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CHARACTERIZATION OF AN ADENYLYL CYCLASE ACTIVITY IN PARTICULATE PREPARATIONS FROM EPIMASTIGOTE FORMS OF *TRYPANOSOMA CRUZI*

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Summary

Particulate preparations from epimastigote forms of *Trypanosoma cruzi* contain an adenylyl cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) which could be stored at −20°C and resisted 5 cycles of freezing and thawing over 10 days without significant loss of activity. The enzyme reaction strictly required Mn^{2+}, had a pH optimum of 7.7 and was not inhibited or stimulated by NaF. Particles prepared in the presence of 10 mM Mn^{2+} or Mg^{2+} were 3—4 times more active than particles prepared in the absence of these cations. However, Mg^{2+} could not substitute for Mn^{2+} during enzyme assay nor did it enhance activity in the presence of saturating concentrations of Mn^{2+}. The binary complex Mn · ATP^{2-} was shown to be the true substrate for the adenylyl cyclase and free ATP was highly inhibitory. Plots of enzyme activity against equimolar concentrations of ATP · Mn gave sigmoid curves with n values in Hill plots ranging between 1.5 and 2.0. Excess Mn^{2+} activated the cyclase catalyzed reaction at low but not at high concentrations of ATP · Mn. In the presence of an excess of 1 mM Mn^{2+}, which transforms 97% of the added ATP to productive Mn · ATP^{2-} complex, the substrate saturation curve assumed a Michaelian pattern with an apparent K_m = 0.2 mM.

Introduction

The pathogenic digenetic protozoon *Trypanosoma cruzi*, responsible for Chagas disease in man, is known to occur in several stages of differentiation during residence in mammalian hosts and insect vectors. The non-reproducing

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trypomastigote forms undergo transformation into the reproducing epimastigote forms in the insect midgut, following blood suction from an infected host. This transformation is accompanied by large morphological and biochemical changes. The epimastigote forms, after several cycles of division, differentiate into the infective trypomastigote forms which are ready for another cycle of infection in the vertebrate host (for a detailed review of the biology of *T. cruzi* see ref. 1).

The growth cycle of *T. cruzi* in the invertebrate host can be fairly well reproduced in vitro through the use of complex liquid media [2]. However, efficient in vitro differentiation of epimastigotes into trypomastigotes is difficult and erratic by virtue of the almost total lack of knowledge on the conditions which may regulate such transformation.

Adenylyl cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) an enzyme known to be modulated by a variety of different external signals in higher vertebrates, could potentially be a mediator for environmental control in microorganisms including the trypanosomatids. In fact, changes in the intracellular levels of adenosine 3′,5′-monophosphate and in the activities of cyclic AMP-phosphodiesterase and adenylyl cyclase have been correlated with cell growth and differentiation in many eukaryotic microorganisms [3—12]. In the field of trypanosomatids a report has been recently published [13] which correlates the ablastin-induced transformation of rapidly reproducing to non-reproducing forms of *T. lewisi* with cyclic AMP intracellular changes.

As part of an overall effort to understand the role of cyclic AMP and related enzymes in growth and differentiation of *T. cruzi* we have initiated studies on the metabolism of this nucleotide in the various forms of the protozoon. The present work describes the properties of the adenylyl cyclase of the epimastigote forms of *T. cruzi*.

Materials and Methods

Materials. Pyruvate kinase, phospho*enol*pyruvate, bovine heart cyclic nucleotide phosphodiesterase, adenylate kinase and unlabeled nucleotides were obtained from Sigma Chemical Co. 2′,3′-isopropylidene adenosine and neutral chromatographic alumina were purchased from Calbiochem and Merck, respectively. Dowex 50 W-X8 (H^+) 100—200 mesh, was a product of BioRad. Carrier-free, radioactive inorganic phosphate was supplied by the Instituto de Energia Atomica, Sao Paulo. [α-^{32}P]ATP was synthesized essentially by the method described by Symons [14]. The purity of the [α-^{32}P]ATP was checked by thin layer chromatography in polyethyleneimine-(PEI)-cellulose (Merck) precoated plates [15] followed by radioautography. Cyclic [^{3}H]AMP (specific activity 33.2 Ci/mmol) was obtained from New England Nuclear.

