SUPPLEMENTAL MATERIAL

MECHANISM OF PDK1-CATALYZED T229 PHOSPHORYLATION OF THE S6K1 PROTEIN KINASE*

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Running Title: PDK1 Phosphorylation of S6K1 Kinase

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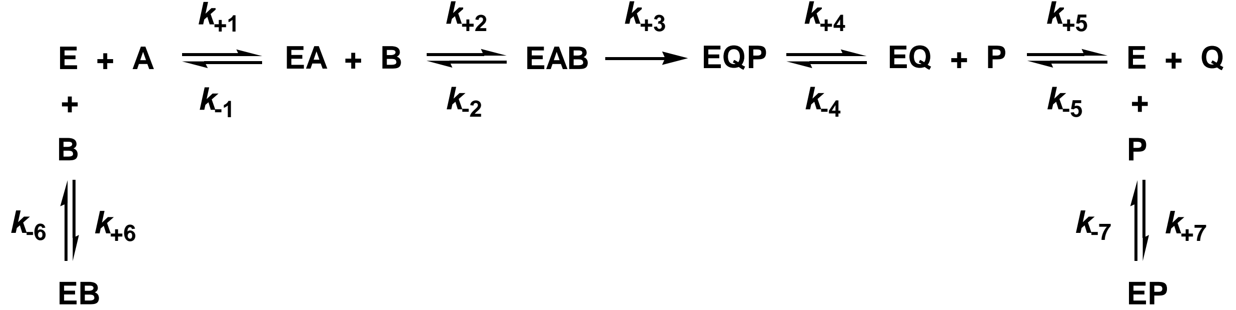
1. Formulation of an Ordered Bi Bi Mechanism with Competitive Substrate Inhibition

1.1. King-Altman Derivation

Kinetic formulations are simultaneously described for (i) an Ordered Bi Bi system (Scheme 1; steps 1−5) and (ii) an Ordered Bi Bi system with competitive substrate (and product) inhibition (Scheme 1; steps 1−7), taking into account an irreversible chemical phosphorylation step in the ternary complex (Scheme 1; step 3).



SCHEME S1

The schematic method of King and Altman (1) was used to derive the complete rate equation for the mechanism in Scheme S1 (steps 1−7), which is given by Equation S1,

$$\frac{v\,(\mathrm{M\ s^{-1}})}{[\mathrm{E}]\,(\mathrm{M})} = k\,(\mathrm{s^{-1}}) = \frac{n_1\,[\mathrm{A}][\mathrm{B}]}{d_1 + d_\mathrm{A}\,[\mathrm{A}] + d_\mathrm{B}\,[\mathrm{B}] + d_\mathrm{AB}\,[\mathrm{A}][\mathrm{B}] + d_\mathrm{Q}[\mathrm{Q}] + d_\mathrm{BQ}[\mathrm{B}][\mathrm{Q}] + d_\mathrm{QP}[\mathrm{Q}][\mathrm{P}] + d_\mathrm{BQP}[\mathrm{B}][\mathrm{Q}][\mathrm{P}] + d_\mathrm{ABP}[\mathrm{A}][\mathrm{B}][\mathrm{P}] + \{d_\mathrm{P}[\mathrm{P}] + d_\mathrm{BB}[\mathrm{B}][\mathrm{B}] + d_\mathrm{BP}[\mathrm{B}][\mathrm{P}]\}} \tag{S1}$$

whereby [A], [B], [P], and [Q] represent the concentrations of the first binding substrate (ATP), the second binding substrate (S6K1), the product of the second binding substrate (pT229-S6K1), and the product of the first binding substrate (ADP), respectively. The concentration terms and coefficients {in brackets} are additional terms that distinguish this equation from the standard equation derived for an Ordered Bi Bi mechanism (Scheme S1; reaction steps 1−5) (2). In other words, the terms {in brackets} derive from the two additional steps, whereby free enzyme can form binary complexes with either S6K1 substrate (step 6; E + B <=> EB) or pT229-S6K1 product (step 7; E + P <=> EP). The microscopic rate constants that comprise the coefficients in Equation S1 are defined:

$$n_1 = k_{+1}\,k_{+2}\,k_{+3}\,k_{+4}\,k_{+5}\{k_{-6}\,k_{-7}\}$$

$$d_1 = k_{-1}\,k_{-2}\,k_{+4}\,k_{+5}\{k_{-6}\,k_{-7}\} + k_{-1}\,k_{+3}\,k_{+4}\,k_{+5}\{k_{-6}\,k_{-7}\}$$

$$d_\mathrm{A} = k_{+1}\,k_{-2}\,k_{+4}\,k_{+5}\{k_{-6}\,k_{-7}\} + k_{+1}\,k_{+3}\,k_{+4}\,k_{+5}\{k_{-6}\,k_{-7}\}$$

$$d_\mathrm{B} = k_{+2}\,k_{+3}\,k_{+4}\,k_{+5}\{k_{-6}\,k_{-7}\} + \{k_{-1}\,k_{-2}\,k_{+4}\,k_{+5}\,k_{+6}\,k_{-7} + k_{-1}\,k_{+3}\,k_{+4}\,k_{+5}\,k_{+6}\,k_{-7}\}$$

