

Supplementary Material for

A price to be paid for two-metal catalysis: Magnesium ions that accelerate chemistry unavoidably limit product release from CDK2 kinase.

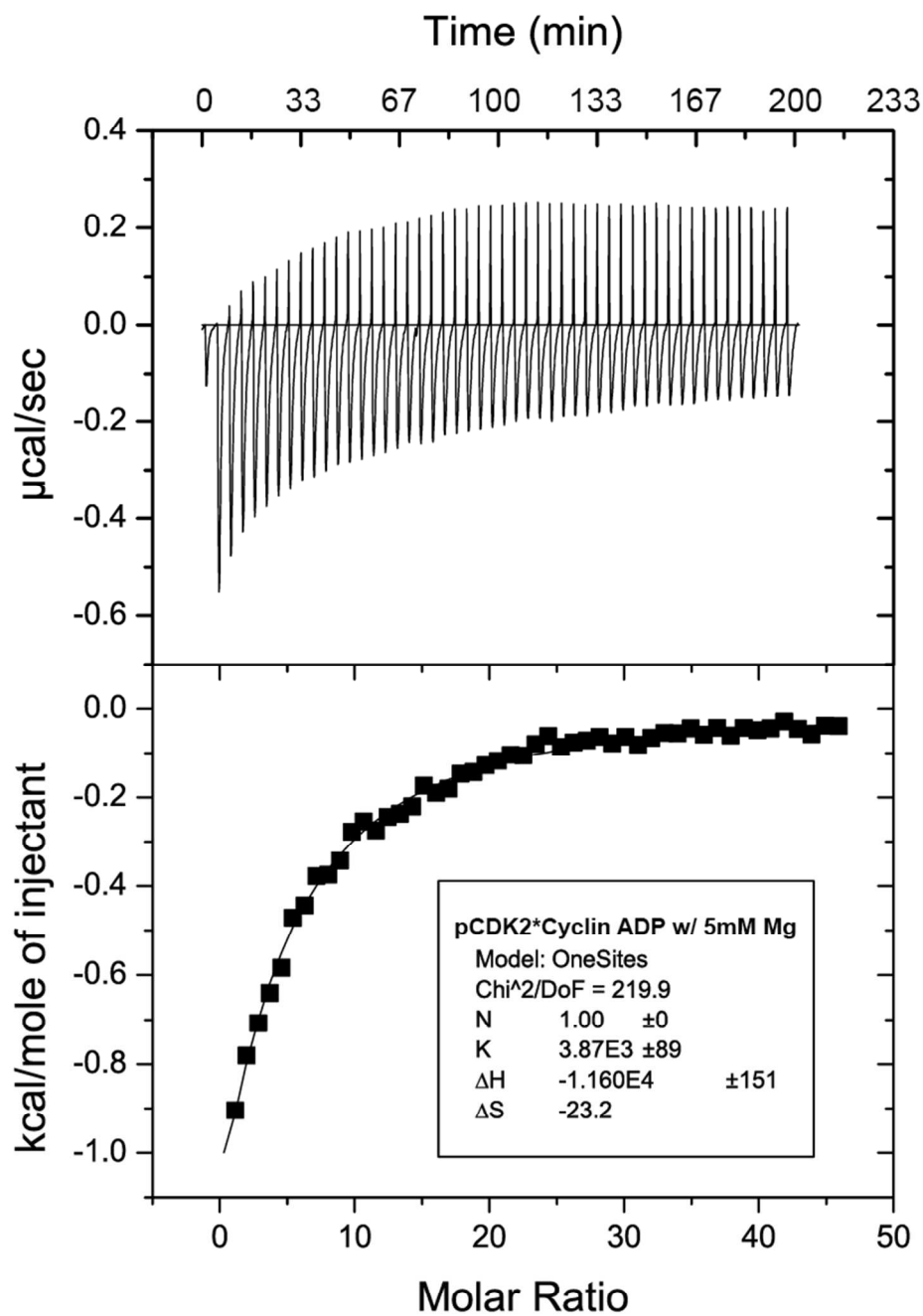
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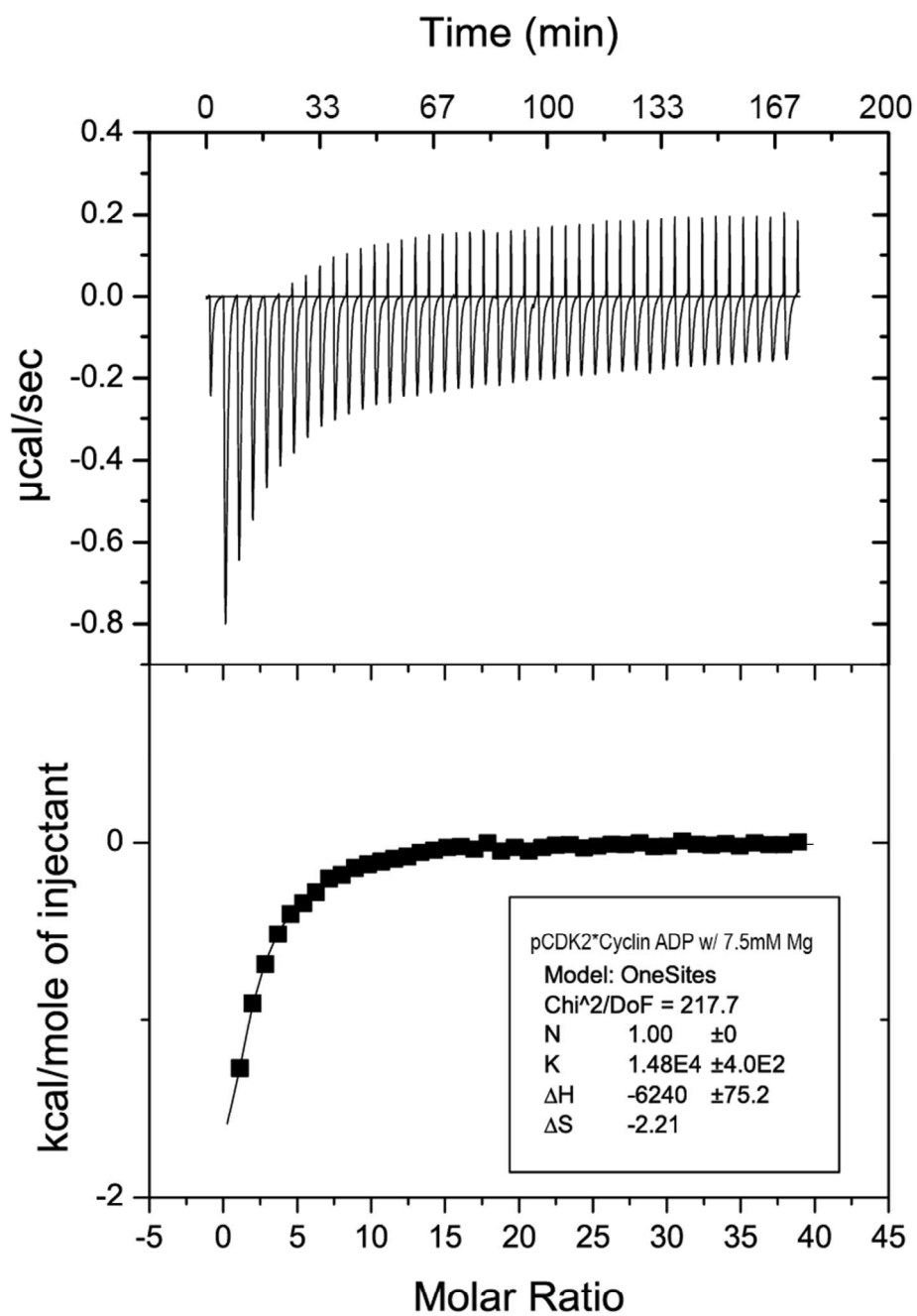
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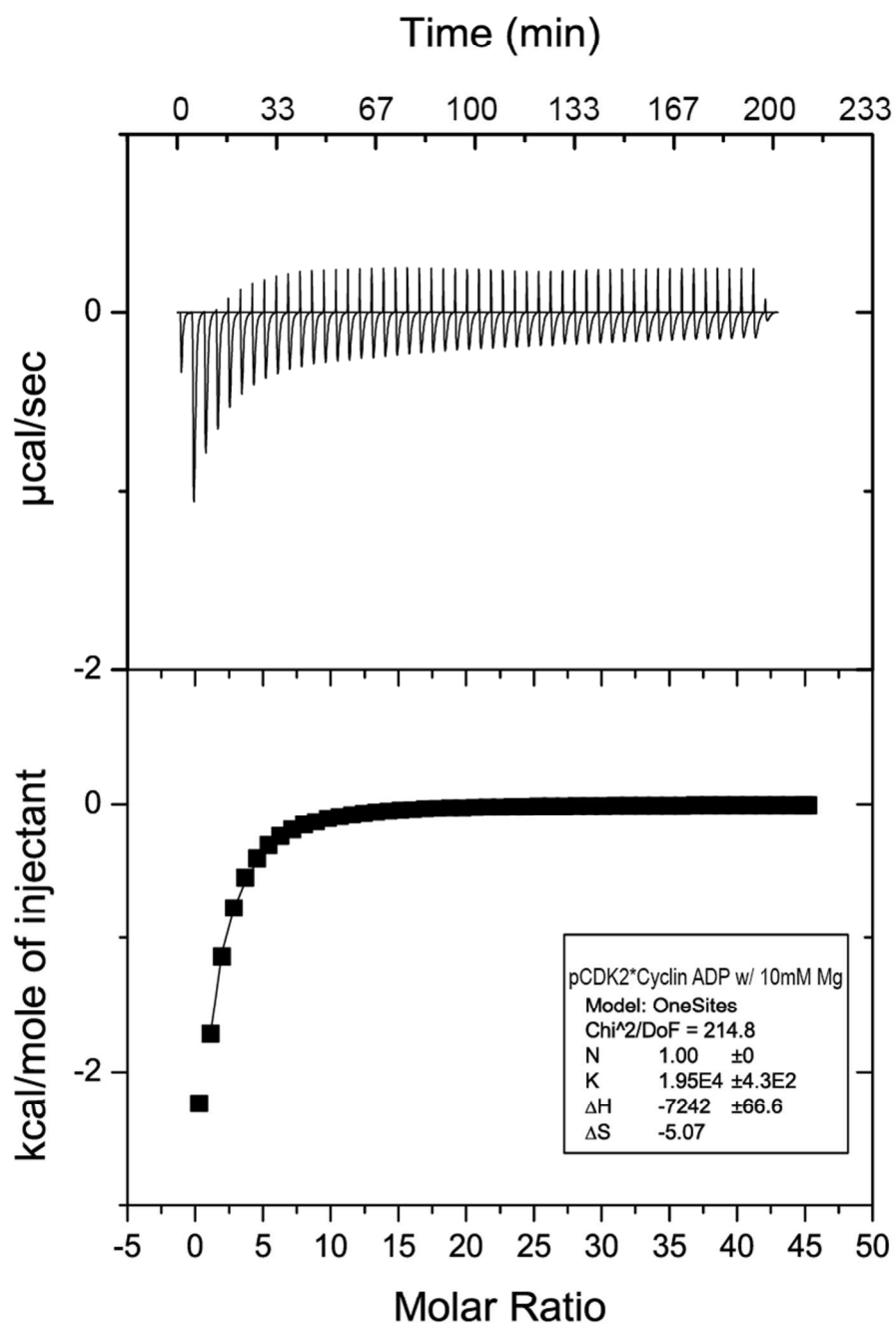
SUPPLEMENTARY FIGURES



Supplemental Figure 1 ITC of pCDK2•CyclinA binding ADP with 5 mM $[\text{Mg}^{2+}]_{\text{Total}}$.