Cell cultures. Epimastigote forms of *T. cruzi*, Y strain [16] were cultured in a liquid medium [2] in a rotary shaker (New Brunswick) at 120 rev./min, 28°C Cells (approx. 5×10^9) were harvested during late exponential growth (80—100 h) or during early stationary phase (120 h) by centrifugation at $1000 \times g$ (4°C). The protozoa were washed 3 times with 30 ml of 0.9% (w/v) NaCl solution.

Enzyme preparation. The washed cellular pellet was resuspended in 5 ml of 10 mM Tris · HCl, pH 7.5, containing 10 mM $MgCl_2$, unless otherwise specified,

and sonicated for 15 s in a Branson sonifier (5-s pulses with a 1-min interval between pulses, at medium power). The homogenate was centrifuged at 105 000 × *g* for 60 min at 4°C in a Spinco L3-50 centrifuge. The pellet was then washed with 10 ml of 10 mM Tris · HCl, pH 7.5, and recentrifuged under the same conditions. The washed pellet was resuspended in 10 mM Tris · HCl, pH 7.5, and diluted to a protein concentration of 2 mg/ml. No enzymatic activity was detected in the 105 000 × *g* supernatant. Protein was measured by the method of Lowry et al. [17] using bovine serum albumin as standard.

Adenylyl cyclase assay. Unless otherwise specified the assay mixture contained: 1 mM [α-^{32}P]ATP (13—50 · 10^3 cpm/nmol), 2 mM $MnCl_2$, 0.5 mM cyclic AMP, 1 mM KCl, 5 mM phospho*enol*pyruvate, 10 μg of pyruvate kinase and 50 mM Tris · HCl, pH 7.5, in a final volume of 0.1 ml. The incubation was carried out at 30°C for 8 min, or as specified. The reaction was initiated by the addition of the particulate fraction containing the adenylyl cyclase (40—50 μg of protein). The reaction was stopped by the addition of 0.1 ml of a solution containing 40 mM ATP and 12.5 mM cyclic AMP followed by incubation for 2 min in a boiling water bath. The radioactive cyclic AMP produced was eluted from neutral alumina columns (0.5 × 6 cm) with 3 ml of 10 mM Tris · HCl, pH 7.4 [18]. A parallel column with cyclic [^{3}H]AMP (20 000 cpm) as standard was used to estimate the efficiency (50—70%) in each set of experiments. The results reported were always an average of duplicate experiments. Variation between duplicates was less than 4%. Radioactivity of the total eluate was determined using a system of naphthalene/dioxane (PPO, 5 g; naphthalene, 100 g, dioxane, to a final volume of 1000 ml) in a Beckman liquid scintillation counter. Blanks of the reaction carried out in the absence of the enzyme gave values between 300 and 500 cpm.

Identification of the reaction product. The labeled material eluted from the neutral alumina column, as described above, was chromatographed on a Dowex 50 W X-8 (H^+) column (0.6 × 8.5 cm). Elution was performed with water [19]. The radioactive eluate was lyophilized and resuspended in 0.1 ml of 1 mM Tris · HCl, pH 8.0. A portion of this solution was directly applied to Whatman No. 1 paper together with optical markers. The other portion was incubated with 50 μg of bovine heart cyclic nucleotide phosphodiesterase in a reaction mixture containing 40 mM Tris · HCl, pH 8.0, 5 mM $MgCl_2$, 10 mM KCl, 1 mM mercaptoethanol and 200 μg bovine serum albumin in a final volume of 0.1 ml. The reaction was allowed to proceed for 60 min at 30°C and was terminated by boiling. The supernatant obtained after low speed centrifugation was also applied to the Whatman No. 1 paper. The descending paper chromatogram was developed with isopropanol/ammonia/water (7 : 2 : 1) as running solvent [20]. After 20 h the paper was thoroughly dried and the markers were visualized under ultraviolet light. Strips of paper (1 × 2 cm) were cut and counted in 5 ml of scintillation fluid (PPO, 4 g; POPOP, 0.1 g; toluene, to a final volume of 1000 ml).