$$d_\mathrm{AB} = k_{+1}\,k_{+2}\,k_{+4}\,k_{+5}\{k_{-6}\,k_{-7}\} + k_{+1}\,k_{+2}\,k_{+3}\,k_{+5}\{k_{-6}\,k_{-7}\} + k_{+1}\,k_{+2}\,k_{+3}\,k_{+4}\{k_{-6}\,k_{-7}\}$$

$$d_\mathrm{Q} = k_{-1}\,k_{-2}\,k_{+4}\,k_{-5}\{k_{-6}\,k_{-7}\} + k_{-1}\,k_{+3}\,k_{+4}\,k_{-5}\{k_{-6}\,k_{-7}\}$$

$$d_\mathrm{BQ} = k_{+2}\,k_{+3}\,k_{+4}\,k_{-5}\{k_{-6}\,k_{-7}\}$$

$$d_\mathrm{QP} = k_{-1}\,k_{-2}\,k_{-4}\,k_{-5}\{k_{-6}\,k_{-7}\} + k_{-1}\,k_{+3}\,k_{-4}\,k_{-5}\{k_{-6}\,k_{-7}\}$$

$d_{BPQ} = k_{+2}\, k_{+3}\, k_{-4}\, k_{-5}\{k_{-6}\, k_{-7}\}$

$d_{ABP} = k_{+1}\, k_{+2}\, k_{+3}\, k_{-4}\{k_{-6}\, k_{-7}\}$

$\{d_{P} = k_{-1}\, k_{-2}\, k_{+4}\, k_{+5}\, k_{-6}\, k_{+7} + k_{-1}\, k_{+3}\, k_{+4}\, k_{+5}\, k_{-6}\, k_{+7}\}$

$\{d_{BB} = k_{+2}\, k_{+3}\, k_{+4}\, k_{+5}\, k_{+6}\, k_{-7}\}$

$\{d_{BP} = k_{+2}\, k_{+3}\, k_{+4}\, k_{+5}\, k_{-6}\, k_{+7}\}$

Again, the coefficients and microscopic rate constants {in brackets} are additional terms that distinguish this Equation S1 (Scheme S1; steps 1–7) from the standard equation derived for an Ordered Bi Bi mechanism (Scheme S1; steps 1–5). According to the general rules of Cleland (3–5), the coefficient terms are converted to coefficient forms composed entirely of kinetic constants by first dividing each coefficient term by the coefficient of all reactants, d_{AB}, as shown by Equation S2.

$$k = \frac{\frac{n_1}{d_{AB}}[A][B]}{\frac{d_1}{d_{AB}} + \frac{d_A}{d_{AB}}[A] + \frac{d_B}{d_{AB}}[B] + \frac{d_{AB}}{d_{AB}}[A][B] + \frac{d_Q}{d_{AB}}[Q] + \frac{d_{BQ}}{d_{AB}}[B][Q] + \frac{d_{QP}}{d_{AB}}[Q][P] + \frac{d_{BPQ}}{d_{AB}}[B][Q][P] + \frac{d_{ABP}}{d_{AB}}[A][B][P] + \left\{\frac{d_P}{d_{AB}}[P] + \frac{d_{BB}}{d_{AB}}[B][B] + \frac{d_{BP}}{d_{AB}}[B][P]\right\}} \tag{S2}$$

1.2. Two-Substrate Steady-State Kinetics

In the absence of any added products and when measuring initial velocities (i.e., [Q] = [P] = 0), Equation S2 reduces to Equation S3.

$$k = \frac{\frac{n_1}{d_{AB}}[A][B]}{\frac{d_1}{d_{AB}} + \frac{d_A}{d_{AB}}[A] + \frac{d_B}{d_{AB}}[B] + [A][B] + \left\{\frac{d_B}{d_{AB}}[B]^2\right\}} \tag{S3}$$

Although the n_1, d_1, d_A, and d_{AB} coefficients in Equation S3 each contain two additional microscopic rate constants $\{k_{-6}k_{-7}\}$, these constants cancel when the n_1/d_{AB}, d_1/d_{AB}, and d_A/d_{AB} ratios are calculated, yielding expressions of k_{cat} ($=n_1/d_{AB}$), K_m^B ($=d_A/d_{AB}$), and $K_m^B K_d^A$ ($=d_1/d_{AB}$) that are identical between the standard Ordered Bi Bi mechanism (Scheme S1; steps 1–5) and the Ordered Bi Bi mechanism with competitive substrate-product inhibition (Scheme S1; steps 1–7) as depicted by Equations S4–S6.

$$k_{cat} = \frac{n_1}{d_{AB}} = \frac{k_{+1}k_{+2}k_{+3}k_{+4}k_{+5}\{k_{-6}k_{-7}\}}{k_{+1}k_{+2}k_{+4}k_{+5}\{k_{-6}k_{-7}\} + k_{+1}k_{+2}k_{+3}k_{+5}\{k_{-6}k_{-7}\} + k_{+1}k_{+2}k_{+3}k_{+4}\{k_{-6}k_{-7}\}} \tag{S4a}$$

$$k_{cat} = \frac{k_{+3}k_{+4}k_{+5}}{k_{+4}k_{+5} + k_{+3}k_{+5} + k_{+3}k_{+4}} \tag{S4b}$$

$$K_m^B = \frac{d_A}{d_{AB}} = \frac{k_{+1}k_{-2}k_{+4}k_{+5}\{k_{-6}k_{-7}\} + k_{+1}k_{+3}k_{+4}k_{+5}\{k_{-6}k_{-7}\}}{k_{+1}k_{+2}k_{+4}k_{+5}\{k_{-6}k_{-7}\} + k_{+1}k_{+2}k_{+3}k_{+5}\{k_{-6}k_{-7}\} + k_{+1}k_{+2}k_{+3}k_{+4}\{k_{-6}k_{-7}\}} \tag{S5a}$$

$$K_m^B = \frac{k_{+4}k_{+5}(k_{-2} + k_{+3})}{k_{+2}(k_{+4}k_{+5} + k_{+3}k_{+5} + k_{+3}k_{+4})} \times \frac{k_{+3}}{k_{+3}} \tag{S5b}$$

