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
- p66α 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, serine residue that becomes phosphorylated is shown in **bold**

1. PKAc

MGSSHHHHHHSSGLVPR//GSHMGNAAAAKKGSEQESVKEFLAKAKEDFLKKWETPSQNTAQLDQFDRIKTLGT
GSFGRVMLVKHKESGNHYAMKILDKQKVVKLKQIEHTLNEKRILQAVNFPFLVKLEFSFKDNSNLYMVMEYVAGG
EMFSHLRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDQQGYIQVTDFGFAKRVKGRTWTLCGTPE
YLAPEIILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQIYEKIVSGKVRFPSHFSSDLKDLLRNLLQVDLTKRF
GNLKNGVNDIKNHKWFATTDWIAIYQRKVEAPFIPKFKGPGDTSNFDDYEEEEIRVSINEKCGKEFTEF

2. MBD2-$(GS)_n$-PKAc

MGSSHHHHHHSSGLVPR//GSHMVTDEDIRKQEERAQQVRKKLEEALMADAS$(GS)_n$GTGNAAAAKKGSEQESVKE
FLAKAKEDFLKKWETPSQNTAQLDQFDRIKTLGTGSFGRVMLVKHKESGNHYAMKILDKQKVVKLKQIEHTLNEK
RILQAVNFPFLVKLEFSFKDNSNLYMVMEYVAGGEMFSHLRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKP
ENLLIDQQGYIQVTDFGFAKRVKGRTWTLCGTPEYLAPEIILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQI
YEKIVSGKVRFPSHFSSDLKDLLRNLLQVDLTKRFGNLKNGVNDIKNHKWFATTDWIAIYQRKVEAPFIPKFKGPG
DTSNFDDYEEEEIRVSINEKCGKEFTEF

