Problem session

210417 Koichi Kamiya

Please read the description below and answer the questions

Dissociation constant (k_D) is important value to understand the protein-protein, protein-small molecule, etc interaction. There are two approaches to obtain k_D value, which are equilibrium approach and kinetic approach. Following shows some examples.

Question 1

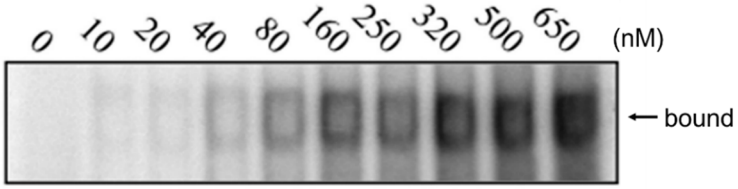
Here shows one example of equilibrium approach (describing two molecules as A and B, and describing concentration of each molecule as [A] and [B]). **Please calculate k_D value and explain what you need to concern when you obtain k_D value**

conditions

A: $[A]_{tot}$ = 125 nM, $[A]_{tot}$ is the concentration of A at the start (t = 0)

B: $[B]_{tot}$ = 0 nM, 10 nM, 20 nM, 40 nM, 80 nM, 160 nM, 250 nM, 320 nM, 500 nM, and 650 nM

analysis: native PAGE



*Gel image was taken from Bhardwaj, A.; Myers, M. P.; Buratti, E.; Baralle, F. E. *Nucleic Acids Research*, **2013**, *41*, 5062.

$[B]_{tot}$ (nM)	650	500	320	250	160	80	40	20	10	0
intensity (bound)	198	196	194	180	147	98	58	30	12	0

*Intensities were virtually set, independent of the original paper.

Question 2: kinetic approach (describing two molecules as C and D, and describing concentration of each molecule as [C] and [D]).

$$C + D \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} CD$$

(i) **Please calculate association rate constant (k_{on}) from the following**

conditions

C: [C]<<[D], $[C]_{tot}$ = constant, $[C]_{tot}$ is the concentration of C at the start (t = 0)

D: $[D]_{tot}$ = 210 pM, 340 pM, 480 pM, 770 pM, 1.2 nM, and 2.0 nM

Time of detection: 0 min, 5 min, 10 min, 20 min, 30 min, 40 min, and 50 min

Experiment was conducted using radioisotope labelled D (details are not mentioned here)

210 pM							
time (min)	0	5	10	20	30	40	50
bind (%)	0	6	12	23	31	34	35

340 pM							
time (min)	0	5	10	20	30	40	50
bind (%)	0	11	21	36	47	51	50

480 pM							
time (min)	0	5	10	20	30	40	50
bind (%)	0	16	30	51	61	64	63

770 pM							
time (min)	0	5	10	20	30	40	50
bind (%)	0	27	45	67	78	80	80

1.2 nM							
time (min)	0	5	10	20	30	40	50
bind (%)	0	39	66	87	93	94	93

2.0 nM							
time (min)	0	5	10	20	30	40	50
bind (%)	0	65	88	98	101	101	100

*Percentage of binding was normalized by the binding at 2.0 nM, 50 min as 100%

At the begining of accosiation experiment ($0 \leq t \leq 10$ min on this problem),
the following equation can be set

$$\frac{d[CD]}{dt} = k_{on}[C][D]$$

When [C]<<[D], [D] = constant. Therefore, if you set $k_{on}[D] = k_{obs}$, the following equation is obtained.

$$[CD] = [CD_{max}](1-e^{-k_{obs}t})$$ $[CD_{max}]$ is specific binding reaching to equilibrium

(ii) **Please calculate dissociation rate constant (k_{off}) from the following**

conditions
binding CD: prepared before the experiment. C and radioisotope labelled D were mixed 1 h before the experiment. percentage of $[CD]_0$ is set as 100% (t = 0)
start of the experiment: When 10 μM non-labelled D was added, the experiment started.

time (min)	0	40	60	90	120	180	240	300	360	420
binding (%)	100	88	81	72	64	52	43	36	31	27

At the dissosiation experiment, the following equation can be set

$$\frac{d[CD]}{dt} = -k_{off}[CD]$$

Therefore, the following equation is obtained

$$[CD] = [CD]_0 e^{-k_{off}t}$$

(iii) (a) **Please calculate k_D value from k_{on} and k_{off}**

When an experiment of equilibrium approach (conditions are shown below) was conducted, k_D value was calculated as 200 pM which is different from that of kinetic approach.

