We report evidence that adenylate kinase (AK) from *Escherichia coli* can be activated by the direct binding of a magnesium ion to the enzyme, in addition to ATP-complexed Mg$^{2+}$. By systematically varying the concentrations of AMP, ATP, and magnesium in kinetic experiments, we found that the apparent substrate inhibition of AK, formerly attributed to AMP, was suppressed at low magnesium concentrations and enhanced at high magnesium concentrations. This previously unreported magnesium dependence can be accounted for by a modified random bi-bi model in which Mg$^{2+}$ can bind to AK directly prior to AMP binding. A new kinetic model is proposed to replace the conventional random bi-bi mechanism with substrate inhibition and is able to describe the kinetic data over a physiologically relevant range of magnesium concentrations. According to this model, the magnesium-activated AK exhibits a 23-±3-fold increase in its forward reaction rate compared with the unactivated form. The findings imply that Mg$^{2+}$ could be an important affector in the energy signaling network in cells.

Adenylate kinase (AK) is a ~24-kDa enzyme involved in cellular metabolism that catalyzes the reversible phosphoryl transfer reaction (1) as in Reaction 1.

\[
\text{forward: } \text{Mg}^2+ \cdot \text{ATP} + \text{AMP} \leftrightarrow \text{Mg}^2+ \cdot \text{ADP} + \text{ADP}
\]

\[
\text{reverse: } \text{Mg}^2+ \cdot \text{ADP} + \text{ADP} \leftrightarrow \text{Mg}^2+ \cdot \text{ATP} + \text{AMP}
\]

Here, we report a comprehensive kinetic study of the forward reaction of AK, exploring concentrations of nucleotides and Mg$^{2+}$ that are comparable to those inside *E. coli* cells, [Mg$^{2+}$] ~ 1–2 mM (38) and [ATP] up to 3 mM (39). We discovered a previously unreported phenomenon: an increase in the forward reaction rate of AK with increasing Mg$^{2+}$ concentrations, where the stoichiometry of Mg$^{2+}$ to the enzyme is greater than one. The new observation leads us to propose an Mg$^{2+}$-activation mechanism augmenting the commonly accepted random bi-bi model for *E. coli* AK. Our model can fully explain AK's observed kinetic behavior involving AMP, ATP, and Mg$^{2+}$ as substrates, out-performing the previous model requiring AMP inhibition. The new Mg$^{2+}$-activation model also explains the dis-
crepancies in AMP inhibition behavior and currently available *E. coli* AK kinetic data. Given the central role of AK in energy regulation and our new experimental evidence, it is possible that Mg$^{2+}$ and its regulation may participate in respiratory network through AK (40–42), an exciting future research direction.

**EXPERIMENTAL PROCEDURES**

*Sources*—AMP, ADP, ATP, pyruvate kinase (PK), lactate dehydrogenase, bovine serum albumen (BSA), phosphoenolpyruvate (PEP), dithiothreitol, and EGTA were purchased from Sigma-Aldrich and used without further purification. Unless specified, other reagents were also from Sigma. Nicotinamide adenine dinucleotide disodium salt (NADH) was from Indofine Chemical Co.

**Purification of AK**—The wild-type *E. coli* AK gene from the K-12 strain was a generous gift from Prof. Michael Glaser's group. The wild-type adenylate kinase was purified in a manner similar to that described earlier (28). The protein was first purified with an Affi-Gel® Blue Gel column (100–200 mesh, Bio-Rad). Further purification was performed with a Q-Sepharose anion exchange column (Amersham Biosciences) and a 1-m S-200 gel-filtration column (Amersham Biosciences).

