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Cyclic AMP-dependent protein kinase activity in *Trypanosoma cruzi*

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A cyclic AMP-dependent protein kinase activity from epimastigote forms of *Trypanosoma cruzi* was characterized. Cytosolic extracts were chromatographed on DEAE-cellulose columns, giving two peaks of kinase activity, which were eluted at 0.15 M- and 0.32 M-NaCl respectively. The second activity peak was stimulated by nanomolar concentrations of cyclic AMP. In addition, a cyclic AMP-binding protein co-eluted with the second kinase activity peak. Cyclic AMP-dependent protein kinase activity was further purified by gel filtration, affinity chromatography on histone–agarose and cyclic AMP–agarose, as well as by chromatography on CM-Sephadex. The enzyme ('holoenzyme') could be partially dissociated into two different components: 'catalytic' and 'regulatory'. The 'regulatory' component had specific binding for cyclic AMP, and it inhibited phosphotransferase activity of the homologous 'catalytic component' or of the 'catalytic subunit' from bovine heart. Cyclic AMP reversed these inhibitions. A 'holoenzyme preparation' was phosphorylated in the absence of exogenous phosphate acceptor and analysed by polyacrylamide-gel electrophoresis. A 56 kDa band was phosphorylated. The same preparation was analysed by Western blotting, by using polyclonal antibodies to the regulatory subunits of protein kinases type I or II. Both antibodies reacted with the 56 kDa band.

INTRODUCTION

In eukaryotic organisms the molecular basis for cyclic AMP function is located at the level of the activation of cyclic AMP-dependent protein kinases. There is no direct evidence to indicate that cyclic AMP may act in these organisms by a mechanism that does not involve the activation of such protein kinases.

However, the impossibility to demonstrate the existence of these enzyme activities in the *Trypanosomatidae* family might indicate that these organisms constitute an exception in the evolution of the *Protoctista* kingdom (Walter & Ebert, 1977; Walter, 1978; Rangel-Aldao *et al.*, 1983).

Besides the conceptual importance of this problem, it is clear that therapeutics of Chagas' disease, African trypanosomiasis and leishmaniasis might be conditioned by the existence in *Trypanosomatidae* of alternative mechanisms for the regulation of metabolism by the cyclic nucleotide.

This paper describes the purification and characterization of a cyclic AMP-dependent protein kinase in *Trypanosoma cruzi*, the etiological agent of Chagas' disease. A preliminary report of this work has been published (Ulloa *et al.*, 1986).

MATERIALS AND METHODS

Cell cultures and extracts

Except where otherwise indicated, operations were performed at 2–5 °C. *Trypanosoma cruzi* epimastigote forms (Tulahuen 2 strain) were cultured for 7 days at 28 °C, up to the late-exponential phase, in biphasic (Gerez de Burgos *et al.*, 1976) or monophasic axenic media (Wynne de Martini *et al.*, 1980). All the components of these media, including salts, haemin, peptone, tryptose and liver, brain, heart, or yeast extracts were autoclaved before use for 15 min at 118 °C.

Cells were collected by centrifugation at 1000 *g* for 10 min and washed three times with 0.25 M-sucrose in 5 mM-KCl. The cells were homogenized in the same solution (10 ml/g of material) with a Sorvall–Ribi press operated at 34.5 MPa (5000 lb/in^2) under N_2 atmosphere. Cell debris was discarded by centrifugation at 1000 *g* for 10 min. The supernatant, previously adjusted to 0.1 mM-phenylmethanesulphonyl fluoride and 25 units of Trasylol/ml, was further centrifuged at 105000 *g* for 60 min. The supernatant ('crude extract') was immediately processed to avoid proteolytic degradation.

Purification of cyclic AMP-dependent protein kinase activity

The purification protocol refers to 'crude extracts' purified from cells grown in monophasic axenic media. This medium lacked any protein kinase or cyclic AMP-binding activities.

1. DEAE-cellulose column chromatography. A column (2.5 cm × 11 cm) was equilibrated with 25 mM-Tris/HCl buffer, pH 7.4, containing 0.1 mM-phenylmethanesulphonyl fluoride and 25 units of Trasylol/ml (buffer A) and loaded with 160 ml of 'crude extract' (2.7 mg of protein/ml). The column was washed with 200 ml of buffer A and eluted with a linear gradient of 0–0.7 M-NaCl in buffer A (425 ml); 10 ml fractions were collected

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at a rate of 0.5 ml/min. Fractions corresponding to the cyclic AMP-stimulated protein kinase activity peak (PKII), eluted at about 0.32 M-NaCl, were pooled and precipitated by the addition of 2 vol. of a saturated and neutralized $(NH_4)_2SO_4$ solution. The mixture was left on ice for 15 min, and then centrifuged at 10000 *g* for 10 min. The precipitate was resuspended in a small volume of buffer A or buffer C (see below) and dialysed against the same buffer (referred to as 'DEAE preparation').

2. Gel filtration through Sephacryl S-300. The column (46 cm × 0.8 cm) equilibrated and eluted with buffer A containing 2 mM-β-mercaptoethanol, 5% (v/v) glycerol and 0.1 M-NaCl (buffer B) was loaded with 1 ml of 'DEAE preparation' (3.5 mg of protein/ml); 1 ml fractions were collected at a rate of 0.2 ml/min. The fractions with the highest specific activity, referred to as 'Sephacryl preparation', were combined and stored at −20 °C.