(b) **Please explain why these are different and what we need to do to obtain accurate k_D value**

conditions
C: $[C]_{tot}$ = constant, $[C]_{tot}$ is the concentration of C at the start (t = 0)
D: $[D]_{tot}$ = various concentration from 50 pM to 5 nM
detection time: 2 h later

Problem session (answer)

210417 Koichi Kamiya

Topic: Interaction analysis

Question 1 was created from Bhardwaj, A.; Myers, M. P.; Buratti, E.; Baralle, F. E. *Nucleic Acids Research*. **2013**, *41*, 5062.
Question 2 was created from Sullivan, S. K.; Hoare, S. R. J.; Fleck, B. A.; Zhu, Y.; Heise, C. E.; Struthers, R. S.; Crowe, P. D. *Biochem Pharmacol.* **2006**, *72*, 838.

Dissociation constant (k_D) is important value to understand the protein-protein, protein-small molecule, etc interaction. There are two approaches to obtain k_D value, which are equilibrium approach and kinetic approach. Following shows some examples.

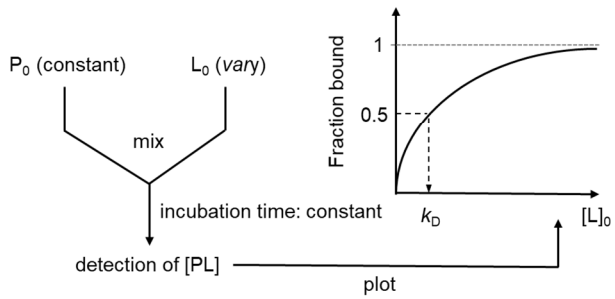
How to measure k_D?

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

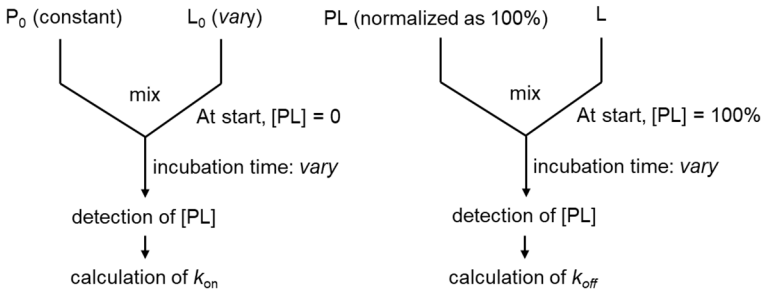
when $k_{on}[P][L] = k_{off}[PL]$

$$k_D = k_{off}/k_{on} = \frac{[P][L]}{[PL]}$$

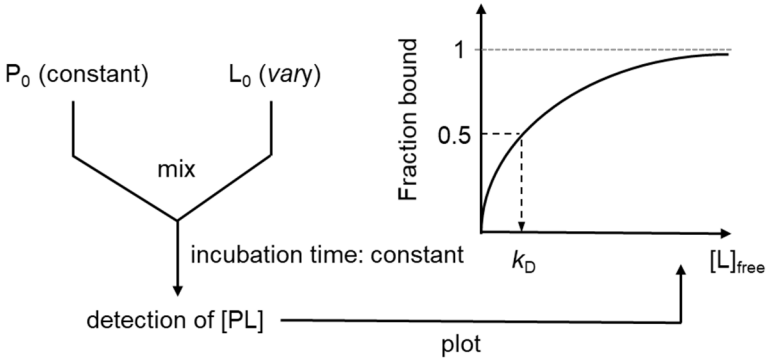
equilibrium (thermodynamic) approach



kinetic approach



Question 1 equilibrium (thermodynamic) approach



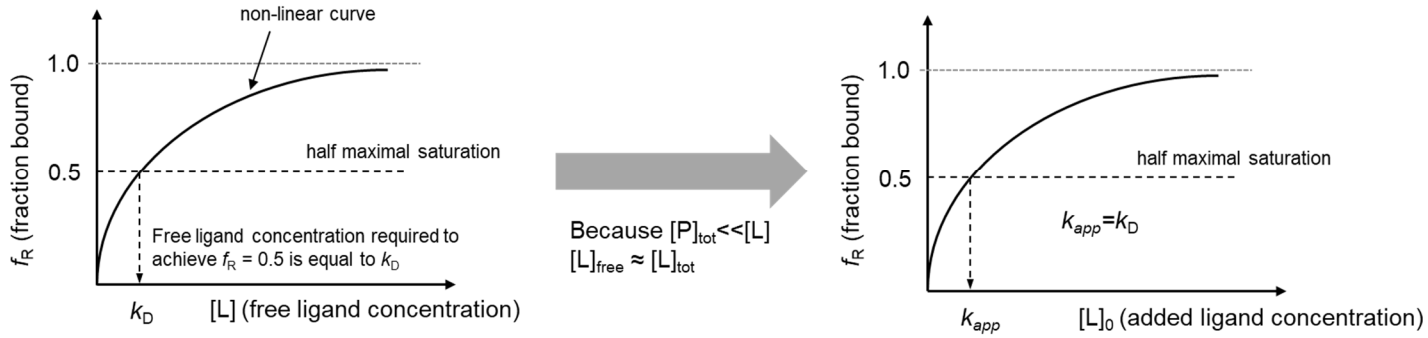
When fraction rate is 0.5, $[P]_{free} = [PL]$.
From $k_D = [P][L]/[PL]$, $k_D = [L]_{free}$

・The most common approach to measuring affinity is to vary the concentration of one component, while keeping the concentration of the other binding partner constant. [P] needs to be constant, while [L] needs to be varied started from lower then k_D and ended at higher than k_D.