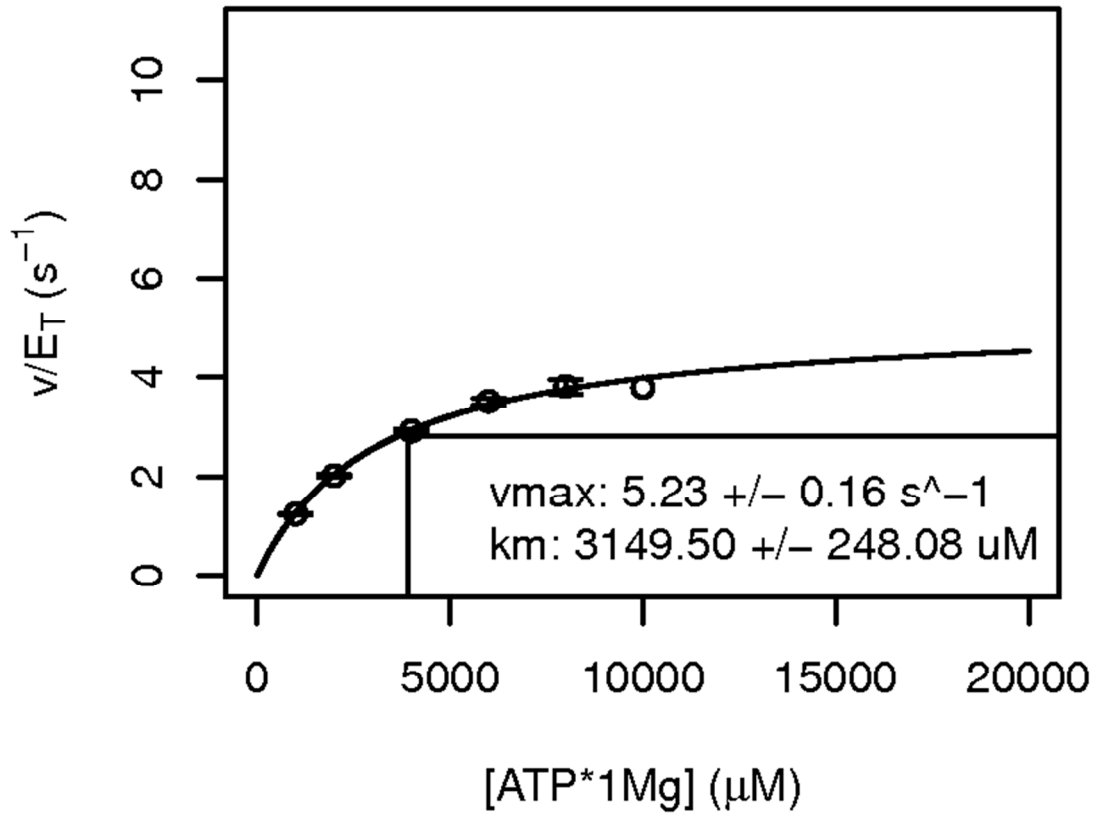


Supplemental Figure 2 ITC of pCDK2•CyclinA binding ADP with 7.5 mM $[Mg^{2+}]_{Total}$.



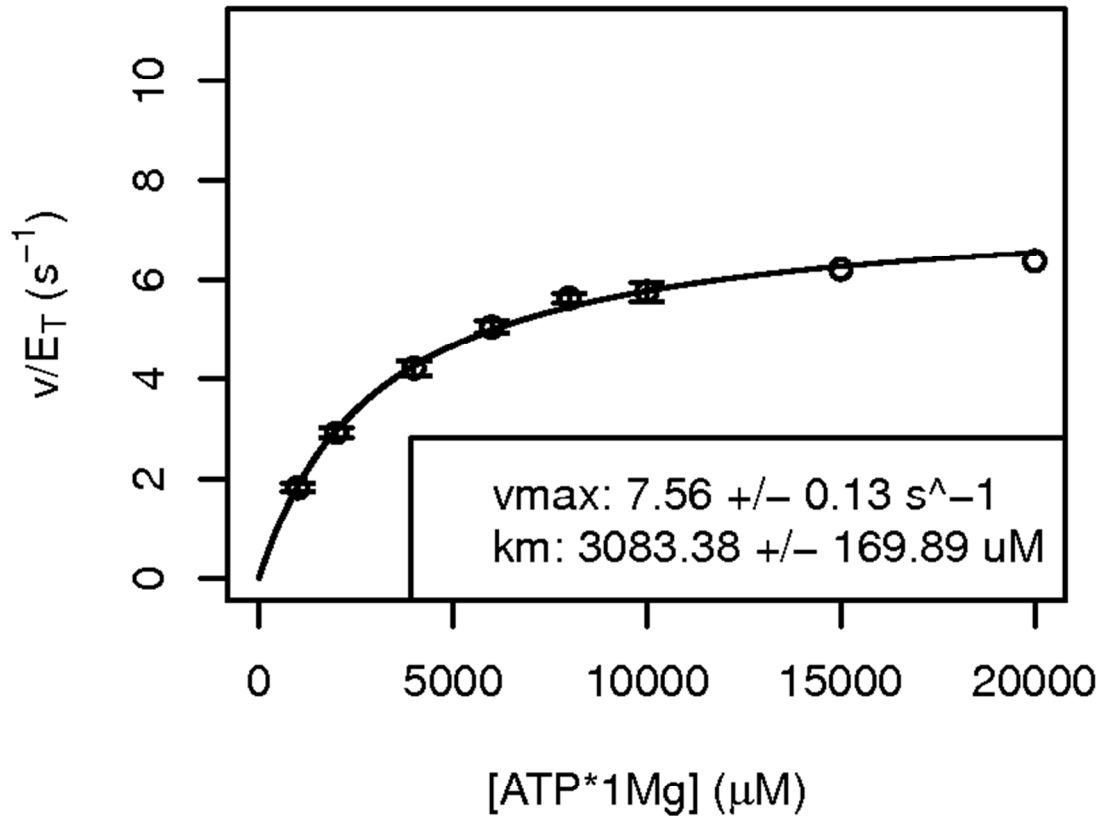
Supplemental Figure 3 ITC of pCDK2•CyclinA binding ADP with 10 mM $[Mg^{2+}]_{Total}$.

KM ATP*Mg 0.025mM [MgFree]



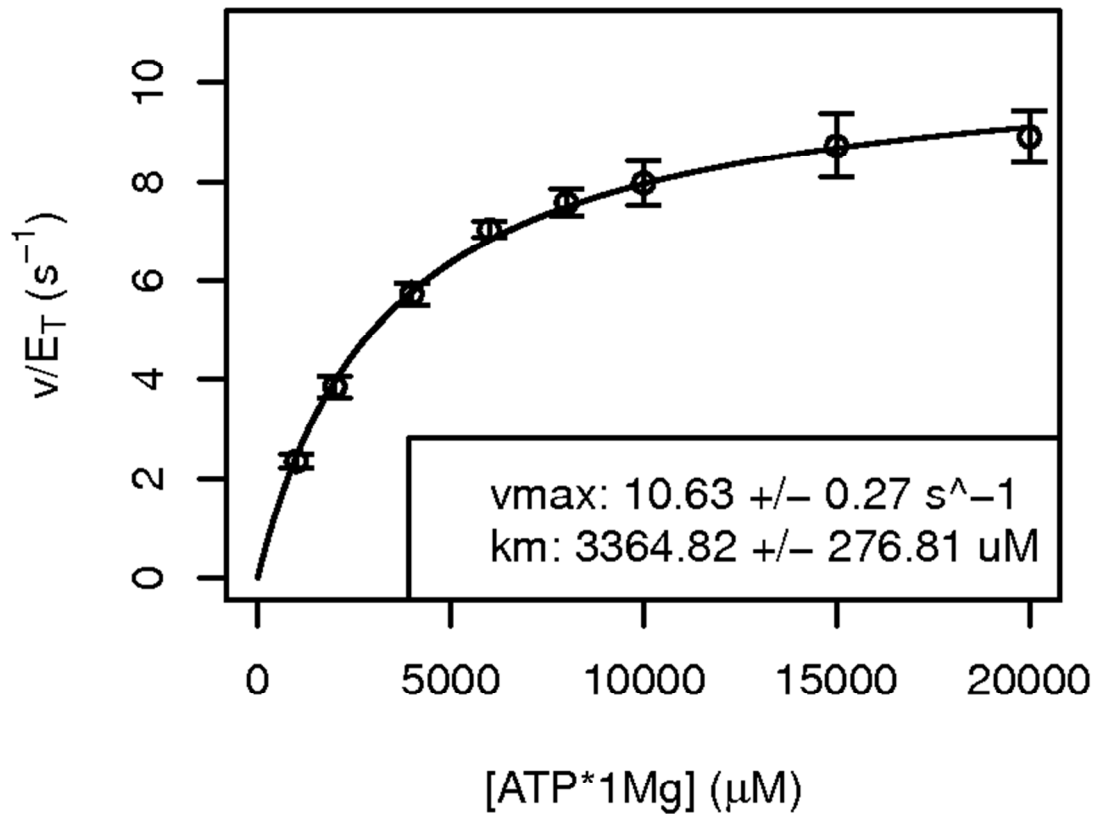
Supplemental Figure 4 K_M for ATP•Mg substrate at 200 μM [H1], 0.025 mM $[Mg^{2+}]_{Free}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{max} \cdot [ATP \cdot 1Mg]) / (k_m + [ATP \cdot 1Mg])$. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$.

KM ATP*Mg 0.05mM [MgFree]



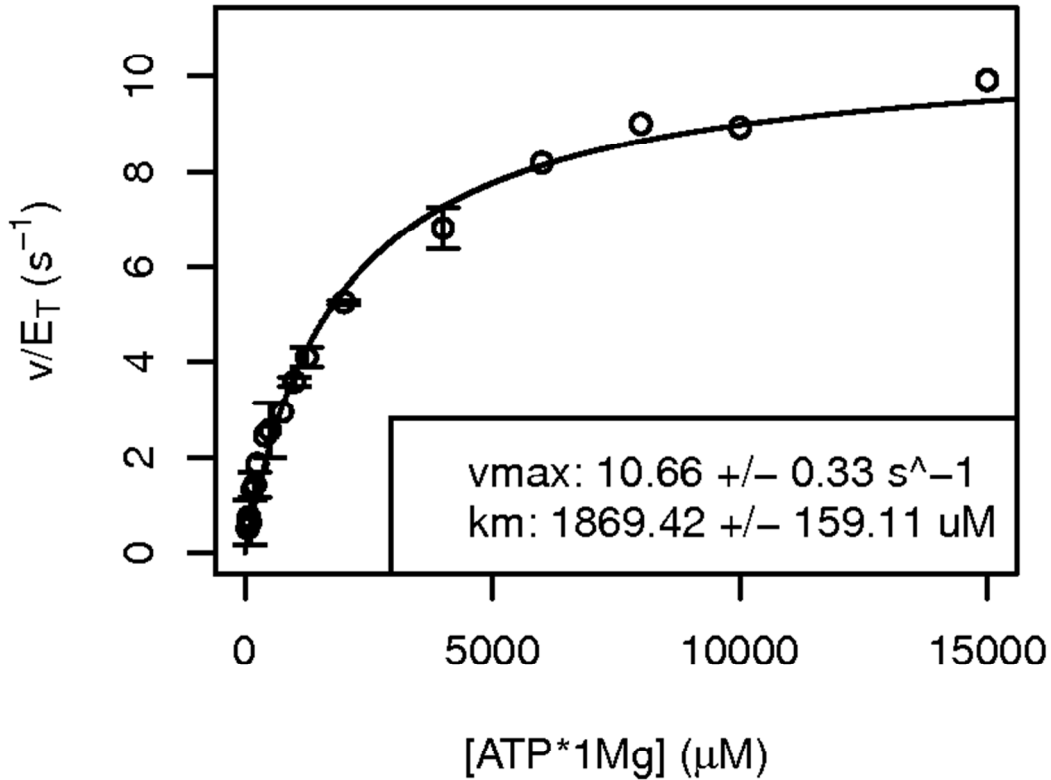
Supplemental Figure 5 K_M for ATP•Mg substrate at 200 μM [H1], 0.05 mM $[Mg^{2+}]_{Free}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{max} \cdot [ATP \cdot 1Mg]) / (k_m + [ATP \cdot 1Mg])$. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$.

