ESC4
Advanced kinetics approaches to unravel protein structure and function

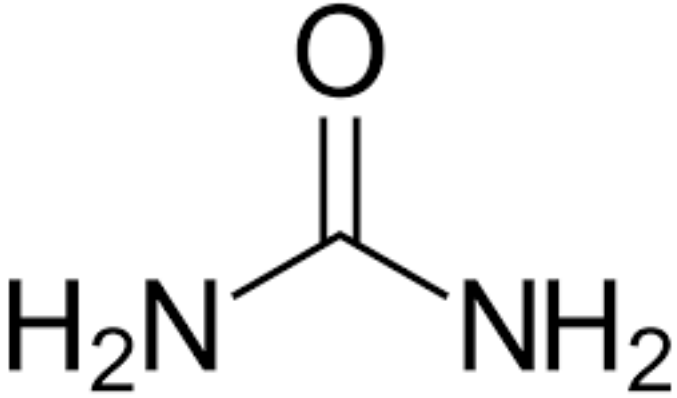
Experimental characterization of folding and binding reactions

Dr. Angelo Toto

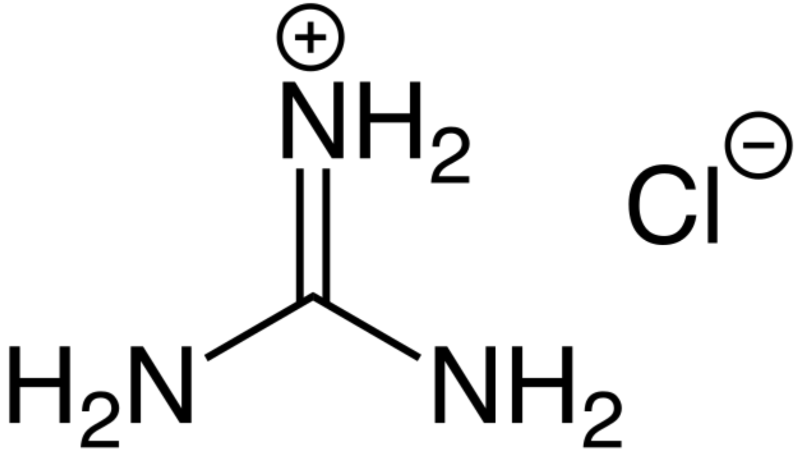
Protein Folding Kinetic Experiments

Protein (un)folding is a highly cooperative reaction!!
Only two states can be (often) detected!!

$$U \rightleftharpoons N$$



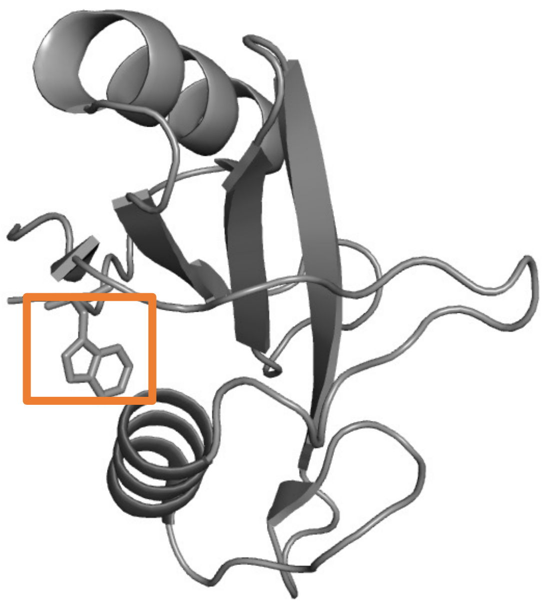
UREA

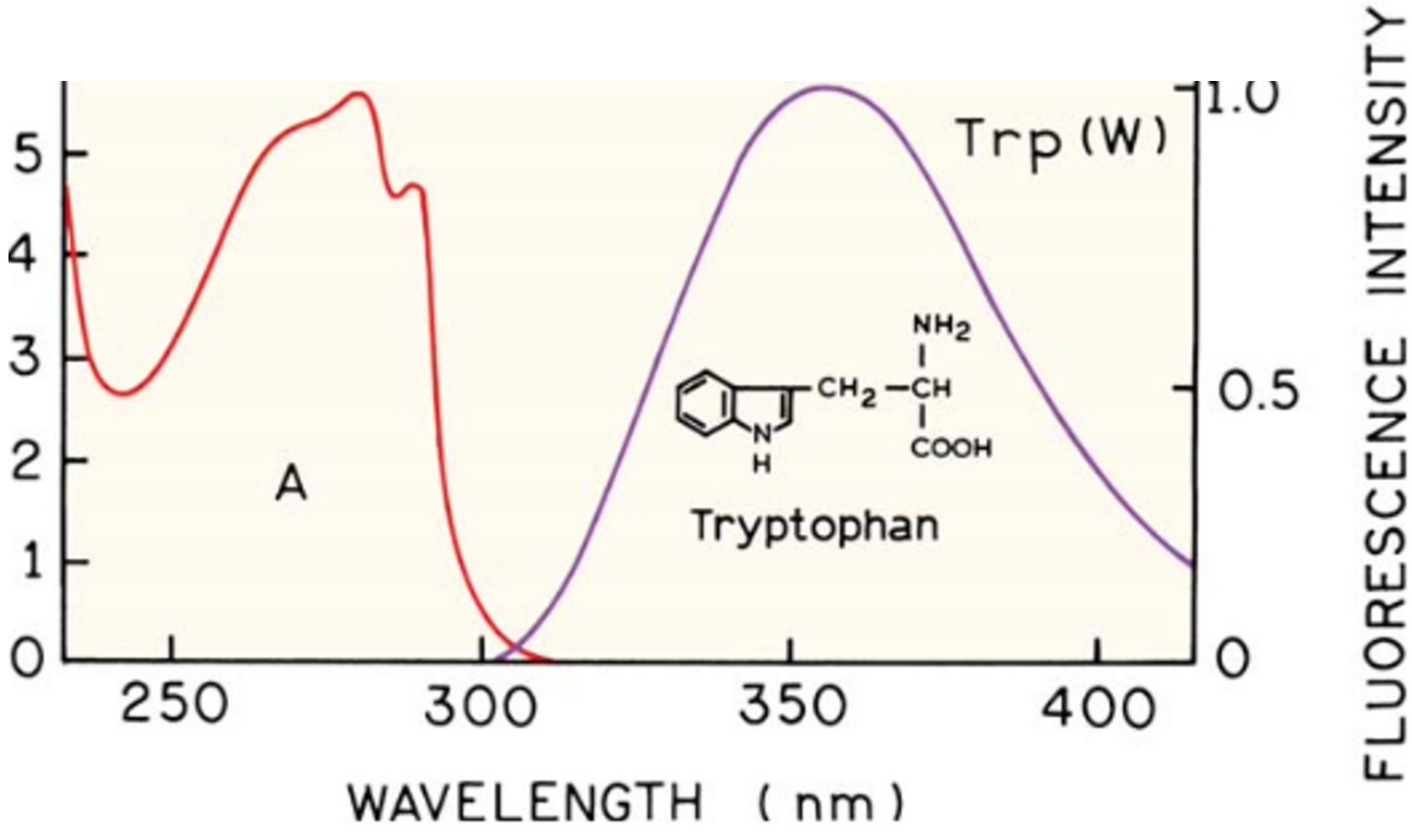


GUANIDINIUM ION

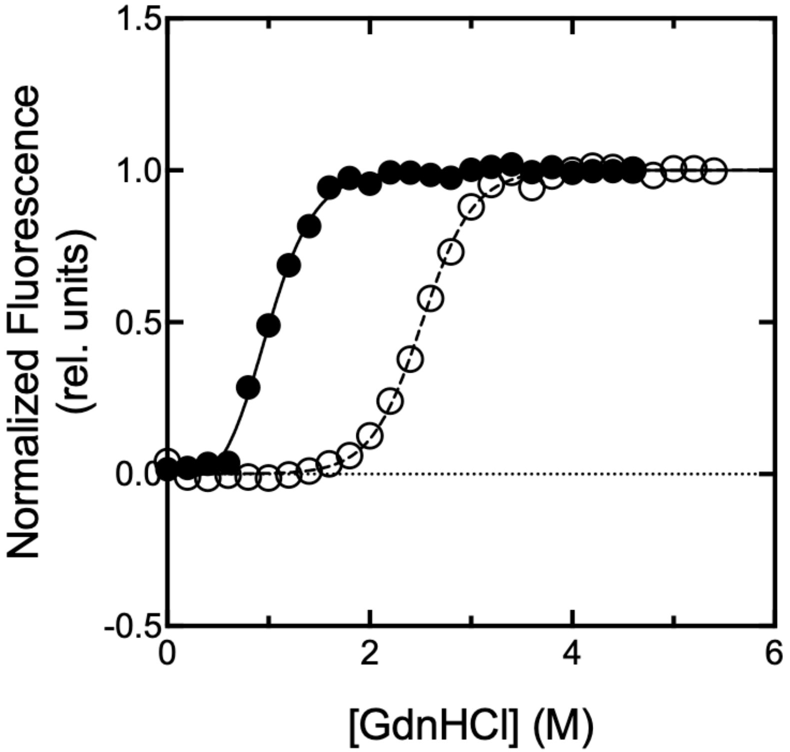
Chaotropic Agents

trp residue

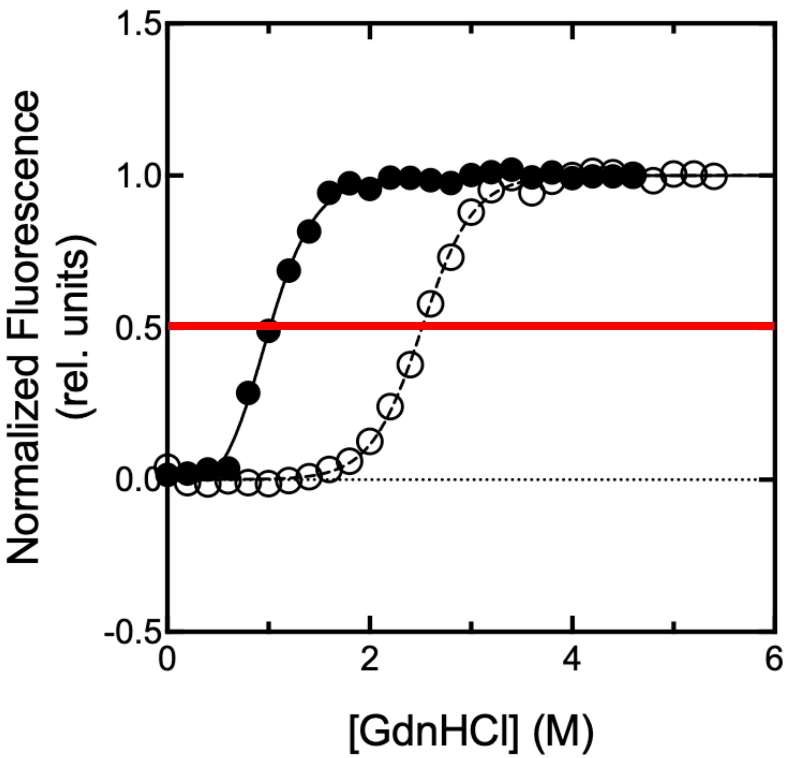




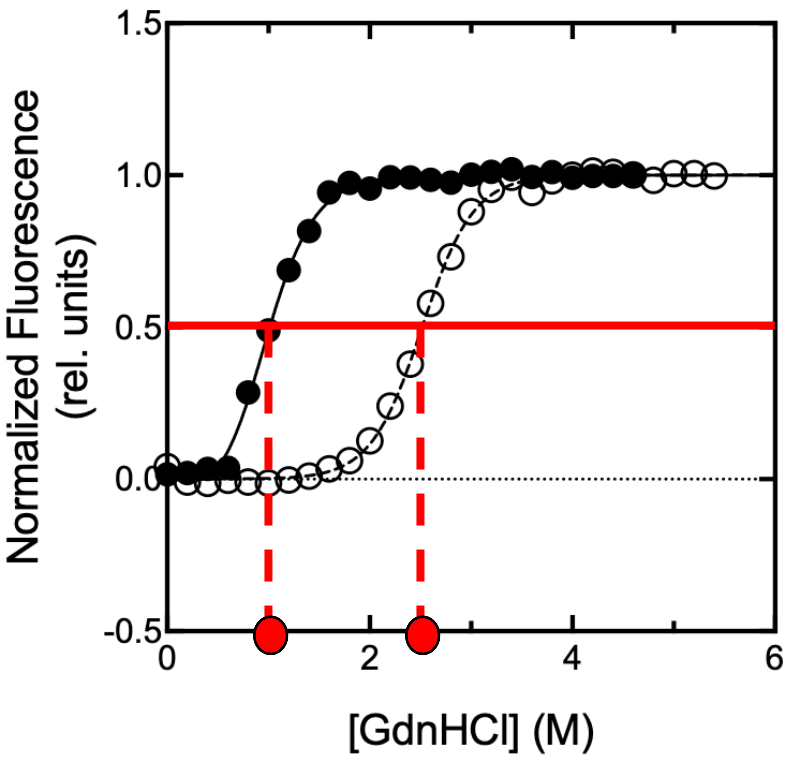
5
4
3
2
1
0
1.0
0.5
0
Trp (W)
A
NH_2
CH_2 — CH
N
H
COOH
Tryptophan
250
300
350
400
WAVELENGTH (nm)
FLUORESCENCE INTENSITY



