Effects of Mg$^{2+}$ Ions on the Plasma Membrane [H$^+$]-ATPase of Neurospora crassa

II. KINETIC STUDIES*

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The rate of ATP hydrolysis by the Neurospora plasma membrane [H$^+$]-ATPase has been measured over a wide range of Mg$^{2+}$ and ATP concentrations, and on the basis of the results, a kinetic model for the enzyme has been developed. The model includes the following three binding sites: 1) a catalytic site at which MgATP serves as the true substrate, with free ATP as a weak competitive inhibitor; 2) a high affinity site for free Mg$^{2+}$, which serves to activate the enzyme with an apparent $K_M$ of about 15 $\mu$M, and 3) a separate low affinity site at which Mg$^{2+}$ causes mixed type inhibition, lowering the $V_{max}$ while raising the $K_M$ for MgATP at the catalytic site. The $K_M$ for Mg$^{2+}$ at the low affinity site is about 3.5 $\mu$M. The model satisfactorily explains the activity of the enzyme as Mg$^{2+}$ and ATP are varied, separately and together, over a wide range. It also can account for the previously reported effects of Mg$^{2+}$ and ATP on the inhibition of the Neurospora [H$^+$]-ATPase by N-ethylmaleimide (Brooker, R. J., and Slayman, C. W. (1982) J. Biol. Chem. 257, 12051–12055; Brooker, R. J., and Slayman, C. W. (1983) J. Biol. Chem. 258, 8827–8832).

The plasma membrane [H$^+$]-ATPase of Neurospora is completely inactivated by the reaction of NEM$^-$ with a single amino acid residue on the $M_r = 104,000$ polypeptide (1, 2). From the way in which inactivation varies as a function of the ligands (ATP, ADP, Mg$^{2+}$) present in the incubation medium (1–3), it has been possible to identify three distinct ligand binding sites. (i) The catalytic site of the enzyme binds MgATP or MgADP, either of which confers protection against NEM inhibition; the $K_M$ values for binding, calculated from the inhibition data, equal the $K_M$ of the enzyme for MgATP and MgADP, respectively. (ii) There is also a high affinity site for free Mg$^{2+}$ which, when occupied, leads to protection against NEM inhibition. (iii) And finally, the binding of Mg$^{2+}$ to a low affinity site has the opposite effect, enhancing the sensitivity of the enzyme to NEM.

It is possible that the high and low affinity Mg$^{2+}$ sites, like the catalytic site, participate directly in the reaction cycle of the ATPase, but inactivation studies with NEM are not capable of revealing such a role. Therefore, we have carried out a series of kinetic experiments to measure the effects of free Mg$^{2+}$, free ATP, and MgATP, varied over a wide concentration range, on ATPase activity. Particular emphasis has been directed towards sorting out which ligands are required as substrates or activators and which serve as inhibitors, and towards determining the corresponding kinetic constants. The results confirm that all three ligand binding sites are involved in the reaction cycle of the Neurospora [H$^+$]-ATPase, and make it possible to derive a rate equation that satisfactorily accounts for enzyme activity as a function of ligand concentrations.

MATERIALS AND METHODS

For kinetic studies, the standard assay mixture contained 50.0 mM PIPES, adjusted to pH 6.7 with Tris base; 0.0025% asolectin; 5.0 mM KC$_2$ and combinations of MgCl$_2$ and ATP in a total volume of 1.5 ml. Enzyme was added and the assay was carried out at 30°C for 5–10 min. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 1.1%. Under these conditions, hydrolysis was a linear function of time for at least 10 min. Inorganic phosphate production was determined by the method of Dryer et al. (4).

All other methods were as described in the preceding paper (3).

RESULTS

Effects of Mg$^{2+}$ and ATP on ATP Hydrolysis

In order to investigate the kinetic behavior of an enzyme, it is customary to vary the concentration of each ligand at several fixed concentrations of the others. With enzymes that use MgATP, however, such an analysis becomes more difficult. Since Mg$^{2+}$ and ATP combine to form the MgATP complex, the concentrations of Mg$^{2+}_{free}$, ATP$_{free}$, and MgATP cannot be varied independently of each other. Instead, as discussed by London and Steck (5), the desired information must be extracted from experiments in which the total concentration of one ligand (e.g. Mg$^{2+}$) is fixed and enzyme activity is observed to vary with changes in the concentration of the second (e.g. ATP).

