"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

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:

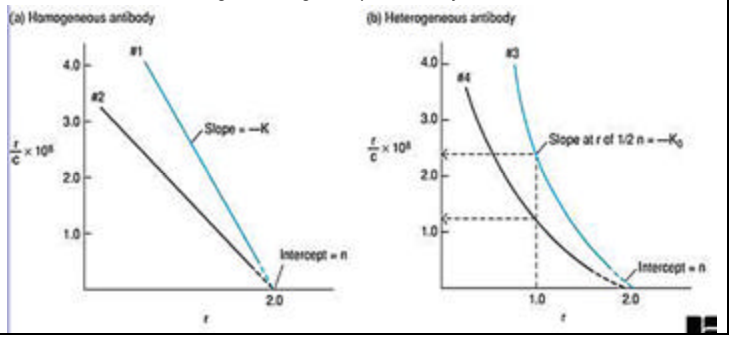
$$\theta = n - \frac{\upsilon K_d}{[S]}$$

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

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.

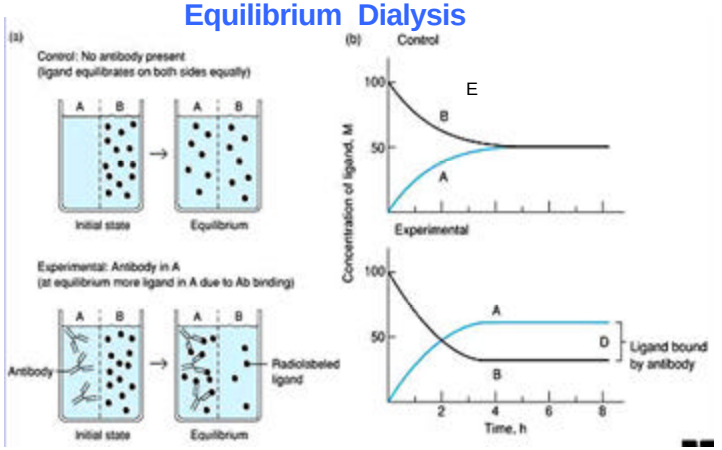
- ***gel filration chromatography*** **- Add M to a given concentration of L. Then elute the mixture on a gel filtration column, eluting with the free ligand at the same concentration. The ML complex will elute first and can be quantitated. If you measure the free ligand coming off the column, it will be constant after the ML elutes with the exception of a single dip near where the free L would elute if the column was eluted without free L in the buffer solution. This dip represents the amount of ligand bound by M.**
- ***membrane filtration*** **- Add M to radiolableled L, equilibrate, and then filter through a filter which binds M and ML. For instance, a nitrocellulose membrane binds proteins irreversibly. Determine the amount of radiolabeled L on the membrane which equals [ML].**
- ***precipitation*** **- Add a precipitating agent like ammonium sulfate, which precipitates proteins and hence both M and ML. Determine the amount of ML.**

EXPERIMENTAL DETERMINATION OF Kd

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

- ***equilibrium dialysis*** **- Place M in a dialysis bag and dialyse against a solution containing a ligand whose concentration can be determined using radioisotopic or spectroscopic techniques. At equilibrium, determine free L by sampling the solution surrounding the bag. By mass balance, determine the amount of bound ligand, which for a 1:1 stoichiometry gives ML. Repeat at different ligand concentrations.**
- ***gel shift assay*** **– Use autoradiography and gel electrophoresis with radiolabeled macromolecule.**
- ***spectroscopy*** **- Find a ligand whose absorbance or fluorescence spectra changes when bound to M. Alternatively, monitor a group on M whose absorbance or fluorescence spectra changes when bound to L.**

Equilibrium Dialysis



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/AmRa 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.

Micro-Equilibrium Dialyzer™ (Continued)

Micro-Equilibrium Dialyzers			
Volume per Chamber (µl)	Total Volume (µl)	Qty. of 1	Qty. of 5
25	50	MB 74-1606	MB 74-1600
50	100	MB 74-1607	MB 74-1601
100	200	MB 74-1608	MB 74-1602
250	500	MB 74-1609	MB 74-1603
500	1,000	MB 74-1610	MB 74-1604
Additional Chambers for 3-Chamber System			
25	-	MB 74-1619	MB 74-1620
50	-	MB 74-1611	MB 74-1615
100	-	MB 74-1612	MB 74-1616
250	-	MB 74-1613	MB 74-1617
500	-	MB 74-1614	MB 74-1618

Ultra-Thin Membranes for Micro-Equilibrium Dialyzer		
Membrane MWCO (Daltons)	Qty. of 24	Qty. of 96
For Use with 25, 50 and 100µl Volume Chambers		
5,000	MB 74-1704	MB 74-1700
10,000	MB 74-1705	MB 74-1701

Plate Rotator

A Plate Rotator with variable rotation rates is available for use with Harvard/AmiKa's Equilibrium Dialyzer-96™. The Rotator speeds up the equilibrium dialysis process by keeping the sample in constant motion ensuring higher reproducibility of results.