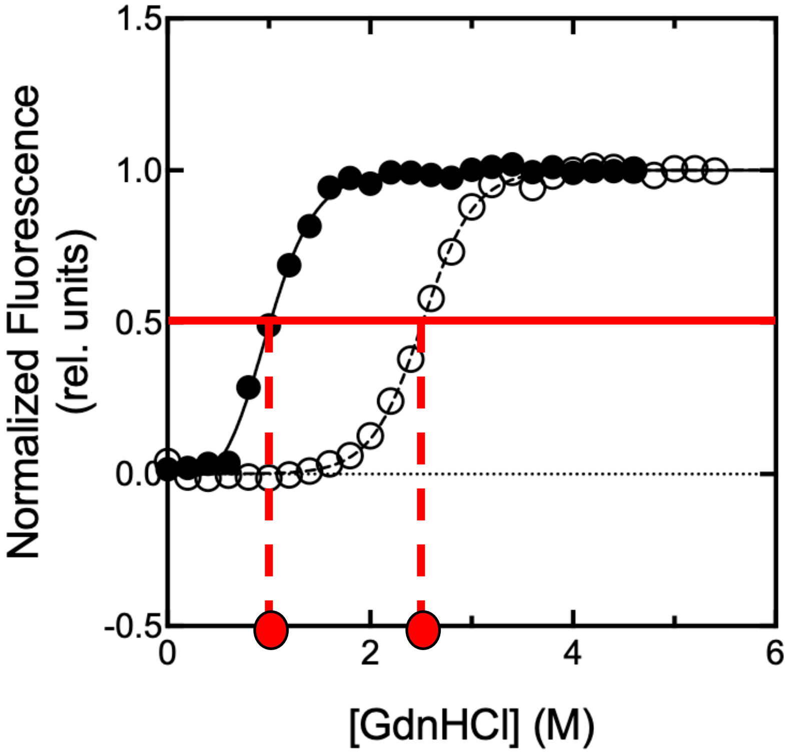
Normalized Fluorescence
(rel. units)
1.5
1.0
0.5
0.0
-0.5
0
2
4
6
[GdnHCl] (M)



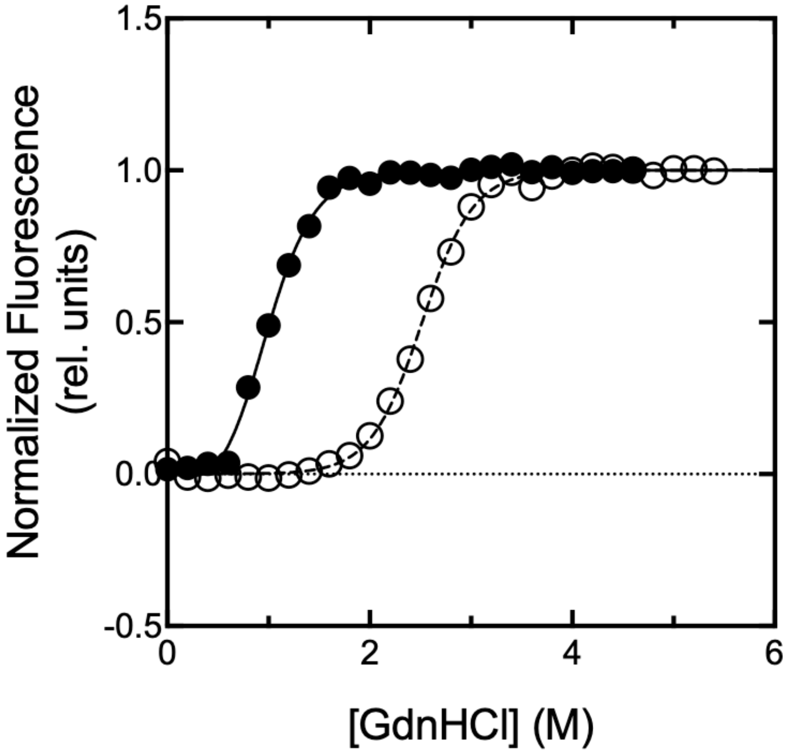
Normalized Fluorescence (rel. units)
1.5
1.0
0.5
0.0
-0.5
0
2
4
6
[GdnHCl] (M)



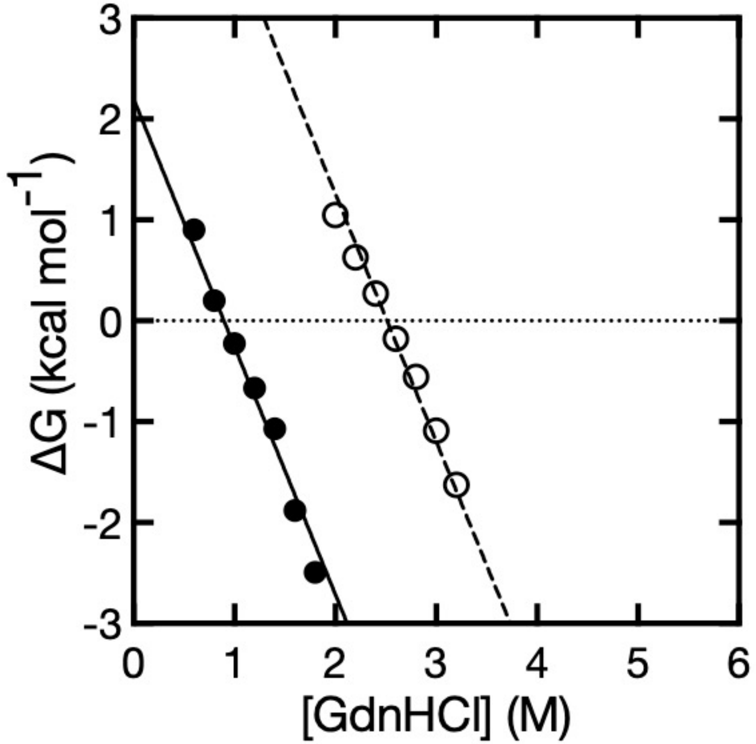
1.5
1.0
0.5
0.0
-0.5
0
2
4
6
Normalized Fluorescence (rel. units)
[GdnHCl] (M)



$$Y_{\mathrm{obs}} = Y_{\mathrm{N}} + Y_{\mathrm{D}} \frac{e^{(m_{\mathrm{D-N}}([\mathrm{urea}]-[\mathrm{urea}]_{1/2}))}}{1 + e^{(m_{\mathrm{D-N}}([\mathrm{urea}]-[\mathrm{urea}]_{1/2}))}}$$

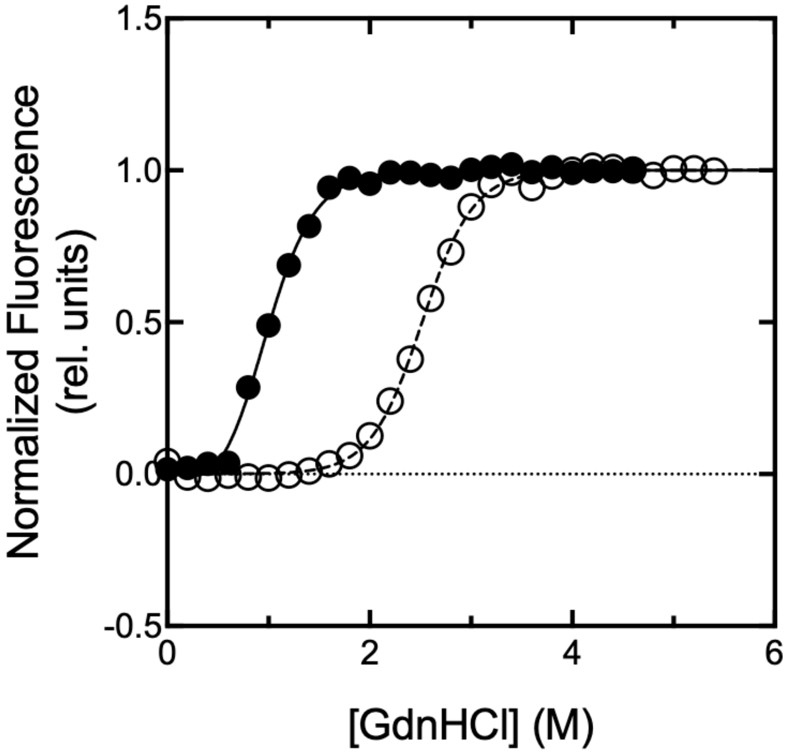


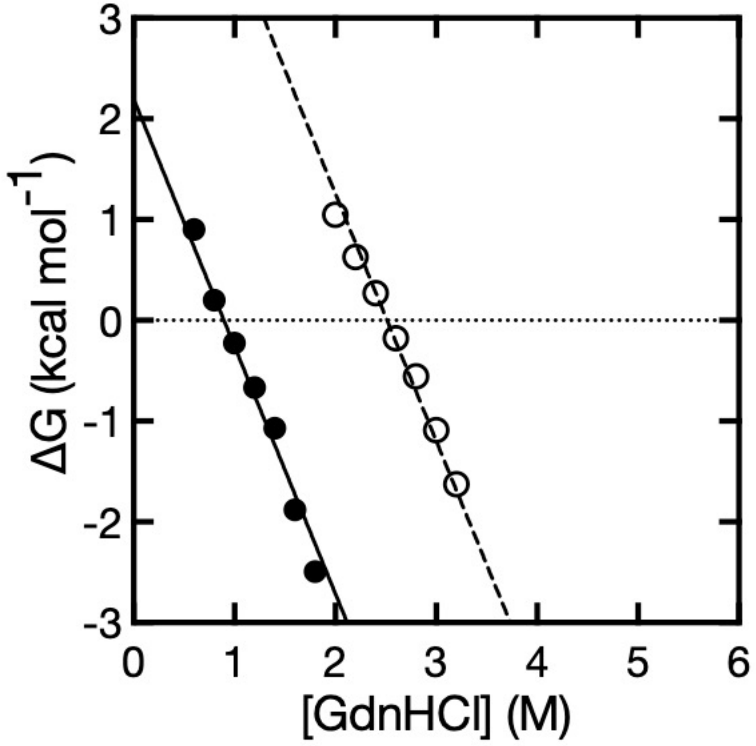
$$Y_{obs} = Y_N + Y_D \frac{e^{(m_{D-N}([urea]-[urea]_{1/2})}}{1 + e^{(m_{D-N}([urea]-[urea]_{1/2})}}$$



$$\Delta G = \Delta G(H_2O) - m[urea]$$

We now have info about the thermodynamic stability. What about “mechanism”?

