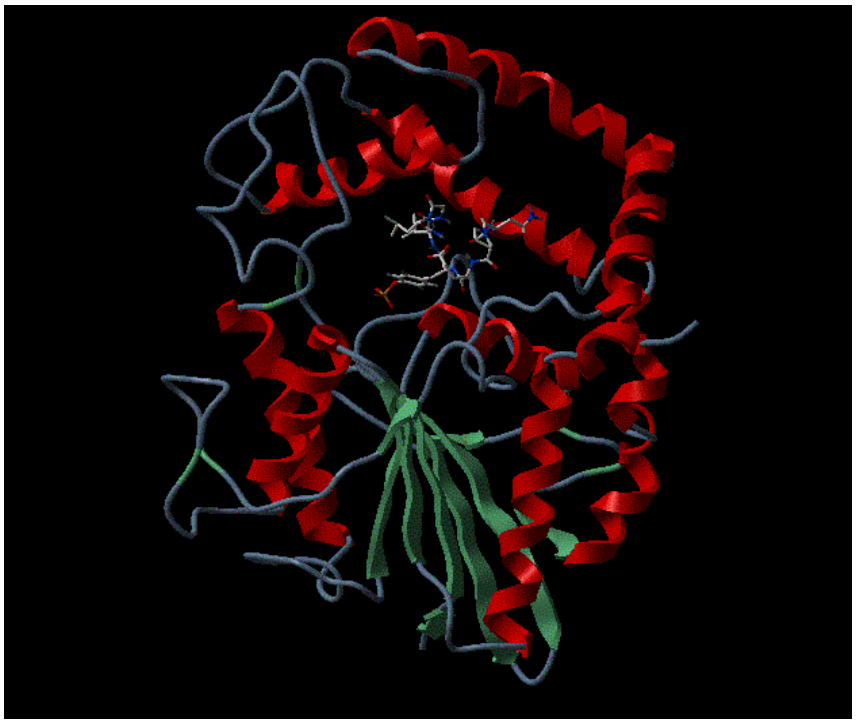
Physical Biochemistry

Protein – ligand binding

Protein-ligand association

- Protein have the fundamental ability to selectively bind to other molecules.
- Important for:
  - Enzyme function.
  - Receptor actions (membrane).
  - Self-organization cellular structures and multicomponent protein complexes.
- Important to understand, both quantitatively and qualitatively.

Protein-ligand binding



$$P + L \Leftrightarrow PL$$

- Protein ligand binding is a spontaneous process.
- Similar forces as in protein folding are at work.
- Function of proteins is defined through its interactions with other molecules.

Overview

- Dissociation constant
- One or more possibly independent binding sites
- Cooperative effect:
  - The binding of the first ligand may affect the binding of the next ligand
  - Positive, negative cooperative effect
- Multivalent interactions:
  - Multiple possibly weak interactions between ligand and protein can lead to a strong affinity

Single binding site

$$P + L \Leftrightarrow PL$$

$$K_b = \frac{[PL]}{[P][L]}$$

$$\Delta_b G^{\Theta} = -RT \ln K_b$$

$$K_d = \frac{[P][L]}{[PL]} = \frac{1}{K_b}$$

$$\Delta_d G^{\Theta} = -RT \ln K_d$$

- Simplest case: one ligand species L:
  - [PL] complex
  - [P] : Free protein (**not** total protein)
  - [L] : Free ligand (**not** total ligand)
  - [P] + [PL] : **Total** protein
  - [L] + [PL] : **Total** ligand
- K_b is binding constant:

  $\Delta_b G^{\Theta}$ is the standard Gibbs free energy of binding
- Biochemistry: reported is the **dissociation constant K_d**:

  $\Delta_d G^{\Theta}$ is the standard Gibbs free energy of dissociation.
  - Free energy usually not reported.

Single binding site

- Protein - ligand solution:
  - P, L, PL are given as concentrations
- Average number of ligand molecules bound to each protein:

$$\text{General: } \bar{n} = \frac{\text{concentration of L bound to P}}{\text{Total concentration of P}}$$

$$\text{Single binding site, one ligand species: } \bar{n} = \frac{[\mathrm{PL}]}{[\mathrm{P}] + [\mathrm{PL}]}$$

Single binding site

$$\bar{n} = \frac{[\mathrm{PL}]}{[\mathrm{P}] + [\mathrm{PL}]} \qquad K_d = \frac{[\mathrm{P}][\mathrm{L}]}{[\mathrm{PL}]} \Rightarrow [\mathrm{PL}] = \frac{[\mathrm{P}][\mathrm{L}]}{K_d}$$

$$\bar{n} = \frac{[\mathrm{PL}]}{[\mathrm{P}] + [\mathrm{PL}]} = \frac{\frac{[\mathrm{P}][\mathrm{L}]}{K_d}}{[\mathrm{P}] + \frac{[\mathrm{P}][\mathrm{L}]}{K_d}} \times \frac{[\mathrm{P}]^{-1}}{[\mathrm{P}]^{-1}}$$

Single binding site, one ligand species:

$$\bar{n} = \frac{[\mathrm{L}]}{K_d + [\mathrm{L}]}$$

$$0 \le \bar{n} \le 1$$

'titration curve'

$a \times a^{-1} = 1$

Fractional saturation

Single binding site, one ligand species: $\bar{n} = \dfrac{[\mathrm{L}]}{K_d + [\mathrm{L}]}$

$$0 \le \bar{n} \le 1$$

Occupancy, fractional saturation: $\theta = \dfrac{\bar{n}}{n}$

General definition.
n = Total number of binding sites.

$$0 \le \bar{n} \le n$$

$$0 \le \theta \le 1$$

Single binding site, one ligand $\Rightarrow \bar{n} = \theta$ species

Scatchard plot

Single binding site, one ligand species: $\theta = \frac{[PL]}{[P]+[L]} = \frac{[L]}{K_d + [L]}$

Hughes-Klotz plot: $\frac{1}{\theta} = 1 + \frac{K_d}{[L]}$ $\frac{1}{\theta}$ versus $\frac{1}{[L]}$

Scatchard plot: $\frac{\theta}{[L]} = \frac{1}{K_d} - \frac{\theta}{K_d}$ $\frac{\theta}{[L]}$ versus θ

Straight line

Scatchard plot: derivation

$$\theta = \frac{[\mathrm{L}]}{K_d + [\mathrm{L}]} \Rightarrow \frac{\theta}{[L]} = \frac{1}{K_d + [\mathrm{L}]}$$

$$\frac{1}{K_d + [\mathrm{L}]} = \frac{1}{K_d} - \frac{1}{K_d} + \frac{1}{K_d + [\mathrm{L}]} =$$

$$\frac{1}{K_d} - \frac{K_d + [L] - K_d}{K_d(K_d + L)} = \frac{1}{K_d} - \frac{[L]}{K_d(K_d + L)} \Rightarrow$$