Results

Requirements for adenylyl cyclase activity. The synthesis of cyclic 3',5'-AMP was proportional to the incubation time up to approximately 12 min. The addi-

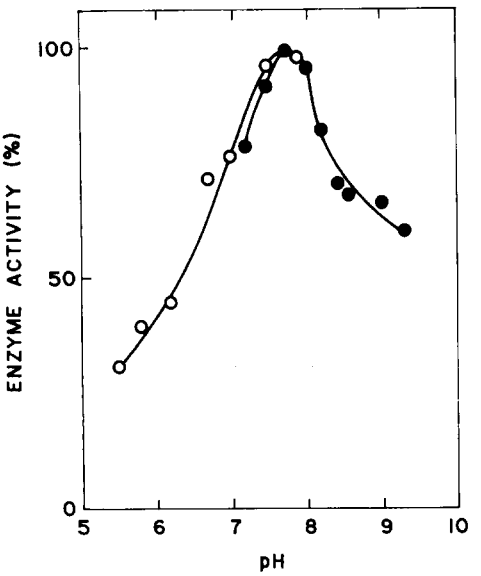

Fig. 1. Adenylyl cyclase activity as a function of pH. The enzyme preparation was assayed in the presence of 50 mM Tris/maleate (○) or Tris · HCl (●) at the indicated pH. The assay conditions were as described in Materials and Methods.

tion of an ATP-regenerating system ensured linearity up to 24 min. These results suggest the presence of poorly active ATPases in *T. cruzi* extracts, under the assay conditions. The synthesis of cyclic AMP was also proportional to protein up to a concentration of 100 μg. Linearity with respect to protein was not affected by the presence or absence of the ATP-regenerating system. No enzymatic activity was detected in the 105 000 × *g* supernatant.

Fig. 1 shows adenylyl cyclase activity as a function of the pH of the reaction mixture. Maximal activities were observed in a narrow pH range between 7.5 and 8.0, under the conditions tested. At pH 7.0 or 8.5 the enzyme activity was only 70% of the activity observed at pH 7.7.

Effect of ions on the adenylyl cyclase activity. The enzyme reaction requires Mn^{2+} for maximal activity. Substitution of this cation by Mg^{2+}, Ca^{2+}, or Co^{2+} resulted in much lower or negligible activities, independent of whether the particles were prepared in the presence (Table I) or absence of Mg^{2+}. When the enzyme was assayed in the presence of 2 mM $MnCl_2$, Zn^{2+} (2 mM) and Co^{2+} (2 mM) were inhibitory and Ca^{2+} (2 mM) or Mg^{2+} (2 mM) had no effect.

The adenylyl cyclase of *T. cruzi* was not affected by F^- under several conditions tested. Preincubation of the enzyme with 5 mM NaF (30 min at 4°C) or addition of this salt to the assay mixtures in concentrations up to 10 mM, in the presence of Mg^{2+} or Mn^{2+} (1—10 mM), did not stimulate or inhibit cyclase activity.

Identification of the reaction product. The stability of the product was assured by the addition of an excess of non-radioactive cyclic AMP to the reaction mixture. We have observed that in the first 10 min of reaction the presence or absence of cyclic AMP in the assay mixture did not alter the levels of cyclic AMP synthesis. After 10 min, reaction rates were decreased in the absence of

TABLE I

EFFECT OF DIVALENT CATIONS ON ADENYLYL CYCLASE ACTIVITY

The enzyme preparation (50 μg) was incubated for 8 min at 30°C as described in Materials and Methods. The concentrations of substrate and cations were 1 mM and 2 mM, respectively. A different enzyme preparation was used in each experiment.

Expt. No.	Substrate	Additions	Enzyme activity	
			$nmol \cdot mg^{-1} \cdot min^{-1}$	% Control
1	ATP	Mn^{2+}	0.77	100
		Mg^{2+}	0.06	8
		Ca^{2+}	0	0
		Co^{2+}	0.09	12
		None	0	0
2	ATP + Mn^{2+}	None	0.62	100
		Mn^{2+}	0.56	90
		Mg^{2+}	0.57	92
		Ca^{2+}	0.61	98
		Zn^{2+}	0.04	6
		Co^{2+}	0.14	23

cyclic AMP. This is in agreement with observations made in our laboratory that 10—15% of a highly active cyclic 3',5'-AMP phosphodiesterase remains associated with the 105 000 × *g* pellet after two washing cycles (Goncalves, M.F. and Colli, W., unpublished observations).

