The energy landscape of adenylate kinase during catalysis

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Kinases perform phosphoryl-transfer reactions in milliseconds; without enzymes, these reactions would take about 8,000 years under physiological conditions. Despite extensive studies, a comprehensive understanding of kinase energy landscapes, including both chemical and conformational steps, is lacking. Here we scrutinize the microscopic steps in the catalytic cycle of adenylate kinase, through a combination of NMR measurements during catalysis, pre-steady-state kinetics, molecular-dynamics simulations and crystallography of active complexes. We find that the Mg2+ cofactor activates two distinct molecular events: phosphoryl transfer (>103-fold) and lid opening (103-fold). In contrast, mutation of an essential active site arginine decelerates phosphoryl transfer 103-fold without substantially affecting lid opening. Our results highlight the importance of the entire energy landscape in catalysis and suggest that adenylate kinases have evolved to activate key processes simultaneously by precise placement of a single, charged and very abundant cofactor in a preorganized active site.

Phosphate esters and anhydrides are high-energy linkages that are extremely resistant to nucleophilic attack and therefore are fundamental to genomic stability, long signaling-state lifetimes and storage of biochemical energy1–4. Although stability is critical, organisms must respond rapidly and effectively to their environment and have therefore evolved enzymes that catalyze transfer of phosphoryl groups with exquisite specificity and enormous rate accelerations relative to the uncatalyzed reactions in solution5–10. Impressive progress has been made in understanding protein phosphorylation in cellular processes11,12, and fundamental work on nonenzymatic phosphoryl transfer (P-transfer) has delivered a deep mechanistic understanding of P-transfer in solution5–10; however, a comprehensive understanding of kinase-catalyzed phosphorylation is still lacking despite the wealth of literature on many different P-transfer enzymes5–13. This includes crystal structures that reveal conservation in the active site and accompanying domain architecture14; kinetic studies that establish the rate-limiting steps and the order of events13; and NMR that links local fluctuations to global dynamics15,16. However, catalysis is typically composed of multiple microscopic steps spanning a hierarchy of time and space, and this often obscures the underlying molecular mechanisms. This has led to persistent controversies regarding, for example, the role of the Mg2+ cofactor in kinase catalysis. Whereas some kinases are activated by a single Mg2+ ion, additional Mg2+ binding may be required for full activation, may result in inhibition or may be involved in structural stabilization13,17,18. The variety of different mechanisms attributed to the Mg2+ cofactor has resulted in arguments about the kinetic schemes to account for the role of Mg2+ in catalysis19. Other heated debates have focused on the role of conformational changes in the enzymatic reaction20–25. Quantitative descriptions of the entire energy landscape of catalysis are necessary to reconcile these different mechanisms including the canonical role of Mg2+ in kinase catalysis.

To address this, here we have performed a comprehensive investigation of the adenylate kinase (Adk) energy landscape and have quantified multiple kinetic states along the reaction pathway at atomic resolution. Adk is a ubiquitous and essential P-transfer enzyme found in all cells. Adks reversibly transfer a phosphoryl group from ATP to AMP, thereby maintaining the equilibrium between cytoplasmic nucleotides (Fig. 1a). During the enzymatic cycle, Adk undergoes large conformational changes by opening and closing of the ATP lid and AMP lid, defined as the regions that close over the Mg-ATP–AMP-binding sites, respectively (Fig. 1a). Unlike many protein kinases that are activated by protein–protein interactions or covalent modifications4, Adk is fully active in the presence of its nucleotide substrates, and it catalyzes a reversible reaction. This provides a tractable framework for quantitative analysis of the reaction-energy landscape. Using complementary techniques to examine the enzyme during catalysis across many orders of temporal and spatial resolution, we separated the microscopic steps of P-transfer and conformational motions with pre-steady-state kinetics and NMR dynamics experiments, investigated the mechanism of transition-state stabilization by crystallography and explored the active site dynamics by molecular dynamics (MD) simulation. Combined, these results reveal the major

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players responsible for the overall rate acceleration and address the key question of how the enzyme dramatically lowers the energy barrier of P-transfer and accelerates conformational changes essential for both efficient catalysis and suppression of detrimental hydrolysis.

RESULTS

Mg$^{2+}$ as electrostatic ’pivot’ for phosphoryl transfer

Expanding on previous structural studies and to set the stage for experiments to characterize the free-energy landscape, we obtained a number of Adk crystal structures in multiple biochemical states (for example, with substrates, with and without metals and with a transition-state mimic). Many available structures of Adks have provided mechanistic insight into the catalysis of P-transfer, the Mg$^{2+}$ cofactor, was not detected in the X-ray structures of only the phosphoryl group of the donor ADP and not the nucleotide acceptor ADP was held rigidly (for example, with substrates, with and without metals and with a transition-state analog). 32–34, a structure of the AAdk–Mg$^{2+}$–ADP–AMP–AlF$_4^-$ quaternary complex was solved, and it revealed first that a square planar tetrafluoroaluminate (AlF$_4^-$) and not a neutral AlF$_3$ was in the active site (AAdk8). This agrees with the fundamental work by Baxter and colleagues on the importance of balancing charge in active sites, a general principle that has been demonstrated for many P-transfer enzymes, including β-phosphoglucomutase, phosphoglycerate kinase, protein kinase A and UMP–CMP kinase 2,35–37.

Second, the AlF$_4^-$ ion derived from anomalous signal collected with the X-ray source at the Co$^{2+}$ edge, together with the electron density, confirmed that the positions of the Co$^{2+}$ and Mg$^{2+}$ ions are identical (AAdk11; Fig. 1d). From these structures, we propose that the metal cofactor helps to anchor the flexible donor phosphoryl group for a more favorable attack by the oxygen nucleophile of the acceptor nucleotide.