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

Example

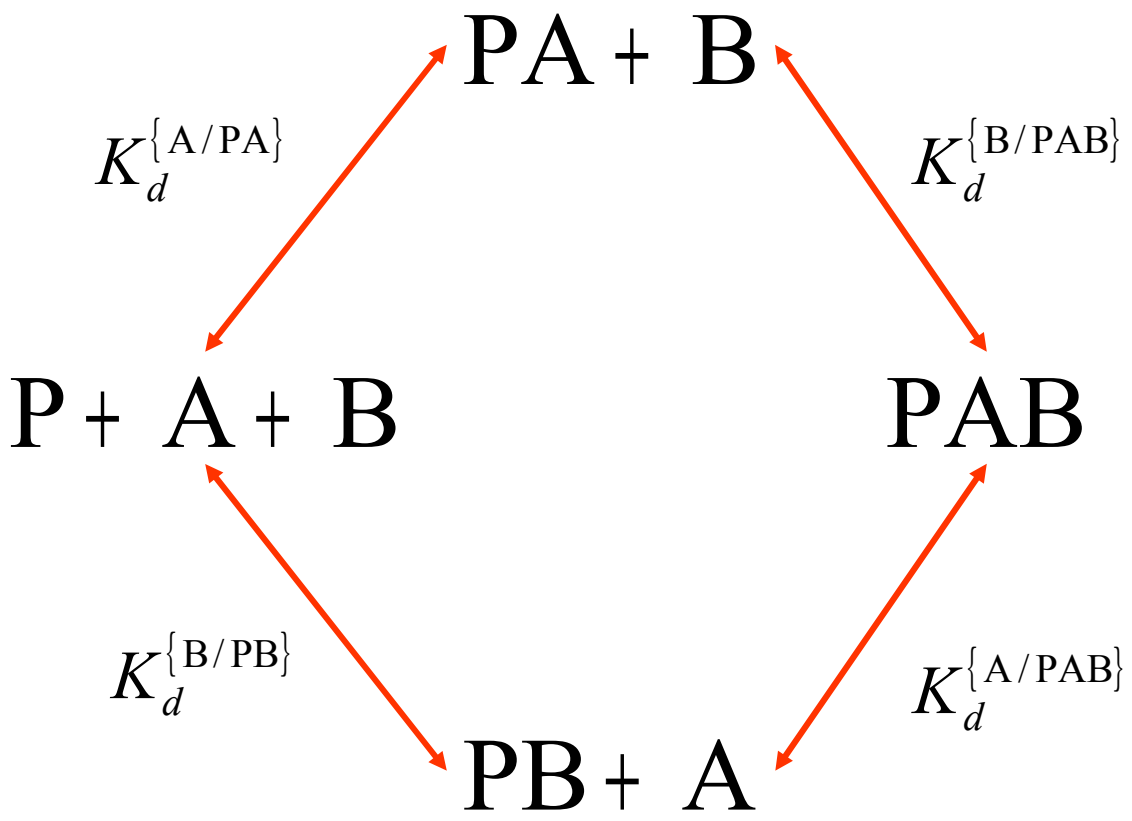
- Mg^{2+} and ADP form a 1:1 complex. In an binding experiment, the total concentration of ADP was kept constant at 80 μM. The following results were obtained. Determine K_d.

Microsoft Excel Worksheet

Kd-single-binding-site.xsl

Total Mg^{2+} (μM)	Mg^{2+} bound to ADP (μM)
20	11.6
50	26.0
100	42.7
150	52.8
200	59.0
400	69.5

Binding of different ligands to protein



$$K_d^{\{A/PA\}} = \frac{[P][A]}{[PA]}$$

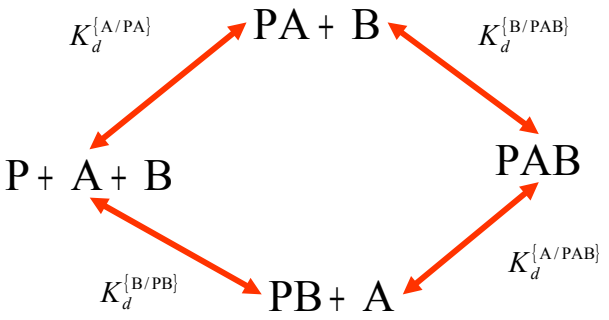
$$K_d^{\{A/PAB\}} = \frac{[PB][A]}{[PAB]}$$

Independent binding

- Binding of A does NOT affect the binding of B and vice versa:

$$K_d^{\{A/PA\}} = K_d^{\{A/PAB\}}$$

$$K_d^{\{B/PB\}} = K_d^{\{B/PAB\}}$$



- Fractional saturation independent: $\theta_{AB} = \theta_A \times \theta_B$
- Independent binding can be treated as before.

Dependent binding

- Binding of A depends on the binding of B and vice versa:
  - Binding constants are different
- Positive cooperativity:
  - Binding of A (B) enhances (makes stronger) binding of B (A) $\Rightarrow$

$$K_d^{\{\mathrm{A/PA}\}} > K_d^{\{\mathrm{A/PAB}\}}$$

$$K_d^{\{\mathrm{B/PB}\}} > K_d^{\{\mathrm{B/PAB}\}}$$

Dependent binding

- Negative cooperativity:
  - Binding of A (B) makes the binding of B (A) weaker.

$$K_d^{\{A/PA\}} < K_d^{\{A/PAB\}}$$

$$K_d^{\{B/PB\}} < K_d^{\{B/PAB\}}$$

- Affects fractional saturation:
  - Positive cooperativity: Increase of [B] increase θ_A
  - Negative cooperativity: Increase of [B] decreases θ_A

Reasons for cooperativity

- Conformational changes induced by ligand binding
- (Un)favorable interactions between ligands.

Binding of L to multiple sites

- For example:
  - Binding of proton H^+ to multiple titrating sites in proteins
  - Acid dissociation constants
  - Titration curves

$$\mathrm{P} + n\mathrm{L} \Leftrightarrow \mathrm{PL} + (n-1)\mathrm{L} \Leftrightarrow \mathrm{PL}_2 + (n-2)\mathrm{L} \Leftrightarrow \cdots \Leftrightarrow \mathrm{PL}_{n-1} + \mathrm{L} \Leftrightarrow \mathrm{PL}_n$$

$\theta = 0$

$\bar{n} = 0$

Complete dissociation of L

Fully saturated

$\theta = 1$

$\bar{n} = n$

Macroscopic versus microscopic

- Macroscopic dissociation constants are experimentally determined values.
- For example, two binding sites or $n = 2$

$$P + 2L \Leftrightarrow PL + L \Leftrightarrow PL_2$$

$$K_d^{\{L/PL\}} = \frac{[P][L]^2}{[PL][L]} = \frac{[P][L]}{[PL]} \qquad K_d^{\{L/PL_2\}} = \frac{[PL][L]}{[PL_2]}$$

Generally: $K_d^{\{L/PL_n\}} = \frac{[PL_n][L]}{[PL_n]}$ $\qquad PL_0 = P$ No L bound

Macroscopic versus microscopic

$$\mathrm{P} + n\mathrm{L} \Leftrightarrow \mathrm{PL} + (n-1)\mathrm{L} \Leftrightarrow \mathrm{PL}_2 + (n-2)\mathrm{L} \Leftrightarrow \cdots \Leftrightarrow \mathrm{PL}_{n-1} + \mathrm{L} \Leftrightarrow \mathrm{PL}_n$$

$$\bar{n} = \frac{\text{concentration of L bound to P}}{\text{Total concentration of P}} = \frac{[\mathrm{PL}] + 2[\mathrm{PL}_2] + \cdots + n[\mathrm{PL}_n]}{[\mathrm{P}] + [\mathrm{PL}] + [\mathrm{PL}_2] + \cdots + [\mathrm{PL}_n]}$$

Macroscopic versus microscopic

- K_d is the microscopic dissociation constant for the binding of a single L to a specific site on the protein:
  - More insight into binding since they relate directly to how strongly a binding site interacts with the ligand.
  - Usually not measurable.
- K_d is not the same as $K_{d:}$
  - Macroscopic constant contains probability of any given L to bind to one of several binding sites.
- When all sites are identical, then their microscopic dissociation constants are identical.
- Both macroscopic and miscroscopic dissociation constants can be expressed in rate constants.

