Mechanistic Characterization and Evolution of Adenylate Kinase

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Department of Biochemistry
Dorothee Kern, Advisor

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by
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ABSTRACT

Mechanistic Characterization and Evolution of Adenylate Kinase

A thesis presented to the Department of Biochemistry

Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts

By Padraig Murphy

In order to maintain homeotic nucleotide concentrations in the cell, the essential and ubiquitous adenylate kinase (ADK) catalyzes the reversible reaction of ATP and AMP to 2 ADPs. The mechanism, both conformational and chemical, was investigated extensively to characterize the role of the magnesium ion (Mg$^{2+}$) cofactor and probe the transition state bonding scheme. Kinetic and NMR experiments performed with high concentrations of EDTA allowed for the determination of microscopic rate constants in the absence of a metal cofactor for the conformational step, lid opening, and the chemical step, phosphoryl transfer.
The presence of a metal cofactor accelerated both lid opening and phosphoryl transfer by a factor of at least 10^3 and 10^4, respectively, with Mg^{2+} exhibiting the greatest chemical step enhancement on the order of 10^6. The lid opening and phosphoryl transfer rate constants for other divalent metals (Ca^{2+}, Co^{2+}, Mn^{2+}... etc.) were also measured. When the rate constant of phosphoryl transfer was related to charge density of the metal ion, proximity to the charge density of Mg^{2+} was correlated to in an increase in rate constant. These results suggest that although lid opening is generally accelerated by the presence of a 2+ charge, the cofactor’s role in phosphoryl transfer is highly specialized for a cation of specific charge and size.

The character of the transition state of phosphoryl transfer was examined through site-directed mutagenesis of highly conserved active site arginines in a thermophilic ADK. Mutations of catalytic arginines to lysines were used to interrupt residue-specific nucleophile and leaving group activation of the nucleotide substrates, contingent upon the direction of the reaction. The ratios of the forward and reverse rate constants for two mutants, R124K and R161K, were hypothesized to indicate the bonding scheme of the transition state, be it more pentavalent-like (tight) or metaphosphate-like (loose) in character. Because all of the observed rate constants were within an order of magnitude, on-enzyme equilibrium experiments were performed to establish relative populations of substrate-bound ADK at equilibrium. ADKs preference for the E•ADP•ADP state was maintained between the wild-type protein and the three arginine mutants, thereby demonstrating that nucleophile and leaving group activation was not specifically interrupted by our
mutations. Therefore, it was impossible to discern the transition state bonding scheme from these experiments.

Phylogenetic sequencing and ancestral reconstruction were carried out using three extant isoforms of ADK. By resurrecting ADK ancestors, we hoped to show that as the Earth cooled, entropic and enthalpic contributions to catalysis adjusted over time to account for the loss in available thermal energy. Through temperature dependences of two ADK ancestors, it was determined that the ancestors were not significantly active, likely due to the large phylogenetic distance between the starting protein sequences. For future investigations, an ancestral tree is being created from a single, bacterial genus, Bacillus.
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List of Abbreviations

aADK = Aquifex isoform of Adenylate Kinase

cADK = Cowellia isoform of Adenylate Kinase

eADK = E. Coli isoform of Adenylate Kinase

ADK = Adenylate Kinase

ATP = Adenosine Triphosphate

ADP = Adenosine Diphosphate

AMP = Adenosine Monophosphate

Apo = Free, unbound to any substrate form of protein

BSA = Bovine Serum Albumin

EDTA = Ethylenediaminetetraacetic acid

FPLC = Fast Protein Liquid Chromatography

HPLC = High Pressure Liquid Chromatography

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

MOPS = 3-(N-morpholino)propanesulfonic acid buffer

TRIS = Trisaminomethane

KCl = Potassium Chloride

MD = Molecular Dynamics

Mg$^{2+}$ = Magnesium Ion

mM = milimolar

uM = micromolar
NADH = Nicotinamide Adenine Dinucleotide

NMR = Nuclear Magnetic Resonance

P-transfer = Phosphoryl transfer

R124K = Arginine to Lysine mutant at residue 124

R150K = Arginine to Lysine mutant at residue 150

R156K = Arginine to Lysine mutant at residue 156

WT = Wild Type

ΔG‡ = Gibbs Free Energy of Activation

ΔH‡ = Enthalpy of Activation

ΔS‡ = Entropy of Activation
**Introduction**

**Kinases and Phosphates**

As life developed on Earth, nature selected the phosphate group as a novel tool to facilitate a wide variety of vital biologic and chemical processes. Arguably, the most important and interesting property of this combination of phosphorus and oxygen is its kinetic stability and thermodynamic instability. Natural selection has shaped this molecular character into a mechanism for energy transfer in the polymerized form of nucleotides, such as ATP and ADP. As shown in the vast amount of literature, the ATP molecule is utilized when the highly stable bond between the β- and γ-phosphates is broken, leading to a large amount of available energy that the cell harnesses to facilitate cellular signaling, molecular localization, and protein regulation (1). Specialized proteins referred to as kinases evolved over time to mediate these important chemical reactions in which the phosphate group is transferred from a high-energy donor molecule, like ATP or GTP, to a lower-energy receptor, like AMP or a serine, threonine, or tyrosine residue on a protein. The addition of the phosphate to a protein often affects the protein’s structure, thereby activating, deactivating, or altering the protein’s activity. Due to the high kinetic stability of phosphate complexes, nature employs the enzymatic activity of kinases to lower the activation energy barrier, allowing the reactions to proceed in a biologically relevant time period, some which have rates of up to 1000 s⁻¹. Because
of the extraordinary role that these enzymes plays in an inordinate number of biological reactions, characterizing and understanding how these integral proteins achieve biocatalysis will help us understand how the cell works at large, including applications in pharmacology, synthetic enzyme design, and disease pathology.