Multiplying by k_{+3}/k_{+3} allows Equation S5b to be expressed as Equation S5c,

$$K_m^B = \frac{k_{cat}(k_{-2} + k_{+3})}{k_{+2}k_{+3}} \tag{S5c}$$

which can be further rearranged to the form given by Equation S5d.

$$K_m^B = k_{cat}\left(\frac{K_d^B}{k_{+3}} + \frac{1}{k_{+2}}\right) \tag{S5d}$$

$$K_m^B\, K_d^A = \frac{d_1}{d_{AB}} \times \frac{d_A}{d_A} = K_m^B \frac{k_{-1}k_{-2}k_{+4}k_{+5}\{k_{-6}k_{-7}\} + k_{-1}k_{+3}k_{+4}k_{+5}\{k_{-6}k_{-7}\}}{k_{+1}k_{-2}k_{+4}k_{+5}\{k_{-6}k_{-7}\} + k_{+1}k_{+3}k_{+4}k_{+5}\{k_{-6}k_{-7}\}} \tag{S6a}$$

$$K_m^B\, K_d^A = K_m^B \frac{k_{-1}k_{+4}k_{+5}(k_{-2} + k_{+3})}{k_{+1}k_{+4}k_{+5}(k_{-2} + k_{+3})} = K_m^B \frac{k_{-1}}{k_{+1}} \tag{S6b}$$

As expected, the ability of the second binding substrate (B) to initially bind and prevent binding of the first substrate alters the expression of K_m^A ($=d_B/d_{AB}$) compared to the standard Ordered Bi Bi mechanism. In this case, the d_B coefficient contains numerous additional rate constants. In Equation S7, the microscopic rate constants {in brackets} are additional terms that distinguish the expression for K_m^A in the Ordered Bi Bi mechanism with competitive substrate inhibition (Scheme S1; steps 1–7) from that derived for a standard Ordered Bi Bi mechanism (Scheme S1; steps 1–5).

$$K_m^A = \frac{d_B}{d_{AB}} = \frac{k_{+2}k_{+3}k_{+4}k_{+5}\{k_{-6}k_{-7}\} + \{k_{-1}k_{-2}k_{+4}k_{+5}k_{+6}k_{-7} + k_{-1}k_{+3}k_{+4}k_{+5}k_{+6}k_{-7}\}}{k_{+1}k_{+2}k_{+4}k_{+5}\{k_{-6}k_{-7}\} + k_{+1}k_{+2}k_{+3}k_{+5}\{k_{-6}k_{-7}\} + k_{+1}k_{+2}k_{+3}k_{+4}\{k_{-6}k_{-7}\}} \tag{S7a}$$

$$K_m^A = \frac{k_{+3}k_{+4}k_{+5}}{k_{+1}(k_{+4}k_{+5} + k_{+3}k_{+5} + k_{+3}k_{+4})}\left\{\frac{k_{+2}k_{+3}k_{-6} + k_{-1}k_{-2}k_{+6} + k_{-1}k_{+3}k_{+6}}{k_{+2}k_{+3}k_{-6}}\right\} \tag{S7b}$$

$$K_m^A = \frac{k_{cat}}{k_{+1}}\left\{\frac{k_{+2}k_{+3}k_{-6} + k_{-1}k_{-2}k_{+6} + k_{-1}k_{+3}k_{+6}}{k_{+2}k_{+3}k_{-6}}\right\} \tag{S7c}$$

$$K_m^A = \frac{k_{cat}}{k_{+1}}\left\{1 + \frac{k_{-1}K_d^B}{k_{+3}K_i^B} + \frac{k_{-1}}{k_{+2}K_i^B}\right\} \tag{S7d}$$

In final consideration, Equation S3 contains the one additional coefficient × concentration term $\{d_{BB}/d_{AB} \times [B]^2\}$; and the unitless coefficient is defined by Equation S8 $\{K_{BB} = d_{BB}/d_{AB}\}$.

$$K_{BB} = \frac{d_{BB}}{d_{AB}} = \frac{k_{+2}k_{+3}k_{+4}k_{+5}k_{+6}k_{-7}}{k_{+1}k_{+2}k_{+4}k_{+5}k_{-6}k_{-7} + k_{+1}k_{+2}k_{+3}k_{+5}k_{-6}k_{-7} + k_{+1}k_{+2}k_{+3}k_{+4}k_{-6}k_{-7}} \tag{S8a}$$

$$K_{BB} = \frac{k_{+3}k_{+4}k_{+5}k_{+6}}{k_{+1}k_{-6}(k_{+4}k_{+5} + k_{+3}k_{+5} + k_{+3}k_{+4})} \tag{S8b}$$

$$K_{BB} = \frac{k_{cat}k_{+6}}{k_{+1}k_{-6}} = \frac{k_{cat}}{k_{+1}K_i^B} \tag{S8c}$$

