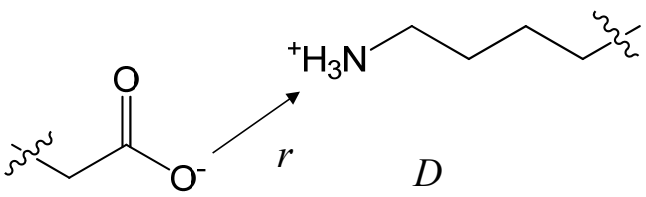
Protein denaturation by pH

- Proteins can also be denatured by pH



Contribution to protein stability:

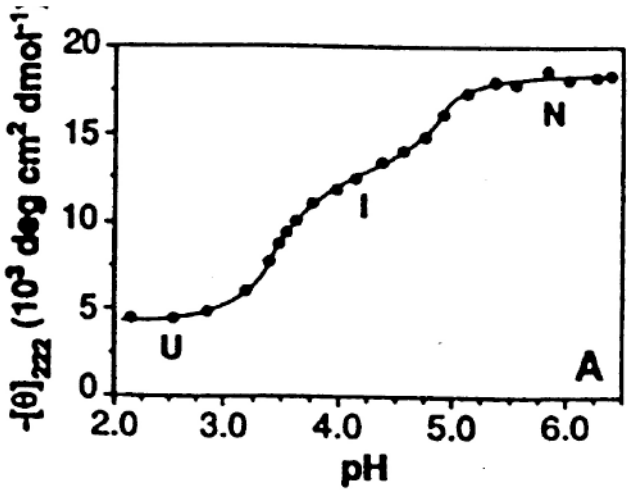
- ion pairs are mostly on the surface of the protein
- the interactions are short range due to **screening**
- disruption of water shell reduces stability contribution

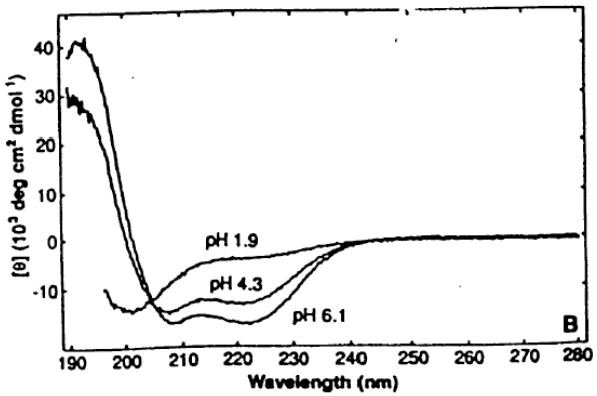
Potential from the ion-pair interaction results in a **shift in the pKa** of the involved residues:

$$\Delta G^0 = -2.303\, RT\, \Delta\mathrm{pK}$$

ΔpK = pK(D) – pK(N) for acidic side chains
ΔpK = pK(N) – pK(D) for basic side chains

pH denaturation of proteins





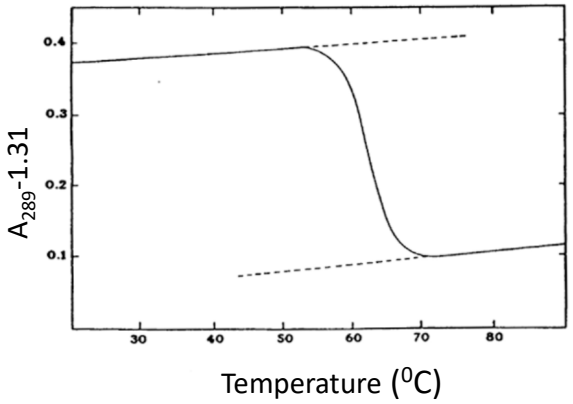
pK values of specific groups

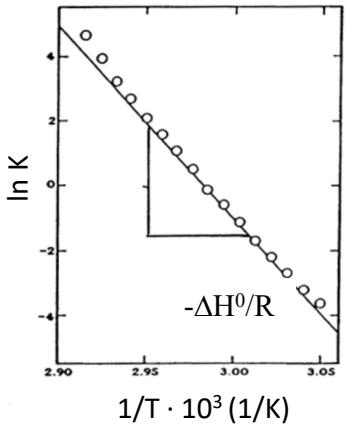
	pK'_1 α-COOH	pK'_2 α-NH_3^+	pK'_R R-Gruppe
Glycin	2,34	9,6	
Alanin	2,34	9,69	
Leucin	2,36	9,60	
Serin	2,21	9,15	
Threonin	2,63	10,43	
Glutamin	2,17	9,13	
Asparaginsäure	2,09	9,82	3,86
Glutaminsäure	2,19	9,67	4,25
Histidin	1,82	9,17	6,0
Cystein	1,71	10,78	8,33
Tyrosin	2,20	9,11	10,07
Lysin	2,18	8,95	10,53
Arginin	2,17	9,04	12,48

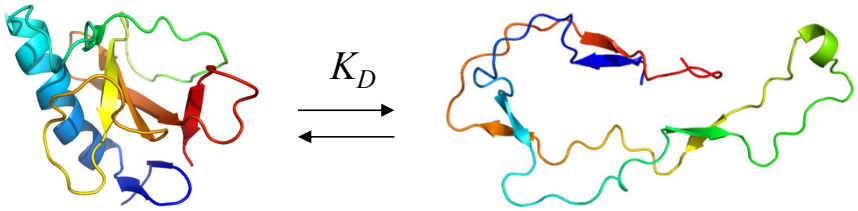
pH denaturation of apo-myoglobin

Thermal denaturation

Ribonuclease T1







N *D*

the T-dependence of K is given by:

$$\ln K(T) = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R}$$

derivative for T:

$$\frac{d \ln K}{dT} = \frac{\Delta H^0_{vH}}{RT^2}$$

$$\frac{d \ln K}{d1/T} = -\frac{\Delta H^0_{vH}}{R}$$

van't Hoff equation

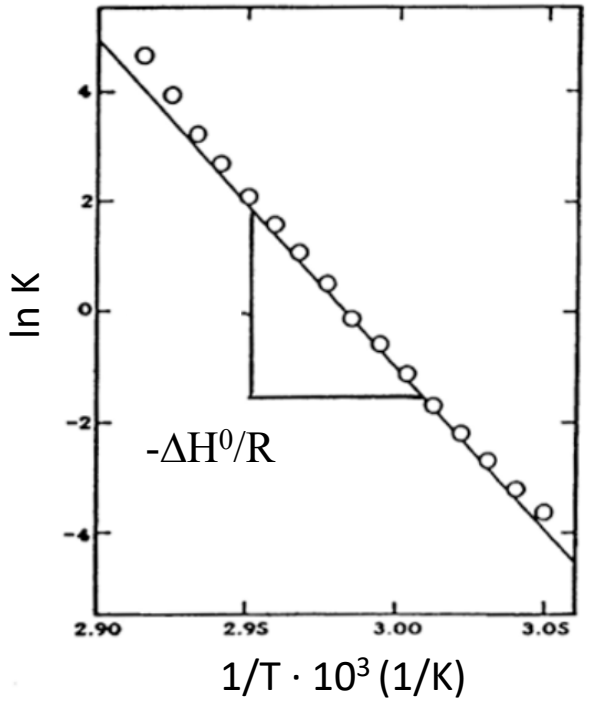
Thermodynamic parameters of protein denaturation

Enthalpy:

Determined from slope of Van't Hoff plot

$$\frac{d \ln K}{d1/T} = -\frac{\Delta H^0_{vH}}{R}$$

within window of linear relationship



Entropy:

At T_m, the temperature of the mid-point of the transition ($\Delta G^0 = 0$)

$$\Delta G^0 = \Delta H^0 - T\Delta S^0$$

$$\Delta S^0 = \Delta H^0 / T_m \quad \text{(for } \Delta G^0 = 0\text{)}$$

Quiz 2: Thermal transitions

- Another small protein is 99% folded at 328K and 1 % folded at 340 K
- what is the standard enthalpy (ΔH^0) of its folding transition?
- also estimate the entropy of the transition (ΔS^0)!

Heat capacity changes in protein denaturation

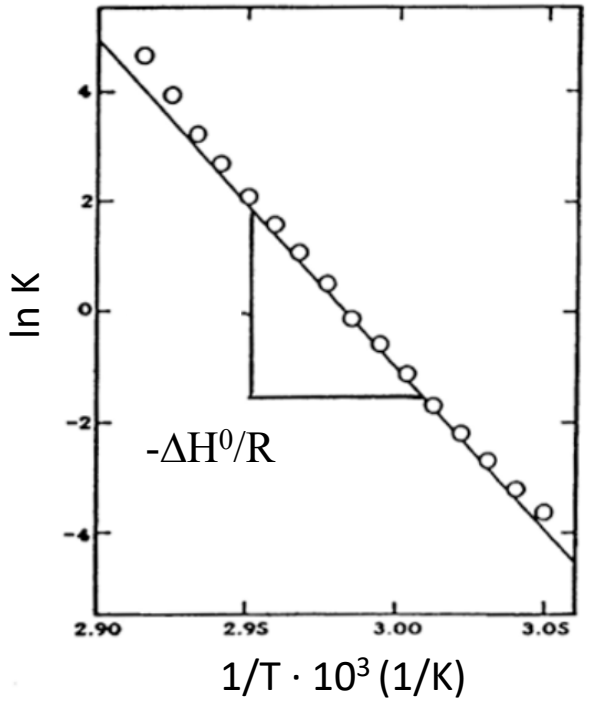
Heat capacity: $$C_P = \left(\frac{\partial H}{\partial T}\right)_P = \left(\frac{\partial Q}{\partial T}\right)_P$$

Change of internal energy upon heating, property of each global state

$$\Delta C_P^0 = C_{P,unfolded}^0 - C_{P,folded}^0$$

Curvature in Van't Hoff relations: The native state and the denatured state have different heat capacities.

Protein unfolding heat capacity is large and positive



$$\Delta C_p^{\,0} > 0$$

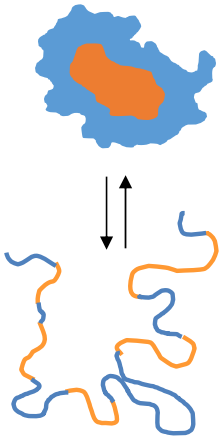
Heat capacity of protein denaturation

Definition of heat capacity:

$$\Delta C_p{}^0 = \frac{d\Delta H^0}{dT}$$

$$\Delta C_p{}^0 / T = \frac{d\Delta S^0}{dT}$$

both, ΔH^0 and ΔS^0 are temperature dependent



The molecular origin of $\Delta C_p{}^0 > 0$:

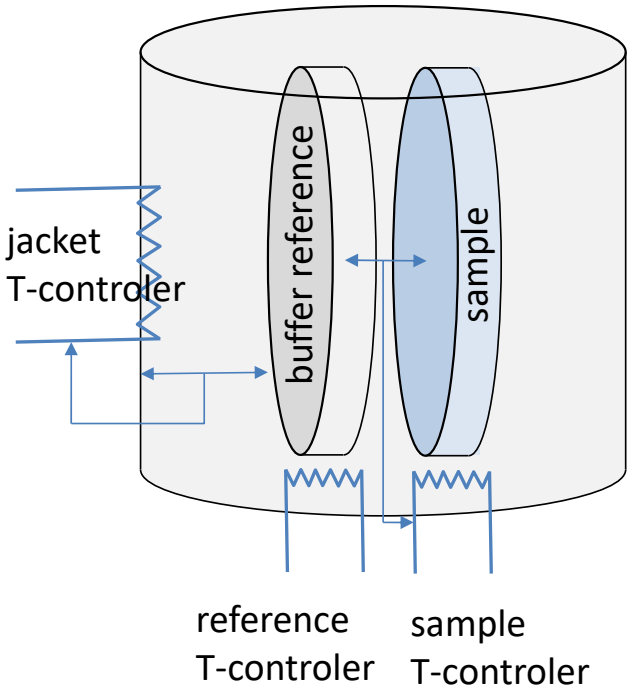
The native and the denatured state exhibit differences in **solvation**

in D, hydrophobic residues are exposed. The **water structure** leads to high heat capacity (compare to Lecture 1)

$\Delta C_p{}^0$ is usually 40-80 J mol^{-1} K^{-1} per residue

related to m-value (also dependent on ASA)

Measuring thermodynamic parameters: Differential Scanning Calorimetry (DSC)



Measures **amount of heat** required to change the **temperature** in the sample vs. the reference.

For an adiabatic isolated microcalorimeter, no heat exchange takes place with the environment

$$\delta Q = 0$$

according to first law

$$dU = dW$$

changes in internal energy solely depend on work within instrument

The partial molar heat capacity Cp

The heat capacity is defined as the **amount of heat** (Q) required to **change the temperature** by dT with constant pressure.

$$C_P = \left(\frac{\partial Q}{\partial T}\right)_P = \left(\frac{\partial H}{\partial T}\right)_P = \frac{dH}{dT}$$

Heat capacity of a protein solution $C_{P,sol}$ is a composite of **partial molar heat capacity** terms:

- Heat capacity of the solvent $C_{P,1}$
- Heat capacity of the protein (e.g. non-covalent interactions) $C_{P,2}$

From $C_{P,2}$ we can determine the enthalpy of protein folding/unfolding (n denotes the molar amounts)

$$C_{P2} = \left(\frac{\partial C_{PSol}}{\partial n_2}\right)_{T,p,n_i \neq 2}$$

Using calorimetry to determine protein stability

Problem: $C_{P,2}$ cannot be directly measured

From calorimetry, the **apparent molar heat capacity** ($C_{P,app}$) is obtained

this is the **difference** between the heat capacity of the solution ($C_{P,sol}$) and of the solvent ($C_{P,1}$)

$$C_{Papp} = \frac{C_{PSol} - n_1 C_{P1}}{n_2}$$

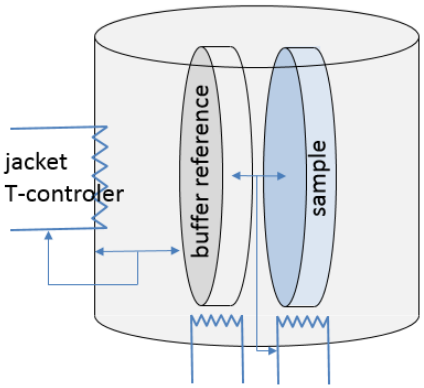
with: $$C_{P2} = \left(\frac{\partial C_{PSol}}{\partial n_2} \right)_{T,p,n_i \neq 2}$$