3. p66α -$(GS)_n$-WT substrate

MGSSHHHHHHSSGLVPR//GSHMTSPEERERMIKQLKEELRLEEAKLVLLKKLRQSQIQKEATAQKAS$(GS)_n$GTPG
SGSGSGSLRRA**S**LGGGGGY

4. p66α -$(GS)_n$-R-2K substrate

MGSSHHHHHHSSGLVPR//GSHMTSPEERERMIKQLKEELRLEEAKLVLLKKLRQSQIQKEATAQKAS$(GS)_n$GTPG
SGSGSGSLRKA**S**LGGGGGY

5. p66α -$(GS)_n$-R-3K substrate

MGSSHHHHHHSSGLVPR//GSHMTSPEERERMIKQLKEELRLEEAKLVLLKKLRQSQIQKEATAQKAS$(GS)_n$GTPG
SGSGSGSLKRA**S**LGGGGGY

Supplementary figures

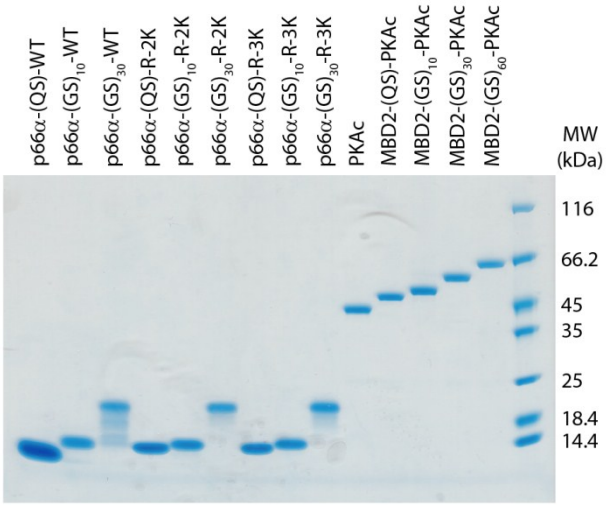
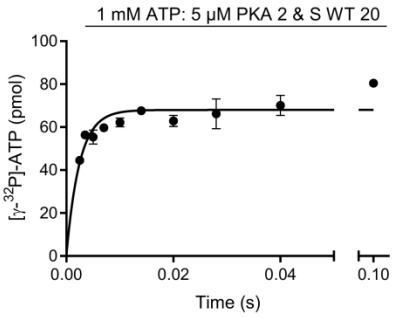
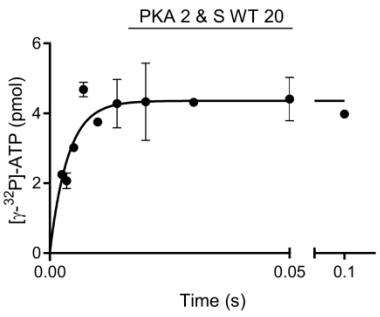
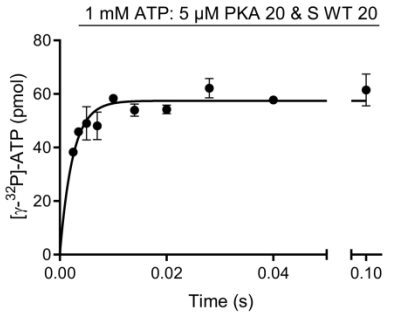
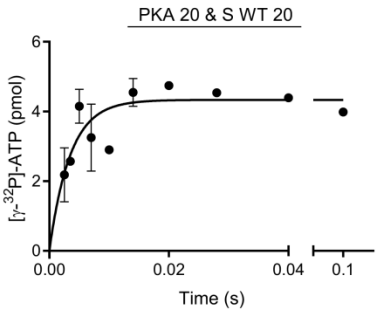
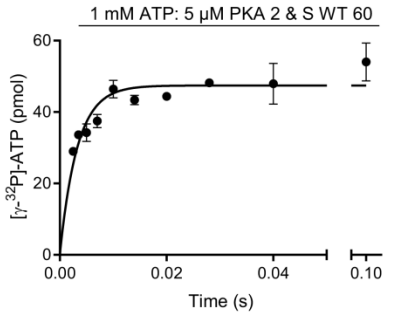
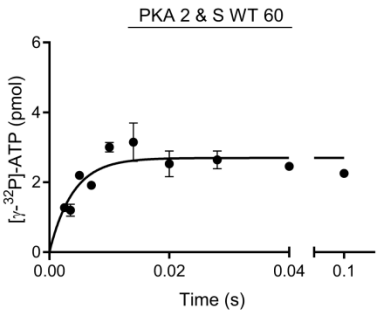
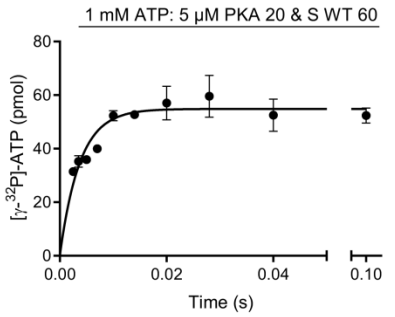
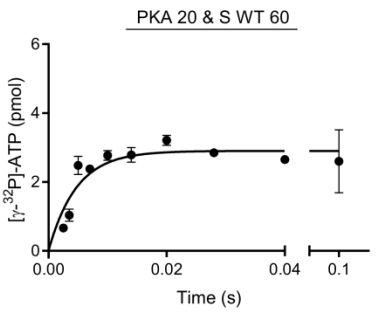


Fig. S1: Protein variants used in this study. SDS-PAGE gel of purified proteins. The substrates with a 120 residue GS linker $(GS)_{60}$ could not be purified to sufficient purity.

Next four pages:

Fig. S2: Primary data from quench-flow experiments. The data are fitted to one-phase association model using GraphPad Prism 8.3, with best-fit parameter values and standard errors shown for each experiment.

PKA & S WT

Linker length:	1 mM ATP+ 5 µM PKA & S	0.1 mM ATP+ 0.5 µM PKA & S
20		
40		
60		
80		

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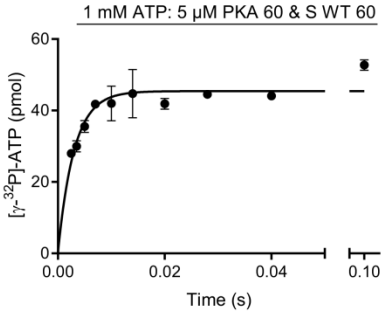
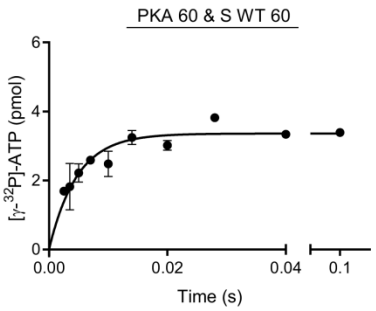


