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DIFFERENTIAL EFFECTS OF MANGANESE IONS ON *BLASTOCLADIELLA EMERSONII* ADENYLATE CYCLASE

SUELY LOPES GOMES and JOSÉ CARLOS DA COSTA MAIA *

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, C.P. 20780, São Paulo 05508, S.P. (Brazil)

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Summary

Adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) activity in *Blastocladiella emersonii* is associated with particulate subcellar fractions. Solubilization after treatment with detergent suggests its localization in a membrane fraction of the zoospore homogenate.

The enzyme specifically requires Mn^{2+} for activity and is not stimulated by NaF. The kinetic characteristics of substrate utilization by *B. emersonii* adenylate cyclase were investigated with various concentrations of ATP and Mn^{2+}, and in the presence of inhibitors. Plots of enzyme activity versus the actual concentration of the $MnATP^{2-}$ complex give sigmoid curves. An excess of Mn^{2+} activates the enzyme at low concentrations of substrate and leads to a modification of the enzyme kinetics. The nucleotides 5′-AMP and GTP were shown to be competitive inhibitors of the enzyme. In addition, kinetic data, obtained under conditions in which an inhibitor (ATP) is added in constant proportion to the variable substrate ($MnATP^{2-}$) concentration, produced reciprocal plots that were linear and intersecting to the right of the ordinate, and secondary replots that were hyperbolic. These kinetic patterns support a model in which: $MnATP^{2-}$ is the substrate; free Mn^{2+} is an activator at low substrate concentrations, but an inhibitor at high substrate concentrations; and free ATP is not an efficient inhibitor ($K_i > 1 \cdot 10^{-4}$ M).

* To whom correspondence should be sent.

Introduction

Many cellular responses are mediated in part by the activation of a membrane-bound enzyme adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1).

In animal cells, the enzyme system is known to include hormone-specific receptors at the cell surface, a GTP regulatory site, a divalent cation site and a catalytic site [1,2]. Although a comprehensive picture of the adenylate cyclase functions in these cells is now emerging, studies on the regulatory properties of the enzyme have few antecedents in lower eukaryotes, with the notable exceptions of *Neurospora crassa* [3], *Trypanosoma cruzi* [4] and *Dictyostelium discoideum* [5].

In *Blastocladiella emersonii*, whose life cycle is characterized by two transitional stages, germination and sporulation, the cyclic AMP seems to be involved with the control of germination. Thus, the transition of zoospore to round cell is accompanied by a rapid decrease in cyclic AMP phosphodiesterase activity [6], with concomitant, though transient, cyclic AMP accumulation [7]. Moreover, this cytodifferentiation can be induced by methylxanthines and adenine [8], competitive inhibitors of the enzyme cyclic AMP phosphodiesterase. These data suggest that germination may be mediated by cyclic AMP and that the initial stimulus that leads to zoospore germination might somehow involve the enzyme adenylate cyclase.

We have recently demonstrated that particulate subcellular fractions of *Blastocladiella* zoospore contain an adenylate cyclase activity which is developmentally regulated [9]. In contrast to the animal enzymes, the zoospore adenylate cyclase specifically requires Mn^{2+} for activity and is not stimulated by either NaF or catecholamines. In view of these peculiarities and in order to evaluate the role of cyclic AMP and related enzymes in the regulation of metabolic events which result in the differentiation of the zoospore, in the present work, we describe studies of the kinetic properties of adenylate cyclase activity in particulate preparations of zoospores of *B. emersonii*.

Materials and Methods

Cells and growth conditions. Stock cultures of *B. emersonii* were kept at 27°C on Difco agar plates. Zoospores were obtained by flooding first generation cultures grown for 15—16 h at 23°C on Difco Cantino PYG agar plates.

Enzyme preparation. Zoospores were harvested by centrifugation for 3 min at 1000 × *g*, resuspended in 100 mM Tris-HCl (pH 8.5) and broken in a French Press cell at 1000 lb/inch^2. The homogenate was centrifuged at 1000 × *g* for 30 min and the supernatant centrifuged at 105 000 × *g* for 90 min. The pellets were resuspended in 100 mM Tris-HCl (pH 8.5). All centrifugation steps were performed at 4°C. Protein concentration was determined by the method of Lowry et al. [10].