KM ATP*Mg 0.1mM [MgFree]



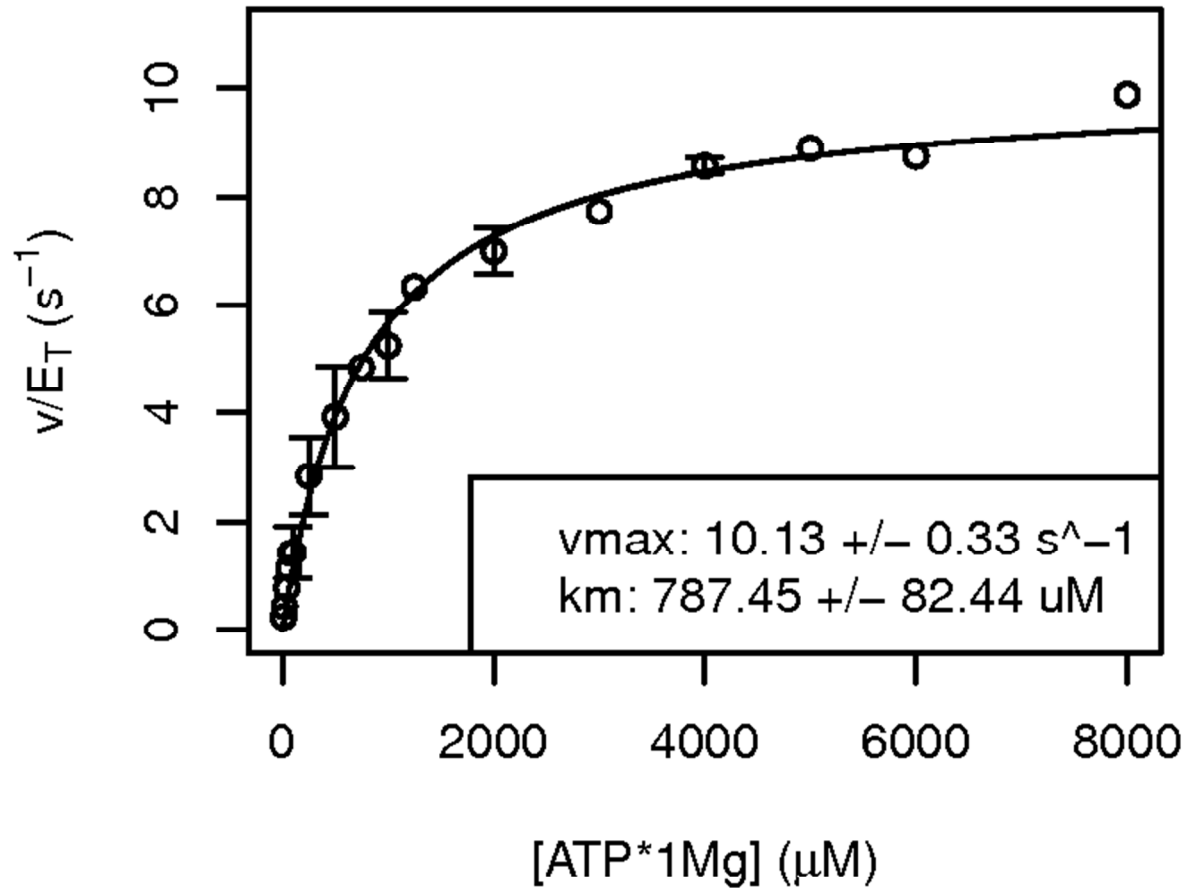
Supplemental Figure 6 K_M for ATP•Mg substrate at 200 μM [H1], 0.1 mM $[Mg^{2+}]_{Free}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{max} \cdot [ATP \cdot 1Mg]) / (k_m + [ATP \cdot 1Mg])$. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$.

KM ATP*Mg 0.5mM [MgFree]



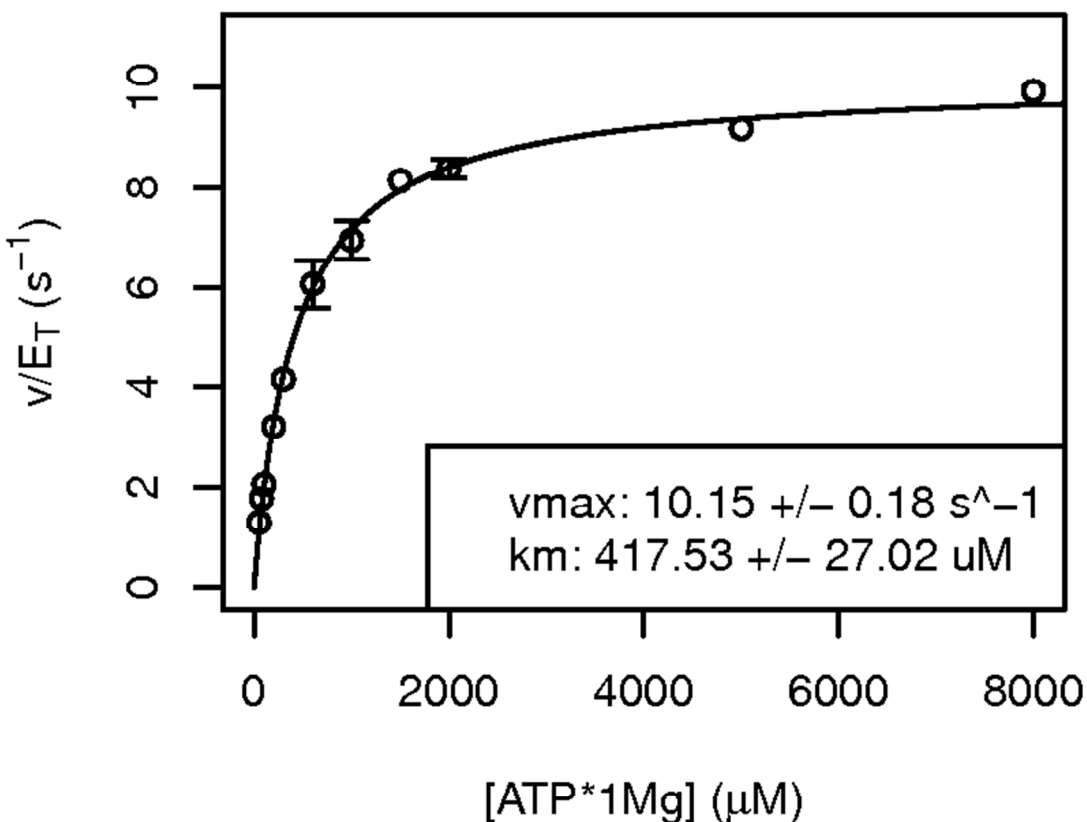
Supplemental Figure 7 K_M for ATP•Mg substrate at 200 μM [H1], 0.5 mM $[Mg^{2+}]_{Free}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{max} * [ATP \cdot 1Mg]) / (k_m + [ATP \cdot 1Mg])$. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$.

KM ATP*Mg 1mM [MgFree]



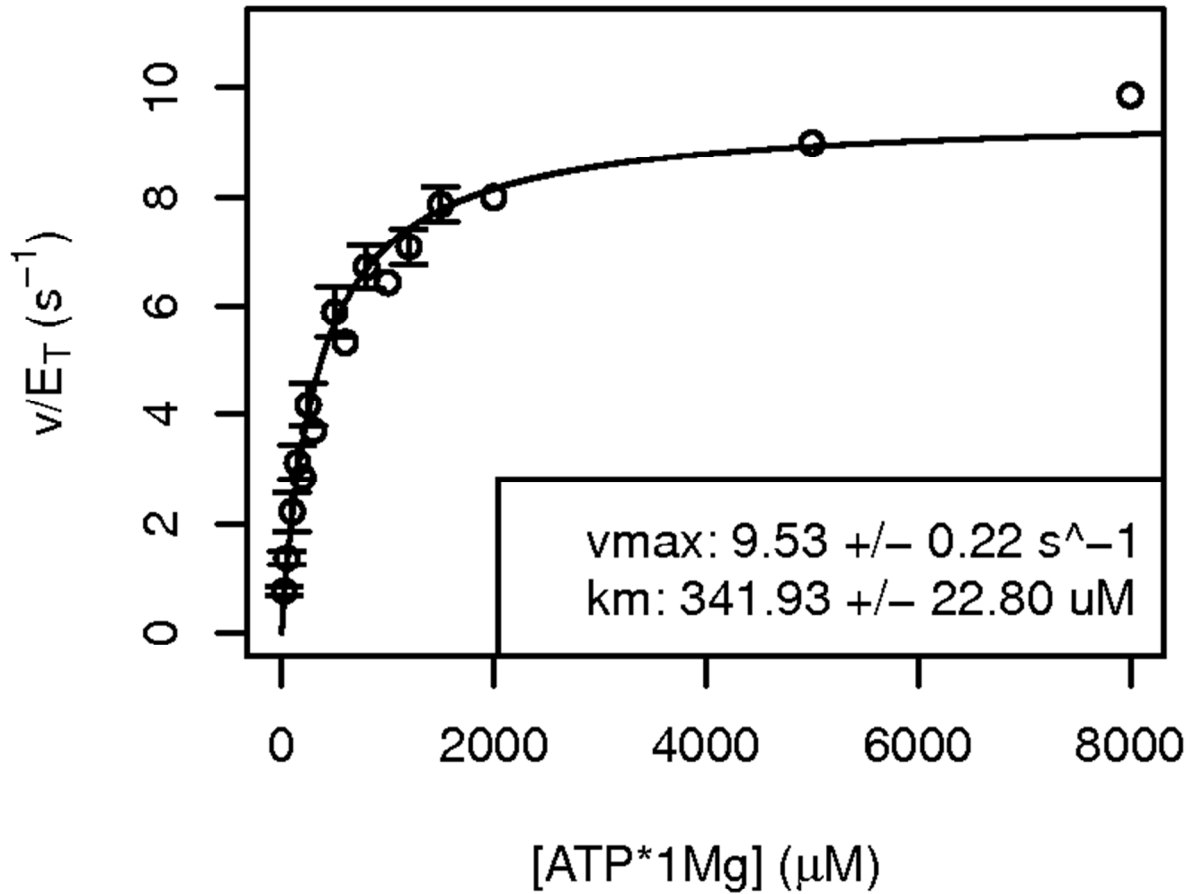
Supplemental Figure 8 K_M for ATP•Mg substrate at 200 μM [H1], 1 mM $[\text{Mg}^{2+}]_{\text{Free}}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{\text{max}} \cdot [\text{ATP} \cdot 1\text{Mg}]) / (k_m + [\text{ATP} \cdot 1\text{Mg}])$. Total amounts of ATP/MgCl₂ calculated using $K_D(\text{ATP} \cdot 1\text{Mg}) = 28.6 \mu\text{M}$.