There are three conditions to obtain k_D value according to the relation between k_D and concentration of the constant.

1. ***Condition A*** *($[P]_0 < 0.1\ k_D$)*

In this condition, the concentration of one component (P) is a lot below the k_D value. In this case, the concentration of variable component (L) around k_D is much higher than the concentration of P ($[L] >> [P]_0$). This means the concentration of L which binds to P is very small. Therefore, [L] can be approximated as $[L]_0$, which is the concentration of the added L.



At condition A, the following formula holds:

$$k_{on}[P][L] = k_{off}[PL]$$

$$\frac{k_{off}}{k_{on}} = k_D = \frac{[P][L]}{[PL]}$$

$$k_D = \frac{([P]_{tot} - [PL])[L]}{[PL]} \quad when\ [P] = [P]_{tot} - [PL]$$

$$[PL] = \frac{[P]_{tot}[L]}{k_D + [L]}$$

$$[PL] = \frac{[P]_{tot}[L]_{tot}}{k_{app} + [L]_{tot}} \quad when\ [L] \approx [L] + [PL] = [L\}_{tot}$$

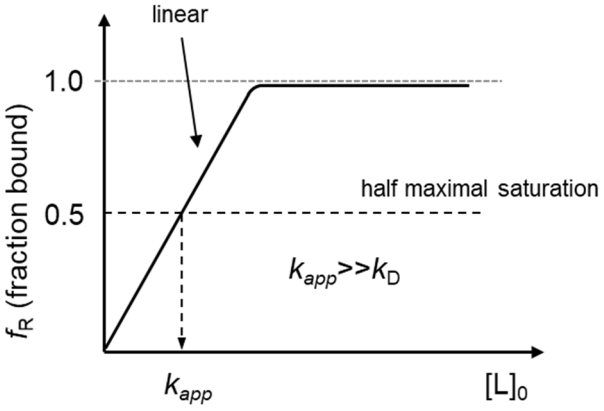
The above equation can be converted to log-logistic functions:

$$[PL] = \frac{[P]_{tot}}{\frac{k_{app}}{[L]_{tot}} + 1} = [P]_{tot}/\{1 + \exp\big(ln\big(k_{app}\big) - ln([L]_{tot})\big\}$$

The k_{app} value calculated by the above equation equals k_D.

2. ***Condition B*** *($[P]_0 > 100\ k_D$)*

In this condition, the concentration of the constant component (P) is much larger than k_D. Therefore, all added L binds to P until no more free P left. In this case, the concentration of P which gives half binding does not equal to the k_D. Moreover, at high concentration of P, the shape of the plotted chart become linear, and k_{app} value is simply half of the concentration of P.



*3. **condition C** (0.1 k_D < $[P]_0$ < 100 k_D)*

In this condition, the concentration of P is not low enough to be able to ignore the concentration of bounded L. Thus [L] cannot be approximated as $[L]_{tot}$. In limiting situations, $[L]_{free}$ can be calculated. However, in other situations, a more complex quadratic binding equation can be considered:

$$\frac{d[PL]}{dt} = k_{on}[P][L] - k_{off}[PL]$$

$$[P] = [P]_{tot} - [PL], \qquad [L] = [L]_{tot} - [PL]$$

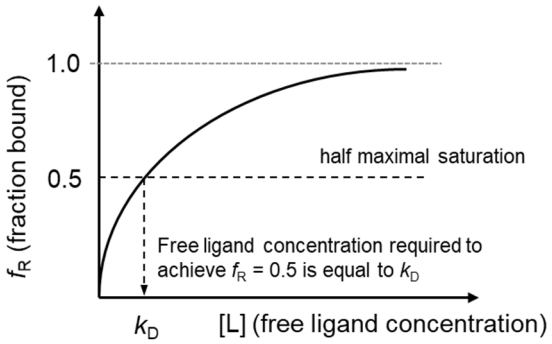
$$\frac{d[PL]}{dt} = k_{on}([P]_{tot} - [PL])([L]_{tot} - [PL]) - k_{off}[PL]$$

