Trypanosoma cruzi adenylate cyclase activity

Purification and characterization

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Adenylate cyclase activity associated with *Trypanosoma cruzi* sedimentable fractions was solubilized by treatment with the non-ionic detergent Lubrol PX and 0.5 M-(NH₄)₂SO₄. The following hydrodynamic and molecular parameters were established for a partially purified enzyme-detergent complex: sedimentation coefficient 6.2 S; Stokes radius 5.65 nm; partial specific volume 0.83 ml/g; *M_r* 244000; frictional ratio 1.33. A *M_r* of about 124000 was calculated for the detergent-free protein from these parameters. The pI of this enzyme activity was 6.2. A monoclonal antibody to *T. cruzi* adenylate cyclase was obtained, which inhibited cyclase activities from several lower eukaryotic organisms. The *T. cruzi* adenylate cyclase was further purified by using this antibody in immunoaffinity chromatographic columns. Fractions obtained after this chromatography showed, on SDS/polyacrylamide-gel electrophoresis, a main polypeptide band with an apparent *M_r* of about 56000, which specifically reacted with the monoclonal antibody.

INTRODUCTION

The enzyme system responsible for cyclic AMP synthesis in lower eukaryotic organisms may be an important tool for functional and structural studies of the catalytic entity of adenylate cyclase. In *Neurospora crassa*, *Saccharomyces cerevisiae*, *Mucor rouxii*, *Trypanosoma cruzi* and *Blastocladiella emersonii*, adenylate cyclase activity has two interesting properties: dependency on Mn²⁺ and insensitivity to fluoride (Flawiá & Torres, 1972; Paveto *et al.*, 1975; Varimo & Londesborough, 1976; da Silveira *et al.*, 1977; López-Gómez *et al.*, 1978). Moreover, only crude fresh preparations from *Neurospora* and yeast show a significant activity in assays performed with Mg²⁺ and guanine nucleotides (Casperson *et al.*, 1983; Rosenberg & Pall, 1983).

In *Neurospora crassa*, the adenylate cyclase catalytic entity, which has a loose association with membranes, may interact with regulatory factors from avian erythrocyte membranes. The reconstituted cyclase system is active with Mg²⁺ and shows stimulatory responses to guanine nucleotides, isoprenaline and fluoride (Flawiá *et al.*, 1983). In addition, *Neurospora* adenylate cyclase may interact with calmodulin, which induces a Ca²⁺-dependent stimulation of this enzyme activity in the presence of Mg²⁺ (Reig *et al.*, 1984). As a general hypothesis, it may be postulated that some adenylate cyclases from lower eukaryotic organisms consist of catalytic entities similar to those found in avian or mammalian membranes, but lacking most of the regulatory factors which are characteristic of adenylate cyclases in these membranes. In fact, some functional and physical properties of *Neurospora* cyclase are strikingly similar to the enzyme system found in the variant of S49 lymphoma cells

deficient in the guanine-nucleotide-binding protein N_s (Flawiá *et al.*, 1983; Reig *et al.*, 1982; Ross *et al.*, 1978).

Immunological studies might extend evidence on the similarities between adenylate cyclases from lower and higher eukaryotic organisms. A previous paper from our laboratory reported the preparation of a monoclonal antibody against *Neurospora* adenylate cyclase (Molina y Vedia *et al.*, 1983). The antibody inhibits *Neurospora* cyclase activity as well as other cyclases from lower eukaryotic organisms, but not adenylate cyclases from avian or mammalian tissues. This may indicate that some epitopic areas in the enzyme molecule were not conserved during evolution.

The present paper extends these studies to *Trypanosoma cruzi* adenylate cyclase activity, i.e. purification, characterization and interaction of the enzyme with a monoclonal antibody.

EXPERIMENTAL

Materials

The sources of materials used in this work have been given in previous papers (Flawiá *et al.*, 1983; Reig *et al.*, 1982; Kornblihtt *et al.*, 1981). Immunoglobulins were obtained from Cooper Biomedical, Malvern, Philadelphia, PA, U.S.A., or from Zymed Laboratories, South San Francisco, CA, U.S.A.

Trypanosoma cruzi membranes

Epimastigotes of the Tulahuen 2 strain were obtained from axenic biphasic cultures (Jerez de Burgos *et al.*, 1976). Cells were collected by centrifugation at 10000 *g* for 20 min, and homogenized in 0.25 M-sucrose containing

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5 mM-KCl (10 ml/g of material) with a Sorvall-Ribi press operated at 34.5 MPa (5000 lb/in²) under a N₂ atmosphere. Cell debris was discarded by centrifugation at 10000 *g* for 10 min and the supernatant fluid was further centrifuged at 105000 *g* for 60 min. Pellets ('crude membranes') thus obtained were stored at -20 °C.