KM ATP*Mg 2.5mM [MgFree]



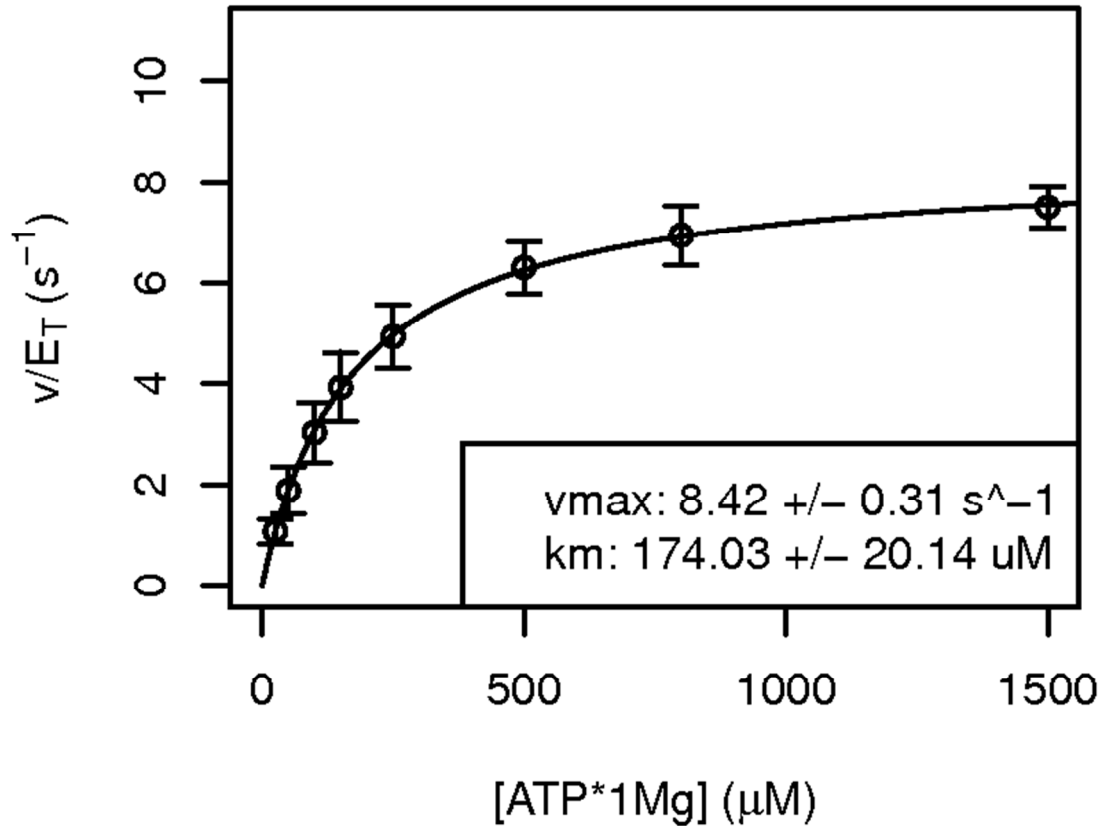
Supplemental Figure 9 K_M for ATP•Mg substrate at 200 μM [H1], 2.5 mM $[Mg^{2+}]_{Free}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{max} \cdot [ATP \cdot 1Mg]) / (k_m + [ATP \cdot 1Mg])$. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$.

KM ATP*Mg 5mM [MgFree]



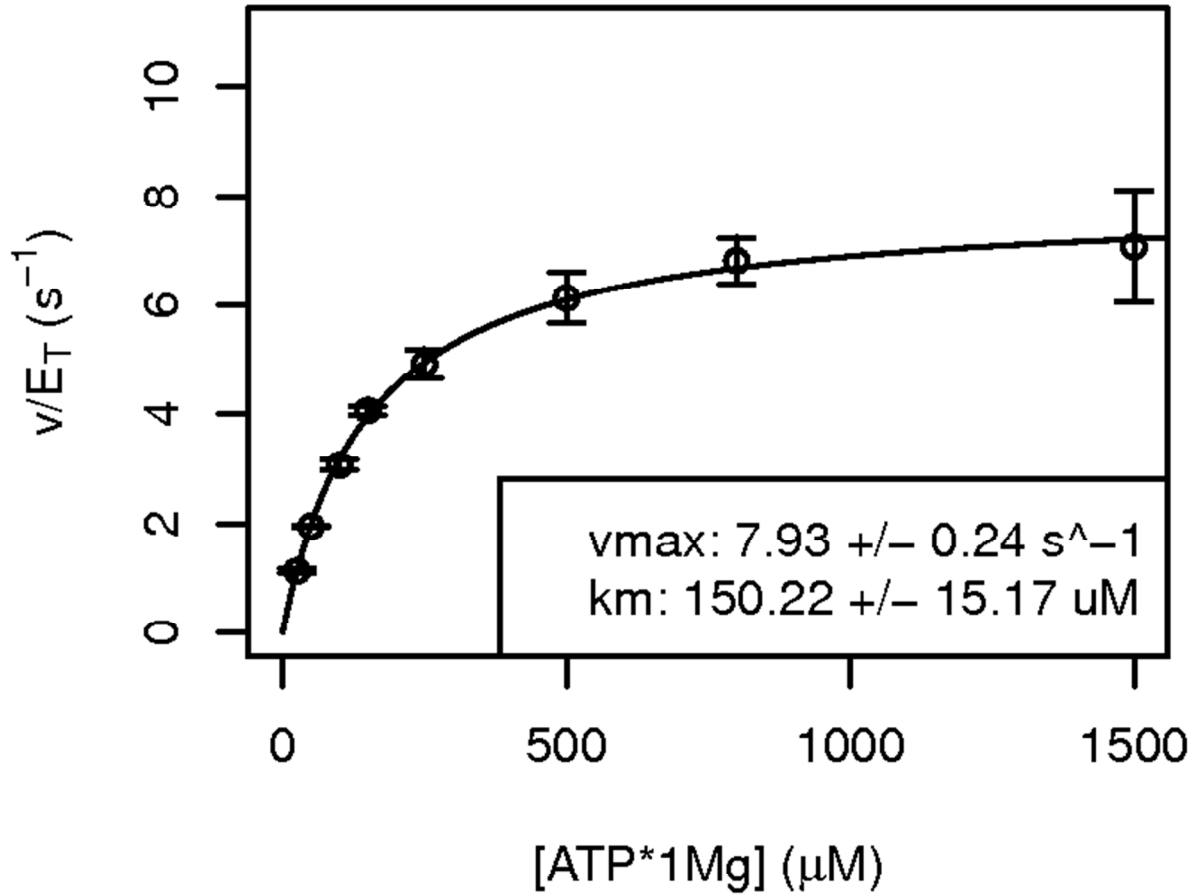
Supplemental Figure 10 K_M for ATP•Mg substrate at 200 μM [H1], 5 mM $[\text{Mg}^{2+}]_{\text{Free}}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_T = (v_{\text{max}} \cdot [\text{ATP} \cdot 1\text{Mg}]) / (k_m + [\text{ATP} \cdot 1\text{Mg}])$. Total amounts of ATP/MgCl₂ calculated using $K_D(\text{ATP} \cdot 1\text{Mg}) = 28.6 \mu\text{M}$.

KM ATP*Mg 7.5mM [MgFree]



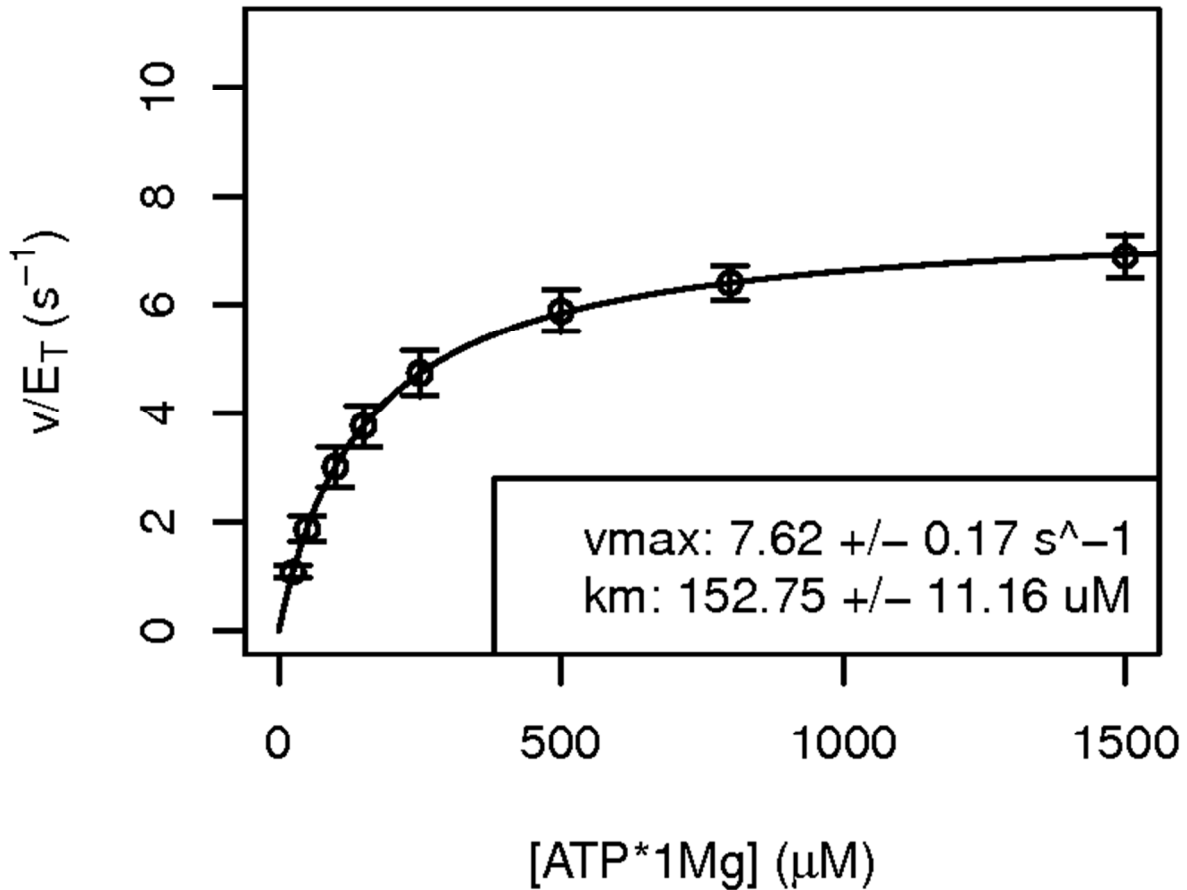
Supplemental Figure 11 K_M for ATP•Mg substrate at 200 μM [H1], 7.5 mM $[Mg^{2+}]_{Free}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{max} \cdot [ATP \cdot 1Mg]) / (k_m + [ATP \cdot 1Mg])$. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$.