**Forward Activity Assay of Adenylate Kinase**—All of the work was done with the assay buffer: 100 mM Tris (pH by acetic acid), pH 7.5, 100 mM KCl, 0.05 mg/ml (0.75 μM) BSA, and 0.6 mM TCEP. The production of ADP was monitored with the coupling enzymes PK and lactate dehydrogenase. Each ADP produced caused the oxidation of one NADH to NAD$^+$ and was detected by the absorbance change at 340 nm (29). The coupling reagent concentrations used were: 4 mM PEP and 0.25 mM NADH with 1.5 unit of PK and 2 units of lactate dehydrogenase per 100-μl reaction. The concentration of AK in each reaction was 0.89 μM. The forward reaction rates of AK were assayed at AMP and ATP concentrations between 0 to 3 mM, and magnesium acetate (MgOAc$_2$) concentrations from 0 to 1.5 mM. All the measurements were carried out in 96-well plates and read with a microplate reader (Molecular Devices SpectraMax M5) at room temperature (24 °C). All of the rates reported in this work are initial reaction rates. Each experiment was repeated at least three times and averaged to determine error bars. All error bars are reported to one standard deviation. Although PK is known to utilize Mg$^{2+}$ as a cofactor, the amount of PK in each reaction is at least two orders of magnitude lower than the lowest Mg$^{2+}$ concentration used (43). Therefore, PK should have negligible effect on the Mg$^{2+}$ concentration in each reaction. To ensure that BSA does not affect reactivity readout, a control experiment was done where AK reactivity was assayed while systematically varying BSA concentrations between 0 and 1 mg/ml (15 μM). The results showed that BSA does not affect AK reactivity, which in turn indicates that BSA does not affect the Mg$^{2+}$ concentration in the solution (see supplemental materials).

**Mg$^{2+}$ Ion-activated Random bi-bi Mechanism for the Forward Reaction**—To illustrate magnesium ion activation of AK, we propose a model with two different forms of the enzyme with distinct catalytic rates, the original unactivated enzyme (E) and the Mg$^{2+}$-activated form (E$^\theta$). Note that this is different from the iso-random bi-bi mechanism proposed earlier for rabbit muscle AK (32). In that work, the two putative enzyme forms were attributed to two different conformations of AK, in which each form follows its own random bi-bi mechanism with distinct dissociation constants and catalytic rates. In the present work, the relative population of the activated enzyme, E$^\theta$, is determined by the Mg$^{2+}$ affinity of unactivated enzyme. Judging from the sigmoidal shape of initial rate curves with increasing AMP concentrations, we propose that Mg$^{2+}$ can only bind to the enzyme prior to AMP binding, but may dissociate from all the activated E$^\theta$ species. Therefore, the Mg$^{2+}$ affinity cannot be described by a simple dissociation constant between activated and unactivated species. We employ a hybrid rapid equilibrium and steady-state approximation to describe this ordered binding (45, 46).

**Mass Balance of Mg$^{2+}$**

The experiments were performed in the same plate reader in 384-well plates at room temperature (24 °C). Each well contained 2 mM Mg$^{2+}$ and 20 μM mag-fluo-4 indicator. The concentration of AK was assayed between 0 and 1.5 mM. Each experiment was blanked at the same protein concentration but zero Mg$^{2+}$, and all the measurements were repeated at least three times and averaged to determine error bars. The binding constant was calculated by fitting the titration curve to the numerical solution of coupled equations: AK + Mg$^{2+}$ ↔ AK·Mg$^{2+}$, $K_d = \frac{[AK][Mg^{2+}]}{[AK·Mg^{2+}]}$ and Ind + Mg$^{2+}$ ↔ Ind·Mg$^{2+}$, $K_d = \frac{[Ind][Mg^{2+}]}{[Ind·Mg^{2+}]}$, where Ind denotes the indicator, and $K_d$ for the indicator is 4.7 mM.

A series of control experiments on BSA, PEP, and TCEP were carried out in similar fashion to ensure that none of these reagents bind Mg$^{2+}$ strongly enough to interfere with the AK kinetic assay. The Mg$^{2+}$ binding assay for BSA was done in the same condition as AK, but the concentration of BSA was varied between 0 and 2.9 mM. The $K_d$ measurements for PEP and TCEP were done in a buffer containing 100 mM KCl, 100 mM Tris/HCl, pH 7.5, 20 μM EGTA, and 0.01% Tween 20. The surfactant Tween 20 was necessary to minimize scattered light during top read fluorescence measurements in the absence of BSA.

