"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

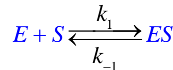
Binding is the first step necessary for a biological response. Before macromolecules can perform a function, they normally must interact with a specific ligand. In some cases like myoglobin, binding and subsequent release of the ligand might be the sole function of the macromolecule. To understanding 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
  - When you can simply by assuming [S] ~ [So]
  - Hyperbolic vs. Quadratic Equations
- **Complex equilibrium binding**
  - ~~Multiple sites / independent or cooperative~~
  - ~~Diff. Microscopic vs. Macroscopic binding constants~~
  - ~~Scatchard plots and Hill Plots~~
- **Techniques to determine K_d**
  - van't Hoff plots
  - Simple (Equil. Dialysis; Fluor) / ITC / SPR
  - How to derive Kd from Equil. Dialysis data
  - How to interpret Fluor, ITC and SPR data

Equilibrium Binding



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES]$$

k_{-1} is a first order rate constant with units of s^{-1}
k_1 is a second order rate constant with units of $M^{-1}s^{-1}$

At Equilibrium

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] = 0$$

$$k_1[E][S] = k_{-1}[ES]$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] = 0$$

$$Ms^{-1} = (M^{-1}s^{-1})\cdot(M)\cdot(M) - (s^{-1})\cdot(M)$$

$$K_a = \frac{[ES]}{[E][S]} = k_1 / k_{-1} \quad \text{units of } M^{-1} \qquad K_d = \frac{[E][S]}{[ES]} = k_{-1} / k_1 \quad \text{units of M}$$

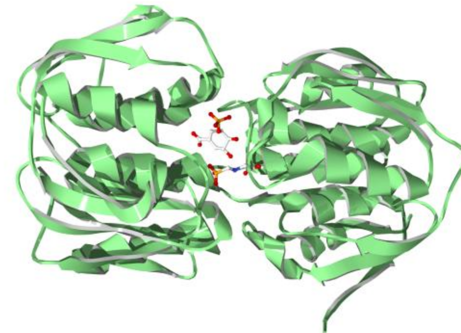
Typical values for substrates binding to proteins:

k_1 = 0.1 to 100 x 10^6 $M^{-1}s^{-1}$ = 0.1 to 100 $\mu M^{-1}s^{-1}$

k_{-1} = 0.01 to 1000 s^{-1}

K_d = nM to mM

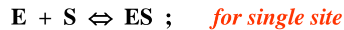
EPSP Synthase with S3P and glyphosate bound



Native tryptophan fluorescence change due to changes in protein structure upon binding ligands.

Anderson, K. S., Sikorski, J. A. and Johnson, K. A. (1988) Evaluation of EPSP Synthase Substrate and Inhibitor Binding by Stopped-Flow and Equilibrium Fluorescence Measurements. *Biochemistry* 27, 1604-1610

Simplification of Key Equations



E + S ⇔ ES ; *for single site*

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

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

$\theta = [S]/(K_d + [S])$ Hyperbolic Equation

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

then $K_d [ES] = [E][S] = [E_o - ES][S_o]$

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

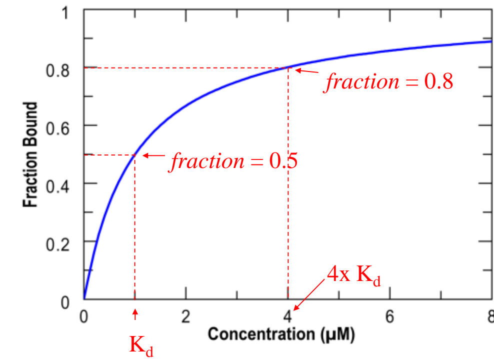
define **Fractional Occupancy** of sites

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

thus when $[S_o] = K_d$, then $\theta = 0.5$

Hyperbola

$$\theta = \frac{[S]_0}{K_d + [S]_0} = \frac{[S]_0 / K_d}{1 + [S]_0 / K_d}$$



Manipulations of Equations

Hyperbolic: $\theta = [S]/(K_d + [S])$

a) double reciprocal plot

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

b) Scatchard Plot: $\theta = [S]/(K_d + [S])$ or

$\theta K_d + \theta[S] = [S]$ or $\theta = 1 - \theta K_d/[S]$

plot θ vs. $\theta/[S]$ 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

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

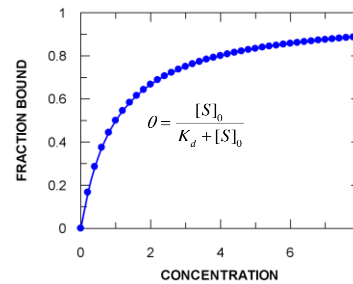
Or for multiple sites:

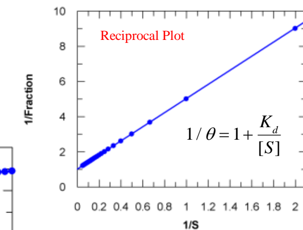
$$\theta = n - \frac{\upsilon K_d}{[S]}$$

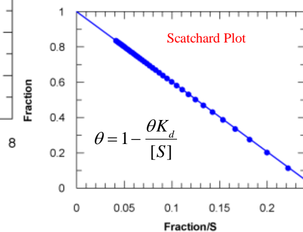
$$\upsilon = \frac{\text{moles bound}}{\text{mole E}} = n\theta$$