What happens to the active site players in the transition state (TS) of the P-transfer reaction? Using aluminum fluoride as a transition-state analog 2–34, a structure of the AAdk–Mg$^{2+}$–ADP–AMP–AlF$_4^-$ quaternary complex was solved, and it revealed first that a square planar tetrafluoroaluminate (AlF$_4^-$) and not a neutral AlF$_3$ was in the active site (AAdk8).

To obtain a high-resolution structure of AAdk–ADP–ADP ternary complex in the presence of Mg$^{2+}$ at an elevated pH of 9. A comparison between the structures with and without Mg$^{2+}$ bound showed that they are very similar (Fig. 1c) except for the extra electron density of the Mg$^{2+}$ and its coordination to the six ligands. It is notoriously difficult to distinguish Mg$^{2+}$ from structural water by electron density alone, and therefore the position of the Mg$^{2+}$ in the presence of natural substrates is ambiguous in many cases. Consequently, in order to unequivocally determine the position of the metal cofactor in the active site of the enzyme–substrate complex, we also obtained a structure of the ternary AAdk–ADP–ADP complex in the presence of Co$^{2+}$ (AAdk7; Fig. 1d). The position of the Co$^{2+}$ ion derived from anomalous signal collected with the X-ray source at the Co$^{2+}$ edge, together with the electron density, confirmed that the positions of the Co$^{2+}$ and Mg$^{2+}$ ions are identical (AAdk11; Fig. 1d). From these structures, we propose that the metal cofactor helps to anchor the flexible donor phosphoryl group for a more favorable attack by the oxygen nucleophile of the acceptor nucleotide.

Figure 1 Adk free-energy landscape of catalysis and exploration of the P-transfer step by X-ray crystallography. (a) Overall Adk reaction, minimal reaction scheme and corresponding schematic of the catalytic energy landscape based on the measured enzyme kinetics (Table 3). E represents the Adk enzyme in the scheme. Rate-limiting lid opening ($k_{opening}$) is shown in red and is visualized by the open and closed structures. (b) Superposition of Adk structures with ADPs bound. Conformational heterogeneity of the donor phosphate group and R150 is highlighted in color. (c) Superposition of Adk structures with bound ADPs in the presence (PDB 4CF7, blue) and absence (PDB 4JL5, red) of Mg$^{2+}$. (d) Superposition of Adk complexed with Mg$^{2+}$–ADP–ADP (blue) and Co$^{2+}$–ADP–ADP (PDB 4JKY, orange). The anomalous scattering of the electron density at the Co edge (σ = 1.609 Å) is shown as anomalous difference map contoured at 5.5σ (orange). (e) Superposition of Mg$^{2+}$–ADP–AMP–AlF$_4^-$ (PDB 3SRK, green) with Mg$^{2+}$–ADP–ADP (blue). Detailed structures of the active site of both the substrate–enzyme complex (blue) and transition-state analog (green) showing metal coordination and relevant O-P or O-Al distances (dashed lines; values in black) and the covalent O-P bond length (value in blue).
to execute P-transfer efficiently and avoid unproductive hydrolysis is impressive. From an evolutionary perspective, this striking difference in hydrolysis rates can be rationalized because Adks are abundant proteins that are always active to maintain nucleotide homeostasis, whereas many protein kinases are activated only transiently while participating in signaling cascades.

What possible mechanisms does Adk use for such efficient suppression of destructive hydrolysis? An obvious way to prevent hydrolysis would be to exclude water from the active site; however, X-ray structures showed that about 20 water molecules were trapped in the AAdk active site cavity. It is likely that these waters helped reduce the energetic penalty associated with desolvating the highly charged nucleotide substrates when binding to the active site. Rather than excluding water from the active site to minimize hydrolysis, Adk tightly coordinates these water molecules, thereby sequestering them from the phosphoryl groups. We investigated the stability of water molecules, present at the same positions in the active site of all crystal structures (AAdks 1–11, in long MD simulations). Although the average positions occupied by these water molecules inside the pocket agreed well with those identified in the crystal structures, the dynamic behavior of these waters was very different in the presence and absence of Mg$^{2+}$. Without Mg$^{2+}$, the waters initially present inside the active site exchange with bulk solvent on a timescale of 100 ns. However, in the presence of the metal cofactor the water molecules inside the active site pocket were prevented from exchanging with bulk solvent throughout the simulations. The terminal phosphates of the substrates, Mg$^{2+}$, and the four water molecules coordinating the metal ion form a very stable complex, serving as a cap to block water exchange. In all simulations we performed

Table 1 Data collection and refinement statistics

|                | AAdk1 | AAdk2 | AAdk3 | AAdk4 | AAdk5 | AAdk6 |
|----------------|-------|-------|-------|-------|-------|-------|
|                | ADP–ADP | ADP–ADP | ADP–ADP | ADP–ADP | AMPPN–AMPPN | ADP–ADP |
| Space group    | $P2_12_12_1$ | $P2_12_12_1$ | $P2_12_12_1$ | $P2_12_12_1$ | - | $P2_12_12_1$ |
| Complete (%)   | 99.4 (96.2) | 97.6 (93.5) | 100.0 (99.8) | 100.0 (100.0) | 99.1 (98.0) | 91.1 (99.9) |
| Resolution (Å) | 29.0–1.24 (1.31–1.24) | 42.9–1.55 (1.64–1.55) | 37.6–1.53 (1.61–1.53) | 38.0–1.79 (1.88–1.79) | 43.2–1.65 (1.74–1.65) | 43.9–2.12 (2.23–2.12) |
| $R_{merge}$    | 0.062 (0.427) | 0.139 (1.287) | 0.072 (1.342) | 0.105 (0.566) | 0.065 (0.653) | 0.174 (1.698) |
| Completeness (%) | 99.4 (96.2) | 97.6 (93.5) | 100.0 (99.8) | 100.0 (100.0) | 99.1 (98.0) | 91.1 (99.9) |
| Reﬁnement     | Resolution (Å) | 29.0–1.24 | 36.1–1.55 | 36.6–1.53 | 38.0–1.79 | 42.4–1.65 | 43.9–2.12 |
| No. refl.      | 111,278 | 57,161 | 62,870 | 42,421 | 49,227 | 22,909 |
| $R_{merge}$    | 0.130 / 0.159 | 0.185 / 0.229 | 0.173 / 0.232 | 0.180 / 0.232 | 0.208 / 0.241 | 0.186 / 0.256 |

The number of crystals for each structure is one.