Thus, incorporation of these steady-state kinetic constants into Equation S3 yields the overall steady-state kinetic rate Equation S9 for the Ordered Bi Bi mechanism with competitive substrate inhibition.

$$k = \frac{k_{cat}[A][B]}{K_m^B K_d^A + K_m^B[A] + \{K_m^A\}[B] + [A][B] + \{K_{BB}[B]^2\}} \tag{S9}$$

Equation S9 (Scheme S1; steps 1–7) is identical to the Equation S10 derived for a standard Ordered Bi Bi mechanism (Scheme S1; steps 1–5),

$$k = \frac{k_{cat}[A][B]}{K_m^B K_d^A + K_m^B[A] + K_m^A[B] + [A][B]} \tag{S10}$$

except that in Equation S9, $\{K_m^A\}$ is modified according to Equation S7 and $\{K_{BB}[B]^2\}$ is an additional term defined by Equation S8. As well documented (6), Equation S10 for the standard Ordered Bi Bi mechanism is not mathematically distinguishable from the equation derived for a Random Bi Bi mechanism using the rapid equilibrium assumptions for all substrate binding steps (Equation S11).

$$k = \frac{k_{cat}[A][B]}{\alpha K_d^B K_d^A + \alpha K_d^B[A] + \alpha K_d^A[B] + [A][B]} \tag{S11}$$

Here, $\alpha K_d^B = K_m^B$ and $\alpha K_d^A = K_m^A$, and the symbol α is a proportionality constant, which quantifies the degree that the binding of one substrate either increases ($\alpha < 1$) or decreases ($\alpha > 1$) the affinity of the enzyme for the other substrate. In the following sections, it will be shown how study of two-substrate steady state kinetics can distinguish the Ordered Bi Bi mechanism with competitive substrate inhibition (Equation S9) from a standard ternary complex mechanism involving either ordered or random addition of substrates (Equation S10).

1.2.1. Varying [A] at fixed [B]

Dividing by [B] and collecting like terms, Equation S9 is rearranged to Equation S12,

$$k = \frac{k_{cat}[A]}{\frac{K_m^B K_d^A}{[B]} + \frac{K_m^B[A]}{[B]} + \{K_m^A\} + [A] + \{K_{BB}[B]\}} \tag{S12a}$$

$$k = \frac{k_{cat}[A]}{K_m^A\left(1 + \frac{K_m^B K_d^A}{K_m^A[B]} + \left\{\frac{K_{BB}[B]}{K_m^A}\right\}\right) + [A]\left(1 + \frac{K_m^B}{[B]}\right)} \tag{S12b}$$

which is further arranged to the Michaelis-Menten hyperbolic form given by Equation S13 for when [A] is the varied substrate at different fixed [B].

$$k = \frac{k_{cat(app)}[A]}{K_{m(app)}^A + [A]} = \frac{\dfrac{k_{cat}}{1 + \frac{K_m^B}{[B]}}[A]}{K_m^A\left(\dfrac{1 + \frac{K_m^B K_d^A}{K_m^A[B]} + \left\{\frac{K_{BB}[B]}{K_m^A}\right\}}{1 + \frac{K_m^B}{[B]}}\right) + [A]} \tag{S13}$$

Thus, Equation S13 yields expressions for the apparent values of $k_{cat(app)}$ and $K_m{}^A{}_{(app)}$ at different fixed [B] given by Equations S14 and S15, respectively.

$$k_{cat(app)} = \frac{k_{cat}}{1 + \frac{K_m^B}{[B]}} = \frac{k_{cat}[B]}{K_m^B + [B]} \tag{S14}$$

$$K_{m(app)}^A = K_m^A\left(\dfrac{1 + \frac{K_m^B K_d^A}{K_m^A[B]} + \left\{\frac{K_{BB}[B]}{K_m^A}\right\}}{1 + \frac{K_m^B}{[B]}}\right) = \frac{K_m^A[B] + K_m^B K_d^A + \left\{K_{BB}[B]^2\right\}}{K_m^B + [B]} \tag{S15}$$

The dependences of apparent $k_{cat(app)}$ on fixed [B] (Equation S14) are identical between the Ordered Bi Bi mechanism with competitive substrate inhibition (Scheme S1; steps 1–7) and the standard Ordered Bi Bi mechanism (Scheme S1; steps 1–5). Similarly, the dependences of apparent $K_m{}^A{}_{(app)}$ on fixed [B] (Equation S15) are nearly identical except for the one additional term $\{K_{BB}[B]^2\}$ that accounts for competitive substrate inhibition. In the absence of this term (standard ternary complex mechanism), $K_m{}^A{}_{app}$ approaches a maximum or asymtopic value with increasing [B]. In contrast, the additional $\{K_{BB}[B]^2\}$ term in the numerator allows for $K_m{}^A{}_{app}$ to continue increasing to higher values, as expected for competitive type inhibition.

