"Ligand" 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.

Hackert – CH370

Goals for this Unit

- **Understand basic ligand binding equation**
  - essential terms and equations
  - equilibrium binding / meaning of Kd / van't Hoff plots
  - When you can simply by assuming [S] ~ [So]
- **Complex equilibrium binding**
  - Multiple sites / independent or cooperative
  - Diff. Microscopic vs. Macroscopic binding constants
  - Scatchard plots and Hill Plots
- **Techniques to determine K_d**
  - Simple (Equil. Dialysis; Fluor) / ITC / SPR
  - How to derive Kd from Equil. Dialysis data
  - How to interpret Fluor / ITC and SPR data

Summary of Key Equations / Relationships

ES Û E + S; ***for single site***

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

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

define Fractional Occupancy of sites

$q = [ES]/[E_o] = [ES]/([E] + [ES]) \times [S]/[S] \times [1/ES]/[1/ES]$

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!!

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, M or 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 $?G^o = - R T \ln K_d$

?G, ?G° of an reaction at equilibrium

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

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

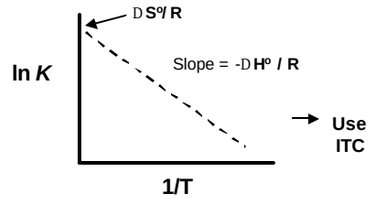
$$DG^0 = -RT \ln\left(\frac{[G]^g[H]^h \ldots}{[A]^a[B]^b \ldots}\right)_{Eq} = -RT \ln K$$

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

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

van't Hoff Equation

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



K_d values in biological systems

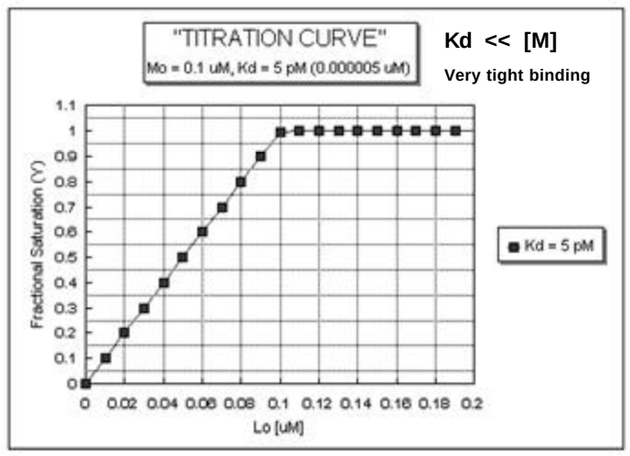
Movovalent ions binding to proteins or DNA have **K_d 0.1 mM to 10 mM**

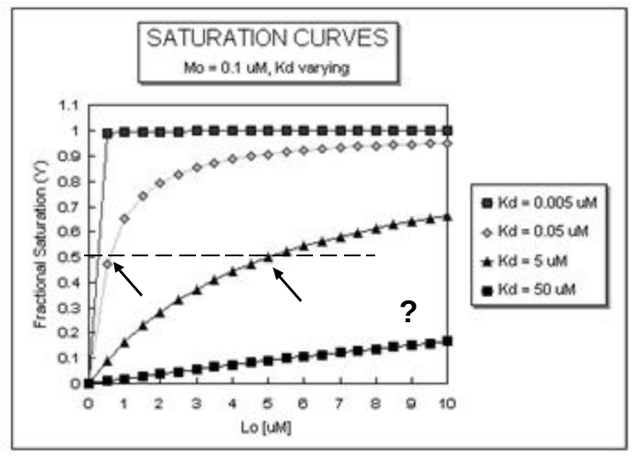
Allosteric activators of enzymes e. g. NAD have **K_d 0.1 µM to 0.1 mM**