Independent binding of L to multiple identical sites

- Single ligand species.
- All sites same $K_{d,}$.
- No change if more L bind.
- Site are independent.
- Fractional saturation identical.

microscopic

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

Site

Average number of ligands bound per protein:

$$\bar{n} = n\theta = \frac{n[L]}{K_d + [L]}$$

$$\theta = \frac{\bar{n}}{n}$$

Determination of number of sites

$$\bar{n} = n\theta = \frac{n[\mathrm{L}]}{K_d + [\mathrm{L}]} \rightarrow \frac{\bar{n}}{[L]} = \frac{n}{K_d} - \frac{\bar{n}}{K_d}$$

$$y = ax + b$$

$$x = \bar{n}$$

$$y = \frac{\bar{n}}{[\mathrm{L}]}$$

Scatchard plot

$$a = -\frac{1}{K_d}$$

$$b = \frac{n}{K_d}$$

Suppose : $y = 0$

$$x = -\frac{b}{a} \Rightarrow x = -\frac{\frac{n}{K_d}}{-\frac{1}{K_d}} = n$$

$$\bar{n} = \frac{\text{concentration of L bound to P}}{\text{Total concentration of P}}$$

Worked example

- In an experiment the concentration of an enzyme was kept constant at 11 μM and the total concentration of inhibitor was varied. The following results were obtained. Determine the dissociation constant and the number of ligand binding sites. Assume independent sites.

Microsoft Excel Worksheet

Kd-inhibitor.xls

$[I]_{total}$ μM	$[I]_{free}$ μM
5.2	2.3
10.4	4.8
15.6	7.95
20.8	11.3
31.2	18.9
41.6	27.4
62.4	45.8

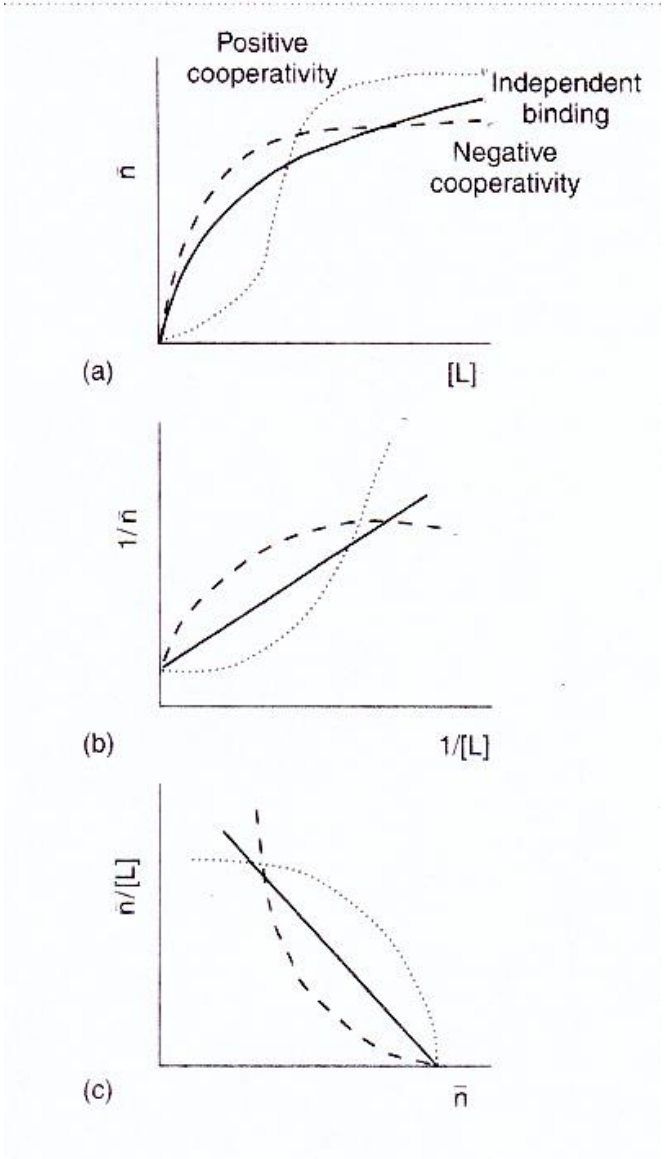
Dependent binding to identical sites

- Microscopic dissociation constants are the same but dependent on the level of occupancy of the other tites.
- Reason:
  - Conformational change upon binding of a ligand
  - Ligand - ligand interaction.
- Consequence:
  - The Scatchard plot is not linear (curved instead of a straight line).

Dependent binding to identical sites

- Positive cooperativity: increased binding with increasing concentration of ligand

$$n = \frac{n[\mathrm{L}]}{\mathrm{K}_d + [\mathrm{L}]}$$



Limiting case of positive cooperativity

- Dependent binding to identical sites:
  - Each site has the same microscopic dissociation constant.
- The only species present are P and PL_n.
- PL_1, PL_2, …, PL_{n-1} are present at very low concentrations.
- The binding of the first ligand increases the affinity at the other sites such that they become immediately fully saturated.
- Remaining equilibrium: $\mathrm{P} + n\mathrm{L} \Leftrightarrow \mathrm{PL}_n$

Limiting case of positive cooperativity

- Same microscopic dissociation constant:
  - Now equal to macroscopic dissociation constant for all ligands because dissociation of one ligand causes dissociation of all.

$$K_d = \mathrm{K}_d = \frac{[\mathrm{P}][\mathrm{L}]^n}{[\mathrm{PL}_n]}$$

$$\bar{n} = \frac{\text{concentration of L bound to P}}{\text{Total concentration of P}} = \frac{n[\mathrm{PL}_n]}{[\mathrm{P}] + [\mathrm{PL}_n]}$$

Limiting case of positive cooperativity

$$K_d = \frac{[P][L]^n}{[PL_n]} \Rightarrow [PL_n] = K_d^{-1}[P][L]^n$$

$$\bar{n} = \frac{n[PL_n]}{[P]+[PL_n]} = \frac{n[P][L]^n K_d^{-1}}{[P]+[P][L]^n K_d^{-1}} = \frac{n[L]^n K_d^{-1}}{1+[L]^n K_d^{-1}} = \frac{n[L]^n}{K_d+[L]^n}$$

The limiting never happens: protein with some of the binding sites occupied can be observed experimentally.

The Hill coefficient

$$\bar{n} = \frac{n[\mathrm{L}]^n}{K_d + [\mathrm{L}]^n} \quad \text{Modeled by} \quad \bar{n} = \frac{n[\mathrm{L}]^h}{K_d + [\mathrm{L}]^h}$$

- The quantity *h* is the Hill coefficient.
- Measure for cooperativity:
  - Positive cooperativity: $h > 1$:
    - Infinite cooperativity: $h = n$.
  - Negative cooperativity: $h < 1$
  - No cooperativity: $h = 1$
    - Equation is identical to that binding to independent sites.

$$\mathrm{P} + n\mathrm{L} \Leftrightarrow \mathrm{PL}_n$$

The Hill coefficient

$$\bar{n} = \frac{n[\mathrm{L}]^h}{\mathrm{K}_d + [\mathrm{L}]^h} \rightarrow \ln\frac{\bar{n}}{n-\bar{n}} = h\ln[L] - \ln \mathrm{K}_d$$

$$\ln\frac{\bar{n}}{n-\bar{n}} \text{ versus } \ln[\mathrm{L}]$$

- Should be a straight line with a gradient (slope) equal to the Hill coefficient *h*.
- In experiment: never the case, since *h* varies with the ligand concentration.
- When [L] → 0 or [L] → ∞ : h → 1 because:
  1. Conformational changes responsible for cooperative effect can occur only when some ligand has bound.
  2. No more cooperativity can taken place when sites but 1 are occupied.

