DEMONSTRATION OF A Mg^{2+}-ACTIVATED ADENOSINE TRIPHOSPHATASE IN *TRYPANOSOMA CRUZI*

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1. Introduction

The Mg^{2+}-activated ATPase (ATP-phosphohydrolase, EC 3.6.1.4) plays a crucial role in cell metabolism because dephosphorylation of ATP is the reversal of synthesis of ATP during the process of oxidative phosphorylation [1]. In this communication is demonstrated the presence of a Mg^{2+}-activated ATPase in the culture (epimastigote) form of *Trypanosoma cruzi*, the agent of Chagas disease. The point is relevant because energy-yielding reactions of parasites may be susceptible targets for parasiticides [2].

2. Material and methods

2.1. *Organism*

T. cruzi (Tulahuen strain) was grown as described in [3].

2.2. *Enzyme preparation*

This was based on cell disruption, after freezing in liquid nitrogen [4]. The cells were washed in the centrifuge with buffer solution containing 0.25 M sucrose, 40 mM Tris-HCl, 1 mM EDTA, pH 7.4. The pellet was suspended in 10 vol of a hypotonic solution (6.0 mM NaCl, 1.5 mM Tris-HCl, pH 7.4), at 0°, frozen in liquid nitrogen and allowed to thaw at 1–3°. The freezing operation was performed in less than 1 min. Phase contrast microscopy showed complete disruption of epimastigotes. The homogenate was subjected to differential centrifugation. Fractions sedimenting between 200–1000 *g* (F-1; 15 min centrifugation); 1000–30,000 *g* (F-2; 30 min centrifugation); 30,000–105,000 *g* (F-3; 4 hr centrifugation) and the supernatant (F-4) were collected. The pellets were washed once in the centrifuge with 10 vol of 75 mM Tris-HCl buffer, pH 7.4 and finally resuspended in 0.1 M Tris-HCl buffer, pH 7.4. Most of the experiments were performed with F-1.

2.3. *Determination of enzymes activities*

ATPase activity was measured by the release of P_i in the presence of ATP. Unless stated otherwise the reaction mixtures contained 6.0 mM ATP, 6.0 mM $MgCl_2$ and 0.1 M Tris-HCl buffer, pH 7.2. Duplicate samples were equilibrated at 37°, the enzyme was added (final vol, 1.0 ml) and incubation was continued for 20 min (*standard experimental conditions*). The reaction was stopped by addition of TCA (final conc. 5.0% (w/v)) and after centrifugation, the supernatants were analyzed for P_i. Zero-time samples were added TCA before the enzyme. Inorganic pyrophosphatase [5], phosphomonoesterase [6], glucose-6-P phosphatase [7], phenyl-P phosphatase [8] and succinate dehydrogenase [9, ferricyanide method] were measured as described in the respective references.

Abbreviations:
In addition to standard abbreviations, the following were used: TCA, trichloracetic acid, PMB, *p*-chloromercuribenzoate; NEM, N-ethylmaleimide; GSH, reduced glutathione; DNP, 2,4-dinitrophenol; CCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluormethoxyphenylhydrazone.

2.4. *Analytical methods*

P_i [10], adenosine phosphates [11, 12] and protein concentration [13] were measured as described in the references.

2.5. *Reagents*

Nucleotides, creatine-P, creatine kinase, oligomycin, PMB, NEM, iodoacetic acid, GSH and Tris were purchased from Sigma Chemical Company. Kits for measurement of ATP, ADP and AMP, some samples of ATP and yeast hexokinase were purchased from Boehringer und Soehne, Mannheim. CCP and FCCP were gifts of Dr. P.G. Heytler, E.I. Dupont de Nemours & Co. (Inc.).

3. Results

3.1. *Distribution of ATPase in cell fractions.*

It can be seen in table 1 that ATPase activity was maximal in the particulate, low-speed sedimenting fraction F-1 while the reverse occurred with the soluble pyrophosphatase, which activity was maximal in the supernatant (F-4). Under adequate experimental conditions [ref. 6–8; in parenthesis pH at which activities were measured], F-1 and F-2 did not dephosphorylate glycerol-2-P (pH 7.5; 8.5), glucose-6-P (pH 6.5), phenyl-P (pH 9.8) or *p*-nitrophenyl-P (pH 7.8). A fairly good correlation was found between ATPase and succinate dehydrogenase activities in fractions (table 1).

Table 1
Distribution of enzyme activities in *T. cruzi* fractions.

