Binding

"**The secret of life is molecular recognition**; the ability of one molecule to "recognize" another through weak bonding interactions."

Linus Pauling at the 25th anniversary of the Institute of Molecular Biology at the University of Oregon

Binding is the first step necessary for a biological response.

Before macromolecules can perform a function, they normally must interact with a specific ligand. In some cases like myoglobin, binding and subsequent release of the ligand might be the sole function of the macromolecule. To understanding binding, we must consider the equilbria involved, how binding is affected by ligand and macromolecule concentration, and how to experimentally analyze and interpret binding data and binding curves.

Note: DNA binding examples from Karsten Rippe - Heidelberg

Ligand Binding

- **General description of ligand binding**
  - the essential terms and equations
  - thermodynamics
- **Simple equilibrium binding**
  - stoichiometric titration
  - equilibrium binding / dissociation constant
  - techniques to determine K
- **Complex equilibrium binding**
  - Multiple sites / cooperativity
  - Scatchard plots and Hill Plots

BINDING (COVALENT) OF PROTONS

$HA \leftrightarrow H^+ + A^-$ $\quad pH = pK_a + \log(A^-/HA)$

Low pH — High pH

High $[H^+]$ — low $[H^+]$

$pH = pK_a - 2$	$pH = pK_a$	$pH = pK_a + 2$
~ 100% protonated	50% protonated	~ 100% deprotonated

BINDING (NONCOVALENT) OF LIGANDS

$ML \leftrightarrow M + L$ (written as dissociation) $\quad ML = (M_O)L/(K_D + L)$

High [L] — low [L]

$L = 100\ K_D$	$L = K_D$	$L = .0.1K_D$
~ 100% bound	50% bound	~ 100% unbound

What is the meaning of the dissociation constant (Kd) for binding of a single ligand to its site?

1. K_d has units of concentration, mol / liter

2. **K_d gives the concentration of ligand to saturate 50%** of the ligand binding sites (when the total site concentration is much lower than K_d)