1.2.2. Varying [B] at fixed [A]

Alternatively, dividing by [A] and collecting like terms, Equation S9 is rearranged to Equation S16.

$$k = \frac{k_{cat}[B]}{\frac{K_m^B K_d^A}{[A]} + K_m^B + \frac{K_m^A[B]}{[A]} + [B] + \left\{\frac{K_{BB}[B]^2}{[A]}\right\}} \tag{S16a}$$

$$k = \frac{k_{cat}[B]}{K_m^B\left(1 + \frac{K_d^A}{[A]}\right) + [B]\left(1 + \frac{K_m^A}{[A]} + \left\{\frac{K_{BB}[B]}{[A]}\right\}\right)} \tag{S16b}$$

In this case, Michaelis-Menten hyperbolic kinetics would not be observed on varying [B] at fixed [A]. Rather, the $\{K_{BB}[B]/[A]\}$ term yields substrate inhibition kinetics with respect to varied [B], which diminishes with increasing fixed [A]. Therefore, experimental titrations of [B] at different fixed [A] must be fit directly to Equation S16b.

For the case of a standard Ordered Bi Bi mechanism, the $\{K_{BB}[B]/[A]\}$ term is not present. Therefore, Michaelis-Menten hyperbolic kinetics would be observed on varying [B] at fixed [A], as shown by Equation S17,

$$k = \frac{k_{cat(app)}[B]}{K_{m(app)}^{B} + [B]} = \frac{\dfrac{k_{cat}}{1 + \dfrac{K_m^A}{[A]}}[B]}{K_m^B\left(\dfrac{1 + \dfrac{K_d^A}{[A]}}{1 + \dfrac{K_m^A}{[A]}}\right) + [B]} \quad \text{(S17)}$$

with hyperbolic expressions for the apparent values of $k_{cat(app)}$ and $K_{m\ (app)}^{B}$ at different fixed [A] given by Equations S18 and S19, respectively.

$$k_{cat(app)} = \frac{k_{cat}}{1 + \dfrac{K_m^A}{[A]}} = \frac{k_{cat}[A]}{K_m^A + [A]} \quad \text{(S18)}$$

$$K_{m(app)}^{B} = K_m^B\left(\frac{1 + \dfrac{K_d^A}{[A]}}{1 + \dfrac{K_m^A}{[A]}}\right) = \frac{K_m^B[A] + K_m^B K_d^A}{K_m^A + [A]} \quad \text{(S19)}$$

2. Computational Scripts

2.1. King-Altman Derivation

The King-Altman computer algorithm (BioKin, Ltd., Pullman, WA) generated Equation S1 using the following script:

```
[reaction]
A + B <==> P + Q

[mechanism]
E + A <==> EA
EA + B <==> EAB
EAB −−> EQP
EQP <==> EQ + P
EQ <==> E + Q
E + B <==> EB
E + P <==> EP

[end]
```

2.2. Stopped-Flow Fluorescence Kinetic Analyses of Direct and Competitive Binding

For global fitting of stopped-flow kinetic fluorescence data pertaining to measurements of direct binding of either Mant-ATP (Fig. 4A) or tamra-PIF (Fig. 5A) to PDK1, the following computer script was used for the DynaFit 3.28 software (BioKin, Ltd., Pullman, WA). Estimate values for the fitted parameters were varied, and in each case data analysis converged to a single value for each designated fitted parameter.

```
[task]
data = progress
task = fit

[mechanism]
E + A <==> EA         :  k+1  k−1

[constants]
k+1 = 5 ?, k−1 = 5 ?     ; bimolecular rate constant k+1 is given in μM−1 s−1 ; first-order rate constant k−1
                         ;  is given in s−1; ? indicates fitted parameters; otherwise fixed
[concentrations]
A = 0.5

[responses]
EA = 5 ?                 ; value for the differential molar fluorescence, ΔFmol

[progress]
directory     ./pdk1_bind_direct/data
extension     txt
files         file1, file2, file3, file4, file5, file6
vary conc. E =  1,   2,   3,   5,   7,   10

[output]
directory     ./pdk1_bind_direct/output

[end]
```

2.3. Stopped-Flow Fluorescence Kinetic Analyses of Competitive Binding

For global fitting of stopped-flow kinetic fluorescence data pertaining to measurements of binding of unlabeled ligands that were competitive with either Mant-ATP (Figs. 4B,C) or tamra-PIF (Figs. 5B,C) to PDK1, the following computer script was used for the DynaFit 3.28 software (BioKin, Ltd., Pullman, WA):

```
[task]
data = progress
task = fit

[mechanism]
E + A <==> EA        :  k+1  k–1
E + B <==> EB        :  k+2  k–2

[constants]
k+1 = (fixed measured value), k–1 = (fixed measured value)
                          ; bimolecular rate constants k+1 and k+2 are given in µM⁻¹ s⁻¹ ; first-order rate
k+2 = 5 ?, k–2 = 5 ?      ; constants k–1 and k–2 are given in s⁻¹; ? indicates fitted parameters; otherwise
                          ; fixed
[concentrations]
A = 0.5
B = 10

[responses]
EA = 5 ?             ; value for the differential molar fluorescence, ΔF_mol

[progress]
directory    ./pdk1_bind_competitive/data
extension    txt
files        file1, file2, file3, file4, file5, file6
vary conc. E =  1,    2,    3,    5,    7,    10

[output]
directory    ./pdk1_bind_competitive/output

[end]
```

3. Results

3.1. Two-Substrate Steady State Kinetics

TABLE S1

Comparison of Steady-state Kinetic Constants Obtained for His_6-PDK1(ΔPH)-catalyzed T229 Phosphorylation of Native and T389E Mutant His_6-S6K1αII(ΔAID)–Experiment 1 [a]

kinetic constant	native His_6-S6K1αII(ΔAID)		T389E His_6-S6K1αII(ΔAID)	
	Scheme 1	Scheme 2	Scheme 1	Scheme 2
k_{cat} (s^{-1})	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
αK_d^A or K_m^A (μM)	29	26 ± 4	15	17 ± 2
αK_d^B or K_m^B (μM)	25	28 ± 4	17	16 ± 2
α (K_m/K_d)	21 ± 3	na	11 ± 2	na
k_{cat}/K_m^A (μM^{-1} s^{-1})	0.034	0.042	0.073	0.071
k_{cat}/K_m^B (μM^{-1} s^{-1})	0.040	0.039	0.065	0.075

[a] Experiment 1 was carried out by varying the total concentration of one substrate at different fixed total concentrations of the other substrate as described in the legends of Figures S1 and S2.