Adenylate Kinase

Amongst the many kinases that have been discovered, the ubiquitous adenylate kinase (ADK) is my special interest. ADK is a 23kDa protein that is part of a large family of nucleoside monophosphate kinases that regulate homeostatic nucleotide concentrations in the cell. ADK does this in a reversible process that transfers the γ-phosphate of an ATP molecule to an AMP to create two ADPs or transforms two ADPs into an ATP and an AMP. Catalytic efficiency is achieved with a divalent cation cofactor that is magnesium (Mg$^{2+}$) in life systems. The structure of ADK is broken down into three domains: an ATP lid, a NMP lid, and a Rossmann folded core (2). The core domain contains the phosphate loop (P-loop), necessary for nucleotide triphosphate binding, which is highly conserved in all kinases and even many ATPases (3-4). The two lids are highly dynamic substrate binders that exhibit large conformational changes around the active site, with the NMP lid displaying less selective binding than the ATP lid.
Without biocatalysis, the transfer of a phosphate group between the two nucleotides would take on the order of 8000 years (5). ADK facilitates the reaction in milliseconds and an isoform of the protein is believed to exist in every cell. ADK’s pervasive presence in all life signals its biological importance and our investigations have wide-reaching implications for both kinases and other enzyme-catalyzed reactions.
Figure 2: The total reaction scheme for ADK and the catalytic energy landscape as determined from kinetic and NMR data. The depth and height of energy landscape are estimated from known conformational and chemical step microscopic rate constants. ADK dramatically decreases the energy of activation to complete the phosphoryl transfer reaction (5).

Phosphoryl Transfer

Although the scientific community has probed kinases and phosphotransferases for many years, the character and energies of the transition state of phosphoryl transfer have been elusive. Much work has been done on non-biocatalyzed phosphoryl transfer reactions in solution, but the short lifetime of the transition state (on the order of picoseconds) and the complicated ensembles of proteins have made characterization of the chemical process in enzymes challenging. Specific determinations of the motions and energy necessary to achieve biocatalysis are therefore essential in creating a comprehensive energy landscape for ADK (6).

A number of hypotheses exist regarding the character of the transition state in enzyme-mediated phosphoryl transfer. In a concerted reaction pathway, the
transition state is described as being tight, synchronous, or loose, differentiated by the manner of bond breaking and formation. The tight transition state refers to a pentavalent, phosphorane bonding scheme in which bonds are formed between the donor and receptor phosphate molecules prior to bond breakage. The opposite is true for a loose transition state, where the phosphate first breaks bonds with the donor in a metaphosphate-like state before forming bonds with the receptor nucleotide. In between these two extremes exists a synchronous transition state that is formed when bond breakage and formation occurs in unison. There is likely a continuum between these three schemes, meaning that the transition state of the pathway could occur at any point between the loose and tight bonding schemes. Because of the illusive nature of the transition state, many techniques have been suggested, including $^31$P NMR, but none have been able to delineate a bonding scheme for any particular enzyme (1,6).
Figure 3: A 2D plot of the continuum of transition states between the tight and loose bonding scheme geometries. The character of the transition state can be more associative (tight) or dissociative in bond geometry. The tight scheme has a pentavalent (phosphorane) transition state, which the loose scheme has a metaphosphate-like transition state (6).

Through the use of NMR spectroscopy and kinetic experiments, it is possible to distinguish the individual steps of ADK's catalytic cycle. The steps of the enzyme are: binding of substrates, a conformational lid-closing step, the phosphoryl transfer, a conformation lid-opening step, and product release. It has been previously shown that the lid-opening step is rate-limiting in the presence of Mg$^{2+}$, however this does not give insight into how the protein is able to facilitate the chemical step of the reaction (7). Pre-steady state kinetic experiments performed by R. Agafonov, a member of our group, have shown that phosphoryl transfer occurs
within 5\(\mu\)s, the experimental dead time of the quench-flow apparatus. When Mg\(^{2+}\) is chelated from the experiment with high concentrations of EDTA, the rate-limiting step becomes phosphoryl transfer, suggesting Mg\(^{2+}\) integral role in mediating the chemical step of the reaction. It is therefore important to further investigate the roles of metal ion cofactors in the catalytic cycle of ADK.

**Active Site Arginine Residues**

In order to further our understanding of how ADK catalytically achieves phosphoryl transfer, we must utilize structural techniques like x-ray crystallography and site-directed mutagenesis in conjunction with kinetic experiments. This allows us to operate under the guiding biological principle that structure is heavily related to function. Numerous crystal structures of different ADK isoforms have been solved with both natural (ATP, ADP, and AMP) and unnatural substrates (AP5A and AMPPCP) bound. The apo state has also been crystallized, which allows for the comparison between unbound and substrate-bound conformations (8-13). To truly understand the phosphoryl transfer, the active site must be examined to identify the residues important in the chemical step.
It has been previously reported that the mutation R156K in the *E. coli* isoform of ADK (eADK) results in severely reduced activity, but very similar in $K_0$, in comparison to the wild-type protein (5). The mutant was never crystalized for an x-ray structure, which left the question of why and how activity is impeded unanswered. L. Phung and Y. J. Cho, members of our group, were able to make the same mutation in a thermostable isoform of ADK from *A. aeolicus* (aADK), crystallize the protein, and solve its structure. Superpositioning of the aADK WT and R150K mutant structures (analogous to the R156K eADK mutant) showed that due to the lysine’s shorter length, the distance between the amine group and the $\beta$-phosphate
of the donor molecule was larger. As a result, the hydrogen bond between the substrate and R150 that exists in aADK WT was disrupted. Because the spatial arrangement of other active site residues and the charge of the residue were not changed, it was concluded that the decrease in phosphoryl transfer rate (on the order of $10^3$) resulted from the loss of coordination between the enzyme and the reactant, originally accelerated by R150.