**Analytical Procedures**—Data analysis and multiparameter, global chi-squared fitting was done in Matlab (Mathworks, Inc.) with fminuit (44). Error bars for each fit parameter were obtained by a 100-iteration Monte Carlo simulation of the original data set; all errors are reported to one standard deviation. Mass balance of Mg$^{2+}$ ions was achieved numerically by using $K_d$ values measured in this work (PEP, TCEP, and BSA), determined from literature (ATP) or fit as a model parameter (AK).
Direct Mg$^{2+}$ Binding Activates AK from E. coli

Fig. 1a, the proposed Mg$^{2+}$-activation model. The forward AK reactions of the unactivated enzyme (E) and its Mg$^{2+}$ activated counterpart (E*) each follow a random bi-bi model, illustrated by the diamond shapes and assumed to be in rapid equilibrium. The conversion between E and E* (vertical dashed lines) is treated with the steady-state approximation. In the activated form (the upper tier), the dissociation constants $K_{\text{AMP}}$ and the catalytic rate $k_{\text{cat}}$ are increased by a factor of $\alpha$ and $\beta$, respectively ($E = \text{AK enzyme}, E* = \text{AK-Mg}^{2+}$, $T = \text{ATP-Mg}^{2+}, M = \text{AMP}$, and $D = \text{ADP}$. $T$ denotes ATP without Mg$^{2+}$ that has been bound to the enzyme). The blue arrows denote the second order correction to our proposed model, a reduction of the model in a according to the hybrid rapid equilibrium and steady-state assumption used to model the kinetic data.

The relative populations of the unactivated ($X$), and activated species ($X^*$), are determined according to the following steady-state approximation.

$$X = k_{\text{off}}$$

(Eq. 3)

$$X^* = \left( k_{\text{sw}}[\text{Mg}^{2+}] \left( 1 + \frac{[M]}{K_{\text{AMP}}} + \frac{[T]}{K_{\text{ATP}}} + \frac{[D]}{K_{\text{ADP}}} \right) \right) / \sum E$$

(Eq. 4)
The last term in Equation 4 represents the second order correction, which allows the bound Mg\(^{2+}\)·ATP to lose its Mg\(^{2+}\) directly to AK. The fractions of product producing species can be written as in Equations 5–7.

\[
f_{TEM} = \frac{[M]}{K_{AMP} + [T]/\Sigma E}
\]

(Eq. 5)

\[
f_{TEM} = \frac{[M]}{\alpha K_{AMP} [T]/\Sigma E^*}
\]

(Eq. 6)

\[
f_{TEM} = \frac{[M]}{\alpha K_{AMP} [T]/\Sigma E^*}
\]

(Eq. 7)

The initial rate can be written as,

\[
\frac{v}{[E_{tot}]} = \frac{k_{cat} f_{TEM} \cdot (X) + (\beta f_{TEM} + f_{TEM}) \cdot (X_c)}{(X) + (X_c)}
\]

(Eq. 8)

where [E\(_{tot}\)] denotes the total enzyme concentration. Inserting Equations 1–7 into Equation 8, we arrive at Equation 9.

In Equation 9, the Mg\(^{2+}\) association and dissociation rates, \(k_{on}\) and \(k_{off}\) always appear as a ratio. In the results section, we will only report this ratio, because we cannot resolve rates independently of the fitting. This ratio, \(k_{cat}/k_{on}\), can be interpreted as an effective dissociation constant and corresponds to the \(K_d\) measured in the direct binding assay, although the amount of enzymes that bind Mg\(^{2+}\) under reactive conditions also depends on the AMP concentration and the Mg\(^{2+}\) swapping rate constant, \(k_{swap}\). For convenience in the fitting, we define the factor \(\kappa\) to have the relationship \(k_{swap} = \kappa k_{on}\). Here, the factor \(\kappa\) has units of millimolar, because \(k_{swap}\) and \(k_{on}\) carry different units. This proposed model, while highly simplified due to limited set of fitting parameters, illustrates the kind of mechanistic features needed to fully describe the observed kinetics.

