I activity corresponds to clone 17 and was associated to an immunoglobulin of the IgM type.

The effect of clone 17 monoclonal immunoglobulin was tested as shown in fig. 1. Inhibition by the purified IgM was proportional to its concentration, reaching a maximum value of about 60% of

Table 1

Characterization of monoclonal immunoglobulins with the capacity to inhibit *Neurospora* PDE I

Medium	PDE I inhibition (%)	Immunoglobulin chains			
		Heavy		Light	
		2b	μ		
Control	2	–	–	–	–
Clone 17	63	–	+	+	–
Clone 32	45	+	–	–	+
Clone 37	37	–	+	+	–

total enzyme activity at the highest amount of immunoglobulin tested. In contrast, purified IgA or IgG from clone 17 was not inhibitory. When the IgM fraction was pretreated with anti-mouse IgM no inhibition was detected, confirming the specificity of this fraction. In addition, protein fractions from a control medium had no inhibitory effect (not shown).

The purified monoclonal IgM from clone 17, covalently coupled to Sepharose 4B, was used for the immunoaffinity purification of PDE I. After absorption, the enzyme activity was eluted with water according to the procedure of Garberi et al. [9], and thereafter with 0.5 M NaCl (fig. 2). The two phosphodiesterase activity peaks thus eluted were fully activated by Ca^{2+} and calmodulin (not shown). Both fractions analyzed on SDS-PAGE showed only one stained polypeptide band of about 41 kDa (fig. 3).

Moreover, a PDE I DEAE preparation transferred to nitrocellulose membranes was incubated

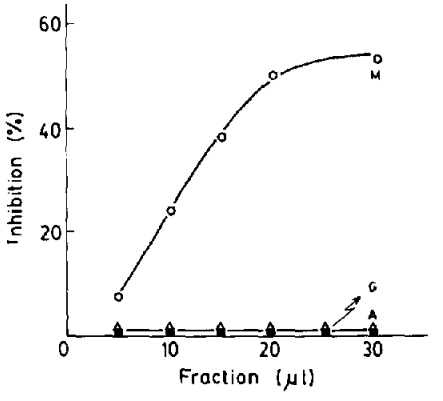

Fig.1. Effect of monoclonal immunoglobulin to *Neurospora* PDE I activity on cyclic AMP phosphodiesterase activity. Samples of a PDE I DEAE preparation (25 μg protein) were preincubated for 180 min at 22°C with variable amounts of the purified monoclonal IgM (○—○), IgA (●—●), IgG (△—△) from clone 17. Other conditions were given in section 2.

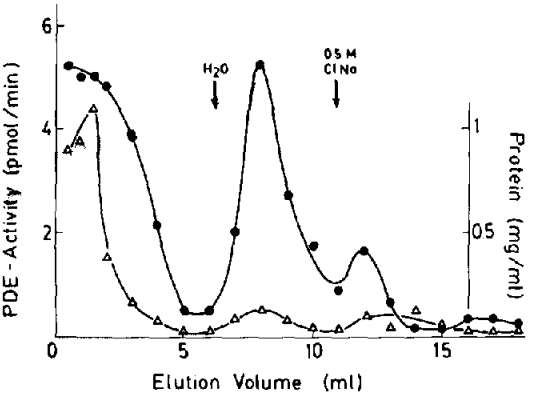

Fig.2. Immunoaffinity chromatography of PDE I activity. (●—●) PDE activity; (△—△) protein. Conditions were as indicated in section 2.

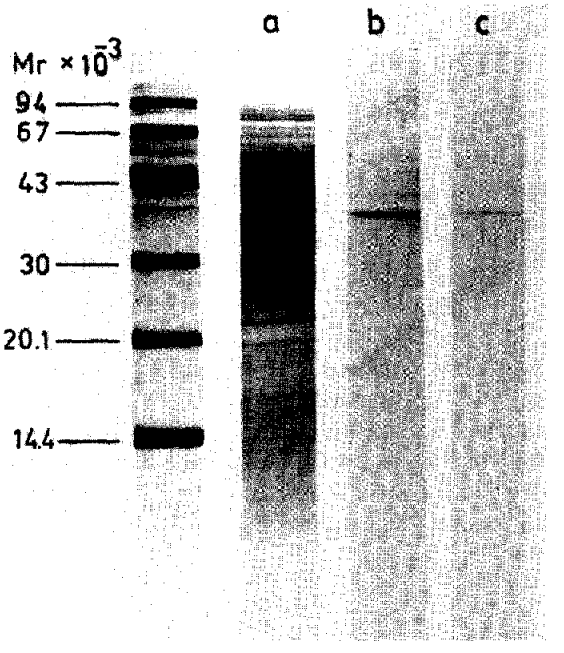

Fig.3. SDS-PAGE of a PDE I DEAE preparation (lane a) and both peaks (lanes b and c) purified by immunoaffinity chromatography described in fig. 1. Molecular mass markers are indicated (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin). Conditions were as indicated in section 2.