KM ATP•Mg 10mM [MgFree]



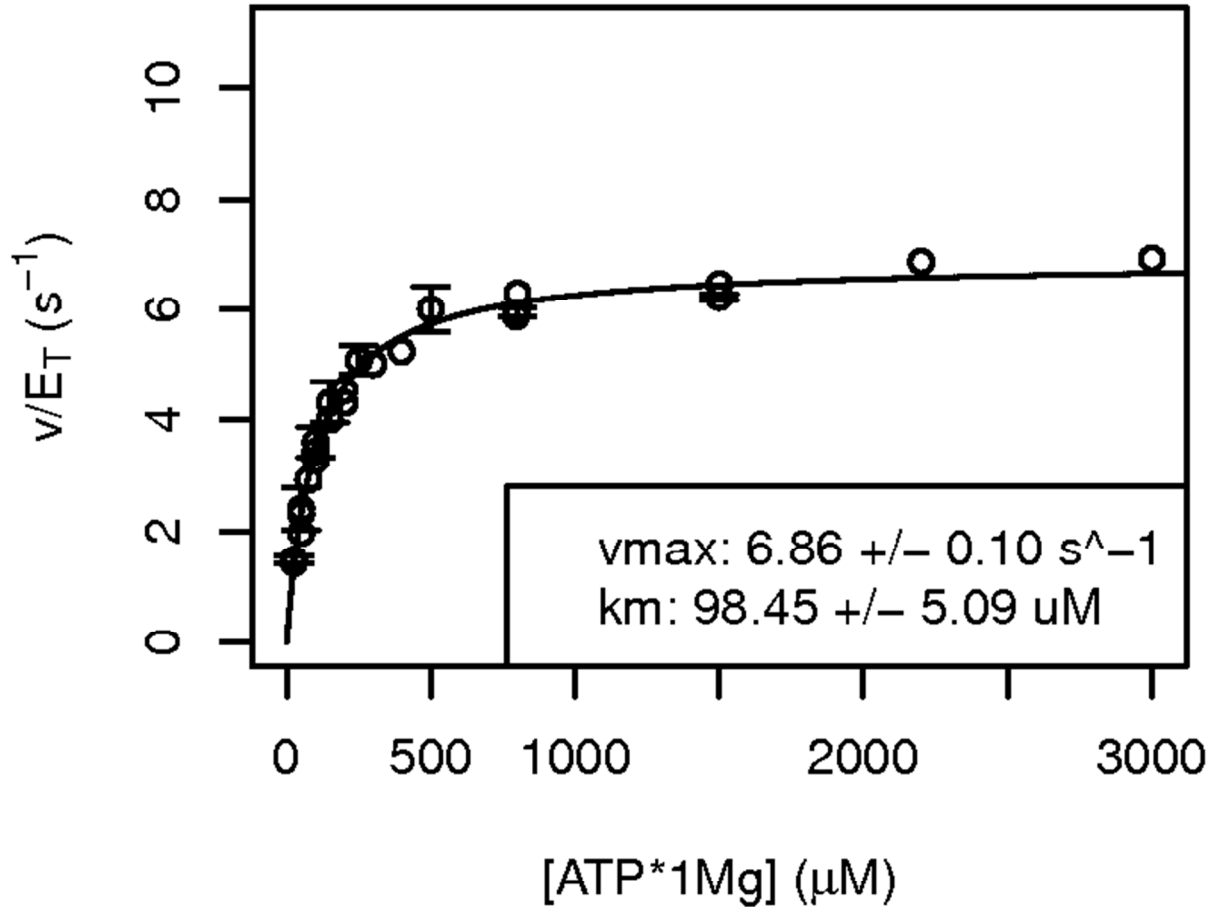
Supplemental Figure 12 K_M for ATP•Mg substrate at 200 μM [H1], 10 mM $[Mg^{2+}]_{Free}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{max} \cdot [ATP \cdot 1Mg]) / (k_m + [ATP \cdot 1Mg])$. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$.

KM ATP*Mg 15mM [MgFree]

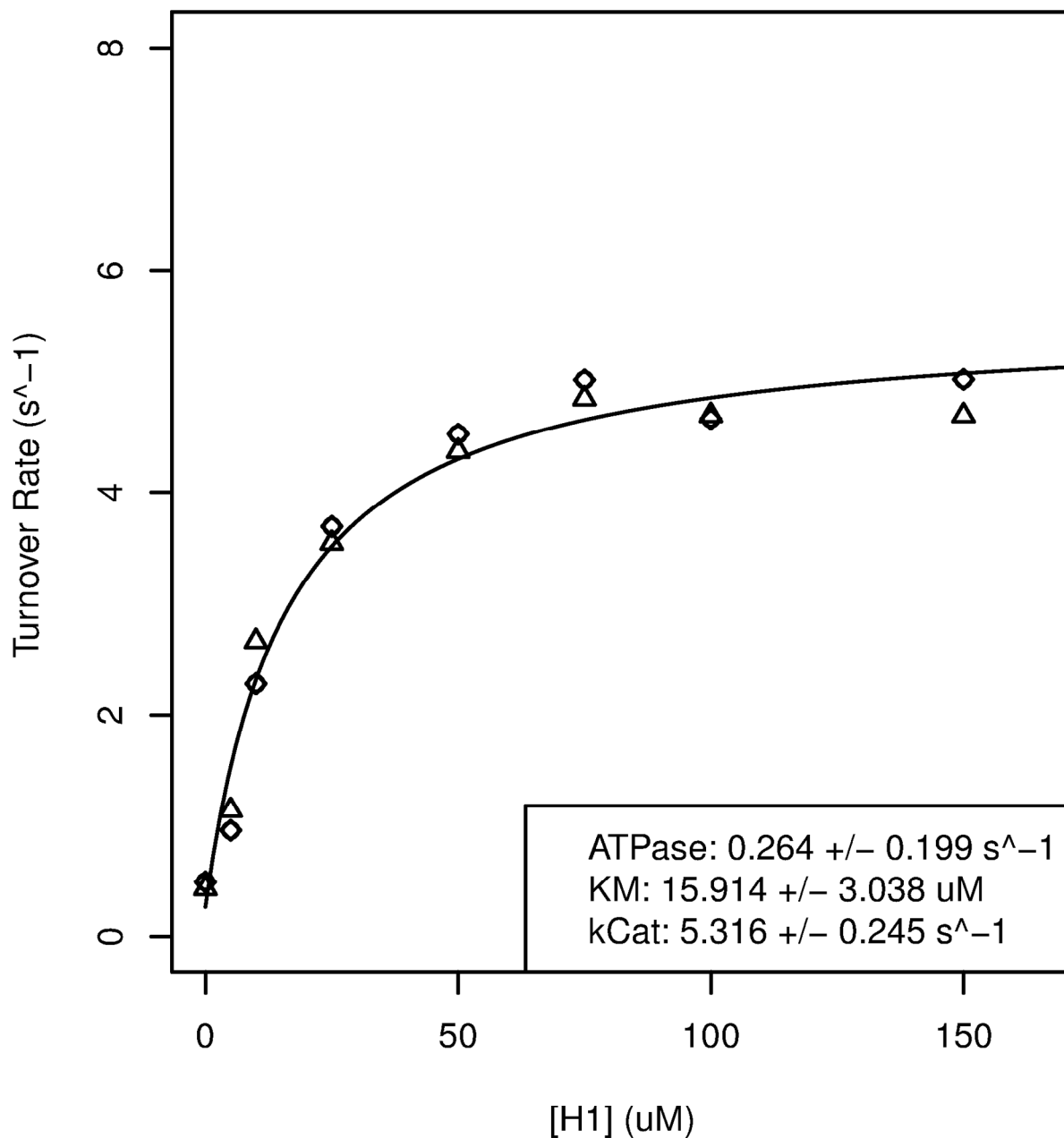


Supplemental Figure 13 K_M for ATP•Mg substrate at 200 μM [H1], 15 mM $[\text{Mg}^{2+}]_{\text{Free}}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{\text{max}} \cdot [\text{ATP} \cdot 1\text{Mg}]) / (k_m + [\text{ATP} \cdot 1\text{Mg}])$. Total amounts of ATP/MgCl₂ calculated using $K_D(\text{ATP} \cdot 1\text{Mg}) = 28.6 \mu\text{M}$.

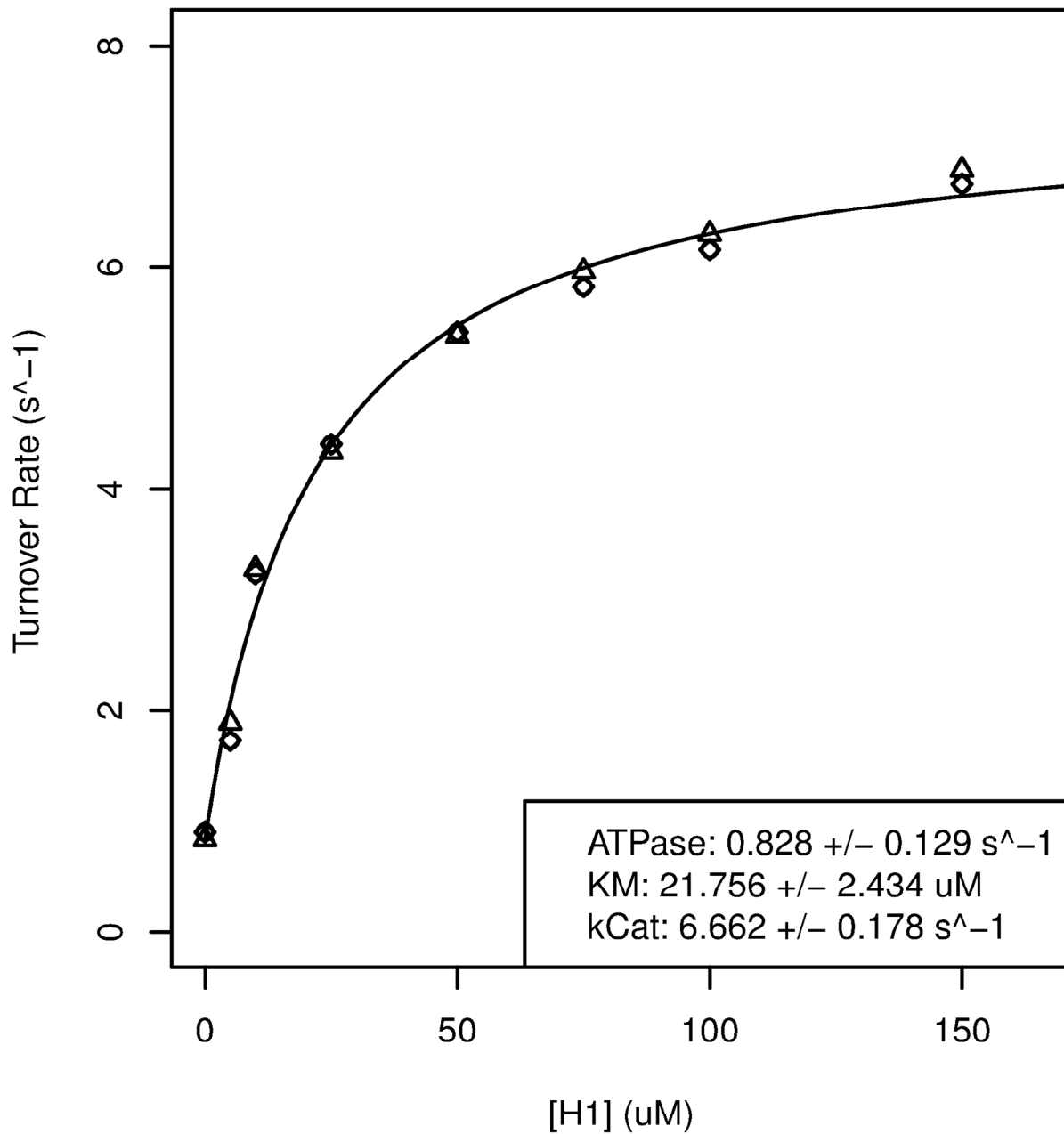
KM ATP*Mg 20mM [MgFree]



Supplemental Figure 14 K_M for ATP•Mg substrate at 200 μM [H1], 20 mM $[Mg^{2+}]_{Free}$. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{max} \cdot [ATP \cdot 1Mg]) / (k_m + [ATP \cdot 1Mg])$. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$.



