

Supplementary Information for:

Intrinsically disordered linkers control tethered kinases via effective concentration

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Protein sequences

Color coding:

- 6xHis-tag
- Thrombin cleavage sequence, // marks the cleavage site
- MBD2 dimerization domain
- p66 α dimerization domain
- GCTAGC (AS) - NheI restriction site
- (GS)_n - variable-length GS linker; n = 1, 10, 30 or 60
- GGTACC (GT) - KpnI restriction site
- PKA substrate motif, catalytic Ser shown in **bold**

1. PKAc

MGSSHHHHHHSSGLVPR//GSHMGNAAAAKKGSEQESVKEFLAKAKEDFLKKWETPSQNTAQLDQFDRIKTLGTGSFGRVMLVKHKESGNHYAMKILDQKQVVKLKQIEHTLNEKRILQAVNFPFLVKLEFSFKDNSNLYMVMEYVAGGEMFSLRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDQQGYIQVTDFGFAKRVKGRWTLCGTPEYLAP EIILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQIYKIVSGKVRFP SHFSSDLKDLLRNLLQVDLTKRFGNLKNGVNDIKNHKWFATTDWIAIYQRKVEAPFIPKFKGPGDTSNFDDYEEEEIRVSINEKCGKEFTEF

2. MBD2-(GS)_n-PKAc

MGSSHHHHHHSSGLVPR//GSHMVTDEDIRKQEERAQQVRKKLEELMADAS(GS)_nGTGNAAA AKKGSEQESVKEFLAKAKEDFLKKWETPSQNTAQLDQFDRIKTLGTGSFGRVMLVKHKESGNHYAMKILDQKQVVKLKQIEHTLNEKRILQAVNFPFLVKLEFSFKDNSNLYMVMEYVAGGEMFSLRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDQQGYIQVTDFGFAKRVKGRWTLCGTPEYLAP EIILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQIYKIVSGKVRFP SHFSSDLKDLLRNLLQVDLTKRFGNLKNGVNDIKNHKWFATTDWIAIYQRKVEAPFIPKFKGPGDTSNFDDYEEEEIRVSINEKCGKEFTEF