3. Affinity chromatography on histone IIA–Sepharose. The column (0.5 cm × 5 cm) equilibrated with buffer A was loaded with 3 ml of the 'Sephacryl preparation' (0.5 mg of protein/ml) and washed with 10 ml of the same buffer. Elution was performed with 0.5 M-NaCl in buffer A; 0.5 ml fractions were collected at a rate of 0.1 ml/min. Eluted fractions were pooled and dialysed for 3 h against buffer A (referred to as 'holoenzyme').

4. Affinity chromatography on cyclic AMP–agarose. Alternatively, the 'DEAE preparation' was subjected to chromatography on agarose–8-(6-aminohexylamino)-cyclic AMP, by the procedure described by Dills *et al.* (1979), modified as follows. The column (0.5 cm × 4 cm) equilibrated with 10 mM-Mops, pH 6.9, containing 15 mM-β-mercaptoethanol, 0.1 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride, 10% glycerol and 25 mM-NaCl (buffer C), was loaded with 6 ml of a 'DEAE preparation' (4.2 mg of protein/ml), and dialysed against buffer C, followed by 15 ml of buffer C containing 0.75 M-NaCl, and thereafter by 5 ml of buffer C. Elution was performed with 10 ml of buffer C containing 10 mM-cyclic AMP; 2 ml fractions were collected every 3 h at a rate of 0.2 ml/min. Flow-through and the first wash fractions with buffer C were pooled (referred to as 'catalytic component'). The fractions eluted with buffer C containing cyclic AMP were exhaustively dialysed against several changes of buffer C (referred to as 'regulatory component').

5. Chromatography on CM-Sephadex. The above-mentioned 'catalytic' and 'regulatory' components were also resolved by chromatography on a column (0.5 cm diameter) containing a lower layer (4 cm high) of Sephadex G-25 (superfine grade) and an upper layer of CM-Sephadex C-50 (1 cm high) as described by Rangel-Aldao (1979). The column was equilibrated with 50 mM-sodium phosphate buffer, pH 6.5, containing 0.1 mM-EDTA, 15 mM-β-mercaptoethanol and 0.1 mM-cyclic AMP (buffer D). The column was loaded with 1 ml of a 'DEAE preparation' (4 mg of protein) containing 0.1 mM-cyclic AMP and washed with 13 ml of buffer D. Elution was performed with 10 ml of buffer D (without cyclic AMP) containing 0.3 M-sodium phosphate buffer, pH 6.5. Under these conditions the 'regulatory component' was obtained in the flow-through and washing fractions, whereas the 'catalytic component' was eluted with 0.3 M-sodium phosphate.

Purification of 'catalytic' and 'regulatory' subunits of cyclic AMP-dependent protein kinase activity from bovine heart

The procedures used for the purification of 'catalytic' and isoenzyme II-derived 'regulatory' subunits were described by Beavo *et al.* (1974) and Rannels *et al.* (1983) respectively. Briefly, the steps of purification from fresh bovine heart (800 g) were: homogenization, centrifugation and DEAE-cellulose column chromatography. Pooled fractions were concentrated by $(NH_4)_2SO_4$ precipitation, dialysis and affinity chromatography on cyclic AMP–agarose. Specific activities of these subunits were about 76.5 nmol of ^{32}P incorporated/min per mg of protein, and 1 nmol of cyclic AMP bound/mg of protein, respectively.

Protein kinase activity assay

Standard assays were performed essentially as described by Roskoski (1983). Unless otherwise indicated, reaction mixtures contained 20 mM-Mops buffer, pH 6.9, 3 mg of histone IIA/ml, 5 mM-$MgCl_2$, 10 mM-NaF, 0.2 mM-3-isobutyl-1-xanthine, 0.1 mM-[γ-^{32}P]ATP (sp. radioactivity 100 c.p.m./pmol), and, when indicated, 1 μM-cyclic AMP. Total volume was 0.1 ml. Incubations were performed at 30 °C for 10 min. Assay conditions were selected so that phosphorylation had a linear dependence on incubation time and enzyme concentration. Reaction was terminated by spotting a 0.05 ml sample from each incubation mixture on to a square piece (2 cm × 2 cm) of P-81 phosphocellulose paper, which was immediately soaked in 75 mM-H_3PO_4, followed by three washes in the same solution. The filters were dried and counted for ^{32}P radioactivity in a Omnifluor/toluene scintillation solution. Some assays were performed with 50 μM-kemptide instead of histone IIA as phosphate acceptor.

Autophosphorylation reaction

Enzyme preparations were subjected to phosphorylation in the absence of exogenous phosphate acceptor. Mixtures contained 20 mM-Mops buffer, pH 6.9, 2.5 mM-$MgCl_2$, 10 mM-NaF, 0.2 mM-isobutylmethylxanthine and 10 μM-[γ-^{32}P]ATP (500 c.p.m./pmol). Mixtures were supplemented or not with 1 mM-cyclic AMP. Total volume was 0.03 ml. Reactions were performed at 30 °C for 1–5 min and stopped by the addition of 0.03 ml of 4% (w/v) SDS containing 10% (v/v) β-mercaptoethanol, 40% (v/v) glycerol, 0.12 M-Tris/HCl buffer, pH 6.5, and 0.004% (w/v) Bromophenol Blue, and boiled for 90 s. Samples were resolved by SDS/polyacrylamide-gel electrophoresis (10%-acrylamide gels) as previously described (Kornblihtt *et al.*, 1981). Gels were fixed, stained with Coomassie Brilliant Blue R250, dried, and exposed at −70 °C to X-ray films with an intensifying screen.