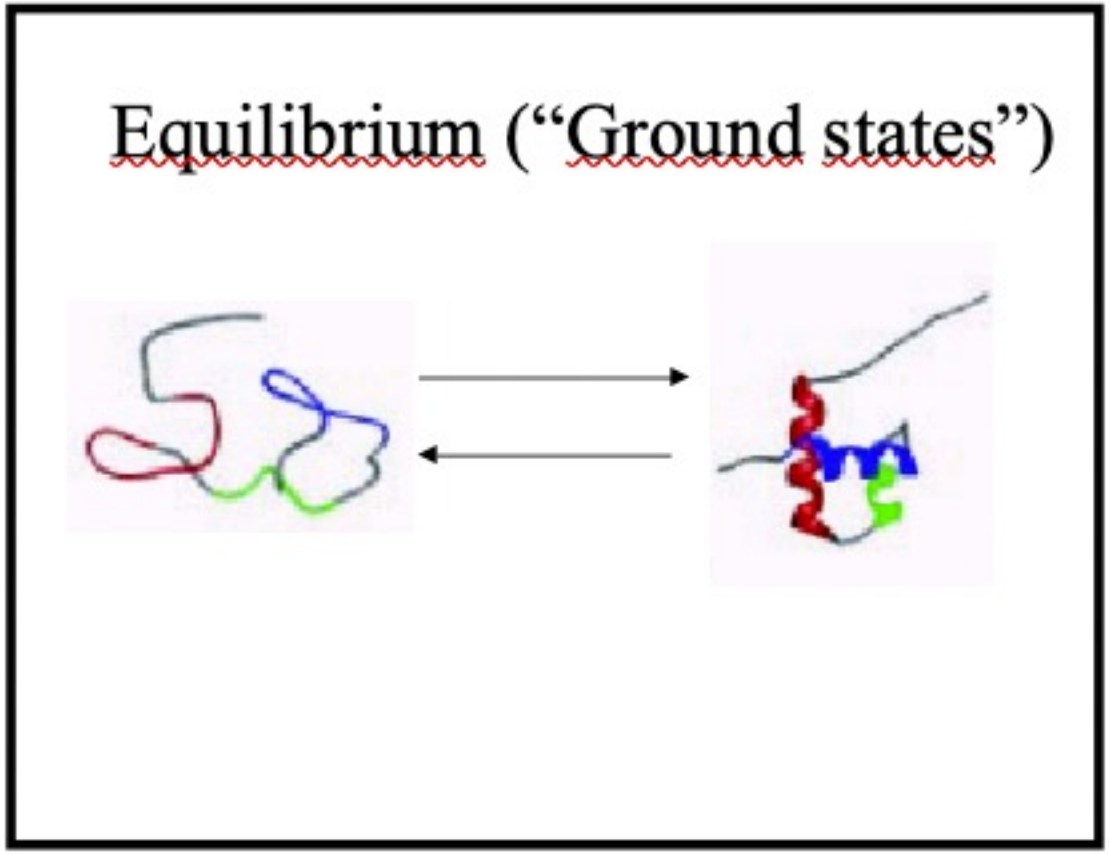


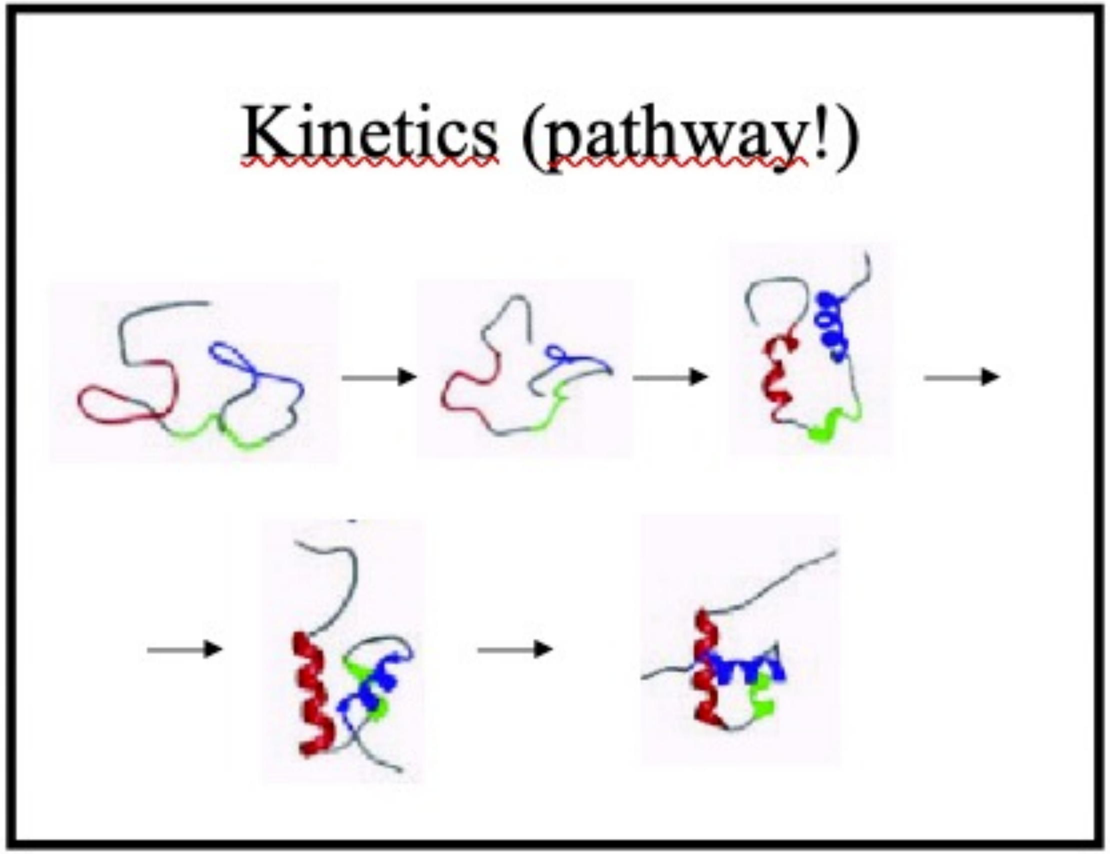


$$Y_{obs} = Y_N + Y_D \frac{e^{(m_{D-N}([\text{urea}]-[\text{urea}]_{1/2})}}{1 + e^{(m_{D-N}([\text{urea}]-[\text{urea}]_{1/2})}}$$

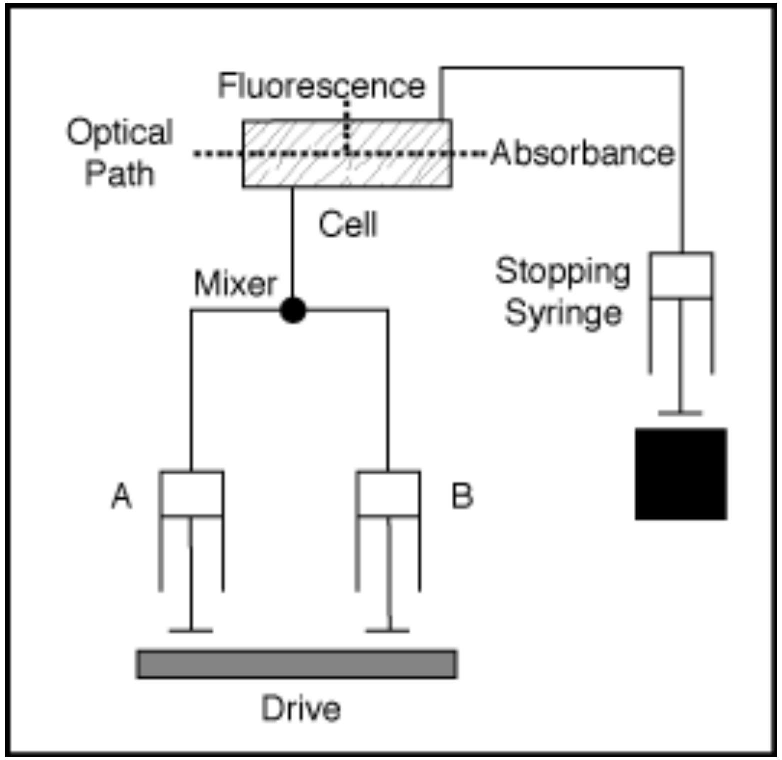
$$\Delta G = \Delta G(H_2O) - m[\text{urea}]$$

From equilibrium denaturation experiments I cannot obtain information about the mechanism of folding

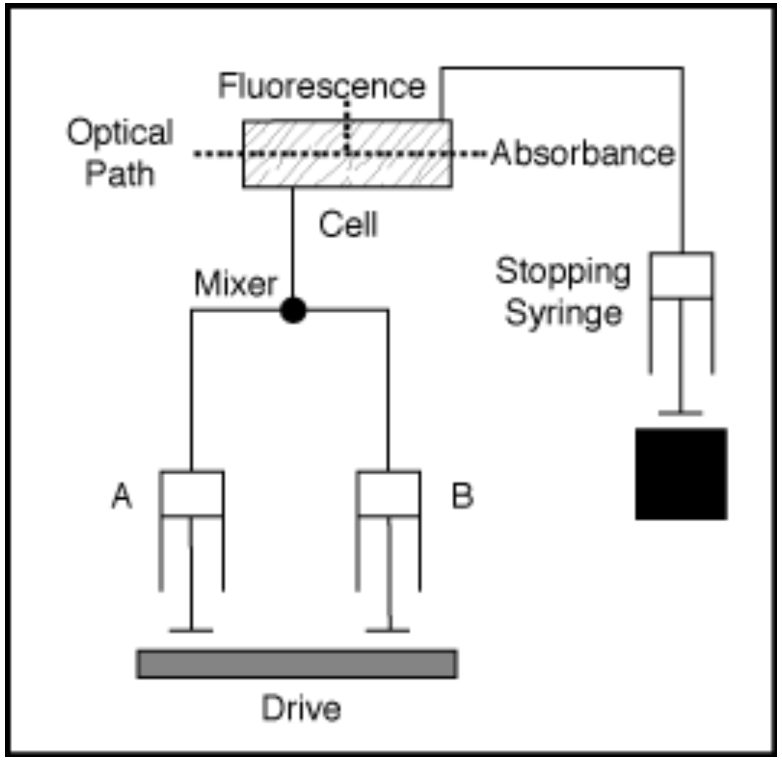




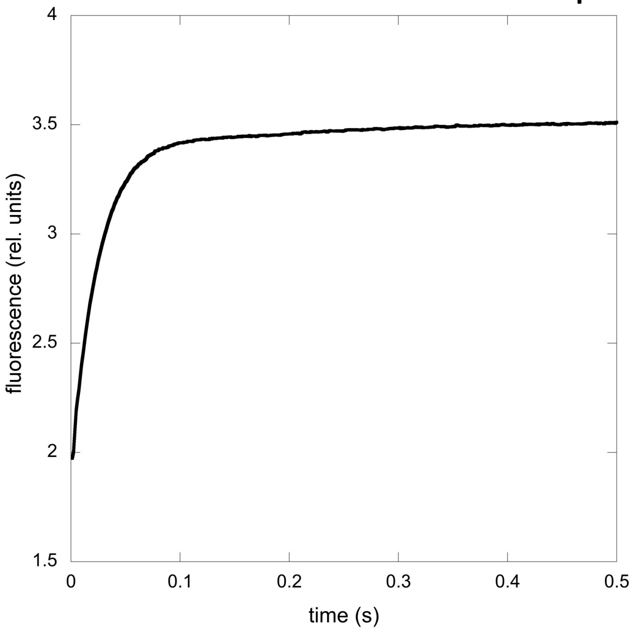
RAPID MIXING METHOD

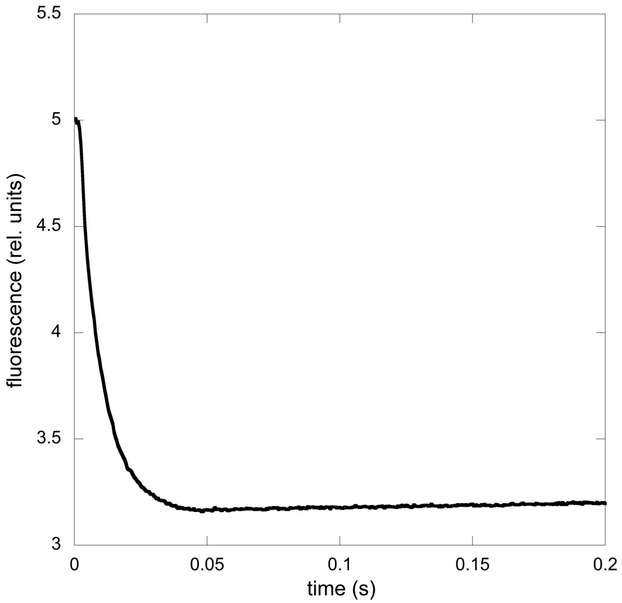


STOPPED-FLOW APPARATUS



Exponential decay





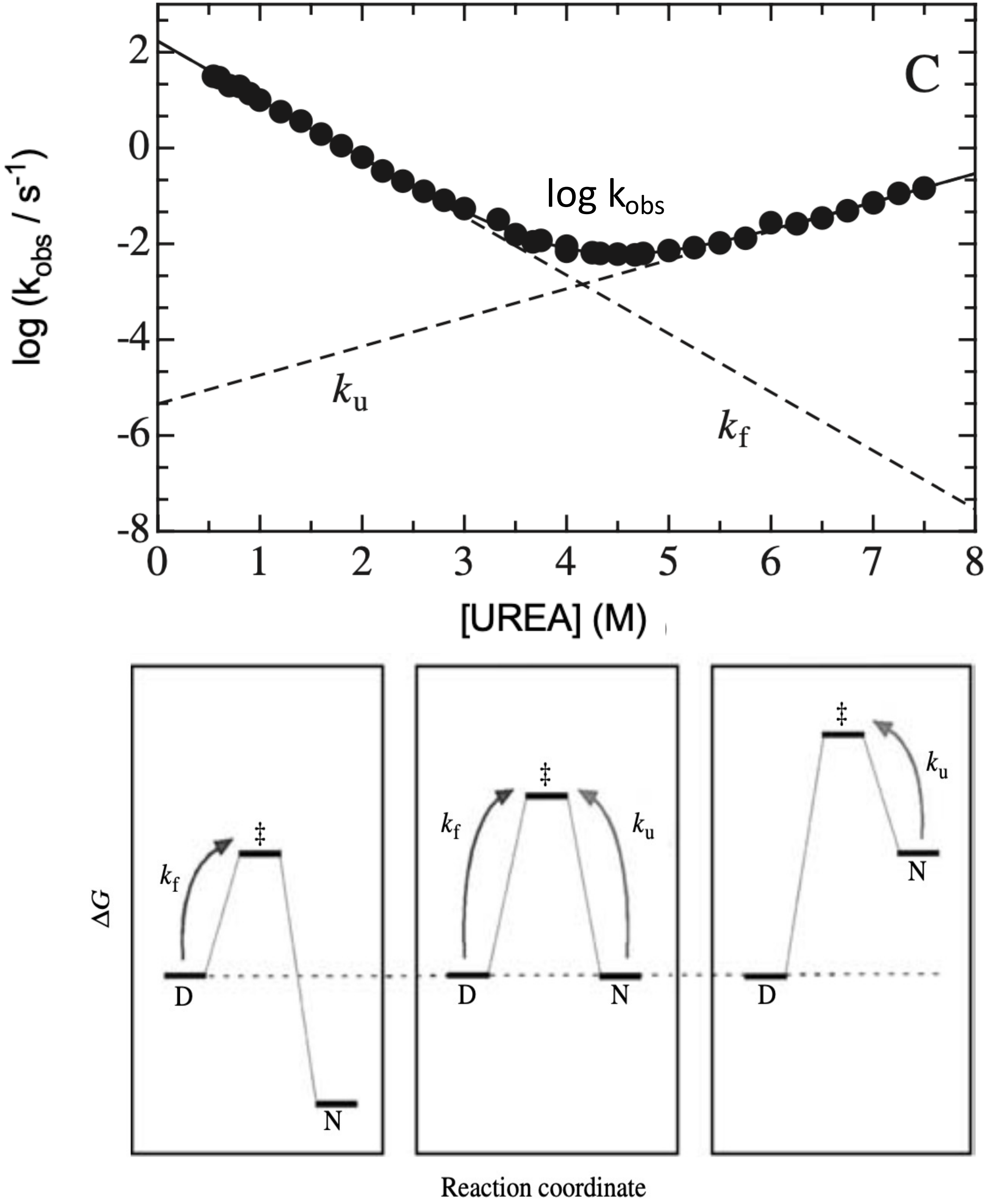
UNFOLDING
(native protein vs denaturant)

