FREE Mg^{2+} AND PROPOSED ISOMERIZATIONS OF THE (Na^+ + K^+)-DEPENDENT ATPase

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1. Introduction

In 1966 Fahn et al. [1] showed that the Na^+-dependent ADP/ATP exchange activity of an eel electric organ (Na^+ + K^+)-dependent ATPase preparation could be demonstrated best at $MgCl_2$ concentrations low relative to the optimum for the ATPase. From these studies a formulation for the reaction sequence arose in terms of a high-energy phosphoenzyme E_1~P, demonstrable in the exchange reaction, and a lower-energy phosphoenzyme E_2–P, formed from E_1~P in the presence of free Mg^{2+} and functioning in the hydrolytic step [2]. In addition, it has generally been proposed that these are obligatory sequential steps in the overall reaction process, with a cyclical binding and release of free Mg^{2+} [2–4]:

$$E_1 \underset{Mg,\,Na}{\overset{ATP}{\rightleftharpoons}} E_1 \sim P \xrightarrow[Mg]{} E_2\text{–}P \xrightarrow[K]{} E_2 + \quad + P_i \xrightarrow[-Mg]{} E_1 \qquad (A)$$

Since the primary structures of E_1~P and E_2–P appear to be the same [2,4], the two phosphoenzymes would thus represent different conformational states; identification is operationally defined in terms of exchange vs hydrolytic activity.

Although this formulation is the usually-presented reaction scheme, the data may be accomodated in other formulations as well. From considerations of the actions of oligomycin (which favors the exchange over the hydrolytic activity [1,2]) and of divalent cations a different formulation in terms of alternative parallel pathways has been proposed [5,6]:

$$\begin{array}{ccccc} E_1 & \underset{Mg,\,Na}{\overset{ATP}{\rightleftharpoons}} & E_1 \sim P & \xrightarrow[K]{(slow)} & E_1 + P_i \\ \downarrow\uparrow & & \downarrow & & \downarrow\uparrow \\ E_2 & \xrightarrow[Mg,\,Na]{ATP} & E_2 - P & \xrightarrow[K]{(fast)} & E_2 + P_i \end{array} \qquad (B)$$

Here higher concentrations of MgATP favor the E_2 pathway, while lower concentrations of MgATP (either from low concentrations of $MgCl_2$ with high ATP or from low concentration of both) favor the E_1 pathway*. A major distinction between these formulations is the requirement in (A) for a cyclical binding and release of free Mg^{2+}; strangely, such a requirement for Mg^{2+} has been largely ignored. Nevertheless, the issue deserves attention for the $E_1 \rightleftarrows E_2$ isomerizations play essential roles in several transport models [3,4], the E_1 form being labeled 'inward facing' and the E_2 form 'outward facing', with Na^+ and K^+ translocation effected through their sequential interconversions.

In the course of studies on nucleotide interactions with a rat brain enzyme preparation, preliminary calculations indicated that if free Mg^{2+} is bound cyclically, then sequential changes in affinity of several orders of magnitude are required [7]. This approach is pursued here with an eel electric organ

* Alternatively, in the presence of low concentrations of ATP high concentrations of free Mg^{2+} may produce certain E_2 characteristics [6]; clearly, this formulation requires multiple interacting substrate sites [7].

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enzyme like that on which the orginal formulation was based. Effects of $MgCl_2$ can be accounted for in terms of the MgATP complex without invoking additional sites for free Mg^{2+}, and if such sites exist then a cyclical change in affinity of nearly five orders of magnitude would be required for the orginal scheme. If this seems improbable, then transport mechanisms based on such isomerizations must seem improbable as well.

2. Materials and methods

The (Na^+ + K^+)-dependent ATPase was isolated from eel electric organ, as described by Dahms and Boyer [8]. Enzyme activity was estimated after brief incubations at 30°C in terms of P_i production [9]. Initial velocities are expressed relative to that of concurrent incubations in the standard medium: 30 mM histidine HCl adjusted to pH 7.8 with Tris (approx. 40 mM), 3 mM ATP, 3 mM $MgCl_2$, 140 mM NaCl and 20 mM KCl. ATP, purchased from Sigma Chemical Company as the sodium salt, was converted to the free acid by treatment with AG-50 resin, and then neutralized with Tris [9].

The MgATP dissociation constant under these experimental conditions was measured by method (a) of Watanabe et al. [10]. The fluorescence of the 8-hydroxyquinoline–magnesium complex was determined at 30°C in an Aminco–Bowman spectrophotofluorometer in the above medium (30 mM histidine buffer, pH 7.8, 140 mM NaCl, and 20 mM KCl) with 0.5 mM 8-hydroxyquinoline and varied concentrations of ATP and $MgCl_2$. Under these conditions a dissociation constant of 32 μM was calculated [10]. This value was then used to estimate the concentrations of the MgATP complex in the following experiments.