For the experiment of Fig. 1A, the activity of the Neurospora [H$^+$]-ATPase was measured at several fixed MgCl$_2$ concentrations (0.5, 1.0, and 2.0 mM) while ATP was increased from 0 to 7.5 mM. At each MgCl$_2$ concentration, ATPase activity rose to a maximum and then fell again. The maximal rate of ATP hydrolysis occurred at a point where the MgATP concentration was high (most of the Mg$^{2+}$ in the MgATP form) but where the excess ATP$_{free}$ concentration was relatively low.

Analogously, for Fig. 1B, enzyme activity was measured at several fixed ATP concentrations (1.0, 2.5, or 5.0 mM) while...
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**Fig. 1.** Effect of varying Mg\(_{\text{total}}\) and ATP\(_{\text{total}}\) on the rate of ATP hydrolysis. ATP hydrolysis was measured under standard conditions (see "Materials and Methods"). For A, the total ATP concentration was varied at the following three fixed MgCl\(_2\) concentrations: 0.5 mM (●), 1.0 mM (▲), and 2.0 mM (■). For B, the total MgCl\(_2\) concentration was varied at the following three fixed ATP concentrations: 1.0 mM (●), 2.5 mM (▲), and 5.0 mM (■).

MgCl\(_2\) was varied from 0 to 20 mM. The results were parallel to those just described; once again, the maximal rate was reached where the MgATP concentration was high (most of the ATP in the MgATP form) but where the excess Mg\(^{2+}\) concentration was relatively low. In a separate experiment, it was found that the addition of choline chloride up to a concentration of 50 mM had no significant effect on ATPase activity. Therefore, the inhibitory effects of high concentrations of MgCl\(_2\) or ATP do not appear to be the result of an alteration in ionic strength.

From the results of Fig. 1, it is evident that maximal ATP hydrolysis depends upon the relative concentrations of both Mg\(^{2+}\) and ATP. One possible mechanism to account for this behavior would be sequential binding of Mg\(^{2+}\) and ATP\(_{\text{free}}\) to the enzyme. Alternatively, the true substrate may be the MgATP complex which binds directly to the enzyme in a single step. These two mechanisms can be differentiated on the basis of kinetic studies (6). If it is found that the apparent \( K_0 \) for ATP\(_{\text{free}}\) \( (K_{\text{ATP}}) \) at a given concentration of Mg\(^{2+}\)\(_{\text{free}}\) is equal to the apparent \( K_0 \) for Mg\(^{2+}\)\(_{\text{free}}\) \( (K_{\text{Mg}}) \) at the same concentration of ATP\(_{\text{free}}\), then the mechanism of the reaction involves the binding of the MgATP complex to the enzyme. It must be noted that the apparent \( K_0 \) values for Mg\(^{2+}\)\(_{\text{free}}\) and ATP\(_{\text{free}}\) determined in this fashion do not reflect actual affinities of the enzyme for these two ligands. Rather, \( K_{\text{ATP}} \) and \( K_{\text{Mg}} \) are related to the dissociation constant of the enzyme for MgATP in the following fashion (6):

\[
\begin{align*}
K_{\text{Mg}} & = K_0 K_{\text{MgATP}}/\text{[Mg}^{2+}\text{free]} \quad (I) \\
K_{\text{ATP}} & = K_0 K_{\text{MgATP}}/\text{[ATP}^{\text{free}}\text{]} \quad (II)
\end{align*}
\]

where \( K_0 \) = dissociation constant of MgATP, and \( K_{\text{MgATP}} \) = dissociation constant for MgATP-enzyme complex.