3. p66 α -(GS)_n-WT substrate

MGSSHHHHHHSSGLVPR//GSHMTSPEERERMIKQLKEELRLEEAKLVLLKKLRQSQIQKEATAQKAS(GS)_nGTPGSGSGSGSLRRASLGGGGGY

4. p66 α -(GS)_n-R-2K substrate

MGSSHHHHHHSSGLVPR//GSHMTSPEERERMIKQLKEELRLEEAKLVLLKKLRQSQIQKEATAQKAS(GS)_nGTPGSGSGSGSLRKASLGGGGGY

5. p66 α -(GS)_n-R-3K substrate

MGSSHHHHHHSSGLVPR//GSHMTSPEERERMIKQLKEELRLEEAKLVLLKKLRQSQIQKEATAQKAS(GS)_nGTPGSGSGSGSLKRASLGGGGGY

Supplementary figures

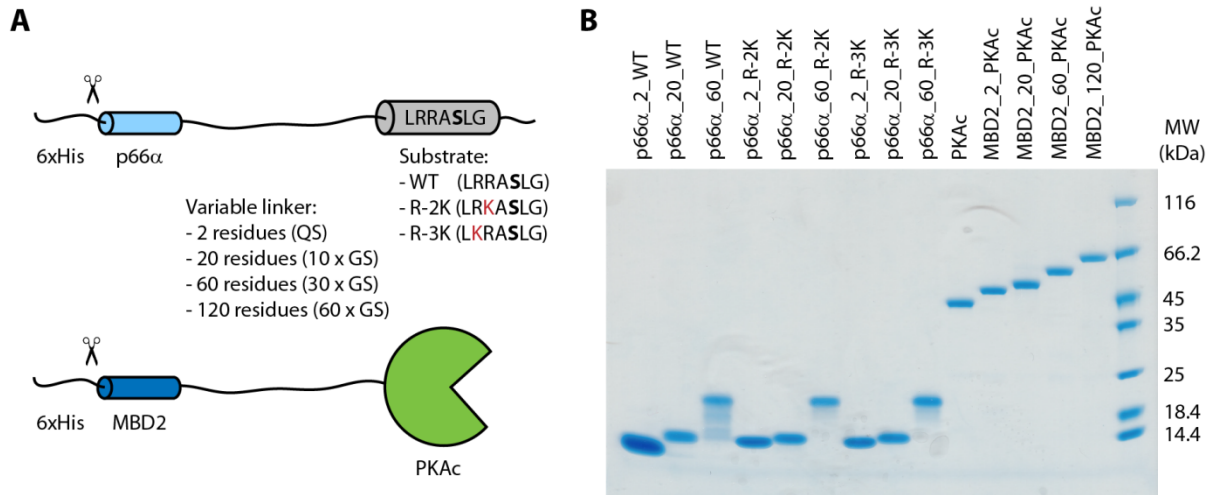


Fig. S1: Protein variants used in this study. (A) Schematic representation of the protein variants used to form the tethered kinase substrate complex. Both constructs contain variable GS-linker. (B) SDS-PAGE gel of purified proteins. The substrate with a 120 residue GS linker could not be purified to sufficient purity.

Next four pages:

Fig. S2: Raw data from quench-flow experiments.

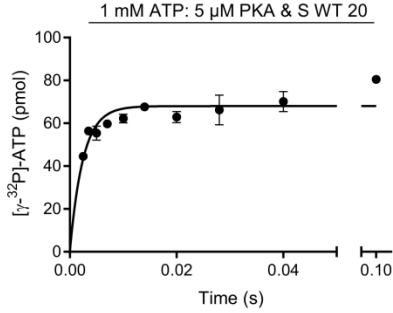
PKA & S WT

Linker length:

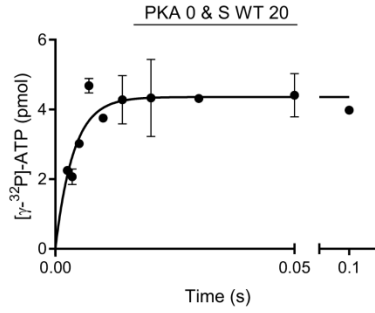
1 mM ATP+ 5 μ M PKA & S

0.1 mM ATP+ 0.5 μ M PKA & S

20

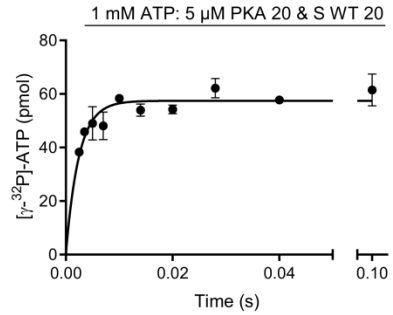


Best-fit values	
Y0	= 0.000
Plateau	68.00
K	406.6
Tau	0.002460
Half-time	0.001705
Span	= 68.00
Std. Error	
Plateau	1.718
K	50.41

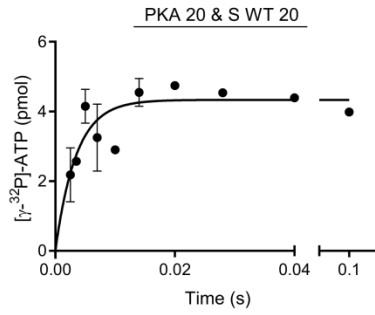


Best-fit values	
Y0	= 0.000
Plateau	4.355
K	263.9
Tau	0.003790
Half-time	0.002627
Span	= 4.355
Std. Error	
Plateau	0.1957
K	47.04

40

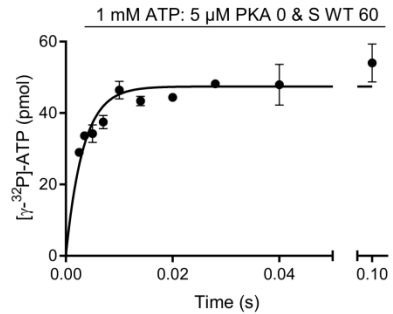


Best-fit values	
Y0	= 0.000
Plateau	57.44
K	411.2
Tau	0.002432
Half-time	0.001686
Span	= 57.44
Std. Error	
Plateau	1.356
K	46.58