Adenylate cyclase assay. The reaction mixture consisted of: $5 \cdot 10^{-3}$—2.5 mM ATP, $6 \cdot 10^{-2}$—2.5 mM $MnCl_2$, 5 mM phosphoenolpyruvate, 0.4 mM cyclic AMP, 10 mM KCl, 10 μg pyruvate kinase, 100 mM Tris-HCl (pH 8.5), 0.8 μCi [α-^{32}P]ATP, and freshly prepared enzyme (105 000 × *g* pellet), in a final

volume of 0.1 ml. Incubation was performed at 30°C for 2.5 min and the reaction stopped by adding 0.1 ml of a solution of 40 mM unlabelled ATP and 12.5 mM cyclic AMP, followed by boiling the tubes for 2 min.

The radioactive nucleotide was isolated according to Ramachandran [11] and counted in a scintillation spectrometer. The recoveries of cyclic AMP (70—90%) were determined by addition of a known quantity of cyclic [^{3}H]-AMP to the reaction mixture. Blanks (without enzyme) were performed in every experiment; the final radioactivity in the blank never exceeded 0.01% of the total radioactivity initially added. Reported values are the average of at least three different and independent experiments.

The synthesis of cyclic AMP was linear with time up to 5 min at all ATP and Mn^{2+} concentrations tested. The use of an ATP regenerating system ensured linearity up to 10 min. The production of cyclic AMP was also proportional to enzyme concentration up to 100 μg protein.

Materials. Phosphoenolpyruvate, pyruvate kinase, adenylate kinase, 2',3'-isopropylidene adenosine, cyclic AMP, ATP, GTP and 5'-AMP were obtained from Sigma Chemical Co. Neutral aluminum oxide was purchased from Merck (Darmstadt). ^{32}P, supplied by the Instituto de Energia Atômica (São Paulo), was used for the preparation of [α-^{32}P]ATP [12]. Its purity was checked by thin-layer chromatography on precoated polyethyleneimine(PEI)-cellulose plates (Merck) [13], followed by radioautography.

Results and Discussion

Effect of Mn^{2+} and Mg^{2+} on enzyme activity

It has long been known that the enzymatic formation of cyclic AMP from ATP requires a divalent cation, which, complexed with ATP (e.g. $MeATP^{2-}$), functions as the active substrate [1]. Contrary to the findings with higher eukaryotes, where $MgATP^{2-}$ and $MnATP^{2-}$ were equally good substrates for the adenylate cyclase, the enzymes of several lower eukaryotes appear to require specifically Mn^{2+} for maximal activity [3,4]. Since it was recently demonstrated that activation of the hepatic adenylate cyclase system by Mg^{2+} requires concentrations 50—100-fold higher than those required for Mn^{2+} [2], we tested *B. emersonii* adenylate cyclase activity as a function of Mg^{2+} and Mn^{2+} concentration. Enzyme activity, assayed in the presence of several ATP (0.1—2 mM) and Mg^{2+} (1—10 mM) concentrations, always resulted in very low reaction velocities (data not shown). Even concentrations of Mg^{2+} 50—100-fold higher than those required for Mn^{2+} activation resulted in negligible activities. The specific substrate for *Blastocladiella* enzyme thus seems to be the manganese salt of ATP.

Effects of ATP and Mn^{2+} on enzyme activity

A plot of enzymatic activity as a function of the concentration of $MnATP^{2-}$ complex in the reaction mixture (calculated using an association constant of 60 300 M^{-1} [14] for ATP^{4-} and Mn^{2+}) is shown in Fig. 1. The corresponding Hill plot gives a value of 1.7 for n, which is an apparent indication of non-michaelian kinetics. Addition of Mn^{2+} in excess over ATP leads to both an activation and a modification of the enzyme kinetics. The reaction rate increases at