After sequential chromatography on neutral alumina and Dowex columns, the product was isolated by paper chromatography. Fig. 2A shows that 98% of

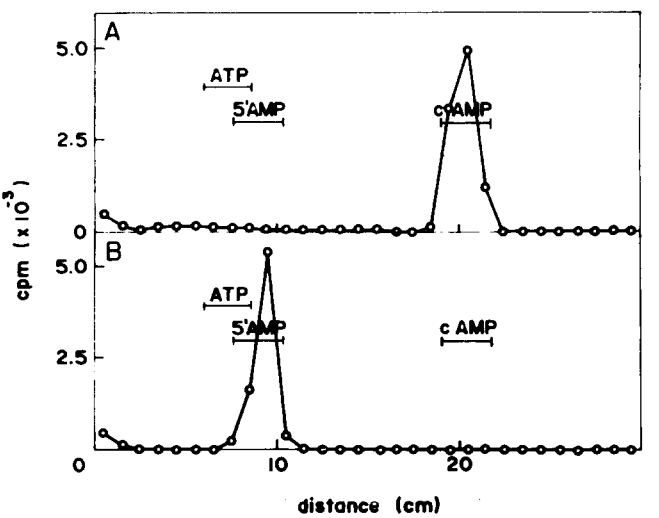

Fig. 2. Paper chromatography of the reaction product of the *T. cruzi* adenylyl cyclase. Details are given in Materials and Methods. (A) Reaction product after sequential chromatography on neutral alumina and Dowex 50 W X-8 (H^+) columns. (B) Reaction product after treatment with bovine heart cyclic nucleotide phosphodiesterase. Descending chromatography was performed on Whatman No. 1 paper, using isopropanol/ammonia/water (7 : 2 : 1) as solvent system. Radioactivity was determined by counting strips of dry paper in a liquid scintillation counter. Recovery of radioactivity in both cases was 95—97%.

the radioactivity obtained in the Dowex column eluate co-chromatographed with authentic cyclic AMP. When the reaction product was incubated with bovine heart cyclic nucleotide phosphodiesterase, 97% of the radioactivity migrated as 5′-AMP (Fig. 2B).

Stability of the adenylyl cyclase. The enzyme preparation could be maintained for several hours at 0—4°C, kept frozen in the presence of 10 mM Tris · HCl, pH 7.5, or maintained at −20°C for 10 days without loss of the original activity. The enzyme activity was also preserved when the preparation was subjected to 5 cycles of freezing and thawing over a period of 10 days. Despite this stability, the majority of the studies presented here were performed with fresh preparations.

Effect of ions added during cell rupture on the adenylyl cyclase activity. We have found that the presence of certain divalent cations (Mn^{2+} or Mg^{2+}) during cell rupture yields particles 3—4 times more active than those obtained in the absence of ions (Fig. 3). This activation was paralleled by an extensive aggregation of the particulate material (as observed by phase contrast microscopy). Particle aggregation and activation of adenylyl cyclase were not reversed by washing the particulate fraction with 10 mM Tris · HCl, pH 7.5. Fig. 3A shows the activation produced by the presence of 10 mM $MnCl_2$ during sonication. The enzyme retained some activity even when no Mn^{2+} was added to the assay mixture. In particulate preparations obtained in the absence of added ions, but preincubated with 10 mM $MnCl_2$ (15 min, 4°C, 2 mg/ml protein concentration) before assay, the enzyme was activated to a level intermediate between the lev-