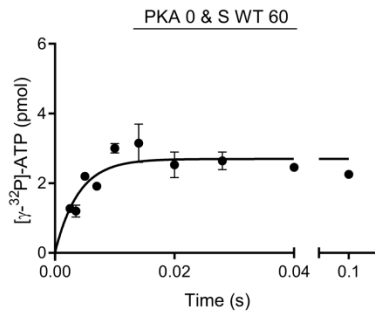


Best-fit values	
Y0	= 0.000
Plateau	4.331
K	287.6
Tau	0.003477
Half-time	0.002410
Span	= 4.331
Std. Error	
Plateau	0.2459
K	58.18

60

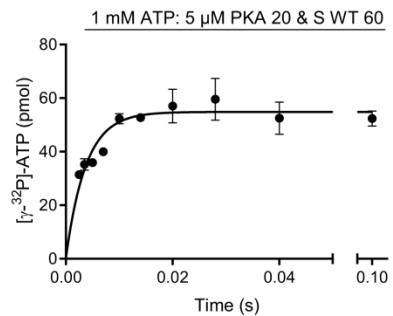


Best-fit values	
Y0	= 0.000
Plateau	47.44
K	306.7
Tau	0.003260
Half-time	0.002260
Span	= 47.44
Std. Error	
Plateau	1.275
K	34.45

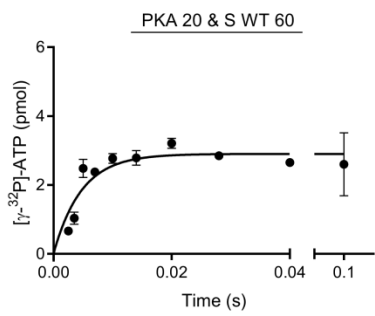


Best-fit values	
Y0	= 0.000
Plateau	2.694
K	254.7
Tau	0.003927
Half-time	0.002722
Span	= 2.694
Std. Error	
Plateau	0.1361
K	47.63

80

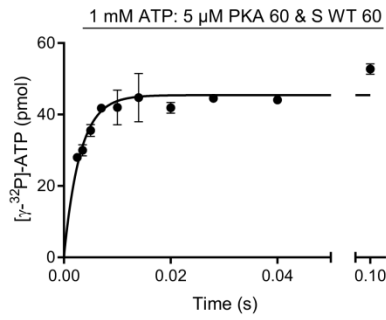


Best-fit values	
Y0	= 0.000
Plateau	54.85
K	267.7
Tau	0.003735
Half-time	0.002589
Span	= 54.85
Std. Error	
Plateau	1.508
K	29.40

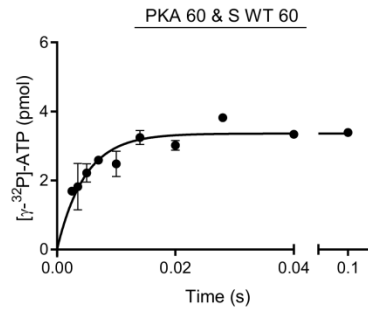


Best-fit values	
Y0	= 0.000
Plateau	2.900
K	212.2
Tau	0.004713
Half-time	0.003267
Span	= 2.900
Std. Error	
Plateau	0.1629
K	40.88

120

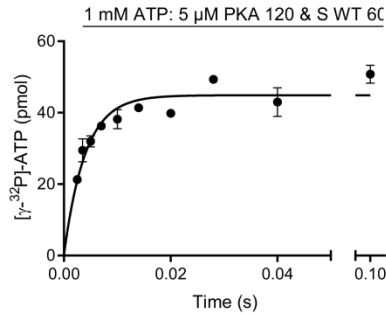


Best-fit values	
Y0	= 0.000
Plateau	45.42
K	330.7
Tau	0.003024
Half-time	0.002096
Span	= 45.42
Std. Error	
Plateau	1.109
K	35.09