![Figure 5: Superposition of aADK WT (grey) and aADK R150K (blue) mutant active site crystal structure. The three arginines R124, R150, and R161 are highly conserved and are important in the chemical step of the reaction. R124 primarily interacts with the ATP-lid and R150 and R161 function at the AMP-lid (5).](image)

Other than R150, two other highly conserved arginines, R124 and R161, are located in the active site of aADK. Seen in L. Phung and Y. J. Cho’s crystal structure, these two residues form specific interactions with the ATP and NMP lids’ substrates, respectively. The reversibility of ADK’s reaction makes R124 and R161 key players with opposite catalytic roles, depending upon which binding site is the donor or acceptor of the phosphate. We hypothesize that determination of the phosphoryl transfer transition state character is possible by mutating these residues to lysines.
and observing the effect on catalytic turnover. Individually, each residue has a role of nucleophile activation or leaving group activation dependent on the direction of the reaction. By comparing forward and reverse kinetic rates of phosphoryl transfer in these mutants (R124K and R161K), the ratio between the two rates will tell us if each arginine is more important as nucleophile activator or a leaving group activator. Because a tight transition state model relies more nucleophile activation and leaving group activation is the important factor in a loose bonding scheme pathway, we can examine the transition state of the enzyme and ask the question: is phosphoryl transfer in ADK more tight or loose in bonding scheme?

![Proposed charge distribution in a loose transition state](image)

![Proposed charge distribution in a tight transition state](image)

Figure 6: The tight and loose transition state bonding schemes with notated charge distribution on the transferred phosphate (6).

**Protein Evolution and a Cooling Earth**

A prominent theory in protein evolution suggests that as life evolved, enzymes developed over time to adapt to a cooling Earth. As the warm Earth
environment of the primordial soup changed, proteins and the way by which they accelerate reactions had to change to accommodate this loss in available thermal energy (16). The First Law of Thermodynamics states

\[
\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger \quad \text{Equation (1)}
\]

that the enzyme can decrease its Gibbs free energy of activation (\(\Delta G\)), thereby increasing its catalytic turnover, in two ways: decreasing its enthalpy of activation (\(\Delta H\)) and/or increasing its entropy of activation (\(\Delta S\)). The Eyring equation allows us to manipulate the First Law to quantify the protein’s contributions from entropy and enthalpy in a plot of \(\ln(K_{\text{cat}})\) with respect to the inverse of temperature.

\[
\kappa = \left(\kappa_B T / h\right) e^{\Delta G^\ddagger / RT} \quad \text{Equation (2)}
\]

\[
R \ln \left( h \kappa / \kappa_B T \right) = -\Delta H^\ddagger / T + \Delta S^\ddagger \quad \text{Equation (3)}
\]

The equations above describe the Eyring plot where the slope is the enthalpic contribution and the y-intercept is the entropic contribution to catalysis, where \(\kappa\) is the turnover rate, \(h\) is Plank’s constant, \(T\) is temperature, \(R\) is the gas constant, and \(\kappa_B\) is Boltzman’s constant.

In congruence with the theory stated above, proteins would have tailored their mechanistic contributions to lower the amount of enthalpy needed to facilitate the chemical reaction (16). L. Phung was able to show with three extant ADK isoforms, a thermophilic, a mesophilic, and a psychrophile, that entropic
contributions to catalysis increased as the organism’s environmental temperature decreased. This was done by performing temperature dependences on the three proteins and comparing the resulting catalytic activity. To further test this theory, it is possible to utilize ancestral protein sequencing and resurrection. If both the theory and the phylogenetic trees are correct, it will be apparent in the decrease of the enthalpy of activation as we move from oldest ancestor to the extant ADKs.
Results and Discussions

Role of the Metal Cofactor in Adenylate Kinase

Adenylate kinase and many other kinases have been shown to use Mg\(^{2+}\) as a metal cofactor in facilitating the catalytic success of phosphoryl transfer. R. Agafonov of our group has previously shown that the addition of Mg\(^{2+}\) changes the rate-limiting step of catalysis from the transfer of phosphate to the conformation step of lid opening through pre-steady state kinetics experiments. It was therefore important to investigate the nature of the effect provided by cationic species.

![Figure 7](image_url)

Figure 7: (A) R. Agafonov’s pre-steady state kinetic experiments with eADK WT at 4mM ADP, pH 7.0. The existence of a burst phase in the presence of 4mM Mg\(^{2+}\) indicates that product release (lid opening) is the rate limiting step of the reaction. (B) The absence of an initial burst suggests that the chemical step, phosphoryl transfer, is rate-limiting in the presence of 50mM EDTA.