$$At\ equilibrium, \frac{d[PL]}{dt} = 0,\ \ k_D = \frac{k_{off}}{k_{on}}$$

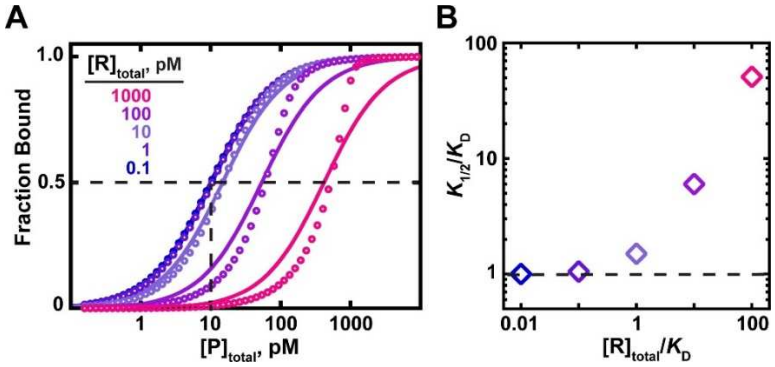
$$[PL]^2 - ([P]_{tot} + [L]_{tot} + k_D)[PL] + [P]_{tot}[L]_{tot} = 0$$

$$[PL] = \frac{([P]_{tot} + [L]_{tot} + k_D) - \sqrt{([P]_{tot} + [L]_{tot} + k_D)^2 - 4[P]_{tot}[L]_{tot}}}{2}$$

Several techniques (for example, isothermal titration calorimetry (ITC)) use this quadratic binding equation for data fitting. Up to 100-fold excess can be useful for data with minimal noise.



Example: equation of condition A (actual k_D = 10 pM)[1)]



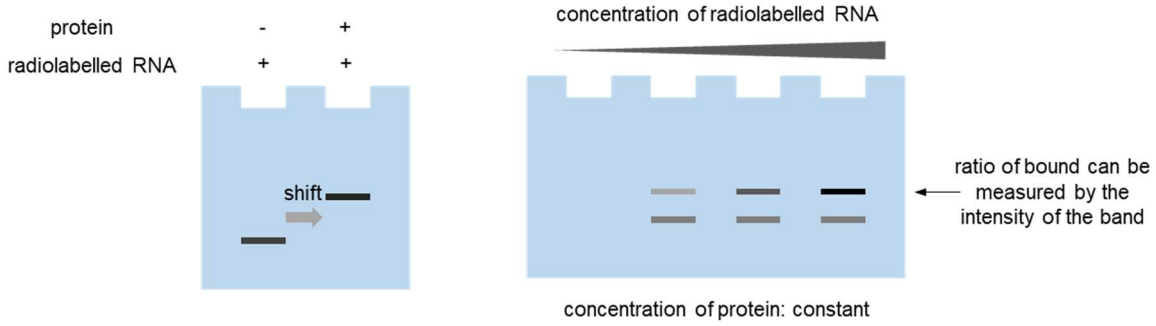
(A) The simulation data ranging 0.1 pM ≤ $[R]_{tot}$ ≤ 1000 pM. When $[R]_{tot}$ ≤ 1 pM, which 10-fold below the k_D, the data are reliable, and $k_{1/2}$ (apparent k_D) equals to actual k_D. At higher concentration of R, the data become unreliable, and $k_{1/2}$ (apparent k_D) becomes much higher than actual k_D. (B)The relationship between $k_{1/2}$ (apparent k_D) and actual k_D.

Answer

Quantitative Electrophoresis mobility shift assay (EMSA)[6)]

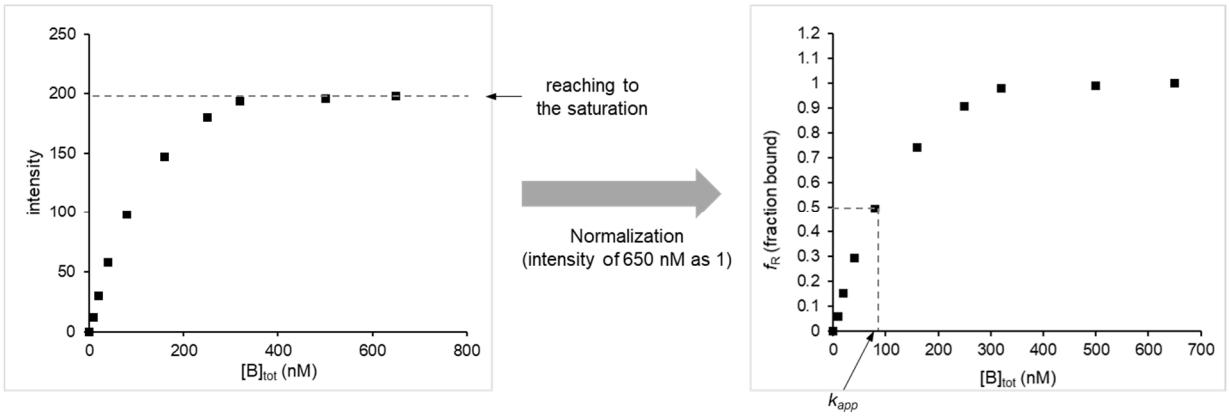
- Used to protein-DNA and protein-RNA interaction
- Usually radiolabeled DNA or RNA are used, analysed by native-PAGE (a method of electrophoresis without denaturing so that complex can be detected), detected by autoradiography