Fraction	Enzyme activities ATPase	Pyrophosphatase	Succinate dehydrogenase
F-1	0.48[a]	0.08[a]	0.33[b]
F-2	0.33	0.10	0.21
F-3	0.10	0.40	0.12
F-4	0.15	0.37	–

[a] P_i formed (μmole per min per mg protein).
[b] Succinate oxidized (μmole per min per mg protein).

Fractions were prepared and enzyme activities were measured as described under Methods. Samples contained 50–100 μg protein.

Table 2
Substrate specificity.

Expt.	Nucleotide (6 mM) and additions	P_i formation (μmole/min/mg protein)
A	ATP	0.27 (100)[a]
	GTP	0.12 (43)
B	ATP	0.14 (100)
	ATP+glu+hex	0.01 (7)
	UTP, CTP or ITP	0.06 (43)
	UTP (or ITP)+glu+hex	0.02 (14)
	CTP+glu+hex	0.03 (21)
C	ATP	0.31 (100)
	ADP	0.04 (13)
	AMP	0.01 (3)
	ATP+ADP (3.0 mM)	0.13 (58)

[a] Percentage of maximal activity

Standard experimental conditions, except nucleotide. 40 μg (expt. A and C) or 60 μg of ATPase preparation (expt. B). Where indicated glu and hex, glucose (50 mM) and hexokinase (16 IU) were added to the reaction mixture.

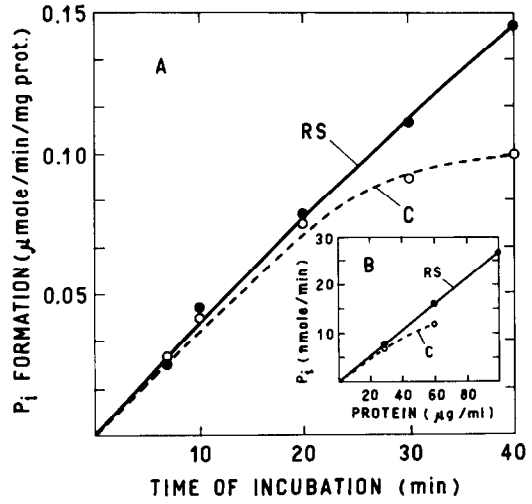

Fig. 1. A) Effect of time of incubation on the rate of ATP hydrolysis. Standard experimental conditions; 70 μg of ATPase preparation (F-1). Incubation for the time stated in the abscissa. *Inset* (fig. 1B). Effect of ATPase concentration on the rate of ATP hydrolysis. Standard experimental conditions. ATPase as stated in the abscissa. Where indicated RS (ATP-regenerating system), samples contained 1.0 mM creatine-P and 6 μg of creatine kinase. C, control samples.

3.2. *Effect of time of incubation and enzyme concentration*

Fig. 1A shows the linear relationship between time of incubation and hydrolysis of ATP, whenever the creatine kinase system (an ATP-regenerating or ADP-trapping system [14]) was added to the reaction mixture. The same occurred with respect to the effect of the enzyme concentration (fig. 1B). In the subsequent paragraph the enzyme inhibition by ADP is demonstrated, which gives account of the effect of the creatine kinase system.

3.3. *Substrate specificity*

It can be seen in table 2 that the enzyme selectively split ATP since the rate of P_i liberation from other nucleotides was significantly less than with ATP. The addition of the hexokinase system, which competed for ATP [15], further reduced the rate of P_i formation from UTP, CTP and ITP suggesting that the hydrolysis of the latter nucleotides occurred in part through the intermediary formation of ATP. ADP and AMP were hydrolysed with rates equivalent to 13 and 3%, respectively, of the rate of hydrolysis of ATP (table 2). Despite the low activity with ADP as substrate, ADP effectively interacted with ATPase as proved by the enzyme inhibition (table 2).

Table 3
Stoichiometry of the ATPase reaction.

ATPase (μg of protein)	Consumption of ATP	Formation of P_i	ADP	AMP
35	0.05[a]	0.06[a]	0.05[a]	0.01
60	–	0.11	0.10	0.01
100	0.19	0.18	0.14	0.01

[a] The figures represent μmole of substrate (or reaction product) consumed (or released) after 20 min incubation and include zero-time values.

Standard experimental conditions. After incubation samples of reaction mixture were analyzed for P_i and adenosine phosphates, as described under Methods.

3.4. *Stoichiometry of ATP hydrolysis*

The enzyme selectively cleaved the terminal phosphate group of ATP (table 3) and accordingly, for 1 mole of ATP that disappeared, 1 mole of P_i and 1 mole of ADP were formed.

Table 4
Effect of cations on ATPase activity.