Table I summarizes the results of experiments in which \( K_{\text{Mg}} \) and \( K_{\text{MgATP}} \) were determined at two fixed concentrations of ATP\(_{\text{free}}\), and \( K_{\text{ATP}} \) and \( K_{\text{MgATP}} \) at two fixed concentrations of Mg\(^{2+}\)\(_{\text{free}}\). \( K_{\text{MgATP}} \) was essentially constant under all four conditions, ranging from 0.83 to 0.91 mM. There was also excellent agreement between \( K_{\text{Mg}} \) at 1.0 mM ATP\(_{\text{free}}\) (0.075 mM) and \( K_{\text{ATP}} \) at 1.0 mM Mg\(^{2+}\)\(_{\text{free}}\) (0.068 mM), and likewise between \( K_{\text{Mg}} \) at 2.0 mM ATP\(_{\text{free}}\) (0.037 mM) and \( K_{\text{ATP}} \) at 2.0 mM Mg\(^{2+}\)\(_{\text{free}}\) (0.035 mM), consistent with the notion that MgATP is the true substrate for the enzyme.

### Inhibition of ATP Hydrolysis by Excess ATP

The inhibitory effect of excess ATP, seen in Fig. 1A, could arise in at least two ways. 1) ATP might act as a competitive inhibitor by binding to the catalytic site of the enzyme, or 2) ATP, by complexing Mg\(^{2+}\)\(_{\text{free}}\), might reduce the concentration of an essential activator. Both of these mechanisms seem plausible on the basis of existing data. In studies with NEM (1), free ATP did protect the Neurospora \( [H^+]-\text{ATPase} \) against inactivation, although its calculated \( K_0 \) (11.4 mM) was considerably higher than that of MgATP (1.5 mM). Thus, ATP\(_{\text{free}}\) might be expected to act as a weak competitive inhibitor of enzyme activity. At the same time, NEM studies have given evidence for a separate high affinity Mg\(^{2+}\)-binding site, which might be involved in activation of the enzyme.

In order to gain further information, the ATP concentration was varied in excess above several fixed MgATP concentrations, and enzyme activity was measured. In Fig. 2, values of 1/\( v_0 \) have been plotted as a function of the concentration of ATP\(_{\text{free}}\). In sharp contrast to previous studies with ADP, where simple competitive inhibition was seen (1), inhibition by ATP\(_{\text{free}}\) was markedly biphasic. Thus, although competitive inhibition at the catalytic site almost certainly takes place, it is not sufficient to explain the observed inhibition of enzyme activity by excess ATP\(_{\text{free}}\).

To see whether activation by Mg\(^{2+}\) might also be involved, the data from Fig. 2 were replotted as a function of Mg\(^{2+}\)\(_{\text{free}}\). At each of the four substrate concentrations, ATPase activity rose along a saturation curve reaching half-maximal values at about 0.015 mM Mg\(^{2+}\). This result supports the idea that Mg\(^{2+}\) serves as an essential activator, and suggests that the effect of excess ATP can be explained largely by the removal of Mg\(^{2+}\) from solution. Indeed, when the curves of Fig. 3 were corrected for the probable contribution of ATP as a competitive inhibitor (by assuming a \( K_0 \) of 11.4 mM, equal to the measured \( K_{\text{ATP}} \); Ref. 1), they were changed in only a minor way, shifting upward slightly and becoming a bit more hyperbolic (see inset to Fig. 3).

### Inhibition by Excess Mg\(^{2+}\)

From Fig. 1B, it is also apparent that excess Mg\(^{2+}\) has an inhibitory effect on ATP hydrolysis. To examine this effect in more detail, ATP hydrolysis was measured as a function of MgATP at several fixed ATP\(_{\text{free}}\) concentrations (1, 2, 5 and 10 mM). Under these conditions, the ATP\(_{\text{free}}\) concentration was very low and its inhibitory effects could be neglected. Fig. 4, in which the results have been plotted in double reciprocal form, shows that increasing the Mg\(^{2+}\)\(_{\text{free}}\) concentration from 1 to 10 mM caused a substantial inhibition of ATPase activity. The inhibition appeared to be of a mixed type (partial competitive and pure noncompetitive), lowering the \( V_{\text{max}} \) while
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Fig. 2. Inhibition of ATP hydrolysis by excess ATP. ATP hydrolysis was measured under standard conditions in the presence of varying excess ATP concentrations and at several fixed MgCl\(_2\) concentrations (0.5, 0.75, 1.0, 1.5 mM). At the lowest ATP\(_{\text{free}}\) concentrations, greater than 90% of the Mg\(^{2+}\) was in the MgATP form, so further increases in ATP resulted in a relatively small change in the MgATP concentration. The data are plotted as \(1/v\) versus ATP\(_{\text{free}}\) at the following four MgATP concentrations: 0.5 mM (○), 0.75 mM (+), 1.0 mM (A), and 1.5 mM (m).

