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Comparison of Different Cations (Mn^{2+}, Mg^{2+}, Ca^{2+}) on the Hydrolytic Activity of Chloroplast ATPase

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The influences of Mn^{2+}, Mg^{2+}, and Ca^{2+} on the enzymic activity of chloroplast ATPase have been compared, using an HPLC method for the separation of ADP. The dissociation constants of the divalent ion–ATP complexes have been determined by a spectrophotometric method, with the dye antipyrylazo III, and enzymic constants (dissociation constant of the ion–enzyme complexes, Michaelis constants, maximum rates) have been calculated. The comparison between the rates obtained, respectively, with Mn^{2+} and Ca^{2+} alone with that given by the mixture of these two ions, allows us to conclude that, as for Mg^{2+}, Mn^{2+} is also an activator of chloroplast ATPase and that metal-free ATP is the true substrate.

KEY WORDS: ATPase; enzymic activity; effect of Mn^{2+}, Mg^{2+}, Ca^{2+}.

INTRODUCTION

The enzymic mechanism of the proton F_0 F_1-ATPase in its bacterial, mitochondrial, or chloroplastic forms has been intensively studied for more than 30 years. The enzyme can catalyze the hydrolysis or the synthesis of ATP (ATP $\leftrightarrow$ ADP + P_i). For the hydrolytic reaction, there is a good agreement on the hypothesis that MgATP is the active substrate, free ATP and free cation being inhibitors (Ulrich, 1964; Adolfsen and Moudrianakis, 1978; Gepshstein *et al*., 1974; Hochman *et al*., 1976; Guerrero *et al*., 1990; Malyan and Makarov, 1977; Anthon and Jagendorf, 1983; Zhou and Boyer, 1992). As stated by Adolfsen and Moudrianakis "the activation is indirect, being the result of complexation of inhibitory-free ATP. Free ATP is a non competitive inhibitor of MgATP hydrolysis. The inhibition by free Mg^{2+} is direct, being the result of Mg^{2+} binding to a specific inhibitory site on the enzyme, which is distinct and separate from the MgATP binding site" (Adolfsen and Moudrianakis, 1978).

We have challenged this interpretation, however, on the basis of comparison of binding and enzymic data (K_m must be superior to K_d) (Berger *et al*., 1994) and of the use of Mg^{2+} chelating agents with different dissociation constants (Berger *et al*., 1998b). The interpretation that fits our data best is that metal-free ATP must be the true substrate of the enzyme and that Mg^{2+} is an activator.

We present here the comparison of enzymic data obtained with different divalent cations Mn^{2+}, Mg^{2+}, and Ca^{2+}, which confirm our interpretation in the case of Mn^{2+}.

MATERIALS AND METHODS

The enzymic activity was determined using an HPLC method based on the separation of ADP and ATP and the quantitation of ADP formed. ATPase activity was measured at 37°C in 75 mM Tris sulfate buffer, pH 8.5, containing variable concentrations of ATP and divalent cation (here Mg^{2+}, Ca^{2+} or Mn^{2+}). Aliquots of the reaction mixture were directly injected on a TSK DEAE 2SW column (4.6 × 250 mm). The reaction was immediately stopped after injection on the column by the buffer used for elution (KH_2 PO_4 0.1 M, NaCl 0.25 M, pH 4.3). The nucleotides were separated in about 3 min, at an elution rate of 1.2 ml/min, with a resolution (mean distance between peaks divided by peak width at half height) better than 2. The concentration of the released ADP was measured by

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the height of the absorption peak at 260 nm, compared to a calibration curve established under the same conditions. The concentrations of ATP and ADP standards were calculated assuming $E^{M}_{1cm} = 15{,}400$ and were corrected for the traces of AMP and ATP present in ADP and of ADP in ATP, measured by HPLC. The amount of ADP released by ATPase-driven ATP hydrolysis increased linearly with the time of action of the enzyme in the reaction mixture up to a percentage of hydrolysis of about 10%. The initial enzymic rate is well defined for a period of at least several minutes. After this time, the rate decreases, because of the consumption of ATP and of the inhibition due to released ADP. No lag time or burst of activity was observed under these experimental conditions, even in the first minute; aliquots were taken every 10 s and frozen in liquid nitrogen before analysis. The initial concentration of ADP in the reaction mixture was determined by extrapolation to zero time and corresponded closely to that brought as an impurity by ATP. Endogenous ADP or ATP that are tightly bound on CF_1 or CF_1-ε were measured after acid denaturation of the protein by the same chromatographic method. These amounts were negligible in the conditions of the measurements of the enzymic activity. It was verified that the quantity of ADP measured by HPLC was completely independent on the quantity of ATP from which it was separated (in the range 0 to 2.5 10^{-6} M ADP, in the absence or presence of 10^{-3} M ATP). The enzymic rate varies linearly with the concentration of CF_1-ε, contrarily to CF_1, which dissociated into CF_1-ε and inhibitory ε at low concentration (Beger *et al*., 1990).