The experiment [Figure 7] demonstrated that the turnover of the enzyme was markedly decreased with the addition of 50mM EDTA, a chelating agent that complexes with divalent cations. The lack of a burst phase indicated that phosphoryl
transfer is rate-limiting in the absence of Mg$^{2+}$, but it was unclear whether or not the apparent rate was a minimum no-metal rate or the result of remaining metal ion contamination. To observe the effect of increasing EDTA concentration with respect to rate, an EDTA dependence was performed.

Figure 8: The EDTA titration for eADK WT at pH 4.5 and 7.0 from the ADK•ADP•ADP direction with 4mM ADP added. The rate remained unchanged after the EDTA concentration exceeded 15mM.

Because the steady state kinetic rate with 4mM ADP reached a minimum at concentrations greater than 15mM EDTA for both pH 4.5 and 7.0, we refer to this as the (-) Mg$^{2+}$ rate. Higher concentrations of EDTA were used as necessary when more nucleotide was used in the reaction, because the stock nucleotides are the origin of most of the metal contamination. The enzyme’s turnover rate at equimolar Mg$^{2+}$ and total nucleotide concentration is the (+) Mg$^{2+}$ rate.

With this information in hand, we were able to measure the pre-steady state and steady-state kinetic rates of eADK WT with different divalent cofactors. The
resulting rate constants were functions of the metal cofactor, the reaction step, and the direction of the reaction.

Table 1: The microscopic rate constants measured for eADK WT using quench-flow kinetics, steady-state kinetics, and NMR relaxation dispersion experiments. All kinetics experiments were performed with 4mM [Metal]ADP and the no metal assays contained 50mM EDTA. The lower limits are estimated using the dead time of the quench-flow apparatus and/or NMR relaxation estimates (5).

<table>
<thead>
<tr>
<th>Rate Constants (s⁻¹)</th>
<th>Metal</th>
<th>Phosphoryl Transfer Rate</th>
<th>Reverse Rate ( (\text{E}^<em>\text{ADP}_\text{ADP}) \Rightarrow \text{E}^</em>\text{ATP}_\text{AMP}) )</th>
<th>Lid Opening Rate ( (\text{E}^<em>\text{ADP}_\text{ADP}) \Rightarrow (\text{E}^</em>\text{ATP}_\text{AMP}) )</th>
<th>Reverse Rate ( (\text{E}^*\text{ATP}_\text{AMP}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>eADK WT</td>
<td>none</td>
<td>0.14±0.1</td>
<td>0.005±0.003</td>
<td>0.05±0.01</td>
<td>0.09±0.05</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>220±30</td>
<td>2800±300</td>
<td></td>
</tr>
<tr>
<td>Co²⁺</td>
<td>&gt;500</td>
<td>140±15.0</td>
<td>190±20</td>
<td>&gt;1900</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>&gt;500</td>
<td>24.1±4.0</td>
<td>260±30</td>
<td>&gt;2600</td>
<td></td>
</tr>
</tbody>
</table>

The table of microscopic rate constants provides insights into how the metal cofactor contributes to catalysis. The phosphoryl transfer rate in the forward reaction direction in the presence of the three metals is faster than the dead time \((5\mu\text{s})\) of the quench-flow apparatus and the lid-opening step is rate-limiting. In contrast, in the reverse direction of the reaction, phosphoryl transfer is only rate limiting for ADK in complex with Co²⁺ and Ca²⁺. All metal cations accelerate the
conformation step, lid opening, on the same order of magnitude, while only in the case of Mg$^{2+}$ is lid-opening rate-limiting in the reverse direction ($E \cdot ADP_{ADP} \rightarrow E \cdot ATP_{AMP}$).

Other divalent metal cations were chosen to investigate the correlation between the rate of phosphoryl transfer in the reverse reaction direction and cationic species through pre-steady state and steady state kinetic experiments. The selected metals, at a concentration of 4mM, were Mn$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Mg$^{2+}$, Co$^{2+}$, and Ca$^{2+}$. The reverse direction rate constants were plotted versus charge density, the charge squared divided by crystal ionic radius (25).

![Figure 9: The reverse reaction rate constants for different cationic species with respect to their charge density (25). Lid-opening rates were measured in NMR relaxation dispersion experiments and steady-state kinetic measurements were performed to determine the rates of phosphoryl transfer for all metals besides Mg$^{2+}$.](image-url)
The lid opening rates for all metal-bound enzymes were catalytically accelerated on the order of $10^3$ over the no metal lid-opening rate. For all metals except $\text{Mg}^{2+}$, phosphoryl transfer in the reverse reaction direction was rate-limiting. In the case of $\text{Mg}^{2+}$, phosphoryl transfer was enhanced the most by a factor of $10^6$ at least, with the other metals experiencing rate enhancements of $10^3$ or $10^4$. The phosphoryl transfer rates of the metals increased in proximity to the charge density of $\text{Mg}^{2+}$.

The above experiments allowed us to dissect the specific catalytic rate enhancements of different cationic species. Dependent on the metal or no metal, different reaction steps were accelerated by different orders of magnitude contingent upon the direction of the reaction. Metal ions catalyze both the conformation step, lid-opening, and the chemical step, phosphoryl transfer. The lid-opening rates of $\text{Ca}^{2+}$, $\text{Co}^{2+}$, and $\text{Mg}^{2+}$ were all similar, but $\text{Mg}^{2+}$ exhibited the fastest phosphoryl transfer rate by at least a factor of $10^2$ over $\text{Ca}^{2+}$ and $\text{Zn}^{2+}$.