3. Almost all binding sites are saturated when the free ligand concentration is 10 x K_d

4. The dissociation constant K_d is related to Gibbs free energy $?G^o$ by the relation $?G^o = - R\ T\ \ln K_d$

Summary of Key Equations / Relationships

E + S Û ES ; ***for single site***

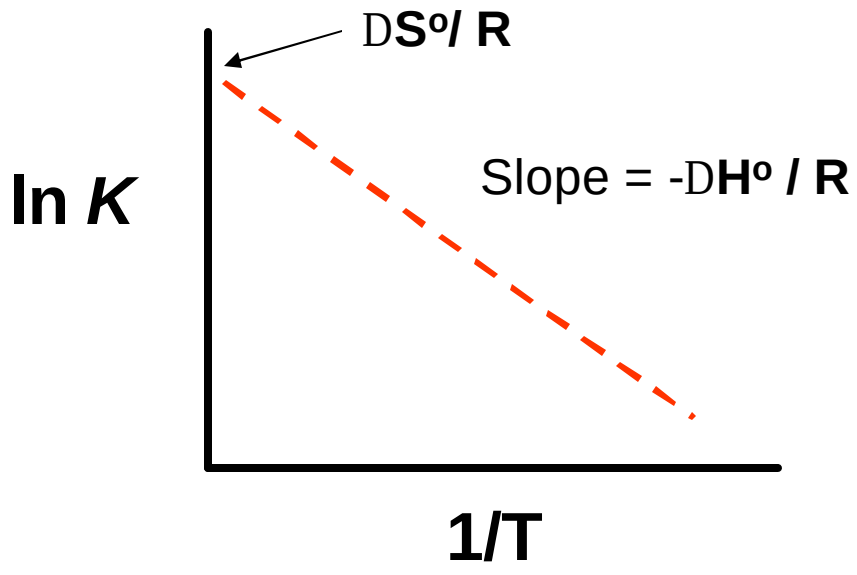
Rate of formation: [E][S] x k_{on}

Rate of breakdown: [ES] x k_{off}

$K_d = k_{off} / k_{on} = [E][S]/[ES]$ and $K_a = 1/ K_d$

van't Hoff Equation

$$\ln K = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R}$$



K_d values in biological systems

Movovalent ions binding to proteins or DNA have **K_d 0.1 mM to 10 mM**

Allosteric activators of enzymes e. g. NAD have **K_d 0.1 µM to 0.1 mM**

Site specific binding to DNA **K_d 1 nM to 1 pM**

Trypsin inhibitor to pancreatic trypsin protease **K_d 0.01 pM**

Antibody-antigen interaction have **K_d 0.1 mM to 0.0001 pM**

Summary of Key Equations / Relationships

$$E + S \hat{U} ES ; \quad \textit{for single site}$$

$$K_d = k_{off} / k_{on} = [E][S]/[ES] \quad \text{and} \quad K_a = 1/ K_d$$

define **Fractional Occupancy** of sites

$$q = [ES]/[E_o] = [ES]/([E] + [ES]) \times [S]/[S] \times [ES]/[ES]$$

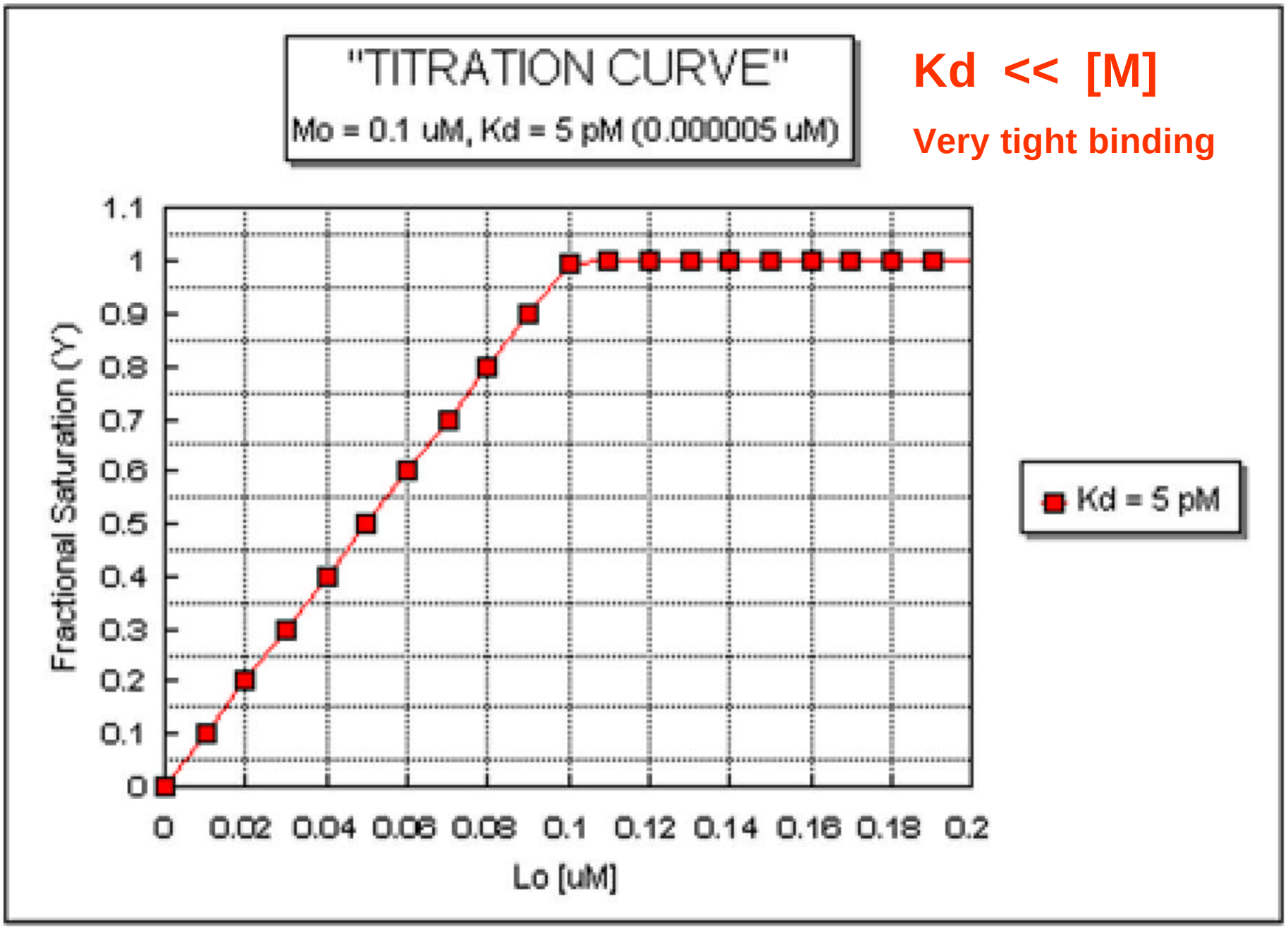
then $q = [S]/(K_d + [S])$

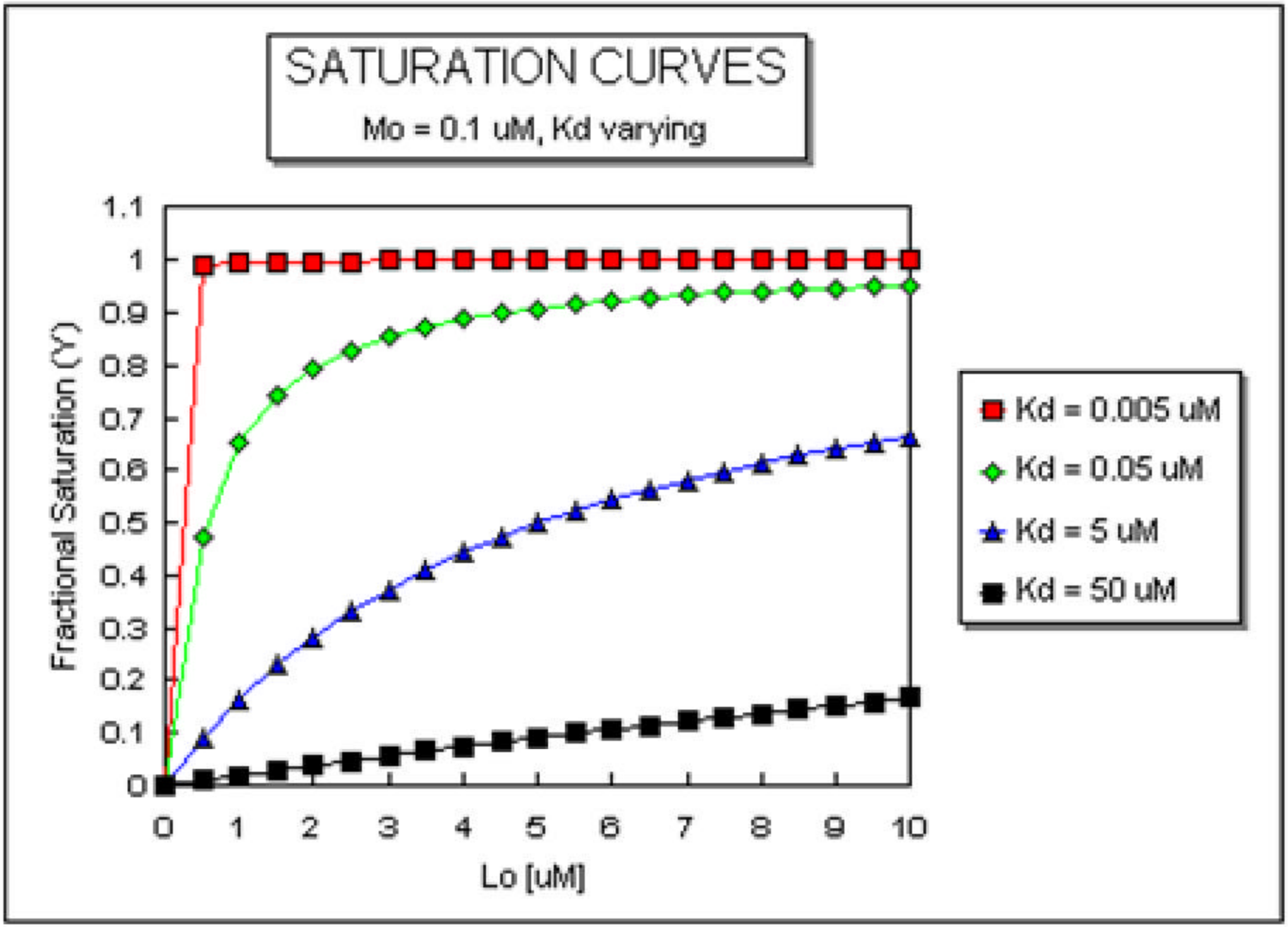
thus when $[S] = K_d$, then $q = 0.50$

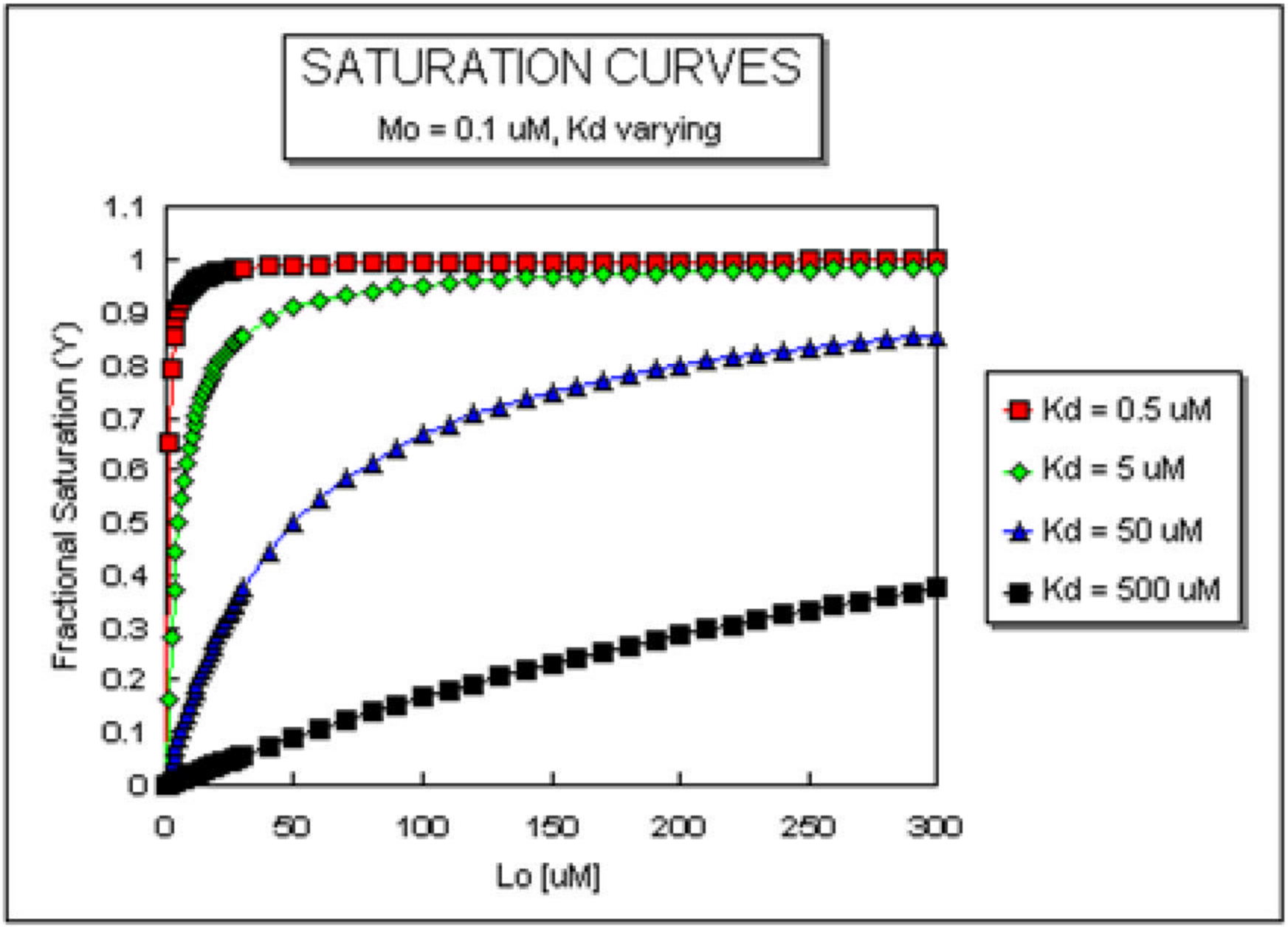
when $[S] = 4K_d$, then $q = 0.80$

when $[S] = 10K_d$, then $q = 0.91$

Note: [S] = conc. of free ligand!!


"TITRATION CURVE"
Mo = 0.1 uM, Kd = 5 pM (0.000005 uM)
Kd << [M]
Very tight binding
Fractional Saturation (Y)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
0.18
0.2
Lo [uM]
Kd = 5 pM


SATURATION CURVES
Mo = 0.1 uM, Kd varying
Fractional Saturation (Y)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
8
9
10
Lo [uM]
Kd = 0.005 uM
Kd = 0.05 uM
Kd = 5 uM
Kd = 50 uM


SATURATION CURVES
Mo = 0.1 uM, Kd varying
Fractional Saturation (Y)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
250
300
Lo [uM]
Kd = 0.5 uM
Kd = 5 uM
Kd = 50 uM
Kd = 500 uM

Simplification of Key Equations

$$E + S \hat{U} ES ;$$

$K_d = k_{off} / k_{on} = [E][S]/[ES]$ and $K_a = 1/ K_d$

$S_o = S + ES$; $E_o = E + ES$

If $S_o >> E_o$, then $S \sim S_o$

then $K_d [ES] = [E_o – ES][S_o]$

$[ES] = E_o S_o/(K_d + S_o)$;