Gel Shift Assay

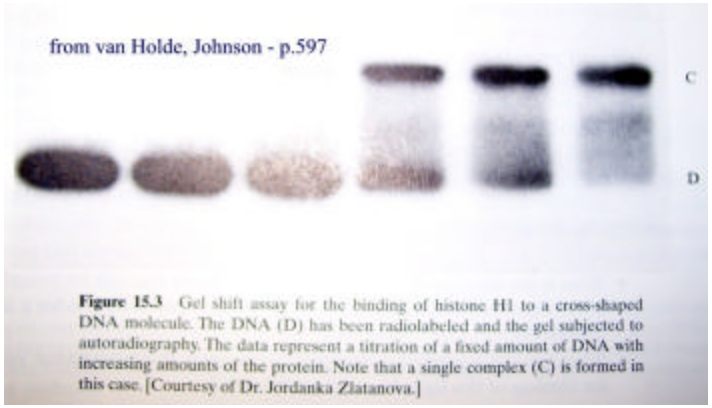


Figure 15.3 Gel shift assay for the binding of histone H1 to a cross-shaped DNA molecule. The DNA (D) has been radiolabeled and the gel subjected to autoradiography. The data represent a titration of a fixed amount of DNA with increasing amounts of the protein. Note that a single complex (C) is formed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

$$\theta = \frac{[S]_0}{K_d + [S]_0} \quad \text{OR.....} \quad \theta = \frac{(E_0 + S_0 + K_d) - \sqrt{(E_0 + S_0 + K_d)^2 - 4 \cdot E_0 \cdot S_0}}{2 \cdot E_0}$$

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

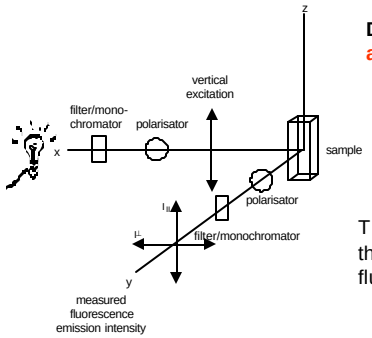
Fluorescence Anisotropy

Definition of fluorescence anisotropy *r*

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

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

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

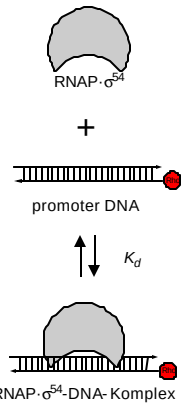


Definition of fluorescence anisotropy *r*

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

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

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



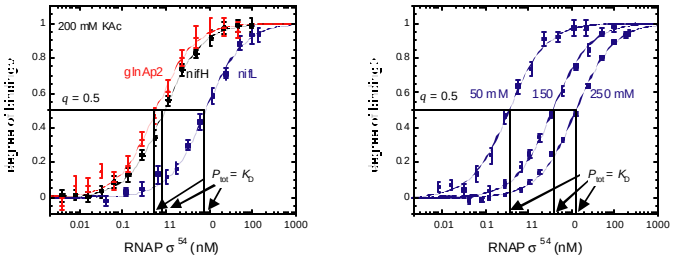
$$q = \frac{[P_{tot}]}{[P_{tot}] + K_D} = \frac{r_{measured} - r_{min}}{r_{max} - r_{min}}$$

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

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



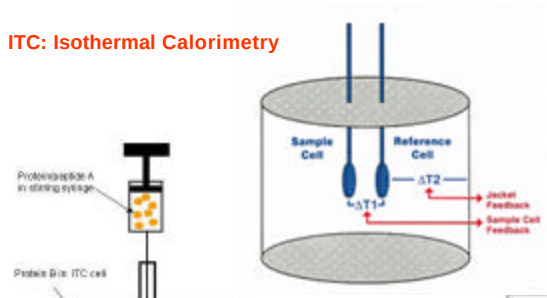
Vogel, S., Schulz A. & Rippe, K.