Active Site Arginine Residues and the Character of the Transition State

Highly conserved, active site arginines have been shown to be key catalytic residues in facilitating the phosphoryl transfer in ADK. The mutation of one of these, $\text{R150K}$, to lysine in eADK and aADK was detrimental to enzyme turnover rate by a factor of $10^3$, with the phosphoryl transfer rate with $\text{Mg}^{2+}$ impacted by at least $10^5$ fold in the reserve reaction direction. In R150K, this rate reduction was attributed to the loss coordination between the $\beta$-phosphate of ADP and the residue due to an increase in distance between the two. By mutating R124 and R161 to lysines, it was
possible to measure the forward and reserve reaction rates for phosphoryl transfer. These two residues primarily interact with ATP-lid substrate and AMP-lid substrate, respectively, allowing us to contribute the resulting loss of activity to a specific site and therefore a specific reaction function.

We hypothesized the following; in the forward reaction direction in a tight transition state bonding scheme, R124 should be responsible for activating the leaving group (bond breakage between the β- and γ-phosphates). In the reverse reaction direction in a tight transition state model, R124 would be responsible for nucleophile activation (bond formation between the β-phosphates of the two ADPs). The individual importance of these reaction roles is switched in a more dissociative or loose bonding scheme and R161 would have the opposite functions to R124 in either scheme. The ratio between the forward and reserve reaction rates tells us which reaction role for a particular residue is more important to catalytic success: nucleophile or leaving group activation. By comparing the magnitudes of rate deceleration, we are able to infer the mechanism of the chemical step of the reaction.
Table 2: The summary of the forward and reverse reaction microscopic rate constants for phosphoryl transfer. The reactions were performed with 4mM MgADP in the continuous UV/Vis assay method. The ratio between the forward and reserve rates in aADK WT could not be used because phosphoryl is not rate-limiting.

<table>
<thead>
<tr>
<th>aADK Mutation</th>
<th>Forward (E•ATP $\rightarrow$ E•ADP $\rightarrow$ ADP)</th>
<th>Reverse (E•ADP $\rightarrow$ E•ATP $\rightarrow$ AMP)</th>
<th>Forward/Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>R124K</td>
<td>0.88±0.06</td>
<td>0.35±0.03</td>
<td>3.52</td>
</tr>
<tr>
<td>R150K</td>
<td>1.08±0.09</td>
<td>0.14±0.01</td>
<td>7.71</td>
</tr>
<tr>
<td>R161K</td>
<td>1.15±0.04</td>
<td>0.29±0.01</td>
<td>3.97</td>
</tr>
</tbody>
</table>

All of the resulting rates for R124K, R150K, and R161K were on the same order of magnitude, a deceleration of at least $10^3$. The result is unexpected, because it was hypothesized that these mutations would affect the forward and reverse rate constants in different amounts due to residue-specific chemical role activity, contingent upon the direction the phosphate group was transferred. Because of the similarity between the rates, we expected that the substrate/product on-enzyme equilibrium was affected differently for each of the mutants, indicating that although the observed kinetic rates were the same, the true kinetic rates were likely different when taking into account ADK’s preference for the E•ADP•ADP state over the E•ATP•AMP state.
In the literature, measuring the on-enzyme equilibrium is done by titrating in enzyme at fixed amount of substrate at a concentration below the $K_D$. For a given enzyme, the ratio between bound substrates vs. bound products indicates the enzyme’s preference at solution equilibrium for a specific enzyme-bound state. In the titration, the maximum value of the hyperbola is the factor by which one state is preferred over the other (5).

Figure 10: The on-enzyme equilibrium ADK titration for the aADK arginine mutants, R124K, R150K, and R161K, and WT in the presence of 600uM MgADP at 25°C. The maximum value of the hyperbola for each curve indicates the enzyme’s preference for the $E\cdot ADP\cdot ADP$ state over the $E\cdot ATP\cdot AMP$ state.

The preference for the $E\cdot ADP\cdot ADP$ state over the $E\cdot ATP\cdot AMP$ state was quite large with all measurements exceeding a 20-fold inclination to $E\cdot ADP\cdot ADP$. All measured values for the arginine mutants were both similar and within error of aADK WT, preferring the $ADP\cdot ADP$ state by a factor of 30. The disadvantage of this method is that it doesn’t account for possibility of having a single bound nucleotide species. For example, if affinity for ADP is higher than affinity for other nucleotides, the shift in equilibrium towards ADP will be overestimated due to significant
fraction of E•ADP species. To account for this possibility, we designed another method to determine the bound-substrate/product equilibrium of the enzyme; that would minimize the formation of single substrate bound enzyme species.

Using a spin concentrator with a 3kDa MWCO, we were able to measure the amount of unbound nucleotide in the reaction aliquot. Instead of a titration, a single measurement was recorded with 300uM ADK and ≥600uM MgADP. This eliminated the issue of the single substrate-bound state and allowed us to measure the concentration of nucleotides not bound to the ADK.

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>[ADP$<em>{\text{bound}}$]/2[ATP$</em>{\text{bound}}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>eADK WT, 600uM MgADP</td>
<td>3.74</td>
</tr>
<tr>
<td>eADK WT, 900uM MgADP</td>
<td>2.48</td>
</tr>
<tr>
<td>eADK WT, 1.5mM Mg ADP</td>
<td>1.70</td>
</tr>
<tr>
<td>eADK WT, 600uM ADP, 50mM EDTA</td>
<td>&gt;15 shifted</td>
</tr>
<tr>
<td>aADK WT, 600uM MgADP</td>
<td>5.43</td>
</tr>
<tr>
<td>aADK R150K, 600uM MgADP</td>
<td>3.11</td>
</tr>
</tbody>
</table>

Table 3: The ratio of ADP$_{\text{bound}}$ to ATP$_{\text{bound}}$ for the arginine mutants measured under different reaction conditions, using the spin concentrator method. A variety of MgADP concentrations were used to establish if the free nucleotide correction is effective.