REFOLDING
(unfolded protein vs refolding buffer)

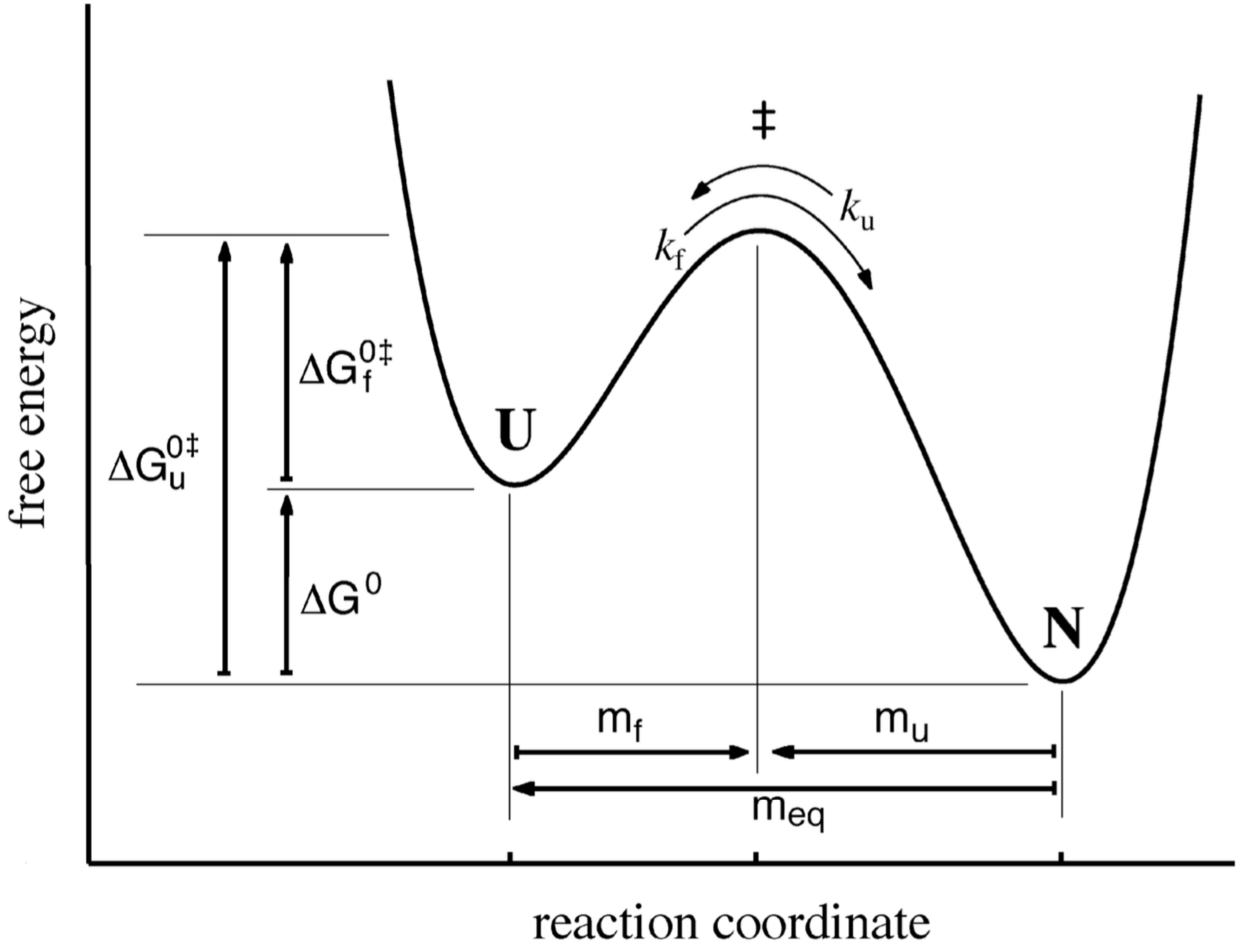
$$y = Aexp^{-k_{obs}t} + c$$

$$U \underset{k_u}{\overset{k_f}{\rightleftharpoons}} N$$

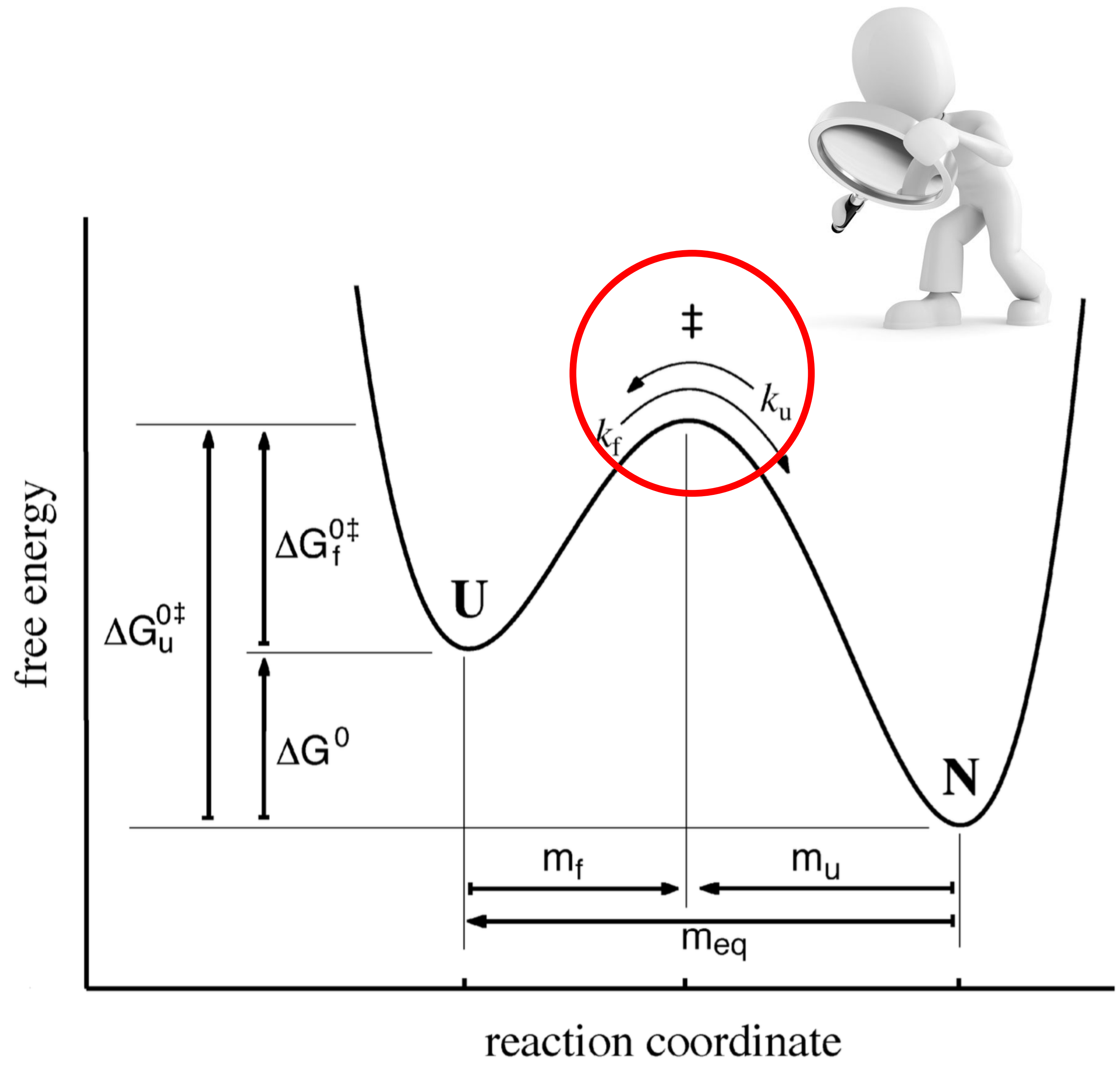
$$k_{obs} = k_f + k_u$$

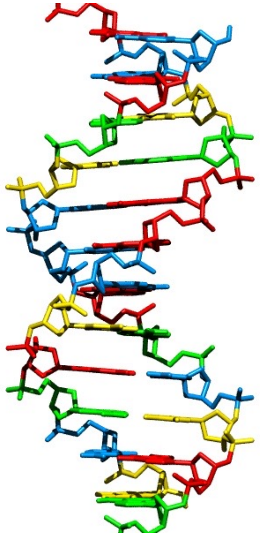


$$k_{obs} = k_f \exp\left(-m_f[urea]/RT\right) + k_u \exp\left(m_u[urea]/RT\right)$$



$$\Delta G^0_{D-N} = -RT\ln\left(\frac{k_F}{k_U}\right) \qquad m_{D-N} = m_F + m_U$$


‡
k_u
k_f
$\Delta G_f^{0\ddagger}$
$\Delta G_u^{0\ddagger}$
ΔG^0
U
N
free energy
m_f
m_u
m_{eq}
reaction coordinate