Best-fit values	
Y0	= 0.000
Plateau	45.42
K	330.7
Tau	0.003024
Half-time	0.002096
Span	= 45.42
Std. Error	
Plateau	1.109
K	35.09



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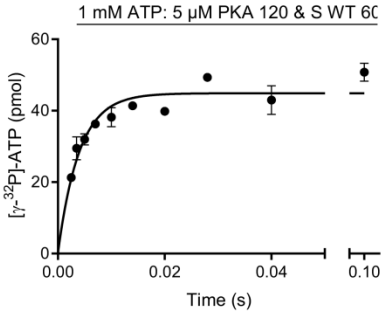
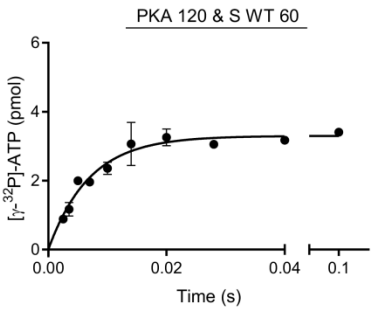
Plateau	3.357
K	211.7
Tau	0.004724
Half-time	0.003275
Span	= 3.357
Std. Error	
Plateau	0.1144
K	25.64

180

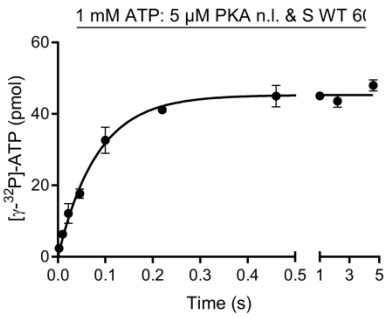


Best-fit values	
Y0	= 0.000
Plateau	44.89
K	250.9
Tau	0.003986
Half-time	0.002763
Span	= 44.89
Std. Error	
Plateau	1.229
K	26.15

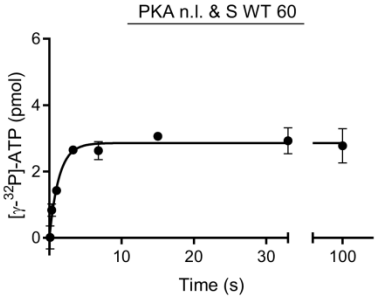


Best-fit values	
Y0	= 0.000
Plateau	3.296
K	143.5
Tau	0.006968
Half-time	0.004830
Span	= 3.296
Std. Error	
Plateau	0.09910
K	13.37