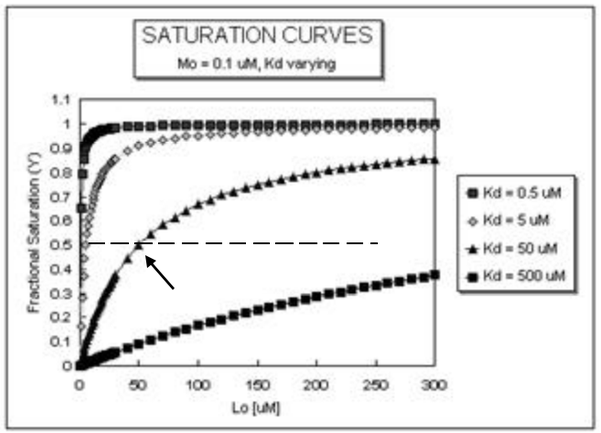
Site specific binding to DNA **K_d 1 nM to 1 pM**

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

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







Simplification of Key Equations

E + S Û ES ; *for single site*

$K_d = k_{off} / k_{on} = [E][S]/[ES]$ 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 $K_d [ES] = [E][S] = [E_o - ES][S_o]$

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

define Fractional Occupancy of sites

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

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

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 $[S]=[S]_0-[ES]$

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

$$[ES]^2-[ES](K_d+[S]_0+[E]_0)+[E]_0[S]_0 = 0$$

☜ 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.

Manipulations of Equations

a) double reciprocal plot

$1/q = K_d/[S] + 1$; plot $1/q$ vs. $1/[S]$

b) Scatchard Plot: $q = [S]/(K_d + [S])$ **or**

$qK_d + q[S] = [S]$ **or** $q = 1 - qK_d/[S]$

plot q **vs.** $q/[S]$ **slope =** $-K_d$

Linearized forms of the equation:

a) Double Reciprocal Plot

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

Or for multiple sites:

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

b) Scatchard Plot

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

Or for multiple sites:

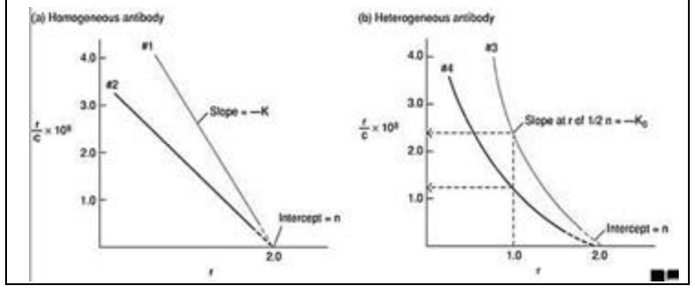
$$\theta = n - \frac{\upsilon K_d}{[S]}$$

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

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

Scatchard plot - determine the equilibrium **association constant** and **valence** of an antibody (**Historical – useful for visualizing data, but not the most accurate way to analyze the data! Use nonlinear regression to fit data**).

- ***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



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.

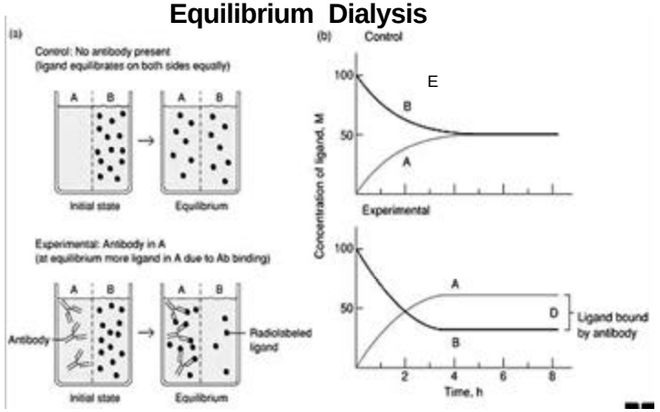
- ***gel filtration chromatography*** **- Add M to a given concentration of L. Then elute the mixture on a gel filtration column, eluting with the free ligand at the same concentration. The ML complex will elute first and can be quantified. If you measure the free ligand coming off the column, it will 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 radiolabeled 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



At equilibrium, determine free [L] by sampling the solution on side "B" and total [L] form side "A". By mass balance, determine the amount of bound ligand. Repeat at different ligand concentrations.