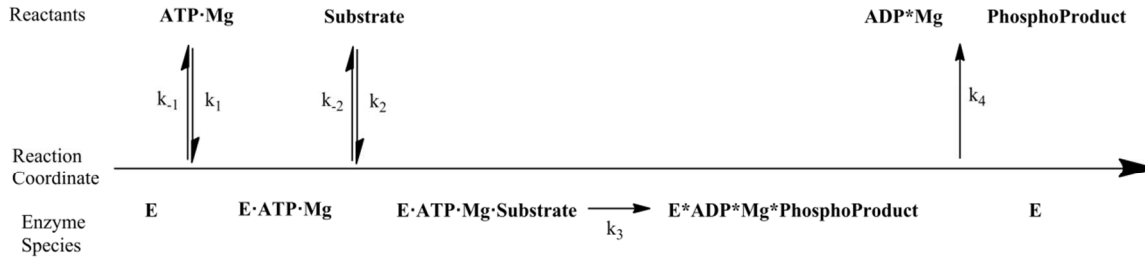
Supplemental Figure 15 K_M for protein substrate at 800 μM [ATP], 1 mM $[\text{Mg}^{2+}]_{\text{Free}}$. K_M curve fit using nonlinear least squares method to equation $v/Et = \text{ATPase} + (k_{\text{Cat}} \cdot [\text{H1}]) / (K_M + [\text{H1}])$. Total amounts of ATP/MgCl₂ calculated using $K_D(\text{ATP} \cdot 1\text{Mg}) = 28.6 \mu\text{M}$.



Supplemental Figure 16 K_M for protein substrate at 800 μM [ATP], 20 mM $[\text{Mg}^{2+}]_{\text{Free}}$. KM curve fit using nonlinear least squares method to equation $v/Et = \text{ATPase} + (k_{\text{Cat}} \cdot [\text{H1}]) / (K_M + [\text{H1}])$. Total amounts of ATP/MgCl₂ calculated using $K_D(\text{ATP} \cdot 1\text{Mg}) = 28.6 \mu\text{M}$.

Solvent Viscosity Effect Experiments

Varying the solvent viscosity can help to discriminate the proportional contribution of specific steps of the catalytic cycle in the overall rate of catalysis. This is because the solvent viscosity alters the rate of diffusive steps (e.g. bi-molecular events such as substrate binding or product release) a great deal more than the rate of non-diffusive steps (e.g. the transfer of the γ -phosphate to the protein substrate).



Scheme 1: Classic protein kinase reaction scheme

Microscopic rate constants are: k_1 (ATP·Mg binding), k_{-1} (ATP·Mg release), k_2 (Protein substrate binding), k_{-2} (Protein substrate release), k_3 (Phosphoryl transfer), and k_4 (Product release). Previous studies of CDK2 and other kinases suggest that the binding order of the ATP·Mg substrate and the protein substrate are randomly ordered. There is less information on when the 2nd Mg binds, but the structures predict it must be concomitant with ATP·Mg binding or after ATP·Mg binding. The apparent effect on $K_M(\text{ATP}\cdot\text{Mg})$ of the 2nd Mg binding further supports the cooperative binding of the ATP·Mg and Mg substrates, so we assume that k_1 and k_{-1} are the effective on and off net rate constants of the combined ATP·Mg and second Mg substrate at each $[\text{ATP}\cdot\text{Mg}]$ and $[\text{Mg}^{2+}]_{\text{Free}}$ condition.

If the ATP·Mg and protein substrates are saturating in the experimental conditions, then the rate of catalysis can be reduced to two sequential steps: phosphoryl transfer (k_3) and then product release (k_4), where only the rate of product release is affected by the solvent viscosity, such that the total flux through the catalytic cycle is given by (Adams, 2001):

$$k_{cat}^{App} = \frac{k_3^{App} * k_4^{App}}{k_3^{App} + k_4^{App}}$$

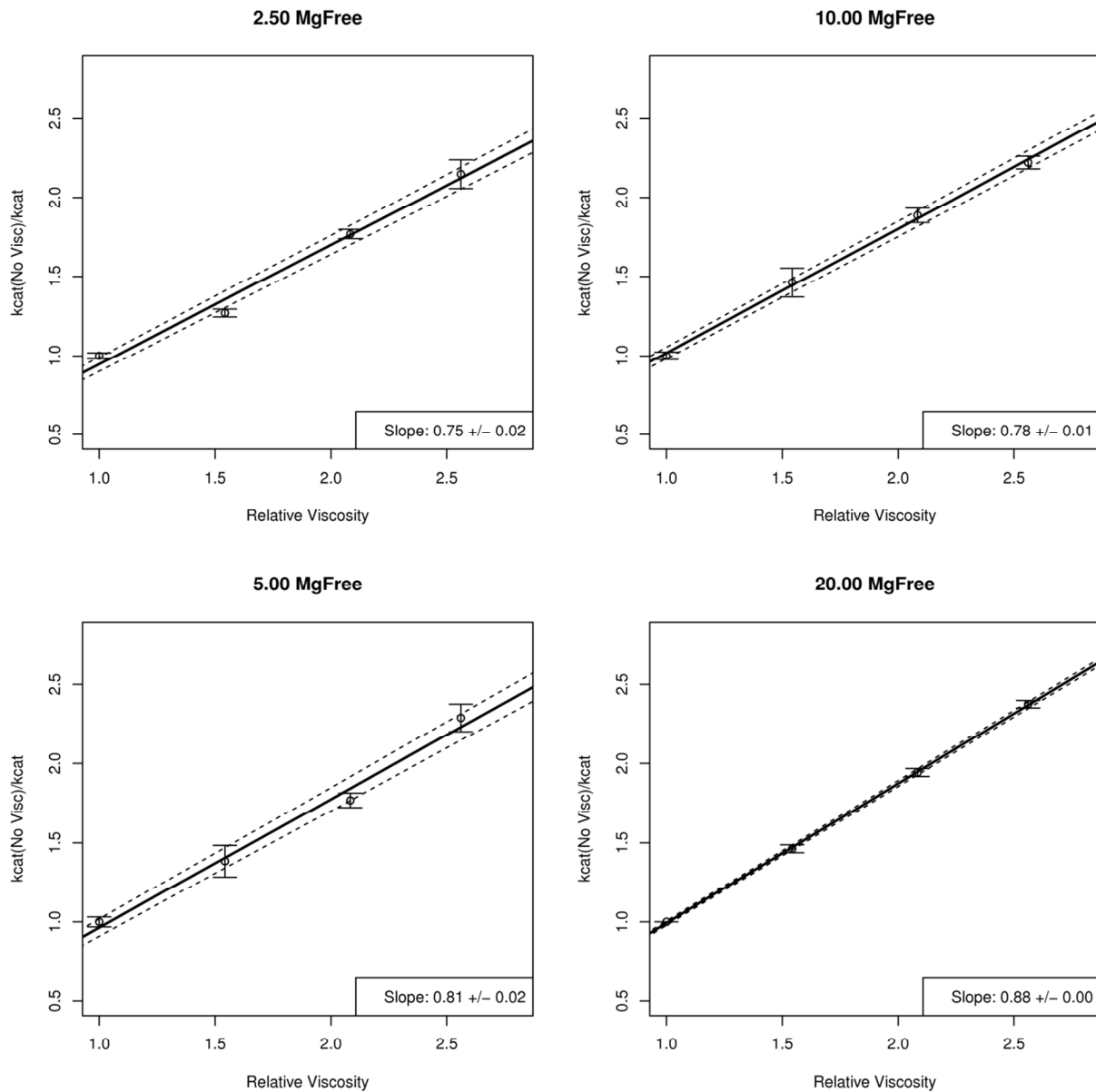
The “App” superscript is used in this case because k_{cat} , k_3 and k_4 are considered separately for each $[\text{Mg}^{2+}]_{\text{Free}}$, and the binding of the 2nd Mg^{2+} ion may not be saturating at any of the tested $[\text{Mg}^{2+}]_{\text{Free}}$. The effect of the solvent viscosity, m , on k_{cat}^{App} is determined by measuring k_{cat} at several different solvent viscosities and then fitting a line to the equation:

$$\eta - 1 = m \left(\frac{k_{cat}^{\circ, App}}{k_{cat}^{App}} - 1 \right) ; \text{ where } \eta = \text{Relative Solvent Viscosity} = \frac{k_4^{\circ, App}}{k_4^{App}}$$

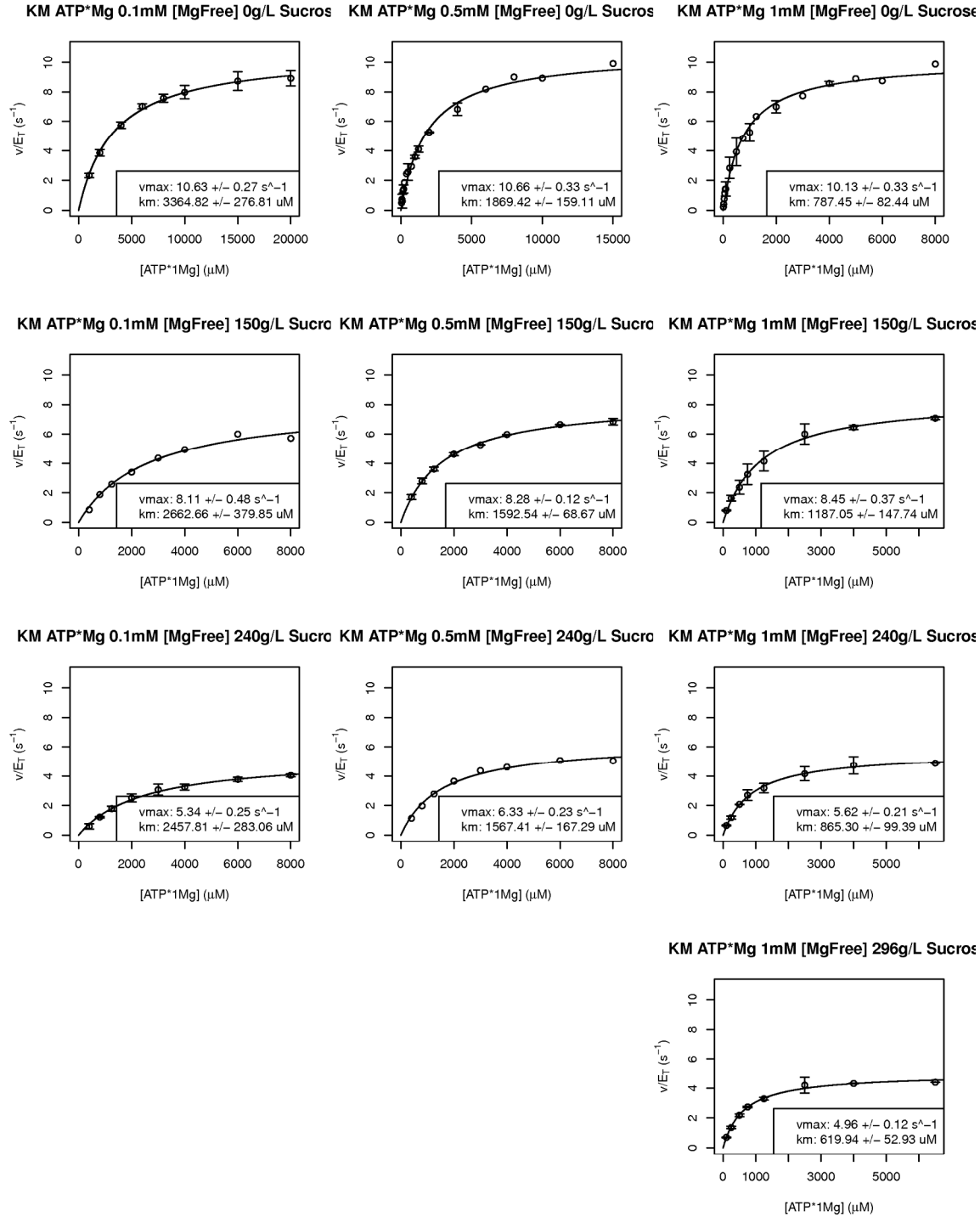
where η is the relative viscosity of the solvent, m is the viscosity effect, k_{cat}^{App} is the measured rate of the reaction at each [sucrose], and $k_{cat}^{\circ,App}$ is the reference viscosity ([sucrose] = 0), and m is the slope of the line, which is the viscosity effect on k_{cat}^{App} . Substituting η with $k_4^{\circ,App}/k_4^{App}$, $k_{cat}^{\circ,App}$ with $k_3^{App} * k_4^{\circ,App} / (k_3^{App} + k_4^{\circ,App})$, and k_{cat}^{App} with $k_3^{App} * k_4^{App} / (k_3^{App} + k_4^{App})$, and then following algebraic manipulation of the above equation and solving for m gives:

$$m = \frac{k_3^{App}}{k_3^{App} + k_4^{\circ,App}} \quad \therefore \quad k_4^{\circ,App} = \frac{k_{cat}^{\circ,App}}{m} \quad \text{and} \quad k_3^{App} = \frac{k_{cat}^{\circ,App}}{1 - m}$$

