Adenylate Cyclase in Bloodstream Forms of *Trypanosoma* (*Trypanozoon*) *brucei* sp.

By B. RICHARD MARTIN, H. PAUL VOORHEIS and EDWINA L. KENNEDY
*Department of Biochemistry, University of Cambridge, Tennis Court Road,
Cambridge CB2 1QW, U.K.*

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1. The adenylate cyclase in *Trypanosoma brucei* is located in the plasma membrane. 2. A partial kinetic analysis of the properties of the enzyme revealed a K_m for ATP of 1.75 mM and a K_m for Mg^{2+} of 4 mM. 3. At low concentrations, Mg^{2+} activated the enzyme directly in addition to its effect of lowering the concentration of inhibitory free ATP species. 4. At high concentrations, Mg^{2+} inhibited the enzyme. Furthermore, the enzyme was inhibited at any Mg^{2+} concentration if the concentration of ATP exceeded that of Mg^{2+} . 5. The opposing effects of Mg^{2+} at low and high concentrations would be consistent with more than one binding site for Mg^{2+} on the enzyme. 6. A study of the patterns of product inhibition revealed little or no effect of 3':5'-cyclic AMP, but a profound inhibition by pyrophosphate, which was competitive with respect to ATP (K_i 0.135 mM). This result suggests that the substrate-binding domain on *T. brucei* adenylate cyclase interacts mainly with the triphosphate portion of the ATP molecule. 7. The enzyme activity was unaffected by the usual mammalian enzyme effectors glucagon, adrenaline, adenosine, GTP and guanyl-5'-yl imidodiphosphate. 8. The enzyme was not activated by fluoride, instead a powerful inhibition was found. The enzyme was also inhibited by relatively high concentrations of Ca^{2+} (1 mM).

Adenylate cyclase has been found in a very wide variety of organisms ranging from bacteria to mammals. It appears to occur throughout the animal kingdom and in most cases a major regulatory role has been established for the enzyme.

Extensive studies have been made in bacteria, particularly in *Escherichia coli*, where adenylate cyclase is involved in the regulation of utilization of carbon sources through catabolite repression (Pastan & Adhya, 1976). A number of systems have been studied in higher animals, where the enzyme mediates the action of many hormone messengers (Birnbaumer, 1973; Rodbell *et al.*, 1975). There have been few reports of the adenylate cyclase of protozoa and those that do exist do not contain any systematic study of the enzyme's properties (Rogensweig & Kindler, 1972; Puchwein *et al.*, 1973; Chlapowski & Butcher, 1973; Walter *et al.*, 1974). We therefore decided to examine the properties of adenylate cyclase in some detail in a protozoon. The aim of the present study was to describe the basic properties of the adenylate cyclase of *Trypanosoma brucei* to provide a foundation for further work to determine its precise regulatory role in the physiology of the organism.

Abbreviations used: p[NH]ppG, guanyl-5'-yl imidodiphosphate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

Experimental

Materials

ATP, 3':5'-cyclic AMP, p[NH]ppG and GTP were obtained from Boehringer (London), Lewes, East Sussex BN7 1LG, U.K. Phosphocreatine, creatine kinase (EC 2.7.3.2), bovine pancreas deoxyribonuclease 1 (grade 1), Tes and phenylmethanesulphonyl fluoride were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Adrenaline was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. Glucagon was a gift from Lilly Research Laboratories, Indianapolis, IN 46206, U.S.A. [^{32}P]P_i and 3':5'-cyclic [^3H]AMP were obtained from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. [α - ^{32}P]ATP was prepared as described by Martin & Voorheis (1977).

Methods

Assay of adenylate cyclase. Adenylate cyclase was assayed by the method of Salomon *et al.* (1974).

Standard conditions were as follows. Membranes (2.0–5.0 μ g of protein) were incubated at 37°C in 0.1 ml of assay medium containing 25 mM-Tris/HCl, pH 7.4, 1 mM-dithiothreitol, 0.1 mM-3':5'-cyclic AMP, 5 mM-phosphocreatine, 50 units of creatine kinase/ml and 0.5 μ Ci of [α - ^{32}P]ATP. Further additions and incubation times are specified in the legends to Figures

and Tables. The reaction was terminated by adding 0.1 ml of stopping solution [2% (w/v) sodium dodecyl sulphate/40 mM-ATP/1.4 mM-3':5'-cyclic AMP. The time course of the reaction was linear up to 20 min. Incubation times were routinely 5 or 10 min. The 3':5'-cyclic AMP formed was proportional to the membrane concentration (20–160 µg/ml).

Preparation of *T. brucei*. The source and history of the organism have been described (Voorheis, 1977). Immediately before the present study the organism was recloned by Dr. G. A. M. Cross and given the revised strain no. 427-12/ICI.060. The growth and preparation of cells from the blood of infected rats was as described by Owen & Voorheis (1976).