(Fig. 1e and Supplementary Fig. 1a,b). Third, the Mg$^{2+}$ cofactor was clearly present and maintained its position and coordination partners relative to the AAdk–Mg$^{2+}$–ADP–ADP quaternary complex (Fig. 1e and Supplementary Fig. 1a,b). The position of the divalent ion (Fig. 1d and Supplementary Fig. 1a–c) was identical while the phosphoryl group was transferred between the donor and acceptor sites, thus suggesting that the Mg$^{2+}$ cofactor acted as an electrostatic pivot during P-transfer. In contrast, the active site R150 shifted in concert with the movement of the phosphoryl group.

Active site dynamics probed by molecular-dynamics simulation

To test our hypothesis that Mg$^{2+}$ helps organize the Adk active site architecture by positioning the donor and acceptor phosphates in proximity, we performed 200-ns MD simulations on the closed AAdk–ADP–ADP ternary complex with and without Mg$^{2+}$.

Simulations with Mg$^{2+}$ showed a stable active site configuration in which both nucleotides experience limited fluctuations (Fig. 2a). Water-molecule ligands to the metal ion were also stable and remained coordinated during the simulation (Supplementary Fig. 1c,d). In contrast, simulations in the absence of Mg$^{2+}$ revealed that the active site, and in particular the donor phosphoryl group, samples a much larger distribution of states (Fig. 2b). These simulations indicate a substantially reduced probability of sampling productive conformations without Mg$^{2+}$, suggesting an appreciable stabilization of pre-catalytic states by the cofactor.

A major challenge for kinases is catalyzing P-transfer efficiently while suppressing the more favorable hydrolysis side reaction. For Adk we measured an extremely slow hydrolysis rate of about $2 \times 10^{-6}$ s$^{-1}$ (Fig. 2c) relative to the productive rate of P-transfer of about $2 \times 10^{2}$ s$^{-1}$. In comparison to other members of the kinase family with hydrolysis rates in the range of $10^{-2}$ to 10 s$^{-1}$ (refs. 39,40), Adk’s ability

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Dissecting microscopic steps by rapid kinetics experiments

Although Mg$^{2+}$ has minimal impact on the Adk structure (Fig. 1c), it is generally known that Mg$^{2+}$ has an enormous effect on the turnover rate in Adk catalysis (44). To determine the energetic contribution of Mg$^{2+}$ to the overall rate acceleration of P-transfer, we measured turnover rates in the absence of Mg$^{2+}$.

For all kinetic and NMR experiments described below, we used Escherichia coli Adk (EAdk), which has identical active site architecture, catalytic mechanism and rate-limiting steps as AAdk (comparison of Fig. 3, Supplementary Fig. 3a–c and refs. 43 and 44), and higher catalytic efficiency at room temperature. This choice was rooted in the inability to measure conformational dynamics for AAdk by NMR, as explained in the next paragraph. To eliminate traces of divalent metals from solution, we included EDTA in excess in all metal-free reactions performed (Supplementary Fig. 3d). For EAdk, Mg$^{2+}$ accelerated overall turnover by about five orders of magnitude, a result in agreement with the general notion that Mg$^{2+}$ is essential for enzyme-catalyzed P-transfer reactions. However, because lid opening and not P-transfer is rate limiting in the presence of Mg$^{2+}$ (ref. 44), the actual rate acceleration of the chemical step by Mg$^{2+}$ must be even greater.

To catch the chemical step directly, we measured the pre-steady-state rate of product formation by quench flow (Fig. 3 and Supplementary Fig. 3e). With Mg$^{2+}$, P-transfer on the enzyme was faster than lid

Figure 2 Role of active site dynamics in efficient P-transfer versus unproductive hydrolysis. (a,b) Superposition of representative snapshots of 200-ns MD simulations (n = 4) of the ADP–ADP ternary complex of AAdk with Mg$^{2+}$ (yellow; a) and without Mg$^{2+}$ (b). (c) Unproductive ATP hydrolysis by 100 µM of EAdk. Fit of the time dependence of nucleotide concentrations yields a hydrolysis rate of $-2 \times 10^{-6}$ s$^{-1}$. (d) Overlay of the active site of the crystal structure of AAdk bound to Mg$^{2+}$–ADP–AMP–AlF$^{4-}$ (green) including bound water molecules (green spheres) and a typical snapshot from a 200-ns MD simulation of the AAdk–Mg$^{2+}$–ADP–ADP complex (yellow). The transparent yellow spheres represent the isosurface of value 0.75 for the fractional occupancy of the water oxygen atoms during the MD simulation. A cavity excluded from access to bulk solvent (transparent green) is solvated by a number of water molecules.