Fig. 3. Dependence of ATPase activity on the Mg\(^{2+}\)\(_{\text{free}}\) concentration. The data from Fig. 2 were replotted as velocity versus Mg\(^{2+}\)\(_{\text{free}}\). For the inset, the data at a single MgATP concentration (1.0 mM) were corrected by subtracting the contribution of ATP\(_{\text{free}}\) as a competitive inhibitor assuming a \(K_i\) of 11.4 mM (Ref. 1). The corrected curve (△, solid line) can be compared with the uncorrected curve (△, dashed line).

Fig. 4. Inhibition of ATP hydrolysis by high concentrations of Mg\(^{2+}\)\(_{\text{free}}\). ATP hydrolysis was measured under standard conditions at several fixed Mg\(^{2+}\)\(_{\text{free}}\) concentrations i.e. 1.0 mM (○), 2.0 mM (●), 5.0 mM (▲), and 10.0 mM (●).

Table II. \(K_{\text{Mg}}\) and \(\alpha K_{\text{Mg}}\) values determined at different Mg\(^{2+}\)\(_{\text{free}}\) concentrations.

| Mg\(^{2+}\)\(_{\text{free}}\) (mM) | Apparent \(K_s\) (MgATP) | Apparent \(V_{\text{max}}\) (μmol of P\(_i\)/min/mg of protein) | \(K_{\text{Mg}}\) (mM) | \(\alpha K_{\text{Mg}}\) (mM) |
|-----------------|----------------|-----------------|--------------|--------------|
| 0.1             | 0.56           | 35.2             | 3.52         | 13.5         |
| 0.2             | 0.96           | 33.3             | 3.89         | 14.8         |
| 0.5             | 1.36           | 28.7             | 3.36         | 15.8         |
| 1.0             | 1.79           | 24.1             | 3.45         | 17.6         |

raising the \(K_s\) for MgATP. This type of inhibition is consistent with the notion that the inhibitor (Mg\(^{2+}\)) and substrate (MgATP) do not compete for the same site, but are bound at different sites which interact via conformational changes (8).

\[
E + S \overset{K_s}{\rightleftharpoons} ES \overset{I}{\rightarrow} E + \text{Product}
\]

\[
K_i = \frac{\alpha K_s}{I}
\]

\[
K_{\text{app}} = \frac{K_s(1 + [I]/K_i)}{1 + [I]/(\alpha K_i)}
\]

\[
\frac{V_{\text{app}}}{K_s} = \frac{V_{\text{max}}}{K_s}/(1 + [I]/(\alpha K_i))
\]

Binding of inhibitor to the enzyme lowers the affinity for substrate and vice versa (\(\alpha > 1.0\)); and both \(V_{\text{app}}\) and \(V_{\text{app}}/K_{\text{app}}\) vary with the inhibitor concentration in the following fashion (8):

\[
V_{\text{app}} = \frac{V_{\text{max}}}{1 + [I]/\alpha K_i}
\]

\[
V_{\text{app}}/K_{\text{app}} = \frac{V_{\text{max}}}{K_s}/(1 + [I]/(\alpha K_i))
\]

where [I] = concentration of inhibitor; \(K_s\) = the dissociation constant of ES; \(K_i\) = the dissociation constant of EI; \(\alpha K_i\) = [ES][I]/[EIS], the dissociation constant of I from the ternary ESI complex. These equations assume that ESI does not break down to yield products and that all the binding reactions can be treated as equilibria (8).

In Table II, the values for \(K_s\) (termed \(K_{\text{Mg}}\)) and \(\alpha K_s\) (termed \(\alpha K_{\text{Mg}}\)) were determined at several Mg\(^{2+}\)\(_{\text{free}}\) concentrations, using the data from Fig. 4. The results show that \(K_{\text{Mg}}\) is about 3.5 mM and remains relatively constant over a broad range of inhibitor concentrations (1–10 mM). As expected, the value
for $aK_{m2}$ is substantially higher (~15.4 mM). Taken together, these results support the notion that the Neurospora [H⁺]-ATPase possesses a low affinity inhibitory site for Mg²⁺. Binding of Mg²⁺ to this site reduces the affinity of the enzyme for MgATP, with the apparent $K_a$ increasing from 0.86 mM at 1.0 mM Mg²⁺free to 1.79 mM at 10 mM Mg²⁺free. At the same time, the apparent $V_{max}$ is reduced from 35.2 to 25.1 µmol of P_i/min/mg of protein.