Cyclic AMP-binding assay

Assays were performed essentially as described by Døskeland & Ueland (1977) at 4 °C for 15 h in mixtures containing 50 mM-potassium phosphate buffer, pH 6.8, 1 mg of bovine serum albumin/ml and 0.2 μM-cyclic [^{3}H]AMP (sp. radioactivity 32 Ci/mmol). Total volume

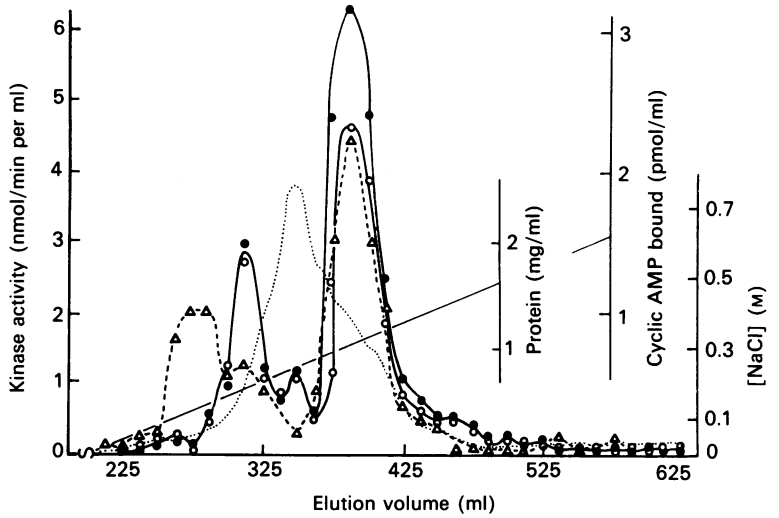

Fig. 1. Elution pattern of *T. cruzi* protein kinase and cyclic AMP binding activities from a DEAE-cellulose column

Protein kinase activity was measured in the presence (●) or absence (○) of 1 μM-cyclic AMP; △, cyclic AMP binding activity; ·····, protein concn.; ——, [NaCl]. Conditions were described in the Materials and methods section.

was 0.1 ml. Non-specific binding of cyclic [^{3}H]AMP was subtracted in each case, measured with a control mixture containing 0.1 mM unlabelled cyclic AMP. After incubation, mixtures received 1 ml of ice-cold 80%-satd. $(NH_4)_2SO_4$, followed by filtration through HAWP-02500 Millipore filters with two 3 ml washings with 60%-satd. $(NH_4)_2SO_4$. The filters were dried and counted for radioactivity with a scintillation mixture containing 0.4% (w/v) Omnifluor and 30% (v/v) Triton X-100 in toluene.

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951). Determination of enzyme markers and procedures for polyacrylamide-gel electrophoresis of protein samples were described elsewhere (Kornblihtt *et al.*, 1981; Reig *et al.*, 1982). Polypeptide transfer from polyacrylamide gels to nitrocellulose sheets was carried out by diffusion and solvent flow (Schaltman & Pongs, 1980; Bowen *et al.*, 1980). Before the immune reaction, the membranes were blocked with a suspension containing non-fat milk (Johnson *et al.*, 1984). Polyclonal antibodies used for immunodetection were: immune serum against regulatory subunit type II (final concn. 4 μg of protein/ml) and an IgG fraction against regulatory subunit type I (final concn. 2 μg of protein/ml). Both preparations were purified by affinity chromatography. These antibodies were kindly provided by Dr. Brian Hemmings, Friedrich Miescher-Institut, Basel, Switzerland. A pre-immune rabbit serum was used as a control. After reaction with the corresponding antibody, detection was carried out with the Vectastain ABC kit.

Reagents

Histone was coupled to Sepharose by the method of Cuatrecasas (1970). Agarose–8-(6-aminohexylamino)-cyclic AMP, Sephadex G-25, CM-Sephadex C-50 and Sephacryl S-300 were from Pharmacia Fine Chemicals (Uppsala, Sweden), and aprotinin (Trasylol) was from Bayer (Leverkusen, West Germany). Phosphocellulose paper (P81) and DEAE-cellulose (DE-52) were from Whatman Chemical Separation (Clifton, NJ, U.S.A.), and Mops, 3-isobutyl-1-methylxanthine, ox liver catalase, horse heart cytochrome *c*, bovine serum albumin, histones, phosvitin, casein and kemptide were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cyclic AMP analogues were given by Dr. Silvia Moreno (Facultad de Ciencias Exactas y Naturales). [γ-^{32}P]ATP was prepared as described by Glynn & Chappell (1964). [^{32}P]P_i and Omnifluor were from New England Nuclear (Boston, MA, U.S.A.).