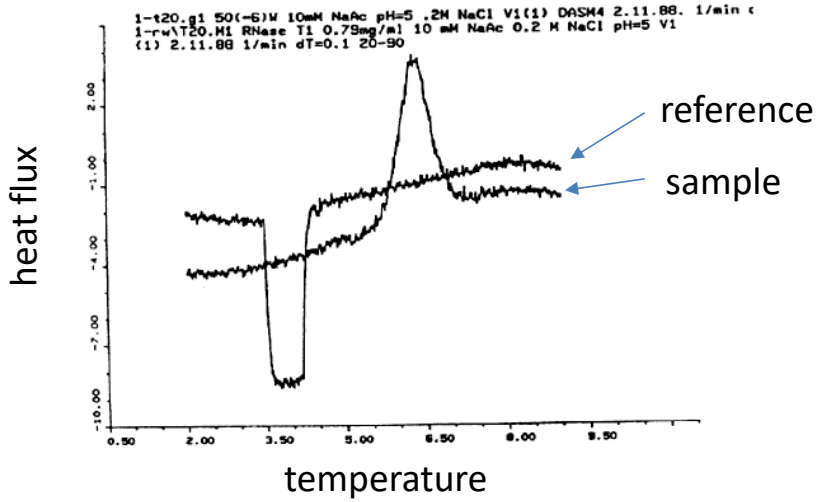
$$C_{P2} = C_{Papp} + \underbrace{n_2 \left(\frac{\partial C_{Papp}}{\partial n_2} \right)_{T,p,n_i \neq 2}}$$

this term can usually be neglected for employed protein concentrations

$$\boxed{C_{P2} = C_{P1} - C_{Papp}}$$

Determining heat capacity from calorimetry





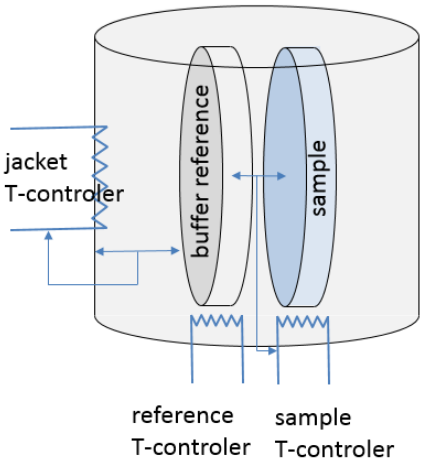
offset correction between sample and reference

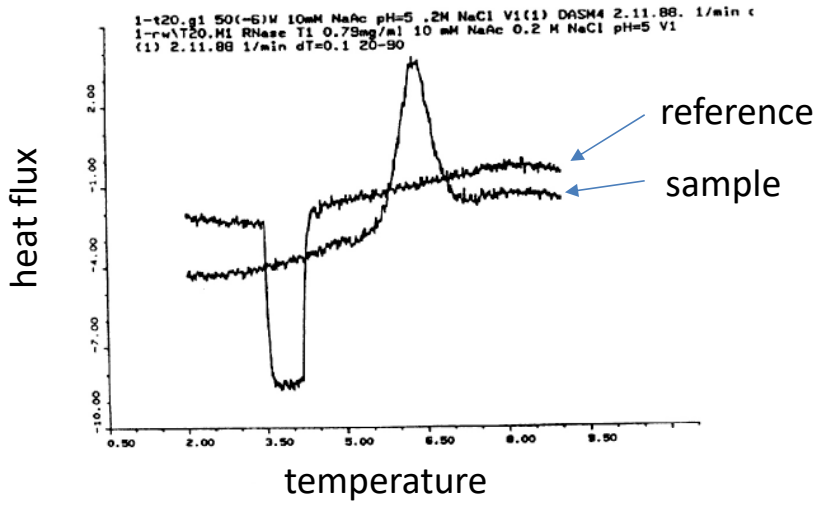
from normalized and calibrated difference follows the **heat capacity change of protein unfolding**

$$C_{P2} = C_{P1}\frac{v_P}{v_1} - \frac{Ck}{m_p}h$$

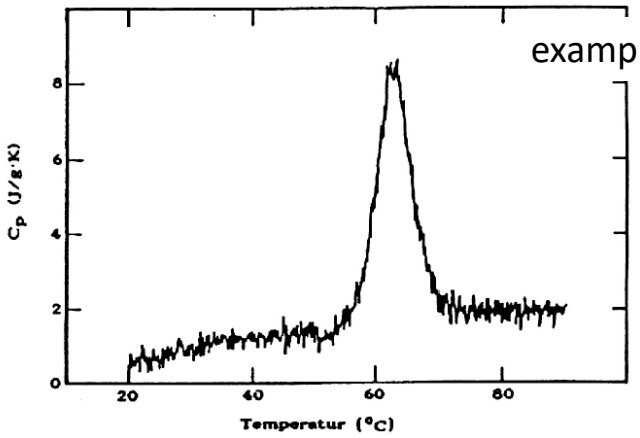
v_P : partial specific volume of protein
v_1 : partial specific volume of solvent
h: normalization
k: calibration constant

Determining heat capacity from calorimetry

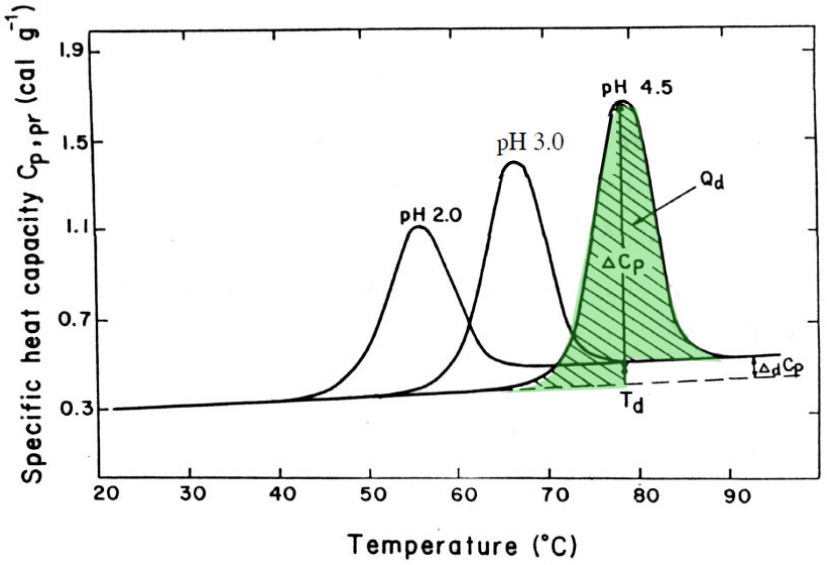




example: RNase T1



Enthalpy of conversion in protein denaturation



$\Delta H_m(cal)$ of lysozyme denaturation as a function of pH

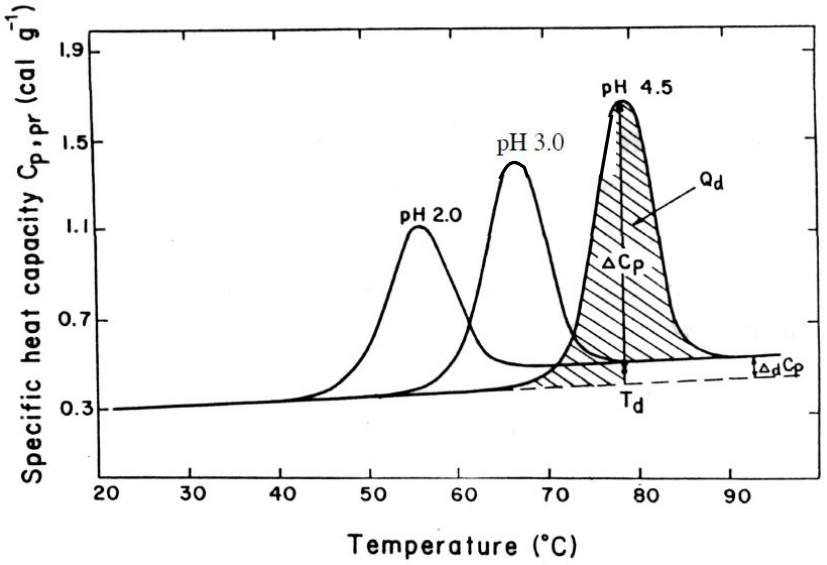
From **integration of the curve** the enthalpy of conversion (folded to unfolded) $\Delta H_m(cal)$ is determined

$$C_P = \frac{dH}{dT}$$

$$Q_d = \Delta H_m(cal) = \int C_p dT$$

model-free determination of enthalpy of state transition

Two-state folding or multistate transition?



$\Delta H_m(cal)$ of lysozyme denaturation as a function of pH

comparing the areas under the curve, an equilibrium constant can be determined.

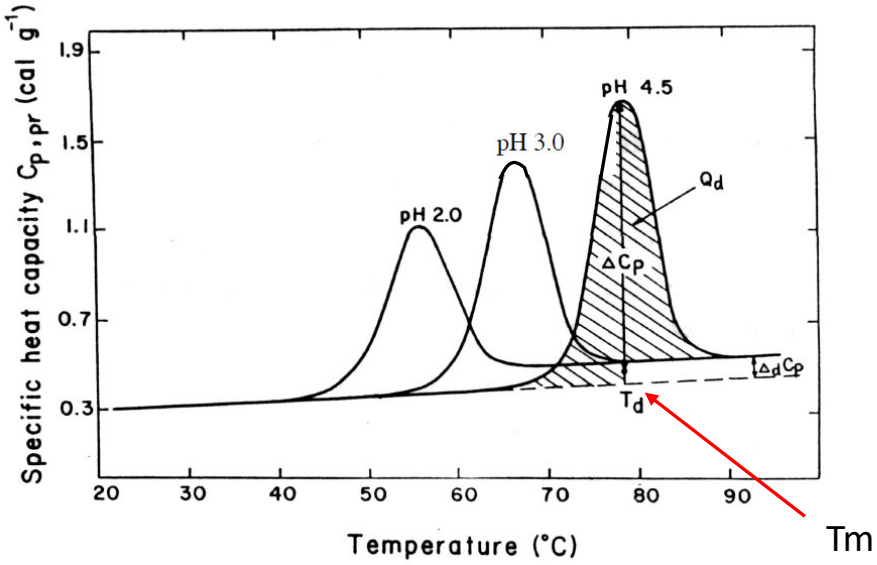
From K, Van't Hoff Enthalpy can be determined immediately, using:

$$\left(\frac{d \ln K}{dT}\right)_p = \frac{\Delta H^0_{v.H.}}{RT^2}$$

This assumes a **two-state transition**

Only in this case, $\Delta H_m(cal) = \Delta H^0_{vH}$

Entropy of conversion



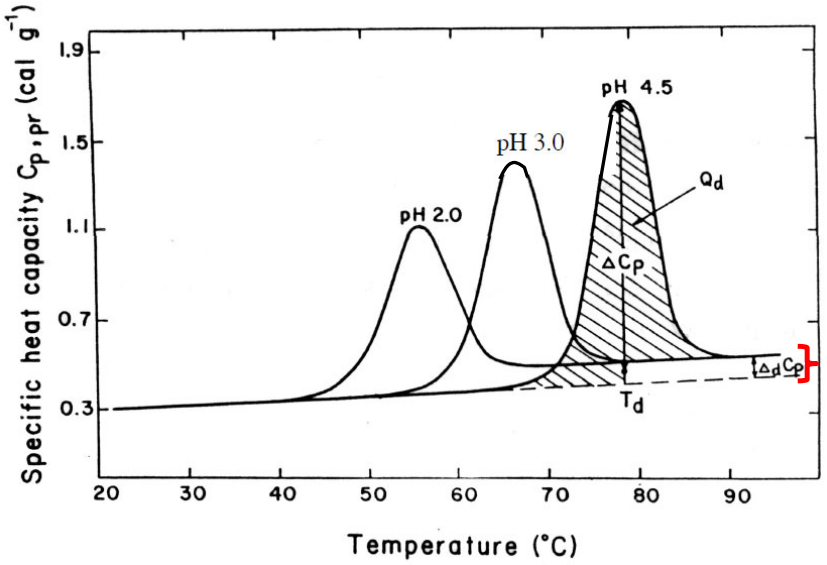
$\Delta H_m(cal)$ of lysozyme denaturation as a function of pH

At the midpoint of the transition (T_m), the entropy can be determined

$$\Delta G = \Delta H - T\Delta S = 0$$

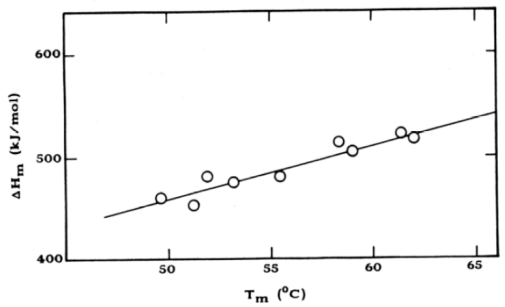
$$\Delta S_m = \frac{\Delta H_m}{T_m}$$

Heat capacity difference of unfolding



ΔC_p can directly be obtained from the measured curve

Can also be determined from the temperature dependence of ΔH_m, e.g. measured under different pH



Rnase T1 denaturation data

The protein stability curve

With heat capacity:

$$\frac{d\Delta H}{dT} = \Delta C_P \qquad \Delta H(T) = \Delta H(T_m) + \Delta C_P (T - T_m)$$

$$\frac{d\Delta S}{dT} T = \Delta C_P \qquad \Delta S(T) = \Delta S(T_m) + \Delta C_P \ln\left(\frac{T}{T_m}\right)$$

With heat capacity, the T-dependence of ΔG^0 becomes:

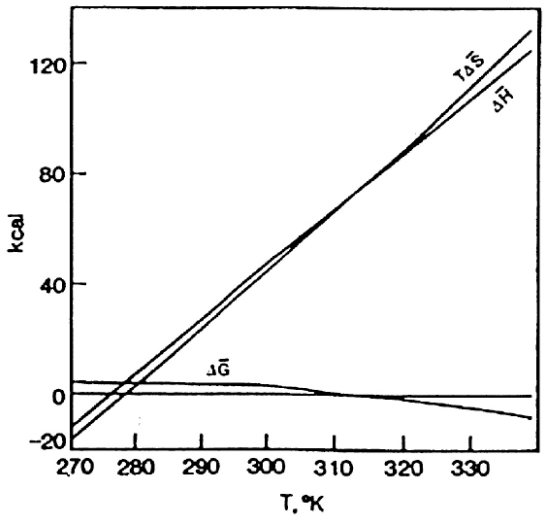
$$\Delta G^0(T) = \Delta H^0(T_m) - \mathrm{T}\,\Delta S^0(T_m) + \Delta C_p{}^0 \left(T - T_m - T \ln\frac{T}{T_m} \right)$$

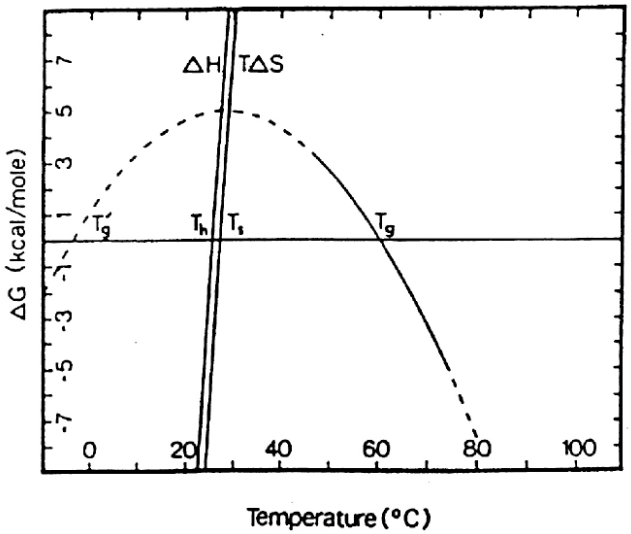
The protein stability curve

With heat capacity, the T-dependence of ΔG^0 becomes:

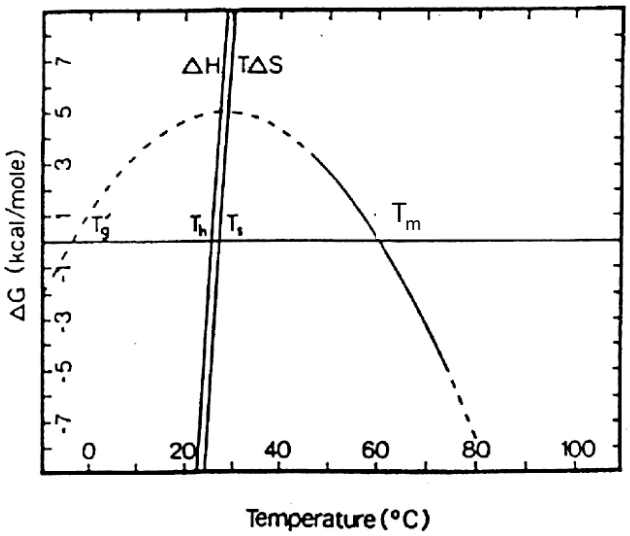
$$\Delta G^0(T) = \Delta H^0(T_m) - \mathrm{T}\,\Delta S^0(T_m) + \Delta C_p{}^0 \left(T - T_m - T \ln \frac{T}{T_m} \right)$$

Example





The protein stability curve



Protein stability is maximal at $\Delta S^0 = 0\ (T_S)$

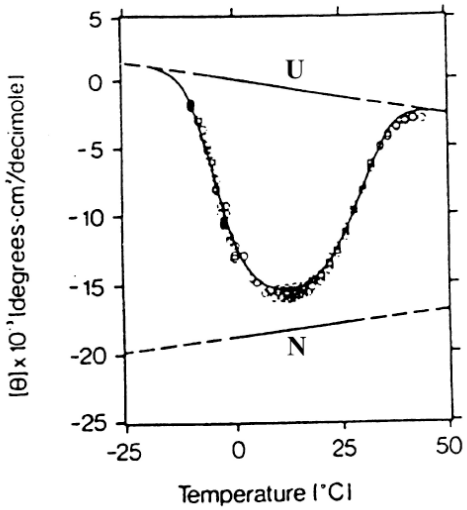
K_D (equilibrium constant) is maximal at $\Delta H^0 = 0\ (T_H)$

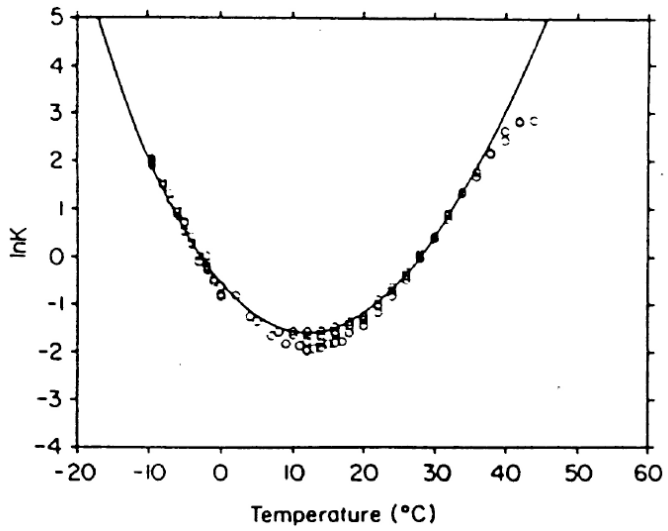
The transition mid-point (T_m) is at $\Delta G^0 = 0$ and $f_N = f_U = 0.5$

Protein cold denaturation

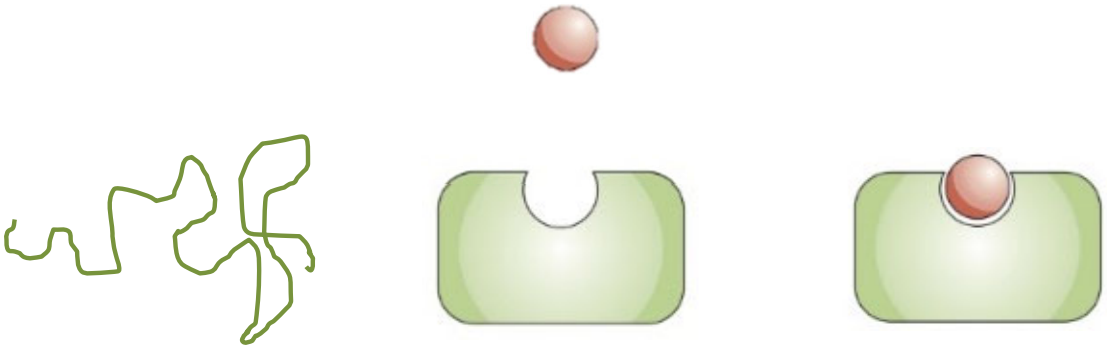
Protein stability curves show second Tm' at low temperature! → cold denaturation

Can be observed for some proteins / mutants:
Cold denaturation of lysozyme (destabilized mutant)





Proteins are stabilized by ligand binding

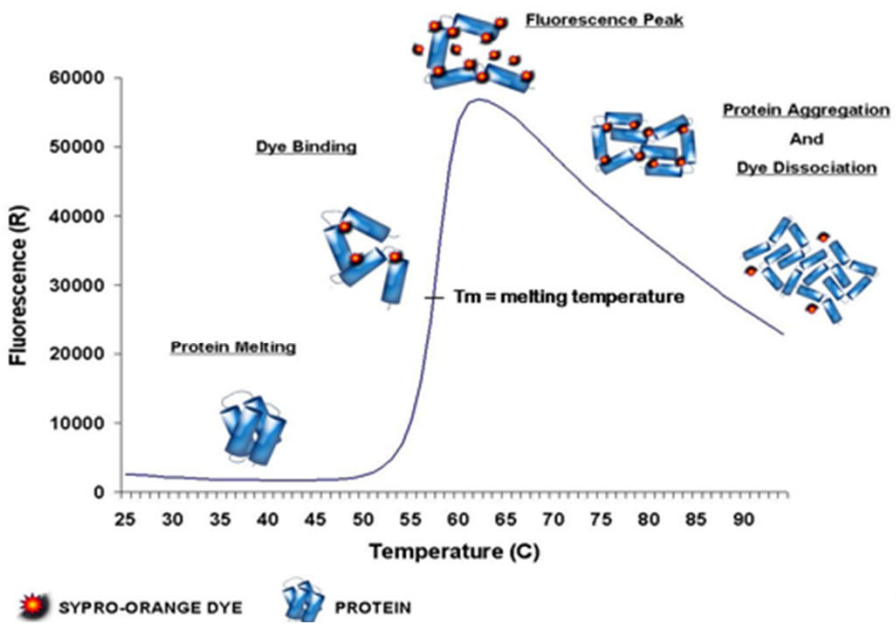


$$R_D \rightleftarrows R_N + L \rightleftarrows R_N : L$$

Protein stabilization by
free energy of ligands

→ measuring protein stability as a
screening tool

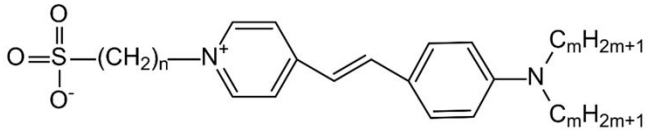
Differential Scanning Fluorimetry: A high-throughput assay to determine protein stability



source: Wikipedia

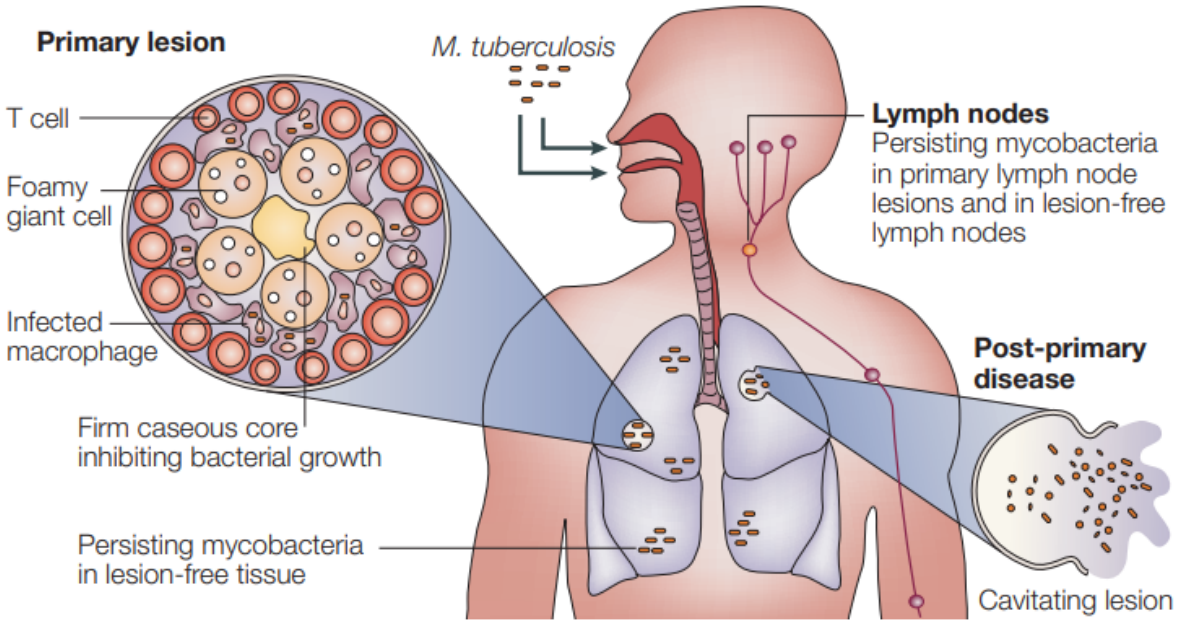
Differential Scanning Fluorimetry: Thermal shift assay

- Addition of a fluorophore
- binding to exposed hydrophobic sites: increase in fluorescence
- dyes: SYPRO orange
- High-throughput method
- ligand binding shifts Tm
- DSC: binding thermodynamics



SYPRO Orange

Case study: protein stability and pharmaceutical research for Tuberculosis



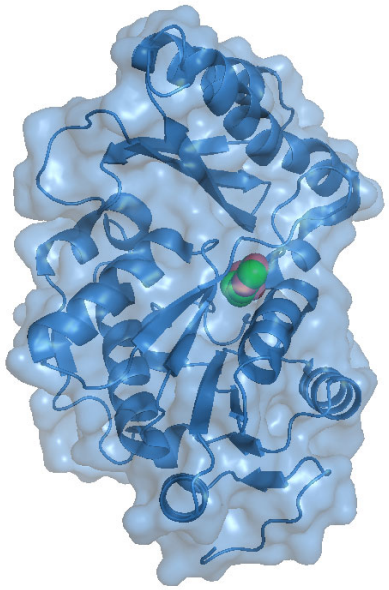
Nat Rev Microbiology 2003

Screening for pantothenate synthetase inhibitors

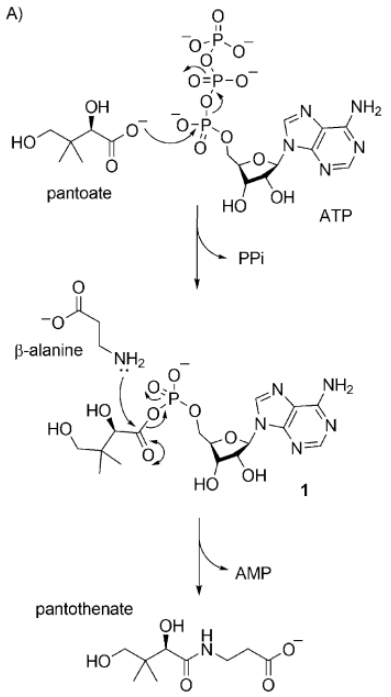
Pantothenate (vitamin B5)

- essential precursor to coenzyme A
- all enzymes absent in mammals
- de novo synthesis important for bacteria, including **M. tuberculosis**
- mutant can get pantotheate through salvage pathway, but no disease

→ **pantotheate synthase is a good target**

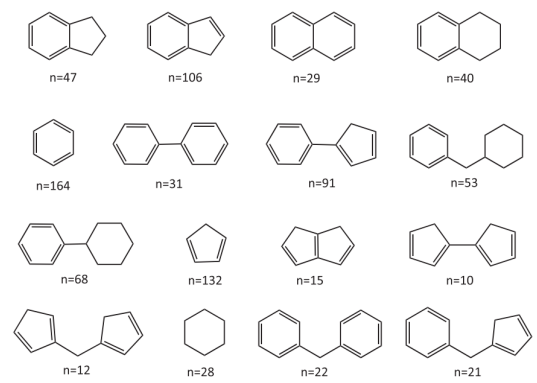


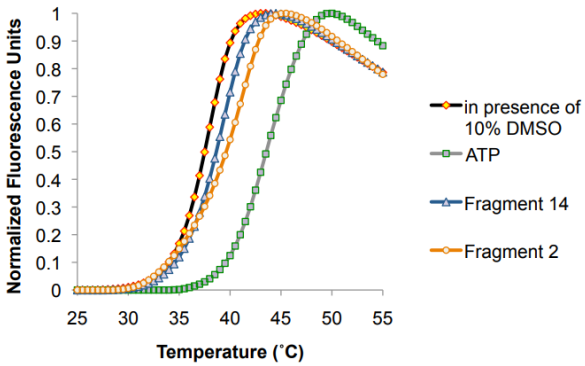
pantothenate synthetase: new target



Ciulli et al. ChemBioChem 2008

Fragment screen to identify binders





Silvestre et al. PNAS 2013

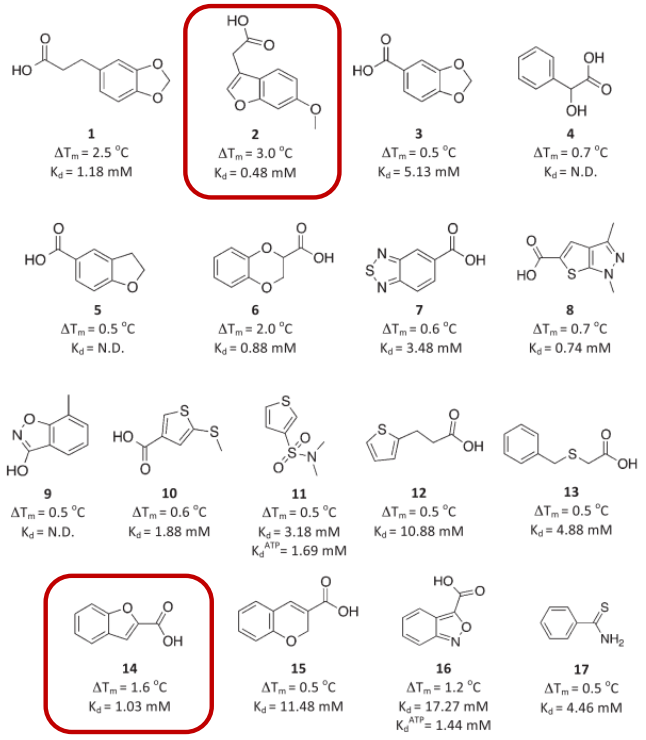


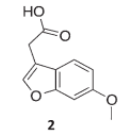
Fig. 1. The hits identified in the primary thermal shift screen and validated by secondary NMR spectroscopy screen, with K_d determined by ITC. The fragments exhibited a varied range in both ΔT_m (from 0.5 to 3.0 °C) and affinities (K_d from 480 μM to 17.3 mM).