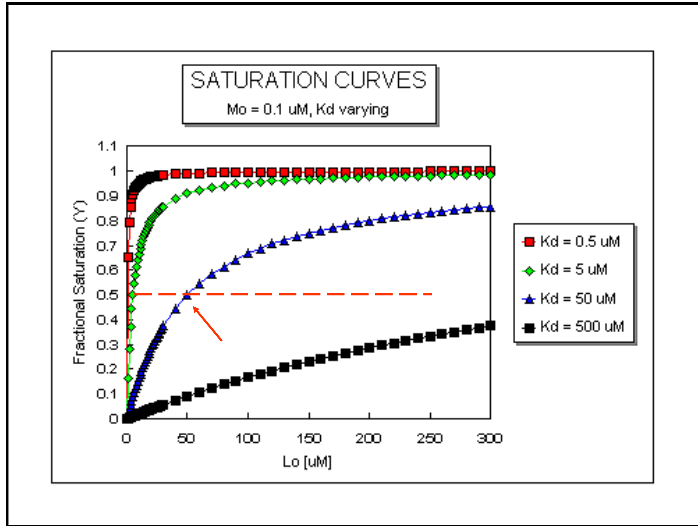
Comparison of three plots

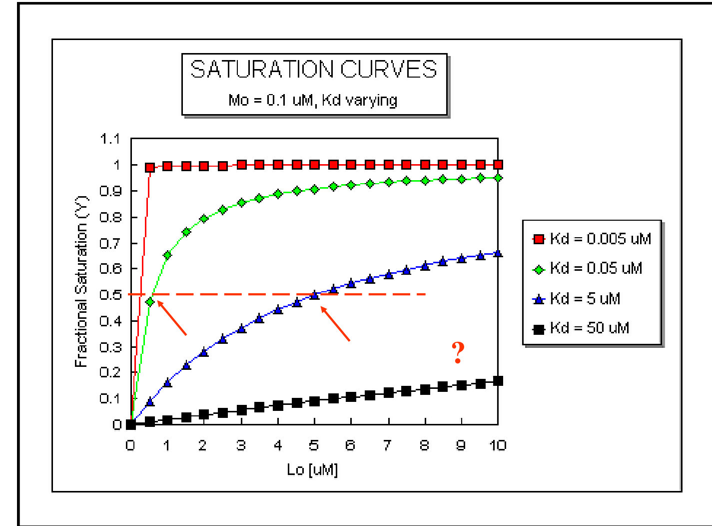
Hyperbolic Plot

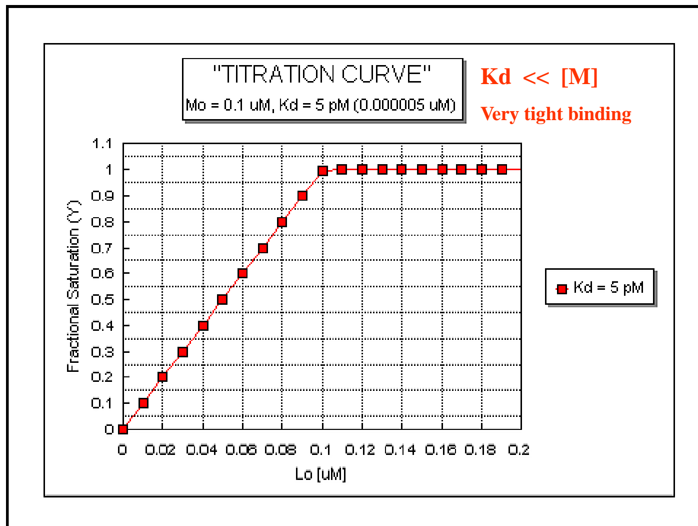












No Assumptions - Key Equations

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

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

Equations taken from Ligand Binding handout of Dr. Ken Johnson.

No Assumptions - Key Equations

Solution is quadratic equation:

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

solution provided by the roots of the quadratic

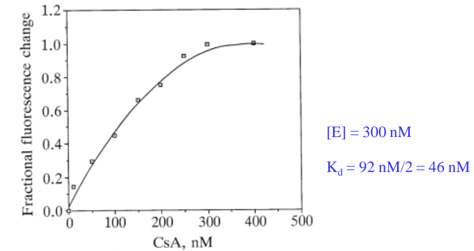
$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

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

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

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.

Example of poorly fit data: Fluorescence titration of cyclosporin A binding to cyclophilin



$[E] = 300$ nM

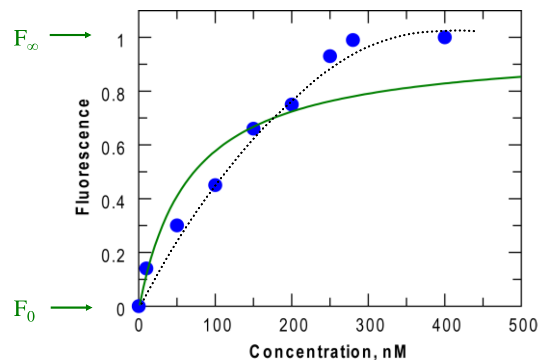
$K_d = 92$ nM/2 = 46 nM

FIG. 2. Titration of CsA binding to CyP by analysis of tryptophan fluorescence enhancement. The CyP concentration was held constant at 300 nM (by Bradford assay) and increasing levels of CsA generated the fluorescence enhancement shown. Given the 1:1 stoichiometry, an apparent K_d can be estimated at a fractional fluorescence change of 0.5. By assuming 50% occupancy, the bound CsA equals free CsA, then the concentration of CsA divided by 2 at 0.5 fluorescence enhancement is the apparent K_d.