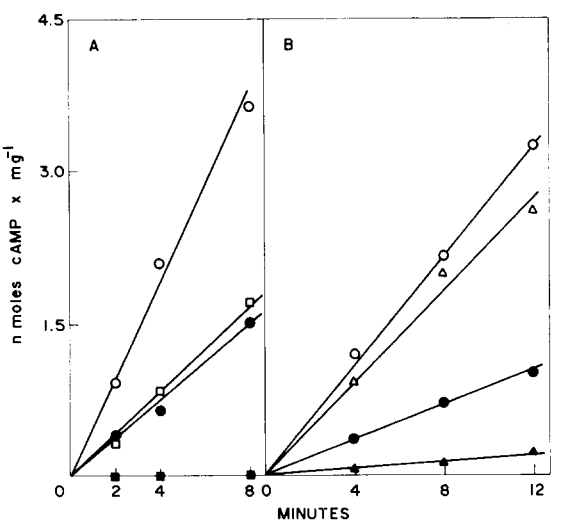

Fig. 3. Effect of ions added during cell rupture on the adenylyl cyclase. Cells were sonicated in the presence or absence of divalent cation as described. The resulting 105 000 × *g* precipitate was washed with 10 mM Tris · HCl, pH 7.5, prior to use. The assay conditions are described under Materials and Methods. In all experiments the enzymatic assays were performed in the absence or in the presence of 2 mM $MnCl_2$ or 2 mM $MgCl_2$, as specified. (A) Cells sonicated in the presence (○, ●) or in the absence (□, ■) of 10 mM $MnCl_2$ and assayed in the presence (○, □) or in the absence (●, ■) of Mn^{2+}. (B) Particles prepared by sonication in the presence of 10 mM $MnCl_2$ and assayed in the presence of Mn^{2+} (○) or Mg^{2+} (●); particles prepared in the presence of 10 mM $MgCl_2$ and assayed in the presence of Mn^{2+} (△) or Mg^{2+} (▲). cAMP, cyclic AMP.

els found with cells broken in the presence or absence of divalent cations. Addition of EDTA to these preincubated preparations reverted enzyme activity to the basal level. These changes in enzyme activity were accompanied by aggregation (Mn^{2+})-disaggregation (EDTA) transitions, which could be followed by phase contrast microscopy (not shown).

Control experiments demonstrated that the adenylyl cyclase activity increased proportionally with the concentration of $MnCl_2$ added to the sonication medium. Saturation tended to occur around 10 mM Mn^{2+}. Similar results were obtained with Mg^{2+} but not with Ca^{2+}. Fig. 3B depicts adenylyl cyclase activity in particulate preparations from cells broken in the presence of 10 mM Mg^{2+} or Mn^{2+} and assayed in the presence of 2 mM Mn^{2+} or Mg^{2+}. From the results it can be concluded that particulate preparations from cells ruptured in the presence of either ion were equally and maximally activated when assayed in the presence of 2 mM Mn^{2+}. The enzyme from cells broken in the presence of Mn^{2+} had some activity (20—30% of the maximal activity) when assayed in the presence of Mg^{2+}. This result suggests that, even after washing, some Mn^{2+} remained bound to the membranes. On the other hand, the enzyme prepared from cells broken in the presence of Mg^{2+} showed negligible or no detectable activity when assayed under the same condition.

In order to rule out the possibility that a specific binding of Mg^{2+} to a putative activating site was responsible for the above results, the experiment in Table II was performed. The particles were prepared in the absence of added ions during cell sonication and were assayed in the presence of 1 mM ATP and 1 mM $MnCl_2$. It can be seen that the addition of Mg^{2+} up to 5 mM did not stimulate cyclase activity while increasing Mn^{2+} up to 3 mM resulted in a 30% stimulation above the control level.

Effect of substrate concentration. The following experiments were performed with washed particulate preparations from cells which had been broken in Tris · HCl, pH 7.5, containing 10 mM $MgCl_2$. The effect of variable ATP concentrations on enzyme activity was studied in the presence of several fixed Mn^{2+} concentrations (Fig. 4). It is apparent that high ATP concentrations result

TABLE II

EFFECT OF Mg^{2+} AND Mn^{2+} IN EXCESS OF ATP : Mn (1 : 1) ADDED TO THE ASSAY MIXTURE IN PARTICLES PREPARED IN THE ABSENCE OF CATIONS

Epimastigote forms of *T. cruzi* were sonicated for 15 s in 10 mM Tris · HCl, pH 7.5 in the absence of cations. The enzyme preparation (50 μg) was incubated for 8 min at 30°C as described in Materials and Methods. The concentrations of ATP and Mn^{2+} in the control were 1 mM each. Concentrations of excess cation were as indicated.

Concentration of excess cation (mM)	Enzyme activity (nmol · mg^{-1} · min^{-1})	
	Mg^{2+}	Mn^{2+}
0	0.31	0.31
0.2	—	0.33
0.5	0.32	0.37
1.0	0.31	0.39
2.0	0.31	0.41
5.0	0.31	—

