HYDRODYNAMICS OF MOLECULES AND SEDIMENTATION

If no change in velocity, net force on particle, \( F_c + F_b + F_d = 0 \)

\[ \omega^2 r m - \omega^2 r m \bar{v} \rho - f v = 0 \]

\[ \omega^2 r m (1 - \bar{v} \rho ) - f \bar{v} = 0 \]

Define SEDIMENTATION COEFFICIENT, \( s = \frac{v}{\omega^2 r} = \frac{M (1 - \bar{v} \rho )}{N_A f} \) units of seconds

but \( f = \frac{R T}{N_A D} \)

\( R = \) Universal gas constant; \( T = \) absolute temperature
\( N_A = \) Avogadro number; \( D = \) Diffusion coefficient

SVEDBERG EQUATION \( M = \frac{R T s}{(1 - \bar{v} \rho ) D} \)

For biological molecules, \( s \) values ~ \( 1 \times 10^{-13} \) seconds or more

1 Svedberg unit (S) = \( 1 \times 10^{-13} \) seconds
As M increases, s increases (sediments faster)

Cytochrome c \( M \sim 15,000 \) s = 1 S
Small ribosomal subunit \( M \sim 900,000 \) s = 30 S
Large ribosomal subunit \( M \sim 1,600,000 \) s = 50 S
Ribosome \( M \sim 2,500,000 \) s = 70 S
Bushy stunt virus \( M \sim 11,000,000 \) s = 132 S
Tobacco Mosaic Virus s = 400 S

s \propto M \) but only approximately, not linear

e.g. 30 S subunit + 50 S subunit = 70 S ribosome

As particle density increases, s increases (sediments faster) 
(ν decreases)

As solvent density increases, s decreases (sediments slower) 
(ρ increases)

As frictional coefficient increases, s decreases (sediments slower) 
(size, shape, hydration)
THE ANALYTICAL ULTRACENTRIFUGE
Analytical Ultracentrifuge = centrifuge + UV/vis spectrophotometer (E.g. Beckman Optima XL-A)

Toroidal diffraction = monochromator (select $\lambda$) grating

Can measure concentration of molecule ($c$) as a function of distance ($r$) from axis of rotation, during centrifugation

Two types of experiment:
1) Sedimentation velocity
   - mass, diffusion coefficient, molecular shape
2) Sedimentation equilibrium
   - mass, interactions

$A = \frac{c}{\varepsilon l}$

distance, $r$
1) Sedimentation equilibrium

Axis of rotation

Meniscus

initial condition (t=0)

particles distributed evenly

centrifuge at moderate speed

time

Reach equilibrium where sedimentation = diffusion

So gradient of particle concentration becomes constant over time

\[
\ln \frac{c_b}{c_a} = \frac{M (1 - \bar{v} \rho) \omega^2 (r_a^2 - r_b^2)}{2 \ R \ T}
\]

Do not need to know \( D \)
Predict curve based on specific models:

e.g. stable monomer, or dimer, or trimer etc

or multiple species in equilibrium:

Use non-linear curve fitting, allowing parameters (mass of species, dissociation constants) to vary. Try to fit measured values as closely as possible, with smallest, most randomly distributed residuals.
Examples of residuals:

- **Good fit**: residuals small and randomly distributed
- **Poor fits**: residuals clearly vary systematically with radius, and/or large

So in published reports, look for residuals and also for details of other possible models to judge if chosen model is really the best.

e.g. Topoisomerase II (Tennyson & Lindsley, 1997, *Biochemistry* 36, 6107-6114)
Advantages of analytical ultracentrifugation:

1) Provides first principle (direct from theory, no reference standards required) information on:
   - molecular mass, diffusion coefficient
   - molecular shape (from sedimentation velocity) and interactions

2) in solution (so true equilibria) and no labelling required

3) solution conditions can be varied (pH, ionic strength, temp.)