Liu et al., PNAS (1990) 87, 2304-2308

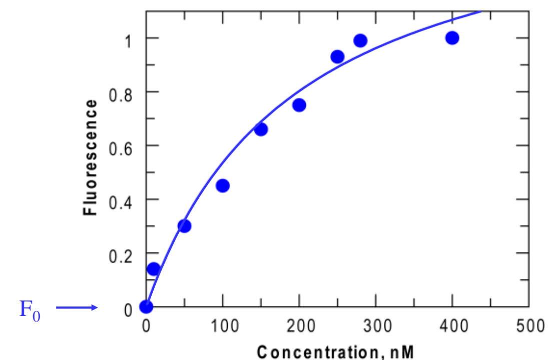
Fitting Equilibrium Binding Data

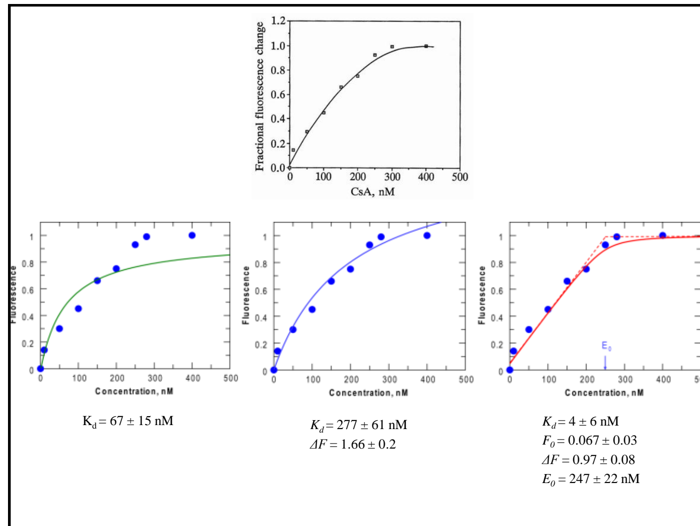
$$F_{normal} = \frac{F + F_0}{F_\infty - F_0} = \frac{[S]}{K_d + [S]} \qquad \Delta F = F_\infty - F_0$$

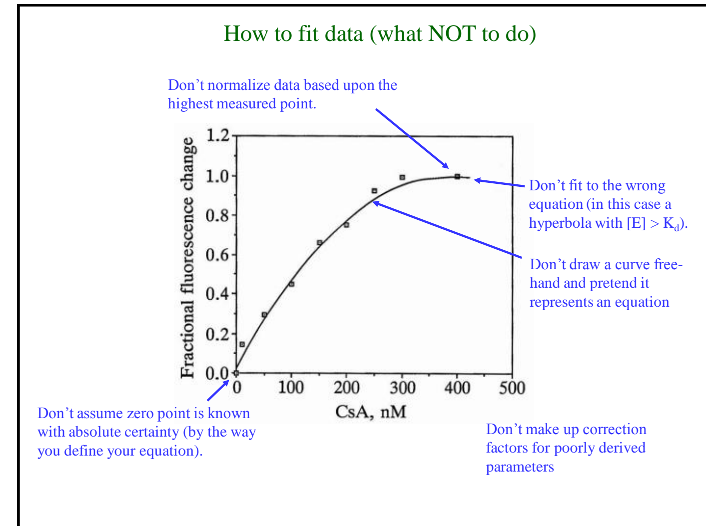


Fitting Equilibrium Binding Data

$$F = F_0 + \Delta F \cdot \frac{[S]}{K_d + [S]} \qquad \Delta F = F_\infty - F_0$$





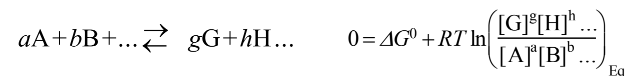


What is the meaning of the dissociation constant (Kd) for binding of a single ligand to its site?

$$K_d = [E][S]/[ES]$$

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, ~[total ligand]).
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 ΔG^o by the relation $\Delta G^o = -RT \ln K_d$

ΔG, ΔG^o of a reaction at equilibrium



$$aA + bB + \ldots \rightleftarrows gG + hH \ldots \qquad 0 = \Delta G^0 + RT\ln\left(\frac{[G]^g[H]^h\ldots}{[A]^a[B]^b\ldots}\right)_{Eq}$$

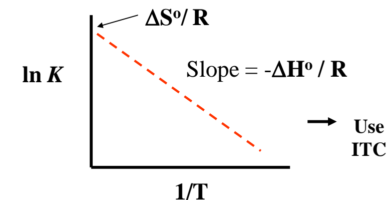
$$\Delta G^0 = -RT\ln\left(\frac{[G]^g[H]^h\ldots}{[A]^a[B]^b\ldots}\right)_{Eq} = -RT\ln K$$

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

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

van't Hoff Equation

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



EXPERIMENTAL DETERMINATION OF Kd

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

•Equilibrium dialysis **- Place M in a dialysis bag and dialyze against a solution containing a ligand whose concentration can be determined using radioisotopic or spectroscopic techniques.**

- Measure [Ligand] total and [Ligand] free → [Ligand] bound = [Protein] bound
- [Protein] total - [Ligand] bound = [Protein] free K_d = [P][L]/[PL]

•Fluorescence 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.**

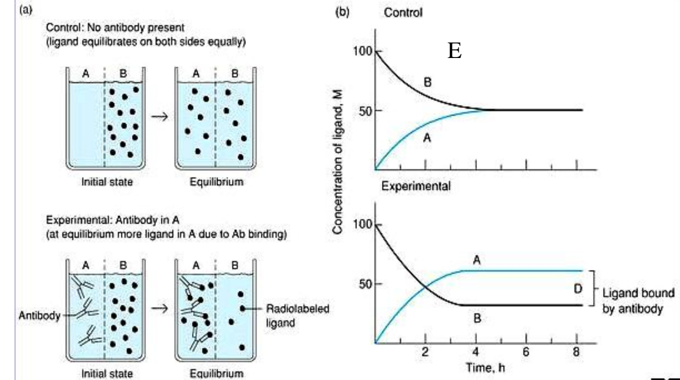
•ITC - Isothermal Titration Calorimetry – **Measure small, incremental heats (Δq) of reaction during binding titration. Obtain ΔH, n and Keq, then calc ΔG and ΔS.**

•Kinetic (higher tech) methods:

SPR – Surface Plasmon Resonance ***kon / koff***

Fast Kinetics **– rate constants**

Equilibrium Dialysis K_d = [E][S]/[ES]



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 auto-claving and the cells can be filled easily with a filling clamp.

Spectroscopy

Fluorescence Spectroscopy

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

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

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

and θ is defined by either:

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

OR.....