Data presented are averages of five or more experiments performed in duplicate.

3. Results and discussion

When $MgCl_2$ and ATP were varied together at a 1:1 molar ratio enzymatic activity increased with the concentration of the MgATP complex, following Michaelis–Menten kinetics (fig. 1). No requirement

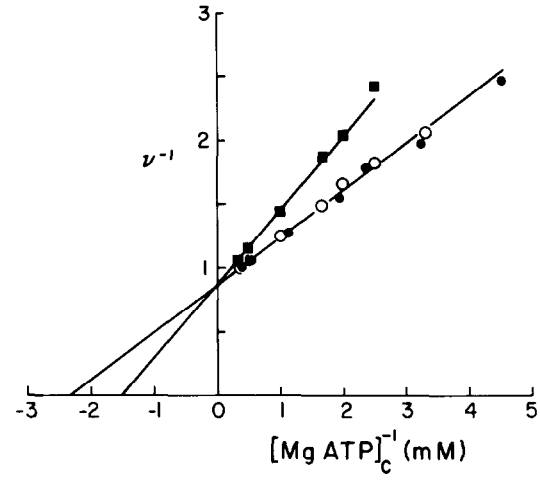

Fig. 1. The MgATP complex and enzyme activity. Initial velocities were estimated from brief incubations (6–8 min) at 30°C in 30 mM histidine HCl (pH 7.8 with Tris), 140 mM NaCl, 20 mM KCl, and equimolar ATP and $MgCl_2$ (●–●–●). Similar experiments were performed varying the ATP concentration with $MgCl_2$ added so that the calculated free Mg^{2+} was 3 mM (○–○–○), or varying the Mg Cl_2 concentration with ATP added so that the calculated free ATP was 3 mM (■–■–■). The calculated MgATP concentration is plotted against velocity, in relative units.

for free Mg^{2+} is apparent, although with the range of $MgCl_2$ and ATP concentration used free Mg^{2+} varied from 0.29 to 0.08 mM. Adding excess $MgCl_2$ increased the concentration of the complex; with 3 mM free Mg^{2+} plots of velocity against the MgATP complex are not distinguishable from the relationship without the constant large excess of Mg^{2+} (fig. 1).

By contrast, adding excess ATP inhibited; at a constant excess of free ATP plots of velocity against the MgATP complex suggest competitive inhibition (fig. 1), as previously noted [7,11]. Dixon plots of free ATP as an inhibitor are consistent with competition, with a K_i for free ATP of 5.4 mM (fig. 2). Thus these data may be interpreted in terms of ATP excluding MgATP from an active site (hindering subsequent addition of Mg^{2+} once the ATP is bound).

However, adding excess ATP must also reduce the concentration of free Mg^{2+}. Thus an alternative explanation for this inhibition could be expressed in terms of ATP reducing the Mg^{2+} required in formulation A for converting $E_1 \sim P$ to $E_2 - P$. This formula-

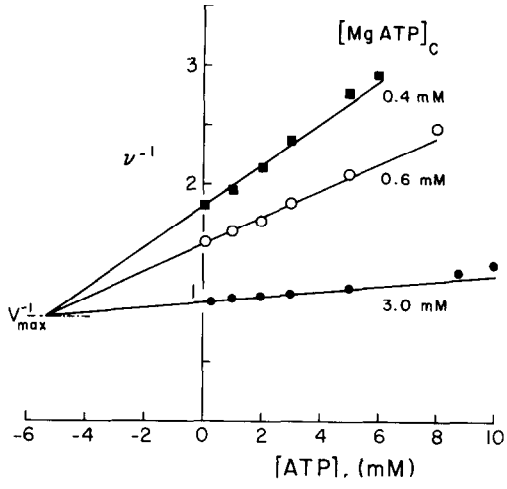

Fig. 2. Inhibition by free ATP. Experiments were performed as in fig. 1, varying the calculated free ATP concentration at three levels of the MgATP complex: 3.0 mM (●–●–●), 0.6 mM (○–○–○) and 0.4 mM (■–■–■).

tion specifies a two substrate kinetic equation with K_{m_1} for MgATP and K_{m_2} for free Mg^{2+}:

$$\frac{V_{max}}{v} = \left[1 + \frac{K_{m_1}}{[MgATP]}\right]\left[1 + \frac{K_{m_2}}{[Mg^{2+}]}\right]$$