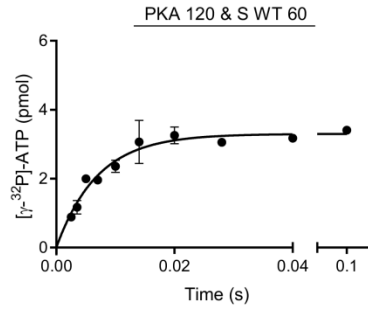


Best-fit values	
Y0	= 0.000
Plateau	3.357
K	211.7
Tau	0.004724
Half-time	0.003275
Span	= 3.357
Std. Error	
Plateau	0.1144
K	25.64

180

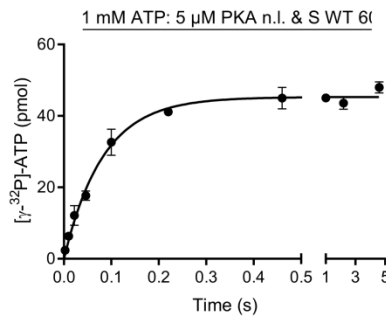


Best-fit values	
Y0	= 0.000
Plateau	44.89
K	250.9
Tau	0.003986
Half-time	0.002763
Span	= 44.89
Std. Error	
Plateau	1.229
K	26.15

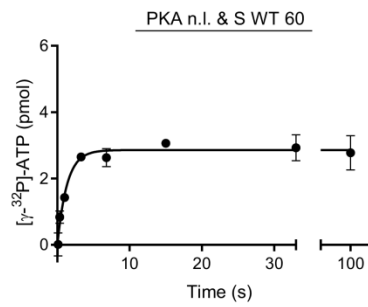


Best-fit values	
Y0	= 0.000
Plateau	3.296
K	143.5
Tau	0.006968
Half-time	0.004830
Span	= 3.296
Std. Error	
Plateau	0.09910
K	13.37

not linked



Best-fit values	
Y0	= 0.000
Plateau	45.27
K	12.16
Tau	0.08226
Half-time	0.05702
Span	= 45.27
Std. Error	
Plateau	0.7081
K	0.7949



Best-fit values	
Y0	= 0.000
Plateau	2.857
K	0.7487
Tau	1.336
Half-time	0.9259
Span	= 2.857
Std. Error	
Plateau	0.09481
K	0.1225

Linker length:

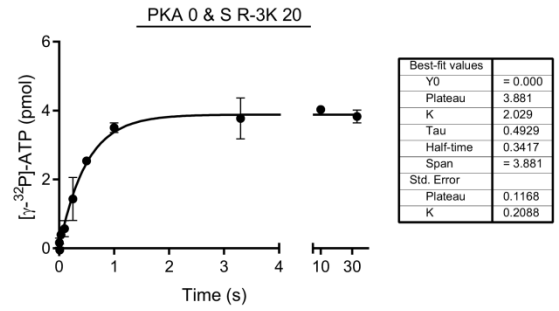
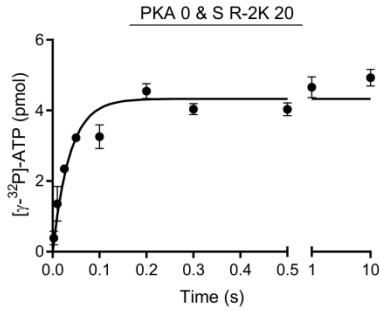
PKA & S R-2K

PKA & S R-3K

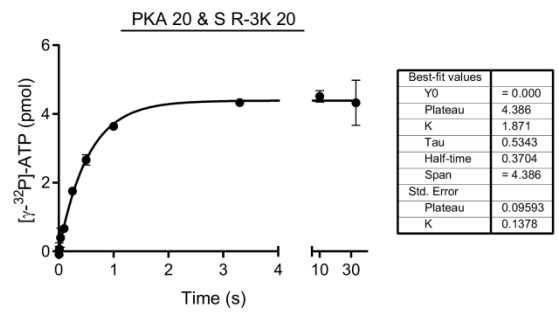
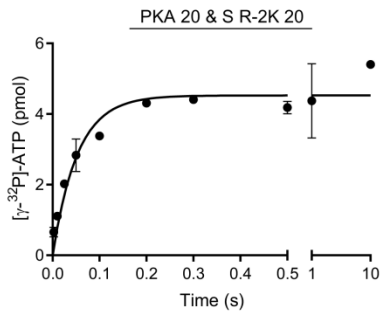
0.1 mM ATP+ 0.5 μM PKA & S