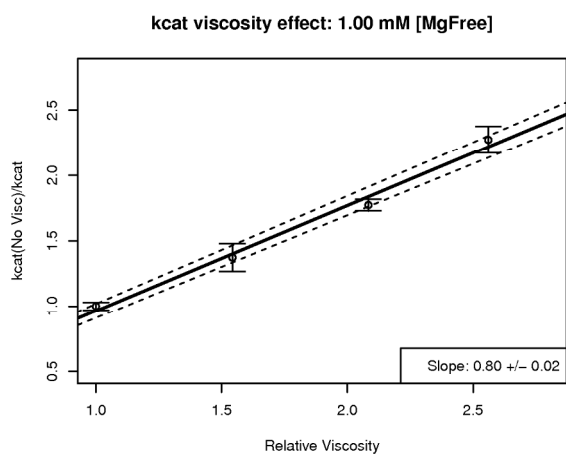
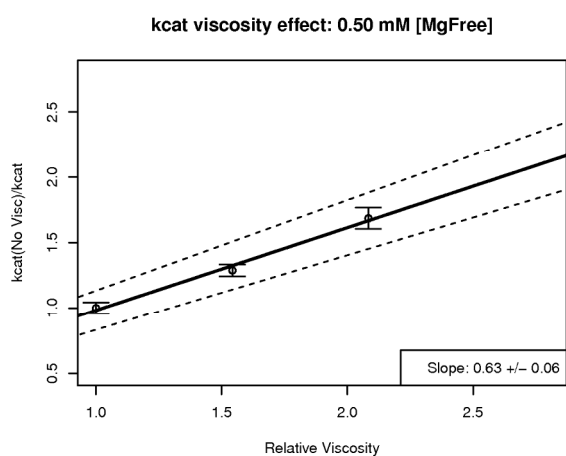
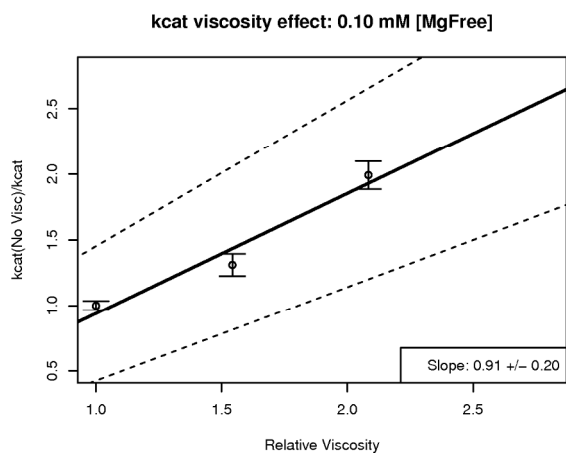
where the $^{\circ}$ superscript denotes that the rate constant is the [sucrose]=0 condition.



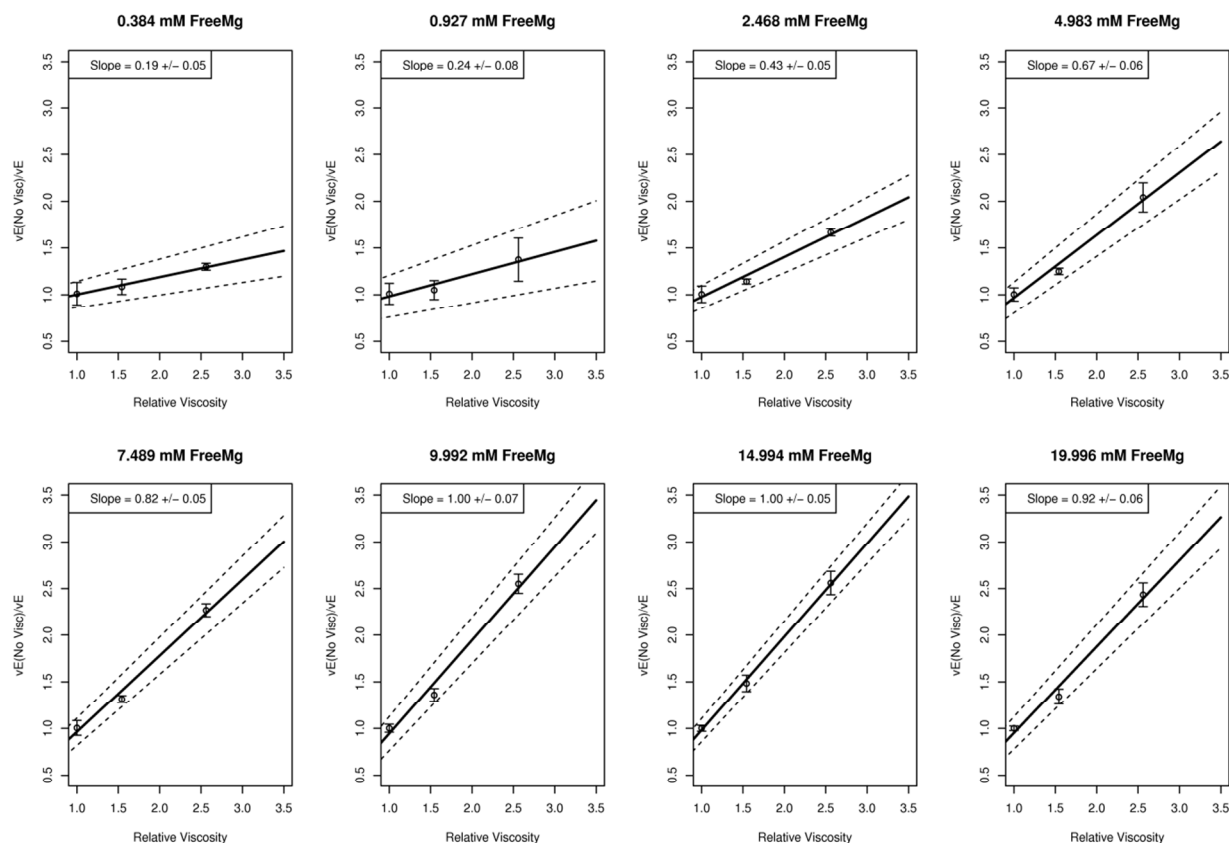
Supplemental Figure 17 Solvent viscosity affect at constant 9.3 mM [ATP] at various $[Mg^{2+}]_{Free}$. This represents $> 20x K_M(ATP \cdot Mg)$ at from 2.5 mM to 20 mM $[Mg^{2+}]_{Free}$. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$. Data points plotted at mean position for observations. Error bars represent standard error for multiple observations of data point. Solid black line is the fit linear model to the equation: $v^0/v = m(\text{relativeViscosity}) + \text{intercept}$, where the slope of the line m is the viscosity effect reported in figure 8A. Dotted lines show the potential variation of the linear fit based on the standard error of the fit intercept and slope m .



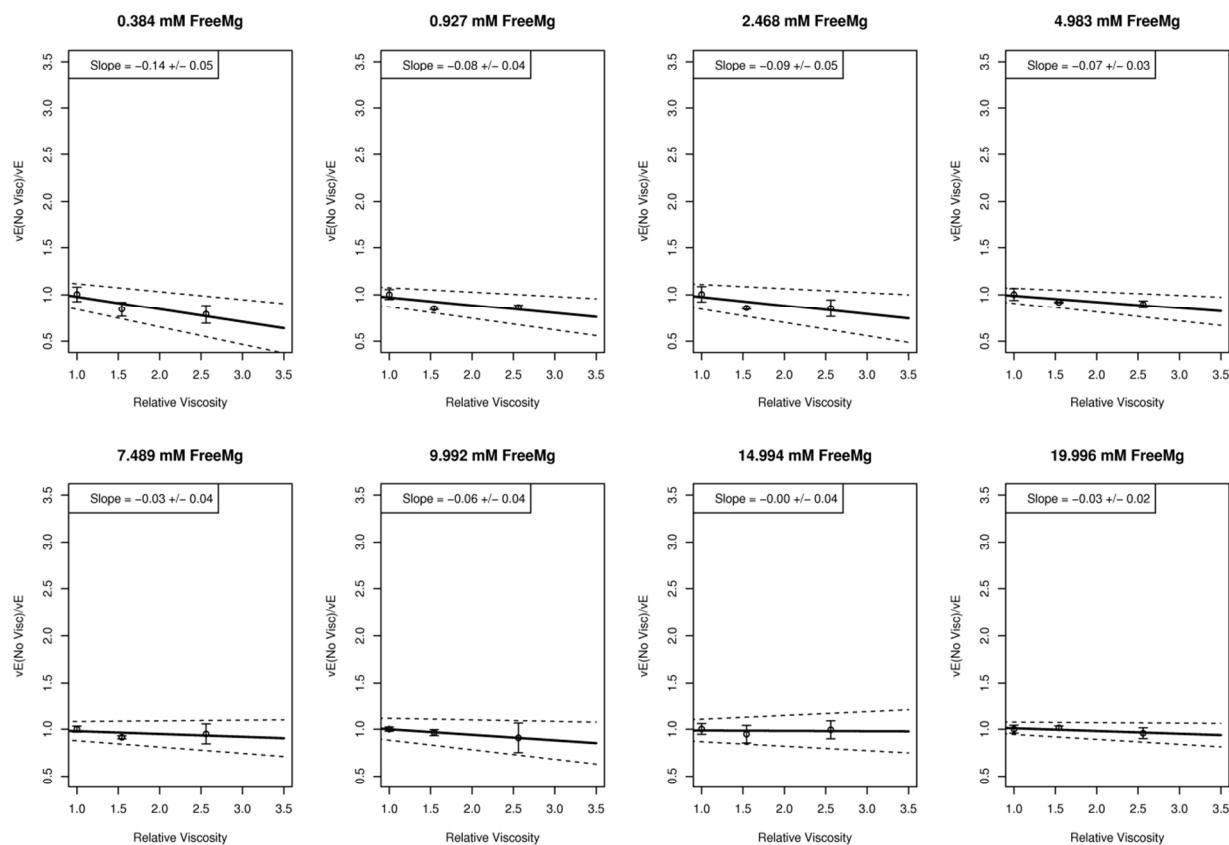
Supplemental Figure 18 K_M for ATP•Mg substrate at 200 μM [H1] at 0.1, 0.5 and 1 $[\text{Mg}^{2+}]_{\text{Free}}$, and 0, 150, 240, 296 g/L Sucrose. Error bars represent standard error for data point among multiple observations. KM curve fit using nonlinear least squares method to equation $v/E_t = (v_{max} \cdot [\text{ATP} \cdot \text{Mg}]) / (k_m + [\text{ATP} \cdot \text{Mg}])$. Total amounts of ATP/MgCl₂ calculated using $K_D(\text{ATP} \cdot \text{Mg}) = 28.6 \mu\text{M}$.