Multi-Equilibrium Dialyzer™

The Harvard/AmiKa Multi-Equilibrium Dialyzer provides highly standardized equilibrium dialysis conditions for up to 20 parallel assays. The instrument offers outstanding uniformity of:

- Membrane Area
- Sample Volume
- Degree of Agitation

The dialyzer cells are made of Teflon, an extremely inert material, and will not interfere with the samples. Multiple cell systems are available (5, 10, 15, 20 cells) at various cell volumes (0.25, 1.0, 2.0 & 5.0ml). The unit can be sterilized by autoclaving and the cells can be filled easily with a filling clamp.

Gel Shift Assay: Binding of Histone H1 to DNA

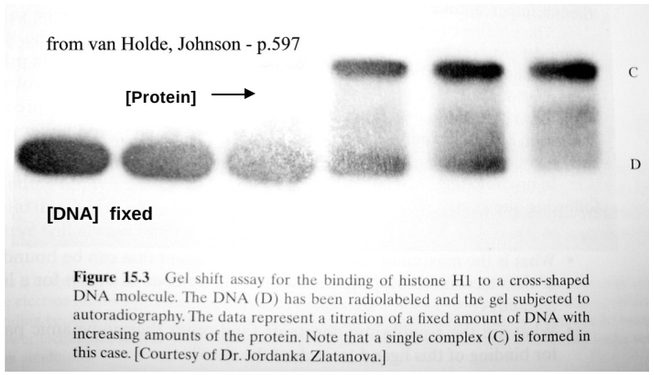


Figure 15.3 Gel shift assay for the binding of histone H1 to a cross-shaped DNA molecule. The DNA (D) has been radiolabeled and the gel subjected to autoradiography. The data represent a titration of a fixed amount of DNA with increasing amounts of the protein. Note that a single complex (C) is formed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

Spectroscopy

Fluorescence Spectroscopy

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

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.

$$q = \frac{F - F_o}{\Delta F = F_\infty - F_0}$$

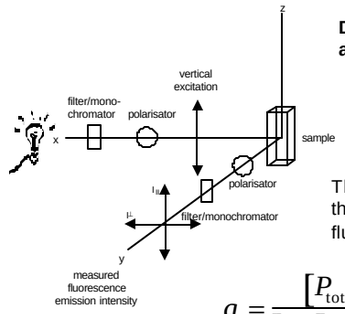
Fluorescence Anisotropy

Definition of fluorescence anisotropy *r*

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

$$q = \frac{r_{measured} - r_{min}}{r_{max} - r_{min}}$$

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



Definition of fluorescence anisotropy *r*

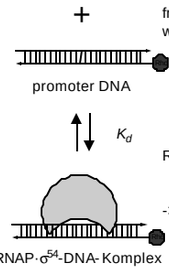
$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

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

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

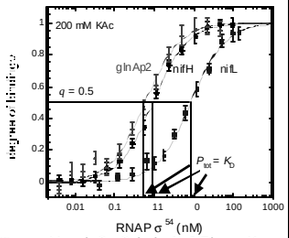
Analysis of binding of RNAP·σ^{54} to a promoter DNA sequence by measurements of fluorescence anisotropy

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



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

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



Note: DNA binding examples from Karsten Rippe - Heidelberg

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

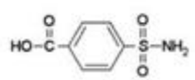
Direct comparison of binding equilibrium, thermodynamic, and rate constants determined by surface- and solution-based biophysical methods

(Binding of Small Molecules CBS & DNSA to Carbonic Anhydrase II)

Protein Science (2002), 11:1017–1025.

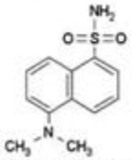
YASMINA S.N. DAY, CHERYL L. BAIRD, REBECCA L. RICH, and DAVID G. MYSZKA

A



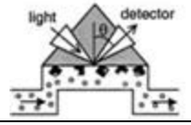
4-carboxybenzenesulfonamide (CBS)

B

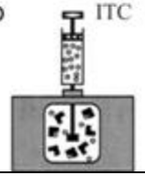


5-dimethyl-amino-1-naphthalene-sulfonamide (DNSA)