define Fractional Occupancy of sites

$q = [ES]/[E_o] = [ES]/([E] + [ES]) = [S_o]/(K_d + [S_o])$

thus when $[S_o] = K_d$, then $q = 0.5$

Manipulations of Equations

a) double reciprocal plot

$$1/q = K_d/S_o + 1 \text{ ; plot } 1/q \text{ vs. } 1/S_o$$

b) Scatchard Plot: $q = S_o/(K_d + S_o)$ **or**

$$qK_d + qS_o = S_o \quad \text{or} \quad q = 1 - qK_d/S_o$$

plot q **vs.** q/S_o **slope =** $-K_d$

Linearized forms of the equation:

Double reciprocal plot:

$$1/\theta = \frac{[S]+K_d}{[S]} = 1 + \frac{K_d}{[S]}$$

Or for multiple sites:

$$1/\upsilon = \frac{[S]+K_d}{[S]} = n + \frac{K_d}{[S]}$$

Skatchard Plot

$$\theta = 1 - \frac{\theta K_d}{[S]}$$

Or for multiple sites:

$$\theta = n - \frac{\upsilon K_d}{[S]}$$

$$\upsilon = \frac{\text{moles bound}}{\text{mole E}} = n\theta$$

No Assumptions - Key Equations

Derivation of quadratic equation--with no assumptions concerning substrate concentration.

$$fraction = \theta = [ES]/[E]_0 = [ES]/([E]+[ES])$$

☜ The derivation starts the same as above

$$\theta = \frac{K_a[E][S]}{[E]+K_a[E][S]} = \frac{K_a[S]}{1+K_a[S]} = \frac{[S]}{1/K_a+[S]}$$

☜ Fraction of sites bound relative to [S]

$$\theta = \frac{[S]}{K_d+[S]} = \frac{[S]_0-[ES]}{K_d+[S]_0-[ES]} = \frac{[ES]}{[E]_0}$$

☜ Substitution of $[S]=[S]_0-[ES]$

$$[ES](K_d+[S]_0)-[ES]^2 = [E]_0[S]_0-[ES][E]_0$$

$$[ES]^2-[ES](K_d+[S]_0+[E]_0)+[E]_0[S]_0$$

☜Form of equation require solution as the roots of the quadratic equation

No Assumptions - Key Equations

Solution is quadratic equation:

$$ax^2 + bx + c = 0$$

solution provided by the roots of the quadratic

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

$$[ES] = \frac{(E_0 + S_0 + K_d) - \sqrt{(E_0 + S_0 + K_d)^2 - 4 \cdot E_0 \cdot S_0}}{2}$$

$$\theta = \frac{(E_0 + S_0 + K_d) - \sqrt{(E_0 + S_0 + K_d)^2 - 4 \cdot E_0 \cdot S_0}}{2 \cdot E_0}$$

This equation must be used rather than the hyperbola whenever the enzyme concentration is comparable or greater than the dissociation constant. As a general rule, if $[E]_0$ is less than 5 times the K_d, the hyperbolic fit is probably adequate.

EXPERIMENTAL DETERMINATION OF Kd

TECHNIQUES THAT REQUIRE SEPARATION OF BOUND FROM FREE LIGAND – Care must be given to ensure that the equilibrium of M + L <==> ML is not shifted during the separation technique.