Cation	P_i formation (μmole/min) / mg prot.
None	0
Mg^{2+}	0.43 (100)[a]
Mn^{2+}	0.38 (88)
Co^{2+}	0.17 (38)
Cd^{2+}	0.13 (29)
Fe^{2+}	0.12 (28)
Zn^{2+}	0.08 (19)
Ca^{2+}	0.07 (17)
Ba^{2+}	0 (0)
Mg^{2+} (12 mM)	0.28 (65)
$Mg^{2+} + Ca^{2+}$	0.16 (43)
$Mg^{2+} + Mn^{2+}$	0.17 (39)

[a] Percentage of maximal activity.

Standard experimental conditions except the cation, which is indicated; 50 μg of ATPase preparation. Cations were added as chloride. Unless stated otherwise, the cation concentration was 6 mM.

3.5. *Requirement of metal ions*

In the absence of metal ions the enzyme was practically inactive (table 4). Addition of divalent cations stimulated the hydrolysis of ATP, with Mg^{2+} and Mn^{2+} being the more active. Co^{2+}, Cd^{2+}, Fe^{2+} and Zn^{2+} could substitute for Mg^{2+} (or Mn^{2+}), in decreasing order of effectiveness. The enzyme was scarcely activated by Ca^{2+} and conversely, in the presence of Mg^{2+} ions, Ca^{2+} inhibited ATPase activity. A similar inhibition was caused by Mn^{2+} (table 4). Fig. 2 shows the relationship between Ca^{2+} concentration and ATPase inhibition. The inhibitory effect of Ca^{2+} clearly differentiated the *T. cruzi* enzyme from myosin ATPase that is activated by Ca^{2+} [16]. On the other hand, the enzyme activity was not affected by addition of 12 mM NaCl, 6 mM KCl or 100 mM NaCl plus 20 mM KCl which contrasts with the activating effect of those cations on the $(Na^+ + K^+)$-dependent ATPases [17].

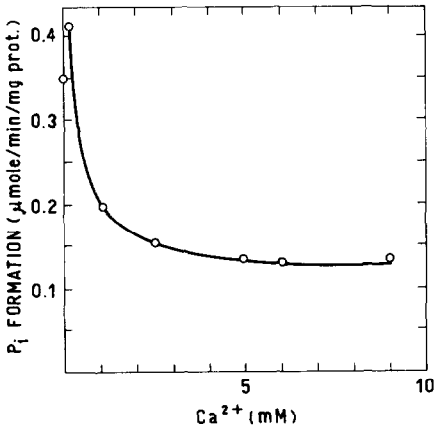

Fig. 2. Inhibition of ATPase activity by Ca^{2+} ions. Standard experimental conditions. 49 μg of ATPase preparation; $CaCl_2$ concentration as stated in the abscissa.

3.6. *Effect of substrate concentration*

When the concentrations of ATP and Mg^{2+} were simultaneously varied, the rate of hydrolysis of ATP followed Michaelian kinetics. This is shown by the double reciprocal plot in fig. 3. The apparent K_m was 2.0 mM, in good agreement with the value reported for the liver mitochondria ATPase [18].

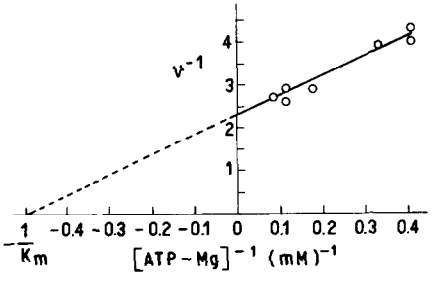

Fig. 3. Effect of substrate concentration on ATPase activity. Double-reciprocal plot. ATP–Mg concentration as stated in the abscissa. 40 μg of ATPase preparation. Other experimental conditions were as stated under Methods. The ATP–Mg substrate was prepared by mixing ATP–Tris and $MgCl_2$ in the stoichiometric proportion. v is expressed as μmole of P_i formed per min per ml of reaction mixture.

3.7. *Optimal pH*

In standard experimental conditions (except pH, that was varied from 5.5 to 8.8), maximal activity was at pH 7.2. A similar value was reported for liver ATPase in the presence of 10 mM Mg^{2+} [19].