An upper limit for K_{m_2} can be estimated from the data: K_{m_1} (MgATP) is about 0.43 mM (fig. 1) and since relative values for V_{max}/v can be determined for different pairs of MgATP and Mg^{2+} concentrations (fig. 2) then the simultaneous equations can be solved for K_{m_2}. For example, with (a) 0.6 mM $MgCl_2$ and 0.6 mM ATP vs (b) 0.6 mM $MgCl_2$ and 5.6 mM ATP the ratio of V_{max}/v (a) to V_{max}/v (b) is 0.717 (fig. 2), $[MgATP]_c$ is 0.477 and 0.596 mM respectively, and $[Mg^{2+}]_f$ is 0.123 and 0.004 mM respectively; thus K_{m_2} is 2.3 μM. This reflects the minimal affinity of a substrate site for free Mg^{2+} in formulation A (to convert $E_1 \sim P$ to $E_2 - P$), assuming that the inhibitory effect of free ATP is *solely* to reduce free Mg^{2+}. (Similar values of K_{m_2} are obtained with other pairs of $MgCl_2$ and ATP concentrations of fig. 2). If free ATP has other inhibitory actions, such as competition with MgATP, then K_{m_2} would be lower and the affinity higher. But the data do not require an action of free Mg^{2+} at all.

Unfortunately, it is not possible to reduce free Mg^{2+} appreciably through additions other than ATP since the governing factor is the MgATP dissociation constant. Additing EDTA only reduced the effective $MgCl_2$ concentration by an equimolar amount (by calculation and in terms of measured velocity), since the affinity of EDTA for Mg^{2+} is so high relative to that of ATP.

In addition, formulation A also requires a cyclical release of Mg^{2+} (to allow re-formation of E_1 and thus predicts that free Mg^{2+} should inhibit. The dissociation constant for Mg^{2+} from that stage of the reaction sequence ($E_2 \rightarrow E_1$) should thus be approachable in terms of the K_i for Mg^{2+}. Excess $MgCl_2$ at high concentrations did inhibit (fig. 3), but the inhibition varied with Na^+ and K^+ concentrations competitively: such competition with both Na^+ and K^+ has been suggested previously [6,12] and can account for the inhibition entirely. However, a lower limit for a dissociation constant can be estimated from the data

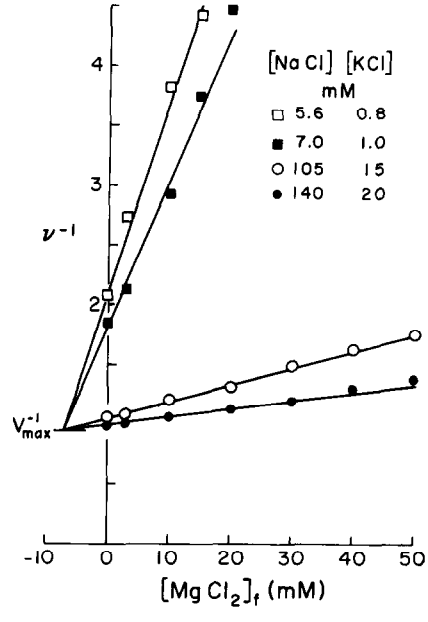

Fig. 3. Inhibition by free Mg^{2+}. Experiments were performed as in fig. 1, except that in all cases 3 mM ATP was present, and excess $MgCl_2$ was added to produce the concentration of free Mg^{2+} indicated. Experiments were performed with 140 mM NaCl and 20 mM KCl (●–●–●), 105 mM NaCl and 15 mM KCl (○–○–○), 7 mM NaCl and 1 mM KCl (■–■–■), and 5.6 mM NaCl and 0.8 mM KCl (□–□–□).

by assuming inhibition is due entirely to blocking an obligatory release of Mg^{2+}. With 140 mM NaCl and 20 mM KCl (the standard ionic composition in figs. 1 and 2) the K_i would be about 150 mM: the intercept with the abscissa in fig. 3. Any competition with Na^+ and K^+, as suggested (fig. 3), would mean a still larger dissociation constant from the hypothetical site.

These limiting values for binding, $K_m \leqslant 2.3\ \mu M$, and for release, $K_i \geqslant 150$ mM, indicate that formulations proposing an obligatory sequential binding and release must require that the affinity for free Mg^{2+} changes at least 60 000 fold with each reaction cycle. By comparison, the change in affinity for K^+, which is transported by the enzyme against a concentration gradient, only undergoes a cyclical change in affinity of about 10^3 [13]

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