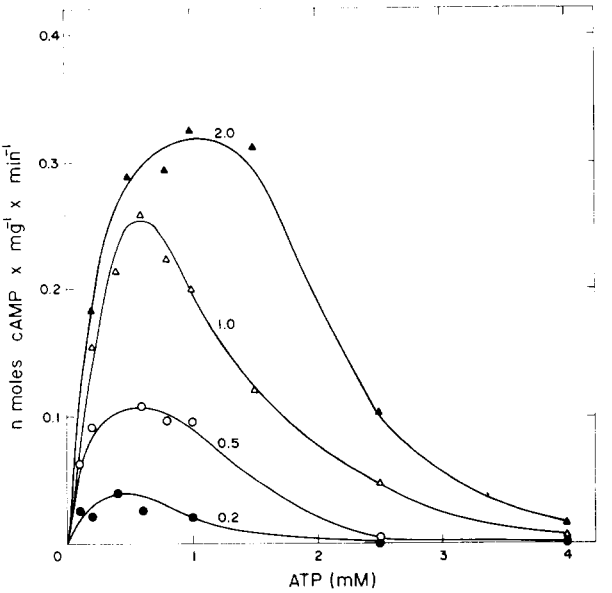

Fig. 4. Effect of ATP at several fixed Mn^{2+} concentrations on adenylyl cyclase activity. Assay conditions were as described under Materials and Methods. Numbers on top of curves refer to the concentration of Mn^{2+} (mM) in each set of experiments. cAMP, cyclic AMP.

in inhibition of the activity. Furthermore, the onset of this inhibition appears to depend on the Mn^{2+} concentration. Since one Mn^{2+} is bound to ATP at the pH of the reaction medium [21], it can be concluded that the substrate for the enzyme is $Mn \cdot ATP^{2-}$ and the free ATP is inhibitory.

The effect of increasing amounts of Mn^{2+} added to the incubation medium at a fixed concentration of ATP (1 mM) is shown in Fig. 5. Saturation of the

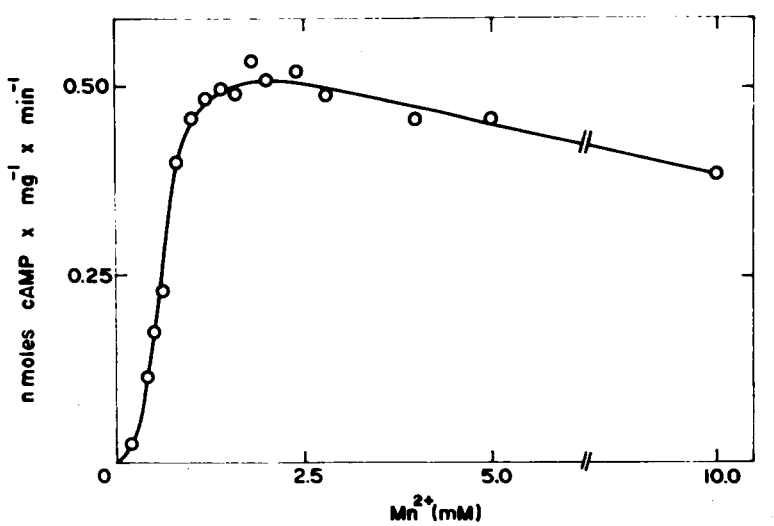

Fig. 5. Effect of Mn^{2+} on adenylyl cyclase activity. Assay conditions were as described under Materials and Methods. The concentration of ATP used was 1 mM. cAMP, cyclic AMP.

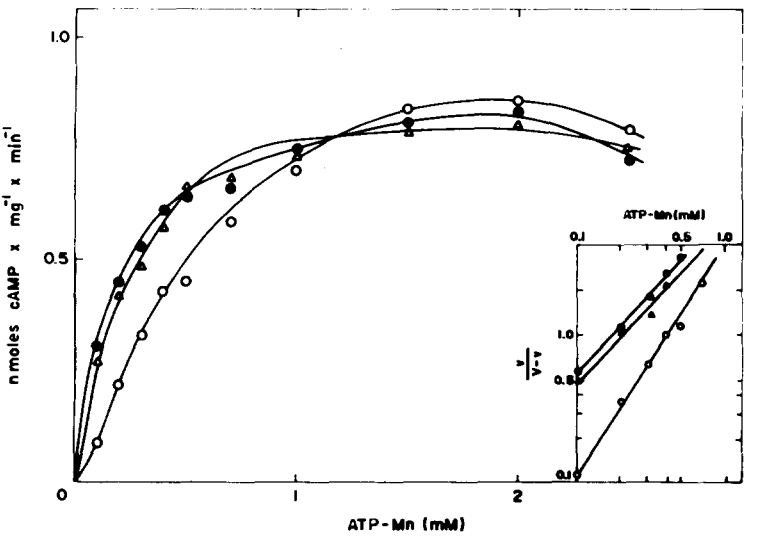

Fig. 6. Adenylyl cyclase activity as a function of ATP : Mn concentration. The incubation medium contained the concentrations of ATP and $MnCl_2$ indicated on the abscissa (○). Excess Mn^{2+} for each experimental point was 1 mM (△) and 2.5 mM (●). Inset: Hill plots. cAMP, cyclic AMP.