- ***gel filration chromatography*** **- Add M to a given concentration of L. Then elute the mixture on a gel filtration column, eluting with the free ligand at the same concentration. The ML complex will elute first and can be quantitated . If you measure the free ligand coming off the column, it will be constant after the ML elutes with the exception of a single dip near where the free L would elute if the column was eluted without free L in the buffer solution. This dip represents the amount of ligand bound by M.**

- ***membrane filtration*** **- Add M to radiolableled L, equilibrate, and then filter through a filter which binds M and ML. For instance, a nitrocellulose membrane binds proteins irreversibly. Determine the amount of radiolabeled L on the membrane which equals [ML].**

- ***precipitation*** **- Add a precipitating agent like ammonium sulfate, which precipitates proteins and hence both M and ML. Determine the amount of ML.**

EXPERIMENTAL DETERMINATION OF Kd

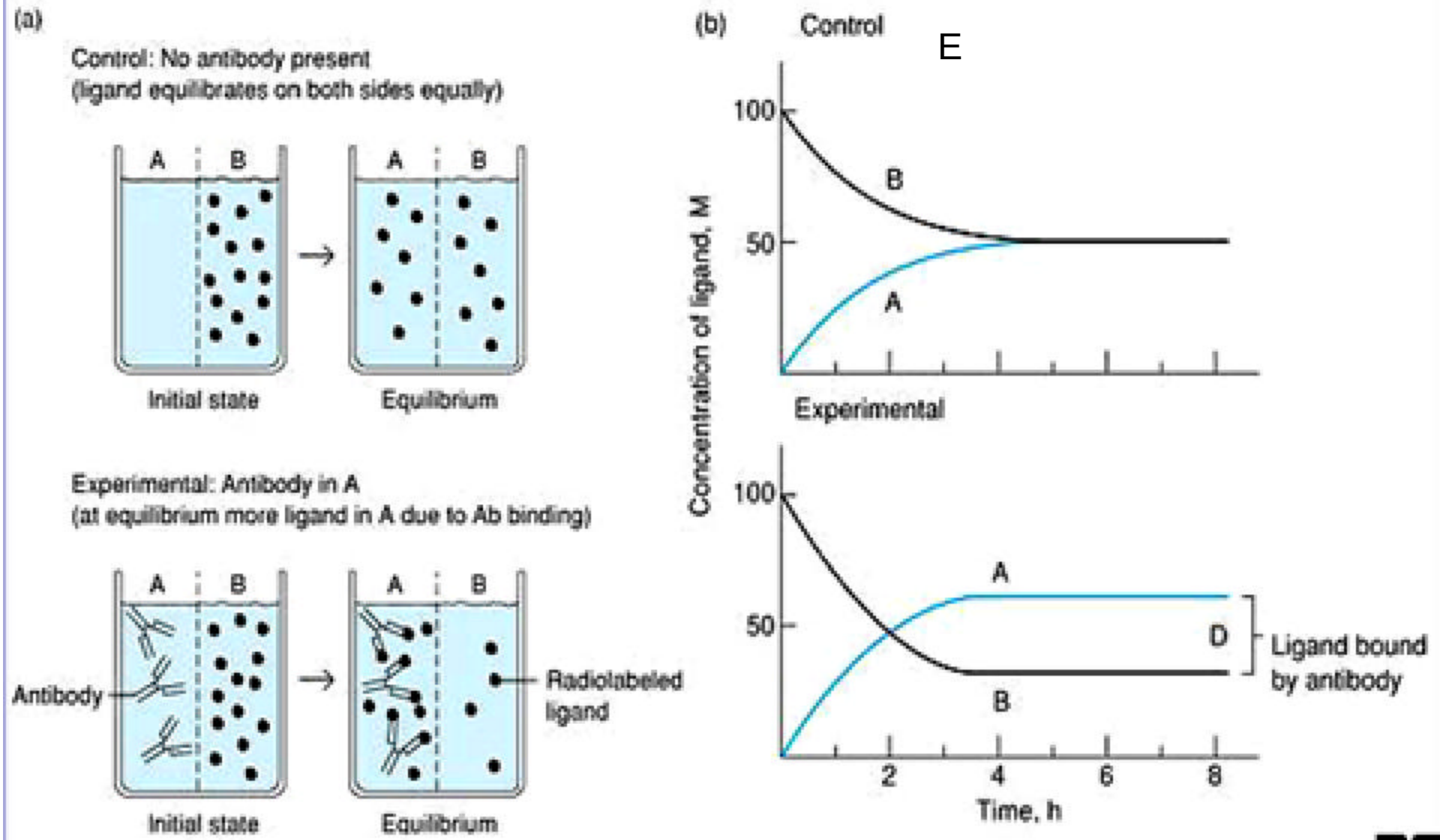
TECH. THAT DO NOT REQUIRE SEPARATION OF BOUND FROM FREE LIGAND

- ***equilibrium dialysis*** - **Place M in a dialysis bag and dialyse against a solution containing a ligand whose concentration can be determined using radioisotopic or spectroscopic techniques. At equilibrium, determine free L by sampling the solution surrounding the bag. By mass balance, determine the amount of bound ligand, which for a 1:1 stoichiometry gives ML. Repeat at different ligand concentrations.**

- ***gel shift assay*** – **Use autoradiography and gel electrophoresis with radiolabeled macromolecule.**

- ***spectroscopy*** - **Find a ligand whose absorbance or fluorescence spectra changes when bound to M. Alternatively, monitor a group on M whose absorbance or fluorescence spectra changes when bound to L.**