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

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

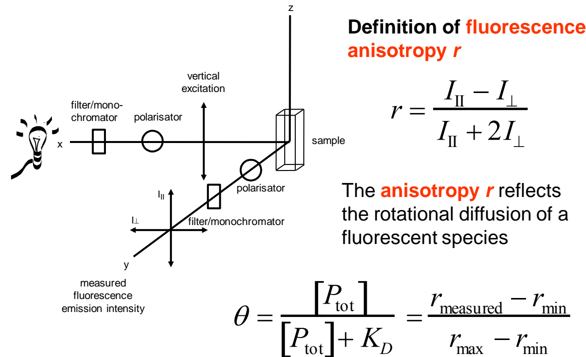
Fluorescence Anisotropy

Definition of fluorescence anisotropy *r*

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

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

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



Definition of fluorescence anisotropy *r*

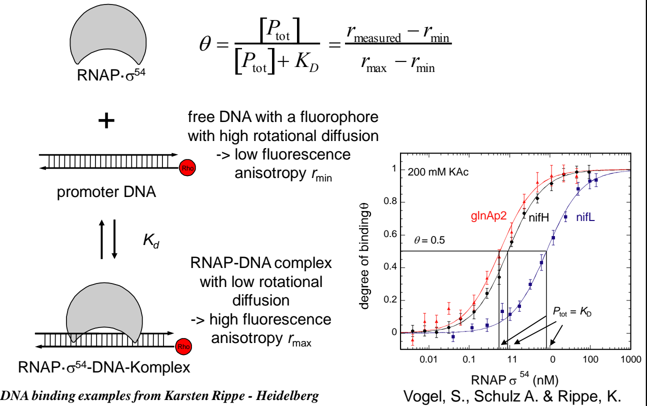
$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}}$$

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

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

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

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



Note: DNA binding examples from Karsten Rippe - Heidelberg

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

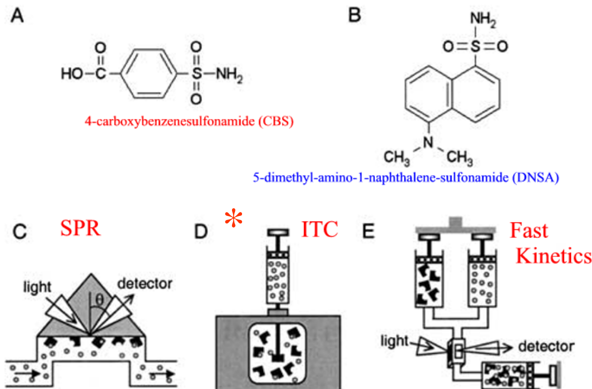
Direct comparison of binding equilibrium, thermodynamic, and rate constants determined by surface- and solution-based biophysical methods

Protein Science (2002), 11:1017–1025.

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

Center for Biomolecular Interaction Analysis, University of Utah, School of Medicine, Salt Lake City, Utah 84132, USA

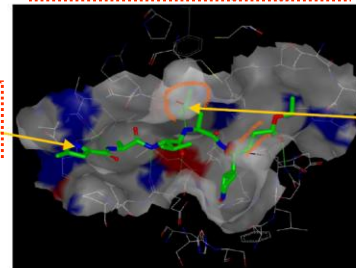
(Binding of Small Molecules CBS & DNSA to Carbonic Anhydrase II)



Microcalorimetry provides a total picture of binding energetics

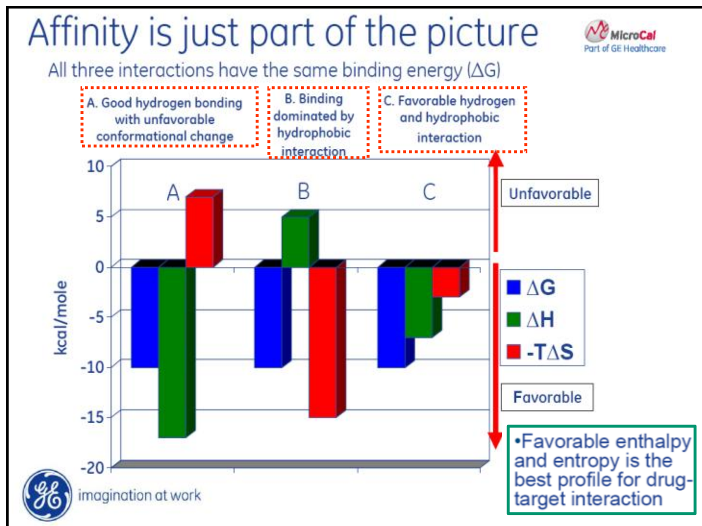
Overall binding affinity K_D correlates with IC_{50} or EC_{50}. This is directly related to ΔG the total free binding energy

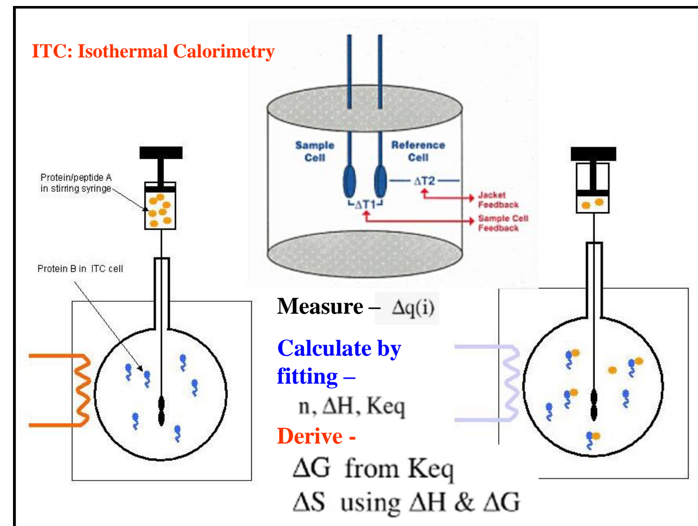
ΔH is a measure of hydrogen and van der Waals bonding