Table S2

Comparison of Steady-state Kinetic Constants Obtained for His_6-PDK1(ΔPH)-catalyzed T229 Phosphorylation of Native and T389E Mutant His_6-S6K1αII(ΔAID)–Experiment 2 [a]

kinetic constant	native His_6-S6K1αII(ΔAID)		T389E His_6-S6K1αII(ΔAID)	
	Scheme 1	Scheme 2	Scheme 1	Scheme 2
k_{cat} (s^{-1})	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
αK_d^A or K_m^A (μM)	25	23 ± 2	13	13 ± 1
αK_d^B or K_m^B (μM)	22	25 ± 2	14	14 ± 1
α (K_m/K_d)	18 ± 2	na	9.3 ± 1.1	na
k_{cat}/K_m^A (μM^{-1} s^{-1})	0.036	0.042	0.077	0.085
k_{cat}/K_m^B (μM^{-1} s^{-1})	0.033	0.042	0.071	0.079

[a] Experiment 2 was carried out by varying the total concentration of one substrate while maintaining fixed free concentrations of the other substrate as described in the legend of Figure 6 in the manuscript.

Figure S1. Two-substrate steady-state kinetics of PDK1 reaction with the native (T389) S6K1. *A*, Direct plots of *k* versus the *total* concentrations of ATP ($[ATP]_{total}$; left panels) and native His_6-S6K1αII(ΔAID) ($[T389]_{total}$; right panels). *B*, Direct plots of *k* versus the *free* concentrations of ATP ($[ATP]_{free}$; left panels) and native His_6-S6K1αII(ΔAID) ($[T389]_{free}$; right panels). Steady-state kinetic assays of His_6-PDK1(ΔPH)-catalyzed T229 phosphorylation of native His_6-S6K1αII(ΔAID) were performed at 25 °C. The total concentrations of either ATP or native His_6-S6K1αII(ΔAID) were 2 μM (●), 5 μM (○), 10 μM (■), 20 μM (□), 30 μM (▲), 50 μM (△), and 100 μM (△); and the free concentrations of the substrates were calculated as described in Experimental Procedures. *Dashed* and *solid* lines were generated using the kinetic constants determined from the global fit of the data to Equations 6 and 7, respectively (Table S1).

Figure S2. Two-substrate steady-state kinetics of PDK1 reaction with the T389E mutant (E389) S6K1. *A*, Direct plots of *k* versus the *total* concentrations of ATP ($[ATP]_{total}$; left panels) and native His_6-S6K1αII(ΔAID) ($[E389]_{total}$; right panels). *B*, Direct plots of *k* versus the *free* concentrations of ATP ($[ATP]_{free}$; left panels) and T389E mutant His_6-S6K1αII(ΔAID) ($[E389]_{free}$; right panels). Steady-state kinetic assays of His_6-PDK1(ΔPH)-catalyzed T229 phosphorylation of T389E mutant His_6-S6K1αII(ΔAID) were performed at 25 °C. The total concentrations of either ATP or native His_6-S6K1αII(ΔAID) were 2 μM (●), 5 μM (○), 10 μM (■), 20 μM (□), 30 μM (▲), 50 μM (△), and 100 μM (△); and the free concentrations of the substrates were calculated as described in Experimental Procedures. *Dashed* and *solid* lines were generated using the kinetic constants determined from the global fit of the data to Equations 6 and 7, respectively (Table S1).