Follow – up on hits to obtain thermodynamics of binding

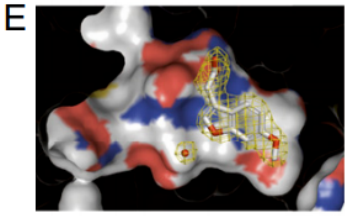
Table 1. Thermodynamic binding parameters determined by ITC

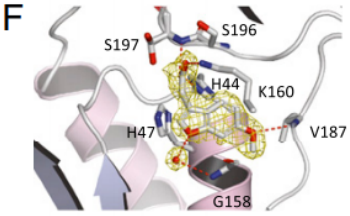
Fragment	ΔH, kcal/mol	ΔG, kcal/mol	K_d, mM	*LE*
1	−13.9 ± 0.1	−4.0 ± 0.1	1.2 ± 0.01	0.29
2	−10.1 ± 0.2	−4.5 ± 0.3	0.5 ± 0.01	0.30
3	−11.6 ± 0.2	−3.1 ± 0.3	5.1 ± 0.2	0.26
6 (racemate)	−10.3 ± 0.05	−4.2 ± 0.1	0.9 ± 0.04	0.32
6 (R-enantiomer)	−7.9 ± 0.1	−4.1 ± 0.1	0.9 ± 0.02	0.32
6 (S-enantiomer)	−12.3 ± 0.2	−4.3 ± 0.2	0.7 ± 0.02	0.33
7	−9.4 ± 0.1	−3.3 ± 0.1	3.5 ± 0.04	0.28
8	−7.51 ± 0.1	−4.2 ± 0.1	0.7 ± 0.02	0.32
10	−7.2 ± 0.1	−3.7 ± 0.1	1.9 ± 0.03	0.37
11	−16.2 ± 0.8	−3.4 ± 0.9	3.2 ± 0.2	0.31
11 (in the presence of ATP)	−1.5 ± 0.05	−3.8 ± 0.1	1.7 ± 0.08	0.34
12	−21.2 ± 1.0	−2.7 ± 1.0	10.9 ± 0.6	0.27
13	−10.7 ± 0.3	−3.1 ± 0.3	4.9 ± 0.2	0.26
14	−8.7 ± 0.2	−4.0 ± 0.2	1.0 ± 0.01	0.33
15	−12.3 ± 0.7	−2.3 ± 0.7	11.5 ± 0.8	0.18
16	−11.3 ± 0.02	−2.6 ± 0.1	17.3 ± 0.8	0.22
16 (in the presence of ATP)	−1.8 ± 0.4	−3.8 ± 0.4	1.4 ± 0.02	0.32
17	−5.3 ± 0.2	−3.1 ± 0.2	4.5 ± 0.2	0.34

binding mode of **2**

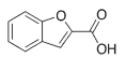


2
ΔT_m = 3.0 °C
K_d = 0.48 mM

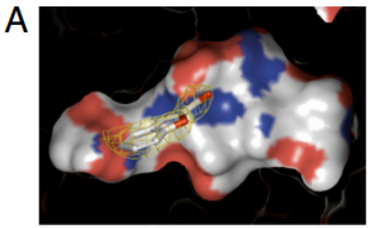


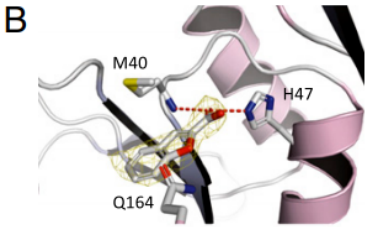


binding mode of **14**

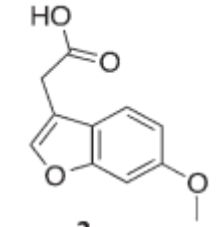


14
ΔT_m = 1.6 °C
K_d = 1.03 mM





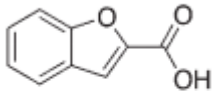
From fragments to inhibitors



2

$\Delta T_m = 3.0\ ^{\circ}C$

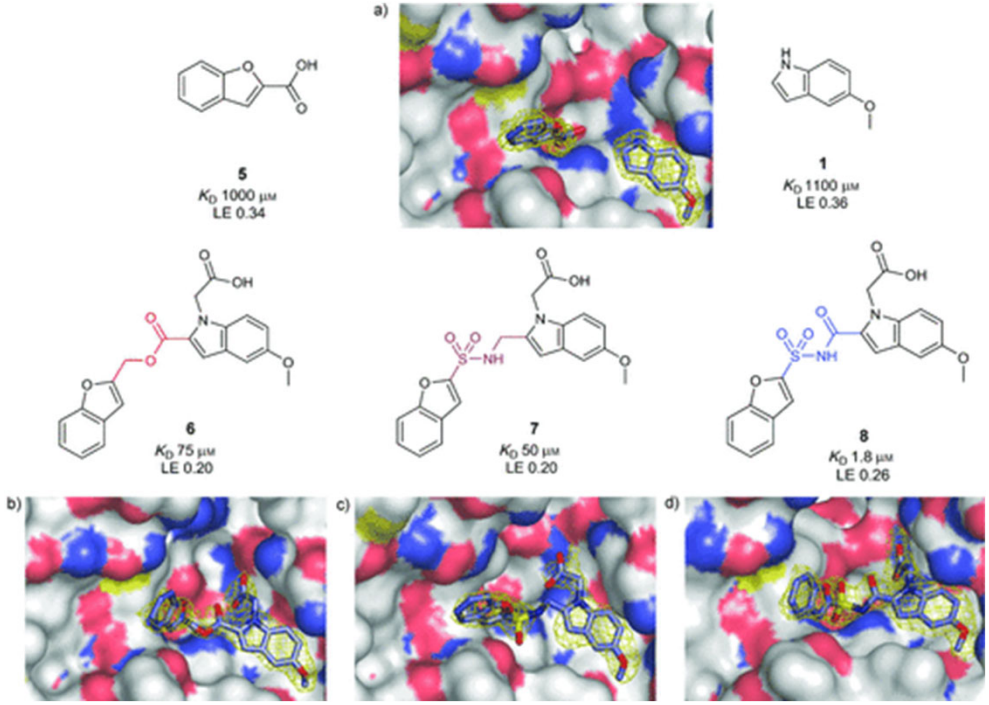
$K_d = 0.48$ mM



14

$\Delta T_m = 1.6\ ^{\circ}C$

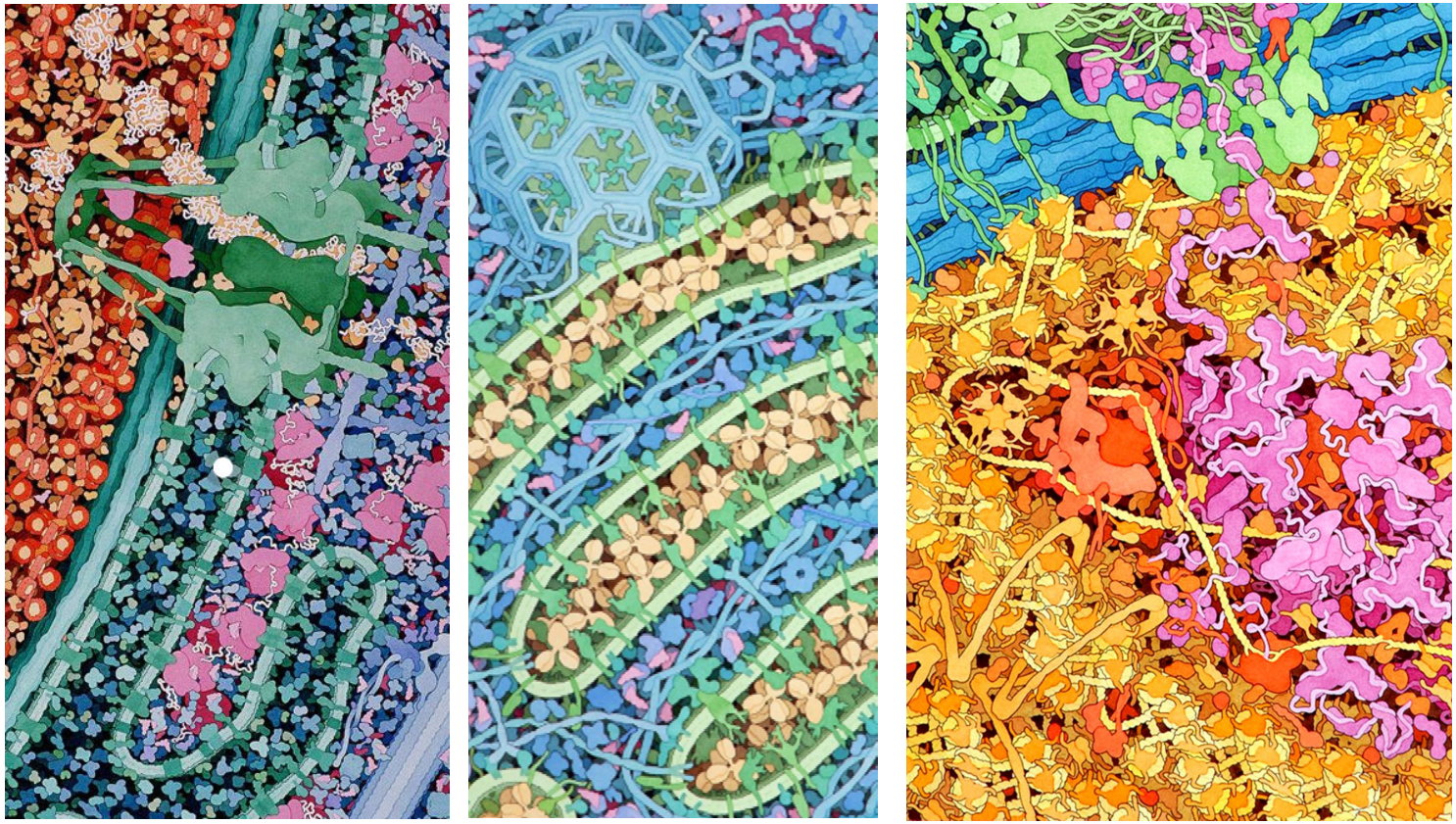
$K_d = 1.03$ mM



A fragment-linking strategy designed for *M. tuberculosis* pantothenate synthetase

Hung et al. Angew Chem 2009

Binding interactions are the foundation of biology



David Goodsell

Cellular signaling – Receptor ligand interactions

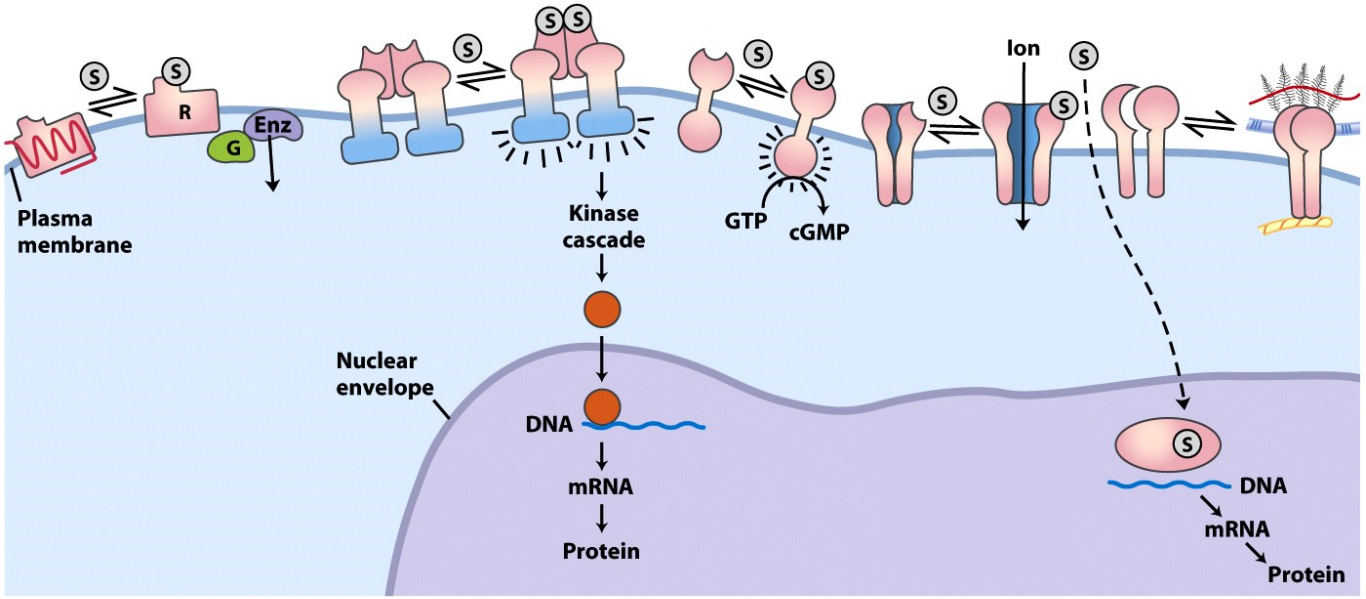
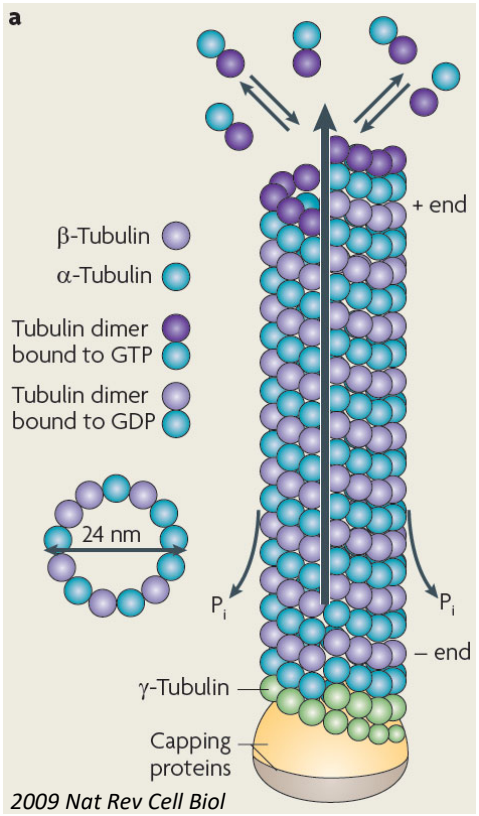
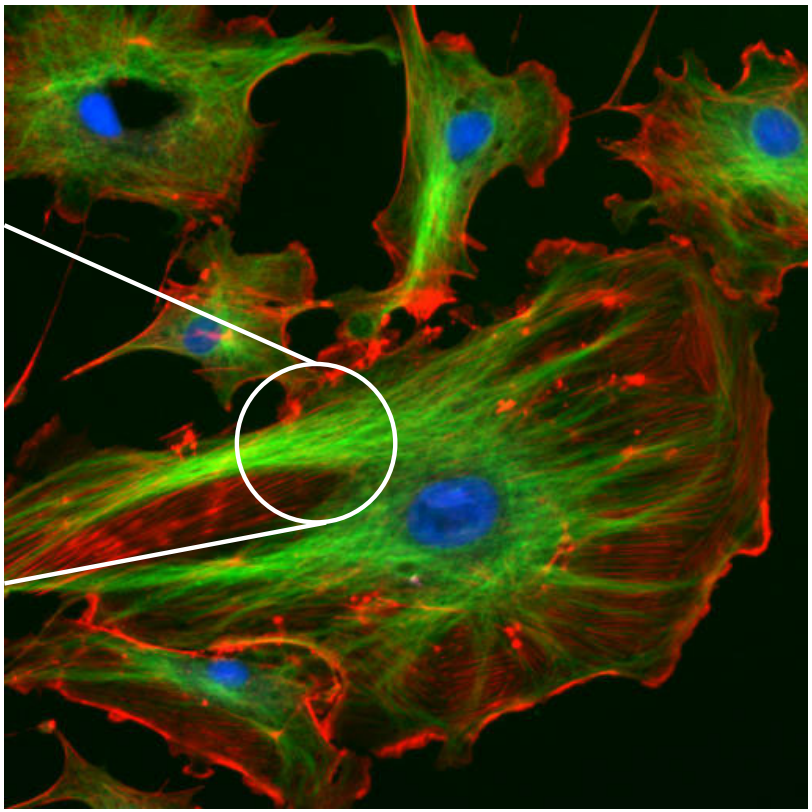