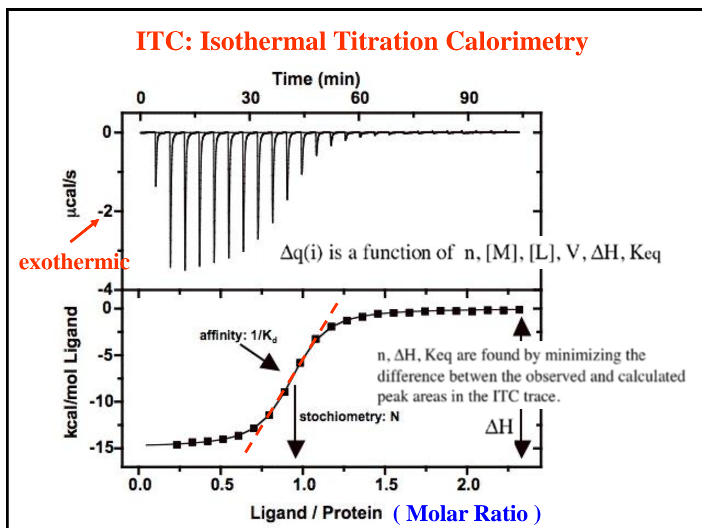


-TΔS is a measure of hydrophobic interaction and conformational changes

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







The iTC$_{200}$

MicroCal

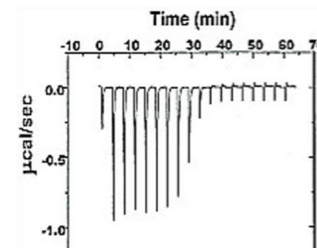
- More Sensitive
  - Up to 7-fold less material
- Faster
  - 2 – 4 times more throughput
- Easier to use
  - Pipetting, filling & cleaning
  - Experiment design wizards
- Automatable

At Rest | Sample Loading | Titrating | Washing

Experimental Design Wizard

iClicker Question 1 – November 8, 2012

Consider typical data from an ITC experiment.



Identify which property is being measured ***directly*** by ITC.

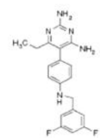
A) n B) ΔG C) Keq D) ΔS E) $\Delta q(i)$

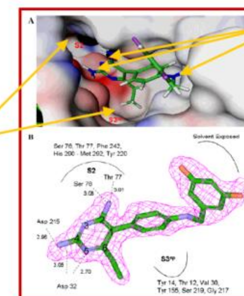
Renin Inhibitor Affinity Improved 45X from Initial 3.6 μM Lead to 79nM

- 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

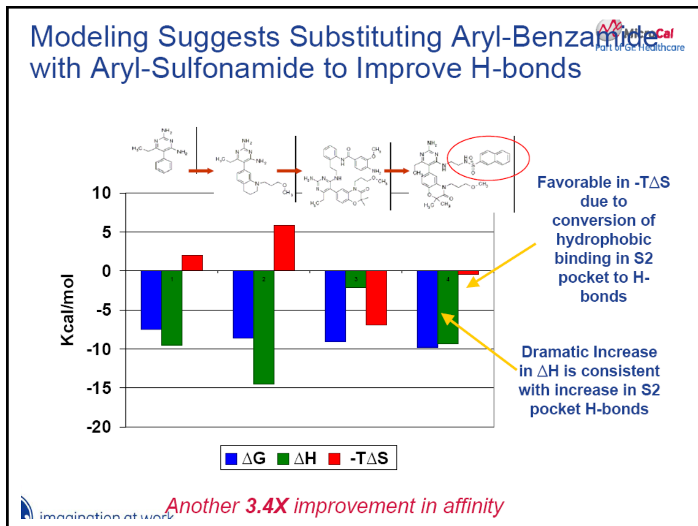
The Binding Orientation for Lead Template to Renin was Determined X-ray Crystallography





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

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



Objectives of the Biacore Experiment

- Yes/No Data
  - Ligand Fishing
- Affinity Analysis: K_D, K_A

- **Kinetic Rate Analysis:**
- **How FAST?**
  - k_a, k_d
  - $K_D = k_d/k_a$, $K_A = k_a/k_d$

- No label
- **Real-time**
- **Unique, high quality data** on molecular interactions
- **Simple assay** design / Robust
- Walk-away automation
- **Small amount of sample** required

Binding - SPR or BIA

"The secret of life is molecular recognition"

"Binding is the first step necessary for a biological response"

Biacore's SPR technology: label-free technology for *monitoring biomolecular interactions as they occur*.

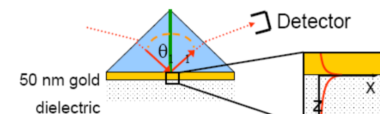
The detection principle relies on surface plasmon resonance (SPR), an electron charge density wave phenomenon that arises at the surface of a metallic film when light is reflected at the film under specific conditions.

The resonance is a result of energy and momentum being *transformed* from incident photons into surface plasmons, and is sensitive to the refractive index of the medium on the opposite side of the film from the reflected light.

Hackert – CH370

Note: Many of these figures/notes were taken from on-line resources from Biacore

Plasmons & SPR "angle"



Measure reflected (polarized) light as function of angle.

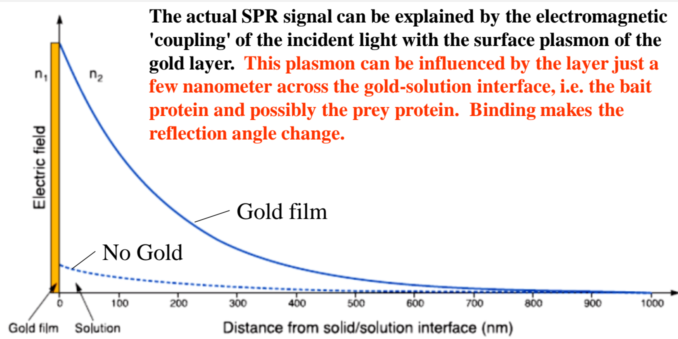
At a certain "Magic Angle" light is not reflected ("total internal reflection") but interacts with free electrons in gold to form a resonant energy wave – or surface plasmon.