overview of EMSA



Using equation of *condition A*

$[B]_{tot}$ (nM)	650	500	320	250	160	80	40	20	10	0
intensity (bound)	198	196	194	180	147	98	58	30	12	0
f_R (fraction bound)	1	0.99	0.98	0.909	0.742	0.495	0.293	0.152	0.061	0



$$k_{on}[A][B] = k_{off}[AB]$$

$$\frac{k_{off}}{k_{on}} = k_D = \frac{[A][B]}{[AB]}$$

$$k_D = \frac{([A]_{tot} - [AB])[B]}{[AB]} \quad when\ [A] = [A]_{tot} - [AB]$$

$$[AB] = \frac{[A]_{tot}[B]}{k_D + [B]}$$

$$[AB] = \frac{[A]_{tot}[B]_{tot}}{k_D + [B]_{tot}} \quad when\ [B] \approx [B] + [AB] = [B\}_{tot}$$

The above equation can be converted to log-logistic functions:

$$[AB] = \frac{[A]_{tot}}{\frac{k_D}{[B]_{tot}} + 1} = [A]_{tot}/\{1 + \exp(ln(k_D) - ln([B]_{tot})\}$$

or

$$[AB] = \frac{[A]_{tot}}{\frac{k_D}{[B]_{tot}} + 1} = [A]_{tot}/\{1 + 10\text{^}(log(k_D) - log([B]_{tot})\}$$

example (GraphPad prism)

・Equation: Sigmoidal dose-response (variable slope)

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(logEC_{50} - X) \times Hillslope}}$$

X is the logarithm of concentration.　Y is the response.　Y starts at Bottom and goes to Top with a sigmoid shape. This is identical to the “four parameter logistic equation”.

・data table

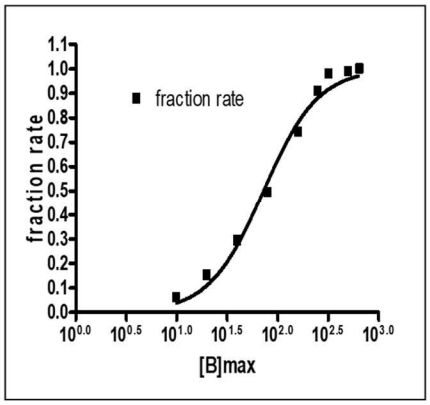
	X Values	A		
	[B]max	fraction rate		
	X	A:Y1	A:Y2	A:Y3
1	650	1.000000		
2	500	0.989899		
3	320	0.979798		
4	250	0.909091		
5	160	0.742424		
6	80	0.494949		
7	40	0.292929		
8	20	0.151515		
9	10	0.060606		

Logarithm of the concentration

	X Values	A		
	X Title	Data Set-A		
	X	A:Y1	A:Y2	A:Y3
1	2.813	1.000		
2	2.699	0.990		
3	2.505	0.980		
4	2.398	0.909		
5	2.204	0.742		
6	1.903	0.495		
7	1.602	0.293		
8	1.301	0.152		
9	1.000	0.061		

・extracted parameters and data plotting

		A
		fraction rate
		Y
1	Sigmoidal dose-response (variable slope)	
2	Best-fit values	
3	BOTTOM	0.0
4	TOP	1.000
5	LOGEC50	1.860
6	HILLSLOPE	1.614
7	EC50	72.48



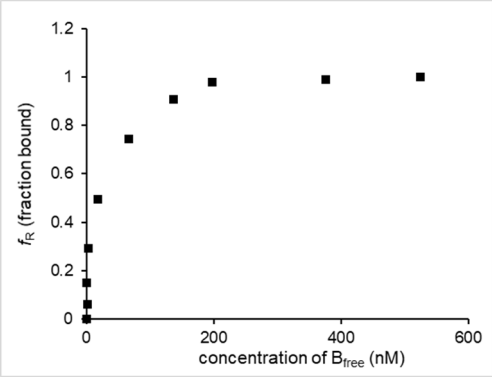
k_{app} is calculated as 72.5 nM.　However, $[A]_{tot}$ is 125 nM.　Therefore, $k_{app} < [A]_{tot}$, which indicated that condition A does not fit to this situation.　The condition C needs to be considered.