0.1 mM ATP+ 0.5 μM PKA & S

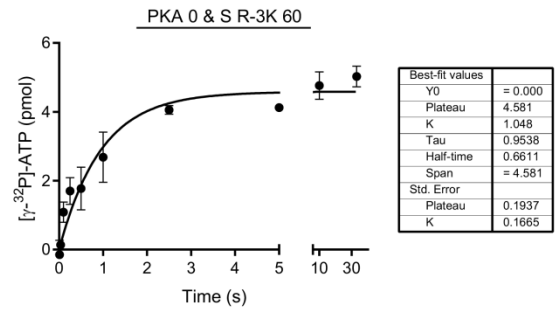
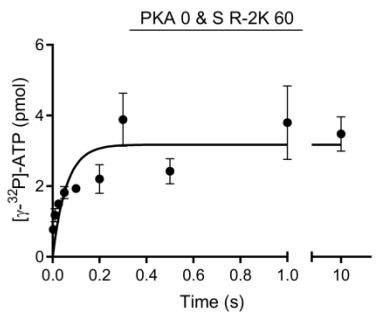
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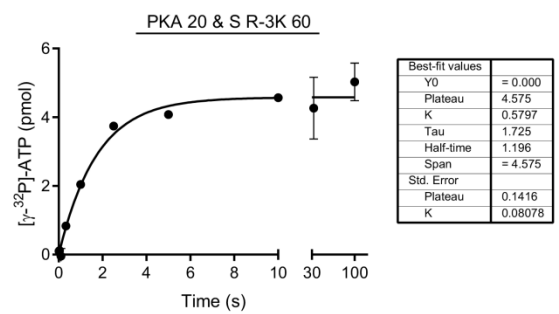
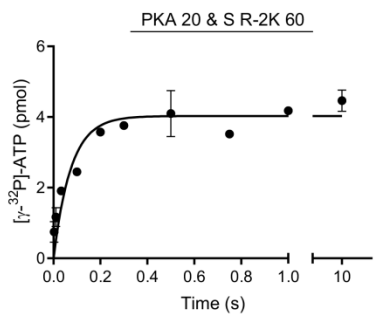
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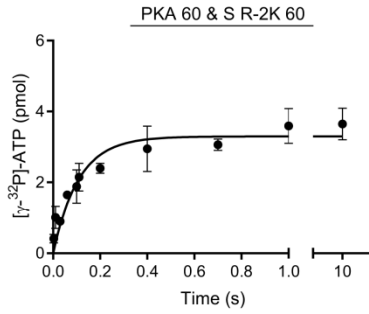
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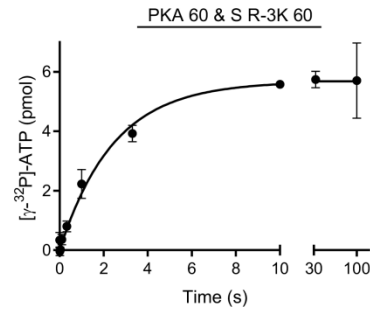
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120

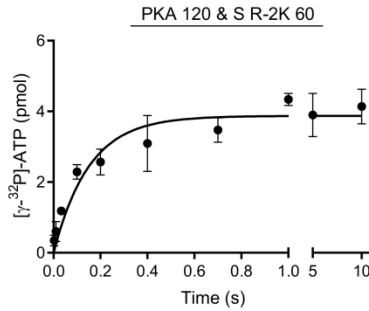


Best-fit values	
Y0	= 0.000
Plateau	3.297
K	9.214
Tau	0.1085
Half-time	0.07522
Span	= 3.297
Std. Error	
Plateau	0.1629
K	1.538