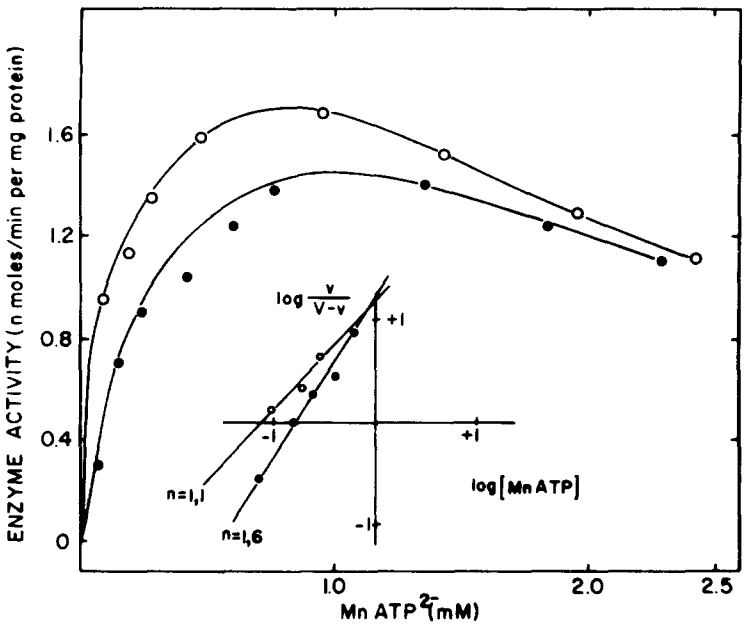

Fig. 1. Adenylate cyclase activity as a function of the calculated concentrations of $MnATP^{2-}$ complex present in the reaction mixture. Excess of added Mn^{2+} over added ATP: ●——●, none; ○——○, 0.25 mM.

low, but not at high, concentrations of substrate. The behavior of the enzyme becomes approximately michaelian ($n \simeq 1$) when the concentration of Mn^{2+} exceeds the concentration of ATP by a value of 0.25 mM. The addition of this excess of Mn^{2+} also results in a decrease in the substrate concentration necessary to reach half of the maximum velocity ($L_{0.5}$) (Table I).

The effect of increasing the concentration of added ATP, at various fixed concentrations of Mn^{2+}, is shown in Fig. 2. Following an initial rise in the reaction velocity at low ATP concentrations, enzyme activity decreased for concentrations of ATP exceeding those of Mn^{2+}. This decrease in enzyme activity can be the result of two distinct factors:

(a) The reaction requires free divalent cation, which at high concentrations of ATP, is largely chelated by the nucleotide.

(b) ATP and $MnATP^{2-}$ compete for the catalytic site. This latter possibility

TABLE I

KINETIC PARAMETERS OF ADENYLATE CYCLASE FROM *B. EMERSONII*

$L_{0.5}$, substrate concentration at which the velocity is one half of the maximum. n, Hill coefficient.

Enzyme source	Mn^{2+} in excess (mM)	$L_{0.5}$ (mM)	n
Sediment (105 000 × *g*)	—	0.16	1.6
	0.25	0.06	1.1
Supernatant (105 000 × *g*) after treatment with 1% Triton X-100	—	0.34	1.7
	0.25	0.25	1.2

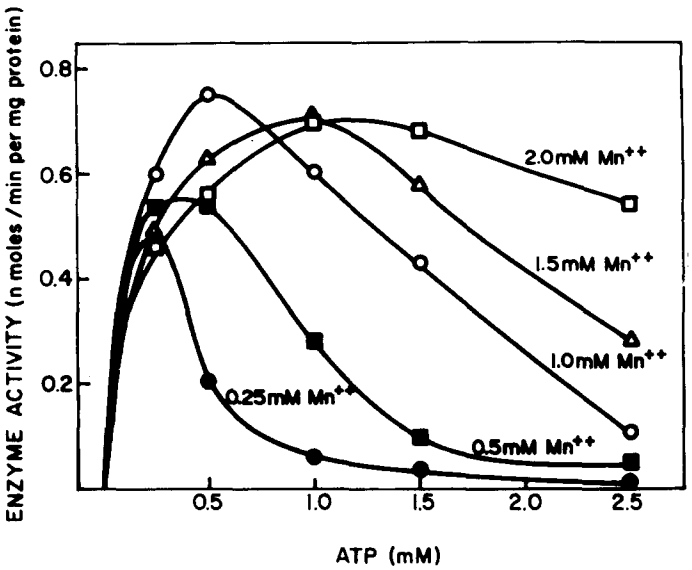

Fig. 2. Effect of various concentrations of added ATP, in the presence of different concentrations of added Mn^{2+}, on adenylate cyclase activity.