enzyme was achieved at concentrations around 2 mM. Excess cation (10 mM) seems to be slightly inhibitory (20%). From this experiment the activation constant for Mn^{2+} was calculated to be approximately 0.6 mM. Hill plots [22] of experiments similar to that described in Fig. 6 gave values between 2.0—2.5. This result indicates that the saturation kinetics for Mn^{2+} activation do not follow a Michaelian pattern. The same observation can be made under conditions of increasing concentrations of ATP with respect to those of Mn^{2+}, as it becomes apparent when the data in Fig. 4 are replotted in terms of Mn^{2+} concentrations for several fixed amounts of ATP (not shown). This was taken as an indication that Mn^{2+} activation depends on the ATP concentration.

Fig. 6 shows a plot of enzymatic activity versus concentration of the active complex ATP · Mn (1 : 1). The curve does not show a typical Michaelian behavior. Hill plots in such experiments gave n values ranging between 1.5 and 2.0 (Table III). Addition of excess Mn^{2+} led to a modification of the enzyme kinetics. It can be seen that the reaction rate increased at low, but not at high, concentrations of ATP · Mn. The curves, however, reached the same maximum velocity. The enzyme kinetics tended to a Michaelian pattern at increasing Mn^{2+} concentrations. Under these conditions the Hill plots gave n values near to 1.0. It could be calculated that for each concentration of ATP : Mn (1 : 1) the addition of 1 mM Mn^{2+} in excess transforms approximately 97% of the ATP to the productive Mn · ATP^{2-} complex (the constant used was log K_{eq}^{M} = 4.5) [21]. The addition of a 2.5 mM excess of Mn^{2+} did not stimulate velocities further. This result further suggests that stimulation by excess Mn^{2+} over the added equimolar concentration of ATP and Mn^{2+} is dependent on ATP concentration. Table III also shows that, in the presence of excess Mn^{2+}, the substrate concentration at which the velocity is one-half of the maximum ($L_{0.5}$) decreases fron an average of 0.4 mM to 0.2 mM.

TABLE III

KINETIC PARAMETERS OF ADENYLYL CYCLASE FROM *T. CRUZI*

The enzymatic activity was measured as a function of equimolar concentrations of ATP and Mn^{2+} in the assay mixture as described under Materials and Methods. The reaction was carried out at 30°C, for 8 min, using 40 μg of the enzyme preparation. The excess of Mn^{2+} in these experiments was as indicated. $L_{0.5}$ and n values (Hill plots) were calculated from the kinetic curves.

Expt. No.	Excess Mn^{2+} (mM)	$L_{0.5}$ (mM)	n
1	—	0.50	1.5
2	—	0.50	2.0
3	—	0.45	1.6
4	—	0.40	1.5
5	1.0	0.20	1.1
6	2.5	0.20	1.0
7	2.5	0.20	1.0

Discussion

The specific substrate for the *T. cruzi* adenylyl cyclase seems to be the manganese salt of ATP since comparable activities could not be attained upon substitution of this ion by any other divalent cation tested. Contrary to what has been found for higher eukaryotes where Mg · ATP^{2-} is the substrate for the adenylyl cyclase, the enzymes of several lower eukaryotes require Mn^{2+} for maximal activity. This seems to be the ion of choice for the enzymes from the fungi *Neurospora crassa* [23], *Mucor rouxii* [24] and *Blastocladiella emersonii* (Lopes Gomes, S. and Maia, J.C.C., personal communication). The maximum activities of such enzymes are of the same order of magnitude as those reported here for the *T. cruzi* enzyme (V = 0.1—0.8 nmol/mg per min). These levels are also similar to the activities shown by the enzymes from higher eukaryotes when fully activated [25]. However, they are 10—50 times higher than those found for the enzymes from several lower eukaryotes [4,6,26—29]. Except for the nuclear enzyme from *Physarum polycephalum* [29], which apparently utilizes either Mg^{2+} or Mn^{2+}, it is interesting to note that these latter enzymes have been reported to be "Mg-enzymes". Since in these papers no explicit reference has been made with regard to the effects of other ions on enzyme activity, it is possible that, at least in some of them, the reported activities might have been underestimated.