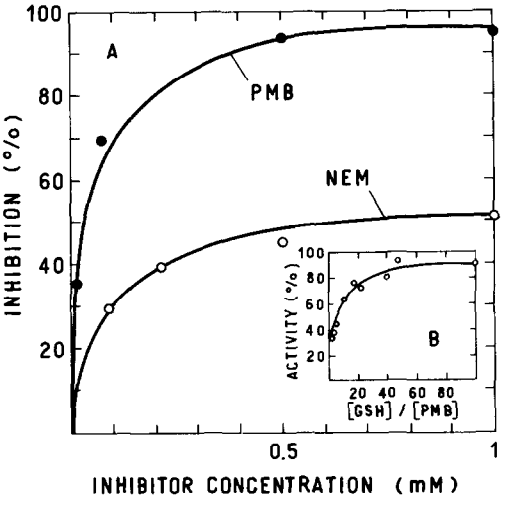

Fig. 4. A) ATPase inhibition by PMB and NEM. Standard experimental conditions; 40 μg of ATPase preparation; inhibitor concentration as stated in the abscissa. The enzyme was preincubated for 4 min with the SH-reagent before the addition of ATP, which was used to initiate the enzyme reaction. *Inset* (fig. 4B). Reversal of PMB inhibition by GSH. ATPase (40 μg protein) in 6.0 mM $MgCl_2$, 0.1 M Tris-HCl buffer, pH 7.2 was preincubated at 0° with 0.5 mM PMB for 4 min. GSH was added (concentration as stated in the abscissa) and after further 5 min the reaction mixture was completed with ATP.

3.8. *Effect of inhibitors*

In standard experimental conditions, the effect of inhibitors was as follows (in parenthesis, substance concentration and ATPase inhibition (%)). Oligomycin (2.5 μg/ml, 31); iodoacetic acid (0.5 mM, 50), sodium fluoride (1.0 mM, 35) and sodium azide (1.0 mM, 58). Iodoacetic acid was preincubated with the enzyme for 4 min before measuring activity. Fig. 4A shows the effect of increasing concentrations of PMB and NEM and fig. 4B shows the almost complete reversibility of PMB inhibition by GSH. Mg^{2+} (6 mM) and the 6 mM ATP–Mg mixture prevented by 50% the inhibition by PMB. Ouabain (2.5 mM) did not affect the enzyme activity (ouabain was assayed in the presence of 100 mM NaCl, 5.0 mM KCl and 6 mM $MgCl_2$).

3.9. *Effect of uncouplers*

In the absence of Mg^{2+}, DNP and CCP significantly stimulated the ATPase activity while in the

Table 5
Effect of uncouplers on ATPase activity.

Expt.	Uncoupler (mM)	P_i formation (μmole/min/mg protein)	
		$MgCl_2$ omitted	$MgCl_2$ added
A	None	0.01	0.32
	DNP (0.10)	0.06	0.30
	CCP (0.01)	0.03	0.31
B	None	–	0.33
	CCP (0.05)	–	0.22 (33)[a]
	FCCP (0.05)	–	0.24 (27)

[a] Percentage inhibition

Standard experimental conditions. 95 (expt. A) or 40 (B) μg of ATPase preparation. Where indicated, 6 mM $MgCl_2$.

presence of Mg^{2+}, the same agents and FCCP did not stimulate and eventually inhibited the hydrolysis of ATP (table 5). Despite the addition of uncouplers, the enzyme activity was very small in the absence of metal ion activator.

4. Discussion

Like mammalian and yeast mitochondrial ATPases [18–22], the *T. cruzi* ATPase, (a) was associated with low-speed sedimenting particles (table 1); (b) split selectively the terminal phosphate group of ATP (tables 2 and 3); (c) was more active with ATP than with other nucleoside phosphates, and (d) was inhibited by ADP (table 2 and fig. 1). Moreover, (e) Mg^{2+} and Mn^{2+} were essential activators; (f) Ca^{2+} and Mn^{2+} inhibited the Mg^{2+}-activated enzyme (table 4 and fig. 2); (g) fluoride, azide and oligomycin were enzyme inhibitors; and (h) free thiol groups were essential for activity (fig. 4). The presence of succinate dehydrogenase in the more active ATPase fractions (table 1) was consistent with the association of ATPase with mitochondrial membranes. On the other hand, the *T. cruzi* ATPase could be distinguished from myosin ATPase [16] and ($Na^+ + K^+$)-dependent ATPases [17] on the basis of the selective requirements for metal activators and substrate specificity. Similarly, the *T. cruzi* enzyme could be distinguished from flagellar ATPases, since dynein, the enzyme isolated from sea urchin sperm [23], (a) does not sediment at 35,000 *g*; (b) is effectively activated by Ca^{2+}, and (c) is inhibited by excess of substrate. These properties were not shared by the *T. cruzi* enzyme (tables 1 and 4; figs. 2 and 3). Nevertheless, our observations do not rule out the presence of other ATPases in *T. cruzi*.