FIGURE S1

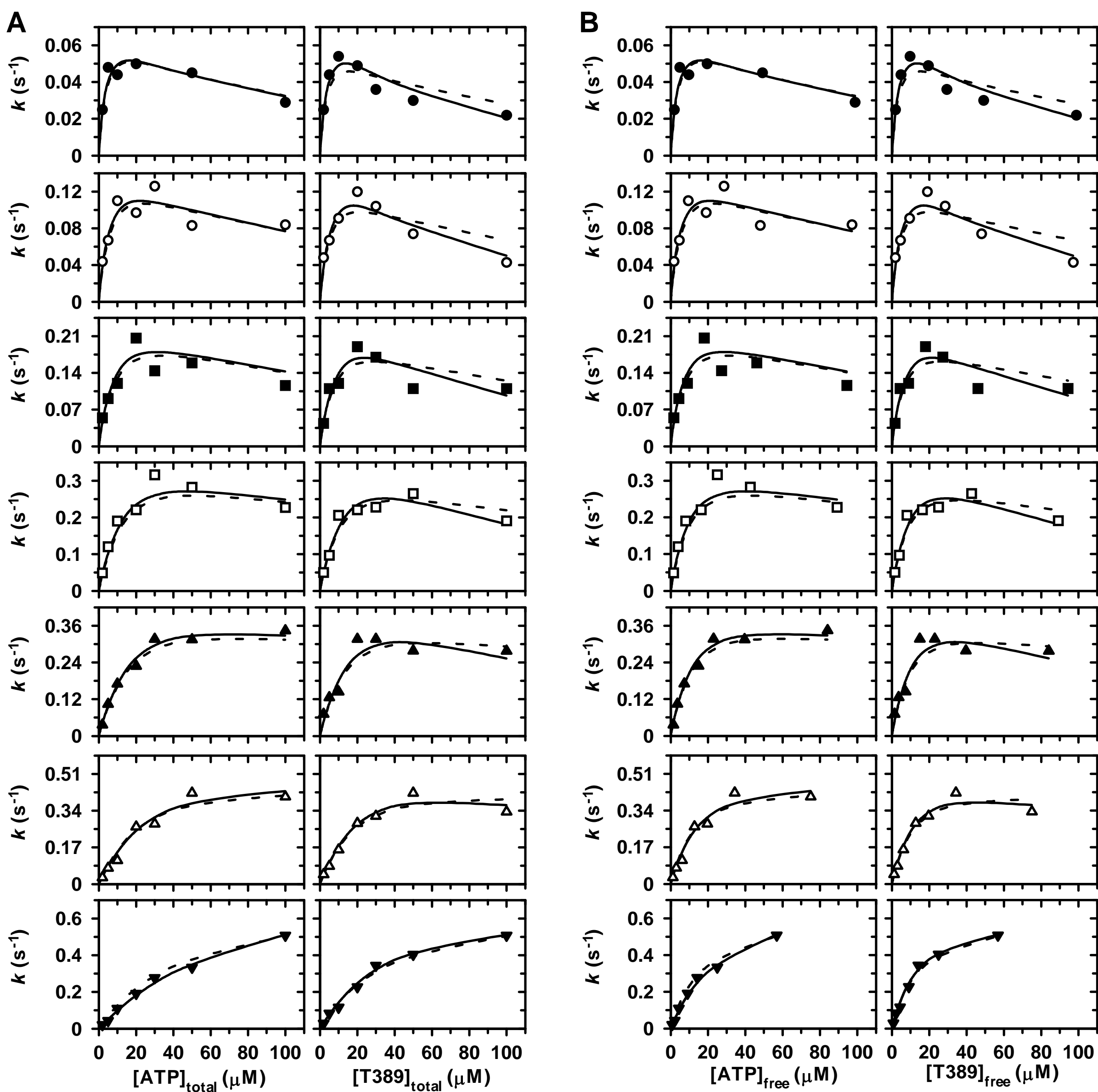
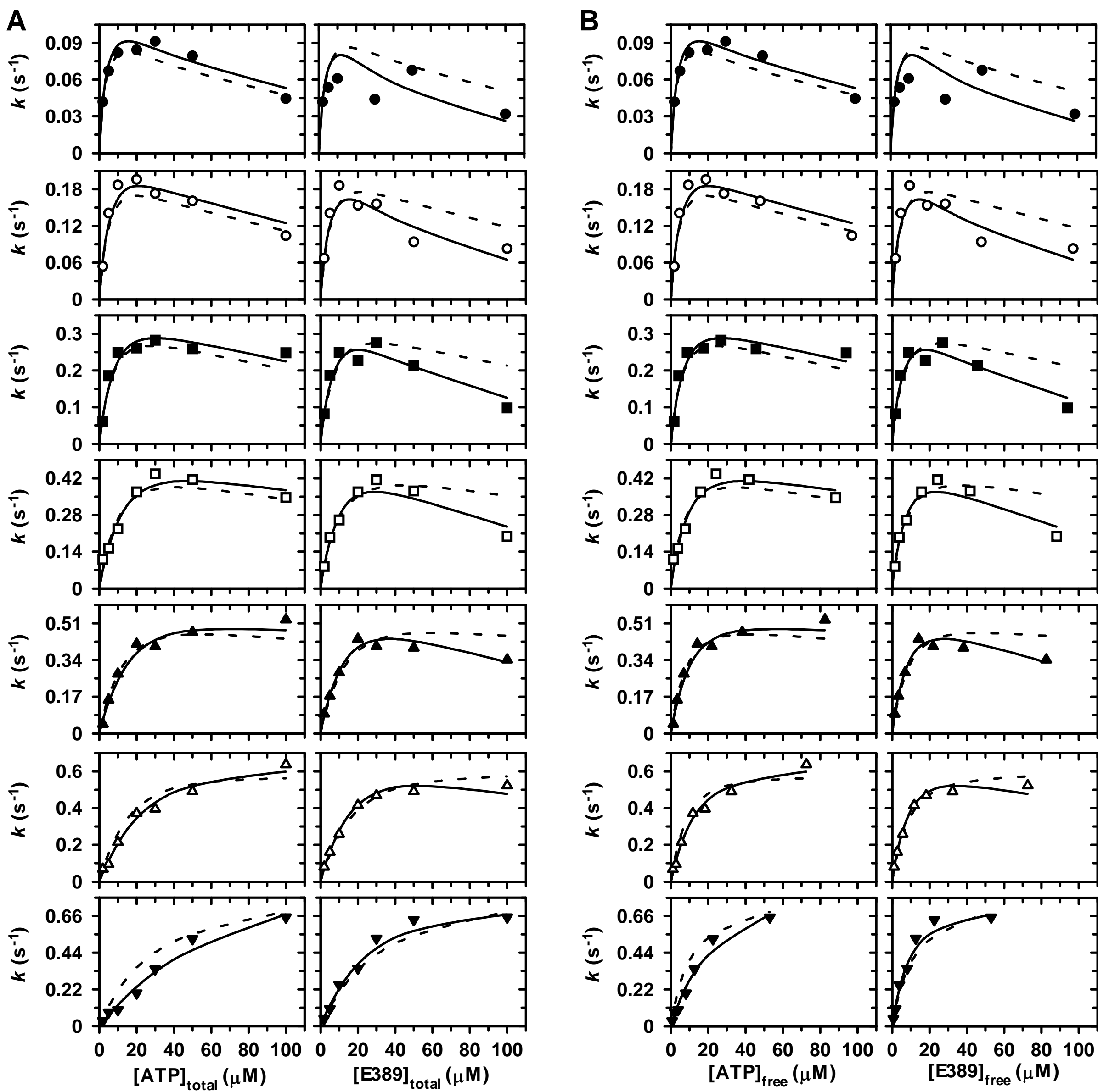