CF_1 and CF_1-ε were purified by HPLC from spinach chloroplasts as described in Berger *et al*. (1987).

Antipyrylazo III was used for Mg^{2+} ligand complexes dissociation constant measurements (Scarpa *et al*., 1978; Ogawa *et al*., 1980) and we have applied this method to ATP Mg (Berger *et al*., 1998b) and extended it to Mn^{2+} and Ca^{2+} complexes (this work). The differences ΔA (523 nm) $-$ ΔA (602 nm), ΔA (603 nm) and ΔA (596 nm) $-$ ΔA (515 nm) were characteristic, respectively, of the Mg^{2+}, Mn^{2+}, and Ca^{2+} antipyrylazo complexes. The K_d of these complexes are superior to 1 mM in 0.075 M Tris buffer pH 8.5. For a 40 μM dye concentration, these differences are quite linear with the metal ion concentration, up to 10 μM. In the presence of a metal ion ligand, such as ATP, with a dissociation constant sufficiently lower than that of antipyrylazo III, the concentrations of the free metal ion are determined by the optical absorption differences mentioned above: the binding of the cation to the dye does not significantly modify the equilibrium between the cation and the chelating agent.

RESULTS

Cation Ligand Binding Data

From curves of ΔA versus total cation concentration (Fig. 1, for Mg^{2+}), free and bound cation concentrations were determined for different total concentrations of added ATP. In the conditions used (Tris buffer 0.075 M, pH 8.5), the mean dissociation constants of ATP complexes were 8.6 10^{-6} M, 14.2 10^{-6}, and 25 10^{-6} M, respectively, for Mn^{2+}, Mg^{2+}, and Ca^{2+}.

Comparison of the Dependence of the Enzymic Rate versus Total ATP Concentration, in the Presence of Mn^{2+}, Mg^{2+}, and Ca^{2+} (Fig. 2)

With Mn^{2+} (1 mM), the rate is proportional to the total ATP concentration up to 1 mM, and limited to very low

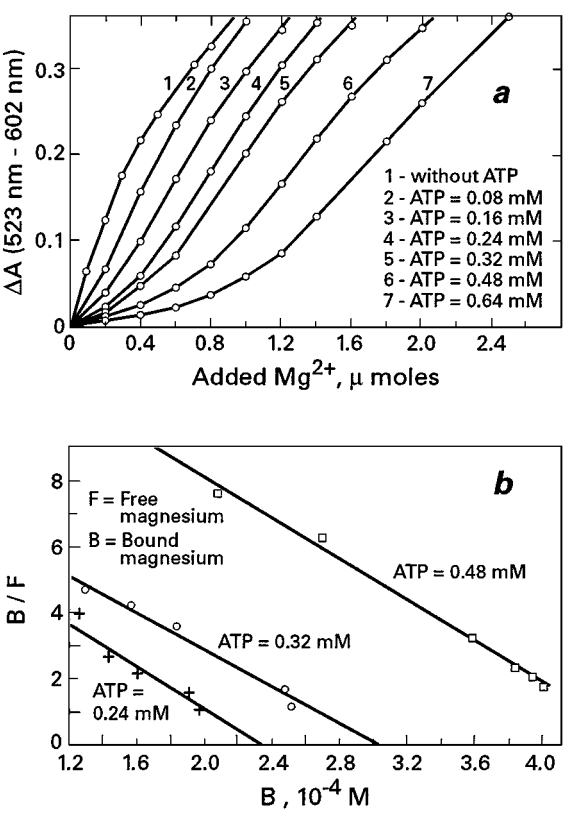

Fig. 1. (***a***) ATP-Mg binding measurements by the antipyrylazo III method. ΔA (523–602 nm) is characteristic of the free magnesium ion concentration. Conditions: Tris buffer 0.075 M, pH 8.5, dye 40 μM, for different ATP_t concentrations. (***b***) Scatchard plot corresponding to (***a***).