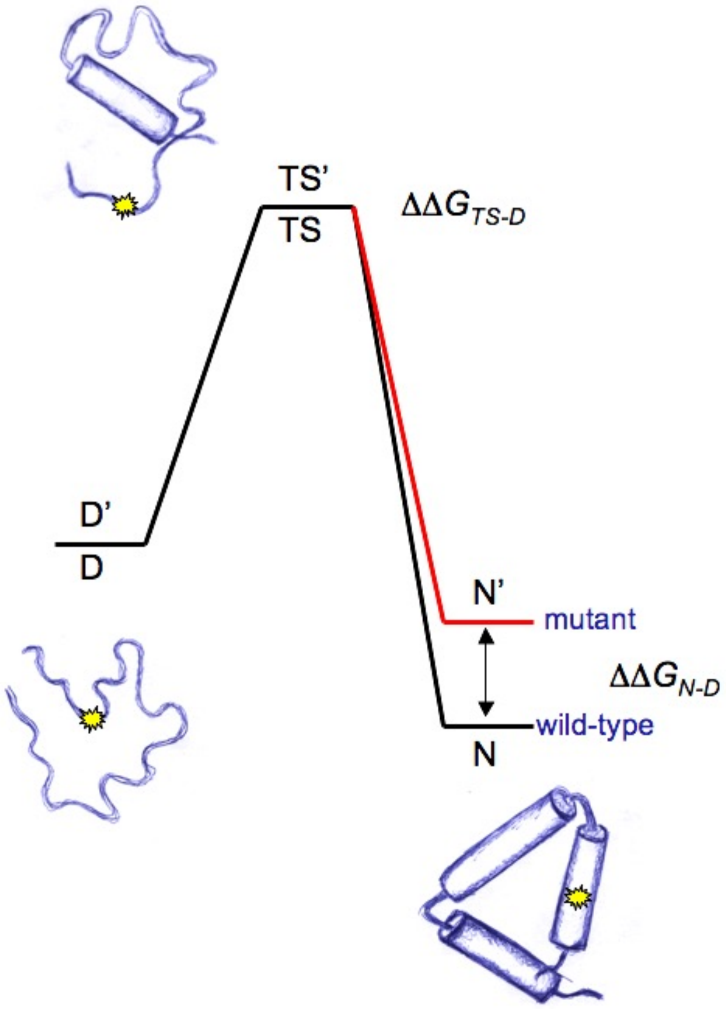
+

Mutagenesis

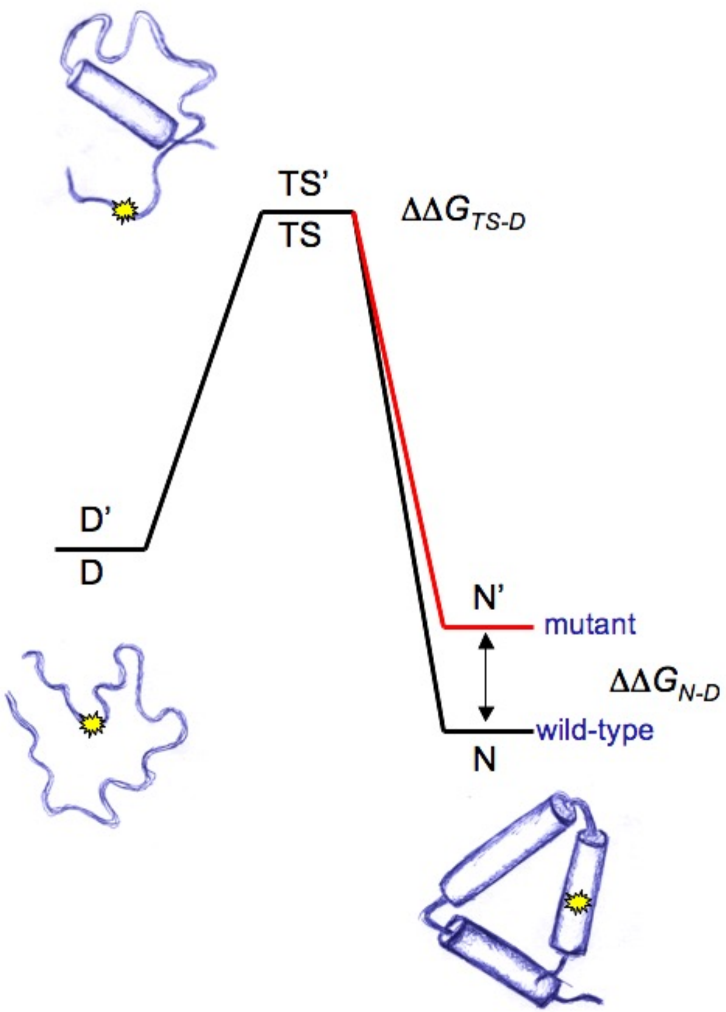
Folding kinetics

THE Φ-VALUE ANALYSIS

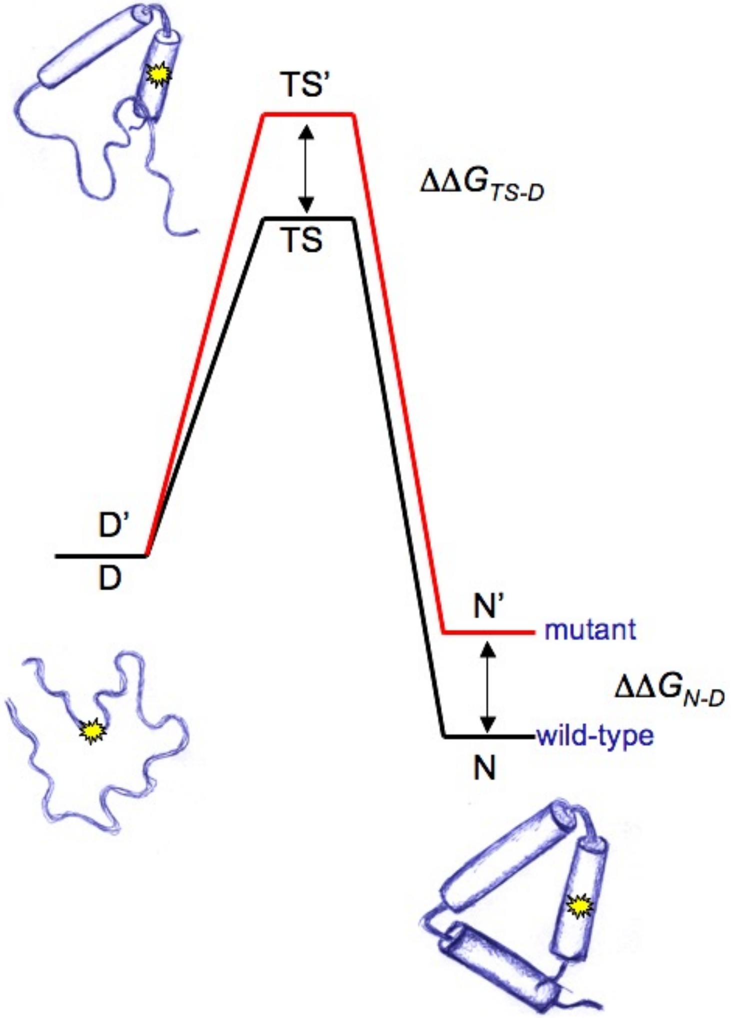
Φ-value



Φ-value

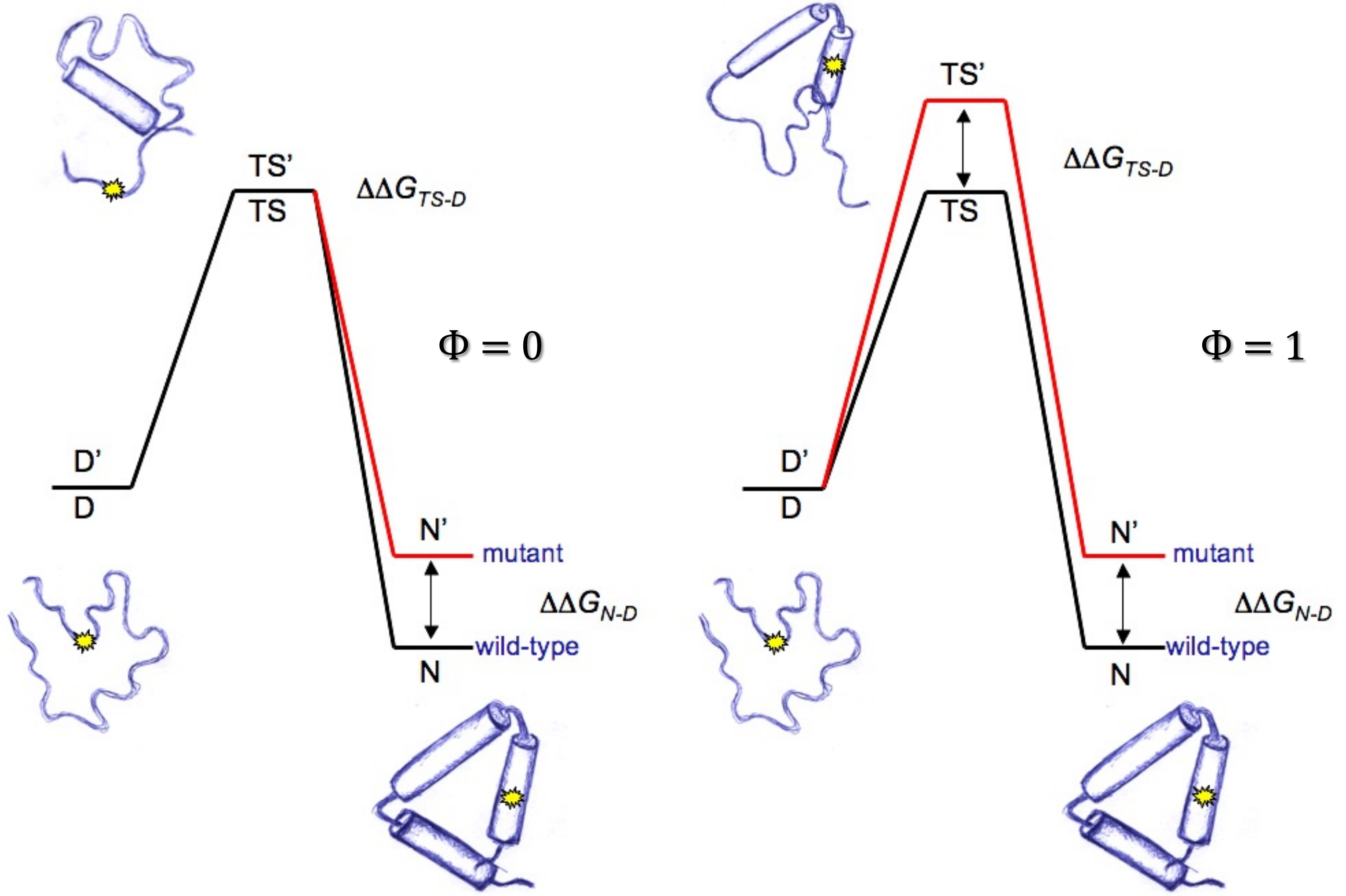


TS'
TS
$\Delta\Delta G_{TS\text{-}D}$
D'
D
N'
mutant
$\Delta\Delta G_{N\text{-}D}$
wild-type
N



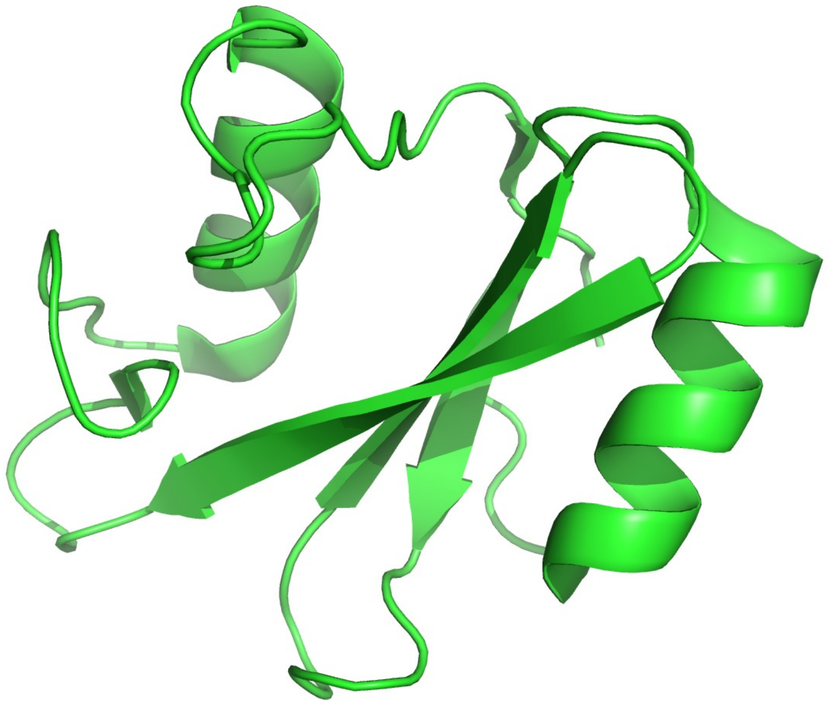
TS'
$\Delta\Delta G_{TS\text{-}D}$
TS
D'
D
N'
mutant
$\Delta\Delta G_{N\text{-}D}$
wild-type
N