RESULTS AND DISCUSSION

Upon chromatography on DEAE-cellulose of a *Trypanosoma cruzi* 'crude extract', two main protein kinase activity peaks were resolved. These peaks, eluted

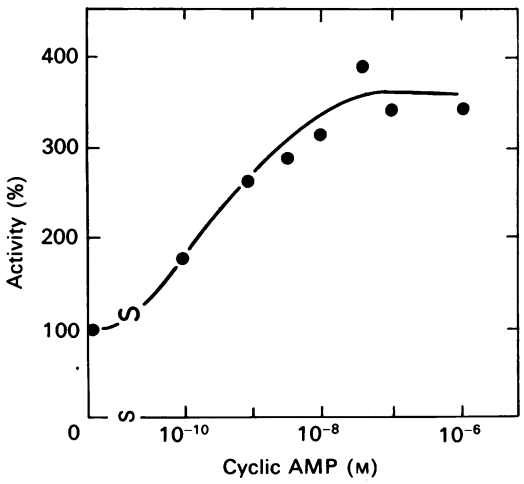

Fig. 2. *T. cruzi* protein kinase activity as a function of cyclic AMP concentration

A 'DEAE preparation' (26 μg of protein/assay) was used as source of enzyme activity. Conditions were described in the Materials and methods section.

Table 1. Specificity of cyclic [^{3}H]AMP binding by *T. cruzi* and bovine heart preparations

Conditions were described in the Materials and methods sections. Abbreviation: nd, not determined.

Additions	Concn. (μM)	Cyclic [^{3}H]AMP binding (%) PKI*	PKII†	PKII‡	RII§
None	—	100	100	100	100
Cyclic AMP	10	34	27	32	12
	100	17	3	5	3
Cyclic GMP	10	103	84	100	nd
	100	85	84	90	nd
C^8-Bromo cyclic AMP	10	nd	nd	33	10
	100	nd	nd	24	4
N^6-Monobutyryl cyclic AMP	10	nd	nd	25	8
	100	nd	nd	22	3
ATP	10	99	88	nd	nd
	100	60	88	nd	nd
5′-AMP	10	89	108	nd	nd
	100	56	96	nd	nd
Adenosine	10	89	64	nd	nd
	100	67	49	nd	nd

* *T. cruzi* 'DEAE preparation'; specific activity 1.7 pmol bound/mg of protein.

† *T. cruzi* 'DEAE preparation'; specific acitivity 3.6 pmol bound/mg of protein.

‡ *T. cruzi* 'regulatory component' preparation purified by affinity chromatography on cyclic AMP–agarose; specific activity 98 pmol bound/mg of protein.

§ Type II 'regulatory subunit' (RII) preparation from bovine heart; specific activity 1 nmol bound/mg of protein.

Table 2. Phosphate acceptor specificity of *T. cruzi* PKII activity

Concentrations of phosphate acceptors were 1 mg/ml for proteins and 50 μM for kemptide. Reaction mixtures contained 10.3 μg of kinase protein and 1 μM-cyclic AMP. Other conditions were described in the Materials and methods section.

Phosphate acceptor	PKII activity (nmol/min per mg of protein)
None	0.13
Kemptide	1.49
Histone VII	1.47
Histone IIA	1.46
Protamine	0.86
Casein	0.54
Histone VI	0.40
Histone VIII	0.36
Phosvitin	0.16

at NaCl concentrations of about 0.15 M and 0.32 M, were designated PKI and PKII respectively (Fig. 1).

PKI activity was insensitive to cyclic AMP, whereas PKII was stimulated by this cyclic nucleotide. Half-maximal effect was observed at about 1 nM-cyclic AMP (Fig. 2). Cyclic GMP had no effect on this kinase activity (results not shown).

The specificity of cyclic [^{3}H]AMP-binding activities corresponding to PKI and PKII was measured in terms of cyclic [^{3}H]AMP displacement by the addition of 10 μM or 100 μM unlabelled cyclic AMP or other analogues. Two different PKII preparations were studied, a 'DEAE preparation' and a 'regulatory component' purified by affinity chromatography on cyclic AMP–agarose. As shown in Table 1, PKI and PKII bind cyclic AMP specifically. In addition, the displacement of cyclic [^{3}H]AMP binding by C^8 and N^6 cyclic AMP derivatives, specific for sites 1 and 2 respectively, was assayed with both the PKII 'regulatory component' and the bovine

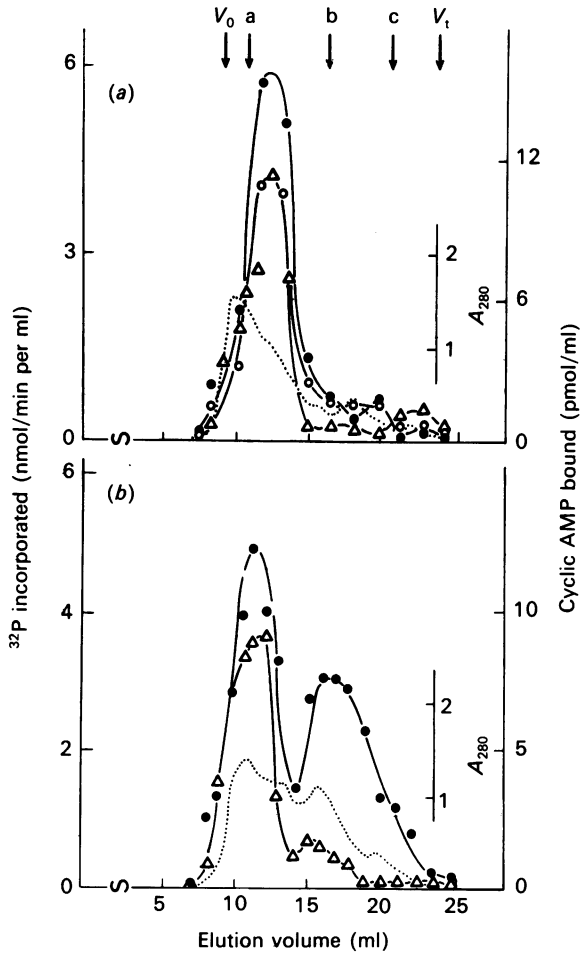

Fig. 3. Elution pattern of *T. cruzi* protein kinase and cyclic AMP binding activities from a Sephacryl S-300 column