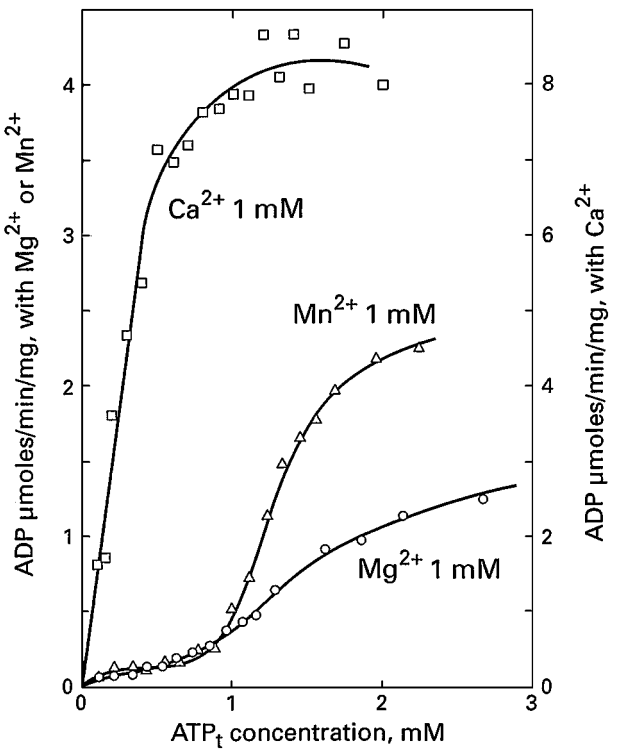

Fig. 2. Influence of the total ATP concentration on the rate of ATP hydrolysis. Conditions: Tris buffer 0.075 M, pH 8.5; total cation concentration 1 mM.

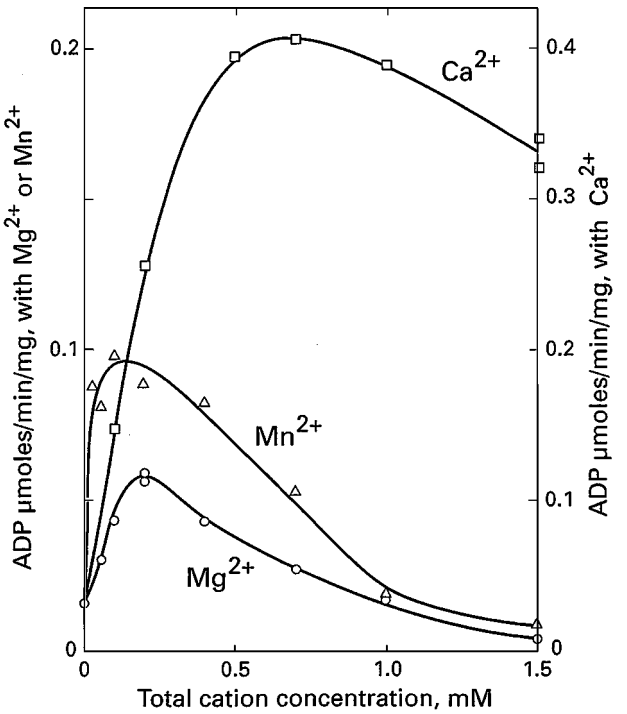

Fig. 3. Influence of the total cation concentration on the rate of ATP hydrolysis. Conditions: total ATP concentration 1 mM; other conditions as in Fig. 2.

values (0.1–0.3 μM ADP/min/mg), then increases steeply at equivalence between ATP and Mn^{2+}, to reach 2.2 μM ADP/min/mg for $ATP_t = 2$ mM.

With Mg^{2+}, (1 mM), the same dependence is also observed, as already described (Berger *et al.*, 1994), but the acceleration of the rate at equivalence of ATP and Mg^{2+} is less pronounced than with Mn^{2+}. The value of the rate for $ATP_t = 2$ mM is around 1 μM ADP/min/mg.

On the contrary with Ca^{2+} (1 mM), the enzymic rate increases steeply and linearly from the low concentrations of substrate, to reach a maximum of 8 μM ADP/min/mg for $ATP_t = 2$ mM.

Comparison of the Dependence of the Enzymic Rate versus Total Divalent Ion Concentration, for a Constant Total Substrate Concentration (1 mM) (Fig. 3)

The three curves present a maximum, more or less pronounced, at different divalent ion concentrations: 0.1, 0.2, and 0.7 mM, respectively, for Mn^{2+}, Mg^{2+}, and Ca^{2+}.