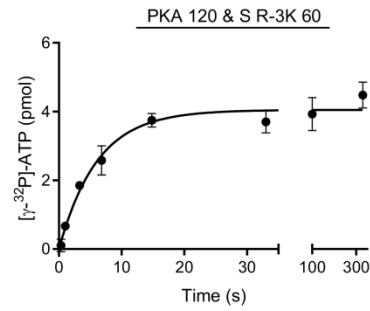


Best-fit values	
Y0	= 0.000
Plateau	5.680
K	0.4094
Tau	2.442
Half-time	1.693
Span	= 5.680
Std. Error	
Plateau	0.1846
K	0.05113

180

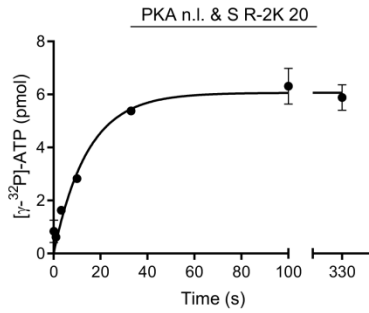


Best-fit values	
Y0	= 0.000
Plateau	3.872
K	6.625
Tau	0.1510
Half-time	0.1046
Span	= 3.872
Std. Error	
Plateau	0.1713
K	1.276

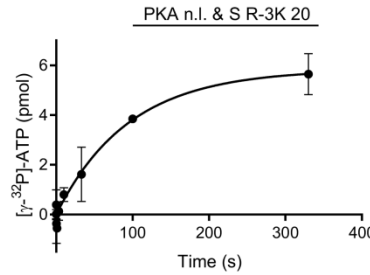


Best-fit values	
Y0	= 0.000
Plateau	4.046
K	0.1644
Tau	6.081
Half-time	4.215
Span	= 4.046
Std. Error	
Plateau	0.1284
K	0.02013

not linked



Best-fit values	
Y0	= 0.000
Plateau	6.061
K	0.06950
Tau	14.39
Half-time	9.973
Span	= 6.061
Std. Error	
Plateau	0.2303
K	0.01012

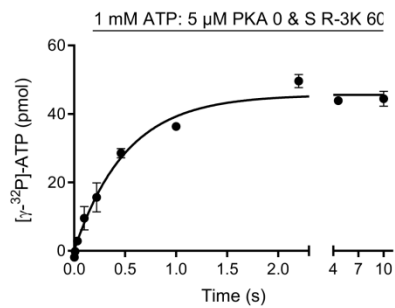


Best-fit values	
Y0	= 0.000
Plateau	5.833
K	0.01056
Tau	94.70
Half-time	65.64
Span	= 5.833
Std. Error	
Plateau	0.4792
K	0.002252

PKA & S R-3K

1 mM ATP+ 5 μM PKA & S

60



Best-fit values	
Y0	= 0.000
Plateau	45.58
K	1.972
Tau	0.5071
Half-time	0.3515
Span	= 45.58
Std. Error	
Plateau	1.113
K	0.1746

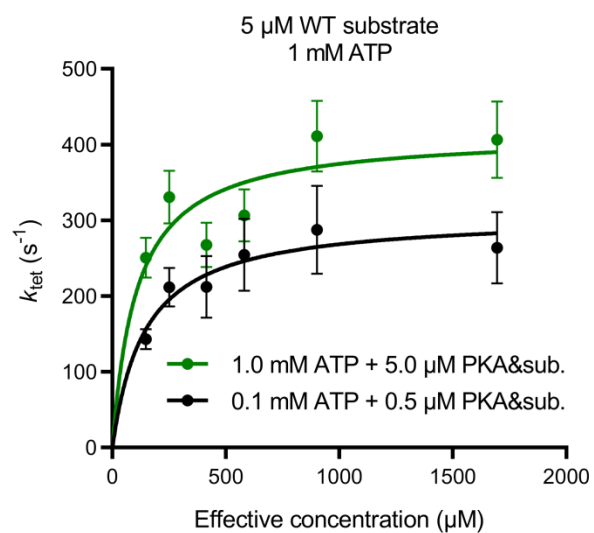


Fig. S3: ATP dependence of the tethered reaction. The ATP dependence of the tethered phosphorylation was tested at 1 mM ATP, whereas all other experiments were conducted at 100 μM . The amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was already at maximally permitted level, so the protein concentration was also increased 10-fold to preserve the same signal to noise.