Using equation of *condition C*

In this situation, the concentration of AB at the maximum ($[AB]_{max}$) can be considered as the total concentration of A ($[A]_{tot}$). Therefore, the concentration of bound ([AB]) is calculated as [AB] = $f_R[A]_{tot}$. The following approximation is set:

$$[B]_{free} \approx [B]_{tot} - f_R[A]_{tot}$$

$[B]_{tot}$ (nM)	650	500	320	250	160	80	40	20	10	0
intensity (bound)	198	196	194	180	147	98	58	30	12	0
f_R (fraction bound)	1	0.99	0.98	0.909	0.742	0.495	0.293	0.152	0.061	0
$[B]_{free}$ (nM)	525	376.3	197.5	136.4	67.2	18.13	3.384	1.061	2.424	0



$$[AB] = \frac{[A]_{tot}}{\frac{k_D}{[B]_{free}} + 1} = [A]_{tot}/\{1 + 10\text{^}\left(log(k_D) - log\left([B]_{free}\right)\right\}$$

example (GraphPad prism)

・Equation: Sigmoidal dose-response (variable slope)

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(logEC_{50} - X) \times Hillslope}}$$

X is the logarithm of concentration. Y is the response. Y starts at Bottom and goes to Top with a sigmoid shape. This is identical to the “four parameter logistic equation”.

・data table

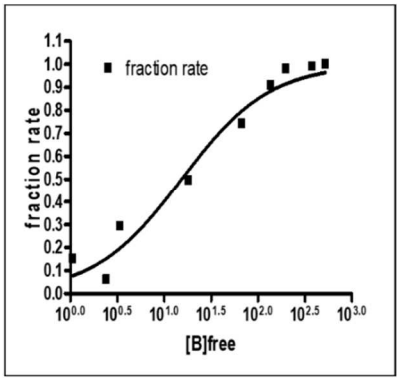
	X Values	A		
	[B]free	fraction rate		
	X	A:Y1	A:Y2	A:Y3
1	525.000000	1.000000		
2	376.262600	0.989899		
3	197.525300	0.979798		
4	136.363600	0.909091		
5	67.196970	0.742424		
6	18.131310	0.494949		
7	3.383838	0.292929		
8	1.060606	0.151515		
9	2.424242	0.060606		

Logarithm of the concentration

	X Values	A		
	X Title	Data Set-A		
	X	A:Y1	A:Y2	A:Y3
1	2.720	1.000		
2	2.575	0.990		
3	2.296	0.980		
4	2.135	0.909		
5	1.827	0.742		
6	1.258	0.495		
7	0.529	0.293		
8	0.026	0.152		
9	0.385	0.061		

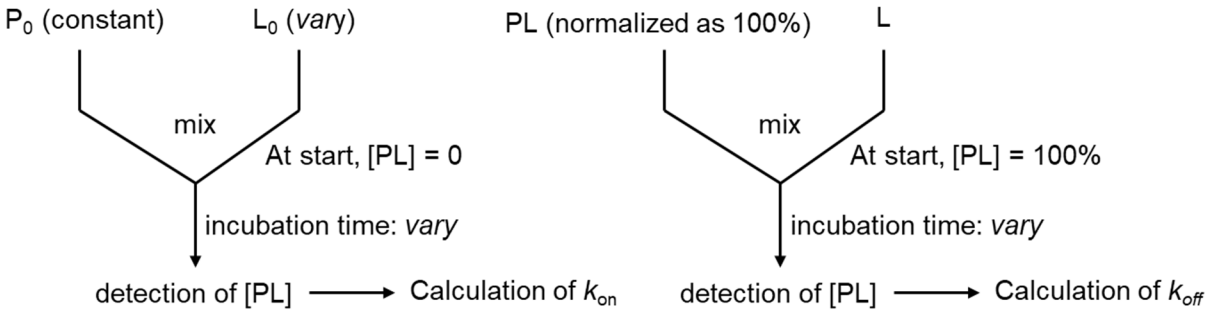
・extracted parameters and data plotting

		A
		fraction rate
		Y
1	Sigmoidal dose-response (variable slope)	
2	Best-fit values	
3	BOTTOM	0.0
4	TOP	1.000
5	LOGEC50	1.187
6	HILLSLOPE	0.9258
7	EC50	15.39



K_D is calculated as 15.4 nM.