Conclusions from Kinetic Analysis

From the kinetic measurements described above, together with the results of previous NEM studies (1, 3) it is possible to derive a rate equation for the Neurospora [H⁺]-ATPase which involves the binding of ligands at three sites. The following assumptions are made.

Catalytic Site—MgATP is the true substrate (Table I, this paper). ATP is a weak competitive inhibitor (Figs. 2 and 3, this paper; Ref. 1).

High Affinity Mg⁺⁺ Site—Mg₄, binding at this site, serves as an essential activator of the enzyme (that is, only $E(Mg₄)(MgATP)$ breaks down to yield products). The binding of Mg₄ is assumed not to alter the affinity of the enzyme for other ligands and vice versa.

Low Affinity Mg⁺⁺ Site—Mg₅, binding at this site, serves as a mixed type inhibitor. $E(Mg₅)(Mg₅)(MgATP)$ does not break down to yield products.

Rapid Equilibrium Conditions Prevail (That Is, Binding Reactions Are Not Rate-limiting)—This assumption appears valid for the catalytic site, where the dissociation constants for MgATP and MgADP (calculated from NEM protection data) have been shown to agree well with the corresponding values of $K_a$ and $K_i$ (measured kinetically) (1). Because of the complex biphasic effect of free Mg⁺⁺ on NEM inhibition (3), dissociation constants for Mg⁺⁺ cannot be calculated reliably. It is worth noting, however, that $K_{M2}$ (measured kinetically) falls within the range of Mg⁺⁺ concentrations that protect against NEM inhibition, and likewise that $K_{M2}$ falls within the range of Mg⁺⁺ concentrations that enhance NEM inhibition. As a working assumption, therefore, rapid equilibrium seems justified at these sites as well.

All Ligands Bind Randomly—The model is illustrated in Fig. 5. For the sake of simplicity, the binding of ATP to the $E(Mg₅)$ form of the enzyme has been omitted. This enzyme state would not be favored under normal conditions because it requires high concentrations of both Mg²⁺free and ATPfree. The rate equation which describes the model of Fig. 5 in terms of ligand concentrations and equilibrium constants is given under "Appendix" (Equation IX).

In order to see whether the model of Fig. 5 can satisfactorily account for the behavior of the ATPase under some of the conditions examined above, Equation IX was used to calculate enzyme activity as a function of ATP at fixed MgCl₂ (analogous to Fig. 1A) and as a function of MgCl₂ at fixed ATP (analogous to Fig. 1B). The following values were assumed for the equilibrium constants: $K_{MaT}$ = 0.72 mM (extrapolated from data in Table II); $K_{ATP}$ = 11.4 mM (Ref. 1); $K_{M2}$ = 0.015 mM (Fig. 3); $K_{M3}$ = 3.56 mM (Table II); and $aK_{M2}$ = 15.4 mM (Table II). Once the calculated enzyme activities had been scaled appropriately to a $V_{max}$ of 30.9 µmol of P_i/min/mg of protein, they could be seen to follow the same general pattern as the experimentally measured activities from Fig. 1 (see Fig. 6). Considering the complexity of the rate equation and the possibility of error in determining the equilibrium constants, the two sets of values are in good agreement.

Equation IX is also relevant to the behavior of the enzyme under a quite different set of conditions. When assayed over a range of equimolar ATP and Mg⁺⁺ concentrations, the Neurospora [H⁺]-ATPase has previously been observed to display sigmoid kinetics with a Hill number of about 1.6–2.0 (Ref. 9 and 10). The sigmoidicity can be explained by pos-

**FIG. 6. Comparison of predicted and observed values for enzyme activity as a function of ATP and Mg²⁺.** The predicted values (open symbols) were calculated using Equation IX and values for $K_{MaT}$, $K_{ATP}$, $K_{M2}$, $K_{M3}$, and $aK_{M2}$ described in the text. The observed values (closed symbols) were taken from the data of Fig. 1. For A, the total ATP concentration was varied at the following three fixed MgCl₂ concentrations: 0.5 mM (○, ○), 1.0 mM (△, △), and 2.0 mM (□, □). For B, the total MgCl₂ concentration was varied at the following three fixed ATP concentrations: 1.0 mM (○, ○), 2.5 mM (△, △), and 5.0 mM (□, □).