Supplemental Figure 19 Solvent viscosity affect at calculated k_{cat} at 0.1 mM, 0.5 mM, and 1 mM $[Mg^{2+}]_{Free}$ (from Supplemental Figure 8). Error bars represent standard error for nonlinear fit of k_{cat} . Solid black line is the fit linear model to the equation: $v^o/v = m(\text{relativeViscosity}) + \text{intercept}$, where the slope of the line m is the viscosity effect reported in figure 8A. Dotted lines show the potential variation of the linear fit based on the standard error of the fit intercept and slope m .

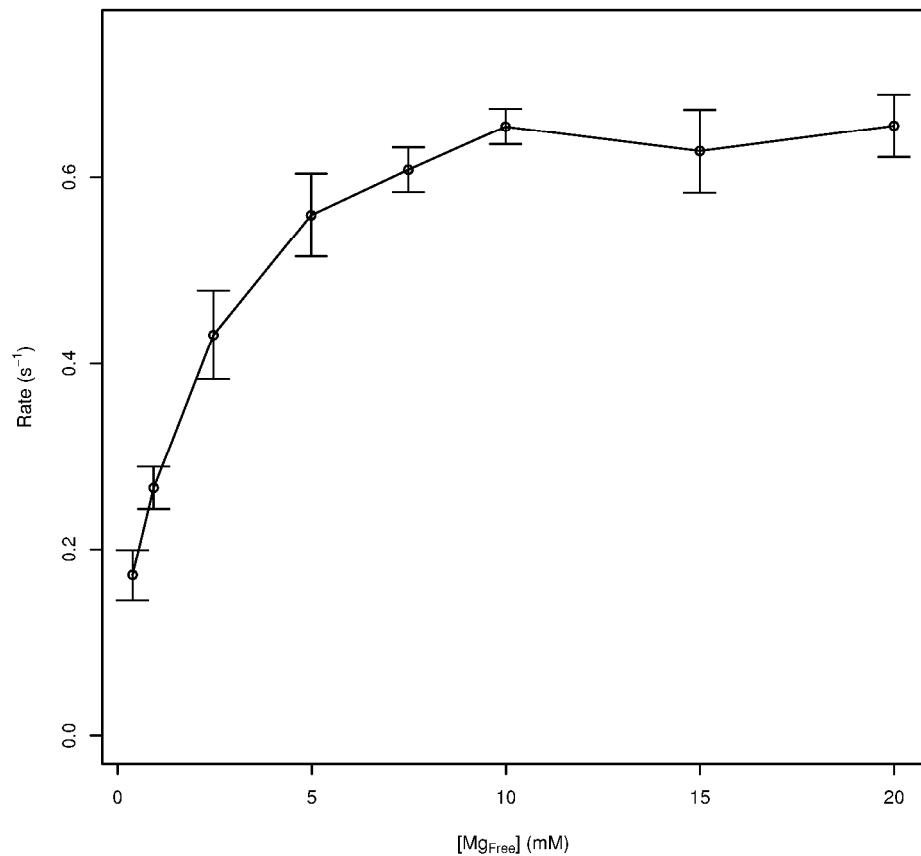


Supplemental Figure 20 Solvent viscosity affect at constant 1.2 mM [ATP] at various $[Mg^{2+}]_{Free}$ with saturating (200 μ M) protein substrate histone H1. At lower $[Mg^{2+}]_{Free}$, 1.2 mM [ATP] is subsaturating, but at high $[Mg^{2+}]_{Free}$, this condition does saturate. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu$ M. Data points plotted at mean position for observations. Error bars represent standard error for multiple observations of data point. Solid black line is the fit linear model to the equation: $v^\circ/v = m(\text{relativeViscosity}) + \text{intercept}$, where the slope of the line m is the viscosity effect reported in figure 8B. Dotted lines show the potential variation of the linear fit based on the standard error of the fit intercept and slope m .

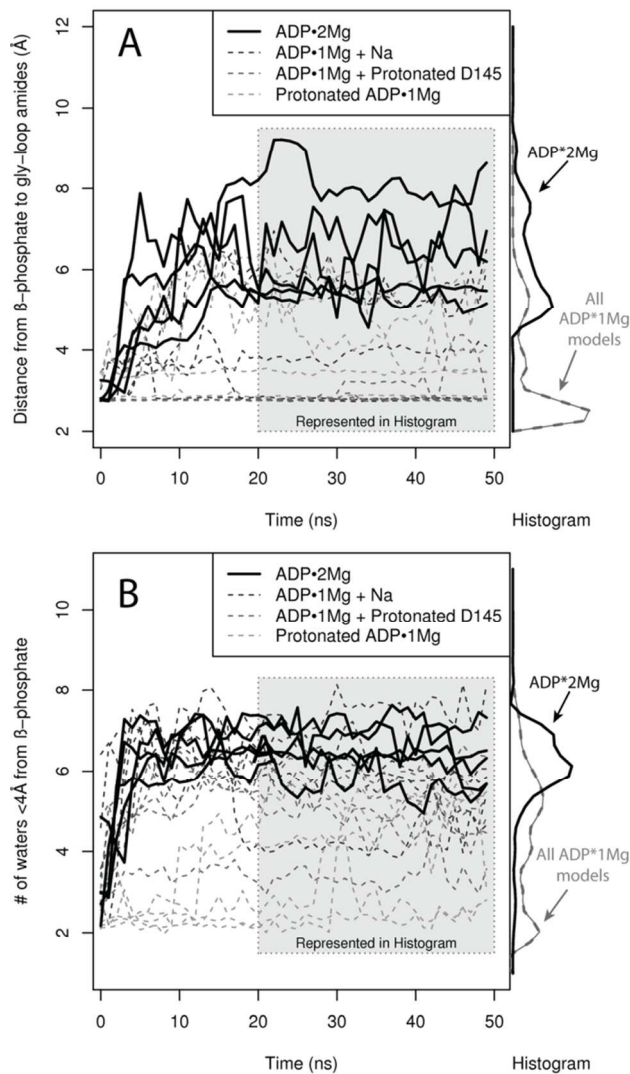


Supplemental Figure 21 Solvent viscosity affect at constant 1.2 mM [ATP] at various $[Mg^{2+}]_{Free}$ with **no protein substrate** (water as substrate). Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$. Data points plotted at mean position for observations. Error bars represent standard error for multiple observations of data point. Solid black line is the fit linear model to the equation: $v^{\circ}/v = m(\text{relativeViscosity}) + \text{intercept}$, where the slope of the line m is the viscosity effect reported in figure 8B. Dotted lines show the potential variation of the linear fit based on the standard error of the fit intercept and slope m .

pCDK2/Cyclin ATPase activity with 1.2mM [ATP]



Supplemental Figure 22 Rate of pCDK2/Cyclin ATPase reaction vs. $[Mg^{2+}]_{Free}$ with 1.2 mM [ATP] and 0 protein substrate H1. Total amounts of ATP/MgCl₂ calculated using $K_D(ATP \cdot 1Mg) = 28.6 \mu M$. Data points plotted at mean position for observations. Error bars represent standard error for multiple observations of data point.



Supplemental Figure 23. Probability of gly-loop opening in MD simulations increased with two Mg^{2+} ions. MD started from closed gly-loop conformation with either ADP•2Mg coordination (solid black lines) or ADP•1Mg-type coordination (dashed gray lines). Each ADP coordination model simulated five times. Each curve in time series plot represents a single trajectory. Histograms integrate the last 30ns from all ADP•2Mg or ADP•1Mg-type trajectories into a single curve. **Panel A)** Distance between gly-loop amides and ADP β -phosphate. ADP•2Mg simulations consistently open gly-loop within simulation timescale, ADP•1Mg-type simulations do not. **Panel B)** Open gly-loop conformation increases solvent accessibility of phosphates. Number of waters around β -phosphate in ADP•2Mg simulations increases rapidly and then maintains a steady population of 6-7 waters around β -phosphate. ADP•1Mg-type simulations sample closed, intermediate, and open conformations which allow 2 to 7 waters to interact within 4Å of β -phosphate.

Table 1. X-Ray Crystallography Statistics

	ADP•2Mg (PDB XXXX)	ADP•1Mg (PDB XXXX)
<i>Data Collection</i>		
Wavelength (Å)	0.97856	0.97856
Resolution range (Å)	43.12 - 2.05 (2.16 - 2.05)	29.56 - 2.15 (2.27 - 2.15)
Space group	P 1 2 ₁ 1	P 1 2 ₁ 1
Unit cell parameters a, b, c (Å)	70.77, 164.13, 73.28	71.14, 164.14, 73.45
Unit cell parameters α , β , γ (°)	90, 107.07, 90	90, 107.04, 90
Total observations	353627 (48178)	299094 (38261)
Unique reflections	98449 (14154)	83146 (11033)
Completeness (%)	98.6 (97.3)	95.5 (87.3)
Mean I/ σ (I)	7.5 (1.6)	6.4 (1.5)
R _{merge}	0.119 (0.857)	0.133 (0.964)
Wilson B-factor (Å ²)	25.3	31.1
<i>Refinement</i>		
R _{cryst}	0.197	0.199
R _{free}	0.227	0.227
Number of protein molecules per asymmetric unit	2 CDK2 + 2 Cyclin	2 CDK2 + 2 Cyclin
Number of nonhydrogen atoms		
Protein	8902	8906
Ligand	114	104
Water	521	517
Average B-factor (Å ²)	39.6	45.2
Ligand Average B-factor (Å ²)	53.3	62.7
RMSD from ideal		
Bond length (Å)	0.0076	0.0103
Bond angle (°)	0.8868	1.1769
Ramachandran plot (%)		
Most favored	99.09	99.18
Additionally allowed	0.91	0.64

Table 2. Summary of Molecular Dynamics Simulations

	Starting Structure & Alterations	Time	Mean Backbone RMSD (Å)
ADP•2Mg	ADP•2Mg crystal	5x 50 ns	1.86 ± 0.22
ADP•1Mg	ADP•1Mg crystal	5x 50 ns	1.88 ± 0.29
ADP•1Mg + Na	ADP•1Mg crystal with Na ⁺ positioned in MgI site	5x 50 ns	1.84 ± 0.20
ADP•1Mg + ProtD145	ADP•1Mg with D145 Protonated	5x 50 ns	1.77 ± 0.21
ProtADP•1Mg	ADP•1Mg with β-phosphate protonated	5x 50 ns	1.75 ± 0.20
Gly-down ADP•2Mg	TS (3QHR) without MgF ₃ or peptide	5x 50 ns	2.16 ± 0.29
Gly-down ADP•1Mg + Na	TS (3QHR) without MgF ₃ , peptide or MgI. Model Na ⁺ in MgI site	5x 50 ns	2.28 ± 0.36
Gly-down ADP•1Mg + Protonated D145	TS (3QHR) without MgF ₃ , peptide or MgI. Protonate D145.	5x 50 ns	2.17 ± 0.26
Gly-down protADP•1Mg	TS (3QHR) without MgF ₃ , peptide or MgI. Protonate β-phosphate.	5x 50 ns	2.32 ± 0.53

References

Due to space restrictions the author list for Gaussian03 was truncated in the original publication. The full reference appears here:

(32) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03, C.02*.