Worked example

- Heamoglobine has four identical sites for O_2. Fractional saturation θ was measured at various partial pressures of O_2. Concentration of the protein is 1.55×10^{-5} μM. Calculate the Hill coefficient at 0%, 50% and 100% saturation.

Microsoft Excel Worksheet

Hill-coefficient.xls

$pO_2 \times 10^3$ (atm)	θ
0.3	0.007
0.5	0.013
1.1	0.030
1.7	0.066
2.8	0.136
3.8	0.273
5.7	0.500
10.1	0.864
15.8	0.953
20.4	0.978
36.6	0.991
109.6	0.007

Example

- Use data from previous example to calculate the microscopic dissociation constant for the tense ($\theta \rightarrow 0$) and relaxed ($\theta \rightarrow 1$) form of heamoglobin. Compute $\Delta\Delta G^{\ominus}$.

- Hint: Use the formula $\ln \frac{\bar{n}}{n - \bar{n}} = h \ln[L] - \ln K_d$

Microsoft Excel Worksheet

Hill-coefficient.xsl

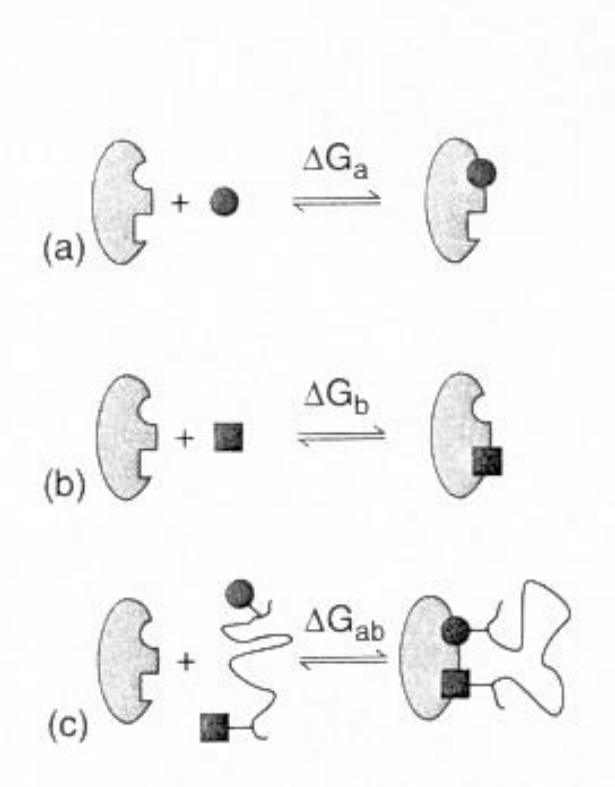
Binding to non-identical sites

- K_d are not the same.
- Sites with the lowest values of K_d will be saturated first
- Fractional saturation still available from

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

- Generally more difficult to handle

Multivalent binding



$$\Delta G_a^{\Theta} + \Delta G_b^{\Theta} < \Delta G_{ab}^{\Theta}$$

$$\Delta G_a^{\Theta} + \Delta G_b^{\Theta} > \Delta G_{ab}^{\Theta}$$

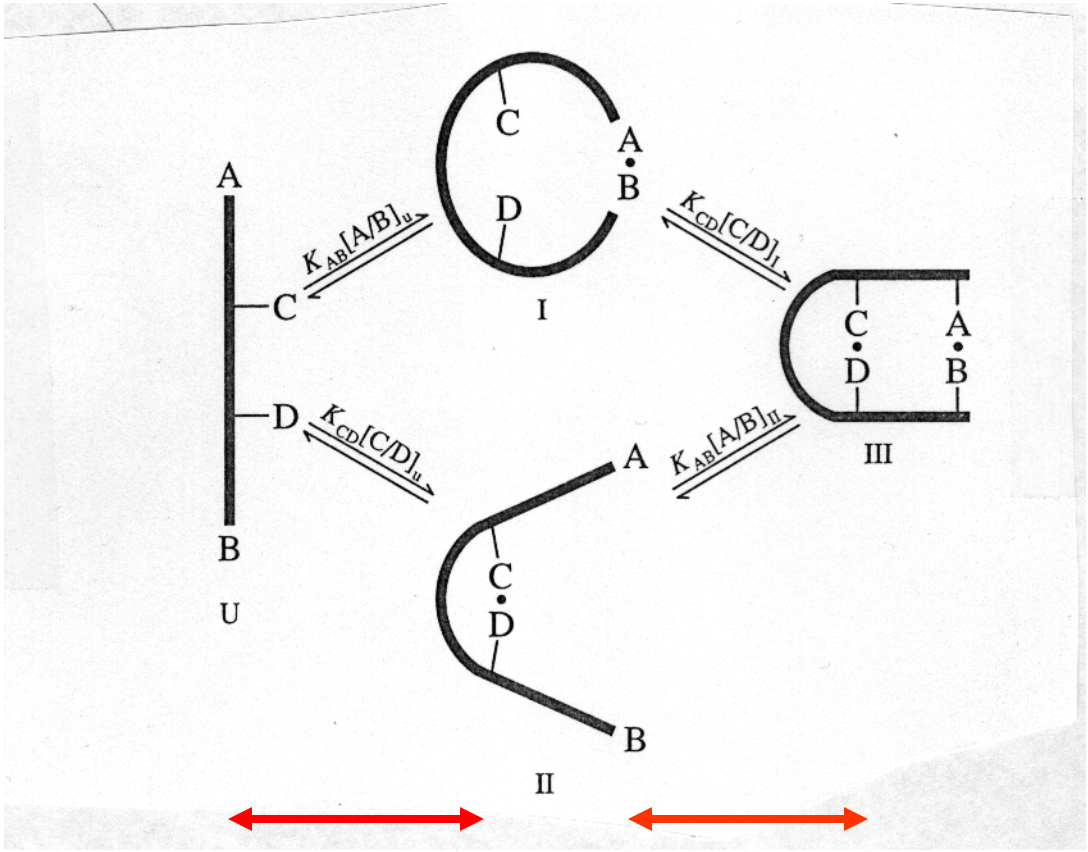
$$\Delta G_a^{\Theta} + \Delta G_b^{\Theta} = \Delta G_{ab}^{\Theta}$$

- Multiple potentially weak interactions could result in a strong affinity.
- Independent binding:

$$\Delta G_a^{\Theta} + \Delta G_b^{\Theta} = \Delta G_{ab}^{\Theta}$$

- If dependent, entropic effects may affect affinity:
  - *E.g.* Loss of degrees of freedom is greater for the independent A and B than for AB.