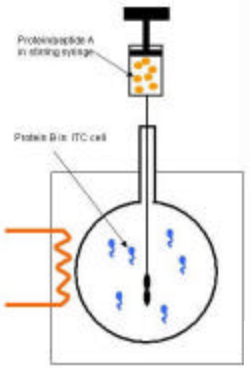
EXPERIMENTAL DETERMINATION OF Kd

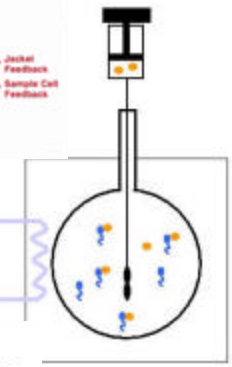
TECH. THAT DO NOT REQUIRE SEPARATION OF BOUND FROM FREE LIGAND

- ***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.**
- ***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.**
- ***Other (higher tech) methods:***
  - ***ITC - Isothermal Titration Calorimetry***
  - ***SPR – Surface Plasmon Resonance***
  - ***Fast Kinetics***

ITC: Isothermal Calorimetry







Measure – $\Delta q(i)$

Calculate by fitting -

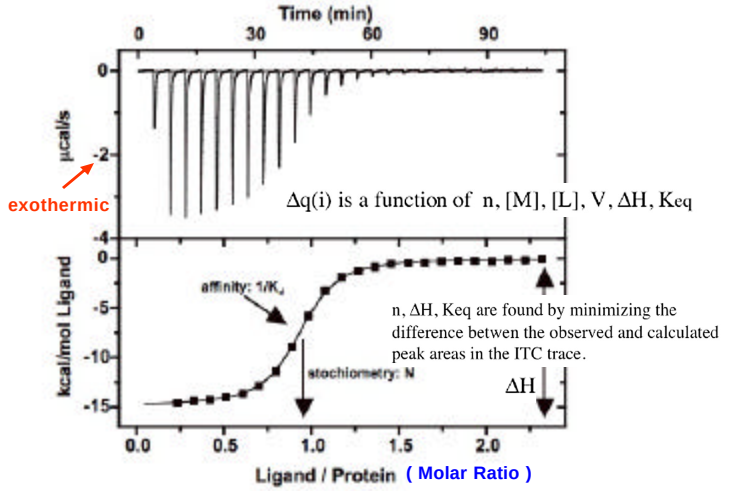
n, ΔH, Keq

Derive -

ΔG from Keq

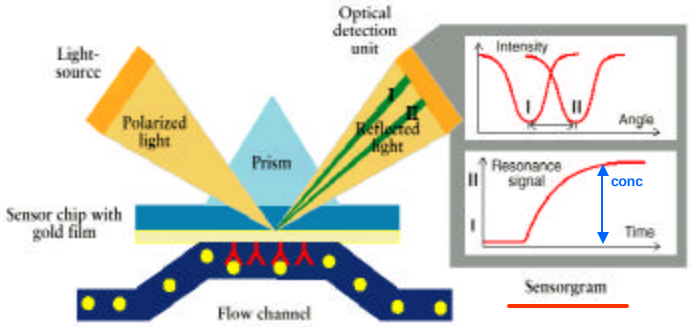
ΔS using ΔH & ΔG

ITC: Isothermal Titration Calorimetry

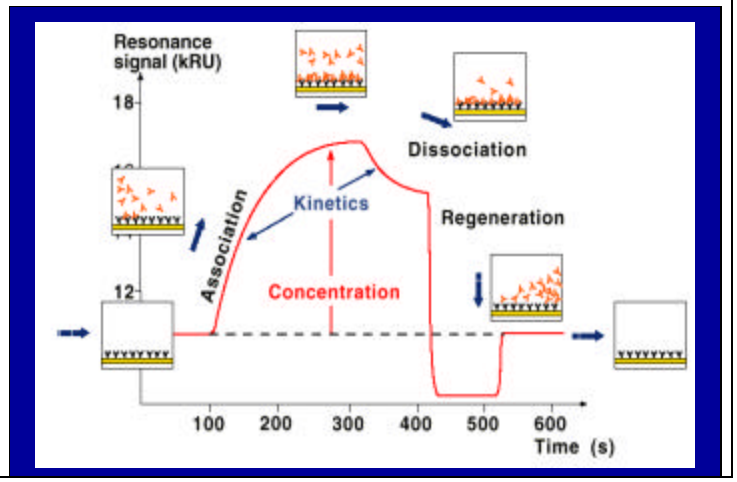


3. Surface Plasmon Resonance Detection: Biomolecular Binding in Real Time

Principle of Detection



The Sensorgram is Information Rich

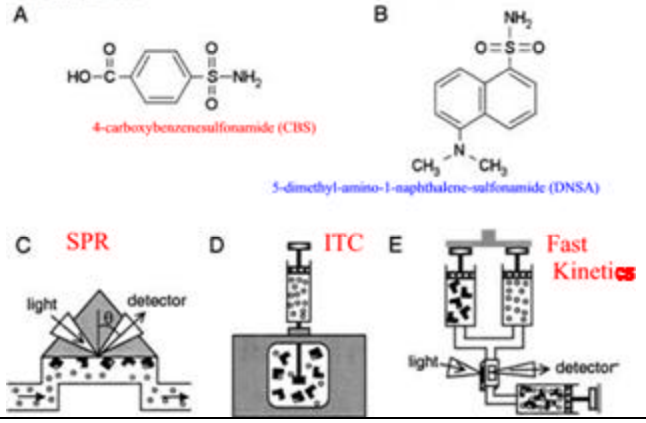


Direct comparison of binding equilibrium, thermodynamic, and rate constants determined by surface- and solution-based biophysical methods

(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



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.