Determination of the Enzymic Constants

The two simplest models, which can account for the data, are the following:

1. the classical model, for which the substrate is the metal ATP complex and the divalent ion is the inhibitor:

$$\underset{\text{active enzyme site}}{E} + \underset{\text{divalent ion}}{A} \Leftrightarrow \underset{\text{inactive}}{EA}$$

$$E + \underset{\text{metal ATP complex}}{AS} \Leftrightarrow EAS \rightarrow EA + \text{products } (ADP + P_i)$$

2. the model we have already proposed (Berger *et al.*, 1994, 1998b) for which the substrate is the

metal-free ATP and the divalent ion is the activator:

$$\underset{\substack{\text{inactive}\\\text{enzyme site}}}{\text{E}} + \underset{\substack{\text{divalent}\\\text{ion}}}{\text{A}} \Leftrightarrow \underset{\text{active}}{\text{EA}}$$

$$\text{EA} + \underset{\substack{\text{metal-free}\\\text{ATP}}}{\text{S}} \Leftrightarrow \text{EAS} \rightarrow \text{EA} + \text{products}$$

It can be shown that the enzymic rate is expressed by:

$$v = \frac{V_m}{1 + \left(1 + \frac{a}{K_a}\right)\frac{K'_m}{as}}$$

or

$$v = \frac{V_m}{1 + \left(1 + \frac{K_a}{a}\right)\frac{K_m}{s}}$$

according to the first or the second model, where V_m = maximum enzymic rate; a = free divalent ion concentration; s = metal-free ATP concentration; as = metal complexed ATP concentration; K_a = activation or inhibition constant, = (E)(A)/(EA); K_m = Michaelis Menten constant, calculated with metal-free ATP; and K'_m = Michaelis Menten constant, calculated with metal complexed ATP.

In fact, the two formula are equivalent, because of the relationship existing between the two kinds of substrate:

A + S $\Leftrightarrow$ AS, with K_{as} dissociation constant,
K_{as} = (A)(S)/(AS)

The dissociation constants with Mn^{2+}, Mg^{2+}, and Ca^{2+} have been determined above, by the use of antipyrylazo III. The K_m, K'_m, and V_m values can be calculated from the enzymic rates (Fig. 2) and the calculated values of the free and complexed ATP concentrations. K_a is deduced from the total ion concentration at the maximum of the enzymic rate, at constant total ATP concentration (Fig. 3).

$$K_a = K_{as}\left(\frac{a_t}{s_t - a_t}\right)^2$$

Because of the equilibrium between A, S, and AS,

$$K'_m = \frac{K_m K_a}{K_{as}}$$

All these constants are brought together in Table I.

These values account for the difference between the two types of dependence of the enzymic rate versus total ATP concentration, at constant total ion concentration (Fig. 2): for Mn^{2+} and Mg^{2+}, the rate is very low when the cation is in excess, then there is an acceleration of the rate beyond equivalence. With the first model, it can be due to the release of inhibition due to the depletion of inhibitory ion. With the second model, it can be due to the emergence of metal-free ATP, beginning from values smaller than K_m. In the case of Ca^{2+}, the rate increases sharply with the total ATP concentration, to reach a maximum. This can be explained by the first model as the release of inhibition, occurring at low values of total ATP, since K_a for Ca^{2+} is much greater than for Mn^{2+} and Mg^{2+} and the EA complex is partially dissociated when free cation begins to decrease. With the second model, the evolution can be due to the increase of metal-free ATP, but as V_m is reached before equivalence ($K_m <$ metal-free ATP at equivalence), no acceleration of the rate is observed.

Table I. Enzymic Constants of Chloroplast ATPase in the Presence of Mn^{2+}, Mg^{2+}, and Ca^{2+}[a]

	Mn^{2+}	Mg^{2+}	Ca^{2+}
V_m (μ mole/min/mg)	3.4	1.87	10.6
K_m (μM)	520	705	12.5
K'_m (μM)	6.4	44	68
K_a (μM)	0.106	0.89	136
K_{as} (μM)	8.6	14.2	25

[a] K_m = Michaelis constant, calculated with metal free ATP; K'_m = Michaelis constant, calculated with metal complexed ATP; K_a = dissociation constant of the enzyme site–metal complex; K_{as} = dissociation constant of the metal ATP complex.

The ratios between the rate values at the maximum (at constant total ATP concentration, versus total ion concentration, Fig. 3) and at the equivalence can be calculated from the constant values of Table I. They agree fairly well with the experimental values (Table II).

