THE ATPase SYSTEM OF ISOLATED RAT LIVER PLASMA MEMBRANES. KINETIC PROPERTIES OF THE MgATP PHOSPHOHYDROLASE

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1. Introduction

Isolated plasma membranes of rat liver contain an enzyme system which hydrolyses ATP to ADP and Pi and which requires Mg^{2+}, Na^+, and K^+ for full activity [1,2]. The kinetic properties of this system in purified membrane preparations have not previously been investigated systematically. Further, in most kinetic studies of membrane ATPases of varying origins only the total concentrations of reactants and effectors have been considered [3–6], except for a few investigations of mitochondrial ATPases [7,8]. In the present study of plasma-membrane ATPase (EC 3.6.1.3) we have used the relevant ionization and stability constants to determine the concentrations of Mg^{2+}, ATP^{4-}, and $MgATP^{2-}$ in the reaction mixtures. The results suggest that the substrate is $MgATP^{2-}$. Free Mg^{2+} ions were not required for maximal enzyme activity but were found to be competitively inhibitory. Free ATP^{4-} had no effect. One of the products, $MgADP^-$, was also a strong competitive inhibitor, indicating that it may share binding sites with $MgATP^{2-}$.

2. Methods and materials

Plasma membranes from rat liver were isolated as described by Emmelot et al. [1] with the following modifications:

(a) Instead of $NaHCO_3$, the homogenization medium contained 10 mM triethanolamine (TRAP) buffer pH 7.4, 10 mM NaCl, and 5 mM KCl.

(b) The time of density-gradient centrifugation (MSE "High Superspeed 50" Model TC, swing-out rotor 3 X 25 ml) was shortened to 20 min at 30,000 rpm.

No change was noted in yield or in membrane contamination as compared to the original method.

Disodium ATP (Reanal, Budapest) was purified on DEAE-Sephadex A25 columns. The contaminants (Pi, AMP, and ADP) were first removed by step-wise elution with 150–300 mM TRAP buffer pH 7.4. The ATP was then eluted with 1 M TRAP buffer pH 7.4 and tested enzymically; it contained less than 0.1% of either ADP or AMP and no detectable Na^+ or K^+. Disodium ADP was purified in the same way using 150 mM TRAP buffer. The initial velocity was measured from the Pi liberated using a sensitive microassay [9]. The reaction mixtures contained 100 mM TRAP-HCl (pH 7.4), 0.05–3.0 mM ATP, 0.01–5.6 mM $MgCl_2$, and 10–30 μg enzyme protein in a total volume of 1.5 ml. In a separate set of experiments 0.04–1.0 mM ADP was also added. The error caused by the very small enzymic hydrolysis of ADP was corrected for by separate blank experiments. In all the experiments varying the concentrations of the effectors did not cause the ionic strength to alter by more than 10%, which is small enough to be neglected. Incubation was at 30° for 5 min. The reaction was stopped by the addition of 0.2 ml of 40% trichloroacetic acid and, after centrifuging, 0.1 ml of the deproteinized supernatant was used for Pi determination. All assays were done in quadruplicate. The rate of reaction was linear with time. Enzyme activity was expressed as μmoles Pi split off from ATP per min per mg protein. Protein was estimated by the method of Lowry et al. [10].

3. Results

The concentrations of $MgATP^{2-}$ were calculated from the quadratic equation

$$[MgATP^{2-}]^2 - \left(\frac{[H^+] + K_a}{K_a K_{MgATP^{2-}}} + [Mg]_{total} + [ATP]_{total}\right)[MgATP^{2-}] + [Mg]_{total}\,[ATP]_{total} = 0\,,$$

where

$$K_a = \frac{[ATP^{4-}]\,[H^+]}{[ATP^{3-}]} = 1.12 \times 10^{-7}\ M\ [11]$$

and

$$K_{MgATP^{2-}} = \frac{[MgATP^{2-}]}{[ATP^{4-}]\,[Mg^{2+}]} = 10^4\ M^{-1}\ [12].$$

The free Mg^{2+} concentration was calculated from the conservation equation

$$[Mg^{2+}] = [Mg]_{total} - [MgATP^{2-}]\,.$$

In the reaction mixtures used, $[MgATP^-]$ could be neglected as it was less than 0.4% of $[MgATP^{2-}]$.

To determine the effects of varying the concentrations of $MgATP^{2-}$, Mg^{2+}, and ATP^{4-} on the enzyme activity, the initial velocity was measured after the addition of (a) increasing amounts of $MgCl_2$ and (b) increasing amounts of ATP to initially equimolar concentrations of ATP and $MgCl_2$.

Fig. 1 shows that at low ATP concentrations (0.1 mM) addition of $MgCl_2$ up to 0.5 mM stimulated enzyme activity but further addition of $MgCl_2$ gradually decreased activity. In contrast, at high ATP concentrations (1.0 mM), inhibition began as soon as additional $MgCl_2$ was added.