(*a*) A column equilibrated with buffer B was loaded with a 'DEAE fraction' and eluted with the same buffer as indicated in the Materials and methods section. (*b*) A column equilibrated with buffer B containing 0.1 mM-cyclic AMP was loaded with a 'DEAE fraction' previously dialysed against buffer B containing 2 mM-cyclic AMP and eluted with the equilibration buffer. Fractions were exhaustively dialysed against buffer B before assay for cyclic AMP binding activity. Symbols are as in Fig. 1 (·····, A_{280}). The column was calibrated with the following markers: Blue Dextran (V_0), catalase (a), bovine serum albumin (b), cytochrome *c* (c) and $CoCl_2$ (V_t). Other conditions were described in the Materials and methods section.

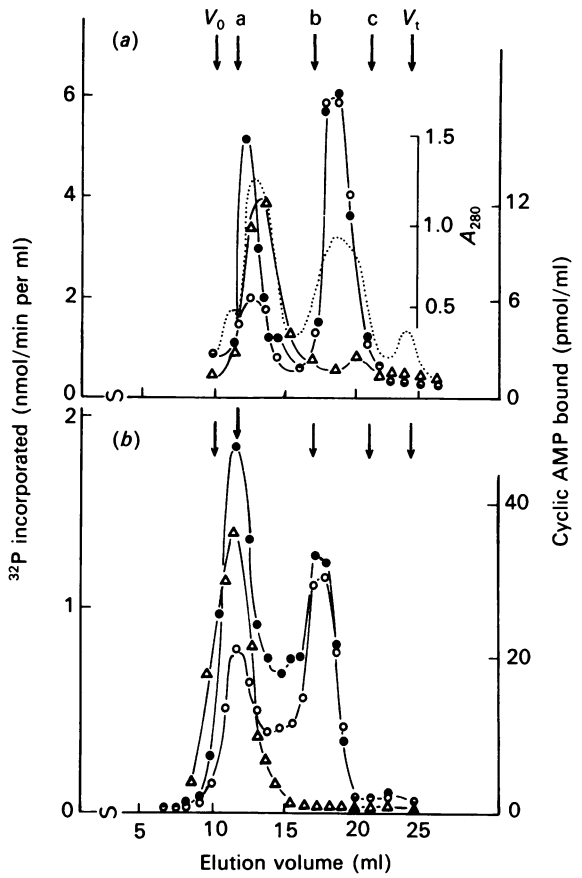

Fig. 4. Elution pattern of *T. cruzi* and bovine heart protein kinase and cyclic AMP binding activities from a Sephacryl S-300 column

(*a*) A column equilibrated with 25 mM-Tris/HCl buffer, pH 7.4, containing 0.5 M-NaCl, was loaded with a *T. cruzi* 'DEAE preparation' preincubated for 30 min at 0 °C in the presence of 1.5 mg of histone IIA/ml, 1 μM-cyclic AMP and 0.5 M-NaCl, and eluted with the equilibrium buffer. (*b*) The same as (*a*), except that a bovine heart 'DEAE preparation' was used. Other conditions were described in the Materials and methods section. Symbols are as in Figs. 1 and 3.

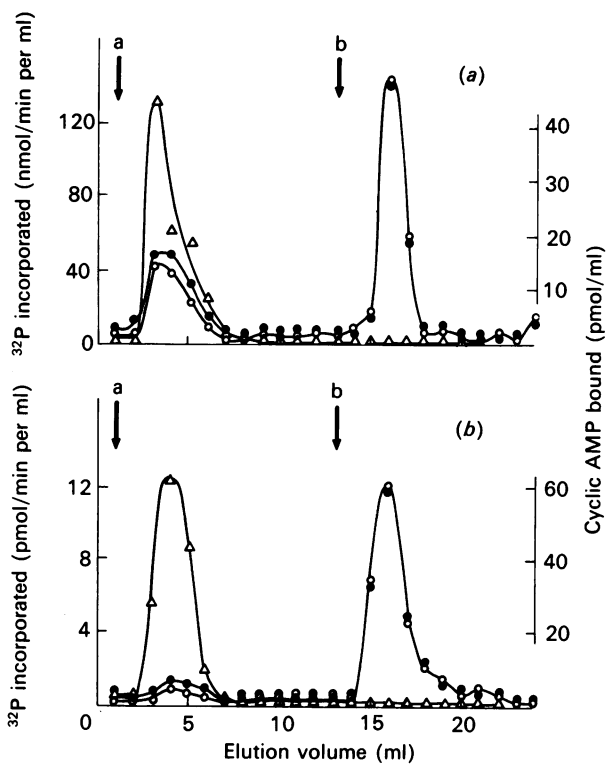

Fig. 5. Elution pattern of *T. cruzi (a)* and bovine heart *(b)* protein kinase and cyclic AMP binding activities from a CM-Sephadex/Sephadex G-25 column

Arrows: a, equilibration buffer; b, elution buffer. Assays were performed in the absence (○) or presence (●) of cyclic AMP. Other conditions were described in the Materials and methods section. Symbols are as in Fig. 1.

heart type II regulatory subunit. At the same concentrations, both analogues compete more efficiently with the cyclic [^{3}H]AMP bound to the heart preparation than to the *Trypanosoma cruzi* 'regulatory component'. The results with the heart preparation were similar to those of Rannels & Corbin (1980).