Table 2 Data collection and refinement statistics

| AAdk7 Co$^{2+}$-ADP–ADP | AAdkB Mg$^{2+}$-ADP–AMP–AlF$^{4-}$ | AAdk9 R150K | AAdk10 R150K | AAdk11 Mg$^{2+}$-ADP–ADP |
|--------------------------|---------------------------------|-------------|-------------|--------------------------|
| Data collection          |                                 |             |             |                          |
| Space group              | $P2_1$$2_1$$2_1$               | $P2_1$$2_1$$2_1$ | $P2_1$$2_1$$2_1$ | $P2_1$$2_1$$2_1$          |
| Cell dimensions          | $a$, $b$, $c$ (Å)               | 67.55, 73.53, 87.47 | 65.95, 69.58, 85.67 | 67.17, 71.01, 85.83          |
| Resolution (Å)           | 56.3–2.37 (2.50–2.37)           | 41.8–1.57 (1.65–1.57) | 48.8–1.73 (1.82–1.73) | 36.6–1.43 (1.51–1.43)          |
| Rmerge (%)               | 0.062 (0.198)                   | 0.085 (0.310) | 0.054 (0.123) | 0.053 (0.268) |
| No. reflections          | 17,150                          | 56,016       | 43,855      | 74,937       |
| Completeness (%)         | 99.7 (97.9)                     | 99.7 (98.3) | 99.9 (100.0) | 99.9 (99.8) |
| Redundancy (%)           | 13.5 (12.7)                     | 6.4 (4.8)    | 6.4 (5.0)   | 6.7 (5.1)    |
| Refinement               |                                 |             |             |                          |
| Resolution (Å)           | 53.5–2.37 (2.50–2.37)           | 41.8–1.57    | 42.4–1.73   | 36.1–1.43    |
| Rmerge / Rfree           | 0.199 / 0.267                   | 0.18 / 0.189 | 0.161 / 0.210 | 0.153 / 0.195 |
| No. atoms                | Protein                         | 3,245        | 6,995       | 3,367        |
|                         | Ligand/ion                      | 110          | 160         | 108          |
|                         | Water                           | 144          | 759         | 518          |
| B factors               | Protein                         | 42.0         | 16.0        | 21.1         |
|                         | Ligand/ion                      | 34.0         | 10.6        | 15.6         |
|                         | Water                           | 37.4         | 28.1        | 32.7         |
| r.m.s. deviations       | Bond lengths (Å)                | 1.276        | 1.515       | 1.222        |
|                         | Bond angles (°)                 | 0.008        | 0.014       | 0.006        |
| The number of crystals for each structure is one.
opening, which resulted in an initial burst of product formation as the enzyme completed its first turnover followed by a linear product increase that was identical to the rate of lid opening (Fig. 3a). Remarkably, P-transfer was faster than ~500 s⁻¹ because the burst phase was completed within the dead time of the instrument (~5 ms). The ratio of the pre-steady-state burst amplitudes reports directly on the on-enzyme equilibrium of EAdk–Mg²⁺–ADP–ADP to EAdk–Mg²⁺–ATP–AMP concentration of about ten, with the sum of the two burst amplitudes equal to the total enzyme concentration (Fig. 3a). This is in contrast to the free-nucleotide equilibrium of about one. We confirmed the values for the on-enzyme nucleotide equilibrium by nucleotide saturation experiments (Supplementary Fig. 3f.g). We note that an on-enzyme equilibrium of 10:1 is equivalent to a small difference in free energy (~1.3 kcal/mol) between the states; this is fully consistent with the idea that optimal enzyme catalytic efficiency is achieved when the free energies of reaction intermediates are balanced.

In the absence of Mg²⁺, P-transfer is severely impaired. In the reverse reaction we observed no burst phase, and the turnover ratio of the pre-steady-state burst amplitudes reports directly on the on-enzyme equilibrium of EAdk–Mg²⁺–ADP–ADP to EAdk–Mg²⁺–ATP–AMP concentration of about ten, with the sum of the two burst amplitudes equal to the total enzyme concentration (Fig. 3a). This is in contrast to the free-nucleotide equilibrium of about one. We confirmed the values for the on-enzyme nucleotide equilibrium by nucleotide saturation experiments (Supplementary Fig. 3f.g). We note that an on-enzyme equilibrium of 10:1 is equivalent to a small difference in free energy (~1.3 kcal/mol) between the states; this is fully consistent with the idea that optimal enzyme catalytic efficiency is achieved when the free energies of reaction intermediates are balanced.

Mg²⁺ greatly accelerates lid opening
Could this other slow step in the absence of Mg²⁺ be the lid opening? ¹⁵N-CPMG NMR relaxation dispersion experiments have previously been used to quantify the millisecond lid-opening rates during catalysis in the presence of Mg²⁺ (ref. 44). Strikingly, when we measured EAdk during catalysis without Mg²⁺, the dispersion profiles were flat (Fig. 4a and Supplementary Note 1), thus indicating that the rate of lid opening could be shifted into the slow-exchange regime and therefore was too slow to be detected. Such NMR relaxation behavior of the enzyme agrees with the rate of lid opening of 0.05 s⁻¹ as determined by pre-steady-state kinetics (Table 3). We note that in the slow-exchange regime, one would expect to see signals for both states in the HSQC spectrum, which we did not observe here for EAdk (Fig. 4b). A plausible explanation for this phenomenon is a small population of the minor, open state that would give rise to weak signals. In addition, the line broadening of signals corresponding to the open state is proportional to the closing rate, which is considerably faster than the opening rate and could broaden the already weak signals beyond detection. However, the absence of NMR dispersion could also be caused by a lack of motions. To differentiate between these scenarios, we measured the temperature dependence of the NMR relaxation dispersion profiles (Table 3). For an exchange process that is slow on the NMR timescale, the exchange contribution (Rex) is equal to the lid-opening rate, whereas for a fast exchange process it is inversely proportional to the sum of the lid-opening and lid-closing rates.