Adenylate cyclase solubilization and purification

Crude membrane pellets were resuspended in ice-cold buffer A (50 mM-Tris/HCl, pH 7.4, 1 mM- β -mercaptoethanol and 0.1% Lubrol PX) containing 0.5 M-(NH₄)₂SO₄ with a Dounce homogenizer, and centrifuged for 120 min at 105000 *g*. The supernatant fluid was then dialysed against buffer A (giving 'solubilized extract').

Hexylamino-Sepharose chromatography

The column (1 cm $\times$ 12 cm) equilibrated with buffer A was loaded with 10 ml of the 'solubilized extract' (4 mg of protein/ml). After washing with 20 ml of this buffer solution, the column was subjected to a stepwise elution with 5 ml portions of NaCl solutions (0.1, 0.2, 0.4, 0.5 and 1.0 M respectively) in buffer A. Fractions (1 ml) were collected at a rate of 1 ml/min. Adenylate cyclase activity was eluted at 0.3 M-NaCl. Fractions containing the activity were pooled and dialysed against buffer A (giving 'hexylamino-Sepharose fraction').

Isoelectric focusing

The 'hexylamino-Sepharose fraction' was further purified on a 110 ml liquid-isoelectric-focusing column by procedures previously described (Reig *et al.*, 1982; Kornblihtt *et al.*, 1981) in Ampholine (LKB, Stockholm, Sweden), pH range 4-7.

Immunoaffinity chromatography

The 'hexylamino-Sepharose fraction' was also purified by chromatography on a column containing a monoclonal antibody to *T. cruzi* adenylate cyclase, bound to Sepharose. The immunoglobulin, prepared and purified by the procedure described below, was bound to Sepharose 4B by the method of Cuatrecasas (1970). The immunoaffinity support contained about 4 mg of bound protein/ml of packed bed volume. The column (7 cm $\times$ 0.75 cm), equilibrated with 50 mM-Tris/HCl containing 1 mM- β -mercaptoethanol (buffer B), was loaded with 2.5 ml of the enzyme fraction previously concentrated in a B15 Minicon ultrafilter (Amicon Corp.) to a protein concentration of about 0.9 mg/ml. The non-adsorbed protein fraction was re-loaded on the column; this step was repeated six times. Then the column was washed with 50 ml of buffer B containing 0.5 M-NaCl and 0.5% Lubrol PX and eluted with 50 mM-triethylamine (pH 11.8); 0.5 ml fractions were collected. The eluted fractions were immediately neutralized by addition of appropriate volumes (about 100 μ l) of 1 M-Mops buffer, pH 7.1.

Molecular and hydrodynamic parameters

Determinations were carried out by the procedures described by Reig *et al.* (1982). Sucrose gradients were made in buffer A (with H₂O or ²H₂O) containing 0.15 M-NaCl. Gel-filtration studies were performed on Bio-Gel A-5m columns (75 cm $\times$ 0.9 cm) equilibrated with buffer A containing 0.2 M-NaCl.

Preparation of monoclonal antibodies

The procedure followed for this has been described elsewhere (Molina y Vedia *et al.*, 1983). Clones were selected for the capacity of culture media to inhibit cyclase activity in a 'Bio-Gel fraction' of *Neurospora* (Reig *et al.*, 1982) or in *T. cruzi* 'crude membranes'. Purification of immunoglobulins from culture media was as described by Fundenberg (1967). Characterization of the secreted immunoglobulins was performed by immunoelectrophoresis as described elsewhere (Williams, 1971).

Adenylate cyclase assay

Enzyme activity was assayed by the method of Flawiá *et al.* (1983) in the presence of 2.5 mM-MnCl₂.

Other analytical procedures

Details of the procedure for determination of protein concentration, activity of enzyme markers and polyacrylamide-gel electrophoresis in the presence of SDS were given in previous papers (Flawiá *et al.*, 1983; Reig *et al.*, 1982; Kornblihtt *et al.*, 1981). Polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 (Reig *et al.*, 1982) or with AgNO₃ by the procedure of Oakley *et al.* (1980). Electrophoretic transfer of polypeptides from polyacrylamide gels to nitrocellulose membranes was performed by the method of Towbin *et al.* (1979) with some modifications. Two samples of the protein were subjected to electrophoresis in a polyacrylamide gel slab in the presence of SDS (Laemmli, 1970). After electrophoresis, the gel was cut with a razor blade and the lanes corresponding to *M_r* standards and to one of the samples were stained with silver by the procedure of Oakley *et al.* (1980). The other portion of the gel was subjected to electrophoretic transfer of polypeptides to a nitrocellulose sheet by the procedure of Towbin *et al.* (1979). A current of 1 A was applied for 3 h in a chamber containing 50 mM-sodium phosphate buffer, pH 6.5. The temperature of the electrophoretic system was maintained at about 25 °C.