Preparation of *T. brucei* plasma membranes. Plasma membranes were prepared by the method of Voorheis *et al.* (1978). Cells were swollen in a low-osmotic-strength buffer (2.0 mM-Tes, 15 mM-KCl, 1.0 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride, 1.0 mM-2-mercaptoethanol, pH 7.4) and ruptured by homogenizing in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 15000g for 15 min and the pellet resuspended in 10 ml of the same buffer. Deoxyribonuclease (0.1 mg) was added and the suspension was incubated for 5 min at 25°C. The suspension was centrifuged for 15 min at 15000g and the pellet washed once. The particulate fraction was suspended in 40% (w/v) sucrose, layered on to a 40%/60% sucrose gradient and centrifuged for 3 h at 86000g. The material from the most prominent band (density 1.3 g/ml) was collected, washed twice in buffer, resuspended and stored frozen in liquid N₂ until used.

Results and Discussion

Cellular location

Table 1 shows the recovery of adenylate cyclase activity in different subcellular fractions from *T. brucei*. Plasma membranes were prepared as

Table 1. Subcellular localization of *T. brucei* adenylate cyclase

Subcellular fractions were prepared as described in the Experimental section. Incubations were for 10 min with 10 mM-MgCl₂ and 0.5 mM-ATP. The results are means ± S.E.M. for three parallel incubations.

	Total activity (nmol/min)	Activity in homogenate (%)	Specific activity (nmol/min per mg of protein)
Homogenate	415 ± 1.6	100	0.21
Supernatant	7.1 ± 0.6	1.7	0.016
Pellet	370 ± 7.0	90	0.32
Purified membranes	197 ± 4.5	47	1.45

described under 'Methods'. About 90% of the enzyme activity present in the homogenate was recovered in the 15000g pellet. Very little activity remained in the 15000g supernatant and all of this activity was sedimented by centrifugation at 100000g for 1 h. This activity was therefore probably bound to small fragments of membrane. The purified plasma-membrane preparation contained 48% of the adenylate cyclase activity and the specific activity was increased 8-fold compared with the homogenate. It seems likely that, in common with most other types of cells, the adenylate cyclase of *T. brucei* is located in the plasma membrane.

Effect of variation of [H⁺] on activity

The pH optima of most adenylate cyclases studied fall between 7 and 8 when ATP is used as substrate (Lin *et al.*, 1975). Fig. 1 shows the effect of variation of [H⁺] on the activity of adenylate cyclase in *T. brucei* plasma membranes. The enzyme has a broad optimum centred between pH 7.0 and 7.5 at a Mg²⁺ concentration of 2 mM and this shifts to a slightly higher value as the Mg²⁺ concentration is increased.

Kinetics

The substrate for adenylate cyclase from rat liver plasma membranes is MgATP²⁻. De Haen (1974)

Fig. 1. Effect of variation of [H⁺] on adenylate cyclase activity

Incubations were for 10 min in the presence of 0.5 mM-ATP and MgCl₂ (○, 2 mM; ●, 5 mM; □, 10 mM; ■, 25 mM). The pH was maintained with 50 mM-Tris/acetate buffer. The pH of reaction mixtures was checked before the assay. The results are means ± S.E.M. for three parallel incubations.

Fig. 2. Variation of adenylate cyclase activity with ATP concentration

Incubations were for 5 min at varying ATP concentration and MgCl_2 ($\circ$, 2 mM; $\bullet$, 5 mM; $\square$, 10 mM; $\blacksquare$, 15 mM). Results are means $\pm$ S.E.M. for three parallel incubations.

Fig. 3. Effect of varying Mg^{2+} concentration on adenylate cyclase activity

Incubations were for 5 min with varying MgCl_2 concentrations and ATP ($\circ$, 0.1 mM; $\bullet$, 0.3 mM; $\square$, 0.5 mM; $\blacksquare$, 1.5 mM). Results are means $\pm$ S.E.M. for three parallel incubations.

suggested that one or more of the other ATP species in solution, ATP^{4-} , ATPH^{3-} or MgATPH^- may be inhibitors. This possibility has been further developed by Rodbell and co-workers (Rodbell *et al.*, 1975; Rendell *et al.*, 1975). The adenylate cyclase from *T. brucei* plasma membranes is markedly inhibited when the concentration of ATP approaches or exceeds that of Mg^{2+} (Fig. 2). This observation is consistent with the possibility that either ATPH^{3-} or ATP^{4-} or both are inhibitors. The stability constant for the formation of MgATPH^- is such that the

Fig. 4. Variation of adenylate cyclase activity with Mg^{2+} concentration (double-reciprocal plot)