We observed an increase in Rex by raising the temperature from 20 to 40 °C, with a uniform amplitude of ~1 s⁻¹ for all residues that experience the exchange process (Fig. 4c). When plotted on the structures (Fig. 4d), these residues are very similar to the ones that report on the lid opening and closing in the presence of magnesium. These results confirm that the lid opening in the absence of magnesium is indeed slow on the NMR timescale. Consequently, Rex reflects the rate of lid opening; however, the dispersion data provide neither its accurate value nor information about the closing rate and the relative population of the states. Therefore, we conclude that a second, unexpected role for the Mg²⁺ cofactor is to dramatically accelerate lid opening by about three orders of magnitude (Table 3).
How can Mg^{2+} lower the activation barriers of the two major steps in the catalytic cycle? Mg^{2+} may simultaneously help shield the strong electrostatic interactions between active site arginines and substrates to facilitate lid opening and may correctly orient the active site players for efficient P-transfer. This suggests that substituting other divalent metals for Mg^{2+} should activate lid opening similarly, whereas P-transfer might be more specifically tuned to the charge density of Mg^{2+}. Indeed, we found that P-transfer for all divalent cations other than Mg^{2+} was much slower and, in fact, was rate limiting, thus indicating that the active site chemistry is optimized for Mg^{2+} (Fig. 5a, Supplementary Fig. 4, Table 3 and Supplementary Table 1). In contrast, the rates of lid opening and closing measured in the presence of Mg^{2+} and Ca^{2+} were similar (Fig. 5b, Table 3, Supplementary Table 2, and Supplementary Note 2), thus implying that electrostatics is the predominant cause for this strong acceleration of lid opening. Simple electrostatic-potential and interaction-energy calculations reveal that the coordination of a divalent cation to the nucleotides opposite to the coordinating arginine side chains weakens the strong electrostatic interactions between the phosphates of the nucleotides and the arginine residues from the lids (Fig. 5c,d). Because these interactions need to be broken for the lids to open, the placement of Mg^{2+} in Adk indeed accelerates lid opening via electrostatics. We note that the lid-opening rates of AAdk without Mg^{2+} were too slow to be measured by 15N-CPMG relaxation dispersion experiments\(^45\); therefore, we used EAdk for all kinetic and NMR experiments.

A conserved arginine accelerates P-transfer 10\(^3\)-fold
In an effort to inventory the contribution of other players in lowering the energy barrier of catalysis, we focused our attention on the

### Table 3 Microscopic rate constants for EAdk catalysis

| Metal | P-transfer Rate constant (s\(^{-1}\)) | Lid opening Rate constant (s\(^{-1}\)) |
|-------|-------------------------------------|--------------------------------------|
|       | Forward | Reverse | Forward | Reverse |
| WT    | None    | 0.14 ± 0.1\(^a\) | 0.005 ± 0.003\(^a\) | 0.05 ± 0.3\(^{10}\) | 0.09 ± 0.05\(^a\) |
| Mg\(^{2+}\) | >5,000\(^c\) | >500\(^i\) | 190 ± 30\(^{10}\) | 2,800 ± 200\(^{10}\) |
| Ca\(^{2+}\) | >500\(^i\) | 24 ± 4\(^a\) | 260 ± 30\(^{10}\) | >2,600\(^f\) |
| Co\(^{2+}\) | >500\(^i\) | 150 ± 15\(^i\) | 190 ± 20\(^{10}\) | >1,900\(^f\) |
| R150K | (4.6 ± 0.2) × 10\(^{-6}\) | (6.5 ± 1.2) × 10\(^{-6}\) | <1\(^a\) | <1\(^a\) |

\(^*\)From the observed forward and reverse rates from quench-flow data and on-enzyme equilibrium.

\(^*\) Lower limit determined from the burst within the dead time of the quench-flow instrument (~5 ms).

\(^*\) From the steady-state (linear) phase of the quench-flow experiment and on-enzyme equilibrium.

### Figure 5

The nature of the divalent cation drastically affects P-transfer but not Adk conformational dynamics. (a) The P-transfer step of EAdk in the reverse reaction measured as a function of different divalent metals. The unit \(e_0\) refers to the elementary charge constant. The rate constant shown for Mg\(^{2+}\) is a lower limit. (b) 15N-TROSY CPMG relaxation dispersion profiles of EAdk with Mg\(^{2+}\) (blue) or Ca\(^{2+}\) (purple). (c) Active site electrostatic potential computed from representative structures extracted from the MD simulations with (top) or without (bottom) Mg\(^{2+}\), plotted on a plane cutting through the active site. (d) Distribution of the electrostatic interaction energy between the ADP molecules and R124 and R150, plotted for four MD simulations with and without Mg\(^{2+}\). The histograms show the electrostatic interaction energies computed for configurations evenly distributed in time along the trajectories. (e,f) The two molecules in the asymmetric unit of AAdk R150K with ADP bound (blue and green), superimposed with WT AAdk (gray). (g) 15N-TROSY CPMG relaxation dispersion at 25 °C on the EAdk R150K mutant saturated with 20 mM nucleotides. Dispersion curves for residues sensitive to lid opening and closing in the presence of Mg\(^{2+}\) (red) are fully suppressed in the absence of Mg\(^{2+}\) (orange). Uncertainties (s.d.) in \(R_2\text{eff}\) in b and g are estimated from the variance for a number of nonexchanging peaks (\(n = 7\)).
conserved active site R150. We have already seen that this residue is flexible and mirrors the donor phosphoryl group position (Fig. 1b). Mutation of R150 has previously been shown to drastically inhibit the rate of enzyme turnover\(^4\). A comparison between the X-ray crystal structures of wild-type (WT) AAdk (AAdk1) and the R150K mutant (AAdks 9 and 10) showed that this mutation disrupts the hydrogen-bonding between R150 and the donor β-phosphate. This implies that a specific coordination of the bidentate guanidinium group of the arginine side chain to the transferring phosphoryl group, rather than a positive charge, is needed to position the reactants for catalysis (Fig. 5e,f). By quantifying the change in the P-transfer and lid-opening rates for the EAdk R150K mutant (AAdk numbering; Supplementary Fig. 3a) we found that this arginine residue has a 10\(^3\)-fold effect on P-transfer but a negligible effect on lid opening (Fig. 5g, Supplementary Fig. 5, Table 3, Supplementary Table 2 and Supplementary Notes 3 and 4). The total acceleration from Mg\(^2+\) and R150 together was therefore larger than 10\(^8\)-fold on the rate of P-transfer (Table 3).