The substrate state preference data yielded from the spin concentrator method allowed us to get reproducible, albeit still conceptually confusing data. As the amount of nucleotide was increased for a given ADK concentration, the shifting
towards the E•ADP•ADP decreased. When 20% excess of Mg\(^{2+}\) over the ADP concentration was used, the resulting values were unchanged, suggesting that all ADP is Mg\(^{2+}\)-bound in the reaction aliquot. The on-enzyme equilibrium is very similar between aADK WT and eADK WT in the presence of Mg\(^{2+}\). In the case of the 50mM EDTA measurement, preference for the E•ADP•ADP state was so high that nearly all ATP was free in solution within error. R150K continued to maintain bound-substrate/product equilibrium similar to that of the wild-type proteins.

The experiments performed above were done to demonstrate the bonding character of the transition state based on ratios of microscopic rate constants for phosphoryl transfer in three arginine mutants. Because the resulting forward and reverse reaction direction rate constants were within one order of magnitude, we probed the substrate/product on-enzyme equilibrium to isolate true rate constants as opposed to observed rate constants. Two methods were used to measure the substrate and product concentrations bound to ADK. The enzyme titration method indicated that the arginine mutants and the wild-type protein had similarly shifted preference for the E•ADP•ADP bound state, but it was unclear if all enzyme species were occupied by substrate. The spin concentrator method demonstrated that eADK and aADK WT had similar on-enzyme equilibrium values, but also showed that the three arginine mutants had different dissociation constants. Based on the data measured, it was not possible to determine the transition state bonding scheme of phosphoryl transfer in ADK using the arginine mutants.
Protein Evolution and Ancestral Protein Resurrection

Figure 11: The genomic phylogenetic tree for all life. The three extant isoforms of ADK are labeled specifically.

Members of our group had previously determined the enthalpic and entropic contributions to catalysis through a temperature dependence of three extant isoforms of ADK: a thermophilic (aADK), a mesophilic (eADK), and a psychrophilic (cADK). Because these three proteins display optimal activity at a variety of environmental conditions, we hypothesized that an ADK ancestral tree created from
these sequences might support the theory that as the Earth cooled, enzymes’
evolved to adapt to the loss in available thermal energy. An ancestral tree was
created from the Maximum Likelihood Method based on the Whelan and Goldman
Model.

Figure 12: The ADK ancestral tree generated from the three extant isoforms of ADK.
The tree was created from the Maximum Likelihood Method based on the Whelan
and Goldman Model.

The ancestral tree led to the identification and resurrection of two ADK
ancestors, N4, a common ancestor of eADK and cADK, and N28, an earlier ancestor
of aADK. N4 and N28 were expressed and purified in order to perform steady state
kinetic experiments. A temperature dependence was performed to probe the turnover rate of the ancestors in hopes of determining the entropic and enthalpic contributions to catalysis through an Eyring plot.

Figure 13: The temperature dependences for ancestors N4 and N28. Both demonstrate a decline in activity above 50° as the protein precipitated.

The observed rates for the two ancestors were at least $10^4$ slower than any of the extant ADKs used to create the tree. Because of their markedly decreased activity, it is likely that these hypothesized ancestors are not novel or accurate representations of ancient proteins; therefore, an Eyring plot was not produced. We hypothesized that this is due to the large genomic separation between the three organisms that express the extant ADKs used in the making of the tree.

An ancestral ADK tree is now being generated for ADKs only from the genus *Bacillus*. We believe that such a tree should yield more significant ancestors because of their more recent common ancestors. With this new tree in hand, we will be able
to resurrect *Bacillus* ancestors and perform an Erying analysis to see how ADK's contributions to catalysis have changed over time.
Conclusions

The pre-steady state and steady state kinetics experiments allowed us to examine the microscopic rate constants for individual steps in the catalytic cycle of ADK. In the forward direction, the conformational step, lid-opening, is rate limiting when a divalent cation, Mg$^{2+}$, Co$^{2+}$, or Ca$^{2+}$ catalyzes the reaction. The presence of any of these metals accelerates forward lid-opening by a factor of $10^4$. Metal cations also increase the phosphoryl transfer rate constant by at least $10^6$, with Mg$^2+$ displaying an increase over the other metals on the order of $10^2$. By using high concentrations of EDTA, we were able to determine a “no metal” rate and demonstrate that in such an environment, phosphoryl transfer becomes the rate-limiting step. When the rate constant of phosphoryl transfer was related to charge density, proximity to the charge density of Mg$^{2+}$ caused an increase in the rate constant. This suggests that although lid-opening is generally accelerated by the presence of a 2$^+$ charge, the cofactor’s role in phosphoryl transfer is highly specialized for a cation of specific charge and size. These results have allowed us to isolate and quantify the metal cofactor’s contribution to the catalytic success of ADK.