Different proteins were assayed as phosphate acceptors of PKII activity. As shown in Table 2, histone VII (formerly designated as H2b), kemptide and histone IIA were the best acceptors; casein and phosvitin were inadequate acceptors, as expected, for a cyclic AMP-dependent protein kinase.

Attempts were made to resolve the PKII 'holoenzyme' into its components. When a 'DEAE preparation' was chromatographed on a Sephacryl S-300 column equilibrated with a low-ionic-strength buffer, both the phosphotransferase and the cyclic AMP binding activities co-eluted as a single peak, indicating the existence of a putative 'holoenzyme' (Fig. 3*a*). However, when the 'DEAE preparation' was previously dialysed against a buffer containing 2 mM-cyclic AMP, to promote dissociation of the 'holoenzyme', and loaded on to a Sephacryl S-300 column equilibrated with a buffer containing 0.1 mM-cyclic AMP, a 'catalytic component' was partially dissociated from the 'holoenzyme' (Fig. 3*b*). The failure to dissociate *Trypanosoma cruzi* holoenzyme fully by cyclic AMP alone was also observed in *Mucor rouxii* (Moreno & Passeron, 1980; Pastori *et al.*, 1981).

A better separation of the 'catalytic component' was obtained preincubating the 'holoenzyme' with histone IIA and cyclic AMP in the presence of 0.5 M-NaCl. This mixture was chromatographed on a Sephacryl S-300 column equilibrated with a buffer solution containing 0.5 M-NaCl (Fig. 4*a*). A comparative experiment was carried out under the same conditions with a 'DEAE preparation' from bovine heart (Fig. 4*b*). As shown, the *T. cruzi* 'holoenzyme' and its 'catalytic component' was eluted slightly after the heart enzyme subunits. The calculated Stokes radii for *T. cruzi* and heart 'catalytic' entities were 2.2 and 2.8 nm respectively. In Fig. 4(*b*), the chromatographic profile on Sephacryl S-300 shows that a high concentration of bovine heart type II protein kinase (6.1 mg of protein) was not fully dissociated by 1 μM-cyclic AMP. As previously described (Rangel-Aldao & Rosen, 1977), concentrations of cyclic AMP greater than 1 mM were necessary to achieve a 100% dissociation when high concentrations of holoenzyme were used. It should be pointed out that Connelly *et al.* (1986) obtained this same profile in a re-association

Table 3. Reconstitution of a protein kinase 'holoenzyme' from 'catalytic' and 'regulatory' components from *T. cruzi* and bovine heart

The mixtures containing the different protein fractions and all the components of the kinase assay (50 μM-kemptide as phosphate acceptor), except [γ-^{32}P]ATP, were preincubated at 0 °C for 20 min. Reactions were initiated by the addition of [γ-^{32}P]ATP. Other conditions were described in the Materials and methods section.

Expt. no.	Source	Component	Protein kinase activity (nmol/min per mg of protein) Without cyclic AMP	With cylic AMP
1	*T. cruzi*	'Catalytic'*	0.403	0.405
	T. cruzi	'Catalytic'* plus	0.105	0.387
	T. cruzi	'Regulatory'*		
2	*T. cruzi*	'Catalytic'†	1.000	0.900
	T. cruzi	'Catalytic'† plus	0.410	1.250
	Bovine heart	'Regulatory'*		
3	Bovine heart	'Catalytic'*	18.200	21.030
	Bovine heart	'Catalytic'* plus	9.330	21.300
	T. cruzi	'Regulatory'*		
4	Bovine heart	'Catalytic'*	23.400	23.000
	Bovine heart	'Catalytic'* plus	3.600	20.050
	Bovine heart	'Regulatory'*		

* Purified by affinity chromatography on cyclic AMP–agarose.
† Purified by chromatography on Sephacryl S-300 as described in Fig. 4(*a*).

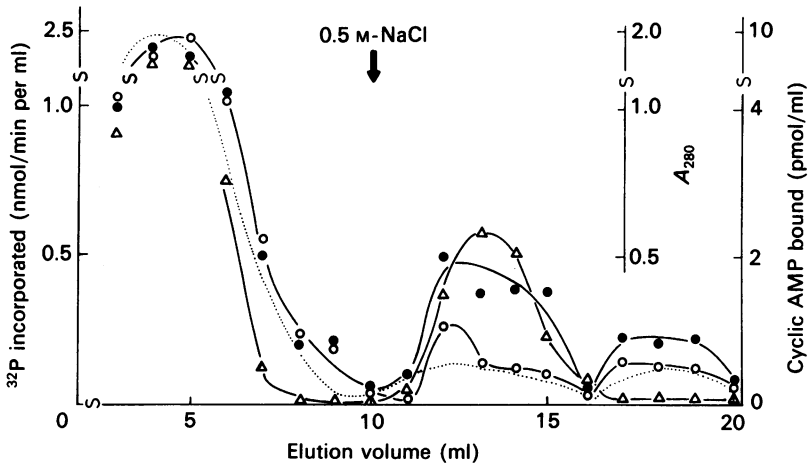

Fig. 6. Affinity chromatography of *T. cruzi* PKII activity on histone–Sepharose

Conditions were described in the Materials and methods section. Symbols are as in Fig. 1.

experiment of purified heart subunits. In addition, these authors argue that R_2C_2 and R_2 molecular entities were difficult to resolve on gel filtration because of their similar Stokes radii.