Plasmon – A plasmon is a collective oscillation of the conduction electrons in a metal - a quasiparticle that can be regarded as a hybrid of the conducting electrons and the photon.

Angle is sensitive to refractive index of dielectric which varies with concentration of molecules on the other side of gold layer!

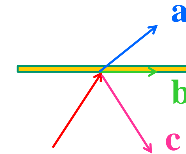
SPR - The need for Gold

The actual SPR signal can be explained by the electromagnetic 'coupling' of the incident light with the surface plasmon of the gold layer. This plasmon can be influenced by the layer just a few nanometer across the gold-solution interface, i.e. the bait protein and possibly the prey protein. Binding makes the reflection angle change.

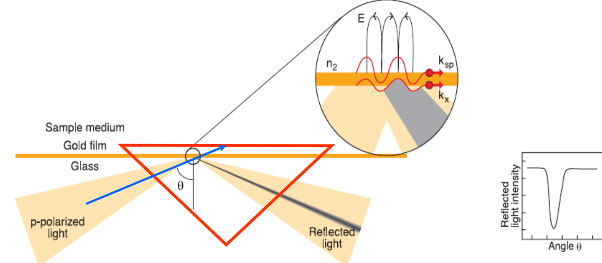


Total Internal Reflection (TIR) for a non-absorbing media

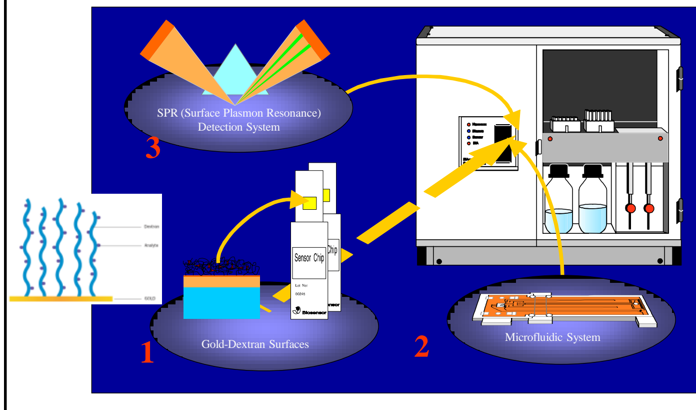
Creating a plasmon causes a decrease in the intensity of the reflected light since - the energy for creating the plasmon comes from the light.



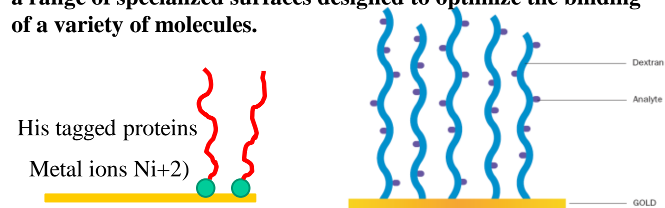
θ - the angle of incidence - depends on the difference in the refractive indices on the two sides, and the refractive index difference depends on [bound ligand] on the binding side.



Three Corner Stones of Biacore Technology

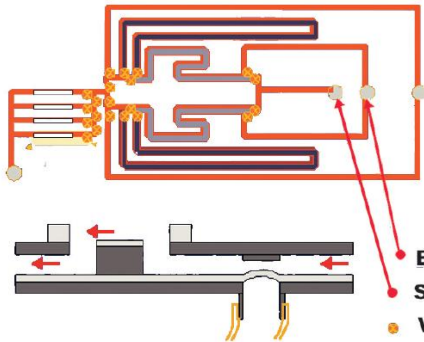


1. The Biacore sensor chip is at the heart of the technology. Quantitative measurements of the binding interaction between one or more molecules are dependent on the immobilization of a target molecule to the sensor chip surface. Binding partners to the target can be captured from a complex mixture, in most cases, without prior purification (for example, clinical material, cell culture media) as they pass over the chip. Interactions between proteins, nucleic acids, lipids, carbohydrates and even whole cells can be studied. The sensor chip consists of a glass surface, coated with a thin layer of gold. This forms the basis for a range of specialized surfaces designed to optimize the binding of a variety of molecules.



2. Integrated micro Fluidics Cartridges (IFC)

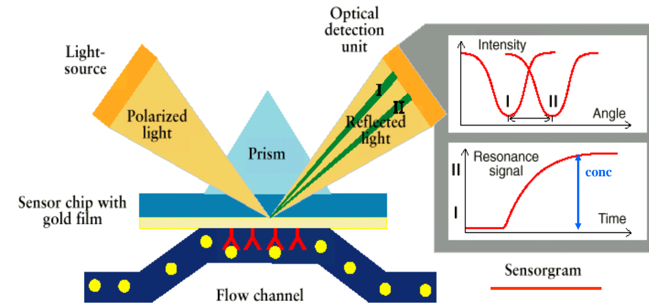
Liquid Handling



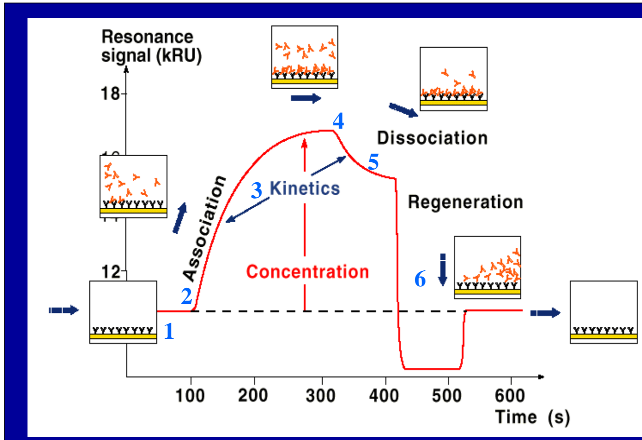
- Minaturized system
- Low volume of reagents
- Intergrated and automated liquid handling