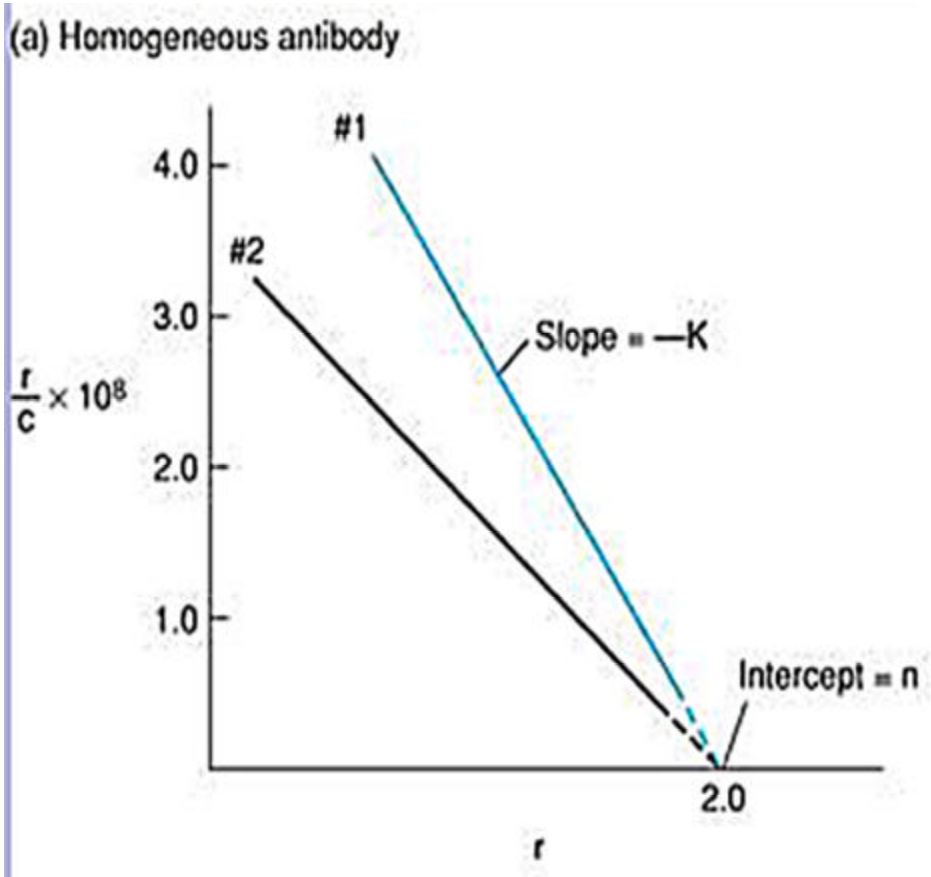
Equilibrium Dialysis

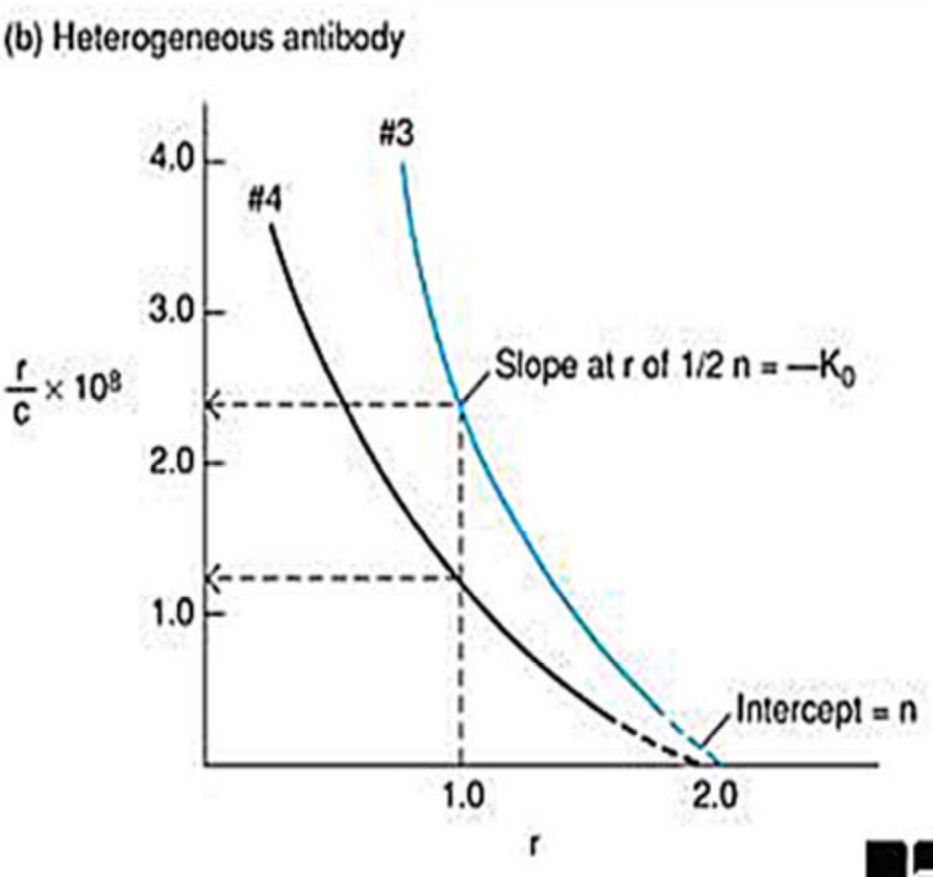


Scatchard equation: $r/c = K(n-r)$

Scatchard plot - determine the equilibrium **association constant** and **valence** of an antibody.

- ***r*** = moles bound ligand/mole antibody at equilibrium;
- ***c*** = free ligand concentration at equilibrium;
- ***K*** = equilibrium association constant; and
- ***n*** = number of antigen binding sites per antibody molecule





Gel Shift Assay

from van Holde, Johnson - p.597

Figure 15.3 Gel shift assay for the binding of histone H1 to a cross-shaped DNA molecule. The DNA (D) has been radiolabeled and the gel subjected to autoradiography. The data represent a titration of a fixed amount of DNA with increasing amounts of the protein. Note that a single complex (C) is formed in this case. [Courtesy of Dr. Jordanka Zlatanova.]

Spectroscopy

Fluorescence data

$$F = F_0 + \Delta F \cdot \theta$$

where $\Delta F = F_\infty - F_0$

and θ is defined by either:

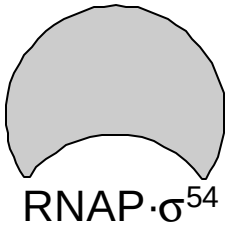
$$\theta = \frac{[S]_0}{K_d + [S]_0}$$

OR.....

$$\theta = \frac{(E_0 + S_0 + K_d) - \sqrt{(E_0 + S_0 + K_d)^2 - 4 \cdot E_0 \cdot S_0}}{2 \cdot E_0}$$

- This equation normalizes the observable signal to a scale that can be related to fractional occupancy, θ.

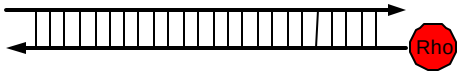
Analysis of binding of RNAP·σ^{54} to a promoter DNA sequence by measurements of fluorescence anisotropy



RNAP·σ^{54}

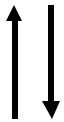
$$q = \frac{[P_{tot}]}{[P_{tot}] + K_D} = \frac{r_{measured} - r_{min}}{r_{max} - r_{min}}$$

+

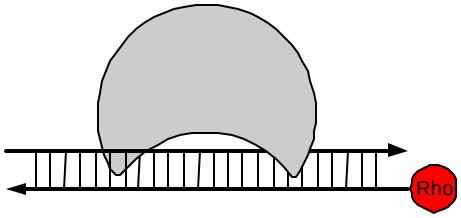


promoter DNA

free DNA with a fluorophore
with high rotational diffusion
-> low fluorescence anisotropy r_{min}