Φ-value



$$\Phi = \Delta\Delta G_{TS\text{-}D} \,/\, \Delta\Delta G_{N\text{-}D}$$

Any given globular domain…



FROM NUMBERS…

	$k_{\text{I-N}}$ (s^{-1})	$k_{\text{N-I}}$ (s^{-1})	$K_{\text{D-I}}$	$\Delta\Delta G_{\text{D-I}}$ (kcal·mol^{-1})	$\Delta\Delta G_{\text{TS2-N}}$ (kcal·mol^{-1})	$\Delta\Delta G_{\text{D-N}}$ (kcal·mol^{-1})	ϕ_{I}	ϕ_{TS}
WT	500 ± 30	1.80 ± 0.11	530 ± 70					
I11V	350 ± 20	3.11 ± 0.19	200 ± 30	0.59 ± 0.11	0.32 ± 0.05	1.12 ± 0.11	0.53 ± 0.11	0.72 ± 0.05
T12S	400 ± 30	2.11 ± 0.13	600 ± 90	−0.07 ± 0.12	0.09 ± 0.05	0.16 ± 0.02	[a]	[a]
V14A	660 ± 40	1.97 ± 0.13	520 ± 80	0.02 ± 0.12	0.05 ± 0.05	−0.09 ± 0.01	[a]	[a]
L19A	480 ± 30	3.38 ± 0.19	60 ± 10	1.26 ± 0.11	0.37 ± 0.05	1.66 ± 0.17	0.76 ± 0.10	0.78 ± 0.04
L21A	320 ± 20	4.10 ± 0.22	30 ± 5	1.72 ± 0.11	0.48 ± 0.05	2.48 ± 0.25	0.69 ± 0.08	0.81 ± 0.03
T22S	460 ± 20	2.20 ± 0.14	350 ± 50	0.26 ± 0.11	0.12 ± 0.05	0.42 ± 0.04	0.61 ± 0.28	0.72 ± 0.13
V25A	420 ± 20	4.00 ± 0.23	180 ± 20	0.66 ± 0.11	0.46 ± 0.05	1.23 ± 0.12	0.53 ± 0.11	0.62 ± 0.06
A31G	300 ± 20	6.18 ± 0.34	90 ± 10	1.06 ± 0.12	0.72 ± 0.05	2.10 ± 0.21	0.51 ± 0.08	0.66 ± 0.04
T42S	500 ± 40	1.34 ± 0.08	60 ± 10	1.33 ± 0.12	−0.17 ± 0.05	1.16 ± 0.12	1.15 ± 0.15	1.14 ± 0.05
V45A	220 ± 20	1.20 ± 0.07	10 ± 2	2.18 ± 0.12	−0.23 ± 0.05	2.45 ± 0.24	0.89 ± 0.10	1.09 ± 0.02
A50G	800 ± 40	1.50 ± 0.09	170 ± 20	0.69 ± 0.12	−0.11 ± 0.05	0.31 ± 0.03	[a]	[a]
T52S	430 ± 20	2.22 ± 0.13	220 ± 30	0.52 ± 0.11	0.12 ± 0.05	0.74 ± 0.07	0.71 ± 0.1	0.83 ± 0.07
I54V	790 ± 60	1.79 ± 0.11	70 ± 10	1.16 ± 0.12	0.00 ± 0.05	0.89 ± 0.09	1.30 ± 0.19	1.00 ± 0.06
I56V	870 ± 60	2.17 ± 0.14	240 ± 30	0.49 ± 0.12	0.11 ± 0.05	0.28 ± 0.03	[a]	[a]
T59S	410 ± 20	2.83 ± 0.17	470 ± 70	0.08 ± 0.12	0.26 ± 0.05	0.47 ± 0.05	0.17 ± 0.25	0.44 ± 0.12
L65A	390 ± 20	1.70 ± 0.10	160 ± 20	0.70 ± 0.11	−0.03 ± 0.05	0.83 ± 0.08	0.85 ± 0.16	1.04 ± 0.06
A72G	420 ± 20	2.15 ± 0.13	250 ± 30	0.46 ± 0.12	0.10 ± 0.05	0.67 ± 0.07	0.68 ± 0.19	0.84 ± 0.08
T73S	650 ± 40	1.76 ± 0.11	320 ± 50	0.30 ± 0.12	−0.01 ± 0.05	0.13 ± 0.01	[a]	[a]
L74A	410 ± 40	3.00 ± 0.16	9 ± 2	2.40 ± 0.12	0.30 ± 0.05	2.82 ± 0.38	0.85 ± 0.10	0.89 ± 0.02
A75G	430 ± 20	2.10 ± 0.13	290 ± 40	0.37 ± 0.12	0.09 ± 0.05	0.56 ± 0.06	0.66 ± 0.22	0.84 ± 0.01
V78A	420 ± 30	15.29 ± 0.82	30 ± 5	1.69 ± 0.12	1.24 ± 0.05	3.06 ± 0.31	0.55 ± 0.07	0.59 ± 0.04
L88A	430 ± 30	32.96 ± 1.92	300 ± 60	0.35 ± 0.14	1.69 ± 0.05	2.15 ± 0.21	0.16 ± 0.07	0.21 ± 0.08
V95A	600 ± 40	4.91 ± 0.30	220 ± 30	0.52 ± 0.12	0.58 ± 0.05	1.01 ± 0.10	0.51 ± 0.13	0.42 ± 0.08
I96V	520 ± 30	2.30 ± 0.15	300 ± 40	0.34 ± 0.12	0.14 ± 0.05	0.47 ± 0.05	0.72 ± 0.26	0.69 ± 0.12

…TO STRUCTURE!

	$k_{I\text{-}N}$ (s^{-1})	$k_{N\text{-}I}$ (s^{-1})	$K_{D\text{-}I}$	$\Delta\Delta G_{D\text{-}I}$ (kcal·mol^{-1})	$\Delta\Delta G_{TS2\text{-}N}$ (kcal·mol^{-1})	$\Delta\Delta G_{D\text{-}N}$ (kcal·mol^{-1})	ϕ_I	ϕ_{TS}
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V45A	220 ± 20	1.20 ± 0.07	10 ± 2	2.18 ± 0.12	−0.23 ± 0.05	2.45 ± 0.24	0.89 ± 0.1	1.09 ± 0.02
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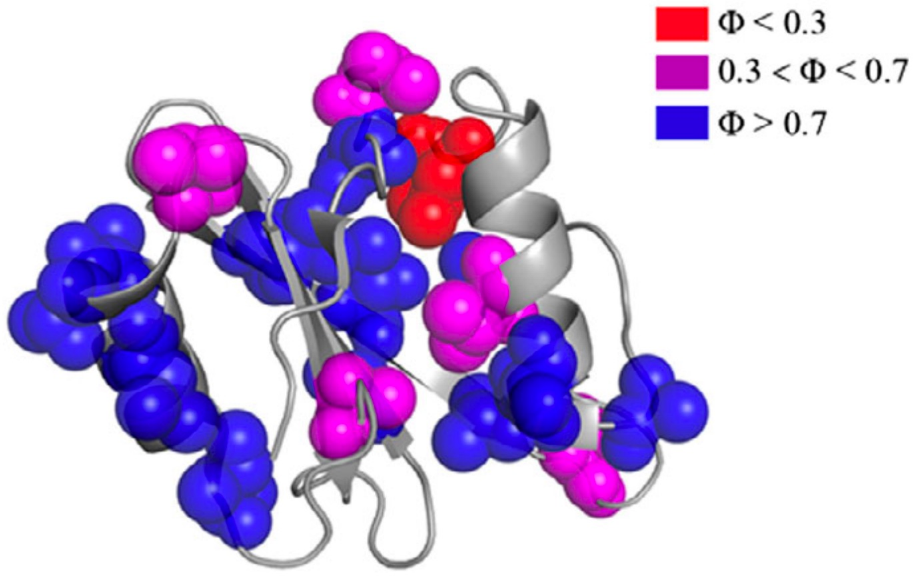
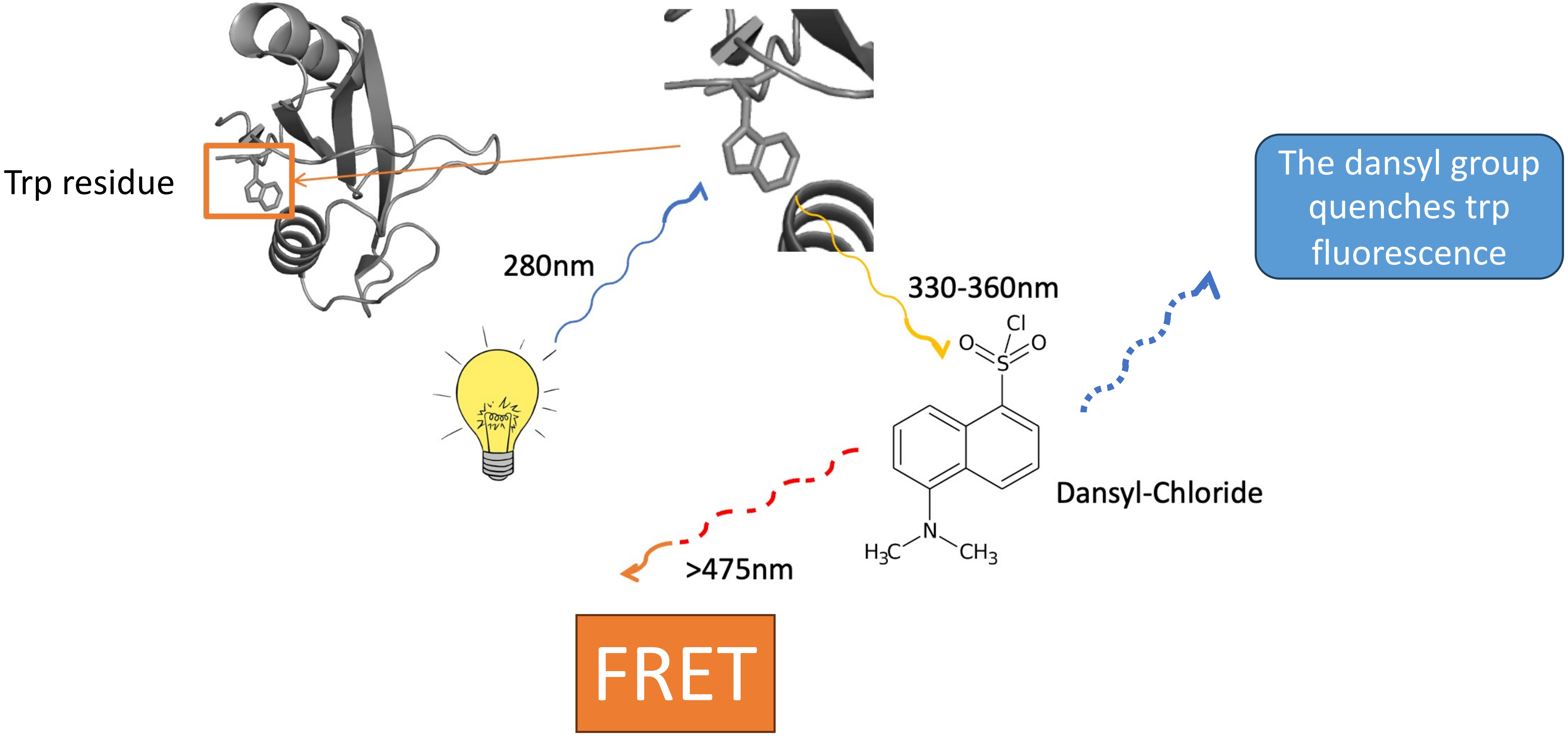
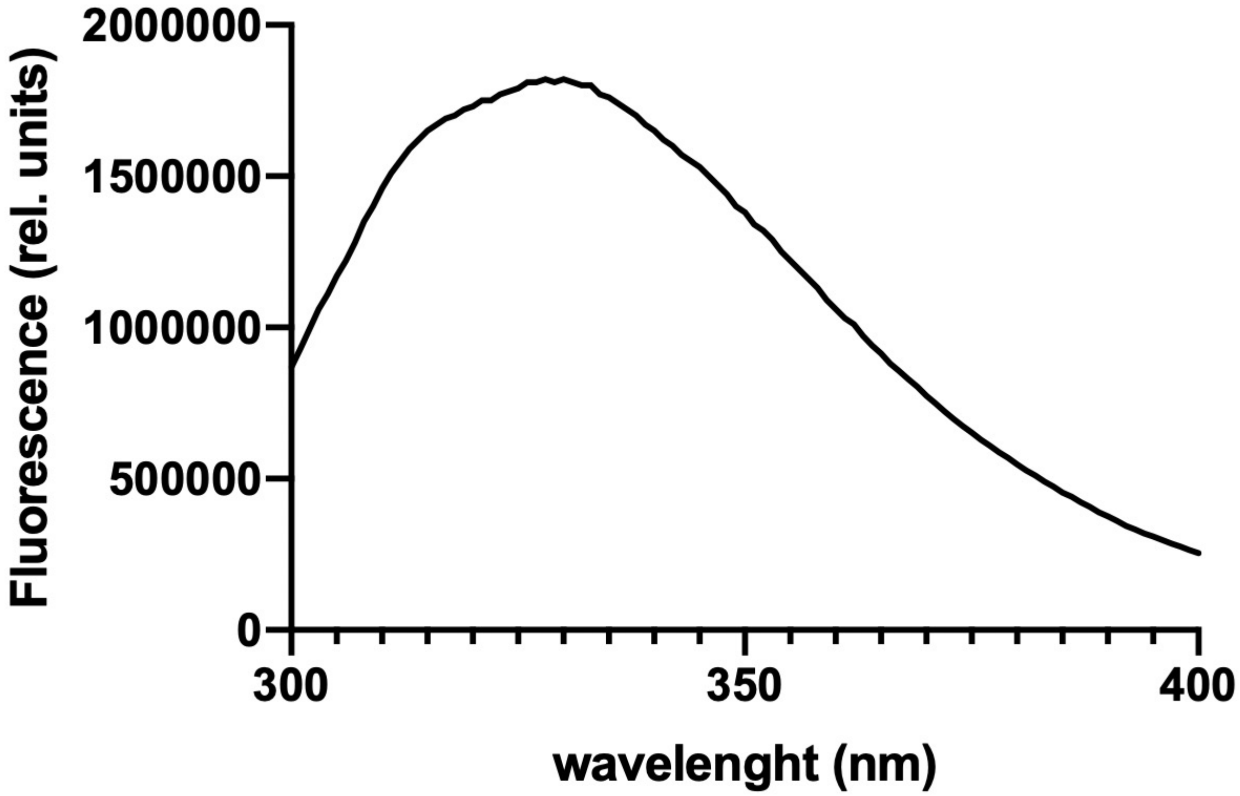