RESULTS AND DISCUSSION

In Fig. 2a, we have plotted initial rate versus [AMP] curves with [ATP] = 0.15 mM at various Mg\(^{2+}\) concentrations. From the dissociation constant for Mg\(^{2+}\)·ATP, we can estimate the Mg\(^{2+}\)·bound ATP concentration at each Mg\(^{2+}\) concentration. The result is plotted in the inset of Fig. 2a assuming the Mg\(^{2+}\)·AMP dissociation constant from Ref. 47 (data plotted in crosses). In the inset, we have also plotted the representative initial rates at 0.2 mM AMP and 0.15 mM ATP. From the inset we can see that the increase of initial rate is much greater than the increase of total [Mg\(^{2+}\)·ATP]. The results indicate that the increase in the concentration of Mg\(^{2+}\)·ATP is not the sole contribution to the Mg\(^{2+}\) dependence of the reaction rates in Fig. 2a. This observation clearly shows that Mg\(^{2+}\) enhances the forward reaction rate. Armed with the knowledge that AK is activated by Mg\(^{2+}\), we next investigate the stoichiometry of the Mg\(^{2+}\) ions. The monophosphate kinase family to which AK belongs is capable of binding to more than one divalent metal ion, as demonstrated by x-ray crystallography (12, 48–51). More broadly, the reactivity of protein kinases are also known to be tuned by a second Mg\(^{2+}\) ion in addition to the ATP-occupied Mg\(^{2+}\) ion (52), also supported by x-ray crystallography (53–55). For protein kinases, the effective stoichiometry of catalytically important Mg\(^{2+}\) binding has been determined by kinetic studies (52). Similarly, to investigate AK’s Mg\(^{2+}\) dependence, we have performed kinetic experiments systematically varying the Mg\(^{2+}\) concentration while keeping [ATP] = [AMP] = 0.2 mM. The experimental results are displayed in Fig. 2b. The dashed line is the best fit from a model in which Mg\(^{2+}\) is only complexed to ATP (a stoichiometry of one, details in supplemental materials). Fig. 2b clearly shows that the model with a single Mg\(^{2+}\) ion starts to level out as magnesium concentrations exceed that of ATP. This is in sharp contrast to the experimental data, which show the reaction rate continues to rise beyond [Mg\(^{2+}\) = 0.2 mM throughout the entire [Mg\(^{2+}\)] range. By contrast the newly proposed Mg\(^{2+}\) activation model, with a stoichiometry of two magnesium ions, the simplest model that accounts for an effective magnesium stoichiometry greater than one, correctly reproduces the experimentally observed Mg\(^{2+}\) dependence. The result shows that more than one magnesium ion participates in catalysis and that additional Mg\(^{2+}\) enhances the reaction rate.

We next determine whether magnesium ions can bind directly to AK. A magnesium binding assay was carried out for this purpose and the results are plotted in Fig. 3. It is clear that the fluorescence level of the indicator, mag-fluo-4, changes with protein concentration. These data strongly support the hypothesis that magnesium ions bind directly to AK. From the reduction of fluorescent intensity with increasing AK concentration, we determine the dissociation constant of Mg\(^{2+}\)·AK to be 4.0 ± 1.5 mM (data and fit shown in the inset of Fig. 3). The same assay was performed on other reagents used in the kinetic assay to determine their respective affinities for magnesium. The \(K_d\) for magnesium ion with TCEP and PEP were determined to be 39.1 ± 4.9 mM and 20.7 ± 1.2 mM, respectively (see
Direct Mg$^{2+}$ Binding Activates AK from E. coli

supplemental materials for data and fit). These Mg$^{2+}$ binding affinities are almost an order of magnitude weaker than AK, so the effects of TCEP and PEP competing for Mg$^{2+}$ with AK should be negligible. The $K_d$ of BSA was determined to be 0.91 ± 0.15 mM. Note that, in this work, we assume the stoichiometry of Mg$^{2+}$ binding for all of the reagents to be one to one. Under the situation that BSA binds to more than one Mg$^{2+}$, the $K_d$ reported would be an underestimation. Because we use 0.05 mg/ml (0.75 μM) BSA in our reaction, the BSA would not bind >0.9 μM of Mg$^{2+}$, which would result in a negligible reduction of the amount of Mg$^{2+}$ in the reaction.