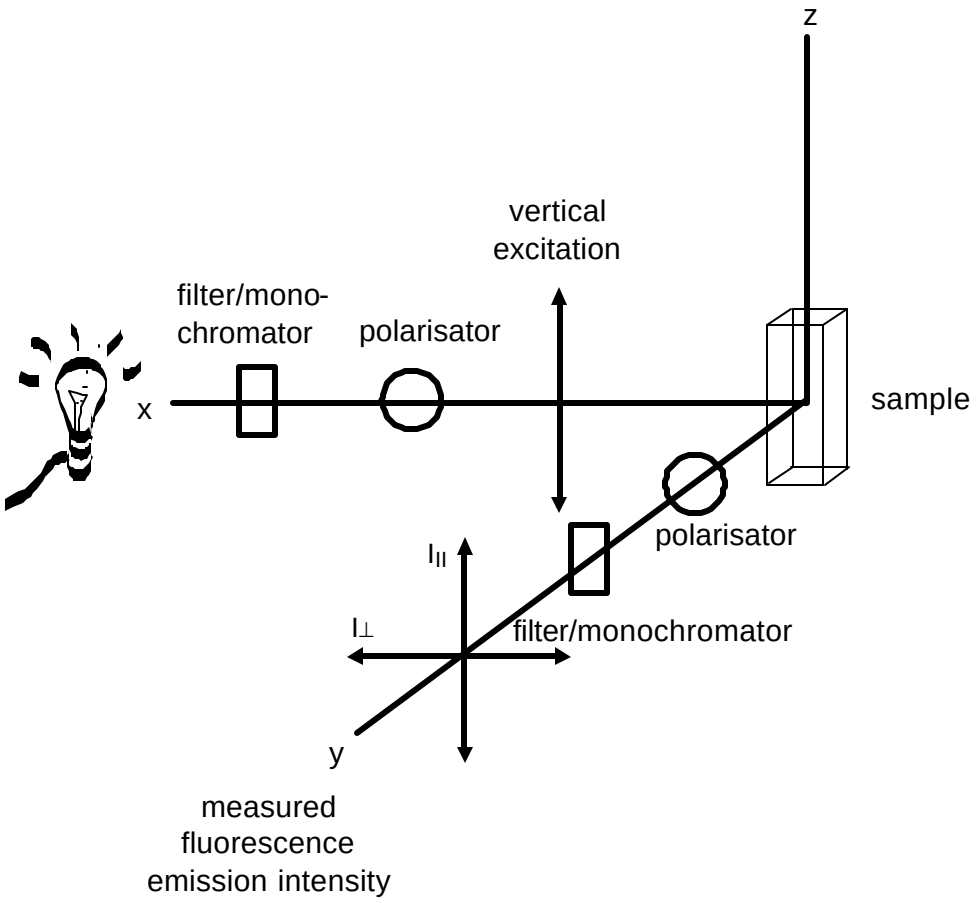
K_d



RNAP·σ^{54}-DNA-Komplex

RNAP-DNA complex
with low rotational diffusion
-> high fluorescence anisotropy
r_{max}

How to measure binding of a protein to DNA?
One possibility is to use fluorescence anisotropy

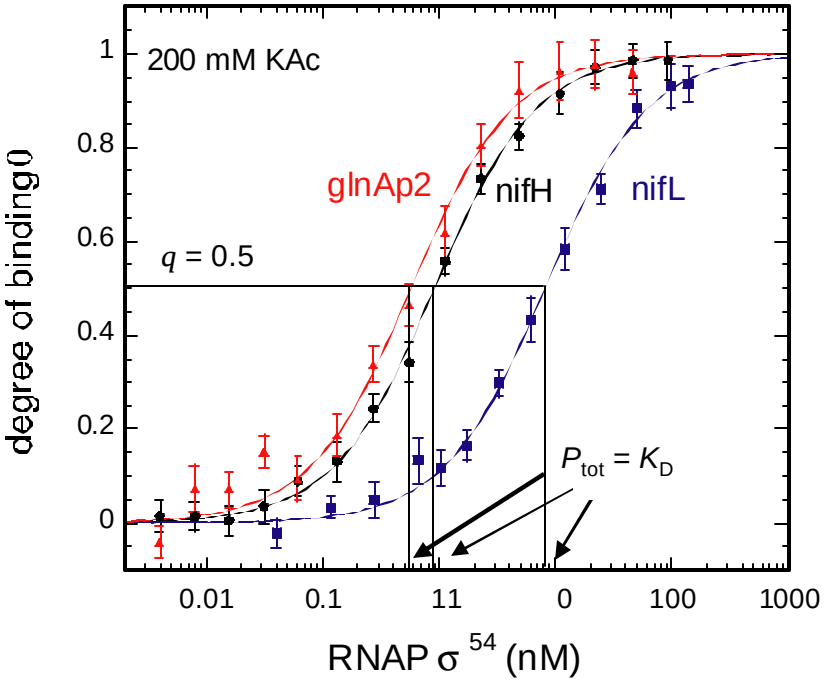


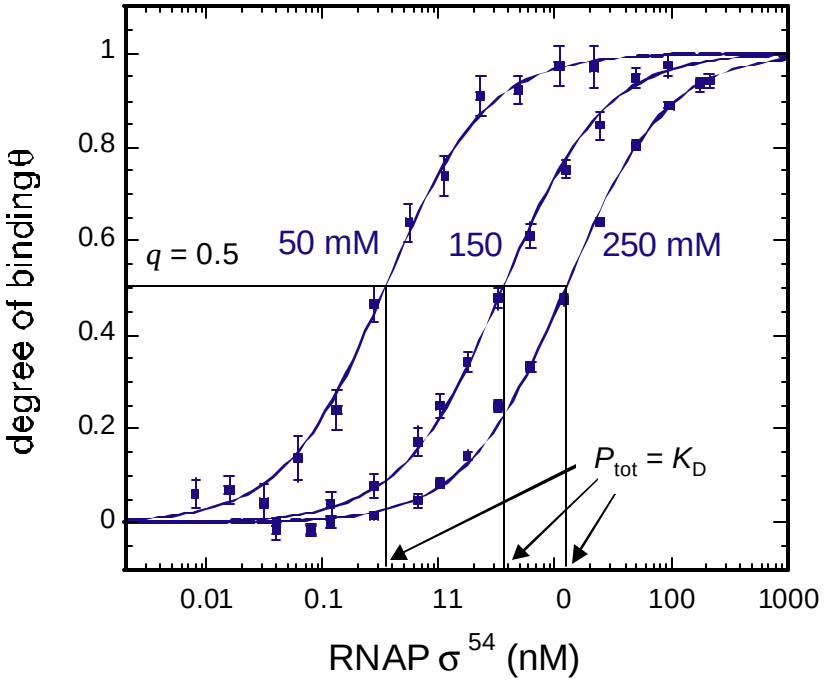
$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}}$$