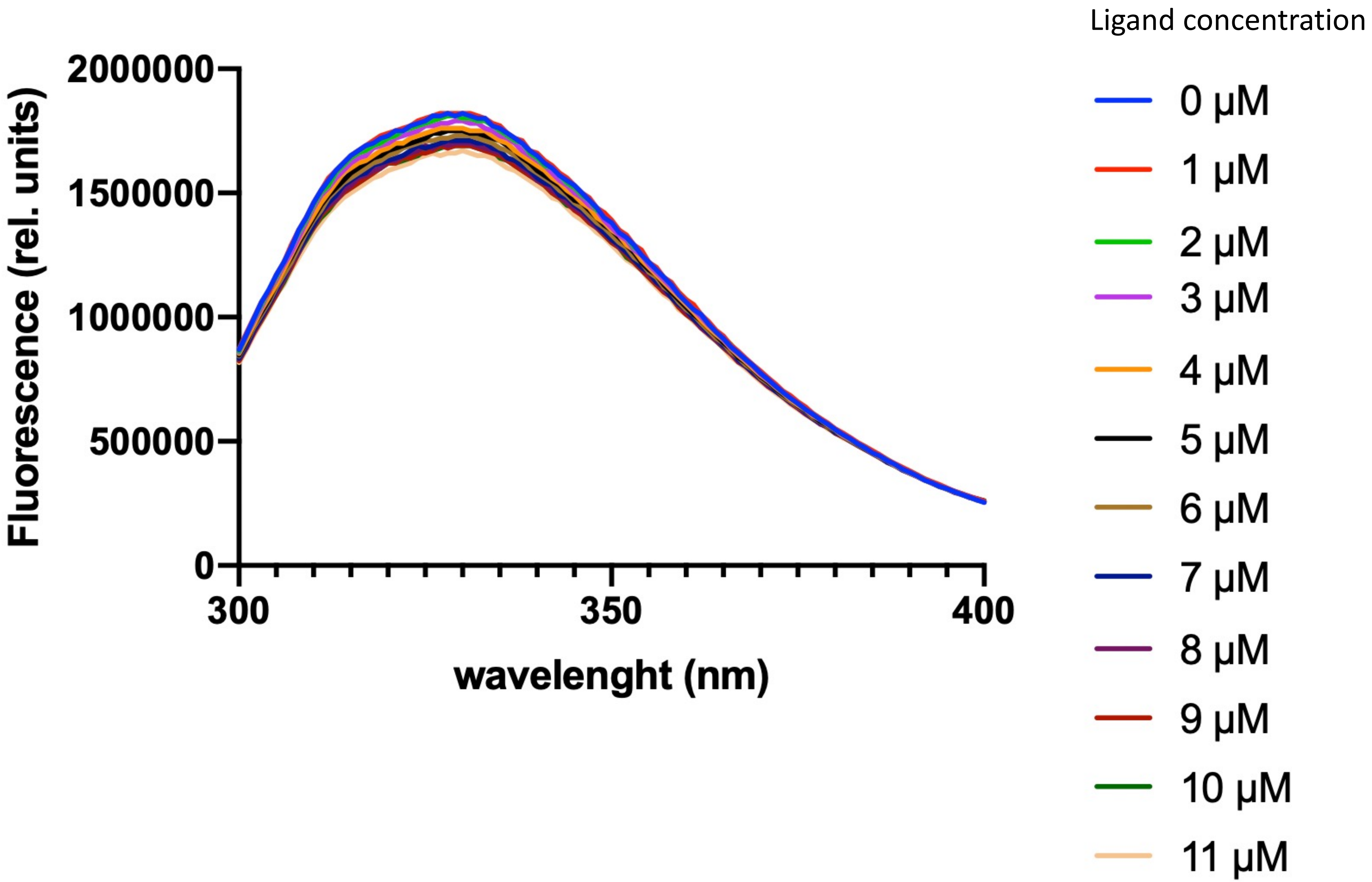
Transition State

Protein-protein interactions kinetics

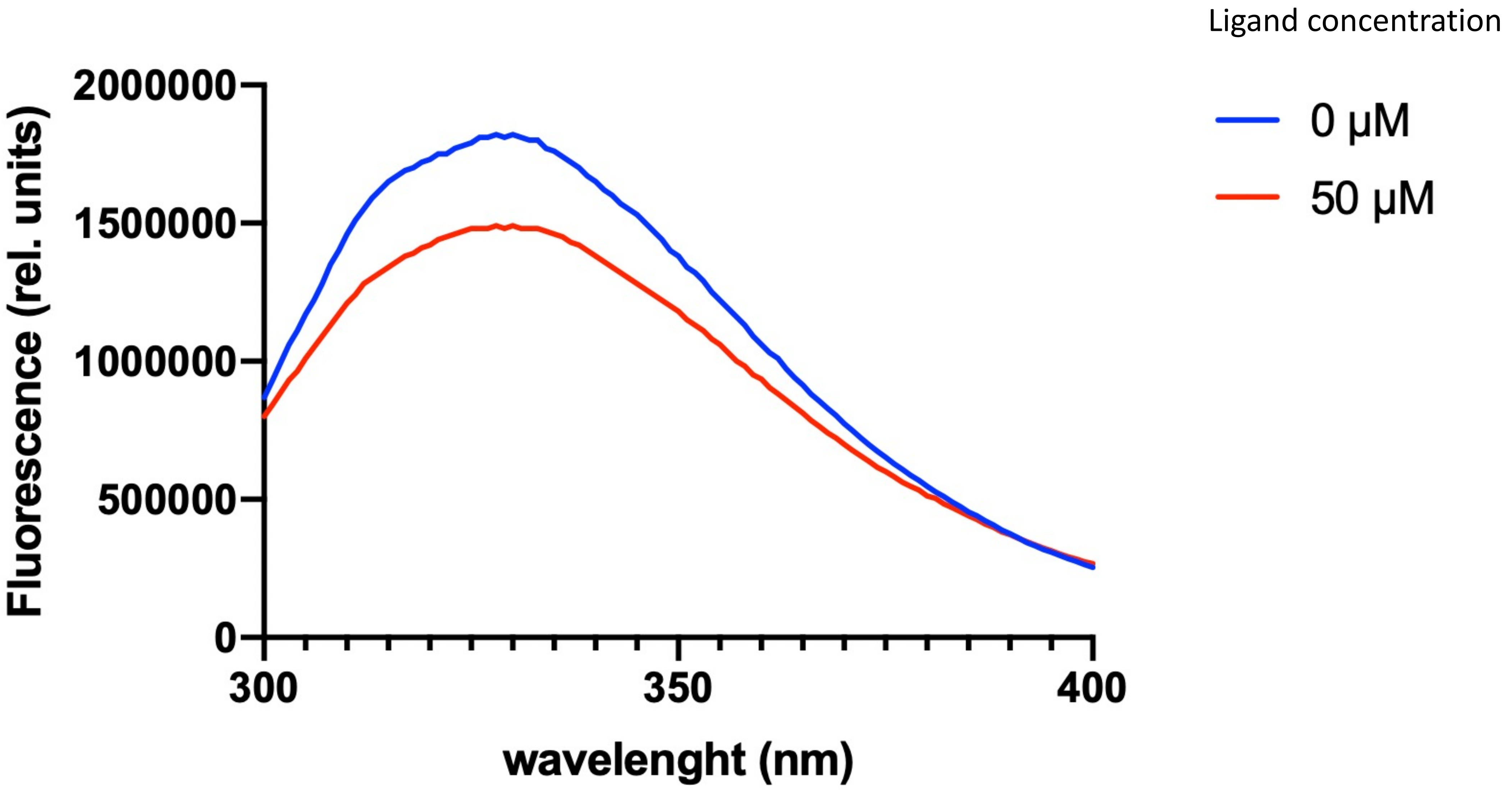

Trp residue
280nm
330-360nm
Cl
O
O
S
N
H_3C
CH_3
Dansyl-Chloride
The dansyl group quenches trp fluorescence
>475nm
FRET



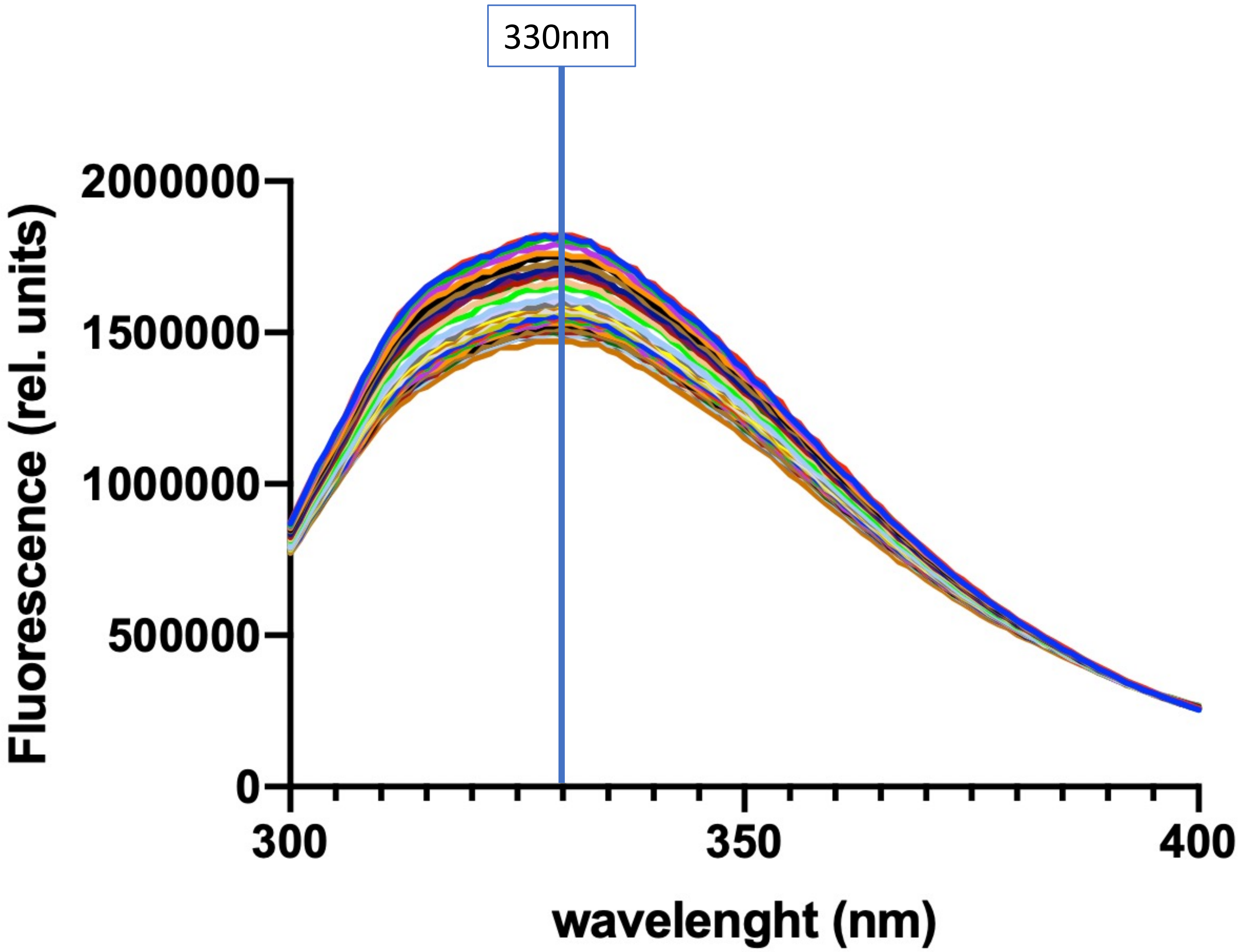
Excitation wavelength = 280nm
Emission recorded between 300nm and 400nm

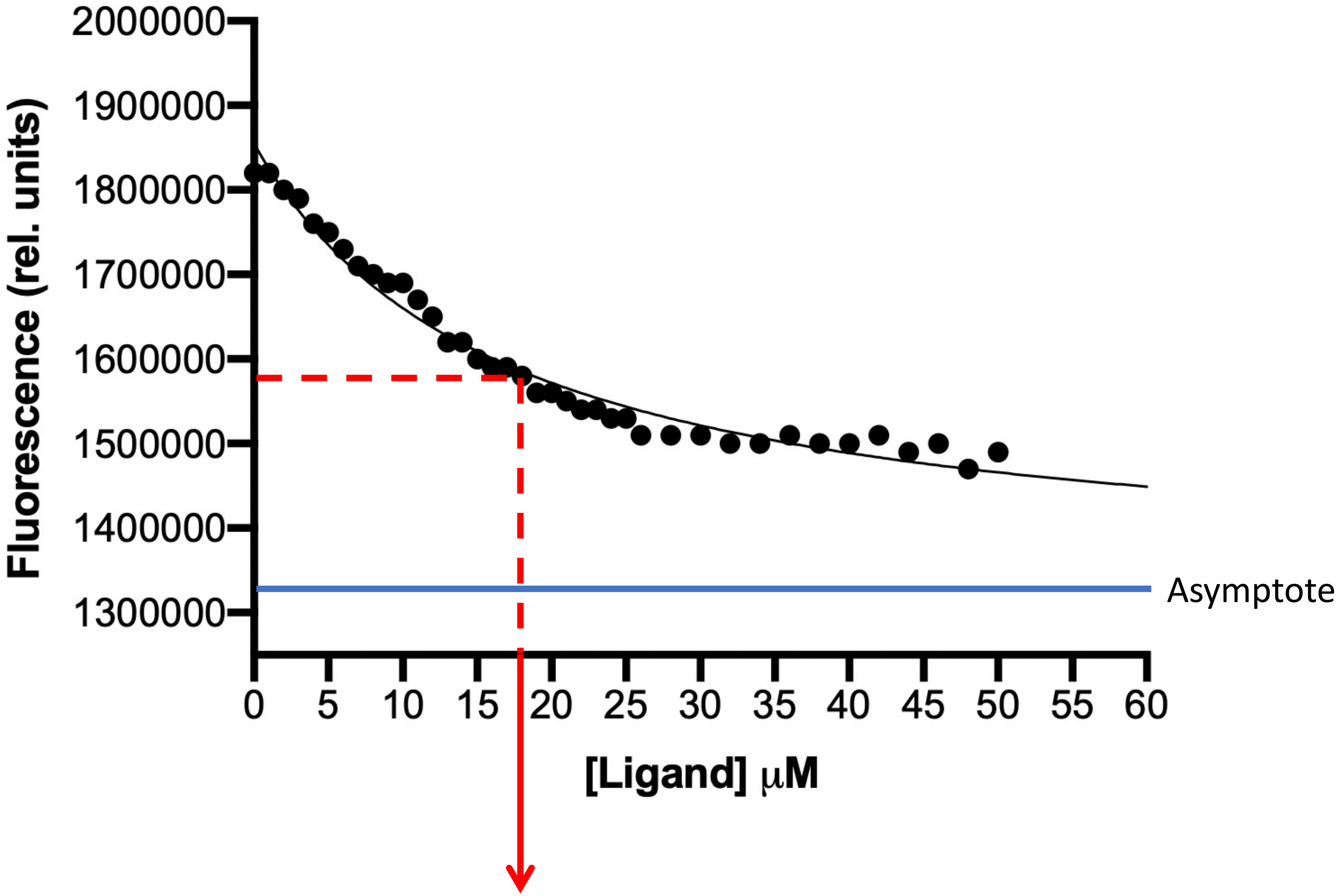


Ligand concentration
0 μM
1 μM
2 μM
3 μM
4 μM
5 μM
6 μM
7 μM
8 μM
9 μM
10 μM
11 μM
Fluorescence (rel. units)
2000000
1500000
1000000
500000
0
300
350
400
wavelenght (nm)