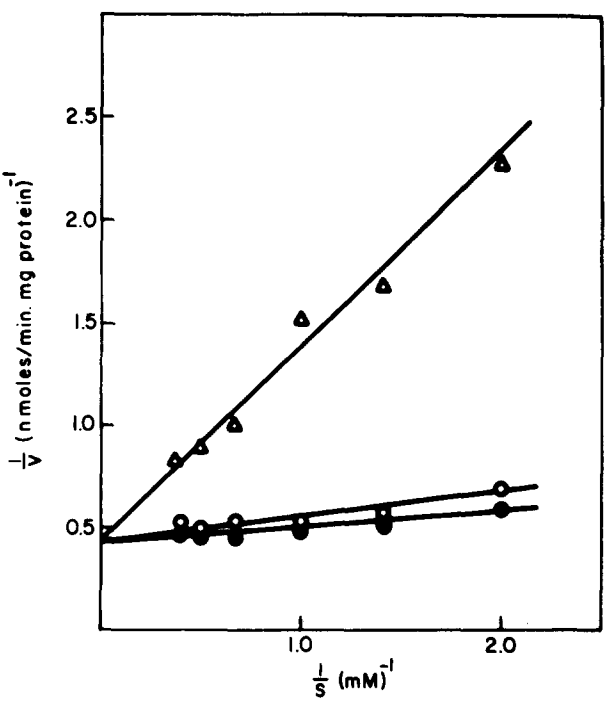

Fig. 3. Reciprocal plots of adenylate cyclase activity against equimolar concentrations of ATP and Mn^{2+}, in the absence and in the presence of inhibitors. ●———●, in the absence of inhibitor; ○———○, in the presence of 0.1 mM 5′-AMP; △———△, in the presence of 0.1 mM GTP.

was examined using 5'-AMP and GTP as ATP analogues. From the data in Fig. 3, it can be seen that the nucleotides 5'-AMP and GTP are indeed competitive inhibitors of the enzyme, with K_i values of 0.5 mM and 0.01 mM, respectively.

However, due to the simultaneous changes in the free Mn^{2+} concentration and in the $MnATP^{2-}$: ATP ratio, these data do not permit a clear distinction between an activation site for Mn^{2+} or a potent inhibitory role for free ATP. The inadequacy of this approach for distinguishing between these two possibilities has been discussed previously [15].

In order to decide whether or not the adenylate cyclase activity is influenced by direct interactions with Mn^{2+} and ATP, the $MnATP^{2-}$: ATP ratio was maintained constant while the total ATP concentration was varied. This was accomplished by maintaining the free Mn^{2+} concentration constant (Fig. 4).

Reciprocal plots of *B. emersonii* adenylate cyclase activity as a function of the $MnATP^{2-}$ concentration, at several concentrations of free Mn^{2+}, are shown in Fig. 5. The curves obtained from the double reciprocal plots are linear, intersecting to the right of the ordinate. Furthermore, the secondary replots of the slopes and intercepts of the double reciprocal plots are hyperbolic (not shown). These kinetic patterns suggest that free Mn^{2+} is an activator at low substrate concentrations, but an inhibitor at high substrate concentrations, and that free ATP is not an efficient inhibitor ($K_i > 1 \cdot 10^{-4}$ M) [15,16]. This implies that, although the affinity of the enzyme for $MnATP^{2-}$ increases upon binding of

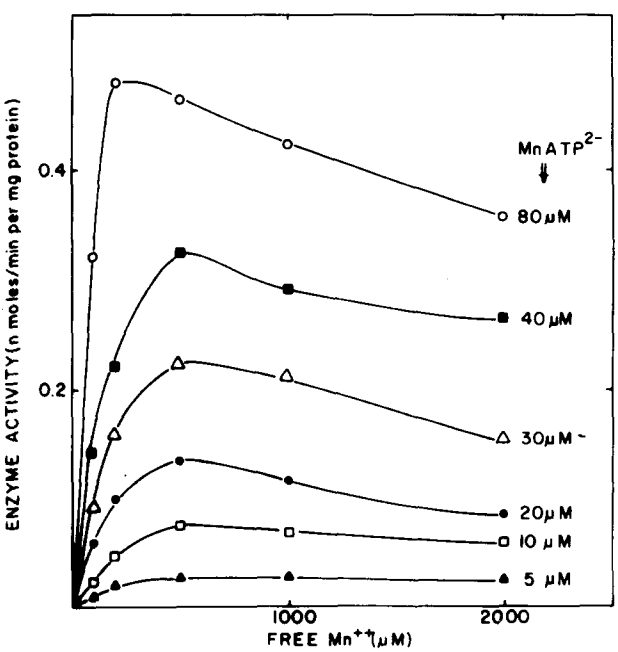

Fig. 4. Effect of adding various concentrations of Mn^{2+}, in the presence of different concentrations of added ATP, on adenylate cyclase activity. The enzyme activity is plotted as a function of the calculated concentration of free Mn^{2+} in the reaction mixture. The calculated $MnATP^{2-}$ concentrations are indicated by the value on each curve.