The effect of added ATP on enzyme activity is shown in fig. 2. At each of the three constant $MgCl_2$ concentrations tested, enzyme activity was stimulated as ATP was increased, the activity reached a maximum at about 1 mM ATP. Enzyme activity increased more rapidly with increasing ATP concentrations at lower than at higher $MgCl_2$ concentrations. No inhibi-

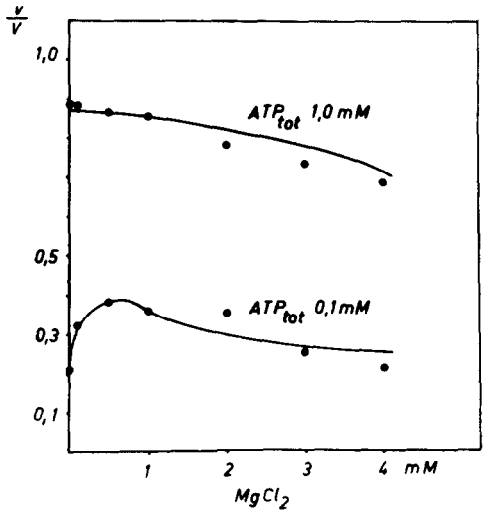

Fig. 1. Effect of $MgCl_2$ on the relative velocity of the ATPase reaction at two different $[ATP]_{total}$. Abscissa: $MgCl_2$ added to an initial equimolar concentration of ATP and $MgCl_2$. Solid lines represent calculated theoretical curves, solid circles indicate measured experimental values (see text).

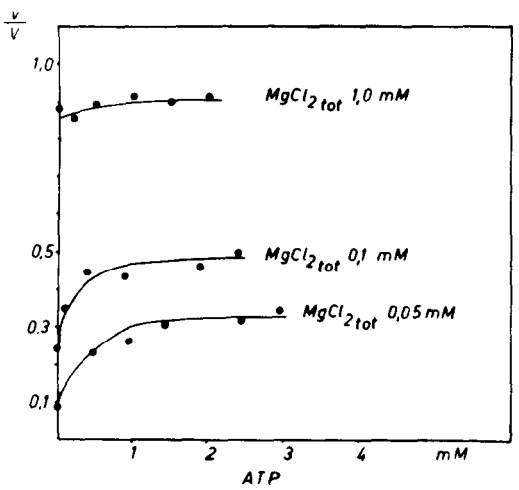

Fig. 2. Effect of ATP on the relative velocity of the ATPase reaction at three different $[MgCl_2]_{total}$. Abscissa: ATP, added to an initial equimolar concentration of ATP and $MgCl_2$. Solid lines represent calculated theortical curves, solid circles indicate measured experimental values (see text).

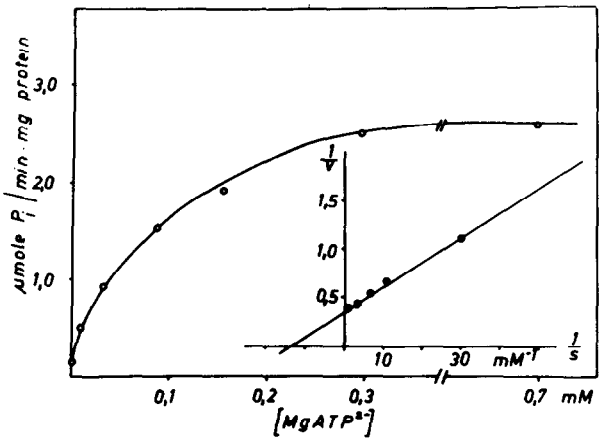

Fig. 3. Effect of $[MgATP^{2-}]$ on the rate of plasma-membrane ATPase. v = μmoles Pi liberated/min/mg protein; $[ATP]_{total}/[Mg^{2+}]_{total} = 1$. Inset: double reciprocal plot for the determination of K_m for $MgATP^{2-}$.

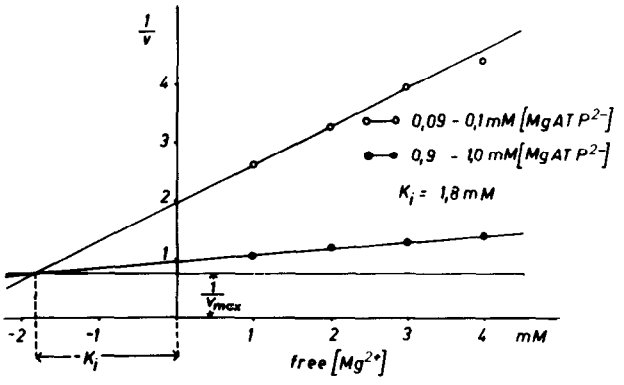

Fig. 4. Dixon plot for the inhibitor $[Mg^{2+}]$. v = μmoles Pi liberated/min/mg protein.

tion by ATP was observed over the concentration range tested.

These results may be explained on the assumptions that $MgATP^{2-}$ is the substrate for the enzyme and that free Mg^{2+} ions inhibit while free ATP^{4-} ions have no effect. When the initial velocity is plotted against $[MgATP^{2-}]$ a Michaelis-type hyperbolic saturation curve is obtained (fig. 3). From the double reciprocal plot, $K_m = 9.5 \times 10^{-5}$ ($1.4 \times 10^{-4} - 7.3 \times 10^{-5}$) M and $V_{max} = 1.69$ (1.02–2.85) μmole/min/mg protein.