Effective concentration



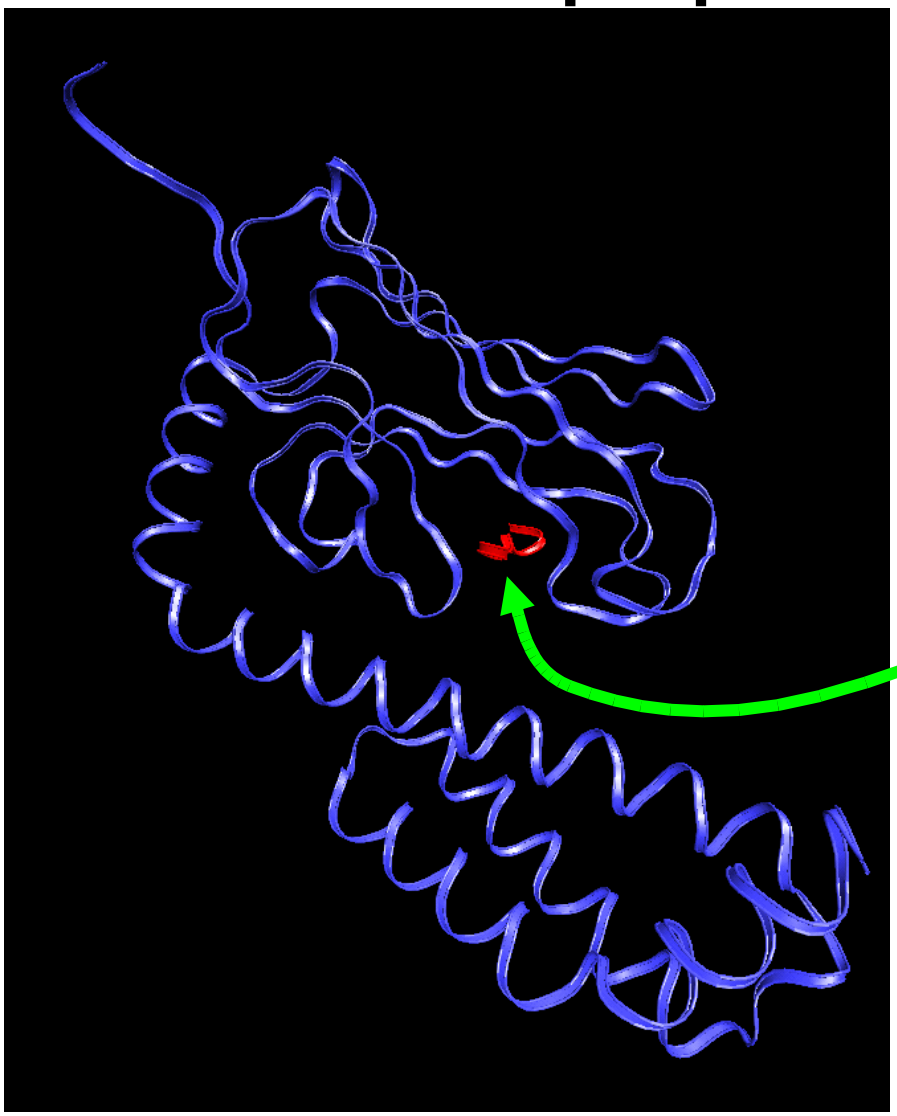
$\Delta S<0$ $\Delta H<0$

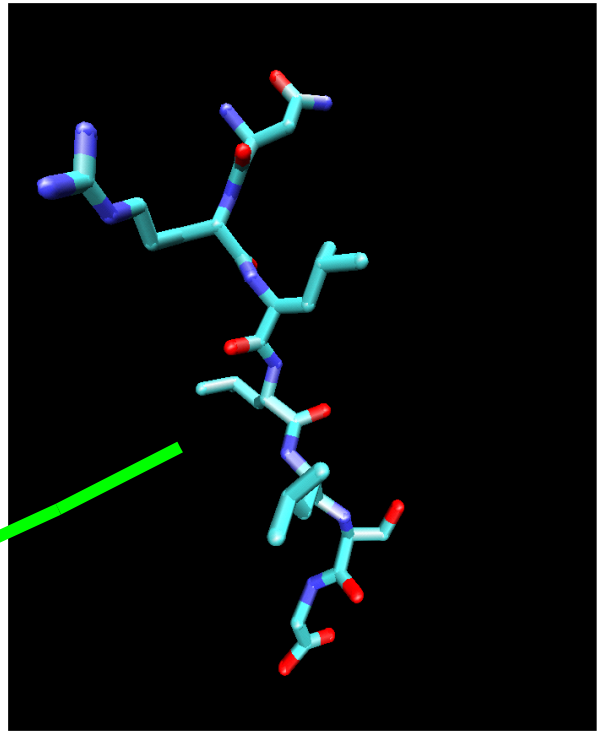
Effective concentrations can be higher than in solution → Initial entropic barriers can be overcome by favorable enthalpic contributions.

Molecular basis of binding

- What types of interactions do play a role in binding?
- Link to thermodynamics.
- How to investigate such a problem:
  - One option is to use computational approaches.