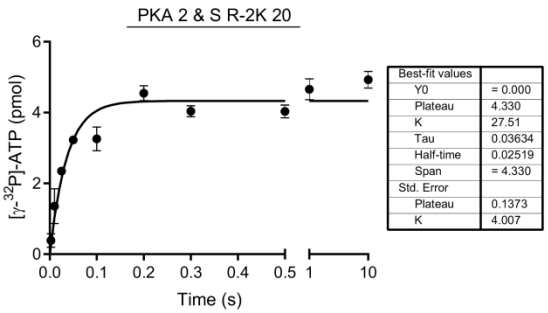
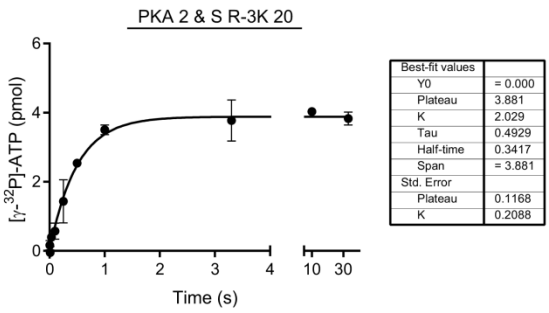
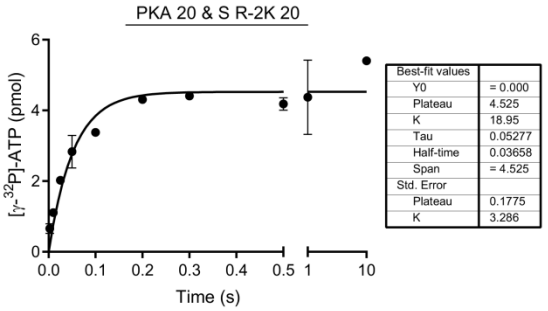
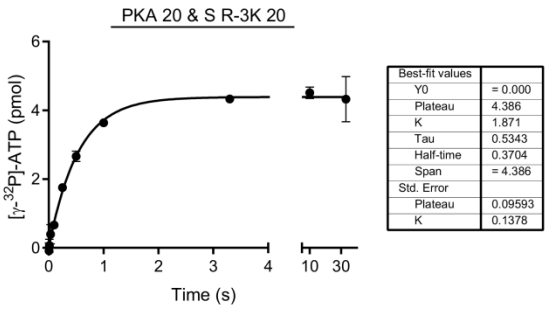
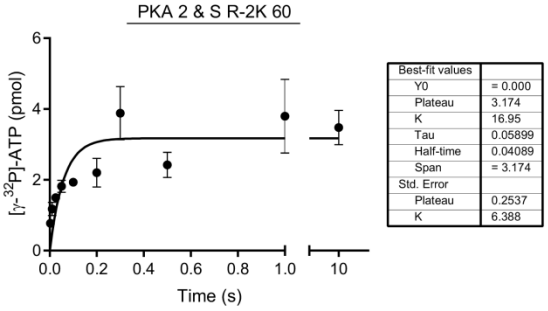
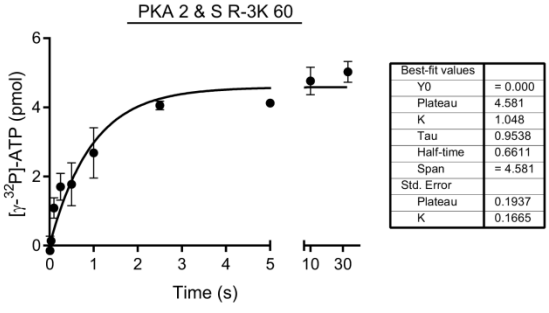
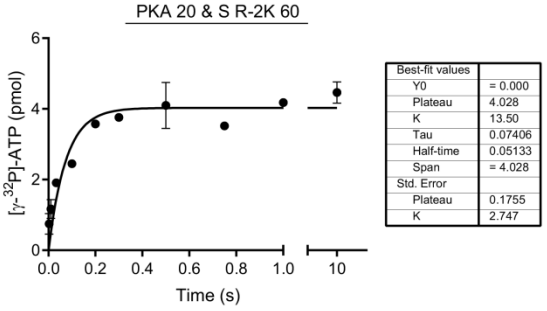
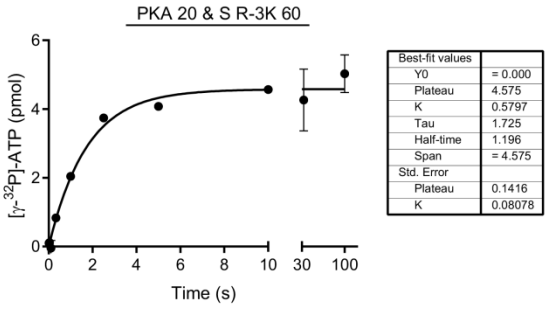
not linked



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

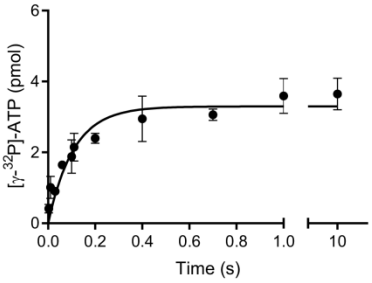


Best-fit values	
Y0	= 0.000
Plateau	2.857
K	0.7487
Tau	1.336
Half-time	0.9259
Span	= 2.857
Std. Error	
Plateau	0.09481
K	0.1225