3. Surface Plasmon Resonance Detection: Biomolecular Binding in Real Time

Principle of Detection

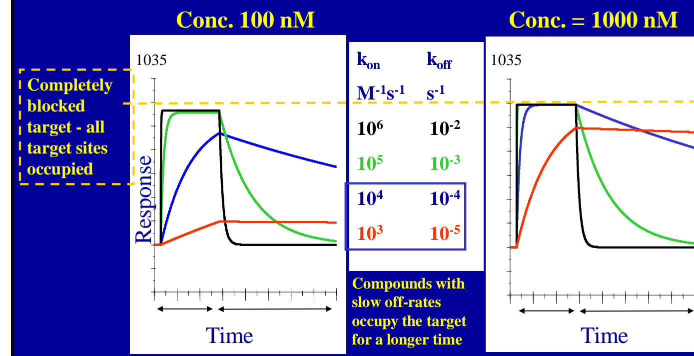


The Sensorgram is Information Rich



Same affinity but different kinetics

- All 4 compounds have the **same affinity** $K_D = 10\ nM = 10^{-8}\ M$
- The binding **kinetic constants vary by 4 orders** of magnitude



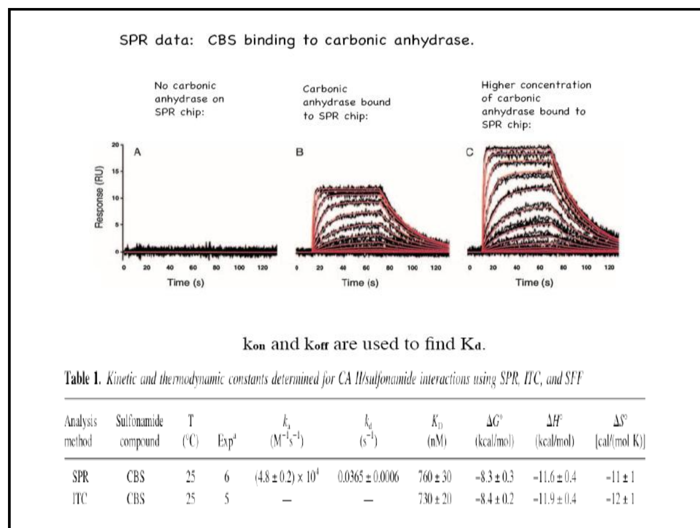


Table 1. *Kinetic and thermodynamic constants determined for CA II/sulfonamide interactions using SPR, ITC, and SFF*

Analysis method	Sulfonamide compound	T (°C)	Exp[a]	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ [cal/(mol K)]
SPR	CBS	25	6	$(4.8 \pm 0.2) \times 10^4$	0.0365 ± 0.0006	760 ± 30	-8.3 ± 0.3	-11.6 ± 0.4	-11 ± 1
ITC	CBS	25	5	—	—	730 ± 20	-8.4 ± 0.2	-11.9 ± 0.4	-12 ± 1

Summary

- **SPR detects binding events as changes in mass at the chip surface**
- **Real-time kinetic measurements**
- **Qualitative rankings**
- **Measurement of *active* concentration**
- **Information about structure-activity relationships**
- **Low volumes of precious samples needed**

BUT !!! -

SPR is not a true solution method (vs. ITC)

Attaching receptor to surface can influence binding properties.

Chemical Kinetics: the study of the rate of reactions

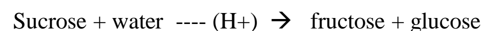
rate measurements + dependence of experimental conditions

Mechanism: Explain what the molecules are doing / a set of reactions showing how molecules collide and make and break bonds.

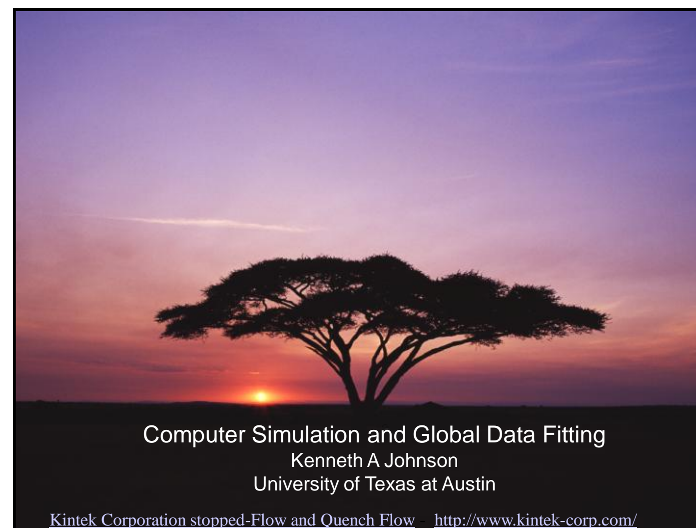
For ***one stoichiometric reaction***, there are ***many mechanisms***.