Having established that Mg$^{2+}$ can directly bind to E. coli AK and that direct interaction between Mg$^{2+}$ and AK elevates its reactivity, we next study the mechanistic roles of the additional Mg$^{2+}$. The kinetic data for the forward reaction at four Mg$^{2+}$ concentrations is shown in Fig. 4a. A multiparameter global fit, which includes all Mg$^{2+}$ concentrations, using the conventional random bi-bi model with competitive AMP and free ATP inhibition is plotted in the red lines ($x^2 = 1.7$). This model is unable to reproduce the characteristic sigmoidal shape seen at higher Mg$^{2+}$ concentrations. If one fits each magnesium concentration individually, the $k_{cat}$ predicted by the conventional model would be magnesium-dependent. This can be illustrated by considering the magnesium dependence of the initial rates when the concentrations of AMP and ATP are the same, as seen in Fig. 2a. If we try to address the Mg$^{2+}$ activation by building a two-tier random bi-bi model, with the upper and lower tiers representing an activated and unactivated AK, respectively, we arrive at a global fitting as shown in Fig. 4b ($x^2 = 1.5$, see supplemental materials for the details of this model). The fits shown in Fig. 4b are not too different from Fig. 4a and are still unable to capture the unique Mg$^{2+}$-dependent features evident in the data (note the regions at both high and low AMP concentrations). The behavior of the entire series of curves argues against the model of AMP inhibition as a major contribution to the sigmoidal curves and suggests the need for a new model to explain the observed Mg$^{2+}$ and AMP dependence of the rates.

The standard random bi-bi with Mg$^{2+}$-activation model failed to capture the steep drop of rates with increasing AMP concentrations. Because the AMP inhibition is already taken into account in this model, there must be additional effects that contribute to the depression of the rates. These results led us to propose a new model in which the increase of AMP will hinder the Mg$^{2+}$ activation. One explanation is that the binding of AMP hinders Mg$^{2+}$ activation, such that activation can only be achieved by a sequential binding of Mg$^{2+}$ followed by AMP.

The newly proposed Mg$^{2+}$-activation modified random bi-bi model can be globally fit to the forward assay data, as shown in Fig. 5. The fitting results are summarized in Table 1. The fitting results reported in Table 1 were obtained by fixing the $K_d$ (i.e. $k_{cat}/k_{on}$ in the model) to the measured value of 4.0 mM (see

![FIGURE 2. Initial rates versus [AMP].](image1)

![FIGURE 3. The fluorescence spectra from the AK Mg$^{2+}$ binding assay.](image2)

**FIGURE 2.** Initial rates versus [AMP]. a, the rates at [ATP] = 0.15 mM for four different total Mg$^{2+}$ concentrations. An increase in initial rate at higher [Mg$^{2+}$] is seen from this comparison. Inset: an estimate of the Mg$^{2+}$-ATP complex concentrations at the assay conditions in a. Using dissociation constants of Mg$^{2+}$-ATP from Ref. 47, the concentrations of Mg$^{2+}$-ATP complex are plotted in crosses. Initial rates from a at [AMP] = 0.2 mM are also plotted for comparison. The scale of these rates is at the right-hand side. These data suggest that the observed Mg$^{2+}$ activation is not solely due to an increase in the available pool of Mg$^{2+}$-ATP, b, the same assay is done at [ATP] = [AMP] = 0.2 mM as a function of [Mg$^{2+}$]. The conventional random bi-bi mechanism (dashed curve) with a stoichiometry of one magnesium (bound to ATP) predicts a Mg$^{2+}$ rate curve, which plateaus when the Mg$^{2+}$ concentration is similar to the ATP concentration, in contrast to the real data. On the other hand, the proposed Mg$^{2+}$ activation model with a stoichiometry of two Mg$^{2+}$ ions (solid line) exhibits excellent agreement with data. Combining the findings of a and b, the data indicate that AK is catalytically activated by Mg$^{2+}$ ions.