Question 2: kinetic approach



(i) calculating k_{on}

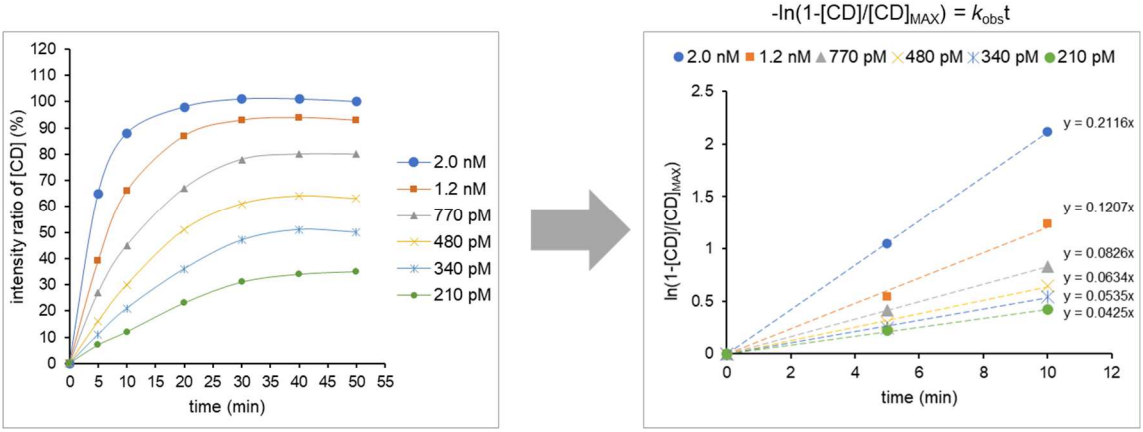
The following equations hold:

$$[CD] = [CD]_{MAX}(1 - e^{-k_{obs}t})$$

$$1 - \frac{[CD]}{[CD]_{MAX}} = e^{-k_{obs}t}$$

$$-\ln\left(1 - \frac{[CD]}{[CD]_{MAX}}\right) = k_{obs}t$$

$when\ -\ln\left(1 - \frac{[CD]}{[CD]_{MAX}}\right)\ is\ fitted\ with\ t\ (time), k_{obs}\ can\ be\ dermined\ as\ slope\ for\ each\ concentration\ ([B])$

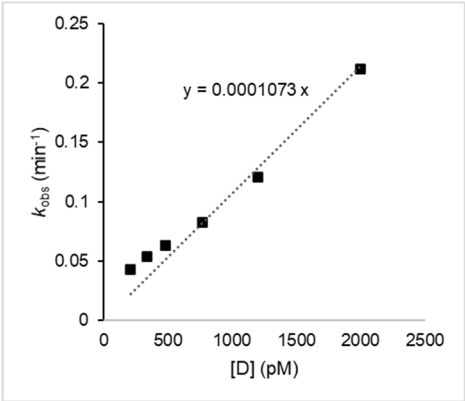


$[D]_{tot}$ (pM)	210	340	480	770	1200	2000
k_{obs} (min^{-1})	0.0425	0.0535	0.0634	0.0826	0.1207	0.2116

$$Also\ the\ fllowing\ equation\ holds$$

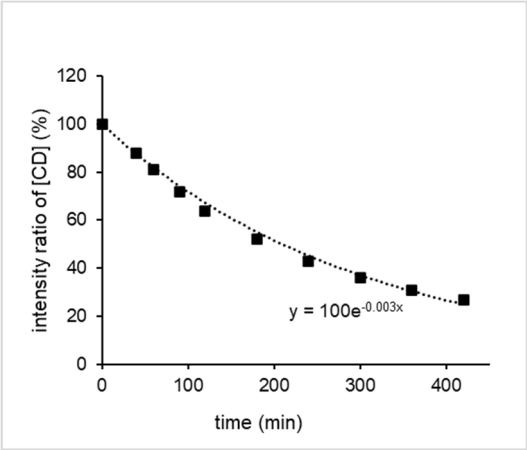
$$k_{obs} = k_{on}[D]$$

$$from\ the\ equation\ above, k_{on}\ can\ be\ determined.$$



k_{on} = 0.000107 ($pM^{-1}min^{-1}$) = 107 ($\mu M^{-1}min^{-1}$)

(ii) calculating k_{off}



The data is fitted as above.

k_{off} = 0.00331 (min^{-1}) = 0.199 (h^{-1})

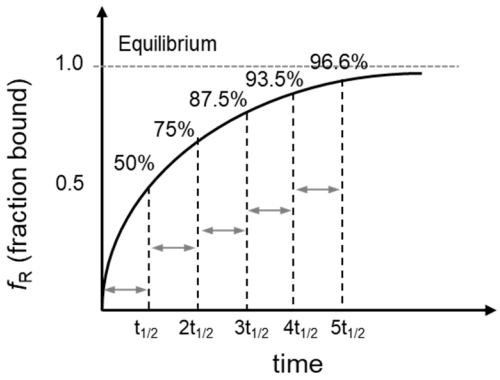
(iii) (a) calculating k_D

$k_D = k_{off}/k_{on}$ = 0.00331/107 = 31 pM

The k_D value is 6.5-fold different from that from the experiment of equilibrium approach (200 pM)

(b) why does k_D value from equilibrium approach from kinetic approach?