Figure 12-2
Lehninger Principles of Biochemistry, Fifth Edition

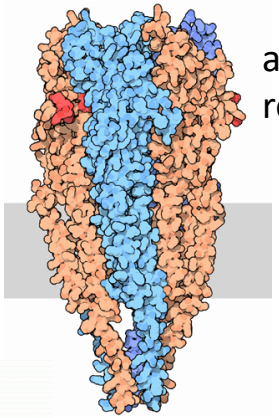
Protein – protein association: Large structures



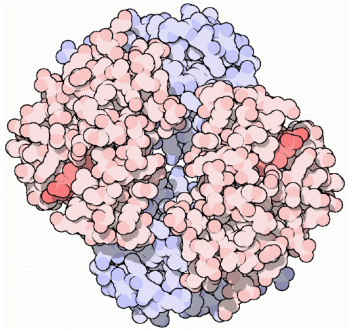


Types of association reactions

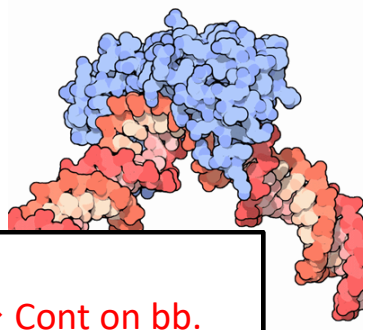
- **Protein – protein binding (homo-oligomers, hetero-oligomers)**
  - oligomeric enzymes
    - metabolic enzymes
    - ribosomes
    - channel proteins and pores
    - molecular machines
  - structural proteins
    - cytoskeleton
    - extracellular proteins
- **Receptor – ligand interactions**
  - signaling and import
  - cell surface receptors
  - intracellular receptor
- **Protein – DNA binding**
  - transcription factors
  - chromatin
  - DNA enzymes



acetylcholine receptor



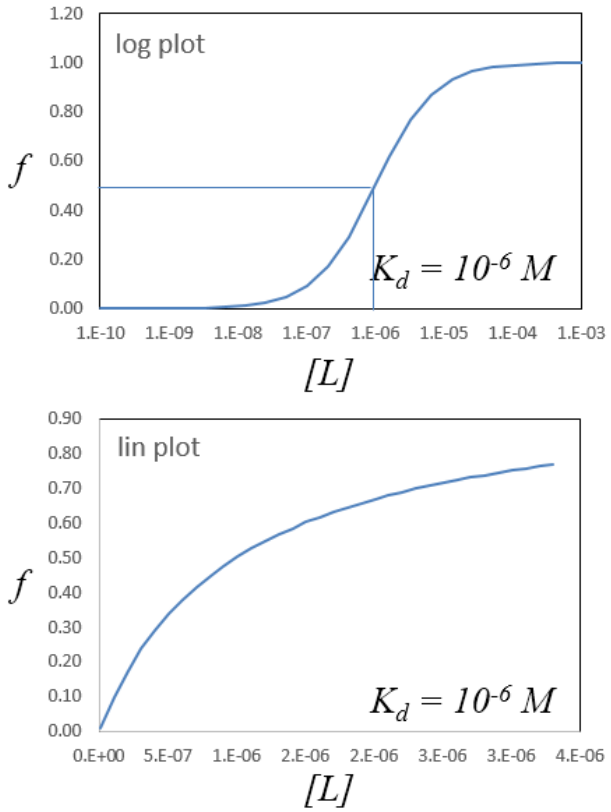
hemoglobine



TATA-bin…
pro…

→ Cont on bb.

Binding curves for one site binding



$$R + L \rightleftharpoons RL$$

sigmoidal binding curve

advantage of semilogarithmic plot:
both baselines are available

The free energy of binding

The energy of a ligand-receptor interaction is determined by ΔG :

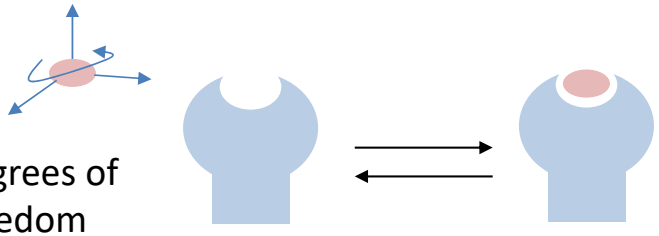
$$\Delta G = RT \ln K_D$$

... whereas ΔG itself can be separated into enthalpy and entropy

$$\Delta G = \Delta H - T\Delta S$$

+ ionic
+ vdW
+ H-bonds

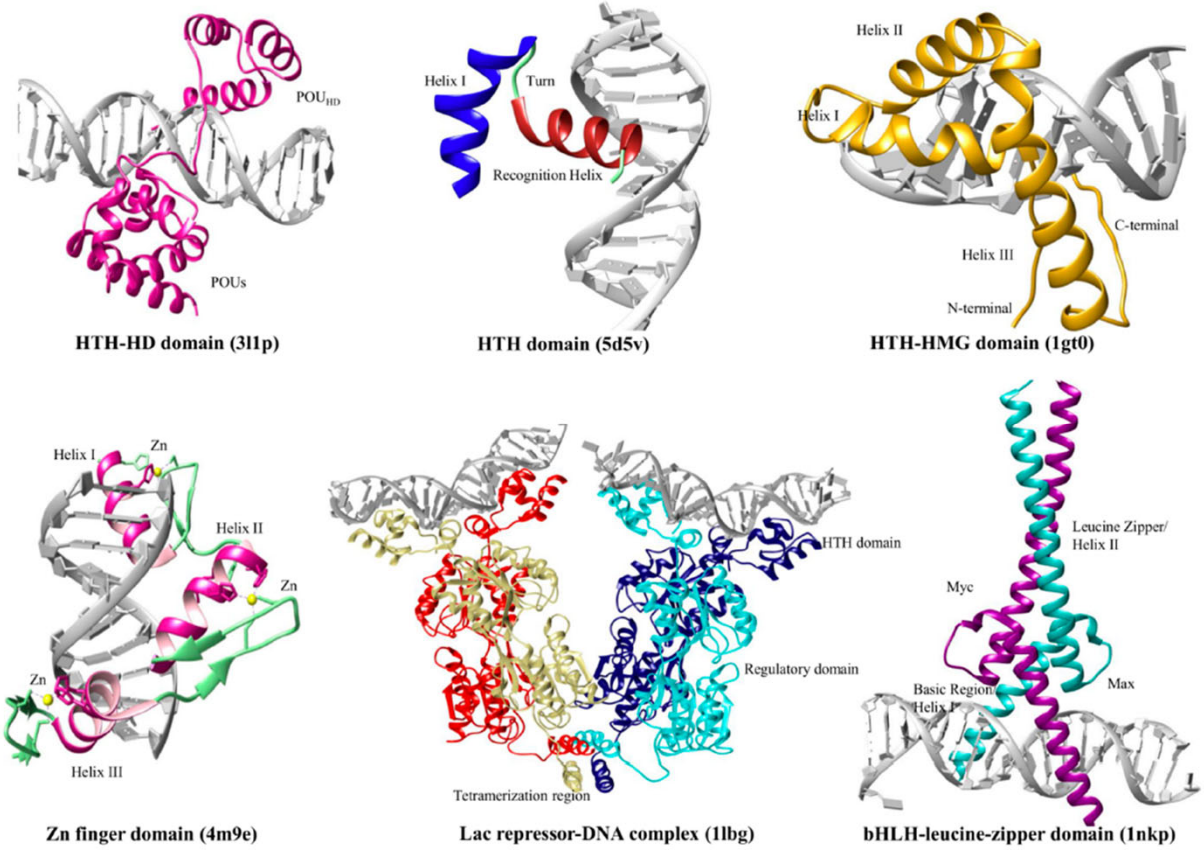
+ hydrophobic effect
- loss of ligand entropy!



degrees of freedom

degrees of freedom fixed!

Calculation example

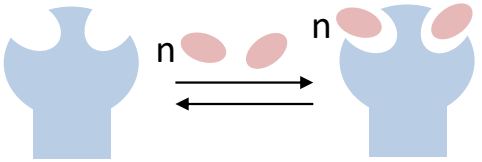


- You have a protein (transcription factor) binding to a particular DNA sequence with a dissociation constant of 10^{-9} M.
- Now, you test a different sequence and find that the protein binds tenfold (10x) better
- what is the **free energy difference** between the two interactions?
- Could you suggest what molecular interaction could account for that difference?

https://doi.org/10.3390/genes8080192

Binding to multiple non-interacting sites

$$R + nL \rightleftarrows \rightleftarrows \ldots \rightleftarrows RL_n$$



for a single site: $L + s \rightleftarrows L_b$ $\quad K_d = \dfrac{[s][L]}{[L_b]}$

s: free binding site

L_b: bound ligand

$$f = \frac{RL + 2RL_2 + 3RL_3 + \ldots}{R + RL + RL_2 + \ldots} = \frac{\sum_{i=1}^{n} iRL_i}{\sum_{i=0}^{n} RL_i}$$

fraction of ligand bound per macromolecule

$$f = \frac{\sum_{i=1}^{n} i[R][L]^i / K'_i}{\sum_{i=0}^{n} [R][L]^i / K'_i} = \frac{\sum_{i=1}^{n} i[L]^i / K'_i}{\sum_{i=0}^{n} [L]^i / K'_i}$$

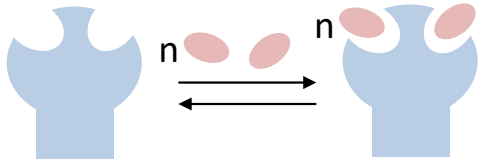
$$\boxed{f = \frac{n[L]}{[L] + K_d}}$$

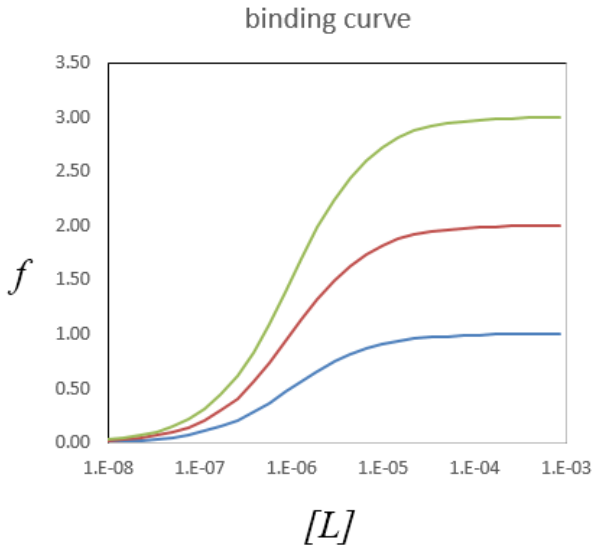
Binding isotherm for multi-site binding

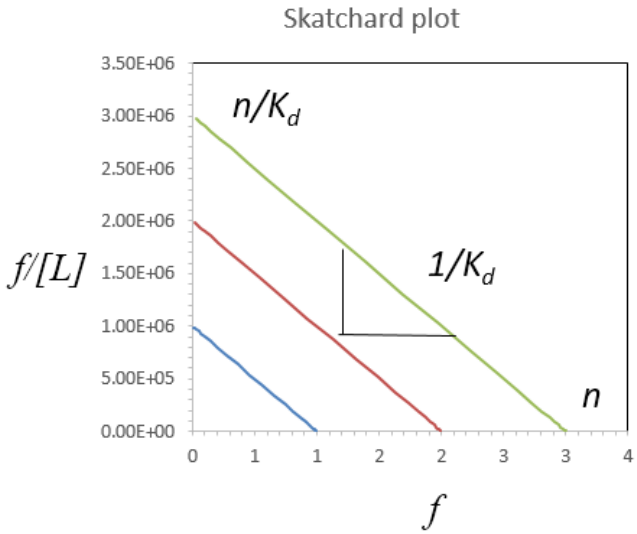
$$K'_i = K_1 \cdot K_2 \cdot K_3 \cdot \ldots K_i = \prod_i K_i$$