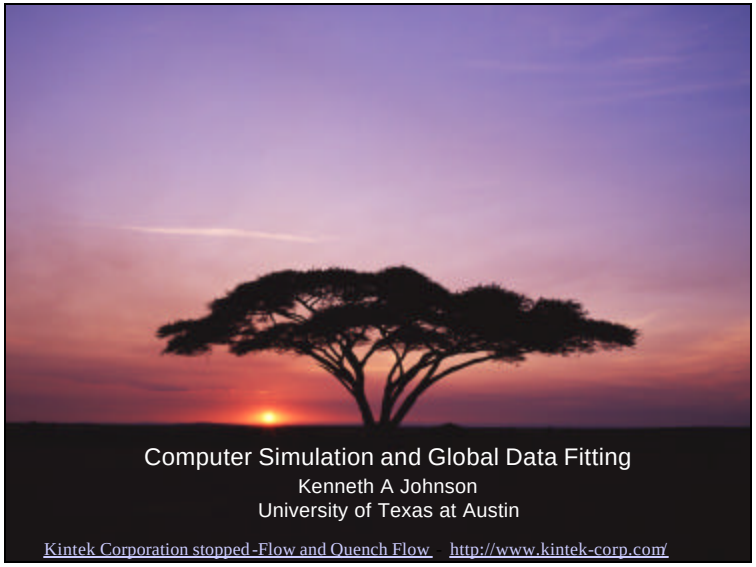
For ***one stoichiometric reaction***, there are ***many mechanisms***.

Principle of microscopic reversibility

Rate Law / Order of Reaction

Sucrose + water ---- (H+) → fructose + glucose

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



Order of a Reaction /Activation Energy /Transition State

Zero Order Reactions:

Rate is constant / [ ] vs. t is linear with slope = k / units of M/s

First Order Reactions:

Rate is proportional to [ ] / ln[ ] vs. t is linear with slope = k (1/s)

Half-life = ln(2)/k = 0.693/k

Relaxation time = time for [ ] to become 1/e of its original value

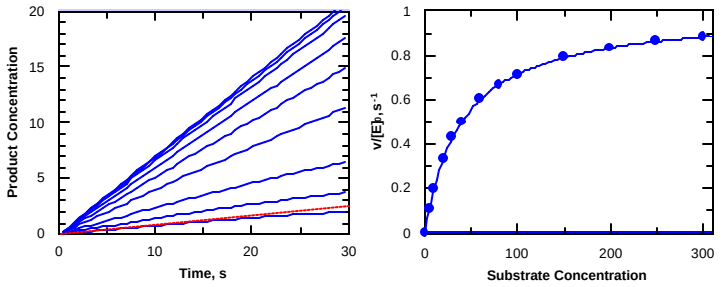
tau = 1/k or since 1/e = 0.368 when [ ] = 0.368[ ]$_o$

Only relative conc needed so any concentration units are OK

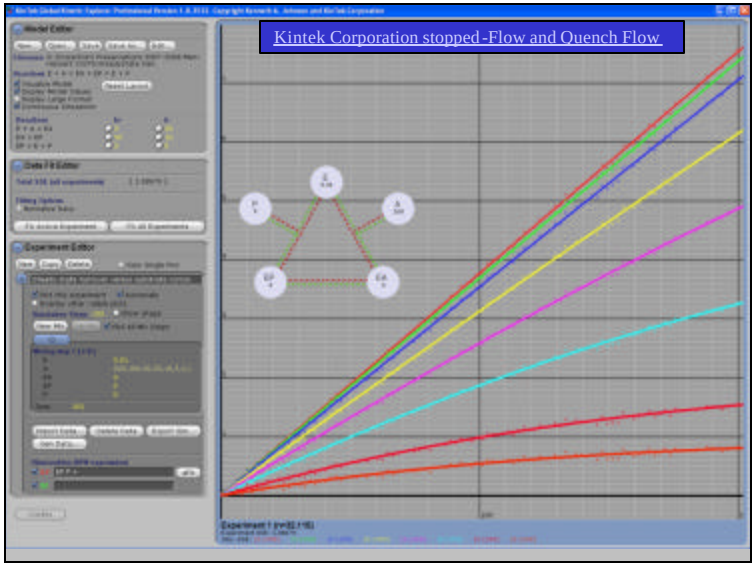
Arrhenius Equation: k = A exp(-E_a/RT) lnk vs. 1/T E_a = -R x slope