Linker length:	PKA & S R-2K 0.1 mM ATP+ 0.5 µM PKA & S	PKA & S R-3K 0.1 mM ATP+ 0.5 µM PKA & S
20	 Best-fit values: Y0 = 0.000; Plateau 4.330; K 27.51; Tau 0.03634; Half-time 0.02519; Span = 4.330; Std. Error: Plateau 0.1373; K 4.007	 Best-fit values: Y0 = 0.000; Plateau 3.881; K 2.029; Tau 0.4929; Half-time 0.3417; Span = 3.881; Std. Error: Plateau 0.1168; K 0.2088
40	 Best-fit values: Y0 = 0.000; Plateau 4.525; K 18.95; Tau 0.05277; Half-time 0.03658; Span = 4.525; Std. Error: Plateau 0.1775; K 3.286	 Best-fit values: Y0 = 0.000; Plateau 4.386; K 1.871; Tau 0.5343; Half-time 0.3704; Span = 4.386; Std. Error: Plateau 0.09593; K 0.1378
60	 Best-fit values: Y0 = 0.000; Plateau 3.174; K 16.95; Tau 0.05899; Half-time 0.04089; Span = 3.174; Std. Error: Plateau 0.2537; K 6.388	 Best-fit values: Y0 = 0.000; Plateau 4.581; K 1.048; Tau 0.9538; Half-time 0.6611; Span = 4.581; Std. Error: Plateau 0.1937; K 0.1665
80	 Best-fit values: Y0 = 0.000; Plateau 4.028; K 13.50; Tau 0.07406; Half-time 0.05133; Span = 4.028; Std. Error: Plateau 0.1755; K 2.747	 Best-fit values: Y0 = 0.000; Plateau 4.575; K 0.5797; Tau 1.725; Half-time 1.196; Span = 4.575; Std. Error: Plateau 0.1416; K 0.08078

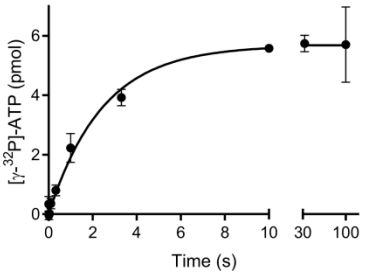
120

PKA 60 & S R-2K 60



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

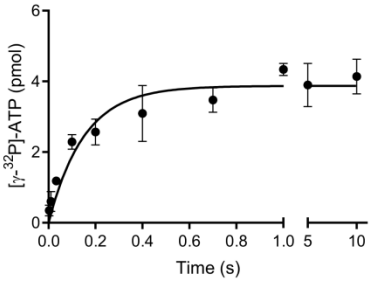
PKA 60 & S R-3K 60



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

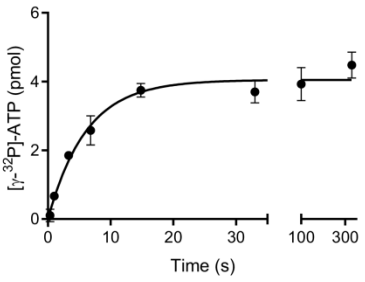
180

PKA 120 & S R-2K 60



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

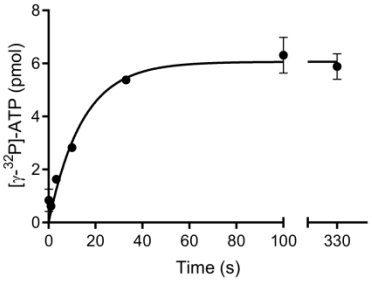
PKA 120 & S R-3K 60



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

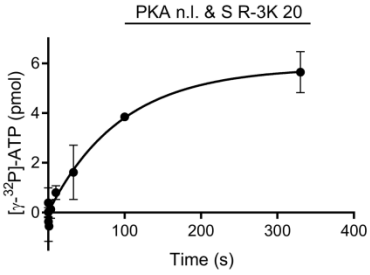
not linked

PKA n.l. & S R-2K 20



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

PKA n.l. & S R-3K 20



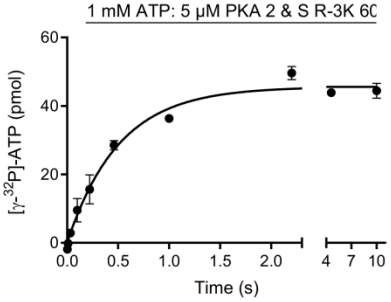
Best-fit values	
Y0	= 0.000
Plateau	5.833
K	0.01056
Tau	94.70
Half-time	65.64
Span	= 5.833
Std. Error	
Plateau	0.4792
K	0.002252