The *Trypanosoma cruzi* 'catalytic component' obtained from the Sephacryl S-300 was used for the reconstitution experiments with the heterologous regulatory subunit from bovine heart. This peak represents free catalytic subunit, as demonstrated by its ability to be reversibly inhibited on addition of purified regulatory subunit (Table 3, expt. 2).

Chromatography on a double-bed CM-Sephadex–Sephadex G-25 column (Rangel-Aldao, 1979) gave a better resolution of the 'catalytic' and 'regulatory' entities from *T. cruzi* and bovine heart (Fig. 5).

Attempts to resolve the components of *T. cruzi* PKII 'holoenzyme' were also accomplished by affinity chromatography by the procedure described by Rutherford *et al.* (1984) for the cyclic AMP-dependent protein kinase activity of *Dictyostelium discoideum*. The 'holoenzyme' preparation obtained after chromatography on Sephacryl S-300 (see Fig. 3*a*) was loaded on a histone–Sepharose

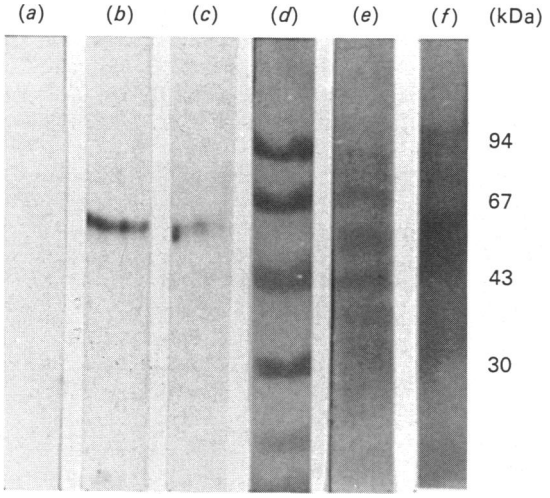

Fig. 7. Immunoblotting of *T. cruzi* and bovine heart preparations after reaction with an anti-(type II heart regulatory subunit) antibody

Lanes: (*a*) *T. cruzi* 'DEAE preparation' treated with a pre-immune serum (control); (*b*) and (*c*), bovine heart 'Sephacryl' and *T. cruzi* 'DEAE' preparations respectively, treated with the anti-(type II 'regulatory subunit') antibody; (*d*), (*e*) and (*f*), stained polypeptide bands corresponding to protein markers, *T. cruzi* and bovine heart preparations respectively.

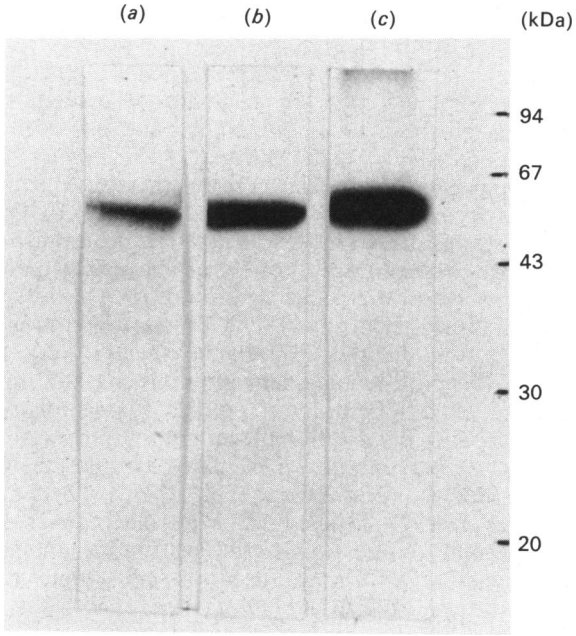

Fig. 8. Autophosphorylation of *T. cruzi* and bovine heart preparations

(*a*) Bovine heart 'Sephacryl preparation' (21 μg of protein); (*b*) and (*c*), *T. cruzi* 'DEAE' and 'Sephacryl' preparations (216 and 115 μg of protein) respectively. Conditions were described in the Materials and methods section.

column. As shown in Fig. 6, a small peak of 'catalytic component' was eluted after the 'holoenzyme'. In addition, the 'catalytic' and 'regulatory' components were also resolved by chromatography on a cyclic AMP–agarose column (results not shown).

Experiments were performed to reconstitute *T. cruzi* PKII 'holoenzyme' from its separate 'catalytic' and 'regulatory' components or to reconstitute heterologous 'holoenzymes' with components from *T. cruzi* PKII and type II protein kinase from bovine heart. As shown in Table 3, the addition of *T. cruzi* 'regulatory component' to its homologous 'catalytic component' led to the inhibition of the phosphotransferase activity; cyclic AMP reversed this inhibition (Expt. 1). The same result was obtained when a type II bovine heart 'regulatory subunit' preparation was used instead of the *T. cruzi* 'regulatory component' (Expt. 2).