After transfer, the nitrocellulose sheet was subjected to successive washings, with 50 mM-Tris/HCl buffer, pH 7.4, containing 0.14 M-NaCl (TBS buffer) (5 min at room temperature), with 3% bovine serum albumin in TBS buffer (30 min at 37 °C), and four times with TBS buffer (10 min each at room temperature). After that, the nitrocellulose membrane was incubated in a polyethylene bag for 14 h at room temperature in the presence of the purified monoclonal antibody (about 1 mg of protein) in 10 ml of TBS buffer containing 3% bovine serum albumin and 10% mouse serum. The sheet was then washed three times with TBS buffer (10 min each, room temperature) and incubated for 3 h at room temperature with a 1:900 dilution of goat anti-(mouse IgG) (heavy- and light-chain-specific) conjugated to peroxidase in TBS buffer containing 3% bovine serum albumin. After washing five times with TBS buffer (5 min each, room temperature) the membrane was treated for 30 min at room temperature with a solution containing 250 μ g of *o*-dianisidine/ml and 0.075% H₂O₂ in 50 mM-Tris/HCl buffer, pH 7.4.

RESULTS AND DISCUSSION

Adenylate cyclase solubilization

Several procedures were used for enzyme extraction from *T. cruzi* particulate fractions. They involved the use

of several detergents (ionic or non-ionic), urea or guanidinium chloride, with or without sonication or freeze-thawing. However, the only method giving a reasonable solubilization of adenylate cyclase activity (about 50%) was extraction in the presence of a non-ionic detergent (0.1% Lubrol PX) in the presence of a relatively high $(\text{NH}_4)_2\text{SO}_4$ concentration (0.5 M). The salt requirement may be attributable to the chaotropic effect of $(\text{NH}_4)_2\text{SO}_4$.

Enzyme purification

Adenylate cyclase activity in the solubilized extracts was purified to provide an adequate antigen to generate monoclonal antibodies. As occurs with *Neurospora* (Reig *et al.*, 1982) and testicular (Kornblihtt *et al.*, 1981) adenylate cyclase, the loss of activity was the major problem in purification of the *T. cruzi* enzyme. Recovery was not improved by the addition of glycerol, by increase in β -mercaptoethanol concentration, by pH change of the elution buffers, or by addition of polyamines etc. After two purification steps such as hexylamino-Sepharose chromatography and isoelectric focusing, 99% of the enzyme activity was lost (Table 1). However, as shown in Fig. 1, isoelectric focusing in a liquid column gave a

reasonable resolution of most of the protein from the enzyme activity.

Molecular parameters

Enzyme samples from hexylamino-Sepharose fractions were centrifuged in sucrose gradients made in H_2O or $^2\text{H}_2\text{O}$, or gel-filtered through Bio-Gel A-5m columns as previously described (Reig *et al.*, 1982). Results from an average of at least five different experiments are shown in Table 2. The calculated M_r for the enzyme-detergent complex was 244000. Assuming that the partial specific volume of the protein is 0.735 and that of the detergent 0.958 (Haga *et al.*, 1977), the M_r for the enzyme should be about 124000. This value is twice that reported for testicular soluble adenylate cyclase and two-thirds that of the *Neurospora* enzyme. These two enzymes are considered to be lipid-free soluble proteins (Kornblihtt *et al.*, 1981; Reig *et al.*, 1982).

Monoclonal antibodies

A *N. crassa* soluble adenylate cyclase preparation was used to test the inhibitory action on enzyme activity of culture media from different clones. The reason for using the *Neurospora* enzyme was the simplicity of obtaining it

Table 1. Purification of *Trypanosoma cruzi* adenylate cyclase activity

Conditions are described in the Experimental section.

Fraction	Protein (mg)	Total activity		Specific activity (pmol/min per mg of protein)	Purification
		(pmol/min)	(%)		
'Homogenate'	507	26922	100	53.1	1
'Crude membranes'*	308	19435	72.2	63.1	1.2
'Solubilized extract'	140	9829	36.5	70.2	1.3
'Hexylamino-Sepharose'	21	5891	21.9	280.5	5.3
'Antibody-Sepharose'	0.005†	185	0.7	37000	96.0
		(1850)‡	(6.9)‡	(370000)‡	(6968)‡
'Isoelectric focusing'§	1.2	216	0.8	180	3.4

* Assay was performed on membranes resuspended in buffer A containing 0.1% Lubrol PX.