**FIGURE 3.** The fluorescence spectra from the AK Mg$^{2+}$ binding assay. The monotonic reduction of fluorescence intensity with increasing AK concentration is a clear indication of direct magnesium ion binding to AK. In the inset we have plotted the fluorescence intensity (at 520 nm) of the indicator versus AK concentration. We then fit the data to a curve by numerically solving the mass balance equations between the free Mg$^{2+}$, Mg$^{2+}$-indicator complex (known $K_d$ = 4.7 mM), and Mg$^{2+}$-enzyme complex (the unknown). The $K_d$ value for $E^*$ was found to be 4.0 ± 1.5 mM.
supplemental materials for results with \( k_{\text{off}}/k_{\text{on}} \) as a free parameter). This model predicts a \( K_{IM} \) (dissociation constant for inhibitory AMP), which is more than two orders of magnitude larger than substrate dissociation constants \( K_{AMP} \) or \( K_{ATP} \), indicating that the effects from AMP inhibition are very weak. Therefore, we have removed competitive inhibition by AMP from the model (fitting results with AMP inhibition considered are reported in the supplemental materials). This result further substantiates our proposal that the sigmoidal rate versus [AMP] curve is not caused by AMP inhibition. Instead, it can be accounted for by the requirement for a sequential binding of the activator \( Mg^{2+} \) followed by AMP. We first fit our data with the model described in Fig. 1 without the second order corrections, illustrated by black lines in Fig. 1a. Without the second order correction, this model can generate a satisfactory fit to the data (dashed lines in Fig. 5, \( \chi^2 = 1.36 \)). The major discrepancy, however, comes from experiments at high \( Mg^{2+} \) and ATP concentrations, where the model predicts a more significant inhibition than was observed experimentally. In this regime, the \( Mg^{2+} \)-ATP concentration is relatively high compared with the low [\( Mg^{2+} \)] conditions. To explain this discrepancy, an alternate channel to mitigate inhibition at higher ATP and \( Mg^{2+} \) concentrations is required. Therefore, as a second order correction, we hypothesize that, once \( Mg^{2+} \)-ATP is bound to AK, the magnesium ion can occasionally be transferred to AK's \( Mg^{2+} \) binding site. The \( T_{E'M} \) complex (the \( Mg^{2+} \)-bound AK complexed with a free ATP and AMP) can, in turn, produce ADP. If we include the effects of the second order correction (blue lines in Fig. 1), we obtain a very satisfactory fit, solid lines in Fig. 5 (\( \chi^2 = 0.71 \)). These results indicate that in the \( Mg^{2+} \)-activated “super-enzyme,” \( E^* \), works 23 ± 3-fold faster than the unactivated form. The AMP binding affinity of the \( Mg^{2+} \)-activated enzyme is 1.4 ± 0.1 times weaker, and this small change in nucleotide affinity can possibly be attributed to a reconfiguration of AK’s active site upon the addition of the activating magnesium ion.

Although the main goals of this report were to report the AK unexpected magnesium-dependent activity and investigate its mechanistic origin, it is nevertheless instructive to consider possible physical origins for the observation. It is common for divalent metal ions to bind to enzymes and, in turn, facilitate catalytic reactions. A number of proteins are known to directly bind \( Mg^{2+} \) (56–60). The crystal structures of \( E. coli \) AKs with substrates (AMP, ATP analog, and \( Mg^{2+} \)) were done with a resolution of \( \sim 2 \) Å (15). However, that work was unable to resolve the precise location of any \( Mg^{2+} \) ions, even though the experimental resolution was sufficient for detection of \( Mg^{2+} \) ions. This is consistent with the low binding affinity (\( \sim 4 \) mM) measured in this work. The lack of \( Mg^{2+} \)-bound crystal structure leaves room for speculating its possible binding site in AK.

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**TABLE 1**

Parameters generated from a global fitting of the magnesium activation model (Equation 9) to the AK forward assay data presented in Fig. 5.

| Parameter | \( Mg^{2+} \) activation model | Model with second order correction |
|-----------|-------------------------------|---------------------------------|
| \( k_{\text{cat}} \) (s\(^{-1}\)) | 605 ± 22 | 319 ± 28 |
| \( K_{AMP} \) (mM) | 0.070 ± 0.005 | 0.207 ± 0.017 |
| \( K_{ATP} \) (mM) | 0.088 ± 0.006 | 0.049 ± 0.003 |
| \( K_{\text{in}} \) (mM) | 0.60 ± 0.12 | 0.28 ± 0.04 |
| \( k_{\text{off}}/k_{\text{on}} \) (mM) | 4.0 ± 1.5 (fixed) | 4.0 ± 1.5 (fixed) |
| \( \kappa = k_{\text{off}}/k_{\text{on}} \) (mM) | NA* | 1.9 ± 0.3 |
| \( \alpha \) | 8.6 ± 1.4 | 1.34 ± 0.13 |
| \( \beta \) | 52.6 ± 6.4 | 23.2 ± 3.0 |
| \( \chi^2 \) | 1.36 | 0.71 |