Definition of fluorescence anisotropy *r*

The anisotropy *r* reflects the rotational diffusion of a fluorescent species

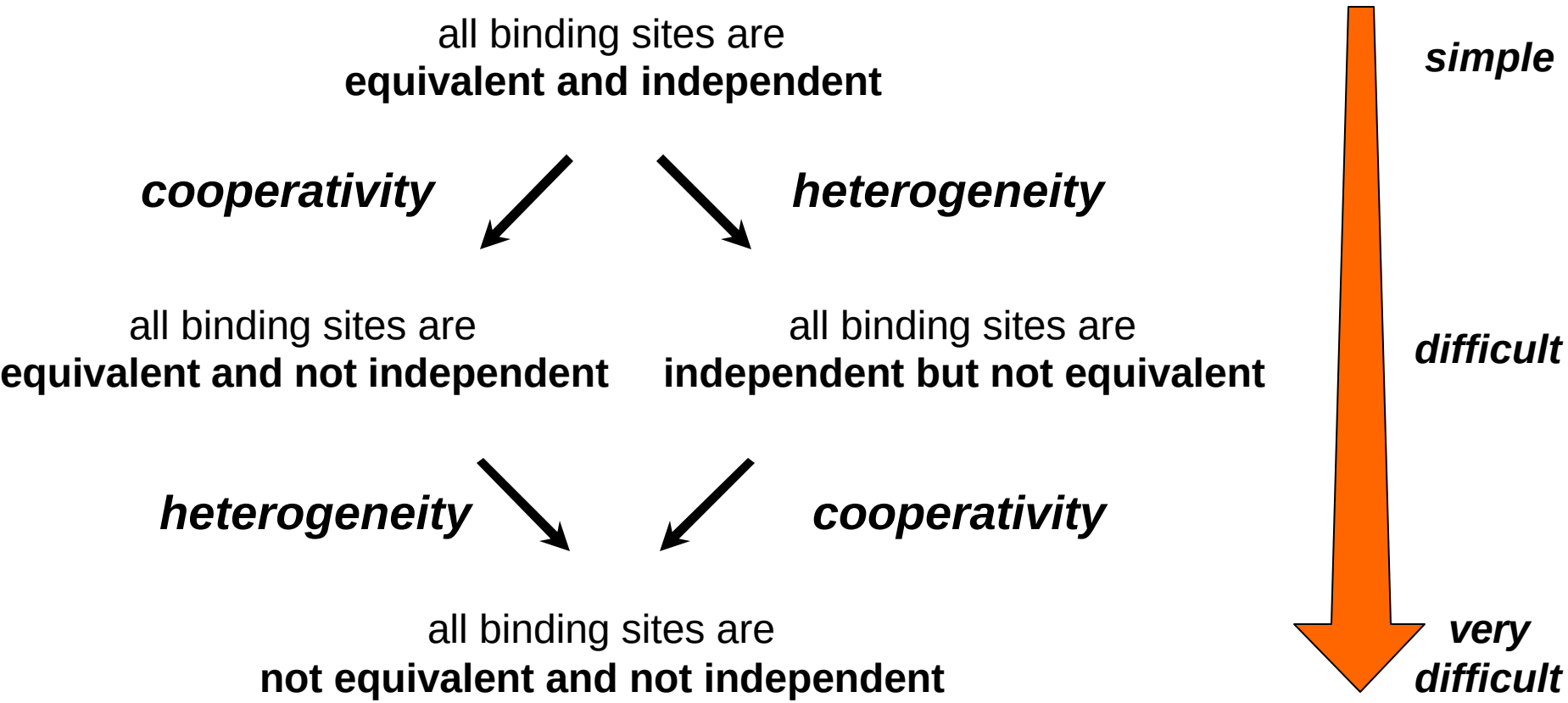
Measurements of fluorescence anisotropy to monitor binding of RNAP·σ^{54} to different promoters





Vogel, S., Schulz A. & Rippe, K.

Increasing complexity of binding



Competitive Binding

$$q = A_o/(K_d + A_o)$$

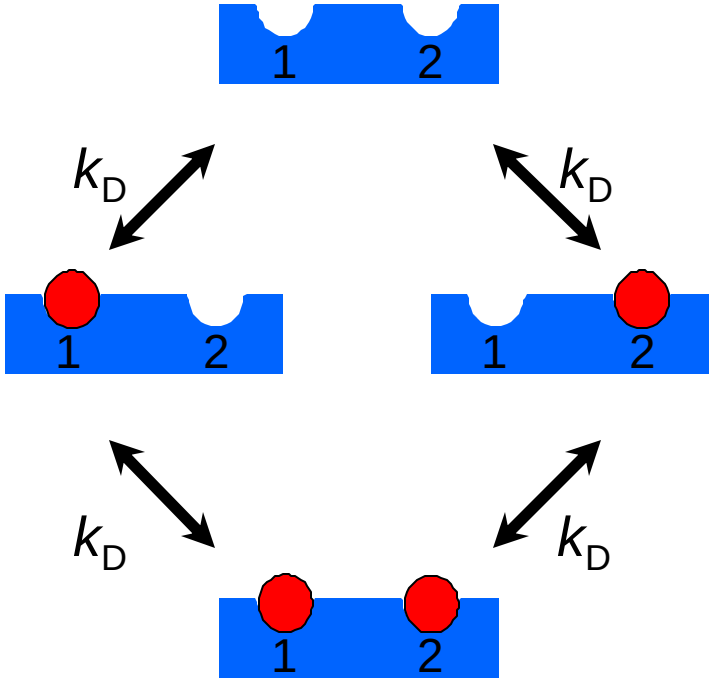
but with a competitive "B" present,

$$K_d = K_{d,A}\,(1 + [B]/K_{d,B})$$

→ replot apparent K_d to obtain $K_{d.B}$

Difference between microscopic and macroscopic dissociation constant

microscopic binding



macroscopic binding

D_{free}

2 possibilities for the formation of *DP*

$K_1 = \frac{k_D}{2}$

DP

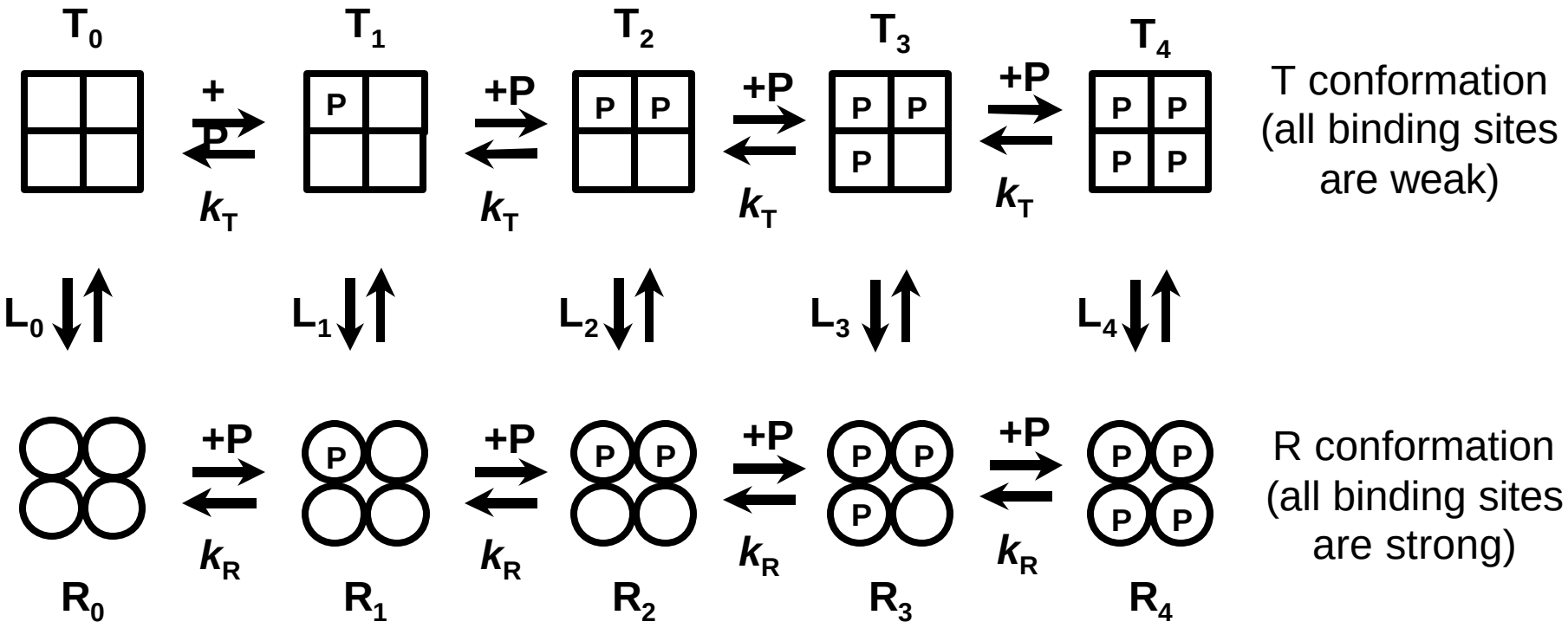
2 possibilities for the dissociation of DP_2