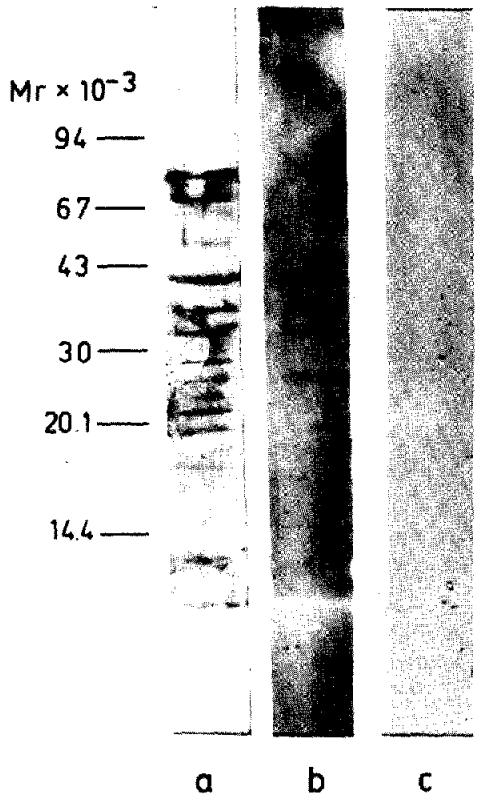

Fig.4. A PDE I DEAE preparation (100 μg protein) was electrophoresed on SDS-PAGE and transferred to nitrocellulose. The strips were incubated with IgM fraction of clone 17 (lane b) or with a monoclonal antibody against surface glycoproteins of *T. cruzi* (lane c). (Lane a) Proteins stained with India ink. Molecular mass markers are indicated. Conditions were described in section 2.

with the monoclonal IgM secreted by clone 17. The antibody recognized a 41 kDa band which is equivalent to the one obtained by the immunoaffinity chromatography and two lesser bands which are probably proteolytic fragments of the enzyme. A monoclonal antibody against surface glycoproteins of *T. cruzi*, used as control, did not react with the enzyme preparation (fig. 4).

Previous work from this laboratory showed that PDE I activity in soluble mycelial extracts presents three aggregation forms, tentatively defined as monomeric, dimeric and tetrameric, having molecular masses of about 57, 126 and 225 kDa [1]. The evidence reported here indicates that the monomeric form may be the result of the association of the 41 kDa polypeptide with a calmodulin molecule. The modulator, as reported by this and other laboratories [3,2], is a polypeptide with a molecular mass of about 16 kDa.

REFERENCES

[1] Téllez-Iñón, M.T., Glikin, G.C. and Torres, H.N. (1982) Biochem. J. 203, 611-616.
[2] Ortega-Pérez, R., Van Tuinen, D., Marmé, D., Cox, A.J. and Turian, G. (1981) FEBS Lett. 133, 205-209.
[3] Téllez-Iñón, M.T., Ulloa, R.M., Glikin, G.C. and Torres, H.N. (1985) Biochem. J. 232, 425-430.
[4] Reig, J.A., Téllez-Iñón, M.T., Flawiá, M.M. and Torres, H.N. (1984) Biochem. J. 221, 541-543.
[5] Molina y Vedia, L., Torruella, M., Flawiá, M.M. and Torres, H.N. (1983) Biochem. Biophys. Res. Commun. 113, 778-783.
[6] Torruella, M., Flawiá, M.M., Eisenschlos, C., Molina y Vedia, L., Rubinstein, C.P. and Torres, H.N. (1986) Biochem. J. 234, 145-150.
[7] Fundenberg, H.H. (1967) in: Methods in Immunology and Immunochemistry (Williams, C.A. and Chase, M.W. eds) vol. I, pp. 306-335, Academic Press, New York.
[8] Williams, C.A. (1971) in: Methods in Immunology and Immunochemistry (Williams, C.A. and Chase, M.W. eds) vol. III, pp. 234-273, Academic Press, New York.
[9] Garberi, J.C., Macario, A.J.L. and Conway de Macario, E. (1985) J. Bacteriol. 164, 1-6.
[10] Laemmli, U.K. (1970) Nature 227, 680-685.
[11] Towbin, H., Staphelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
[12] Hancock, K., and Tsang, V. Anal. Biochem. 133, 157-162.
[13] Thompson, W.J. and Appleman, M.M. (1971) Biochemistry 10, 311-316.
[14] Londesborough, J. (1976) Anal. Biochem. 71, 623-628.