For calculating accurate k_D value by equilibrium approach, the system needs to reach "equilibrium". From the chart at left, it takes five half-times to reach "equilibrium" (96.6%).



The use of an incubation time greater than 5 × $t_{1/2}$ is a safe assumption for ensuring equilibrium.

The half-time depends on the concentration, however, it is useful to consider the limiting case with the protein concentration approaching zero, which is the longest half time calculated from dissociation rate constant. The half-time can be calculated as follows:

$$[CD] = [CD]_{MAX}(1 - e^{(-k_{on}[D] - k_{off})t})$$

$$when\ [CD] = \frac{1}{2}[CD]_{MAX}, \qquad t = t_{\frac{1}{2}}$$

$$\frac{1}{2}[CD]_{MAX} = [CD]_{MAX} e^{(-k_{on}[D] - k_{off})t_{\frac{1}{2}}}$$

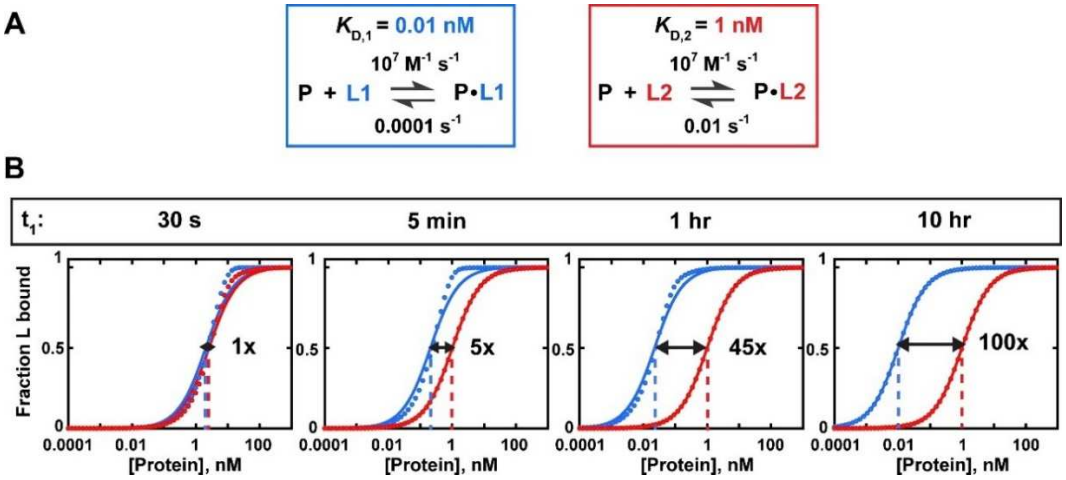
$$t_{\frac{1}{2}} = \frac{ln2}{k_{on}[D] + k_{off}}$$

Considering the limiting case with the protein concentration approaching zero, the following holds:

$$t_{\frac{1}{2},limit} = \frac{ln2}{k_{off}} = \frac{0.693}{k_{off}} = 3.5\ h$$

The experiment this time incubated for only 2 h. Therefore, it needed to incubate longer enough.

Example[1)]



(A) Binding parameters for protein (P) interactions with two ligands, L1 and L2. The dissociation rate constant (k_{off}) for L1 is 100-fold lower than for L2, such that L1 requires much longer to equilibrate than L2. (B) Simulated binding data for L1 and L2 with varying incubation times (t_1). Equilibration of L1 binding is not complete until t_1 = 10 hr (while L2 equilibration only takes ~5 min). Therefore, the observed relative affinity is time-dependent and underestimates the true specificity if the incubation time is shorter than ~10 hr.

Reference

1. Jarmoskaite, I.; AlSadhan, I.; Vaidyanathan, P. P.; Herschlag, D. *eLife* **2020**, *9*, 1.
2. Hulme, E. C.; Trevethink, M. A.; *Br J Pharmacol*. **2010**, *161*, 1219.
3. Carter, C. M. S.; Leighton-Davies, J. R.; Charlton, S. J. *J Biomol Screen*. **2007**, *12*, 255.
4. Chang, K. J.; Jacobs, S.; Cuatrecasas, P. *Biochem Biophys Acta*. **1975**, *406*, 294.
5. Pollard, T. D.; *Mol Biol Cell*. **2010**, *23*, 4061.
6. Hellman, L. M.; Fried, M. G.; *Nat Protoc*. **2007**, *2*,1849