Scatchard plot for multisite binding

Linearization method







Plot of the ratio of bound ligand, L_b and unbound ligand, L vs. L_b

Cooperative binding

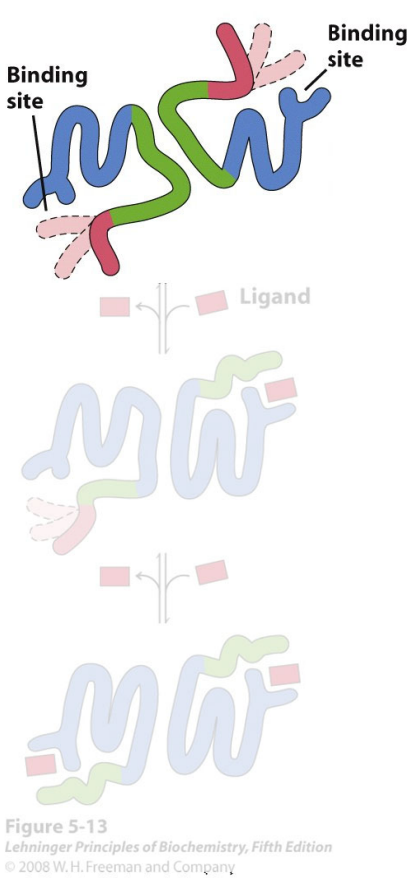


Figure 5-13
Lehninger Principles of Biochemistry, Fifth Edition

no ligand, flexible regions at binding site, interior low stability regions

one site bound
protomer converted into high affinity conformation
second binding site stabilized

second ligand bound with higher affinity
→ positive cooperativity

Cooperativity: Hemoglobin

free

O_2 bound

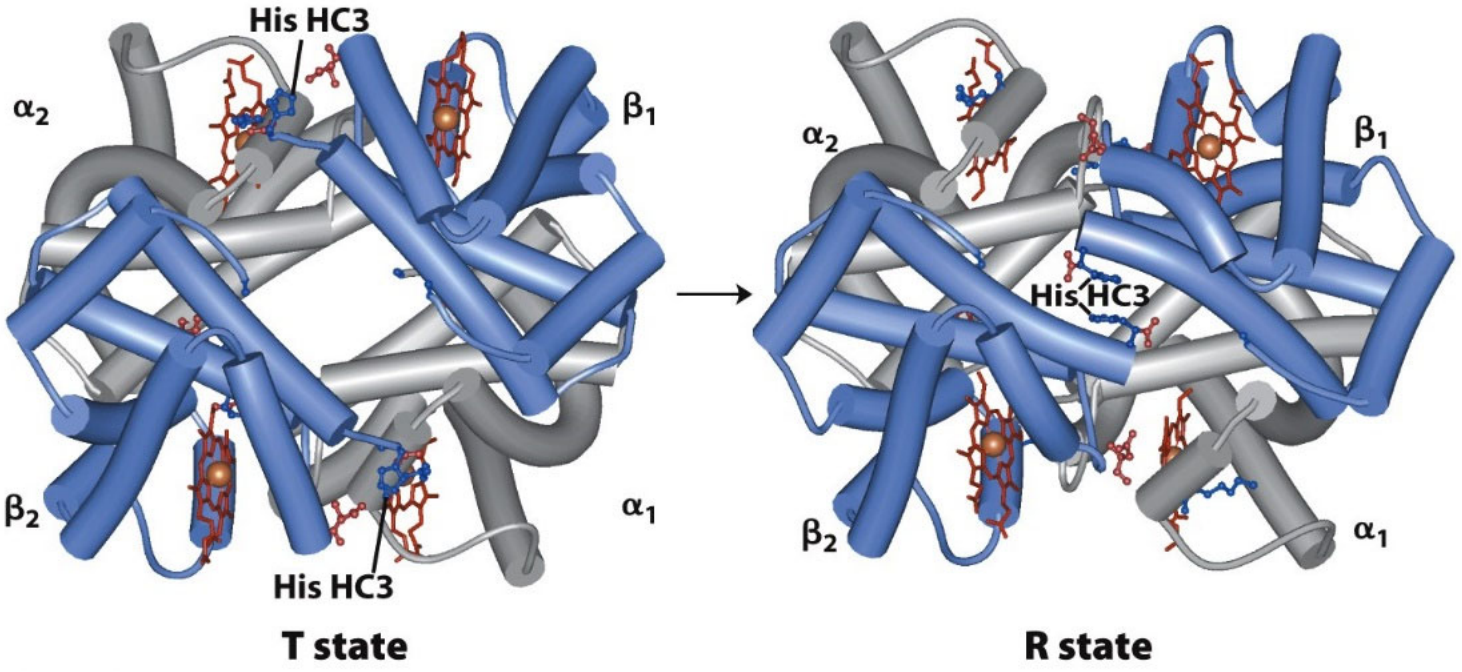
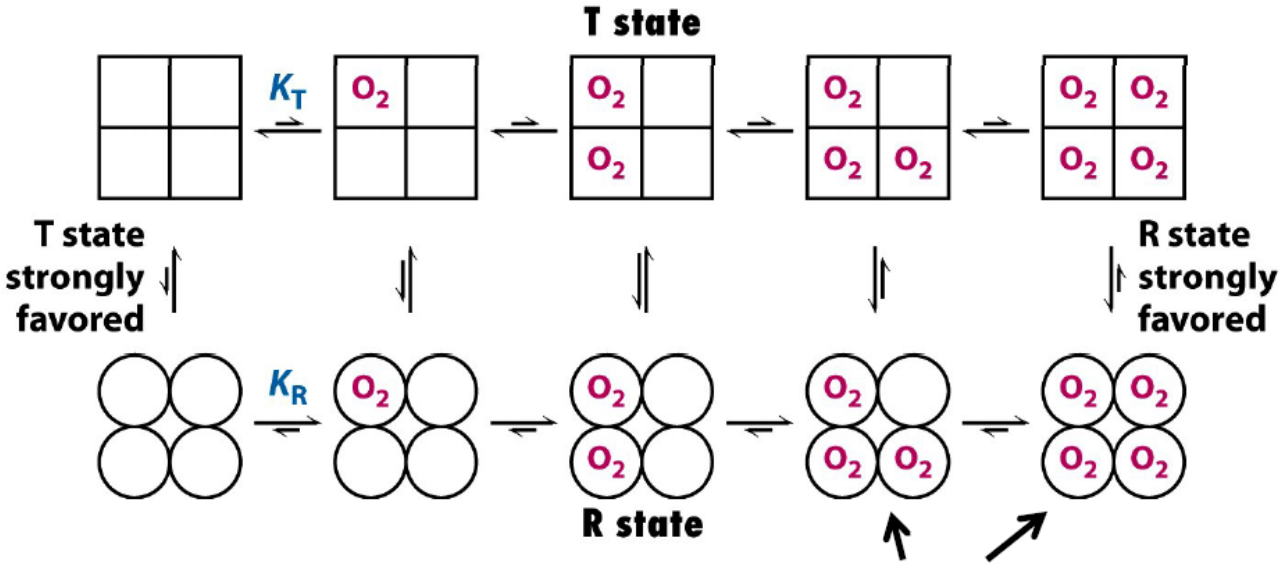


Figure 5-10
Lehninger Principles of Biochemistry, Fifth Edition

Fun fact: 750 g hemoglobin per adult, 10^{22} molecules (10^8 molecules per blood cell).

Cooperativity: Hemoglobin



Cooperativity: Hemoglobin

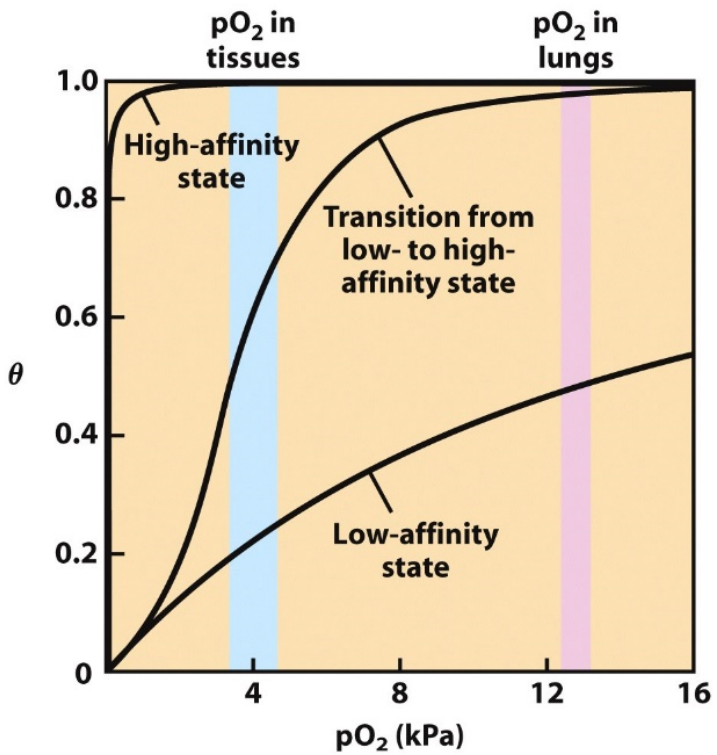


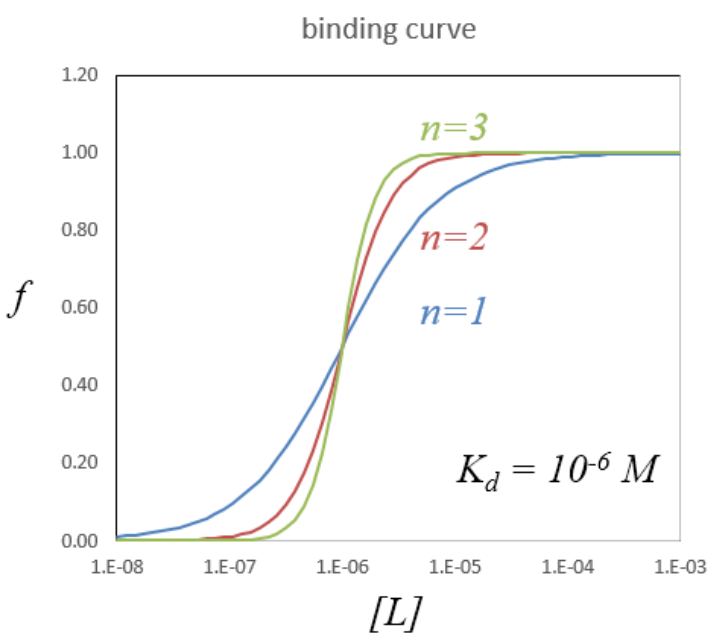
Figure 5-12
Lehninger Principles of Biochemistry, Fifth Edition

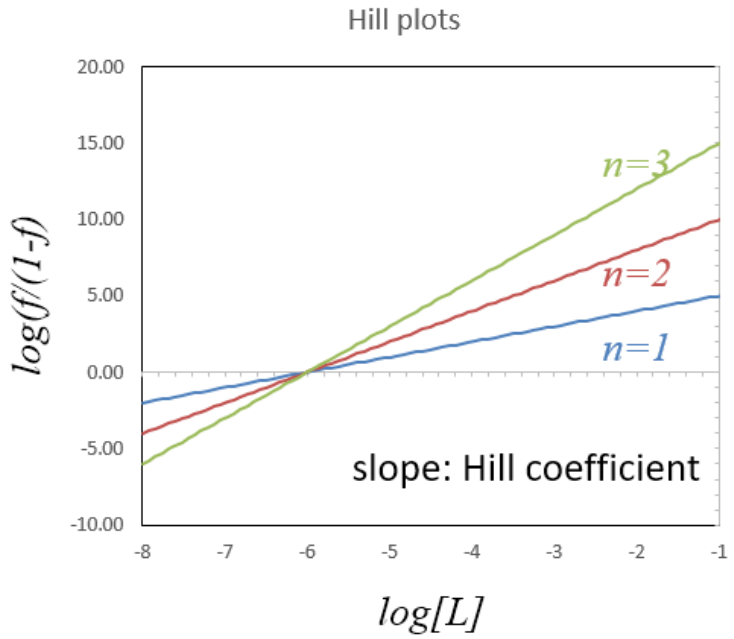
Finely tuned transition between high- to low affinity state

- transport function of hemoglobin
- transfer to myoglobin (only high affinity state)
- blood O2 saturation: critical parameter

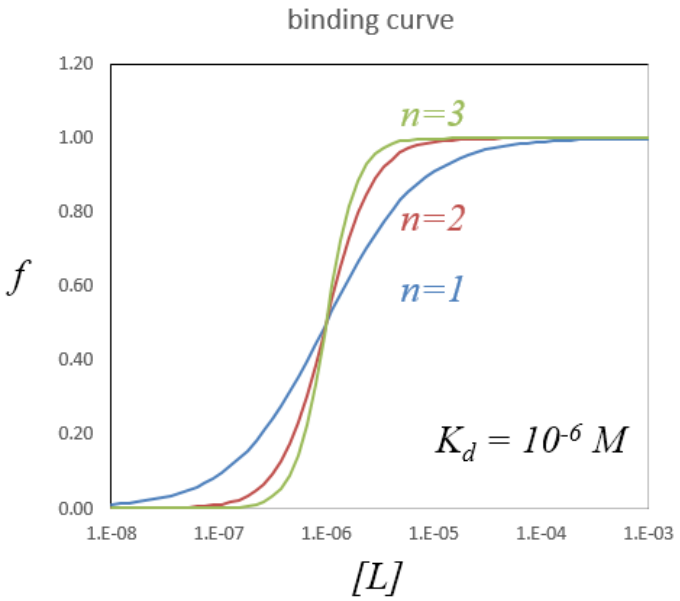
discussion on bb

The Hill plot





Interpretation of the Hill coefficient



For a receptor / protein with x binding sites:

Total cooperativity (all-or-none transition): $n = x$

No cooperativitiy: $n = 1$

All other cases: $1 < n < x$

Empirical description of the behavior

Effect of the receptor concentration

$$R + L \underset{K_d}{\rightleftharpoons} RL$$

Up to this point, we have assumed that [R] is much lower than the K_d

discussion on bb

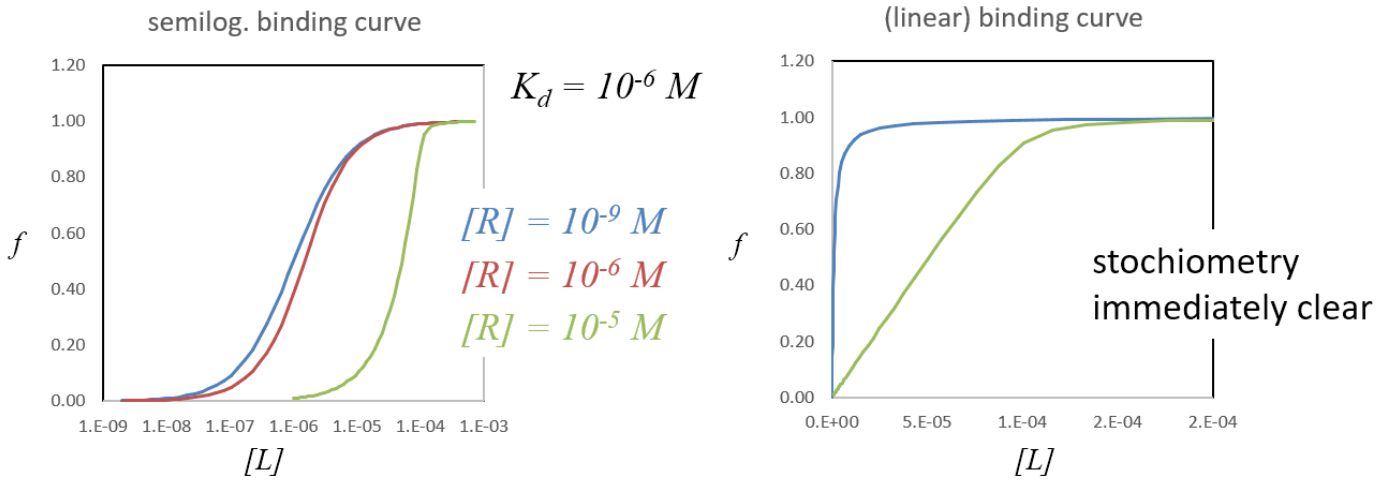
Effect of the receptor concentration

$$R + L \underset{K_d}{\rightleftharpoons} RL$$

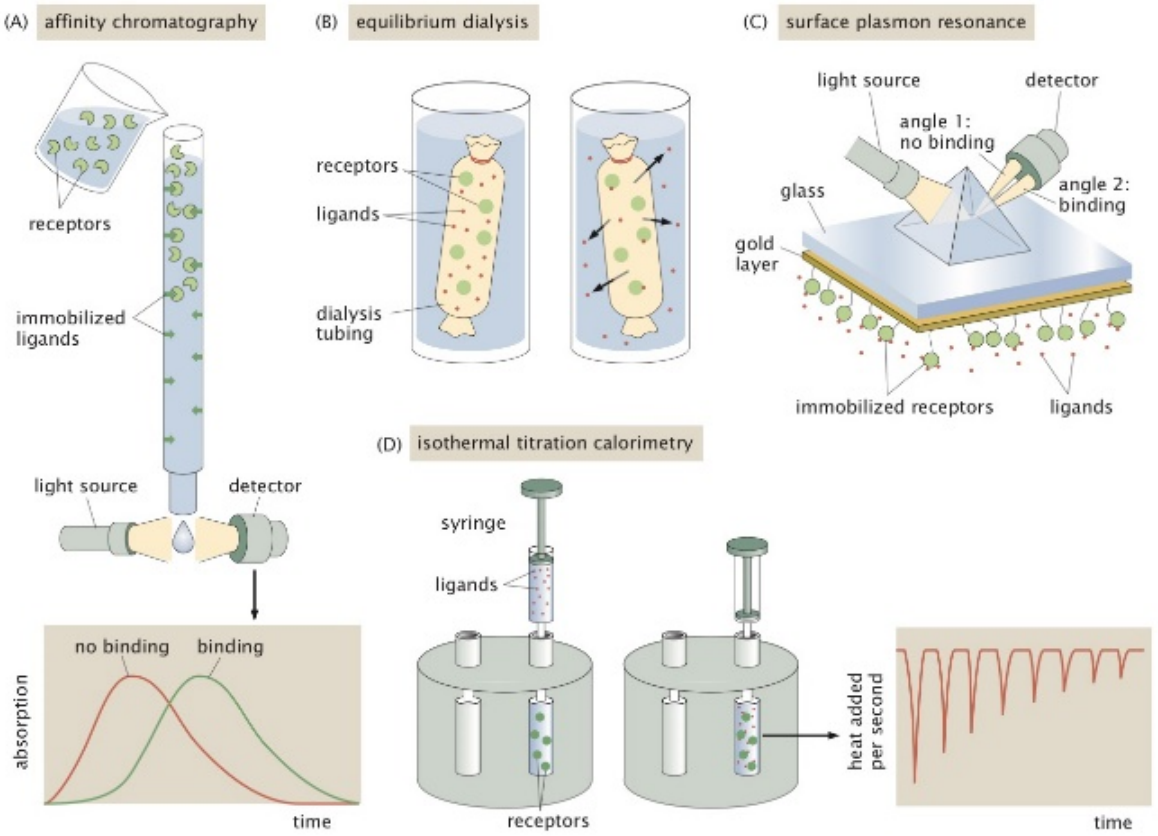
Up to this point, we have assumed that [R] is much lower than the K_d

if this is not the case the fraction bound ligand is expressed as:

$$\frac{[RL]}{[R_{tot}]} = \frac{K_d + [R_{tot}] + [L_{tot}]}{2} - \frac{\sqrt{(K_d + [R_{tot}] + [L_{tot}])^2 - 4[R_{tot}][L_{tot}]}}{2}$$



Measuring binding interactions



Fluorescence anisotropy: Transition dipole moment

Interaction with light:
Incident light ***E*** induces a dipole μ_{ind}:

$$\mu_{ind} = \alpha \bullet E$$

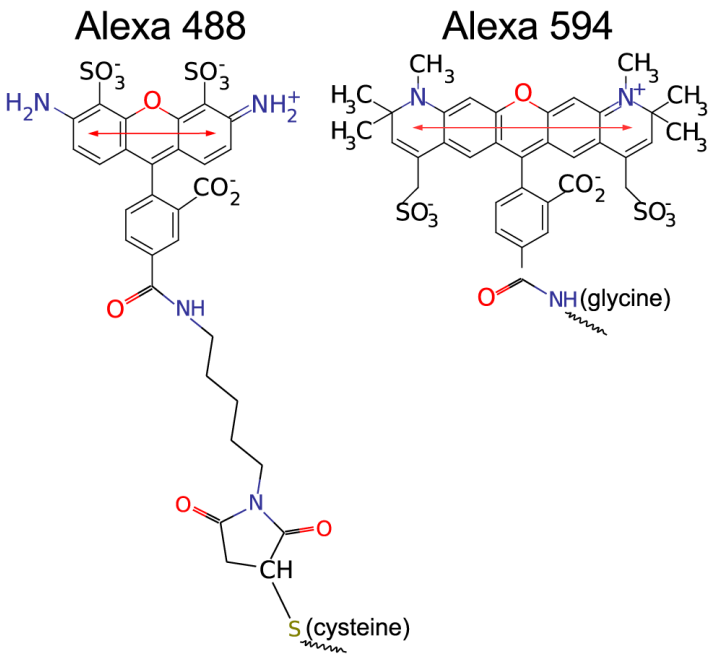
α : polarisability

Transition dipole moment:

Ground state wave funct.: ψ_a
Excited state wave funct.: ψ_b

$$\left\langle \psi_b \left| \underline{\boldsymbol{\mu}} \right| \psi_a \right\rangle$$

can be interpreted as a vector



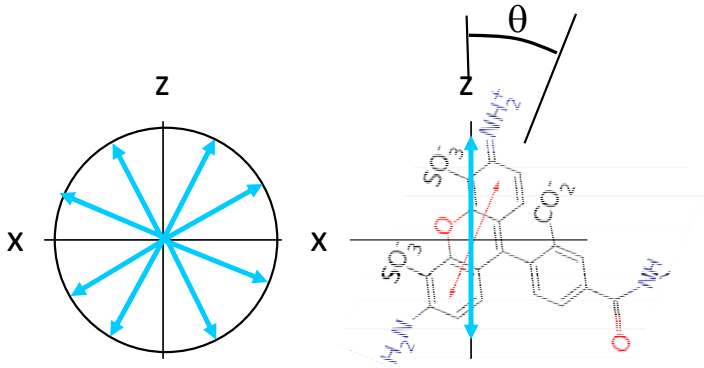
Hoefling et al. PLOS ONE 2011

Excitation of chromophore subpopulation

Conditions:
Immobile chromophores (e.g. embedded in a glass)

Excitation light **vertically polarized**

Probability of absorption: $p \sim cos^2\theta$



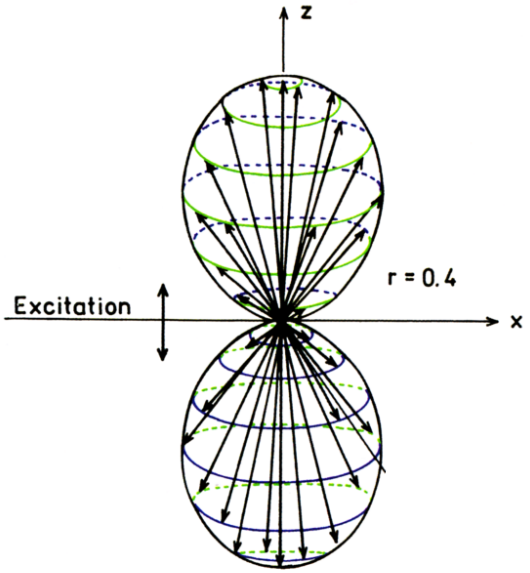


Figure 10.6. Excited-state distribution for immobile fluorophores with $r_0 = 0.4$.

Lakowicz, Principles of fluorescence spectroscopy

Fluorescence emission anisotropy

Conditions:

Emission dipole colinear absorption dipole

No molecular motion

Emission **is polarized**

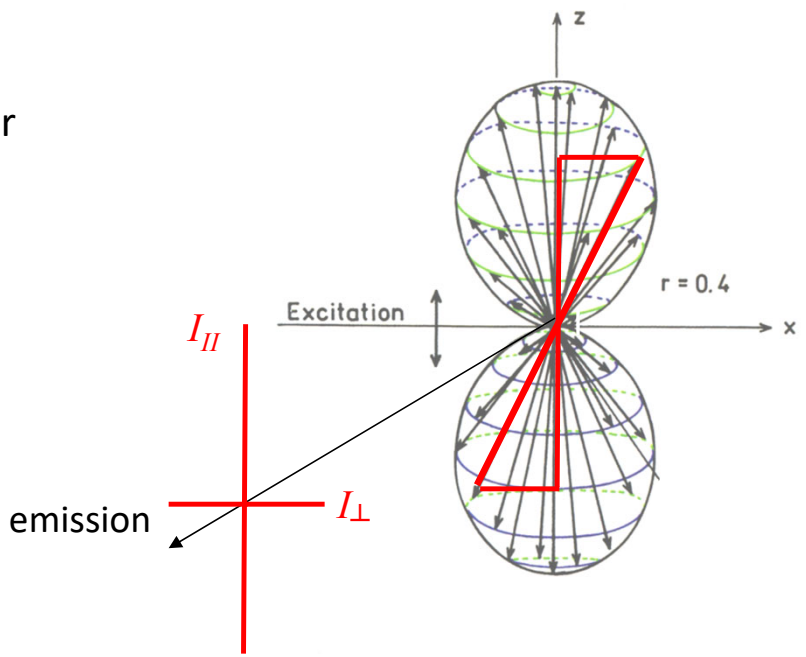
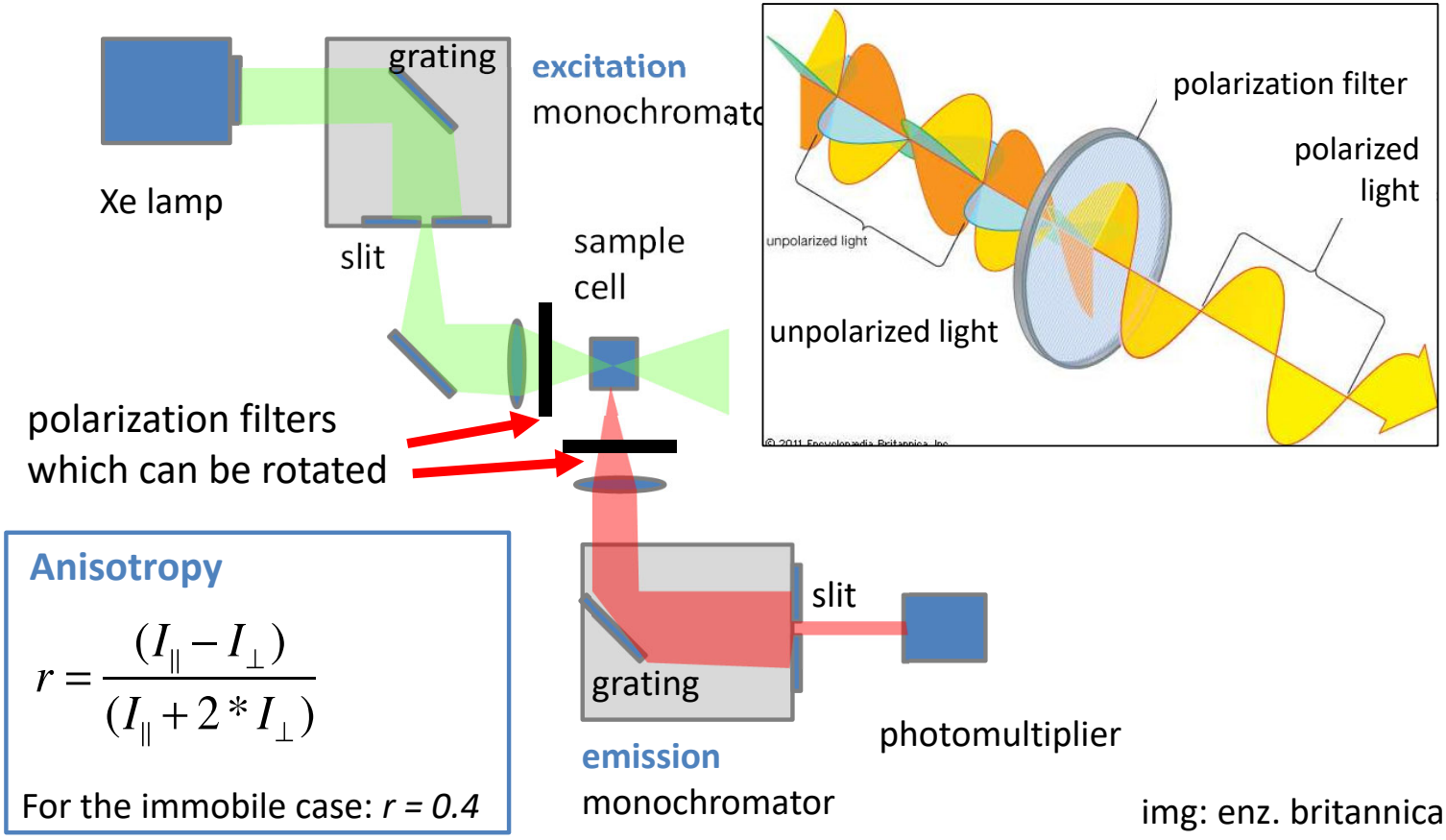


Figure 10.6. Excited-state distribution for immobile fluorophores with $r_0 = 0.4$.