Transition State Theory: $k = k_B T/h \exp(-\Delta G^{++}/RT)$

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



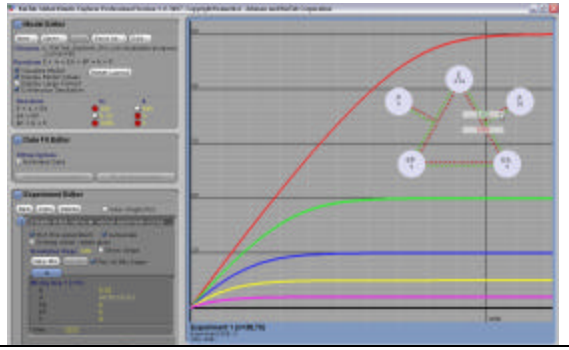
Full Time-course (Progress Curve) kinetics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$$

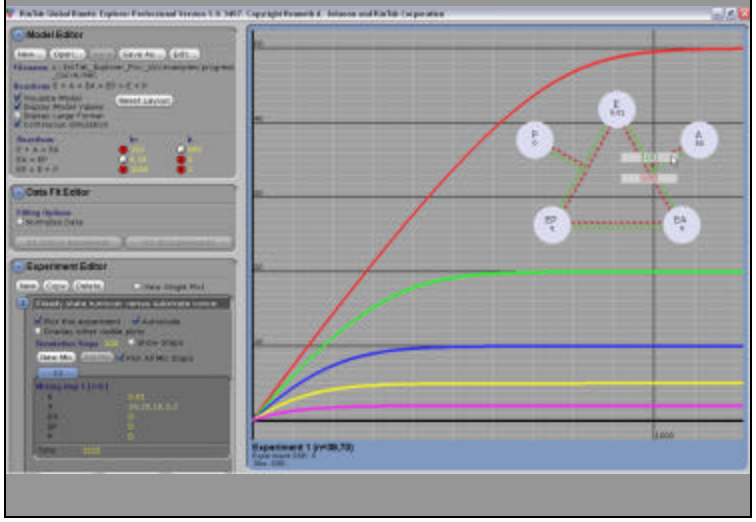
Follow reaction until reaction approaches equilibrium

Decreasing rate of turnover is due to decreasing [S] and increasing [P]

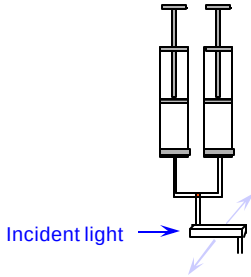
Data can be fit directly by simulation to extract k_{cat} and K_m



Full Progress Curve Kinetics



KinTek Stopped-Flow



Computer-controlled drive syringes

Fluorescence
or
Light scattering

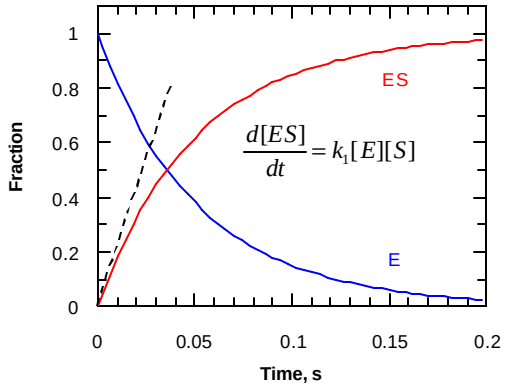
- 30 µl per shot
- 1 msec dead time
- 10 µl observation cell

KinTek SF-2003 Stopped-Flow

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

Kinetics of irreversible substrate binding

$$E + S \xrightarrow{k_1} E\text{-}S$$



Kinetics of substrate binding: *irreversible binding*

$$E + S \xrightarrow{k_1} E\text{-}S$$

$$d[E]/dt = -k_1[E][S]$$

$$d[E]/[E] = -k_1[S]dt$$

$$\int_{E_0}^{E} d[E]/[E] = -\int_0^t k_1[S]dt = -k_1[S]\int_0^t dt$$

$$\ln([E]/[E]_0) = -k_1[S](t - t_0)$$

$$[E]/[E]_0 = e^{-k_1[S]t}$$

$$[ES]/[E]_0 = 1 - e^{-k_1[S]t}$$

k_1 is a second order rate constant, units $M^{-1}s^{-1}$

Assume *[S]* is constant

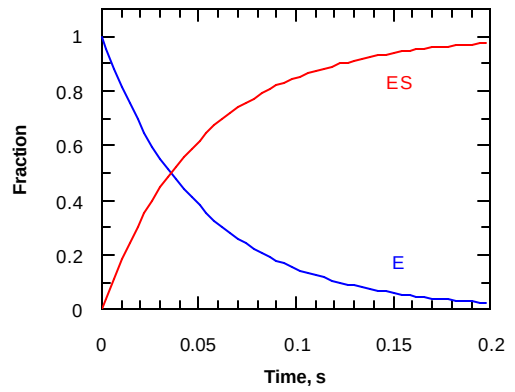
$k_1[S]$ is the pseudo-first order rate constant, unit s^{-1}

E decays and ES appears by an exponential function with rate $k_1[S]$

We often use the units of $\mu M^{-1}s^{-1} = 10^6 M^{-1}s^{-1}$.