Trp fluorescence decreases as the [ligand] increases because of the quenching effect of dansyl group


330nm
Fluorescence (rel. units)
2000000
1500000
1000000
500000
0
300
350
400
wavelenght (nm)



$$Y = \frac{[L]}{K_d + [L]}$$

K_D = 16.6 ± 0.2 μM

Binding reactions are BIMOLECULAR REACTIONS

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL$$

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$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL$$

$$\frac{\partial[PL]}{\partial t} = k_{on}[P][L] - k_{off}[PL]$$

The solution to this is complicated and we need to approximate

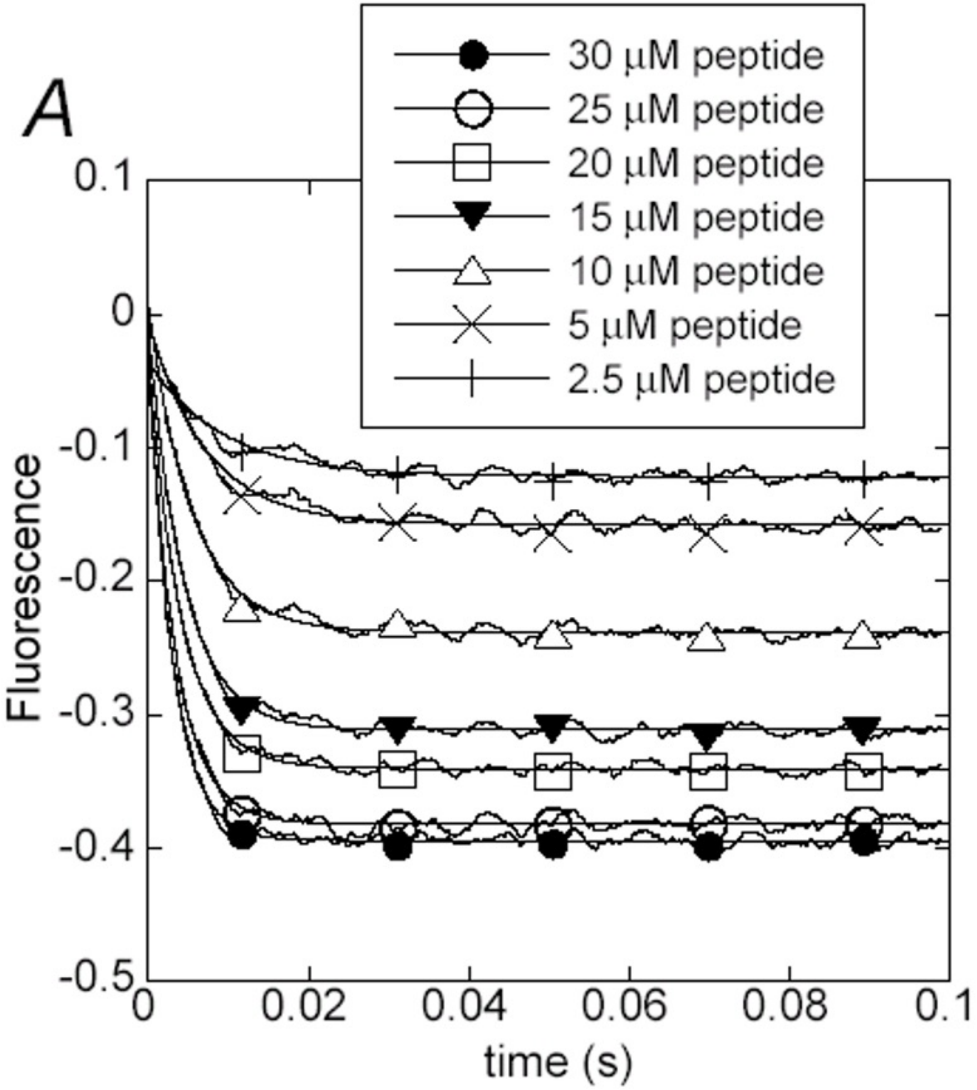
Binding reactions are BIMOLECULAR REACTIONS

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL$$

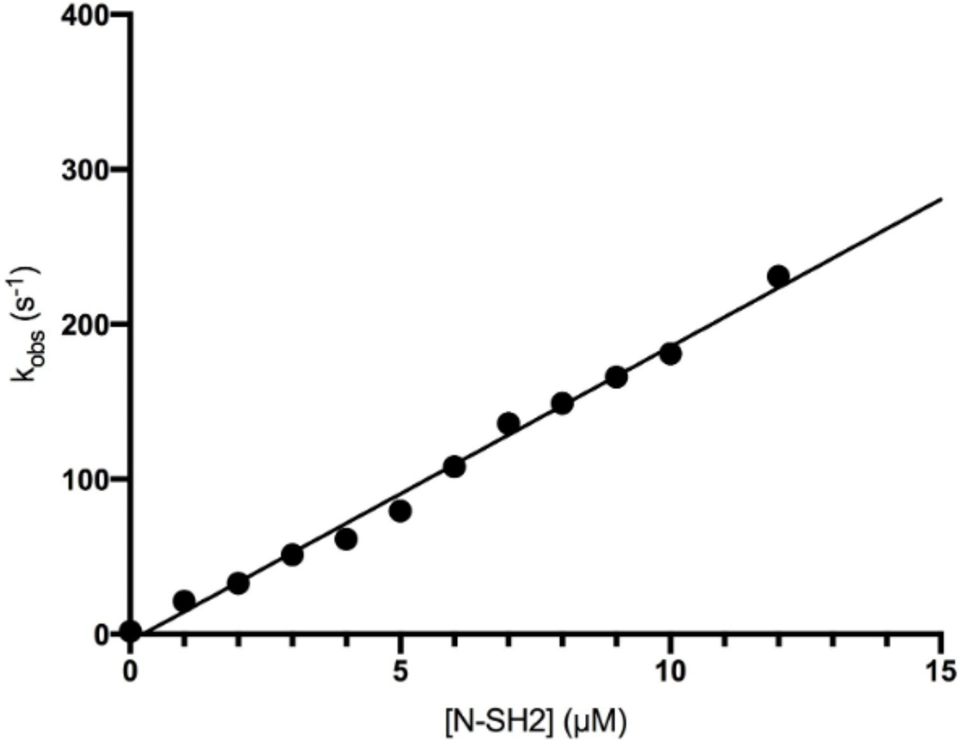
$$\frac{\partial [PL]}{\partial t} = k_{on}[P][L] - k_{off}[PL]$$

Kinetic experiments in PSEUDO-FIRST ORDER conditions

If [L] >> [P] its concentration after time *t* will be equal to $[L]_0$

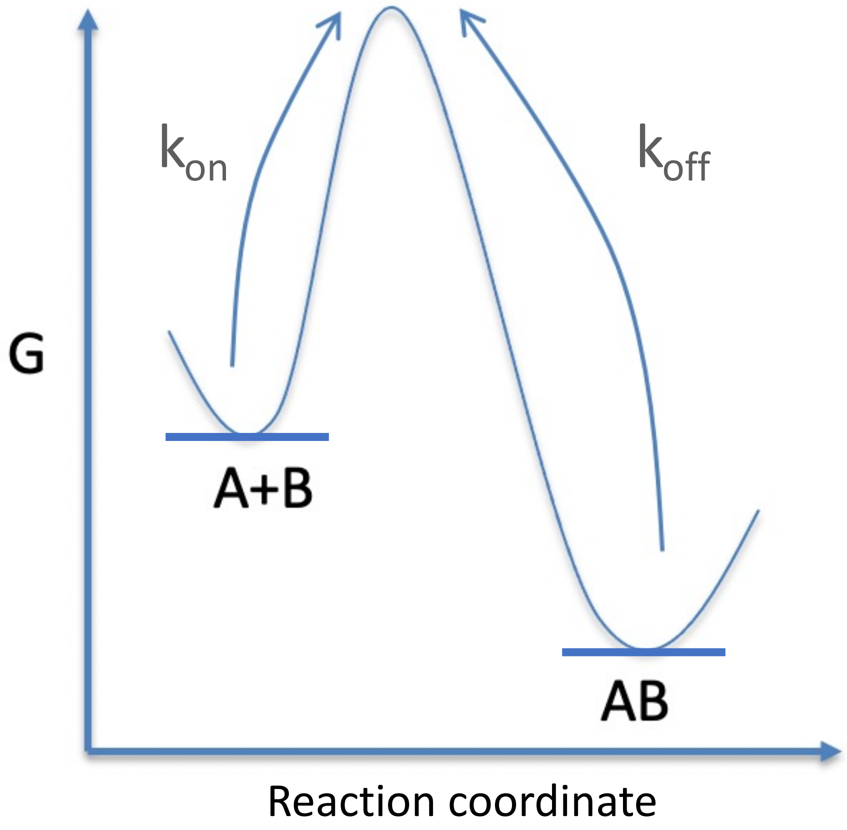


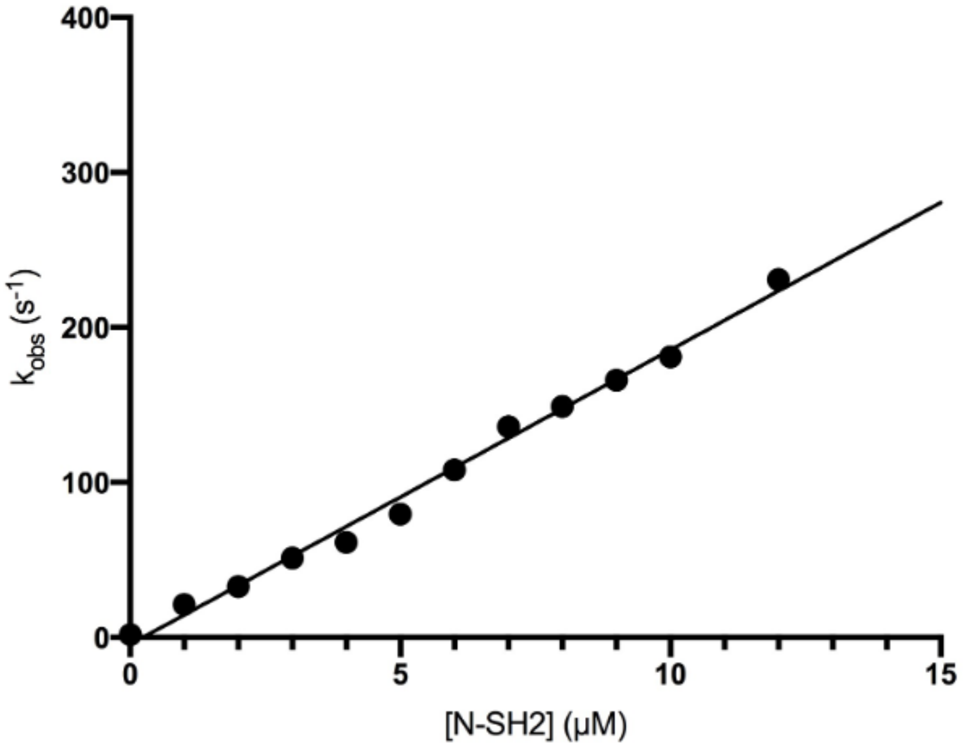
$$y = A * \exp(-k_{obs}*t) + c$$



$$k_{obs} = k_{on}[L] + k_{off}$$

$$K_D = \frac{k_{off}}{k_{on}}$$





$$k_{obs} = k_{on}[L] + k_{off}$$

$$K_D = \frac{k_{off}}{k_{on}}$$