Lakowicz, Principles of fluorescence spectroscopy

Measuring fluorescence anisotropy



Anisotropy

$$r = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + 2 * I_{\perp})}$$

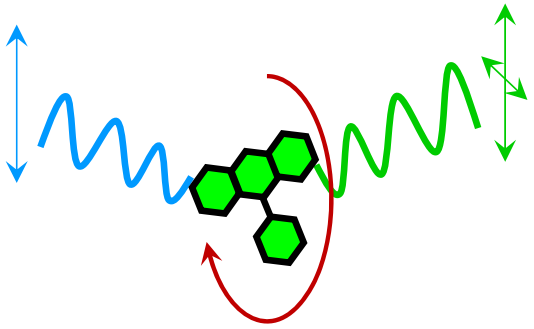
For the immobile case: *r = 0.4*

img: enz. britannica

Loss of fluorescence anisotropy

Anisotropy

$$r = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + 2 * I_{\perp})}$$



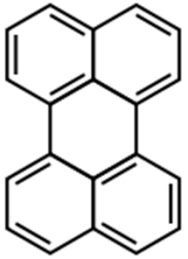
Rotational diffusion:

The Perrin equation

$$\frac{r_o}{r} = 1 + \frac{\tau}{\theta} = 1 + 6D\tau$$

r_0: anisotropy in the absence of motion
τ : fluorescence lifetime
θ : rotation correlation time
D : rotational diffusion coefficient

e.g.: Perylene



r_0: 0.36
τ : 6 ns
Anisotropy in solution (EtOH): 0.005

Loss of fluorescence anisotropy: Proteins

Rotational diffusion:

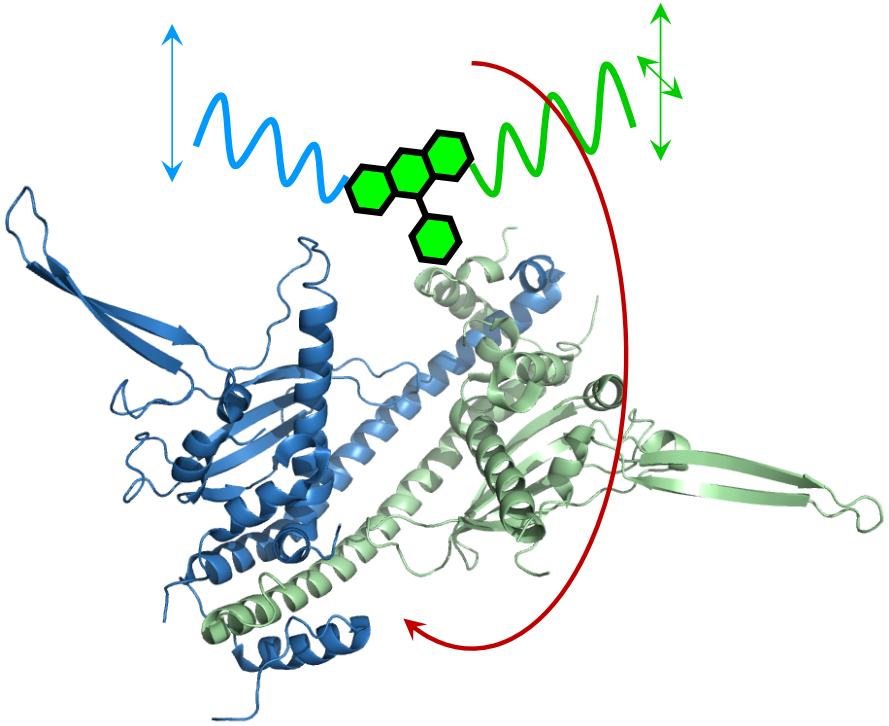
The Perrin equation

$$\frac{r_o}{r} = 1 + \frac{\tau}{\theta} = 1 + 6D\tau$$

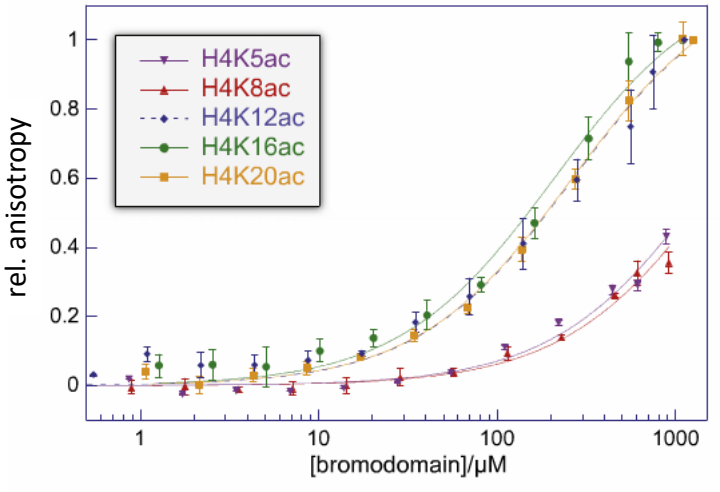
For a 50 kDa protein the rotation correlation time θ = 14 ns

$$\theta = \frac{\eta V}{RT} = \frac{\eta}{RT} \cdot M(\bar{v} + h)$$

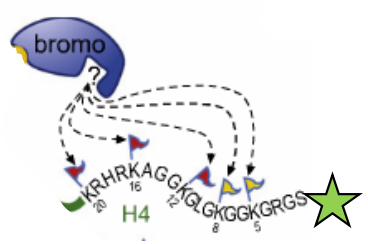
η = viscosity
V = volume
M = molecular weight
v = specific volume protein
h = hydration (g/g protein)



Measuring protein-protein interactions with anisotropy



Ruthenburg et al. Cell 2011



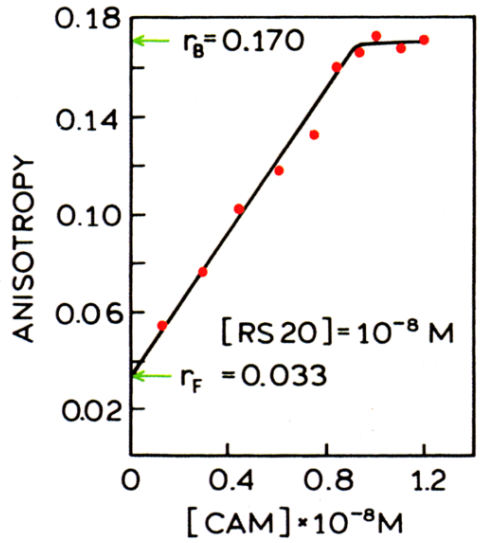
Protein domain (bromodomain) interacting with modified histone peptides

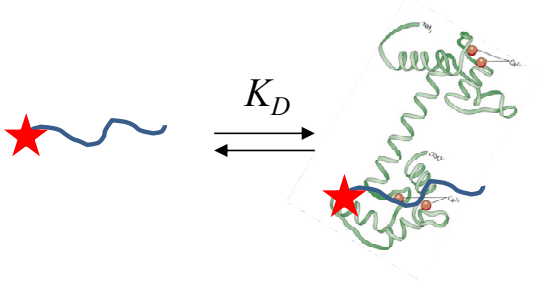
Peptides contain fluorophore, are kept at the same concentration

Protein is titrated and anisotropy is determiend for each concentration

→ Kd is obtained

Stochiometry determined by anisotropy measurement



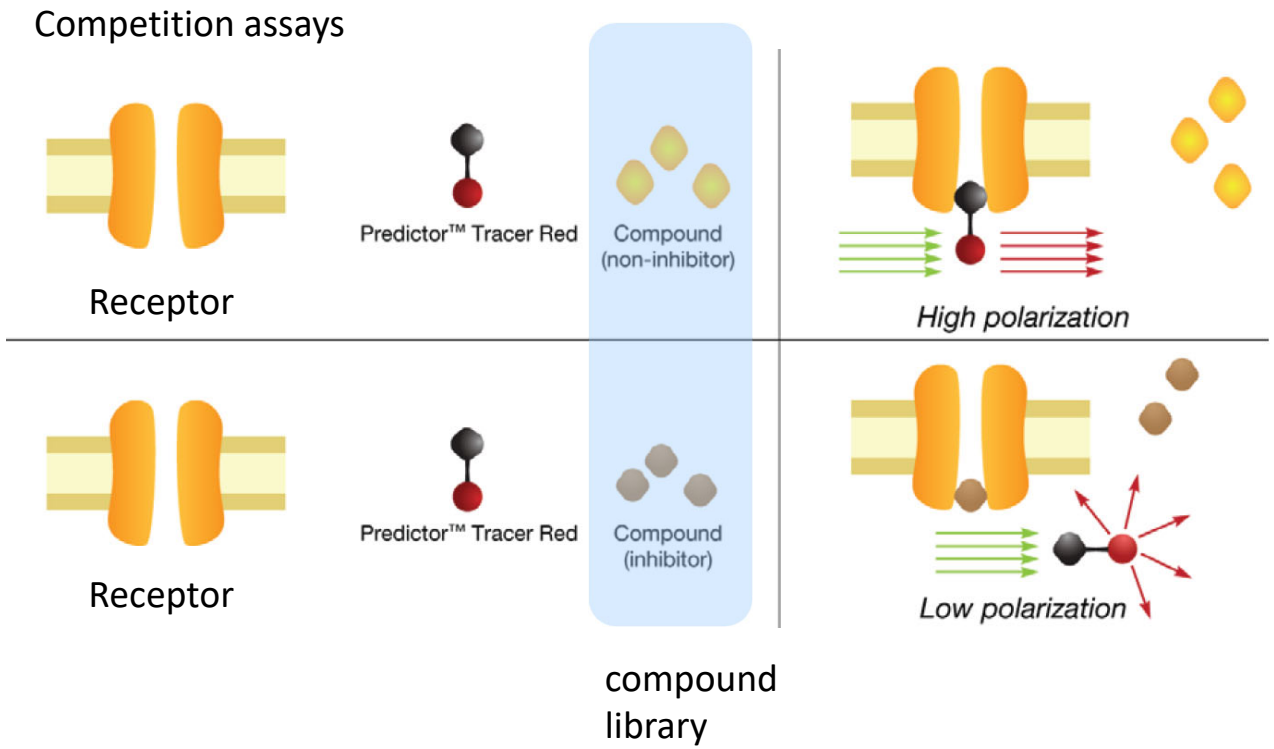


Fluorescence anisotropy measurements can be used to determine binding reactions

Example: MLCK peptide (RS20) binds calmodulin

Determining tryptophan **fluorescence anisotropy** in peptide, the binding constant can be determined in a **titration**.

Fluorescence anistropy in HTS

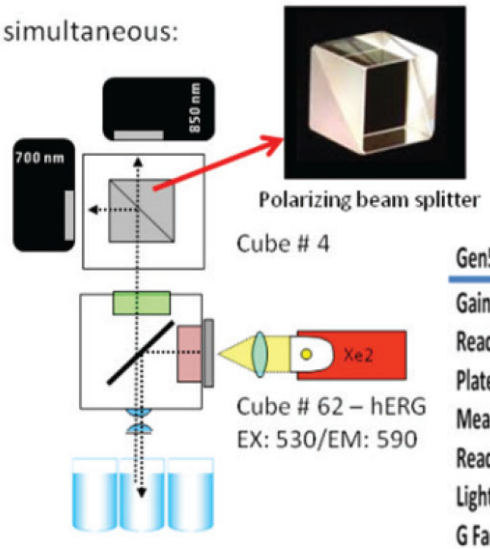


High throughput evaluation using plate readers

Fluorescence Polarization

- Specialized optics:
  - FP cube beam splitter
  - Non-standard high-transmission filters

Top simultaneous:



Polarizing beam splitter

Cube # 4

Cube # 62 – hERG
EX: 530/EM: 590

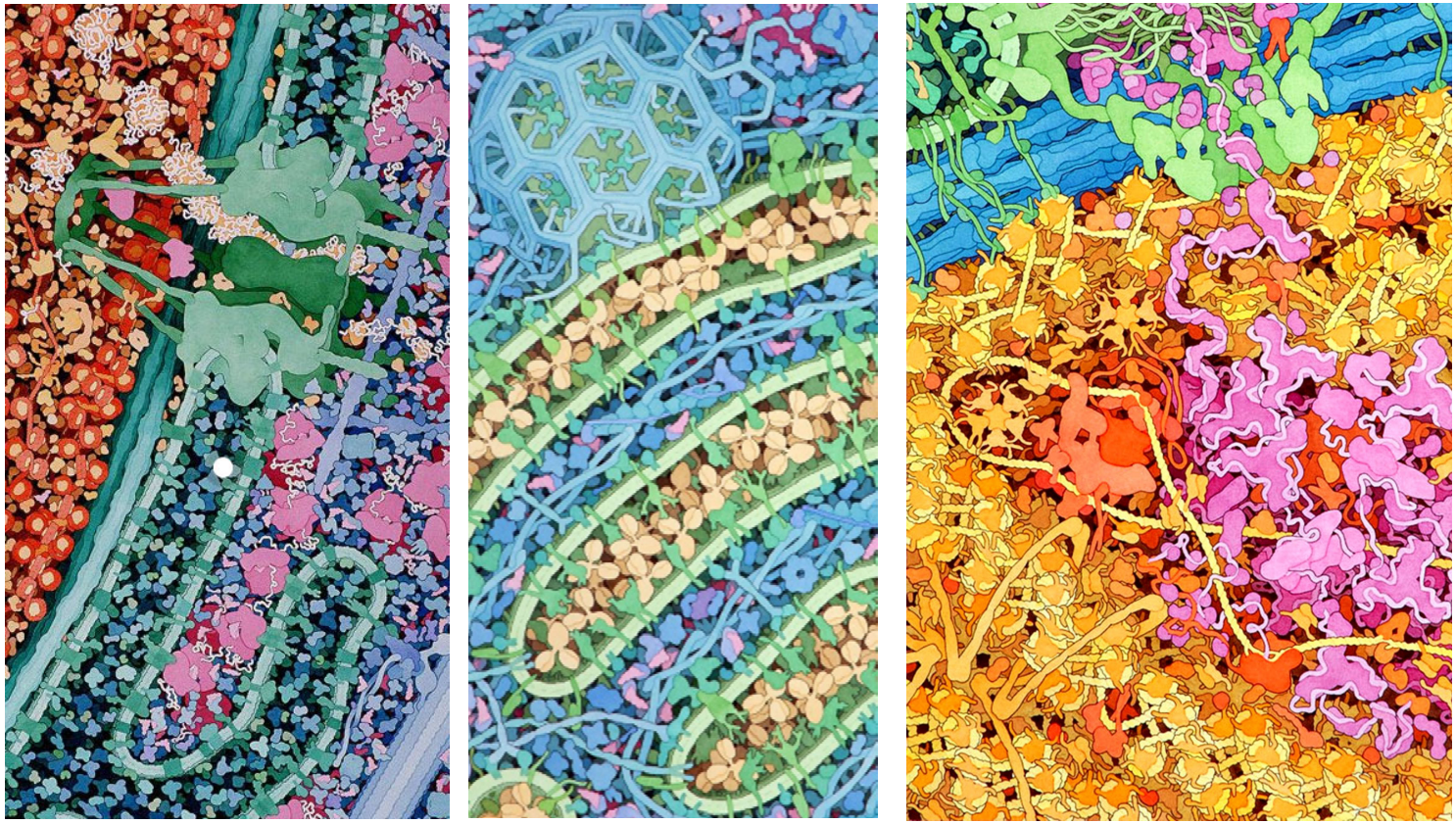
Gen5™ Protocol Parameters	Top PMT	Side PMT	
Gain*	97	119	–
Read Speed	–	–	Normal
Plate Movement Delay	–	–	0
Measurements/well**	–	–	10-200
Read Height (mm)	–	–	10.25
Light Source	–	–	Xenon, Low
G Factor	–	–	Fixed value: 1

** It is recommended Gain be determined for each reader.*

***Higher measurements/well can result in greater precision & specificity but lower read times*

Source: Biotek

In cells, interactions are managed by compartmentalization



David Goodsell