Manganese Ion Is an Activator of ATPase and the Substrate Is Free ATP

The enzymic data presented above do not allow a choice between the two models. Another type of experiment is necessary to settle the question, as it has been done in the case of magnesium ion: determination of the dissociation constant between the enzyme and the substrate, which must be smaller than the K_m (Berger *et al.*, 1994),

Table II. V_{max} to V at Equivalence Ratios in the Presence of Mn^{2+}, Mg^{2+}, and Ca^{2+}, Measured or Calculated from Table I Constants

$V_{max}/V_{equivalence}$	Mn^{2+}	Mg^{2+}	Ca^{2+}
Calculated	4.2	3.03	1.02
Experimental	5.06	3.18	1.05

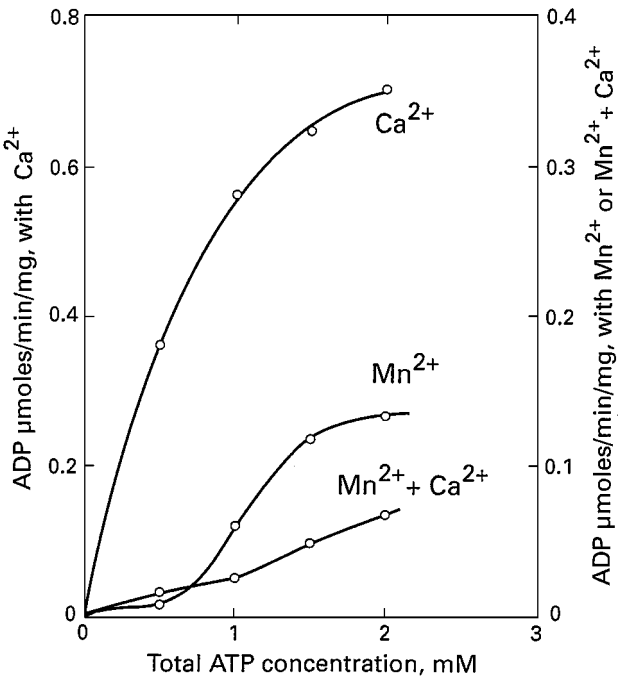

Fig. 4. Influence of the simultaneous presence of Mn^{2+} and Ca^{2+}. Conditions: Ca^{2+}, 1 mM; Mn^{2+}, 1 mM; Ca^{2+} + Mn^{2+}, 1 mM each; other conditions as in Fig. 2.

use of chelators of Mg^{2+}, with dissociation constants of different values (Berger *et al*., 1998b), and inhibition of enzymic activity by monovalent cations Na^+ and K^+ (Berger *et al*., 1994).

We can also remove the ambiguity in the case of Mn^{2+} by measuring the rate of hydrolysis in a mixture of Mn^{2+} and Ca^{2+}. Figure 4 shows that the rate in a buffer containing a mixture of Mn^{2+} and Ca^{2+} (1 mM each) is lower than when each ion is alone. This rather surprising result (one would expect an intermediary rate) can only be explained with the model of ATP as real substrate and Mn^{2+} as activator.

First, the enzyme is quite totally complexed with Mn^{2+} (K_a for $Mn^{2+} = 0.106\ 10^{-6}$ M, K_a for $Ca^{2+} = 136\ 10^{-6}$ M). No Ca^{2+} complexed or free forms are present in the conditions used in the experiment and this assumption is valid whatever the model would be, since the determination of K_a is the same in the two cases.

Second, the presence of Ca^{2+} does not modify the concentration of MnATP, for concentrations of total ATP of 2 mM and beyond. Ca^{2+} modifies the concentration of free Mn^{2+}, but in a range for which there is no modification of complexation of the enzyme by Mn^{2+} (from 9 to130 10^{-6} M for $ATP_t = 2$ mM). Consequently, as there is a neat decrease of the rate by addition of Ca^{2+}, MnATP cannot be the real substrate. Conversely, free ATP is largely decreased by the addition of Ca^{2+}, which accounts for the decrease of the rate only if free ATP is the true substrate of ATPase.

CONCLUSION

The data presented here show that the hydrolytic activity of the chloroplast ATPase is well defined by the model of a Michaelian-type enzyme, activated by Mg^{2+} or Mn^{2+} and acting on metal-free ATP. In the case of Ca^{2+}, however, the two mechanisms are possible and other experiments must be done to decide between them. As K_{as} is inferior to K_a, one could think that the free form of the enzyme acts on the CaATP complex, but the K_{as}/K_a ratio is not small enough to be sure that this mechanism is predominant.

Although contrary to the common opinion which attributes to Mg^{2+} the role of inhibitor and to the MgATP complex the role of substrate, our assertion is not inconsistent with the rotational mechanism. However it implies that ATPase has a Michaelian character. Pedersen *et al*., (1976; Reynafarje and Pedersen, 1996) have shown that no cooperativity exists between the enzymic sites of beef and rat mitochondrial ATPase and we come to the same conclusion with the chloroplast ATPase (Berger *et al*., 1998a).

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