Three active site arginine mutations in aADK, R124K, R150K, and R161K, were characterized with steady state kinetics and substrate/product on-enzyme equilibrium experiments. We hypothesized that the resulting change in the ratio between the forward and reserve phosphoryl transfer rates would identify whether
nucleophile activation (for a tight transition state) or leaving group activation (for a loose transition state) is more important for the chemical step of ADK. The resulting turnover rates for the mutants were all within an order of magnitude, which left us unable to determine the bonding scheme of the transition state. On-enzyme equilibrium experiments were performed with two different methods to establish each enzyme’s preference for a specific substrate complex and in turn establish true kinetic rate constants. The measured on-enzyme equilibrium values for both the mutants and WT proteins were very similar within a given method, indicating that this parameter was not affected by our mutations. Because phosphoryl transfer rates were similar among the arginine mutants, we were unable to impede nucleophile and leaving group activation in a residue-specific way. Early MD simulations have suggested that R124, R150, and R161 are primarily important in the negative charge stabilization of the nucleotides and therefore it is possible that other active site residues are facilitating the activation of the substrates. Due to this unfortunate result, we cannot conclusively suggest the character of the transition state for phosphoryl transfer in ADK.

Phylogenetic sequencing of three extant ADKs, eADK, aADK, and cADK, led to the creation of an ancestral tree. Resurrection of two ADK ancestors, N28 and N4, allowed us to measure their activity through a temperature dependence. Both resurrected ancestors exhibited turnover rates more than $10^3$ slower than the existing ADKs. Because the ancestor proteins demonstrated such poor activity, we hypothesized that the three extant starting sequences are too distantly related to create an accurate ancestral tree. For future investigations in ancestral
reconstruction, we have chosen extant ADKs that are from the same genus, *Bacillus*, in hopes of creating more faithful ancestors.
Materials and Methods

The ATP, ADP, and AMP (99% purity) used in all experiments were purchased from Sigma-Aldrich. NAD+, NADH, HEPES, and IPTG were supplied by Sigma-Aldrich. All metal salts, EDTA, TRIS, and potassium phosphate were acquired from Fisher Scientific. Buffer solutions were created by dissolving the appropriate amount of dehydrated salt. Glucose-6-phosphate dehydrogenase, bovine serum albumin, lactate dehydrogenase, pyruvate kinase, and hexokinase were bought from Sigma-Aldrich. Wild-type Adenylate kinase and all other proteins were expressed and purified in the Kern Laboratory at Brandeis University.

Protein Expression and Purification

Wild-type *A. aeolicus* ADK, *E. coli* ADK, the arginine mutants (R124K, R150K R161K) and the ADK ancestors were expressed and purified as described (7, 18) with the subsequent adjustments. The cells were grown in LB with 100ug/mL ampicillin present (7). The buffers used in purification was 50mM Tris pH 7 and 50mM Tris 1M NaCl pH 7. Following purification, the concentrated protein was stored in 50mM Tris 80mM KCl 1mM TCEP pH 7 at -80°C.

Site Directed Mutagenesis
The wild-type ADK plasmids were obtained as described (7) and were subsequently used to generate the mutant plasmids with 1-3 base pair changes. Primers were acquired from IDT and mutagenesis was performed with polymerase chain reaction as detailed in (17). The resulting plasmid product was transformed in the DH5α strain of *E. coli* and was extracted using BIONEER Plasmid Mini Extraction Kit K-3111. The sequences were confirmed by Genewiz.

**Phylogenetic Ancestral ADK Tree Construction**

A BLAST search was done to compare aADK and eADK amino acid sequences with ADK sequences in the RSCB Protein Data Bank. MUSCLE was used to align the returned sequences (20). For ADKs that also had structural information, a multiple comparison and 3D alignment was performed using PDBeFold (21). This combination approached improved upon the primary sequence alignments, leading to the construction of the phylogenetic tree.

The evolutionary history of the subsequent ADK phylogenetic tree was inferred using the Maximum Likelihood Method based on the Whelan and Goldman Model (22). The consensus tree was bootstrapped for 500 replicates (23). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G parameter = 1.138). The branch lengths on the tree measured the number of substitutions per site. Tree construction and evolutionary analyses were conducted in MEGA5 (24).

**Steady-State Kinetics**
Coupled Enzyme Continuous Spectroscopy Assays

Kinetic rate measurements were done in both reaction directions in the presence of Mg$^{2+}$. The buffer conditions used were 50mM TRIS, 80mM KCl, pH 7.0 and these was maintained across all samples. In the ATP+AMP to ADP direction, the reaction cuvette was prepared to be 4mM ATP, 4mM AMP, 5mM glucose, 200mM NAD$^+$, 1unit/uL hexokinase and 1unit/uL G6P dehydrogenase. For rate measurements in the reverse direction (ADP->ATP+AMP), the conditions were 4mM MgADP, 8mM Mg$^{2+}$, 5mM phosphoenolpyruvate, 200mM NADH, 1unit/uL lactate dehydrogenase, and 1unit/uL pyruvate kinase. The cuvette was incubated at 25\(^\circ\) and final reaction volume was 500uL. Nicotinamide production/consumption was monitored with Cary 100 Bio UV-Visible Spectrophotometer continuously at 340nm with respect to time. The coupled enzyme system allowed for the indirect measurement of ATP/ADP production. For each sample, a control sample with double the enzyme concentration was run at the same time to reaffirm measured rates. Initial background activity was measured before the addition of enzyme to account for any product nucleotide contamination. (19)
Individual Time Point Assays

To determine steady state kinetic rates, time point quench assays were run and quantified by HPLC as described above. The buffer conditions were 50mM Tris 80mM KCl at pH 7.0 with 0.3mg/mL BSA as a non-specific binder. Enzyme concentration varied between 0.5nM and 25nM with adenosine nucleotide concentrations of 4mM with equimolar Mg$^{2+}$. The reaction aliquots were quenched with 10% TCA at different time points and the precipitate was filtered out (as described above). The samples were then neutralized and diluted with 1M potassium phosphate 50mM EDTA pH 8.0. The resulting samples were run over 6 minutes on HPLC to separate reactant and product concentrations.