**DISCUSSION**

Our results reveal how Adks accelerate the rate of P-transfer (\(k_{\text{chem}} >500 \text{s}^{-1}\)) by more than 14 orders of magnitude relative to the uncatalyzed reaction (3.9 × 10\(^{-12}\) s\(^{-1}\); ref. 6) while minimizing the hydrolysis side reaction. We find that Mg\(^2+\) specifically accelerates P-transfer (>10\(^5\)-fold) by reducing nonproductive active site fluctuations (Fig. 2a,b), stabilizing an architecture resembling the TS and serving as an anchor to the phosphoryl group pivoting between donor and acceptor nucleotides (Fig. 1e). In addition, the Mg\(^2+\) cofactor accelerated lid opening (10\(^2\)-fold) by weakening the strong electrostatic interactions of the closed quaternary complex (Fig. 5c,d). The multiple catalytic roles for Mg\(^2+\) are remarkable considering that P-transfer and lid opening are very different molecular events: one involves a subtle 2-Å transfer of a phosphoryl group and the other a large-scale coordinated motion of domains. In contrast, nonenzymic P-transfer was accelerated only ten-fold by Mg\(^2+\) (ref. 6), thus highlighting the evolutionary impact of this highly charged and abundant cation on the enzyme’s energy landscape. R150 contributed an additional 10\(^2\)-fold acceleration to the P-transfer step (Table 3) on top of the large acceleration provided by converting a bimolecular reaction in solution to a unimolecular one with a preferred active site geometry\(^47\). Our results rule out a previously proposed mechanism of activation of Adk by direct binding of a second Mg\(^2+\) (ref. 19); the data from the previous study can be fully explained by our model and the well-known \(K_4\) of Mg\(^2+\) to nucleotides.

We note that there is extensive literature on Adks, and on kinases in general, that can be confusing and contradictory because enzyme catalysis is often reduced to an observed rate constant (\(k_{\text{cat}}/K_m\)) that is a convolution of multiple reaction steps. This has contributed to mechanistic misinterpretations because critical conformational motions are often hidden and therefore are ignored. The importance of characterizing kinase catalysis in molecular detail has been emphasized many times\(^5\),\(^6\),\(^46\) but has unfortunately lagged far behind the understanding of nonenzymatic P-transfer\(^5\). Here we have quantified the microscopic states along the Adk energy landscape (Fig. 1a and Table 3) by analyzing the enzyme during catalysis, shedding light on long-standing controversies\(^1\) and providing a general framework for understanding the role of divalent metals and conformational changes in kinase function. However, we prefer to avoid speculation about associative versus dissociative transition states in enzymatic P-transfer.

We have characterized the free-energy landscape of catalysis and have quantified the energetic contributions of individual players to each microscopic step. Our experiments microscopically assess how enzymes use both preorganize the active site for P-transfer and destabilize the quaternary complex for efficient product release. As elegantly demonstrated by Mayo and collaborators, the principles of reducing unproductive active site fluctuations and minimizing solvent accessibility is essential to understand the design of enzymes\(^48\). However, despite remarkable progress\(^48\)–\(^51\), nature’s enzymes remain far superior, thus highlighting the urgent need to understand the entire energy landscape of enzymatic catalysis.

A complete description of the tremendous rate acceleration achieved by biological catalysts continues to be a captivating topic in biology\(^38\),\(^52\)–\(^55\). A large number of X-ray structures of active complexes, detailed kinetic characterizations and computational studies on kinases have been reported, and these may lead to the assumption that little is left to be discovered about kinase function. However, the understanding of free-energy landscapes underlying the impressive acceleration of P-transfer by kinases has been limited. For hydrogen-transfer enzymes, the role of active site preorganization and conformational sampling in catalysis has been extensively investigated\(^56\)–\(^59\). It has been 27 years since Fierke and co-workers quantified the microscopic steps of the dihydrofolate reductase energy landscape\(^57\), and the exquisite distance sensitivity of hydride transfer continues to make this and similar systems attractive for studies of enzyme catalysis\(^54\). We hope that the comprehensive catalytic framework described here for Adk will provide a similar foundation for understanding the catalytic power of one of the most important class of enzymes, kinases.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 4JL5 (AAdk1), 4JLD (AAdk2), 4JLB (AAdk3), 4JL8 (AAdk4), 4JL6 (AAdk5), 4JLA (AAdk6), 4JKY (AAdk7), 3SR0 (AAdk8), 4JLO (AAdk9), 4JLP (AAdk10) and 4CF7 (AAdk11). \(^1\)H and \(^15\)N chemical-shift assignments have been deposited in the Biological Magnetic Resonance Data Bank under accession codes 19089, 19090, 19091, 19092 and 19093.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