FIGURE S2



3.2. Effective Retention of Radioactive S6K1 by P81 Phosphocellulose Paper

Table S3
Retention of Radioactive S6K1 by P81 Phosphocellulose Paper

[S6K1] [a]		cpm applied [b]		cpm retained [c]		
(μM)	(nmol) [b,c]	data	average	data	average	fraction
30	0.6	1070		902		
30	0.6	1084	1089 ± 18	955	961 ± 45	0.88 ± 0.04
30	0.6	1114	(±1.7%)	1011	(±4.7%)	(±5.0%)
60	1.2	2107		1781		
60	1.2	2184	2165 ± 42	1822	1879 ± 63	0.87 ± 0.03
60	1.2	2203	(±1.9%)	1931	(±3.4%)	(±3.9%)
90	1.8	3184		2794		
90	1.8	3226	3249 ± 65	2914	2924 ± 110	0.90 ± 0.04
90	1.8	3338	(±2.0%)	3063	(±3.8%)	(±4.3%)
120	2.4	4258		3677		
120	2.4	4362	4335 ± 56	3785	3801 ± 108	0.88 ± 0.03
120	2.4	4386	(±1.3%)	3940	(±2.8%)	(±3.1%)
150	3.0	5271		4656		
150	3.0	5412	5388 ± 87	4831	4805 ± 113	0.89 ± 0.03
150	3.0	5481	(±1.6%)	4929	(±2.4%)	(±2.9%)

[a] Purified T389E mutant His_6-S6K1αII(ΔAID) (M_{calc} = 46.5 kDa) was ^{32}P-radiolabeled at T229 by reacting with His_6-PDK1(ΔPH) (M_{calc} = 36.9 kDa). The in vitro phosphorylation reaction was performed at 25 °C in a total reaction volume of 10 mL in reaction buffer comprised of 40 mM MOPS buffer, pH 7, 0.1% 2-mercaptoethanol, 10 mM $MgCl_2$, 1mM EDTA, and 1 mM EGTA. The reaction mixture contained 10 μM T389E mutant His_6-S6K1αII(ΔAID) (4.7 mg or 100 nmol), 10 nM His_6-PDK1(ΔPH) (3.7 μg or 100 pmol), and 100 μM of [γ-^{32}P]ATP (~2000 cpm/nmol). After 30 min, protein was purified from the reaction mixture by His_6-affinity chromatography, concentrated, and adjusted to yield 500 μL of 150 μM (or 75 nmol) ^{32}P-T229 radiolabeled His_6-S6K1αII(ΔAID) (~1780 cpm/nmol; ~89% phosphorylated). Aliquots of this stock solution were diluted into reaction buffer to formulate 150 μL volumes of [S6K1] = 30 μM (4.5 nmol), 60 μM (9 nmol), 90 μM (13.5 nmol), 120 μM (18 nmol), and 150 μM (22.5 nmol). It should be pointed out that in vitro His_6-PDK1(ΔPH)-catalyzed T229 phosphorylation of either native or T389E mutant His_6-S6K1αII(ΔAID) became increasingly inhibited in reactions employing ever higher concentrations of either ATP or the given His_6-S6K1αII(ΔAID) construct. This may likely result from increased formation of nucleotide-bound His_6-S6K1αII(ΔAID), which is not readily phosphorylated.

[b] From each 150 μL protein solution, three 20-μL aliquots were removed for individual direct scintillation counting. The average ± S.E. and (± S.E.%) is given for the total radioactivity (cpm) that would be applied to P81 phosphocellulose paper (Whatman, 2 × 2 cm).

[c] Exactly as performed in steady-state kinetic assays, three additional 20-μL aliquots were individually mixed with 20 μL of 75 mM phosphoric acid and applied to P81 phosphocellulose paper (Whatman, 2 × 2 cm). After 30 s, the papers were washed (3×) in 1 L of fresh 75 mM phosphoric acid for 10 min, then rinsed with 50 mL acetone, and placed in the hood to dry before scintillation counting. The average ± S.E. and (± S.E.%) is given for the total radioactivity (cpm) that was retained on each P81 phosphocellulose paper. For each concentration of T389E mutant His_6-S6K1αII(ΔAID), the fraction retained (± S.E.%) was calculated from the ratio of the retained average (± S.E.%) compared to the applied average (± S.E.%).

4. References

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