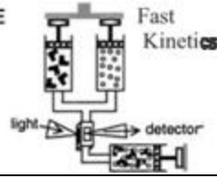
C SPR



D ITC



E Fast Kinetics



The iTC$_{200}$

- More Sensitive
  Up to 7-fold less material
- Faster
  2 – 4 times more throughput
- Easier to use
  Pipetting, filling & cleaning
  Experiment design wizards
- Automatable

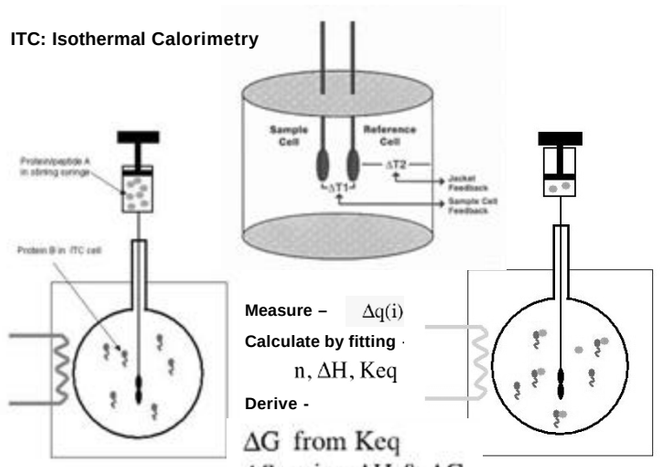
At Rest

Sample Loading

Titrating

Washing

ITC: Isothermal Calorimetry



Measure – $\Delta q(i)$

Calculate by fitting -

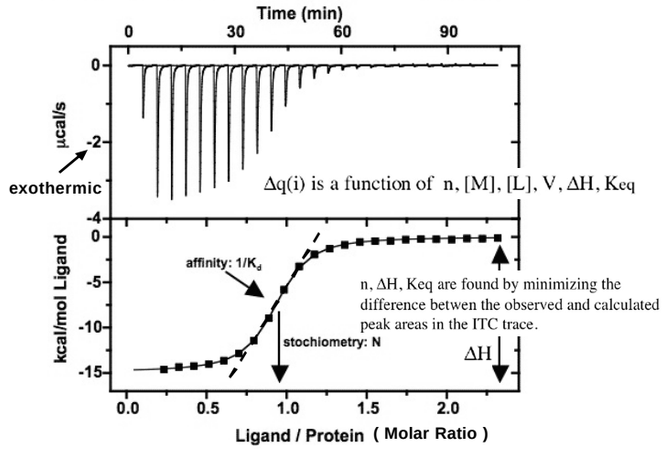
n, ΔH, Keq

Derive -

ΔG from Keq

ΔS using ΔH & ΔG

ITC: Isothermal Titration Calorimetry



Experimental Design Wizard

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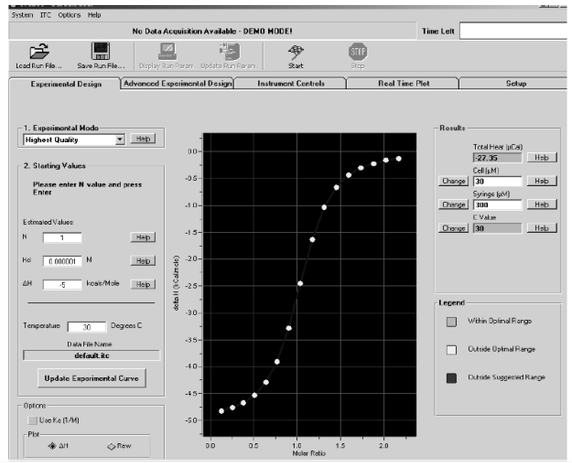

Affinity is just part of the picture

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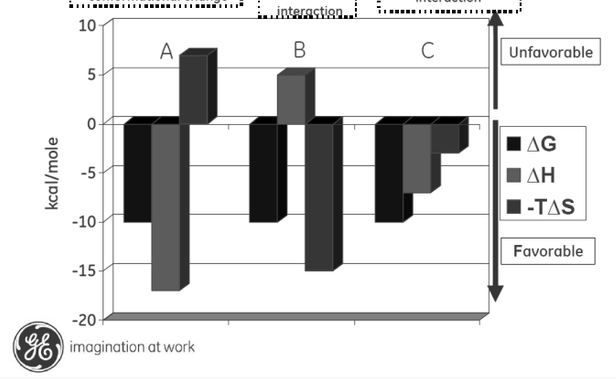
All three interactions have the same binding energy (ΔG)