Derivations of rate equations

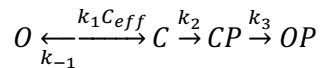
Tethered system

We consider a catalytical model where product release limits steady-state reaction rates and where phosphorylation and product release are two irreversible steps.

Moreover, we use saturating ATP concentrations and assume $k_{\text{ATP binding}} \gg k_{\text{cat}}$. Thus, we define the following states:

- O = open tethered system, bound ATP
- C = closed tethered system, bound ATP
- CP = closed, phosphorylated tethered system, bound ADP
- OP = open tethered system

The tethered system is composed of two interacting partners, and closure of this system is governed by effective concentration, C_{eff} :



The Law of Mass Action applied to the model leads to the following system of nonlinear reaction equations:

$$\frac{d[O]}{dt} = -k_1 C_{\text{eff}}[O] + k_{-1}[C]$$

$$\frac{d[C]}{dt} = k_1 C_{\text{eff}}[O] - (k_{-1} + k_2)[C]$$

$$\frac{d[CP]}{dt} = k_2[C] - k_3[CP]$$

$$\frac{d[OP]}{dt} = k_3[CP]$$

In single turnover experiments both closed and open phosphorylated products are measured, hence:

$$P = CP + OP$$

$$\frac{d[P]}{dt} = \frac{d[CP]}{dt} + \frac{d[OP]}{dt} = k_2[C]$$

From the conservation law, total concentration of the tethered system is constant:

$$\frac{d[O]}{dt} + \frac{d[C]}{dt} + \frac{d[CP]}{dt} + \frac{d[OP]}{dt} = 0$$

$$[O] + [C] + [CP] + [OP] = [E]_T$$

$$[O] = [E]_T - [C] - [P]$$

Rapid equilibrium assumption for the open/closed complex:

$$\frac{d[O]}{dt} = 0$$

$$k_1 C_{eff} [O] = k_{-1} [C]$$

$$k_1 C_{eff} ([E]_T - [C] - [P]) = k_{-1} [C]$$

$$k_{-1} [C] + k_1 C_{eff} [C] = k_1 C_{eff} [E]_T - k_1 C_{eff} [P]$$

$$[C] = \frac{k_1 C_{eff} [E]_T - k_1 C_{eff} [P]}{k_{-1} + k_1 C_{eff}} = \frac{C_{eff} [E]_T - C_{eff} [P]}{\frac{k_{-1}}{k_1} + C_{eff}}$$

Given $K_d = \frac{k_{-1}}{k_1}$:

$$[C] = \frac{C_{eff} [E]_T - C_{eff} [P]}{K_d + C_{eff}}$$

Substituting $[C]$ into the product formation equation:

$$\frac{d[P]}{dt} = k_2 [C] = k_2 \frac{C_{eff} [E]_T - C_{eff} [P]}{K_d + C_{eff}}$$

Integrate product formation rate:

$$\frac{d[P]}{dt} = -\frac{k_2 C_{eff}}{K_d + C_{eff}} [P] + \frac{k_2 C_{eff} [E]_T}{K_d + C_{eff}}$$

$$\frac{1}{-\frac{k_2 C_{eff}}{K_d + C_{eff}} [P] + \frac{k_2 C_{eff} [E]_T}{K_d + C_{eff}}} d[P] = dt$$

$$\int_0^{[P]} \frac{1}{-\frac{k_2 C_{eff}}{K_d + C_{eff}} [P] + \frac{k_2 C_{eff} [E]_T}{K_d + C_{eff}}} d[P] = \int_0^t dt$$

$$-\frac{K_d + C_{eff}}{k_2 C_{eff}} \ln \left| -\frac{k_2 C_{eff}}{K_d + C_{eff}} [P] + \frac{k_2 C_{eff} [E]_T}{K_d + C_{eff}} \right| + \frac{K_d + C_{eff}}{k_2 C_{eff}} \ln \left| -\frac{k_2 C_{eff}}{K_d + C_{eff}} \cdot 0 + \frac{k_2 C_{eff} [E]_T}{K_d + C_{eff}} \right| = t - 0$$

$$\ln \left| \frac{k_2 C_{eff} ([E]_T - [P])}{K_d + C_{eff}} \right| - \ln \left| \frac{k_2 C_{eff} [E]_T}{K_d + C_{eff}} \right| = - \frac{k_2 C_{eff}}{K_d + C_{eff}} t$$

$$\ln \left| \frac{[E]_T - [P]}{[E]_T} \right| = - \frac{k_2 C_{eff}}{K_d + C_{eff}} t$$

$$\frac{[E]_T - [P]}{[E]_T} = e^{-\frac{k_2 C_{eff}}{K_d + C_{eff}} t}$$

Formation of phosphorylated product is described by the following equation:

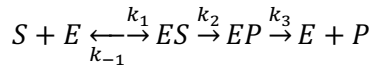
$$[P] = [E]_T \left(1 - e^{-\frac{k_2 C_{eff}}{K_d + C_{eff}} t} \right)$$

Phosphorylation rates in the tethered system obtained from quench flow measurements (k_{tet}) are dependent on effective concentration:

$$k_{tet} = \frac{k_2 C_{eff}}{K_d + C_{eff}}$$

Untethered system

We consider a catalytical model where product release limits steady-state reaction rates and where phosphorylation and product release are two irreversible steps.



The Law of Mass Action applied to the model leads to the following system of nonlinear reaction equations:

$$\frac{d[S]}{dt} = -k_1[S][E] + k_{-1}[ES]$$

$$\frac{d[E]}{dt} = -k_1[S][E] + k_{-1}[ES] + k_3[EP]$$

$$\frac{d[ES]}{dt} = k_1[S][E] - (k_{-1} + k_2)[ES]$$

$$\frac{d[EP]}{dt} = k_2[ES] - k_3[EP]$$

$$\frac{d[P]}{dt} = k_3[EP]$$

From the conservation law for the enzyme, total enzyme concentration is constant:

$$\frac{d[E]}{dt} + \frac{d[ES]}{dt} + \frac{d[EP]}{dt} = 0$$

$$[E] + [ES] + [EP] = [E]_0$$

$$[E] = [E]_0 - [ES] - [EP]$$

Rapid equilibrium assumption:

$$\frac{d[S]}{dt} = 0$$

$$k_1[S][E] = k_{-1}[ES]$$

$$k_1[S]([E]_0 - [ES] - [EP]) = k_{-1}[ES]$$

$$k_1[S][E]_0 - k_1[S][EP] = k_{-1}[ES] + k_1[S][ES]$$

$$[ES] = \frac{k_1[S][E]_0 - k_1[S][EP]}{k_1[S] + k_{-1}}$$

Quasi-steady-state approximation of the $[EP]$ complex:

$$\frac{d[EP]}{dt} = 0$$

$$k_2[ES] - k_3[EP] = 0$$

$$\frac{k_3[EP]}{k_2} = [ES]$$

$$\frac{k_3[EP]}{k_2} = \frac{k_1[S][E]_0 - k_1[S][EP]}{k_1[S] + k_{-1}}$$

$$k_1k_3[S][EP] + k_{-1}k_3[EP] = k_1k_2[S][E]_0 - k_1k_2[S][EP]$$

$$[EP]([S]k_1(k_2 + k_3) + k_{-1}k_3) = k_1k_2[S][E]_0$$

$$[EP] = \frac{k_1k_2[S][E]_0}{[S]k_1(k_2 + k_3) + k_{-1}k_3}$$

$$[EP] = \frac{\frac{k_2[S][E]_0}{k_2 + k_3}}{[S] + \frac{k_{-1}k_3}{k_1(k_2 + k_3)}}$$

Given $K_d = \frac{k_{-1}}{k_1}$:

$$[EP] = \frac{\frac{k_2[S][E]_0}{k_2 + k_3}}{[S] + K_d \frac{k_3}{k_2 + k_3}}$$

Finally, substituting $[EP]$ into the product formation equation:

$$\frac{d[P]}{dt} = k_3[EP] = \frac{\frac{k_2k_3}{k_2 + k_3} [S][E]_0}{[S] + K_d \frac{k_3}{k_2 + k_3}}$$

Hence:

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3}$$

$$K_M = K_d \frac{k_3}{k_2 + k_3}$$