In addition, as also shown in Table 3 (Expt. 3), the *T. cruzi* 'regulatory component' was capable of inhibiting phosphotransferase activity of bovine heart 'catalytic subunits'. Here also, the inhibition was reversed by cyclic AMP. The bovine heart 'catalytic subunit' preparation used in the latter experiment showed a slight stimulation by cyclic AMP, which is attributable to a marginal contamination with 'regulatory subunits'. However, this 'catalytic subunit' preparation showed a clear inhibition by the homologous RII subunit, which is fully reversed by cyclic AMP (Table 3, Expt. 4).

Further support for the existence of authentic 'regulatory subunits' of cyclic AMP-dependent protein kinase in *T. cruzi* was obtained by Western-immunoblotting analysis of 'DEAE preparations'. The blotted nitrocellulose strips were incubated with polyclonal antibodies to bovine type I or type II 'regulatory subunits'. As shown in Fig. 7, the anti-(type II 'regulatory subunit') antibody reacted with a 56 kDa band from both heart and *T. cruzi* preparations. When the anti-(type I 'regulatory subunit') immunoglobulin was used, a faint reaction with the 56 kDa band was observed (results not shown).

Since heart type II cyclic AMP-dependent protein kinase is able to phosphorylate its own 'regulatory subunits', the possibility that *T. cruzi* PKII activity might catalyse a similar reaction was investigated. 'Sephacryl preparations' were subjected to phosphorylation in the absence of exogenous phosphate acceptor, and the fractions were analysed by SDS/polyacrylamide-gel electrophoresis. As shown in Fig. 8, a 56 kDa band was phosphorylated.

Results shown in this paper indicate the existence in *Trypanosoma cruzi* extracts of a cyclic AMP-dependent protein kinase activity. Criteria to assert the authenticity of this enzyme activity are the following: (1) the enzyme is specifically activated by nanomolar concentrations of cyclic AMP; (2) specificity for phosphate acceptors is characteristic of this type of phosphotransferase activity; (3) PKII preparations specifically bind cyclic AMP; (4) 'catalytic' and 'regulatory' components can be resolved from the 'holoenzyme'; (5) the *T. cruzi* 'regulatory component' inhibits the activity of *T. cruzi* or bovine heart 'catalytic' entities, and cyclic AMP reverses these inhibitions; (6) bovine heart type II 'regulatory subunits' inhibit *T. cruzi* 'catalytic component', and cyclic AMP also reverses this inhibition; (7) polyclonal antibodies to bovine type II 'regulatory subunits' react with a 56 kDa polypeptide in *T. cruzi* PKII preparations (this molecular size is identical with that corresponding to bovine heart type II 'regulatory subunits'; Rosen & Erlichman, 1975); and (8) as occurs with mammalian type II cyclic AMP-dependent protein kinase (Rosen & Erlichman,

1975), *T. cruzi* enzyme is able to phosphorylate its own 'regulatory component'.

Other laboratories have reported in *T. cruzi* and in African trypanosomes the existence of protein kinase activities which are not activated by cyclic AMP (Rangel-Aldao *et al.*, 1983; Walter & Ebert, 1977; Walter, 1978). The apparent discrepancies between these results and the work reported here might be attributable to differences in cell strains and/or in the purification protocols. Many differences between strains of *T. cruzi* have been recorded. Authors have detected immunological differences between strains (Dvorak, 1984), different metabolic end products and distinct oxidative pathways, too. Many strains differ in their nutrient requirements and in their capacity to differentiate in culture (Contreras *et al.*, 1985). Strains from different geographic areas differ in the electrophoretic pattern of many enzymes, a characteristic which has been used to typify them in 'zimodemes' (Miles *et al.*, 1977; Miles, 1979; Miles & Cibulskis, 1986). In addition, differences in the restriction analysis of kinetoplastic DNA were used as taxonomic criteria (Mattei *et al.*, 1977; Morel *et al.*, 1986). With this latter technique Frasch *et al.* (1981) differentiated the non-pathogenic strain Tul 0 from the pathogenic one, Tul 2. In our laboratory we have detected differences of one order of magnitude in the extent of phosphorylation of exogenous substrates by the enzymes of both strains, Tul 2 being more efficient (results not shown).

Besides the characterization of the cyclic AMP dependency of PKII activity, another protein kinase, designated PKI, was also resolved by chromatography of *T. cruzi* extracts in DEAE-cellulose columns. This kinase also co-eluted with a cyclic AMP binding activity. However, it was impossible to demonstrate any stimulation of PKI by the cyclic nucleotide. Further experiments are required to characterize these kinase and binding activities. An interesting possibility to be explored may be the comparison of cyclic AMP binding activity of PKI with that studied by Rangel-Aldao *et al.* (1983).

The existence in *Trypanosoma cruzi* epimastigotes of a cyclic AMP-dependent protein kinase activity reported in the present paper, and previous evidence on the control of *T. cruzi* cyclic nucleotide phosphodiesterase activity by homologous calmodulin (Téllez-Iñón *et al.*, 1985), indicate that regulatory schemes involving cyclic AMP, and the metabolism of the cyclic nucleotide in this parasite, are similar to those found in mammalian cells.

R.M.U. and E.M. are Fellows, and H.N.T. and M.T.T.-I. are Career Investigators, of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

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