DnaK-peptide complexation



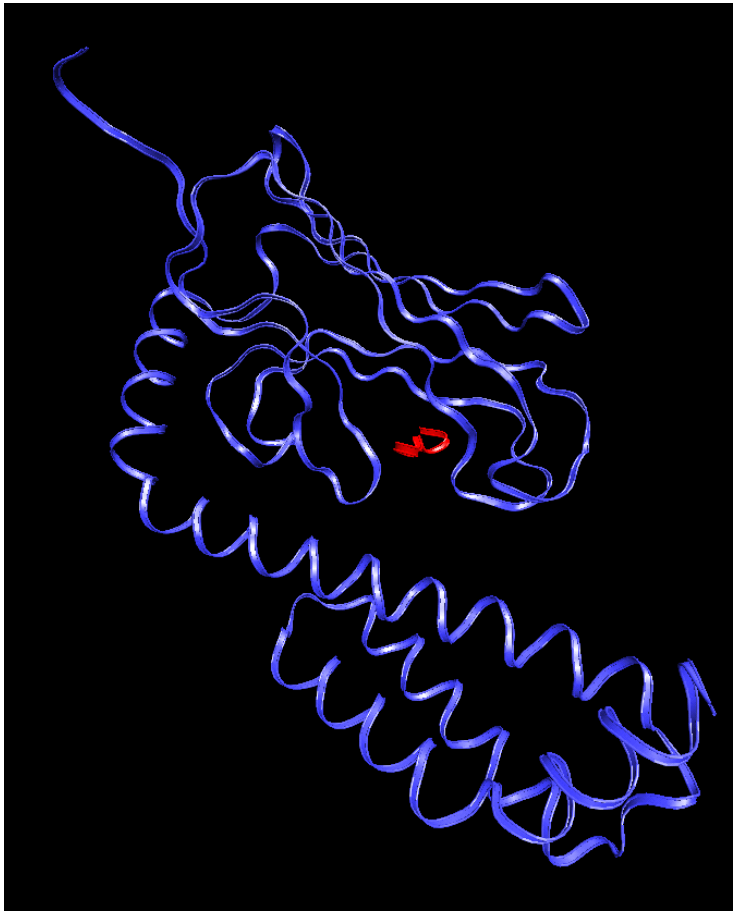


NRL peptide
Asn-Arg-Leu-Leu-Leu-Thr-Gly

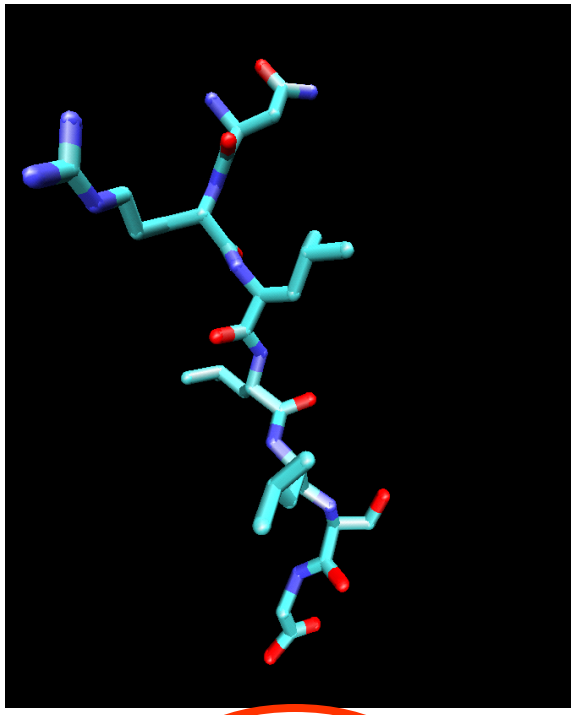
1dkx.pdb from www.rcsb.org

DnaK protein

- Molecular chaperone:
  - It prevents misfolding and aggregation.
- Structure consists two domains:
  - Peptide binding domain (β-subdomain).
  - ATPase (enzyme) domain (α-helical subdomain):
    - No direct contact with β-subdomain, but electric field of the helical subdomain significantly influences peptide binding.
  - Both domains must move apart to allow peptide in or out:
    - ∀ α-helical subdomain acts as a lid.
- Binding and release of peptide is regulated by ATP binding/release to/ from helical subdomain.



NRL peptide



Peptide structure
from 1dkx.pdb

Asn-Arg-Leu-Leu-Leu-Thr-Gly

- Peptide has a hydrophobic core (Leu-Leu-Leu)
- Charged and polar residues flank hydrophobic core:
  - Affinity for these residues is affected by electrostatic field of the DnaK protein.
- Peptide must have a significant effect on stability of protein, since the structure of the free protein could not be resolved.

Experimental determination of affinities

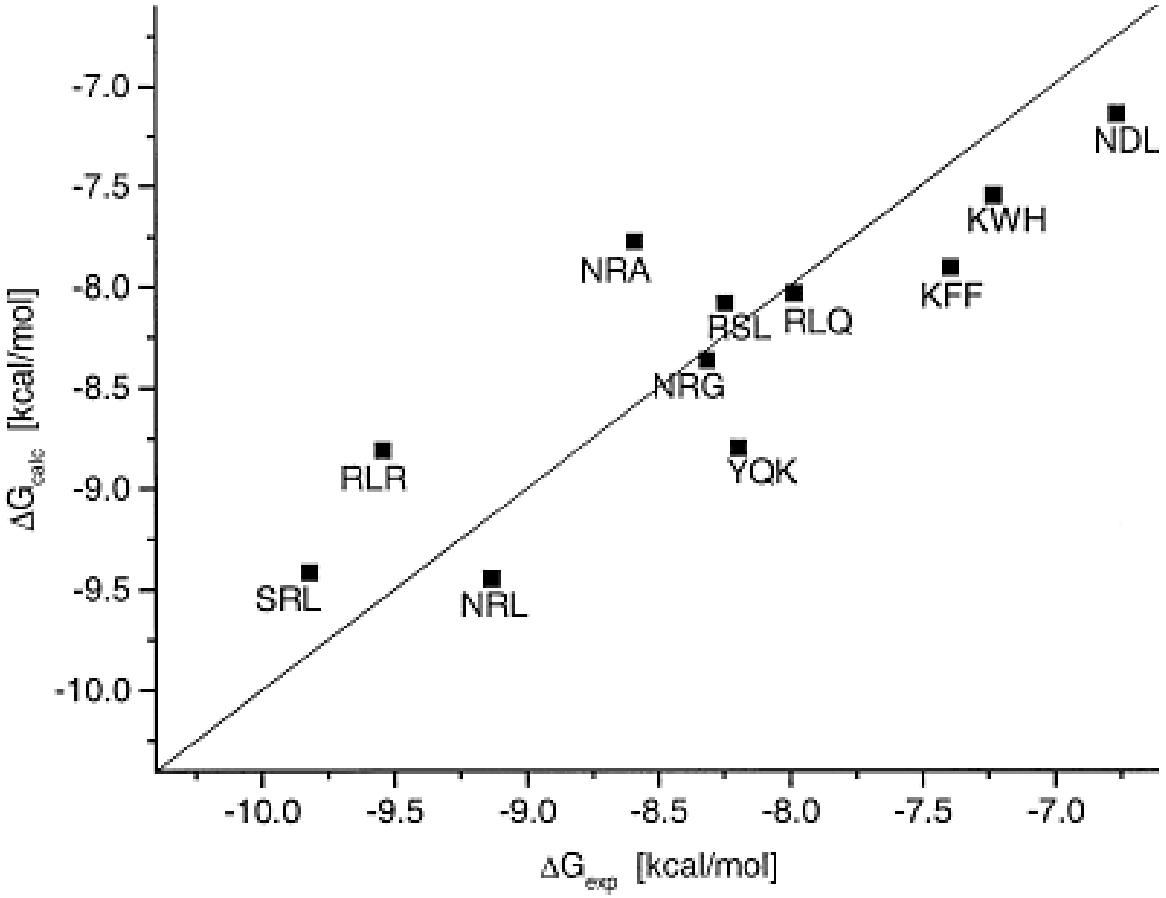
- Mutation of a His in the α-helical subdomain into Cys.
- Measurement of flueresence signal emitted by Cys labelled with a fluorophor (MIANS):
  - Binding of peptides results in a decrease of the signal.
- Affinities have been determined for a mutant instead of the original protein:
  - How representative are the experimental values for wild type DnaK?
  - Computation were carried out for original protein.

Set of peptides

Peptide	Sequence	K_d (exp) (μM)	ΔG_{exp} (kcal/mol)
SRL	LQSRLLLSAPR	0.06 (±0.02)	-9.8
RLR	NRLLLRG	0.1	-9.5
NRL	NRLLLTG	0.2 (±0.02)	-9.1
NRA	NRLALTG	0.5 (±0.1)	-8.6
NRG	NRLGLTG	0.8 (±0.5)	-8.3
RSL	CARSLLLSS	0.9 (±0.3)	-8.2
YQK	FYQLAKTCPV	1.0	-8.2
RLQ	RALLQSC	1.4 (±0.4)	-8.0
KFF	AQRKLFFNLRK	3.8	-7.4
KWH	KWVHLFG	5.0 (±3.0)	-7.2
NDL	NDLLLTG	11.0	-6.8

Calculations were performed for RED residues, experiment for full peptide.