Taking into consideration that, at the pH of the reaction, the concentrations of ATP and Mn^{2+} used could only have led to the formation of a binary complex [21], it was concluded that Mn · ATP^{2-} is the true substrate for the *T. cruzi* enzyme. At an ATP concentration of 1 mM, 10 mM Mn^{2+} inhibited the enzymatic reaction by 20%, suggesting the possibility of an inhibition by either the ternary complex Mn_2 · ATP or by free Mn^{2+} itself.

Concentrations of ATP exceeding those of Mn^{2+} resulted in inhibition of the activity. De Haën [30] has attributed similar inhibitory action of adenylyl cyclase from other systems to free ATP (ATP^{4-}), the binding constants of which are 100-fold higher than those of the productive cation-substrate complex. Although agreeing with De Haën's kinetic analysis, Lin et al. [31] concluded that the most important inhibitory species which accumulates when

ATP exceeds cation concentration is most probably the protonated form $ATPH^{3-}$ rather than ATP^{4-}. Accordingly, a partial inhibition of the enzyme should occur even when the substrate system contains very low concentrations of unchelated ATP. A calculation of this species present when 1 mM Mn^{2+} and 1 mM ATP are added indicates that 80% of the ATP is in the form of the binary complex [21]. In the presence of 2 mM Mn^{2+} and 1 mM ATP, 97% of the ATP is chelated as $Mn \cdot ATP^{2-}$. Therefore, the inhibitory action of unchelated forms of ATP might explain the non-Michaelian behavior of the enzyme at low Mn^{2+} concentrations (Figs. 5 and 6). Indeed, at equimolar concentrations of Mn^{2+} and ATP, Hill plots gave n values ranging between 1.5 and 2.0. Addition of Mn^{2+} in excess up to 2.5 mM brought n values down to 1.0 (Fig. 6 and Table III).

Although these effects might conceivably be due to an allosteric action of Mn^{2+} at a putative regulatory site (see refs. 32 and 33 for the alternative explanation) we do not favor this possibility since the Mn^{2+} activation should have been independent of the ATP concentration. This possibility is further rendered unlikely by the fact that at any given ATP concentration, the amount of additional Mn^{2+} necessary to change the sigmoidal kinetics to Michaelian kinetics was that which should theoretically transform 95—97% of the ATP present into the productive $Mn \cdot ATP^{2-}$ complex.

The effect of Mg^{2+} or Mn^{2+} in enzyme activation when these cations are present in the sonication medium cannot be explained by supposing that they remain bound to specific activating sites of the enzyme. In fact, Mg^{2+} was unable to promote enzyme catalysis or to enhance cyclase activity in the presence of saturating concentrations of Mn^{2+}. Moreover, Mg^{2+} was incapable of stimulating the activity of particles prepared in the absence of cations. However, the activating effects of Mg^{2+} were similar to those observed with Mn^{2+} when the particles were prepared in the presence of either ion. The fact that this activation was abolished by EDTA suggests that, even after washing, some ion remained bound to the particles. Therefore, such activation effects are distinct from true catalytic effects since Mg^{2+} or Mn^{2+} produce the same results at the level of particle aggregation and of stimulation of enzyme activity. This activation might result from transformations at the level of membrane conformation. Assuming that adenylyl cyclase is an enzyme located at one side of the membrane [34—36], it is conceivable that the binding of ions to membrane phospholipids brings about conformational changes in the membrane vesicles which favor substrate access to the enzyme.

In view of the pleiotropic effects which have been attributed to cyclic AMP [37—40] the presence of a highly active adenylyl cyclase in *T. cruzi* raises the intriguing question as to the role of this nucleotide in this pathogenic protozoon. It may be significant to note, in this context, that *T. cruzi* does not store glycogen or other reserve polysaccharides [41]. Thus, regulation of the adenylyl cyclase activity might play an important role in the regulation of other metabolic routes. The absolute requirement for Mn^{2+} and the inhibition of the *T. cruzi* adenylyl cyclase by unchelated forms of ATP could be one of such regulatory mechanisms since activity would depend on intracellular ATP/Mn^{2+} ratios.

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