A. Good hydrogen bonding with unfavorable conformational change

B. Binding dominated by hydrophobic interaction

C. Favorable hydrogen and hydrophobic interaction



imagination at work

Microcalorimetry provides a total picture of binding energetics

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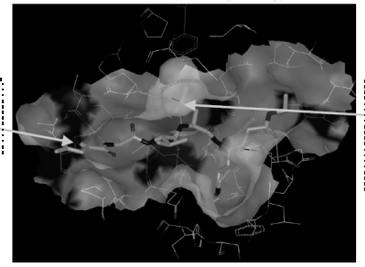
Overall binding affinity K_D correlates with IC_{50} or EC_{50}.This is directly related to ΔG the total free binding energy

ΔH is a measure of hydrogen and van der Waals bonding



$-T\Delta S$ is a measure of hydrophobic interaction and conformational changes

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n = Stoichiometry indicates the ratio of ligand molecules that bind each macromolecule

Drug Discovery – Similar Affinities…Different Mechanisms

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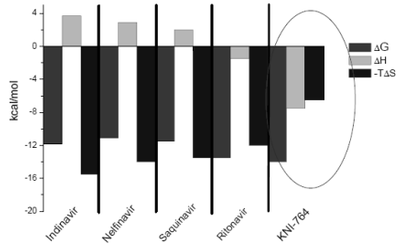

- Binding of inhibitors to HIV-1 protease.
- Favorable enthalpy and entropy is the best profile for drug-target interaction

imagination at work

Carbonic anhydrase-inhibitor titrations with Auto-iTC$_{200}$

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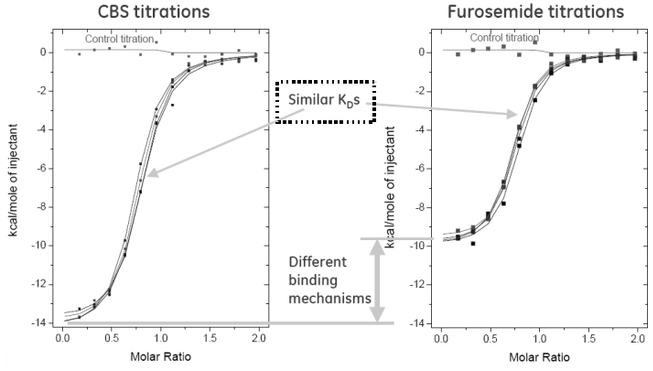

Carbonic anhydrase inhibitors

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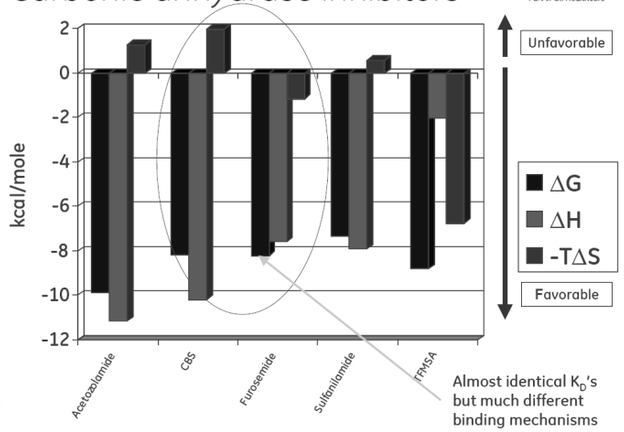
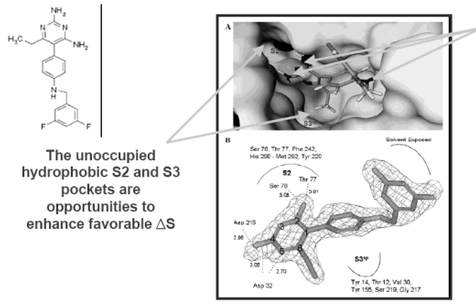