Incubations were for 5 min with varying Mg^{2+} concentration and ATP ($\circ$, 0.1 mM; $\bullet$, 0.3 mM; $\square$, 0.5 mM; $\blacksquare$, 1.5 mM). Results are means for three parallel incubations. The S.E.M. was less than 10% of the mean in all cases.

concentration of this species will be negligible under the conditions used (De Haen, 1974). It has been suggested that the inhibitory species is ATPH^{3-} (Rendell *et al.*, 1975). We have not made any extensive attempts to determine which species inhibit *T. brucei* adenylate cyclase. However, the observation that the position of the pH optimum shifts to a higher pH value with increasing Mg^{2+} concentration is inconsistent with ATPH^{3-} being the only inhibitory species. If ATPH^{3-} were the only inhibitor, the position of the pH optima would shift to a lower pH value with increasing $[\text{Mg}^{2+}]$. It appears therefore that either ATP^{4-} is the inhibitory species or that both ATPH^{3-} and ATP^{4-} are inhibitors. We are unable to distinguish between these two possibilities.

The effects of Mg^{2+} were further examined. The enzyme was activated by increasing Mg^{2+} up to 50 mM. When this concentration was exceeded there was a very slight decrease in activity (Fig. 3). This pattern is similar to that reported for rat liver plasma-membrane adenylate cyclase (Birnbauer *et al.*, 1969). Provided that the Mg^{2+} concentration was well in excess of the ATP concentration and that the Mg^{2+} concentration did not exceed 50 mM, Michaelis-Menten kinetics were observed with respect to both Mg^{2+} and ATP (Figs. 4 and 5). Under these conditions the K_m for Mg^{2+} was 4.1 ± 0.39 mM (mean $\pm$ S.E.M. for four observations). There was no variation in the

Fig. 5. Variation of adenylate cyclase activity with ATP concentration (double-reciprocal plot)

Incubations were for 5 min with varying ATP concentration and MgCl_2 (●, 5 mM; □, 10 mM; ■, 15 mM). The results were means for three parallel incubations. The S.E.M. was less than 10% of the mean in all cases.

Fig. 6. Effect of PP_i on adenylate cyclase activity: plot of $1/v$ against $1/[\text{ATP}]$

Incubations were for 5 min with varying ATP concentration, 10 mM- MgCl_2 , and PP_i (○, none; ●, 0.1 mM; □, 0.3 mM; ■, 0.7 mM; △, 1.0 mM). The results are means for three parallel incubations. The S.E.M. was less than 10% of the mean in all cases.

K_m for Mg^{2+} with varying ATP concentration (Fig. 4). This implies that Mg^{2+} activates the enzyme independently of its effect of decreasing the concentration of inhibitory species of ATP. This conclusion agrees with that suggested for adenylate cyclase from mammalian sources (Garbers & Johnson, 1975; Londos & Rodbell, 1977).

The K_m for ATP was 1.75 ± 0.122 (mean $\pm$ S.E.M. for three observations). This value is much higher than that reported for the enzyme from rat liver plasma membranes, which was found to be 0.055 mM (Lin *et al.*, 1975).

The patterns of product inhibition show considerable differences from adenylate cyclases from mammalian sources. 3':5'-Cyclic AMP is a weak inhibitor of rat liver adenylate cyclase with an apparent K_i of 10 mM (B. R. Martin, unpublished work). The rate of reaction in *T. brucei* plasma membranes was unaffected by 50 mM-3':5'-cyclic AMP and only slightly decreased by 100 mM-3':5'-cyclic AMP, whereas the ATP concentration was 0.5 mM (Table 2). Since concentrations of 3':5'-cyclic AMP in excess of 100 mM interfere with the assay used, the pattern of inhibition for this product was not determined. The second product of the reaction, PP_i is a very powerful inhibitor of *T. brucei* adenylate cyclase, competitive with respect to ATP (Fig. 6) with a K_i of 0.135 ± 0.007 mM (mean $\pm$ S.E.M. for four observations). PP_i is a comparatively weak inhibitor of adenylate cyclase in rat liver plasma membranes (Birnbauer *et al.*, 1971; Eisman *et al.*, 1974).

Table 2. Effects of mammalian adenylate cyclase effectors on *T. brucei* adenylate cyclase

Incubations were for 10 min with 10 mM- MgCl_2 , 0.5 mM-ATP and further additions as described below. The results are means $\pm$ S.E.M. for three parallel incubations.