† Protein was assayed on a pool of fractions from four different immunoaffinity-column chromatographies.

‡ Enzyme activity was corrected for inactivation at pH 11.8.

§ Fraction used for immunization of mice.

Fig. 1. Isoelectric focusing of a *Trypanosoma* 'hexylamino-Sepharose' fraction

▲, pH; ●, adenylate cyclase activity; ■, protein. Conditions were as indicated in the Experimental section.

Table 2. Hydrodynamic and molecular parameters of *Trypanosoma cruzi* adenylate cyclase activity

Determinations were carried out as described in the Experimental section.

Parameter	Value
Sedimentation coefficient in H ₂ O (S)	6.2±0.15
Sedimentation coefficient in ² H ₂ O (S)	5.0±0.10
Stokes radius (nm)	5.65±0.10
Partial specific volume (ml/g)	0.83
M _r	244000
Axial ratio	1.33
pI	6.2
Bound detergent (mg/mg of protein)	0.97
M _r of detergent-free protein	124000

Table 3 Effect of media from different clones on *Neurospora crassa* adenylate cyclase activity

Samples of the *Neurospora* soluble adenylate cyclase preparation purified up to the step of Bio-Gel column chromatography (30 µl; 24 µg of protein) were preincubated with a 20 µl sample of each medium for 180 min at 22 °C and then assayed for enzyme activity. The effect of a control medium from a myeloma-cell culture is also indicated.

Medium	Adenylate cyclase activity (pmol/min per mg of protein)	Inhibition (%)
Clone 36	244	70
Clone 46	285	65
Clone 11	366	55
Clone 81	416	49
Clone 23	489	40
Clone 73	530	35
Clone 30	611	25
Clone 50	614	25
Clone 19	652	20
Control	717	12
None	815	—

and previous evidence indicating that a monoclonal antibody to *Neurospora* adenylate cyclase inhibited both *Neurospora* and *T. cruzi* cyclase activities (Molina y Vedia *et al.*, 1983). Table 3 shows the effect of preincubation with control medium or media from several clones on *Neurospora* adenylate cyclase activity. Clone 36 was selected for these studies and subjected to further subcloning by the limiting-dilution procedure. After subcloning, the immunoglobulin secreted by the culture medium from the selected subclone (designated subclone 36) was identified by immunoelectrophoresis as a mouse immunoglobulin of the type G 2b. Culture medium from the subclone 36 was subjected to a purification protocol (Fundenberg, 1967) which involved precipitation with (NH₄)₂SO₄ followed by chromatography on DEAE-cellulose and dialysis. A similar protocol was used for the purification of a control medium from a myeloma-cell culture. A sample of the immunoglobulin preparation from subclone 36 was treated with rabbit anti-(mouse IgG). The effect of these preparations on *Trypanosoma*

Fig. 2. Effect of monoclonal antibody to *Trypanosoma* adenylate cyclase on enzyme activity

(a) Inhibition of adenylate cyclase activity as a function of antibody concentration. The antibody preparation was pretreated (■) or not (●) with rabbit anti-(mouse IgG) as described elsewhere (Molina y Vedia *et al.*, 1983). The effect of a fraction prepared from a control medium is also indicated (○). Samples of a 'crude membrane preparation' (0.02 ml; 170 µg of protein; adenylate cyclase specific activity 75 pmol/min per mg of protein) were preincubated with variable amounts of the purified antibody preparation [treated or not with anti-(mouse IgG)] or the control medium fraction for 180 min at 22 °C. (b) Inhibition of adenylate cyclase activity as a function of the preincubation time of the enzyme preparation with the antibody. Assays contained 100 µg of protein of the purified antibody preparation. Other conditions were given in the Experimental section.

adenylate cyclase was tested as shown in Fig. 2. Inhibition by the purified fraction from subclone 36 was roughly proportional to the protein concentration. No effect was detected when this fraction was pretreated with anti-(mouse IgG) or when a protein fraction from a control medium was used (Fig. 2a). On the other hand, the inhibitory effect required a preincubation step of the adenylate cyclase preparation with the hybridoma immunoglobulin (Fig. 2b).