Almost identical K_D's but much different binding mechanisms

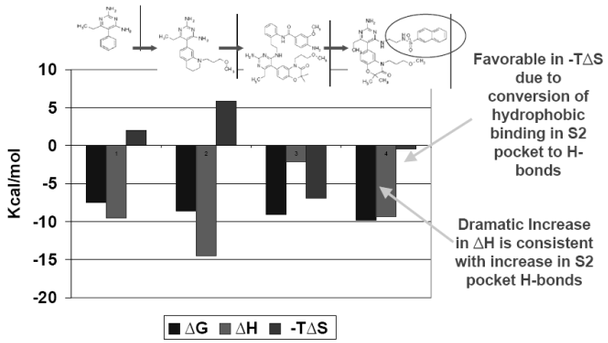
The Binding Orientation for Lead Template to Renin was Determined X-ray Crystallography



The unoccupied hydrophobic S2 and S3 pockets are opportunities to enhance favorable ΔS

favorable ΔH is consistent with the strong network of hydrogen bonds.

Modeling Suggests Substituting Aryl-Benzamide with Aryl-Sulfonamide to Improve H-bonds



Favorable in -TΔS due to conversion of hydrophobic binding in S2 pocket to H-bonds

Dramatic Increase in ΔH is consistent with increase in S2 pocket H-bonds

imagination at work

*Another **3.4X** improvement in affinity*

Renin Inhibitor Affinity Improved 45X from Initial 3.6 μM Lead to 79nM

- S3 Pocket – Ether addition improved enthalpy due to van der Waals bonds
- S2 Pocket – Aryl-Sulfonamide improved binding enthalpy while retaining hydrophobic advantage

imagination at work

Summary: Isothermal Titration Calorimetry (ITC)

Good things about ITC:

Accurate determination of binding (K_d) and thermodynamic (ΔG, ΔH, ΔS) parameters for ligand-macromolecule interactions.

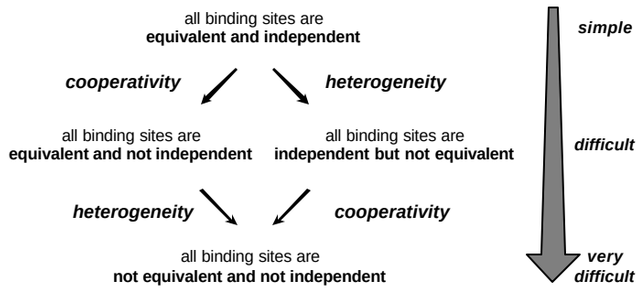
ITC does not make the approximations that are included in a van't Hoff type of analysis.

Not such good things about ITC:

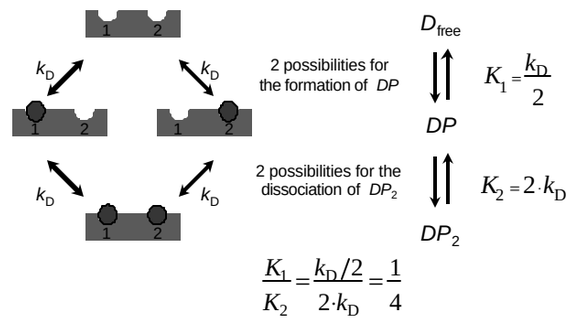
Large (perhaps 10 mg) quantities of material are required, in order to detect the small amount of heat released upon mixing macromolecule and ligand.

In comparison, gel-mobility shift assays can be carried out with << 1 mg of material.

Increasing complexity of binding



Difference between microscopic and macroscopic dissociation constant



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

Multiple Binding Equilibria

Multiple Binding Sites:

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

Adair Equation (1925)

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

Mass balance equations:

$[E]_0 = [E] + [ES] + [ES_2]$

$[S]_0 = [S]$ (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:

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

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

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

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

1 Site

2 Independent Sites

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

Adair Equation (1925)