S.J.K., R.V.A., Y.-J.C., D.V.P., F.P., M.F.H. and D.K. designed experiments; S.J.K., R.V.A., Y.-J.C., E.P., R.O., D.V.P., L.A.P. and P.M.N. performed experiments; S.J.K., R.V.A., Y.-J.C., E.P., R.O., D.V.P., S.K., L.A.P., M.F.H., P.N.M., V.T., T.A. and D.K. analyzed data; S.J.K., R.V.A., F.P., R.O. and D.K. wrote the manuscript.
COMPETING FINANCIAL INTERESTS
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1.7 Å (except for AAdk11), the individual anisotropic ADP-refinement method was refined as isotropic for the AAdk1 and AAdk8 models, whereas other models for AAdk11, for which REFMAC5 was used). Hydrogen atoms were added and before addition of 20 mM MgSO4 and AlFx (from the mixture of Al(NO3)3 • 9 H2O. For AAdk8 crystals, AAdk was premixed with ADP and incubated for 1 week hydrolyzed to AMPPN. Because the electron density of AMPPN’s nitrogen is established from the electron density and from the perfect six-fold coordination lengths for AAdk1–6 and AAdk8–11 were between 0.973900 and 1.00001 Å. Of nearby water molecules and the nucleotides. Moreover, the Mg2+ position is in agreement with the position in the AAdk8 structure and the anomalous density in the AAdk Co2+-structure. We note that there is partial occupancy of ~25% AMP in the donor site in chain B. In chain A, we did not model a Mg2+ because little electron density is seen in this site, and the coordination to the nearby water molecules is not perfect, similarly to what is observed in the crystal structures AAdk1–8 in which no Mg2+ is present.

Structural alignments were done with maximum-likelihood algorithms implemented in the program THESEUS, which produces more accurate results compared to conventional least-square criteria.

Molecular dynamics simulations. The A molecule of the AAdk1 crystal structure (Table 1) in complex with two ADP molecules was used as an initial model for all-atoms classical molecular dynamics (MD) simulations. Parameterization was performed with the CHARMM 22-protein all-atom force field with the CMAP backbone energy correction included68,69 with CHARMM70. The parameters for the bound ADP molecules, singly protonated on the terminal phosphates, were derived by combining parameters for a fully charged ADP and for inorganic phosphate PO4H2− included in the standard force field71. The system was solvated in a truncated octahedral TIP3P72 water box with Solvate (http://www.mibpc.mpg.de/home/grubmueller/downloads/solvate/index.html) and CHARMM73.

Regarding the choice of a classical treatment for the active site, a complete quantitative understanding of the impact of the magnesium ion on the catalyzed reaction may require an accurate quantum-mechanical description of the active site region74–76. However, the size of the system, the timescale of the dynamical properties under study and the difficulties in the characterization of precise structural and geometrical properties of the active site in terms of the magnesium-ion positioning and protonation states of the ADP nucleotides make a direct quantum-mechanical approach infeasible without a previous mechanistic exploration at classical level77,78. Furthermore, even though the classical nonpolarizable force fields with fixed atom charges have limited accuracy, they have been successfully applied to similar metal-ion- and/or nucleotide-dependent problems79–81. A quantitative QM investigation of the catalyzed chemical step of the P-transfer reaction is currently underway, but it is beyond the scope of the present study.

The simulation boxes for both systems, with and without the Mg2+ ion in the active site, were neutralized, and then 50 mM NaCl was added with CHARMM70. The solvated structures were minimized with the conjugate gradient method as implemented in NAMD 2.7b2 (ref. 82) and then were gradually heated to 300 K with a time step of 1 fs while gradually releasing positional restraints in a MD simulation of 2 ns. Periodic boundary conditions were applied to the simulation cell. Electrostatics were treated with the particle mesh Ewald scheme83. The bonds of all hydrogens were constrained with the SHAKE algorithm84. The structures were equilibrated for an additional 20 ns in the NPT ensemble (T = 300 K, P = 1.01325 bar) with NAMD 2.7b2. The temperature was controlled with the Langevin dynamics method85 while keeping the pressure constant with the combined Langevin piston Nose-Hoover method86,87 as implemented in NAMD 2.7b2.

Equilibrated structures were then converted to GROMACS 4.5.3 (ref. 88) with the in-house scripts. For each of the two setups, with and without the Mg2+ ion bound to the active site, four independent replicas of 100 ns each were simulated in the NPT ensemble (T = 300 K, P = 1.01325 bar), constraining the hydrogen bond lengths with LINCS89 and constraining the TIP3P waters with SETTLE90. The temperature was controlled with the Nose-Hoover thermostat91,92, and pressure was controlled with the Parrinello-Rahman pressure coupling93,94, as implemented in GROMACS 4.5.3. Four of the trajectories were further extended to 200 ns.

To define the protein cavities during the analysis of the water dynamics (Fig. 2d and Supplementary Fig. 2), we used an algorithm based on the clustering of the alpha spheres89,96 implemented in Fpocket86. Fpocket relies on the Voronoi tessellation of space implemented in Qvornoi (http://www.qvornoi.org/). The volume of the cavity and the water molecules present inside those cavities were determined with a set of scripts that can partition a complex geometry by defining a convex hull on the basis of atomic positions (kindly provided by P. Varilly57).

The calculations of the electrostatic potential (Fig. 5c) have been performed with APBS86 with the interface built into VMD 1.9 (ref. 99). The linearized form of the Poisson-Boltzmann equation (LPBE) at 298.15 K was solved, thus placing the protein in a cubic box of size 80 Å and 129 grid points in each dimension.
The solvent dielectric constant was set to 78.54. To account for the solution buffer, 50 mM monovalent positive and negative mobile ions were included in the calculation.

All trajectories were analyzed with VMD 1.9 (ref. 99). Grace (http://plasmagate.weizmann.ac.il/Grace/) was used for the plots, and Tachyon ray tracer (built into VMD 1.9, http://jedi.ks.uiuc.edu/~johns/raytracer/) was used for the molecular renderings.