Diffusion limit is approximately $10^8 M^{-1}s^{-1} = 100\ \mu M^{-1}s^{-1}$

Kinetics of irreversible substrate binding



$k_1 = 2\ \mu M^{-1}s^{-1}$

[S] = 10 µM

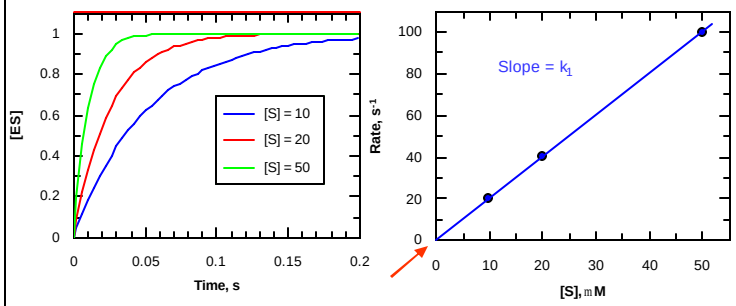
$k_1[S] = 20\ s^{-1}$

$t_{1/2} = (\ln 2)/k$

$= 34.6$ msec

Concentration dependence of binding rate

$k_{obs} = k_1[S]$



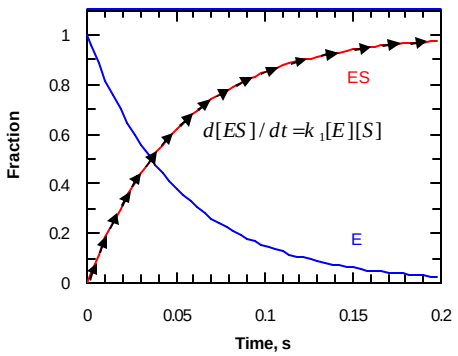
Numerical Integration

$E + S \xrightarrow{k_1} EgS$

$d[E]/dt = -k_1[E][S]$

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

$[S]_0 = [S] + [ES]$



Analytical Solution:

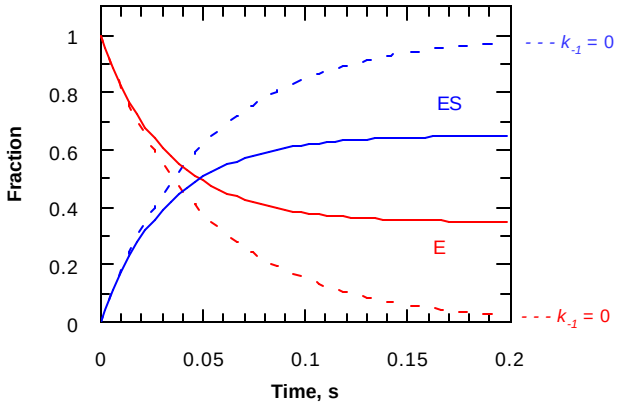
$[E]/[E]_0 = e^{-k_1[S]t}$

$[ES]/[E]_0 = 1 - e^{-k_1[S]t}$

Kinetics of reversible binding

$k_{obs} = k_1[S] + k_{-1}$

Observed rate is the sum of forward and reverse rates.



Kinetics of substrate binding: *Reversible binding*

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

$d[E]/dt = -k_1[E][S] + k_{-1}[ES]$

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

$[E]_0 = [E] + [ES]$ mass balance

$d[E]/dt = -k_1[E][S] + k_{-1}([E]_0 - [E])$

$d[E]/dt = -(k_1[S] + k_{-1})[E] + k_{-1}[E]_0$

$[E]/[E]_0 = Ae^{-k_{obs}t}$

$[ES]/[E]_0 = A(1 - e^{-k_{obs}t})$

$k_{obs} = k_1[S] + k_{-1}$

$k_{obs} = k_1[S] + k_{-1}$ is the sum of the forward and back rates.

$A = K_1[S]/(K_1[S] + 1)$

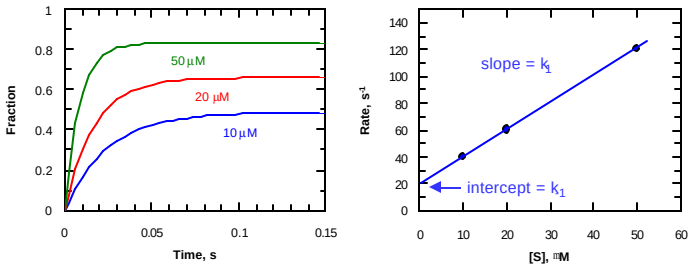
$= k_1[S]/(k_1[S] + k_{-1})$

Amplitude, *A*, is a function of the equilibrium constant for forming the ES complex

General equation for data fitting: $Y = A \cdot e^{-k_{obs}t} + C$

Concentration dependence of binding rate

$$k_{obs} = k_1[S] + k_{-1}$$



NOTE: increase in amplitude and rate as a function of increasing [S]

One experiment can serve to define k_1, k_{-1} and K for S binding.