The Dixon plot [13] in fig. 4 shows that free Mg^{2+} ions compete with $MgATP^{2-}$. An average value of the inhibition constant, $K_{i(Mg^{2+})} = 2.2 \times 10^{-3}$ M, was calculated from the results of three experiments.

Our results may be described by the following equation for competitive inhibition

$$\frac{v}{V_{max}} = \frac{\alpha}{\alpha + \beta + 1},$$

where $\alpha = [MgATP^{2-}]/K_m$ and $\beta = [Mg^{2+}]/K_{i(Mg^{2+})}$. Using $K_{MgATP^{2-}} = 1 \times 10^4$ M^{-1} and values of K_m, $K_{i(Mg^{2+})}$, and V_{max} obtained from one experiment, theoretical curves were calculated to describe the results given in figs. 1 and 2. The theoretical curves (solid lines) are seen to fit the experimental points (solid circles) fairly closely.

The reaction product ADP was found to be a

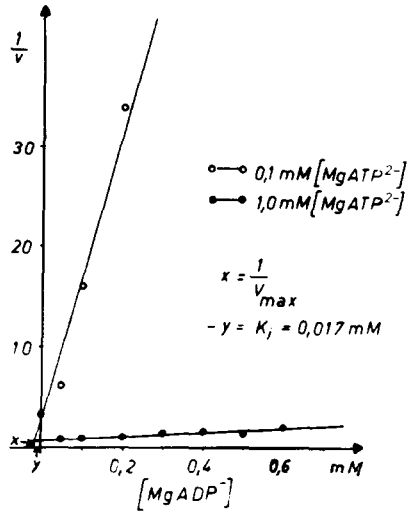

Fig. 5. Dixon plot for the inhibitor $[MgADP^-]$. v = μmoles Pi liberated/min/mg protein. Assay system contained 100 mM TRAP-HCl pH 7.4, 0.04–1.0 mM ADP, 0.1 or 1.0 mM ATP, and 5.6 mM $MgCl_2$. Further additions of $MgCl_2$ were made in equimolar amounts for each unit of ADP and ATP, respectively.

potent competitive inhibitor of the enzyme in the presence of Mg^{2+}, as is seen from the Dixon plot in fig. 5. The inhibition constant had an average value $K_{i(MgADP^-)} = 3.9 \times 10^{-5}$ M; individual values (1.5, 1.7, 8.5) $\times 10^{-5}$ M. A competition between ADP and ATP for Mg^{2+} ions can be excluded since, using

$K_{MgADP^-} = 2.188 \times 10^3\ M^{-1}$ [15], it can be shown that, even in the unfavourable case of ATP 0.1 mM and ADP 1 mM, all the ATP is completely converted into the complex $MgATP^{2-}$. In contrast to ADP, no inhibition was observed with Pi up to 40 mM.

4. Discussion

Stability constants for $MgATP^{2-}$ are cited in the literature from about 3×10^3 to $9 \times 10^4\ M^{-1}$ [14,15]. Theoretical curves describing the results in figs. 1 and 2 were calculated using $K_{MgATP^{2-}} = 1 \times 10^4$, 1.995×10^4, 3.98×10^4, and $8.71 \times 10^4\ M^{-1}$. The best fit to the experimental points was found with $1 \times 10^4\ M^{-1}$.

Our results indicate that $MgATP^{2-}$ is the true substrate of plasma-membrane ATPase, as Ulrich [7] was able to demonstrate for the mitochondrial enzyme. In contrast to ATPases of other origins [16] the substrate is not an allosteric effector for the plasma-membrane enzyme, since the velocity versus substrate concentration curve is a hyperbola of the Michaelis type and the coefficient of the Hill plot is 1.0.

Since the enzyme does not require free Mg^{2+} ions for maximal activity, indeed these ions are competitive inhibitors for the substrate, we would propose that this enzyme be called a MgATP phosphohydrolase instead of a Mg^{2+}-activated or Mg^{2+}-dependent ATPase.

Further, no conclusions about the binding mechanisms of the Mg and ATP to the enzyme can be drawn from our results for the $[Mg]_{total}/[ATP]_{total}$ ratio required for maximal activity. This is because at the same $[Mg]_{total}/[ATP]_{total}$ ratios the proportion of $MgATP^{2-}$ to the total Mg or ATP may differ by as much as one order of magnitude (see fig. 2).

We have shown that $MgADP^-$ is a competitive inhibitor of the enzyme with $K_{i(MgADP^-)} \leqslant K_{m(MgATP^{2-})}$. This suggests the possibility that changes in the physiological concentrations of ADP may control the activity of the enzyme. Also, since $K_{i(Mg^{2+})}$ differs considerably from $K_{i(MgADP^-)}$, it may be concluded that not only the magnesium but perhaps the adenine or the phosphate are concerned in binding of the substrate and of these inhibitors to the enzyme.

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