A special comment deserves the inhibitory effects of CCP and FCCP in table 5. Since inhibition of mitochondrial ATPase by uncouplers can occur after physical alteration of mitochondrial membranes [24], the effects of CCP and FCCP are consistent with the view that the measured enzyme was a mitochondrial ATPase. This assumption agrees with the drastic nature of the procedure employed to disrupt the cells, that in all probability strongly affected the parasite mitochondrion. With intact mitochondria uncouplers elicit latent ATPase activity, even in the presence of Mg^{2+} [24, 25].

On account of well known difficulties [26] we have not been able to isolate mitochondria (or phosphorylating particles) from *T. cruzi*. With trypanosomatidae, only mitochondria from *Crithidia fasciculata* have shown *in vitro* phosphorylation capacity and respiratory control ([27]; J.P. Kusel, personal communication).

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References

[1] E. Racker, Mechanisms in Bioenergetics (Academic Press, New York and London, 1965) p. 129.
[2] G.C. Hill, Exptl. Parasitol. 28 (1970) 356.
[3] J.F. de Boiso and A.O.M. Stoppani, Proc. Soc. Exptl. Biol. Med. 136 (1971) 215.
[4] A. Tzagoloff, J. Biol. Chem. 244 (1969) 5020.

[5] L.A. Heppel, in: Methods in Enzymology, Vol. II, eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York and London, 1955) p. 570.
[6] L.A. Heppel, in: Methods in Enzymology, Vol. II, eds S.P. Colowick and N.O. Kaplan (Academic Press, New York and London, 1955) p. 530.
[7] R.C. Nordlie and W.J. Arion, in: Methods in Enzymology, Vol. IX, ed. W.A. Wood (Academic Press, New York and London, 1966) p. 619.
[8] R.K. Morton, in: Methods in Enzymology, Vol. II, eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York and London, 1955) p. 556.
[9] T.E. King, in: Methods in Enzymology, Vol. X, eds. R.W. Estabrook and M.E. Pullman (Academic Press, New York and London, 1967) p. 322.
[10] C. Fiske and Y. Subbarow, J. Biol. Chem. 81 (1929) 629;
J.B. Sumner, Science 100 (1944) 413.
[11] H. Adam, in: Methods of Enzymatic Analysis, ed. H-U. Bergmayer (Academic Press, New York and London, 1963) p. 539.
[12] H. Adam, in: Methods of Enzymatic Analysis, ed. H-U. Bergmayer (Academic Press, New York and London, 1963) p. 573.
[13] L. Szarkowska and M. Klingenberg, Biochem. Z. 338 (1963) 674.
[14] S. Gatt and E. Racker, J. Biol. Chem. 234 (1959) 1015.
[15] B. Sacktor and D.G. Cochran, J. Biol. Chem. 226 (1957) 241.
[16] V.A. Engelhardt, Adv. Enzymol. 6 (1946) 147.
[17] J.C. Skou, in: Membrane Transport and Metabolism, eds. A. Kleinzeller and A. Kotyk (Academic Press, New York and London, 1961) p. 228;
J.D. Robinson, J. Mol. Pharmacol. 7 (1971) 238;
E. Pfeiler and L.B. Kirschner, Biochim. Biophys. Acta 282 (1972) 301.
[18] C. Cooper and A.L. Lehninger, J. Biol. Chem. 224 (1957) 547.
[19] W.W. Kielley and R.W. Kielley, J. Biol. Chem. 200 (1953) 213.
[20] M.E. Pullman, H.S. Penefsky, A. Datta and E. Racker, J. Biol. Chem. 235 (1960) 3322.
[21] C. Cooper, Biochim. Biophys. Acta 30 (1958) 529.
[22] A. Bennun, E.M. de Pahn and A.O.M. Stoppani, Biochim. Biophys. Acta 89 (1964) 532.
[23] M. Hayashi and S. Higashi-Fujime, Biochemistry 11 (1972) 2977.
[24] E.C. Weinbach, J. Biol. Chem. 221 (1956) 609.
[25] D.D. Tyler and J. Gonze, in: Methods in Enzymology, Vol. X, eds. R.W. Estabrook and M.E. Pullman (Academic Press, New York and London, 1967) p. 75.
[26] S.K. Ray and G.A.M. Cross, Nature New Biology 237 (1972) 174.
[27] J.J. Toner and M.M. Weber, Biochim. Biophys. Res. Commun. 28 (1967) 821;
G.C. Hill and D.C. White, J. Bact. 95 (1968) 2151;
C.J. Bacchi, S.H. Hutner, E.J. Ciaccio and S.M. Marcus, J. Protozool. 15 (1968) 576.