Additions	Activity (nmol/mg of protein per 10 min)
—	16.7 ± 0.81
NaF (10 mM)	4.3 ± 0.32
GTP (10 μM)	16.4 ± 0.78
p[NH]ppG (10 μM)	15.9 ± 0.78
Glucagon (1 μM)	15.3 ± 0.35
Glucagon (1 μM), GTP (10 μM)	15.5 ± 0.99
Adrenaline (50 μM)	15.6 ± 0.78
Adrenaline (50 μM), GTP (10 μM)	15.1 ± 0.34
Glucagon (1 μM), p[NH]ppG (10 μM)	15.0 ± 1.1
CaCl_2 (100 μM)	16.2 ± 0.54
CaCl_2 (1 mM)	5.9 ± 0.6
AMP (1 mM)	13.8 ± 1.2
Adenosine (1 mM)	15.2 ± 1.1
3':5'-Cyclic AMP (2 mM)	17.4 ± 0.3
3':5'-Cyclic AMP (50 mM)	15.1 ± 0.8
3':5'-Cyclic AMP (100 mM)	9.1 ± 0.4

Response to effectors of mammalian adenylate cyclases

There are conflicting reports concerning the ability of adenylate cyclase from the free-living protozoan *Tetrahymena pyriformis* to respond to noradrenaline (Rogensweig & Kindler, 1972; Puchwein *et al.*, 1973). Since *T. brucei* is a parasite living in the bloodstream of mammals, it seemed particularly appropriate to test the possibility that it would respond to mammalian hormones. Table 2 shows the response of *T. brucei* adenylate cyclase to a number of effectors. Neither glucagon nor adrenaline had any effect on the activity of the enzyme either in the presence or absence of GTP or p[NH]ppG. Neither of these guanine nucleotides, which have been reported both to activate adenylate cyclase in higher animals and to potentiate the action of hormones (Rodbell *et al.*, 1975), had any effect. The concentrations used were in excess of those required for maximum effects in mammalian systems (Rodbell *et al.*, 1975) and in the one protozoan where such effects have been reported (Rogensweig & Kindler, 1972). This observation is noteworthy, since there are no other reports of a membrane-bound adenylate cyclase from a eukaryotic cell that does not respond to guanine nucleotides. Activation by guanine nucleotides has been shown in adenylate cyclase from rat liver, adipose tissue and adrenal, from dog heart and bovine thyroid and from erythrocytes of turkey, frog, salmon and pigeon (Harwood *et al.*, 1973; Glossman *et al.*, 1974; Lefkowitz, 1974; Spiegel &

Aurbach, 1974; Londos *et al.*, 1974; Schramm & Rodbell, 1975; Londos & Rodbell, 1975; Pfeuffer & Helmreich, 1975; Salomon *et al.*, 1975). All these higher animal adenylate cyclases respond to hormones and the response is potentiated by guanine nucleotides, whereas the enzyme from *T. brucei* does not respond to either hormones or guanine nucleotides. It is possible therefore that the capacity to respond to hormones and the capacity to respond to guanine nucleotides are in some way associated.

Adenosine has been reported to be an inhibitor of adenylate cyclase from many mammalian sources (Fain *et al.*, 1972; Weinryb & Inge, 1974; Londos & Rodbell, 1977), and to activate adenylate cyclase in others (Blume & Foster, 1975). We were unable to detect any effect of adenosine on the enzyme from *T. brucei*. Ca^{2+} is an inhibitor of the *T. brucei* enzyme, in common with its effect on adenylate cyclase from mammalian sources. Fluoride is a potent activator of adenylate cyclase from mammals at concentrations up to 20 mM, whereas at concentrations above 20 mM, inhibition is observed (Pohl *et al.*, 1971). In contrast, the *T. brucei* enzyme is inhibited by concentrations of fluoride as low as 1 mM and no activation was observed (Fig. 7). This inhibition does not conform to any simple kinetic model and requires further study.

Discussion

The main purpose of the present study was to characterize the adenylate cyclase of *T. brucei* to provide a basis for further work to determine its physiological role. In addition to this it appears that the *T. brucei* enzyme offers considerable advantages as a system in which to study the basic mechanisms of the adenylate cyclase reaction. In common with most mammalian systems, the enzyme from *T. brucei* can be studied with a relatively pure plasma-membrane preparation. Bacterial systems have been studied usually at the level of the whole cell, possibly owing to the technical difficulty of preparing plasma membranes from bacteria.

Londos & Rodbell (1977) have pointed out that a detailed kinetic analysis of adenylate cyclase from mammalian sources is very difficult. This situation is a consequence of the unusually large number of regulatory ligands, among which are GTP, which is often a contaminant of the substrate ATP (Kimura *et al.*, 1976), and adenosine, which is generated from ATP during assay incubations. The adenylate cyclase from *T. brucei* has relatively few effectors and is not affected by either adenosine or GTP. As a result, a detailed kinetic analysis is a much simpler undertaking. The study of this system should increase our understanding of the central mechanism of the adenylate cyclase reaction.

Fig. 7. Effect of fluoride on adenylate cyclase activity. Incubations were for 5 min with varying concentrations of NaF, 10 mM-MgCl₂ and ATP (○, 0.125 mM; ●, 0.5 mM; □, 1.5 mM). The results are means $\pm$ S.E.M. for three incubations.

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