* NA, not applicable.
Earlier studies on the E. coli AK “AMP inhibition” can provide some clues. In the works by Gilles et al. (36), and by Liang et al. (35), the turnover rates of wild-type E. coli AK have been compared with those from mutants Pro-87 → Ser and Phe-86 → Trp, respectively. It is clear from the data shown that the “AMP inhibition” behavior was entirely eliminated by introducing a mutation on residues 86 or 87. If we interpret the same data, not from the AMP inhibition point of view, but under the Mg$^{2+}$-activation model, the mutations can be viewed as abolishing the Mg$^{2+}$ ion binding capability, presumably at a site nearby or allosterically linked to residues 86 and 87. In fact, from available crystal structures (Fig. 6), residues 86 and 87 (in magenta) are directly beneath the AMP binding site. This interpretation therefore provides a tantalizing physical picture for our model in which AMP binding frustrates direct Mg$^{2+}$ binding to AK. In search of the likely Mg$^{2+}$-binding sites, we compared an E. coli AK structure (1ANK (61)) to UMP kinase from Pyrococcus furiosus (2BMU (51)), in which the substrates with two Mg$^{2+}$ were resolved and discussed. From a detailed comparison of the two structures, we can single out the possible Mg$^{2+}$ binding residues. In Fig. 6, Asp-33, Asp-84 (both drawn in yellow), Ser-30 (in deep blue), and the γ-phosphate group may form one of the divalent metal ion sites. The other possible site resides in between Thr-15, β-phosphate group, and shares Asp-84 with the first site. The proposed Mg$^{2+}$ sites are marked in green in Fig. 6. Asp-84 is very close to the two residues, Phe-86 and Pro-87, which were previously shown to abolish the sigmoidal AMP inhibition behavior discussed above (34, 35). This physical picture is also consistent with the proposed second-order correction to the model where Mg$^{2+}$ can exchange binding sites in the highly flexible interior of the enzyme (cf. the pathway indicated by the blue line in Fig. 1).

The demonstration of the Mg$^{2+}$-dependent mechanism of AKs raises an obvious question: What is the biological role of Mg$^{2+}$-activation in adenylate kinase? To discuss this question, we consider the primary biochemical function of AK, to rapidly equilibrate ATP-Mg, AMP, ADP-Mg, and ADP. The [ATP]/[ADP] ratio in its general form has long been recognized as an important parameter in energy regulation in cells (62, 63). In bacteria, for instance, imbalances in the [ATP]/[ADP] ratio have been associated with changes in its DNA super-coiling state (64–66) and also with its carbon source intake efficiency (67). More generally, the energy state when [Mg$^{2+}$] is constant is expected to be regulated by molecular sensing of [ATP-Mg]/[ADP-Mg], where the magnesium-bound nucleotides serve as substrates for various sensing cascades, or by sensing [ATP]/[ADP]$_{free}$ where the unbound nucleotides may act as inhibitors in the energy regulatory network (41). These ratios, however, are mediated by Mg$^{2+}$ concentration through the association between Mg$^{2+}$ and ATP or ADP. Indeed, the possible roles of Mg$^{2+}$ in energy homeostasis have been pointed out by Blair almost four decades ago (68). Because magnesium is required in many biochemical processes and is generally well regulated (69), Blair further hypothesized that the regulatory role that magnesium ion play through AK is during the transient response of a cell’s energy state (68).

The foregoing discussions and the findings in the present work therefore frame a hypothesis that AK serves as a coupling enzyme between the Mg$^{2+}$ regulatory network and bioenergy homeostasis where the Mg$^{2+}$-activated mechanism helps to cope with transient increase in Mg$^{2+}$ concentration, for example, by hormonal stimuli in mammalian cells (70). Therefore, our findings raise the possibility of further investigations of the regulatory roles of AK by examining the transient coupling between Mg$^{2+}$ and energy regulation networks (71).

In conclusion, we have discovered that the E. coli adenylate kinase forward enzymatic reaction can be activated by direct binding a Mg$^{2+}$ ion, prior to AMP binding. When globally fit to AK kinetic data over a range of substrate concentrations, including various Mg$^{2+}$ concentrations, this newly proposed model generates a satisfactory fit. This finding may have implications of the role of AK in the magnesium-regulated energy homeostasis network.

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