PKA & S R-3K

1 mM ATP+ 5 µM PKA & S

60

1 mM ATP: 5 µM PKA 2 & S R-3K 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

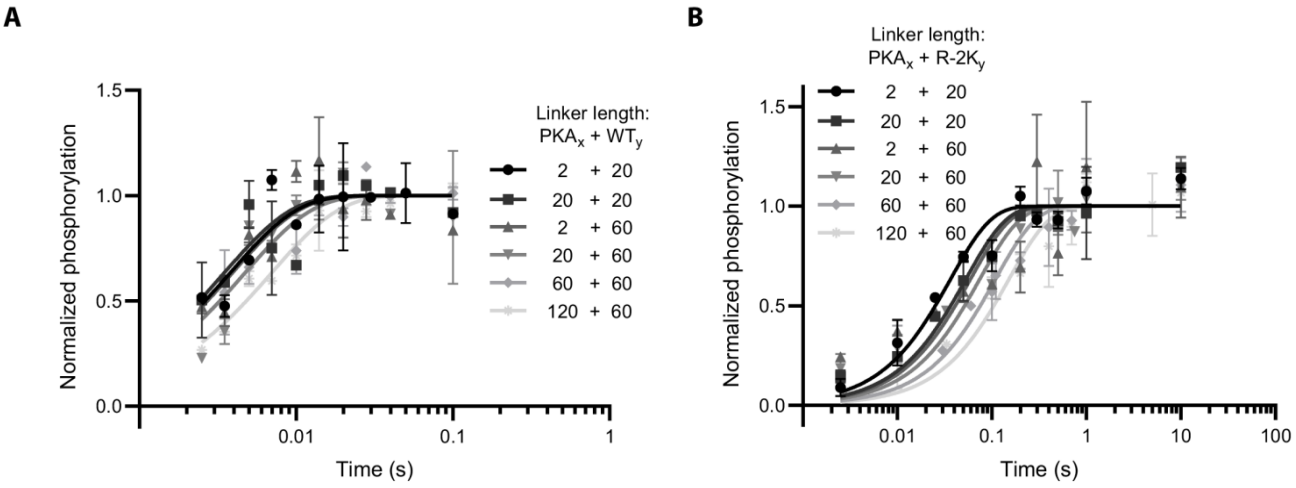


Fig. S3: Linker dependence of single-turnover phosphorylation rates. Quench-flow kinetics of the phosphorylation reactions of (A) WT and (B) R-2K substrate. The total linker length is a combination of contributions from GS-repeats in the two constructs labeled PKA_x and WT_y/R-$2K_y$, where x and y denote linker length in each construct, listed in the figure. k_{tet} is derived from a fit to one-phase association model (black and grey lines). Error bars indicate mean ± s.d., $n = 2$.

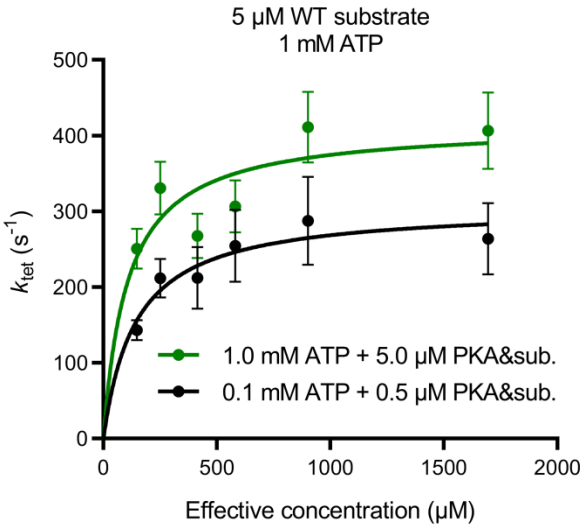


Fig. S4: ATP dependence of the tethered reaction. The ATP dependence of the tethered phosphorylation was tested at 1 mM ATP, whereas all other experiments were conducted at 100 μM. The amount of [γ-^{32}P]ATP was already at maximally permitted level, so the protein concentration was also increased 10-fold to preserve the same signal to noise. Error bars correspond to standard error of the fit to one-phase association model