In determining the rate of phosphoryl transfer in the absence of Mg$^{2+}$, the chelating agent EDTA was employed at a concentration of 50mM in the reaction.
The enzyme concentration used was 100uM and the adenosine nucleotide concentrations were between 8mM and 16mM. Quenching, neutralization, and analysis were performed in the same manner.

**On-Enzyme Equilibrium Measurements**

**Enzyme Titration Method**

The on-enzyme substrate/product equilibrium was measured by increasing ADK concentration while holding the MgADP concentration constant for the arginine mutants and WT ADKs. 600uM MgADP and a range of ADK concentrations (0.2-5.0mM) were mixed in a 20uL reaction tube. The buffer conditions were 50mM TRIS, 80mM KCl, pH 7.0. Amicon Ultra Centrifugal Filters with 3kDa MWCO were used to concentrate ADK up to stock concentrations of 8mM. Each sample was equilibrated over >45 minutes at 25°. The equilibrated reactions were then hand-quenched with 10% TCA and spun down in spin column filters to remove precipitate as described above. The quenched sample was then neutralized with 1.5M HEPES, 75mM EDTA, pH 8.0 to restore neutral pH to the sample. Nucleotide concentrations were then determined by the HPLC method stated above, which allowed for the on-enzyme equilibrium to be determined graphically, using Microsoft Excel.
Figure 15: The enzyme titration to determine the on-enzyme substrate/product equilibrium. The value for $[ADP_{bound}]/2[ATP_{bound}]$ at the horizontal asymptote is the number of ADP-bound complexes for every one ATP-bound complex.

**Spin Concentrator Method**

To measure the equilibrium of substrates bound to the enzyme, equilibrated ADK and MgADP reactions were spun down in Amicon Ultra Centrifugal Filters with 3kDa MWCO. The buffer conditions were 50mM TRIS, 80mM KCl, pH 7.0. Reaction concentrations of 600uM MgADP and 300uM ADK were prepared for a final volume of 200uL. The mixture contained free nucleotides and nucleotides bound to the enzyme. To calculate the contribution of the unbound fraction, the reaction tube was then spun at 6,000 RPM for 2 minutes to allow 15uL of the reaction mixture to filter down. The resulting flow through was then analyzed on a NanoDrop ND-1000 Spectrophotometer to establish solution nucleotide concentration (assumed to be at solution equilibrium ratios). This small fraction of total nucleotides was then used as a correction factor in determining the amount of substrate bound to the enzyme. The remaining 185uL was then hand-quenched with 10% TCA and neutralized with
1.5M HEPES, 75mM EDTA, pH 8.0. Nucleotide concentrations were quantified using the HPLC method detailed previously, which when combined with the correction factor, led to the calculation of the on-enzyme equilibrium in Microsoft Excel.

\[
[Nuc]_{total} = [Nuc]_{bound} + [Nuc]_{unbound} \quad \text{Equation (4)}
\]

\[
A = \varepsilon c L \quad \text{Equation (5)}
\]

\[
[AXP]_{unbound} = [AXP]_{total} - c_{tot} f_{AXP} \quad \text{Equation (6)}
\]

Equations 4-6: Mathematical scheme for calculating the bound and unbound concentrations of nucleotides, where A is the absorbance at 260nm, \(\varepsilon\) is molar extinction coefficient, c is the sum concentration of all nucleotides, L is the path length, \(f_{AXP}\) is the fraction of nucleotide AXP in solution at equilibrium (\(K_{eq} = ([ATP][AMP])/[ADP]^2\)). Total concentration values are determined by HPLC.

**Temperature Dependence Assays**

Assays to determine kinetic rates at temperatures between 0 °C and 100 °C were performed in the individual time point method as described above. The buffer system used was 50mM MOPS, 80mM KCl, pH 7.0. Both ADK stock solution and reaction mixture were incubated at the designated temperature prior to mixing. The reaction was hand-quenched with 10% TCA and then neutralized with 1.5M HEPES, 75mM EDTA, pH 8.0 to re-establish neutral pH. As before, the samples were then run using the HPLC method.

**High-Performance Liquid Chromatography (HPLC) Analysis**

In order to quantitatively measure the amount of nucleotide created in the course of kinetic experiments and for on-enzyme equilibrium measurements, high-
performance liquid chromatography (HPLC) was employed. Protein was precipitated via acid quench and then removed using Spin-X (Costar) centrifugal filter tubes. Samples were then diluted to avoid detector saturation and the pH was brought up to 7.0 to improve separation. The HPLC system used was an Agilent Infinity 1260 with high precision autosampler, injection error <0.1uL, and analytical HPLC column ACE (i.d. 2.4mm, length 250mm, C18-AR, 5Å pore size). The nucleotides were resolved using an isocratic elution, using 100mM potassium phosphate pH 6.1 as the mobile phase. Residual contamination of commercially purchased nucleotides was examined in control experiments.

Figure 16: The separation of nucleotides performed using HPLC. The peak area is proportional to the concentration of nucleotides in each sample.
References

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