**Fig. 7. Predicted and observed velocity curves as Mg²⁺ and ATP were varied at a 1:1 ratio.** The predicted values (open symbols, solid line) were calculated using Equation IX and values for $K_{MaT}$, $K_{ATP}$, $K_{M2}$, $K_{M3}$, and $aK_{M2}$ described in the text. For the observed values (closed symbols, dashed line), ATPase activity was measured under standard conditions at a MgATP ratio of 1:1. The resulting ATPase activities have been plotted as a function of the added concentration of ATP and Mg²⁺.
equations. The numerator and denominator are then expressed in terms of ligand concentra-

The model has been derived principally from measurements of enzyme activity over a wide range of ligand concentrations, but the mode of inhibition appears to vary. For the [H⁺]-ATPase of S. cerevisiae, inhibition has been reported to be "pseudocompetitive," suggesting that the binding of Mg⁺ at the inhibitory site reduces the affinity of the catalytic site for substrate, but that ES and ESI complexes are split with equal velocity (20). For the [Na⁺,K⁺]-ATPase, Mg⁺ has been variously described to act as an uncompetitive (11) or noncompetitive (12, 14) inhibitor. And for the [Ca²⁺]-ATPase of sarcoplasmic reticulum, inhibition by high Mg⁺ concentrations is partially reversible by Ca²⁺, making it difficult to distinguish effects at the putative inhibitory site from effects at the specific Ca²⁺ transport sites on the enzyme (21). Further work will be required to establish the extent to which inhibition by high Mg⁺ concentrations shares a common mechanism in these various cases.

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APPENDIX

From the model of Fig. 5, a rate equation can be derived which expresses the velocity of ATP hydrolysis in terms of the ligand concentrations and equilibrium constants. The method of derivation is analogous to that described by Segel (22). Initially, the velocity dependence equation (VI) is written:

\[ v = k_p E(MgA)(MgATP) \]

where \( E(MgA)(MgATP) \) is the concentration of the only species assumed to yield product and \( k_p \) is the catalytic rate constant. Both sides of the velocity dependence equation are then divided by \( E_T \), where \( E_T \) on the righthand side is expressed as the sum of concentrations of all species (equation VII):

\[ \frac{v}{E_T} = \frac{k_p E(MgA)(MgATP)}{E + E(FTP) + E(MgATP) + E(Mg_A) + E(Mg_B) + E(Mg_A)(MgATP) + \ldots} \]

The numerator and denominator are then expressed in terms of ligand concentra-
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tions and equilibrium constants, assuming \(E = 1\) (equation VII):

\[
\frac{v}{E_T} = \frac{k_p[Mg][MgATP]}{K_{MgA} K_{MgATP}}
\]

\[
1 + \frac{[ATP]}{K_{ATP}} + \frac{[MgATP]}{K_{MgATP}} + \frac{[Mg]}{K_{MgI}} + \frac{[Mg][MgATP]}{\alpha K_{MgATP}}
\]

And finally, the equation is factored and rearranged, taking \(k_pE_T\) to be \(V_{max}\) (equation IX):

\[
\frac{v}{V_{max}} = \frac{[Mg][MgATP]}{K_{MgA} K_{MgATP}}
\]

\[
\left(1 + \frac{[ATP]}{K_{ATP}} + \frac{[MgATP]}{K_{MgATP}} + \frac{[Mg]}{K_{MgI}} + \frac{[Mg][MgATP]}{\alpha K_{MgATP}}\right)^{-1}
\]

\[
\frac{[Mg]}{K_{MgA}} \left(1 + \frac{[ATP]}{K_{ATP}} + \frac{[MgATP]}{K_{MgATP}} + \frac{[Mg]}{K_{MgI}} + \frac{[Mg][MgATP]}{\alpha K_{MgATP}}\right)
\]

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