Results



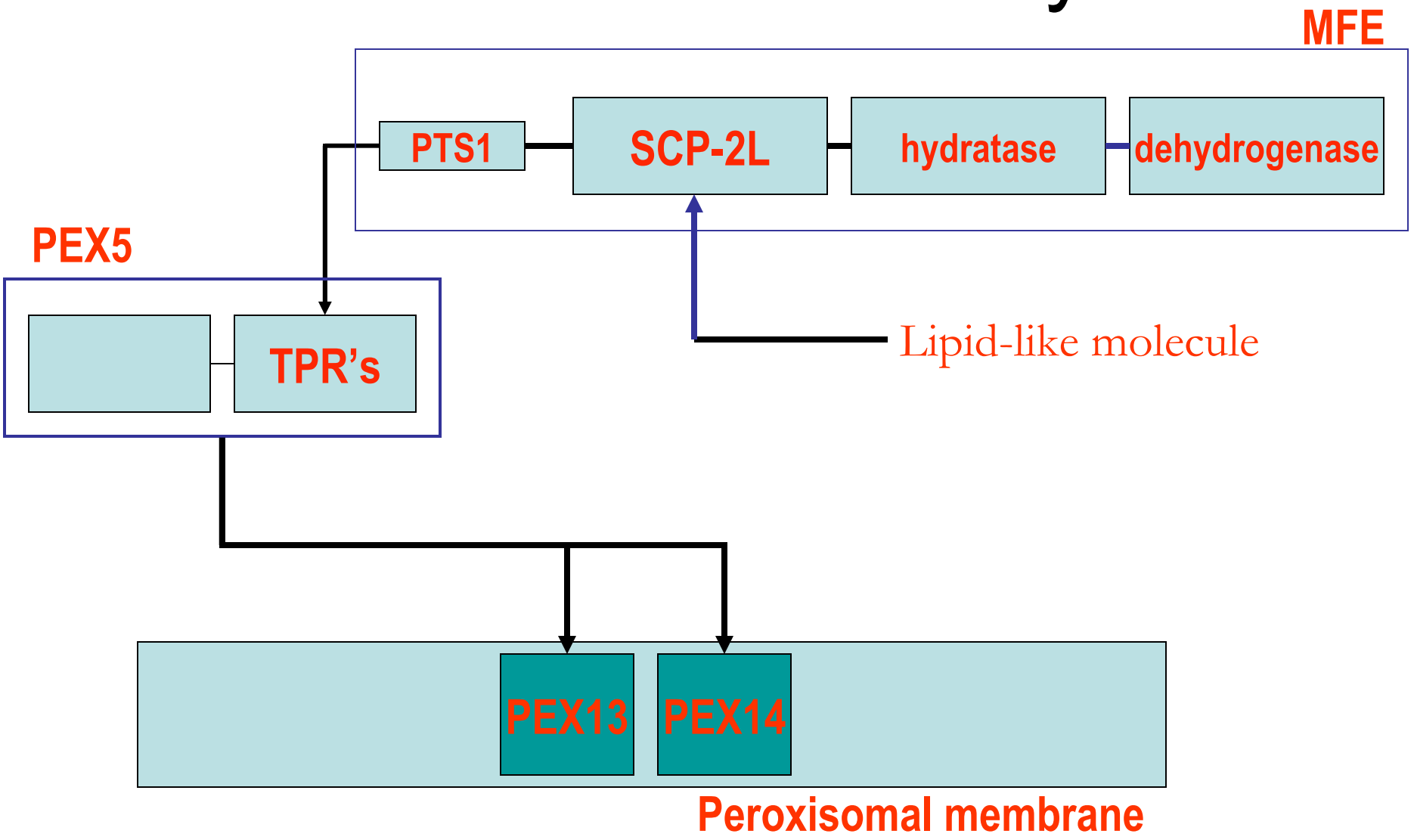
Many contributions were ignored, such as: Rotational, translation and backbone entropies, van der Waals interactions, “strain”.

Many contributions were assumed to cancel, since relative binding free energies were computed.

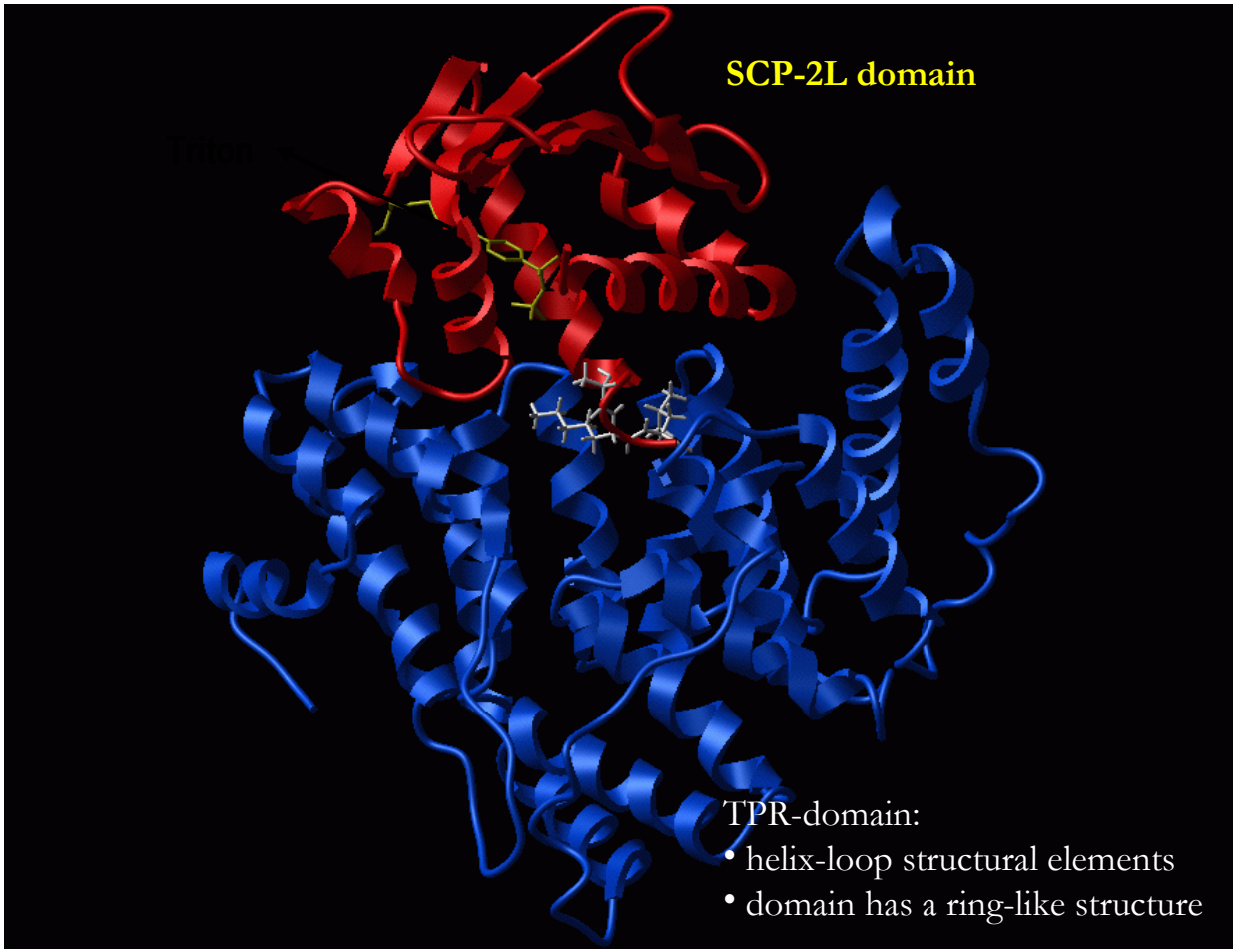
Contributions to affinity

- Affinity is the standard free energy of binding ΔG^{Θ}.
- Contribution due to intermolecular forces between atoms:
  - Dipole – dipole interactions.
  - Ion –dipole interactions.
  - Ion-induced dipole and dipole-induced dipole interactions.
  - Dispersion (or London) interactions.
  - Repulsive interactions
  - Hydrogen bonding
  - Hydrophobic interactions
- Entropic effects:
  - Loss of freedom upon binding:
    - Unfavorable for binding.
  - Changes in conformational flexibility:
    - (Un)favorable for binding.
  - Hydrophobic effect: Release of water from apolar surface are on protein and/or ligand:
    - Favorable for binding.