Kinetics of substrate binding: *Two-steps, four rates*

C. Complete solution $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EgS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EgX$

Each species follows a double exponential

$$[E]_i/[E]_0 = A_1 e^{-l_1 t} + A_2 e^{-l_2 t} + C$$

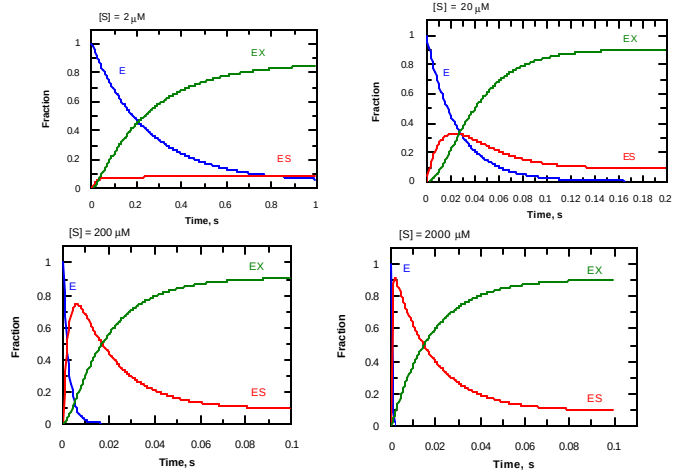
with rates of: $l_1 \approx k_1[S] + k_{-1} + k_2 + k_{-2}$

$$l_2 \approx \frac{k_1[S](k_2 + k_{-2}) + k_{-1}k_{-2}}{k_1[S] + k_{-1} + k_2 + k_{-2}}$$

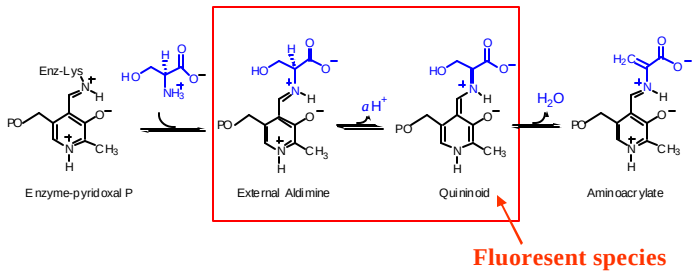
$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EgS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EgX$

$k_1 = 2\ \mu M^{-1}s^{-1}$ $k_{-1} = 2\ s^{-1}$

$k_2 = 50\ s^{-1}$ $k_{-2} = 5\ s^{-1}$



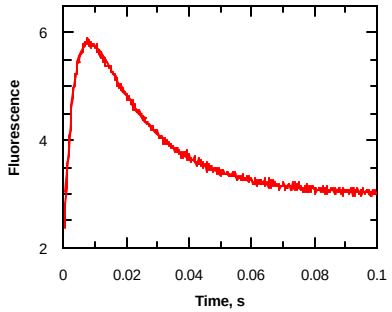
Reaction with serine with pyridoxal phosphate



$$E + Ser \underset{20s^{-1}}{\overset{0.135mM^{-1}s^{-1}}{\rightleftharpoons}} E{\sim}Ser \underset{10s^{-1}}{\overset{45s^{-1}}{\rightleftharpoons}} E{\sim}X$$

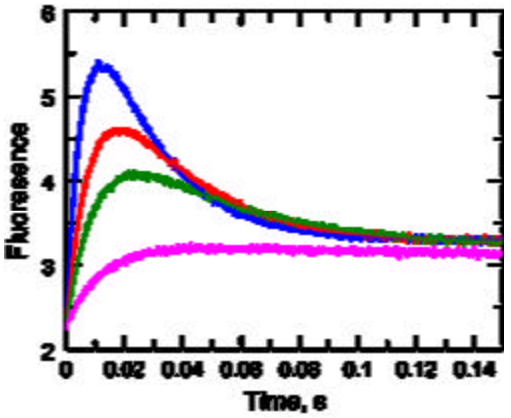
Tryptophan Synthase

$$E + Ser \underset{20\,s^{-1}}{\overset{0.135\,mM^{-1}s^{-1}}{\rightleftharpoons}} E\sim Ser \underset{10\,s^{-1}}{\overset{45\,s^{-1}}{\rightleftharpoons}} E\sim X$$