Reactivity of the subclone 36 antibody with adenylate cyclase from different sources was also studied. As shown in Table 4, the antibody elicited variable extents of inhibition of Mn²⁺-dependent adenylate cyclases from lower eukaryotic organisms, but not on enzyme

Table 4. Effect of control and subclone-36 media on *T. cruzi* adenylate cyclase activity

References: ¹the present paper; ²Reig *et al.* (1982); ³Flawiá *et al.* (1983); ⁴Paveto *et al.* (1975); ⁵Harwood & Peterkofsky (1975); ⁶Kornblihtt *et al.* (1981); ⁷Neville (1968).

Source	Preparation	Adenylate cyclase activity (pmol/min per mg of protein)		Inhibition (%)
		Control medium	Subclone-36 medium	
<i>Trypanosoma cruzi</i>	'Crude membranes' ¹	70	13	81
<i>Neurospora crassa</i>	'Hexylamino-Sepharose' ²	200	96	51
<i>Neurospora crassa</i>	Membranes ³	32	24	25
<i>Mucor rouxii</i>	'Crude membranes' ⁴	45	36	20
<i>Escherichia coli</i>	Extract ⁵	120	117	2
Rat testis	105000 g supernatant ⁶	8	9	0
Liver	Membranes ⁷	200	195	2
Turkey erythrocytes	Membranes ³	25	25	0

preparations from *E. coli*, rat testes, rat liver membranes or turkey erythrocyte membranes assayed with Mn^{2+} .

Purification by immunoaffinity chromatography

Active fractions from hexylamino-Sepharose columns were purified by affinity chromatography on Sepharose 4B columns having the purified monoclonal antibody to *T. cruzi* cyclase bound to this matrix. After absorption, the enzyme activity was eluted by a drastic increase of the pH. Control experiments revealed that the extent of enzyme irreversibly inactivated after such a pH change was about 90%. On the other hand, it was difficult to determine the very low protein concentration in the eluate. For this reason protein was assayed on a pool of fraction from four different immunoaffinity chromatographies. Correcting for enzyme inactivation, the extent of adenylate cyclase purification after this step was over 5000-fold (Table 1). On SDS/polyacrylamide-gel electrophoresis the fraction purified by immunoaffinity chromatography showed polypeptide bands with apparent M_r values of about 56500, 49000 and 34000. After transfer to nitrocellulose membranes, the main polypeptide band (M_r 56500) was the only one reacting with the monoclonal antibody secreted by subclone 36 (Fig. 3).

Evidence reported in this paper indicates that a polypeptide of M_r about 56000 may be an integral part of the *T. cruzi* adenylate cyclase molecule. Other Mn^{2+} -dependent adenylate cyclases, from rat testicular tissue and *Neurospora*, have been characterized in this laboratory. Purified preparations of these cyclases showed the existence of polypeptide components with apparent M_r values slightly higher than that found in the *T. cruzi* enzyme: 69000 and 66000 respectively (Kornblihtt *et al.*, 1981; Reig *et al.*, 1982). Another interesting fact is that the monoclonal antibody to *T. cruzi* adenylate cyclase, which inhibits this enzyme activity, also inhibits *Neurospora* adenylate cyclase activity. As reported elsewhere, the reverse is also true: a monoclonal antibody to *Neurospora* adenylate cyclase inhibits both *Neurospora* and *T. cruzi* adenylate cyclase activities (Molina y Vedia *et al.*, 1983). This evidence strongly indicates that the same epitope exists in the enzyme from these two lower eukaryotic organisms and that this epitope is in some way involved in the catalytic activity of these cyclases. Another characteristic of these monoclonal antibodies is that neither inhibits some adenylate cyclase activities

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the fraction eluted from an immunoaffinity column

(a) Gel stained with $AgNO_3$; (b) electroblot of this gel showing the immunoprecipitation 'in situ' of the monoclonal antibody to *T. cruzi* adenylate cyclase. Positions of the M_r markers (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and trypsin inhibitor) are also given. Conditions were described in the Experimental section.

from higher eukaryotic organisms, indicating that some important epitopic areas of this enzyme are not strictly conserved during evolution.

Closely related to this latter point is the stimulatory

effect of the diterpene forskolin on the catalytic subunit of adenylate cyclases from higher eukaryotic organisms (Seamon *et al.*, 1981). This property was used by Pfeuffer & Metzger (1982) to purify the catalytic entity from cardiac muscle adenylate cyclase. With the *Neurospora* enzyme (Flawiá *et al.*, 1983) and the *T. cruzi* adenylate cyclase (results not shown), it was not possible to see any effect of the diterpene on these enzyme activities. This observation reinforces the view that some structural and functional characteristics of adenylate cyclase may not be maintained through the evolutionary scale of eukaryotic organisms.

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