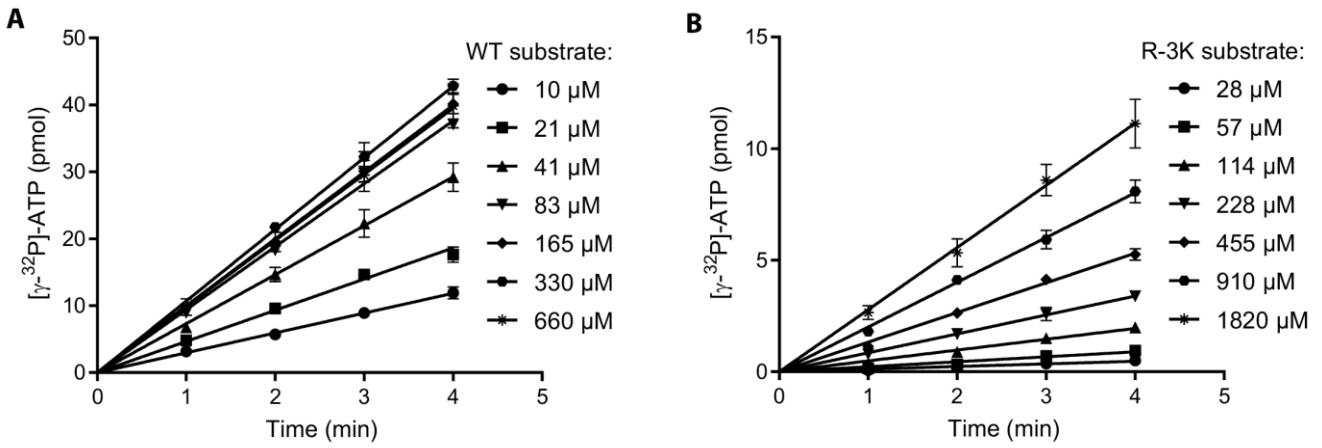


Fig. S5: Quantifying the quality of PKA substrate variants. Primary data from a steady-state kinetic experiment performed at 1 nM PKAc and an indicated concentration of (A) the p66α-(QS)-WT substrate and (B) the p66α-(QS)-R-3K substrate. Error bars indicate mean ± s.d., $n = 3$.

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 $k_{\text{ATP binding}}$ >> k_{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}:

$$O \underset{k_{-1}}{\overset{k_1 C_{eff}}{\longleftrightarrow}} C \overset{k_2}{\rightarrow} CP \overset{k_3}{\rightarrow} OP$$

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

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

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

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

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

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:

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

$$[O] + [C] + [CP] + [OP] = [E]_{\mathrm{T}}$$

$$[O] = [E]_{\mathrm{T}} - [C] - [P]$$

Rapid equilibrium assumption for the open/closed complex:

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

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

$$k_1 C_{eff}([E]_{\mathrm{T}} - [C] - [P]) = k_{-1}[C]$$

$$k_{-1}[C] + k_1 C_{eff}[C] = k_1 C_{eff}[E]_{\mathrm{T}} - k_1 C_{eff}[P]$$

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

Given $K_{\mathrm{d}} = \frac{k_{-1}}{k_1}$:

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

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

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

Integrate product formation rate:

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

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

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

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

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

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

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

Formation of phosphorylated product is described by the following equation:

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

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.

$$S + E \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} ES \overset{k_2}{\rightarrow} EP \overset{k_3}{\rightarrow} E + P$$

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 $K_\mathrm{d} = \frac{k_{-1}}{k_1}$:

$$[EP] = \frac{\frac{k_2[S][E]_0}{k_2 + k_3}}{[S] + K_\mathrm{d}\frac{k_3}{k_2 + k_3}}$$

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

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

Hence:

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

$$K_\mathrm{M} = K_\mathrm{d}\frac{k_3}{k_2 + k_3}$$