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

Concentration dependence of fluorescence transient



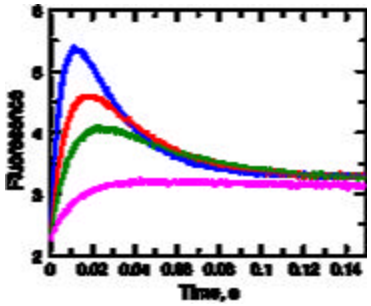
[Serine] μM
1000
500
300
100

Conventional Data Fitting

1. Derive mathematical expressions for time dependence from a model
   - Almost always requires simplifying assumptions
   - Math soon gets complex (one exponential for each step)
2. Fit time dependence to mathematical expression to extract a rate
   - Fit to more independent variables than are relevant to the model
   - Loose relationships between rate and amplitude
3. Re-plot rate as a function of concentration
   - Observe patterns and develop model
4. Derive another mathematical expression to account for concentration dependence of the rate(s)
   - Requires more simplifying assumptions
5. Fit the concentration dependence to the mathematical expression to extract primary kinetic constants (k_{cat}, K_m, or rate constants)
   - Propagate errors through all steps of data fitting

Global Data Fitting based upon Simulation

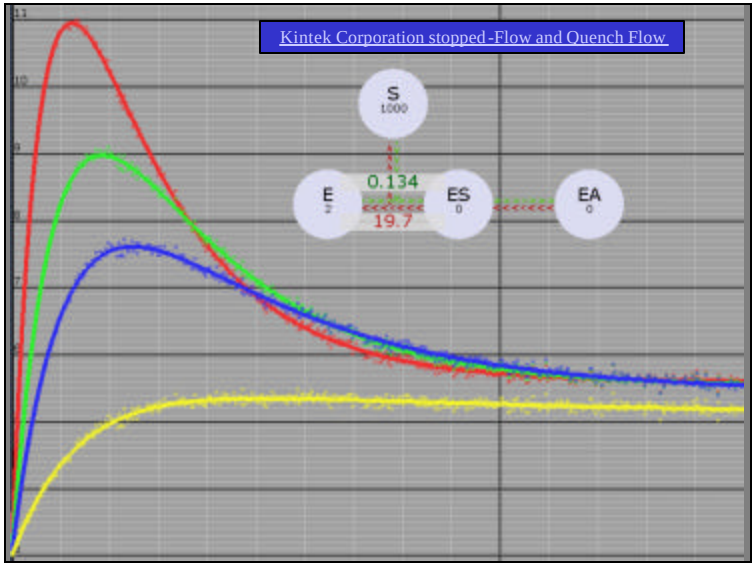
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EX$$



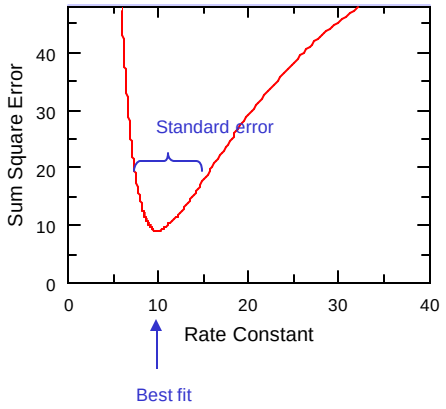
$$F = F_0 + \Delta F \cdot [ES]$$

$$E + S \underset{20\,s^{-1}}{\overset{0.135\,mM^{-1}s^{-1}}{\rightleftharpoons}} E\sim S \underset{10\,s^{-1}}{\overset{45\,s^{-1}}{\rightleftharpoons}} E\sim X$$

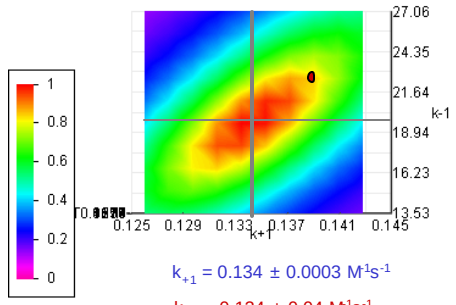
Fit data directly to the model, get 4 rate constants and two fluorescence output factors.



Nonlinear Regression: minimize sum square error



Confidence Contour from Data Fitting

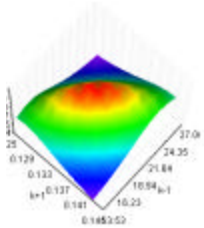


k_{-1} = 19.7 ± 0.09 s^{-1}

k_{-1} = 19.7 ± 3 s^{-1}

k_{+1} = 0.134 ± 0.0003 $M^{-1}s^{-1}$

k_{+1} = 0.134 ± 0.04 $M^{-1}s^{-1}$



FIT = $SSE_{min}/SSE_{x,y}$

Fitting to only one trace, floating output factors