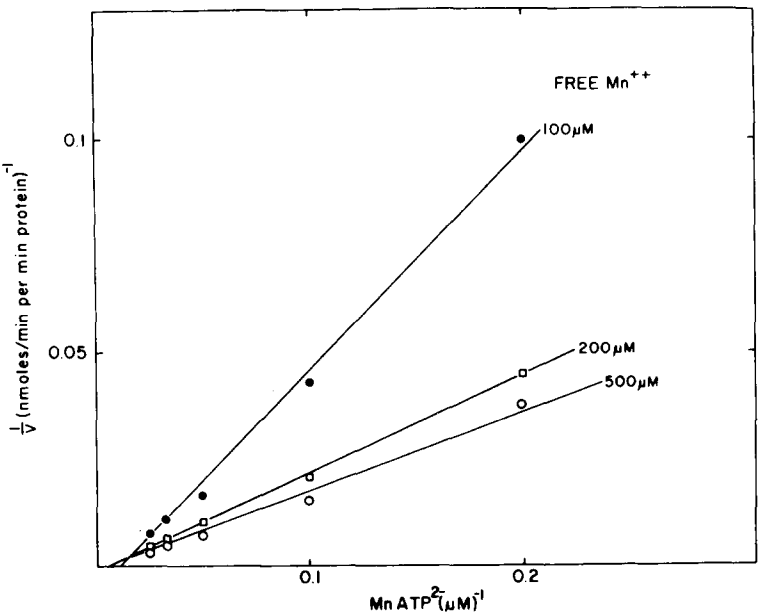

Fig. 5. Reciprocal plots of adenylate cyclase activity versus $MnATP^{2-}$ at the indicated free Mn^{2+} concentrations (under these conditions, free ATP was present in constant proportion to the variable substrate concentrations).

Mn^{2+}, the Mn^{2+}-enzyme-$MnATP^{2-}$ complex is less productive than the enzyme-$MnATP^{2-}$ complex [16].

The results of Fig. 1, showing that Mn^{2+} activates the reaction only at low concentrations of substrate, are consistent with this model.

Inhibition by high concentrations of free Mn^{2+} at a constant substrate concentration (Fig. 4) can also be explained by a lower catalytic efficiency of the Mn^{2+}-enzyme-$MnATP^{2-}$ complex as compared to the enzyme-$MnATP^{2-}$ complex. At Mn^{2+} concentrations where all the $MnATP^{2-}$ is bound to the enzyme, increasing Mn^{2+} would increase the Mn^{2+}-enzyme-$MnATP^{2-}$: enzyme-$MnATP^{2-}$ ratio, thus decreasing enzyme activity.

Kinetics of the enzyme treated with Triton X-100

In the zoospore homogenate, most of the adenylate cyclase activity (73%) is recovered in the 105 000 × *g* pellet. The enzyme activity can be solubilized from the particulate fraction by treatment of the homogenate with 1% of the non-ionic detergent Triton X-100. Upon recentrifugation of the Triton-treated preparation, the supernatant contains 60% of the total enzyme activity [9].

Since treatment with this detergent disrupts the natural membrane structure, the kinetic behavior of the enzyme following solubilization with the detergent is of obvious interest. Table I shows that the kinetic properties of the enzyme obtained after this treatment are similar to those described for the untreated membrane-bound enzyme.

The properties of *B. emersonii* adenylate cyclase, described in this study, are similar in several respects to those reported for *N. crassa* by Flawiá and Torres [3]. Both their work and ours point to the existence of some rather striking

differences between animal cyclases and those of these lower eukaryotes. The zoospore adenylate cyclase specifically requires Mn^{2+}, is not activated by fluoride and is competitively inhibited by ATP, 5'-AMP and GTP, the last being a universal effector of hormonally stimulated adenylate cyclase [17]. On the other hand, as has been observed for mammalian adenylate cyclases [2,15], free Mn^{2+} seems to be an activator of the adenylate cyclase of *B. emersonii* (although only at low concentrations of substrate).

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