$K_2 = 2 \cdot k_D$

DP_2

$$\frac{K_1}{K_2} = \frac{k_D/2}{2 \cdot k_D} = \frac{1}{4}$$

The Monod-Wyman-Changeau (MWC) model for cooperative binding



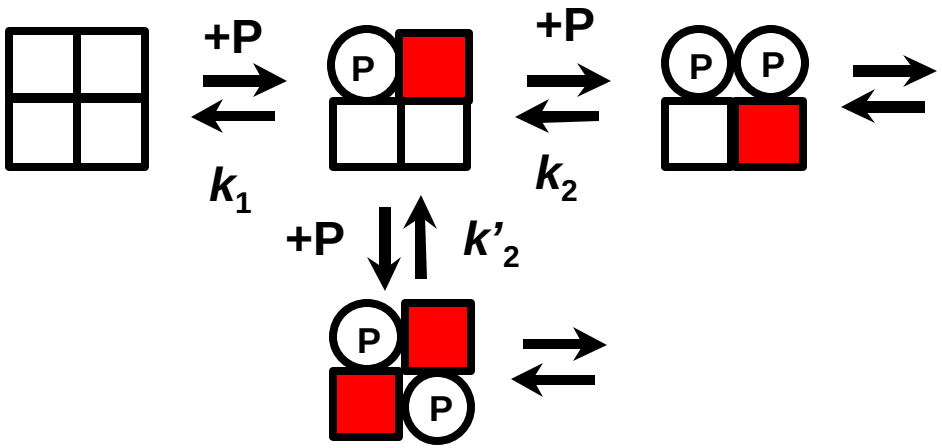
- in the absence of ligand P the the T conformation is favored
- the ligand affinity to the R form is higher, i. e. the dissociation constant $k_R < k_T$.
- all subunits are present in the same confomation
- binding of each ligand changes the T<->R equilibrium towards the R-Form

The Koshland-Nemethy-Filmer (KNF) model for cooperative binding

α-conformation

α-conformation (facilitated binding)

β-conformation (induced by ligand binding)



- **Binding of ligand P induces a conformation change in the subunit to which it binds from the a into the b-conformation ("induced fit").**
- **The bound ligand P facilitates the binding of P to a nearby subunit in the a-conformation (red), i. e. the dissociation constant $k_2 < k'_2$.**
- **subunits can adopt a mixture of a-b confomations.**

Multiple Binding Equilibria

Multiple Binding Sites:

Reaction step	*Association constant*
$E + S \overset{K_1}{\rightleftharpoons} ES$	$[ES] = K_1[E][S]$
$ES + S \overset{K_2}{\rightleftharpoons} ES_2$	$[ES_2] = K_2[ES][S] = K_1K_2[E][S]^2$
$ES_2 + S \overset{K_3}{\rightleftharpoons} ES_3$	$[ES_3] = K_3[ES_2][S] = K_1K_2K_3[E][S]^3$
$ES_3 + S \overset{K_4}{\rightleftharpoons} ES_4$	$[ES_4] = K_4[ES_3][S] = K_1K_2K_3K_4[E][S]^4$

Solution of fraction of sites occupied for a two-step binding sequence.

Mass balance equations:

$$[\mathrm{E}]_0 = [E] + [ES] + [ES_2]$$

$$[S]_0 \simeq [S] \text{ (negligible amount bound)}$$

Fraction of sites bound:

$$\theta = ([ES_1] + [ES_2]) / [E]_0$$

$$= \frac{[ES_1] + [ES_2]}{[E] + [ES_1] + [ES_2]}$$

Substitution of bound states:

$$[ES_1] = K_1[E][S]$$

$$[ES_2] = K_2[ES_1][S] = K_1K_2[E][S]^2$$

$$\theta = \frac{K_1[E][S] + K_1K_2[E][S]^2}{[E] + K_1[E][S] + K_1K_2[E][S]^2}$$

$$\boxed{\theta = \frac{K_1[S] + K_1K_2[S]^2}{1 + K_1[S] + K_1K_2[S]^2}}$$

Fraction of sites bound

If the equations are defined for moles of substrate bound per mole of dimers instead of per mole of active sites, the equations becomes:

$$\upsilon = \frac{K_1[S] + 2K_1K_2[S]^2}{1 + K_1[S] + K_1K_2[S]^2} \quad \text{where } \upsilon = \frac{\text{moles S bound}}{\text{mole of dimers}}$$

Accordingly, the binding equation ranges from 0-2 moles bound rather than from 0-1 fraction of sites occupied.

Changes of the Gibbs free energy *?G* of an reaction

$$a\mathrm{A} + b\mathrm{B} + \ldots \rightleftarrows g\mathrm{G} + h\mathrm{H} \ldots$$

$$DG = G(\text{final state}) - G(\text{initial state})$$

$$DG = g\,m_{\mathrm{G}} + h\,m_{\mathrm{H}} + \ldots - a\,m_{\mathrm{A}} - b\,m_{\mathrm{B}} - \ldots$$

from $m_{\mathrm{i}} = m_{\mathrm{i}}^{0} + RT\ln C_{\mathrm{i}}$ it follows:

$$DG = g\,m_{\mathrm{G}}^{0} + h\,m_{\mathrm{H}}^{0} + \ldots - a\,m_{\mathrm{A}}^{0} - b\,m_{\mathrm{B}}^{0} - \ldots + RT\ln\frac{[\mathrm{G}]^{\mathrm{g}}[\mathrm{H}]^{\mathrm{h}}\ldots}{[\mathrm{A}]^{\mathrm{a}}[\mathrm{B}]^{\mathrm{b}}\ldots}$$

$$DG = DG^{0} + RT\ln\frac{[\mathrm{G}]^{\mathrm{g}}[\mathrm{H}]^{\mathrm{h}}\ldots}{[\mathrm{A}]^{\mathrm{a}}[\mathrm{B}]^{\mathrm{b}}\ldots}$$

?G, ?G° of an reaction at equilibrium

$$aA + bB + \ldots \rightleftarrows gG + hH \ldots$$

$$0 = DG^0 + RT \ln\left(\frac{[G]^g[H]^h \ldots}{[A]^a[B]^b \ldots}\right)_{Eq}$$

$$DG^0 = -RT \ln\left(\frac{[G]^g[H]^h \ldots}{[A]^a[B]^b \ldots}\right)_{Eq} = -RT \ln K$$

$$K = \left(\frac{[G]^g[H]^h \ldots}{[A]^a[B]^b \ldots}\right)_{Eq} = \exp\left(\frac{-\Delta G^0}{RT}\right)$$

van't Hoff Equation

$$\ln K = \frac{-DH^o}{RT} + \frac{DS^o}{R}$$