Multifunctional Enzyme

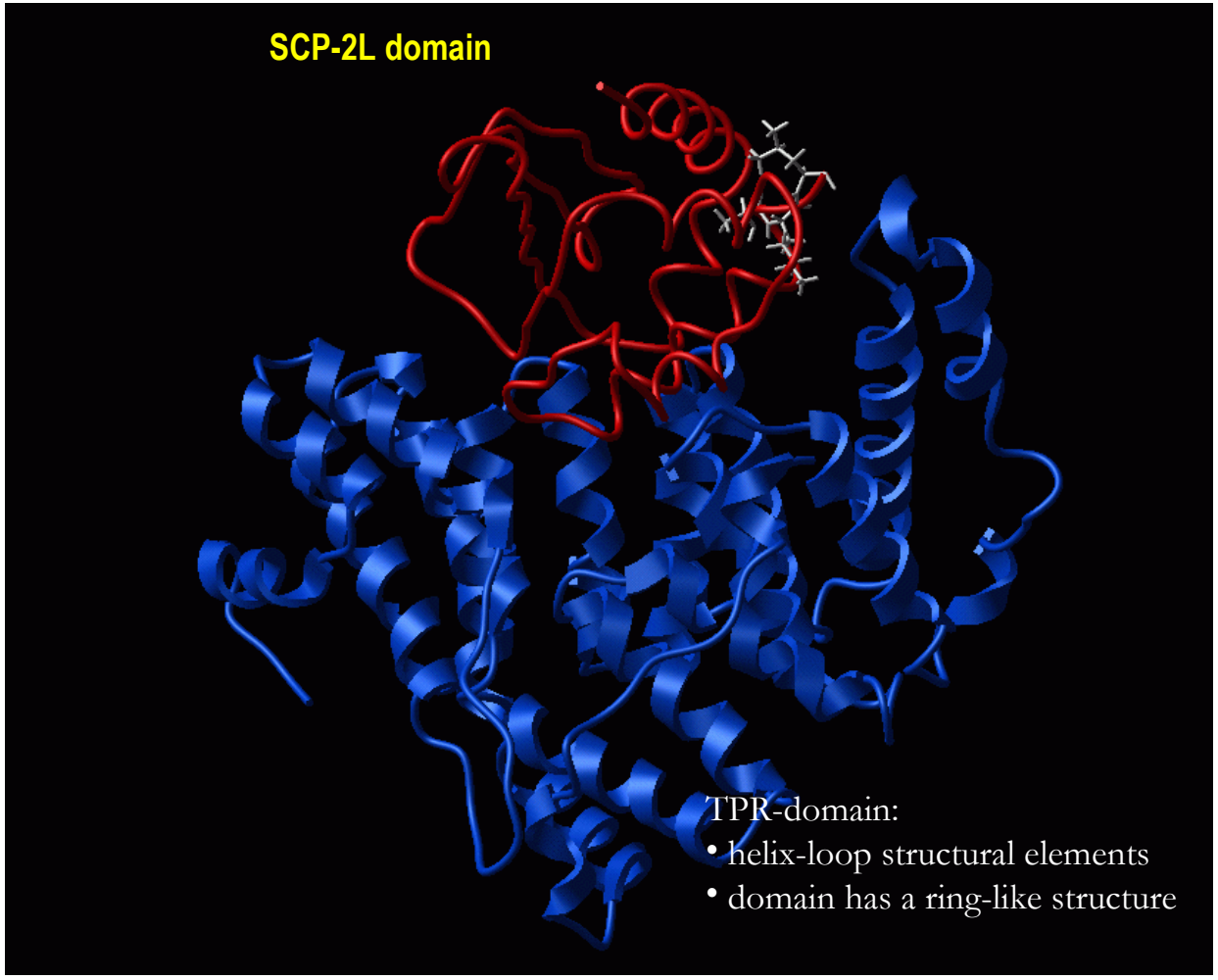


Liganded SCP-2L with TPR domain of PEX5



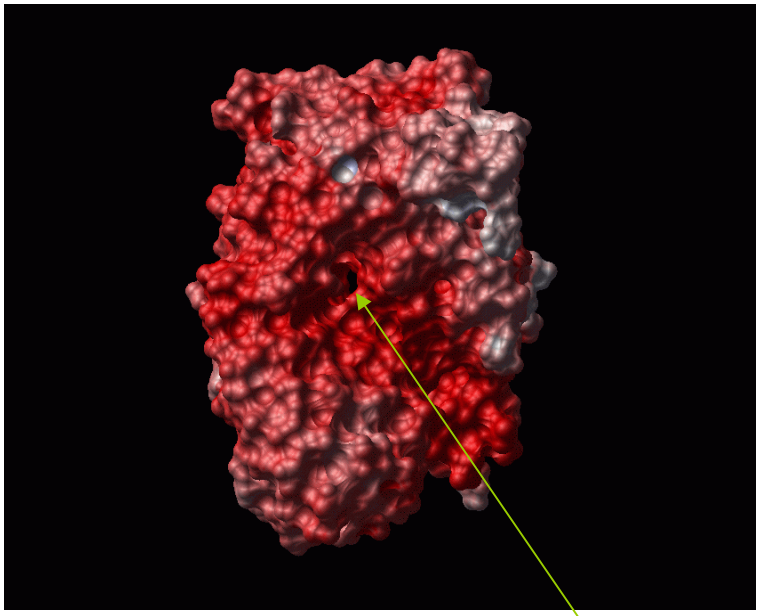
- Binding:
  - PTS has inherent ability to bind to TPR.
  - Electrostatic properties
- Crucial:
  - PTS1 must be accessible for binding.

Unliganded SCP-2L with TPR domain of PEX5

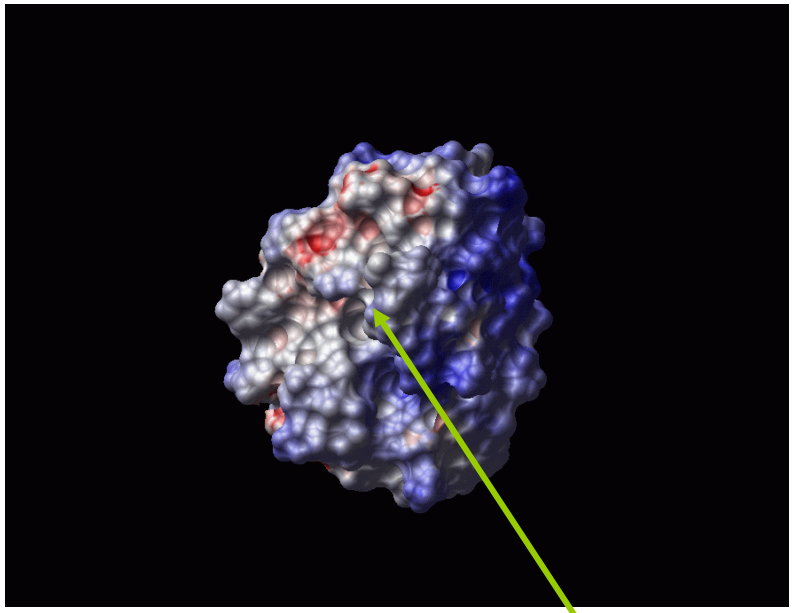


- Mechanism:
  - Lipid-like pushes PTS1 out.

Electrostatics



TPR (PEX5)



SCP-2L

PTS binding site

PTS