Principle of microscopic reversibility

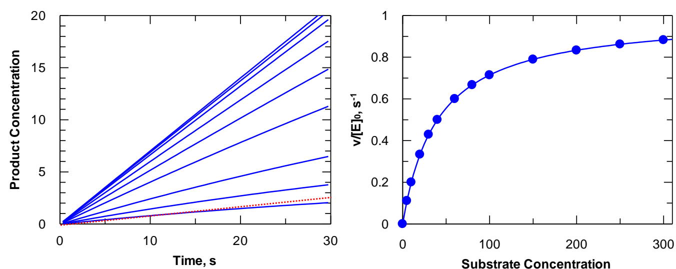
Rate Law / Order of Reaction



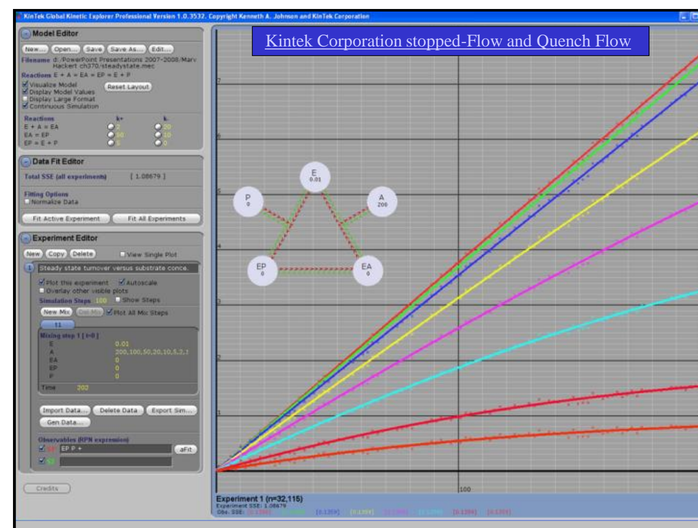
Measuring rate data: [ ] vs. time / "quenching" if time to measure is long compared to rate of reaction. → "Quenched-flow" apparatus



Conventional Steady-State Kinetics

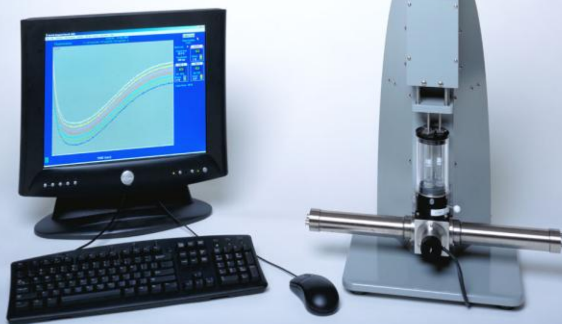


1. Measure initial rate
   a. Restrict data collection to first 10% of reaction
   b. If there is curvature, fit to polynomial to get initial rate
2. Plot rate versus concentration
3. Fit secondary plot to extract k_{cat} and K_m



KinTek SF-2003 Stopped-Flow

- Computer controlled motor drive
- 1 ms dead time
- 10 μL sample volume



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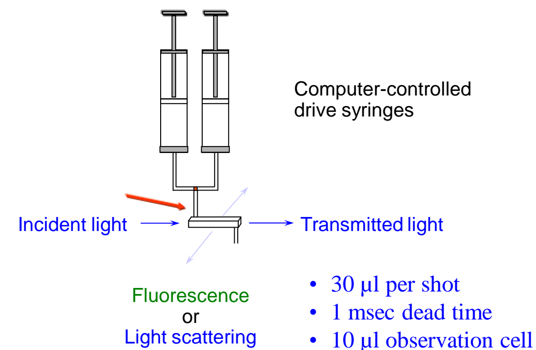
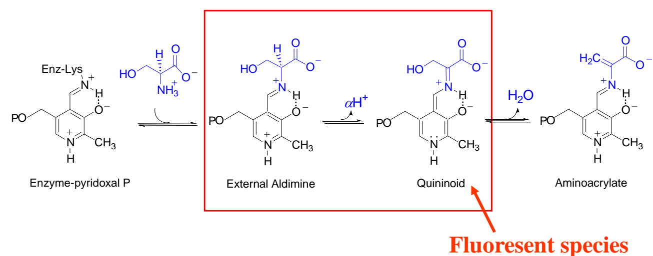
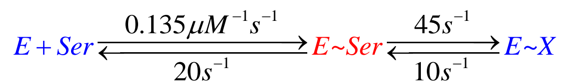
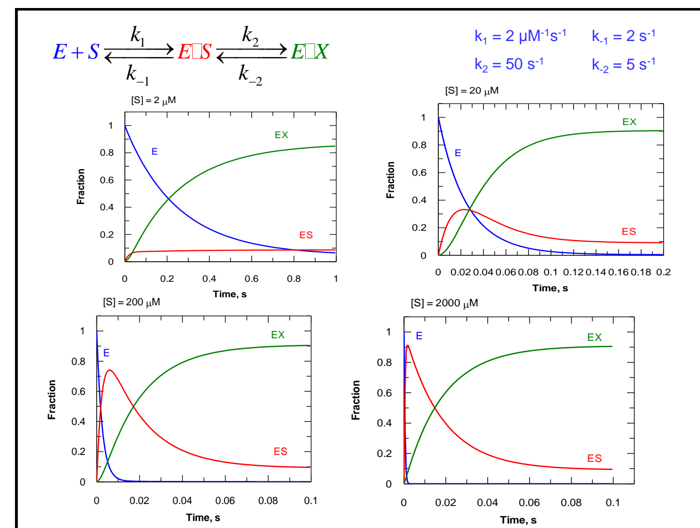
KinTek Stopped-Flow



Reaction with serine with pyridoxal phosphate

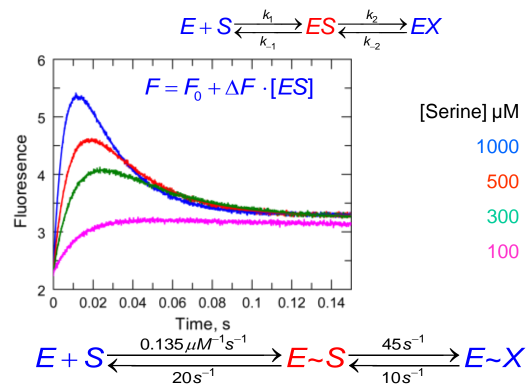






Tryptophan Synthase

Global Data Fitting based upon Simulation



Fit data directly to the model, get 4 rate constants and two fluorescence output factors.

Anderson, K.A., Miles, E. W. and Johnson K. A. (1991) J. Biol. Chem 266, 8020-8033