Pre-steady-state kinetics measurements. Pre-steady-state kinetics was measured with a three-syringe rapid quench-flow apparatus RQF-3 (KinTek Corp.). Protein solution was loaded into one of the syringes, nucleotides (ADP or AMP + ATP mixture with or without divalent ions) into the second syringe and quench solution (30% TCA + 6 M HCl mixture) into the third syringe. The reaction was initiated by a simultaneous push of all three syringes by a high-precision servo-motor. The required delay between the initial mixing of the reagent and the following quench with acid was achieved by selecting aging loops of different length (which increases the path length for the acid component, thus delaying the quench). The forward and reverse reactions were initiated by mixture of ADK with AMP and ATP or ADP, respectively. The protein concentration in the syringe (before mixing) was varied between 40 and 1,000 µM while the nucleotide concentration (in the syringe) was 8–32 mM, depending on the experiment. The buffer contained 100 mM Tris, pH 7.0, 80 mM KCl, and equimolar (with total concentration (in the syringe) was 40–1,000 M) was varied between 40 and 1,000 µM while the nucleotide concentration (in the syringe) was 8–32 mM, depending on the experiment. The buffer was 100 mM Tris, pH 7.0, 80 mM KCl, and equimolar (with total concentration (in the syringe) was 8–32 mM, depending on the experiment. The buffer was 8–32 mM, depending on the experiment. The buffer was 100 mM HEPES, pH 7.0, 80 mM KCl; measurements were collected on either Varian Inova 500 or 600 MHz or Bruker Avance 800 MHz (cryoprobe) spectrometers. Adk concentrations were usually 2 mM. Substrate saturation of Adk was achieved with 10 mM ADP in 100 mM HEPES, 50 mM NaCl, pH 7.0, and 5 mM TCEP. The concentration of divalent metal used in CPMG dispersion experiments was kept equimolar with the concentration of ADP. Samples measured in the absence of divalent metals were conducted in the presence of 5 mM EDTA.

Table 3 were calculated considering (i) the exponential phase of the forward reaction $E + ADP + AMP \rightarrow EADP + ADP$ or $E + ATP + ADP \rightarrow EATP + ADP$ which is determined directly from the slope in Figure 3b, because the equilibrium is highly skewed toward the $EADP$ state.

$\Delta k_{obs} = \Delta k_{EADP} - \Delta k_{EATP}$

$\Delta k_{obs} = \Delta k_{EADP} - \Delta k_{EATP}$

The real quench delay and the delay value specified in the instrument. To take into account this apparent dead time of the instrument, data were corrected, with quenched-flow experiments performed in the forward and reverse directions. Instrumental delay calculations were based on the fact that the sum of the pre-steady-state bursts for forward and reverse reactions must be equal to the total enzyme concentration.

Experimental bursts can be found as:

$$A'_{+} = A_{+} + k_{+} \times t_d$$

$$A'_{-} = A_{-} + k_{-} \times t_d$$

where $A'_{+}$ are experimentally measured bursts in the forward and reverse direction, $k_{+}$ are kinetic rates in the forward and reverse direction, and $A_{+}$ are true bursts in the forward and reverse direction.

Thus the delay time $t_d$ is

$$t_d = [(A'_{+} + A'_{-}) - (A_{+} + A_{-})]/[k_{+} + k_{-}] = [(A'_{+} + A'_{-}) - 1]/[k_{+} + k_{-}]$$

Finally, the true experimental delay in the experimental data can be calculated as

$$t = t' + t_d$$

where $t'$ is a delay without the dead-time correction.

All values reported in Table 3 were obtained from individual fits. Consistent results were also obtained through global fitting with KinTek Explorer, linking the values of all rate constants among the data sets including the values from in-solution and on-enzyme nucleotide equilibrium.

NMR dynamics. $^{15}$N-TROSY CPMG relaxation dispersion experiments were collected on either Varian Inova 500 or 600 MHz or Bruker Avance 800 MHz (cryoprobe) spectrometers. Adk concentrations were usually 2 mM. Substrate saturation of Adk was achieved with 20 mM ADP in 100 mM HEPES, 50 mM NaCl, pH 7.0, and 5 mM TCEP. The concentration of divalent metal used in CPMG dispersion experiments was kept equimolar with the concentration of ADP. Samples measured in the absence of divalent metals were conducted in the presence of 5 mM EDTA.

Typical parameters for $^{15}$N-TROSY CPMG relaxation dispersion experiments were a 40-ns constant time period; 2-ns delay between transients; 12 different refocusing field strengths from 50-1,000 Hz collected interleaved, with 1,024 and 128 direct and indirect points, respectively, and 25 °C. For experiments conducted at 30 °C and 40 °C, the constant time period was increased to 60 and 72 ms, respectively.

Standard $^{15}$N-TROSY-HSQCs were always collected before and after CPMG experiment to check sample stability. CPMG experiments usually took 2.5 d to complete. $^{15}$N-CPMG dispersions collected on the Avance 800 MHz were collected as downfield and upfield pairs to minimize off-resonance effects for the larger sweep width and for lower $^{15}$N pulse powers required on cryoprobes.

Data were processed with the NMRPipe suite and visualized by either CCPN or NMRView. Relaxation dispersion profiles were calculated from peak intensities and analyzed with the general Carver-Richards equation for two-site exchange with in-house scripts. Uncertainties were estimated from the average variation in dispersion for residues not experiencing exchange together with the signal-to-noise ratio of each resonance to be analyzed. Global fit uncertainties were estimated by a jackknife method.

Steady-state kinetics measurements. Steady-state kinetics measurements were collected with 4 mM ADP and equimolar (with nucleotide) concentrations of divalent metal. The enzyme concentration was varied between 0.5 nM and 25 nM; buffer was 100 mM HEPES, pH 7.0, and 80 mM KCl; measurements were collected at room temperature. The amount of product produced over 16 min was quantified with HPLC as described above for pre-steady-state kinetics.