4) relatively little material required (but should be of highest purity possible)

Disadvantages:

1) Slow - full analysis can take days or weeks

2) Heterotypic associations can be more difficult to analyze
THERMODYNAMICS
- systematic description of POPULATION of molecules at EQUILIBRIUM in terms of MACROSCOPIC phenomena

Thermodynamic parameters: temperature (T), pressure (P) volume (V)

enthalpy (H) entropy (S) Heat Capacity (C)

Gibbs free energy (G) $K_d$

Laws of Thermodynamics:
1) Conservation of matter: Heat change, $q$ (constant T, P) = $\Delta H \approx \Delta E$
2) Entropy and order: System always tends towards maximum entropy

Gibbs free energy $G$: $\Delta G^0' = \Delta H^0' - T \Delta S^0'$ (1M, pH 7.0, 298K)

For molecular interactions

\[ \Delta G = \Delta G^0' - RT \ln K_d \]
\[ \Delta G^0' = + RT \ln K_d \]
\[ K_d = e^{\frac{\Delta G^0'}{RT}} \]

Van’t Hoff analysis:
Measure $K_d$ at different T to determine $\Delta H^0'$, $\Delta S^0'$ and $\Delta G^0'$:

\[ \Delta H^0' = R \ln \left( \frac{K_d^1}{K_d^2} \right) \left( \frac{T_2 T_1}{T_2 - T_1} \right) \]
\[ \ln K_d = \frac{\Delta H^0'}{RT} - \frac{\Delta S^0'}{R} \]
Principle:
1. Add small amount of molecule Y ($\Delta Y$) to sample cell in successive injections (titrate)
2. Heat absorbed or evolved ($q$) as a result of interaction causes temperature difference ($\Delta T$) between sample reference cells
3. After each injection, feedback heaters add heat energy to sample or reference cell to bring $\Delta T$ back to zero

Each spike represents one injection of molecule Y. Integrating area of each peak gives total heat released ($\Delta q$) at each titration point (as total concentration of Y increases)

Can obtain information on $\Delta H$, $K_d$ (and $\Delta G$ and $\Delta S$)

Determine $\Delta H$ at different $T$ gives $\Delta C_P = \frac{\Delta H_1 - \Delta H_2}{T_2 - T_1}$
THERMODYNAMICS OF INTERACTIONS

Enthalpy:

Individual components interact with water molecules (and ions) 

$\Delta H$

1) Some interactions with water molecules at interaction surfaces are broken (unfavorable).

2) **New** interactions between X and Y at interface (favorable)

3) Lower heat capacity $\Delta C_p < 0$

Entropy

entropy of rotation and translation of free X and Y

total entropy of bound water molecules low

etropy higher (displaced water)

$+ve$ (unfavorable) $\Delta G$ $-ve$ (favorable)

$-T\Delta S$

rotation/movement

$\Delta H$

interactions

$-T\Delta S$

displaced water (hydrophobic effect)

$-T\Delta S$

NET $\Delta G < 0$
Properties of Interaction Surfaces, Interaction Strength and Enthalpy vs. Entropy Driven Interactions

1) Size and nature (properties) of interface determines magnitude of $\Delta G^0'$ and therefore $K_d$ (strength of binding)

2) Relative contribution of enthalpy ($\Delta H^0'$) and entropy ($-T\Delta S^0$) to a negative (favorable) $\Delta G^0'$ also depends on size and properties of interface

Small surface area of interaction ($Y =$ small molecule e.g. glucose, ATP)

$\Delta G^0' = \Delta H^0' - T\Delta S^0'$

ENTHALPY DRIVEN ($\Delta H^0' > -T\Delta S^0'$)

Fewer displaced water molecules means smaller contribution to favorable entropy

Small molecules also have higher entropy (greater movement) so larger entropic cost when bound in complex

Large surface area of interaction (protein:protein, protein:nucleic acid interactions)

$\Delta G^0' = \Delta H^0' - T\Delta S^0'$

ENTROPY DRIVEN ($\Delta H^0' \leq -T\Delta S^0'$)

More interactions between X and Y means greater favorable enthalpy

More water molecules displaced so larger favorable entropy contribution

Experimentally observed that change in heat capacity ($\Delta C_p$) is related to size (surface area) of interaction surface

Large $\Delta C_p$ can also result from conformation change in molecules upon interaction (proportional to number of residues involved)
**PROTEIN:PROTEIN COMPLEXES**

Interaction surfaces exposed to solvent

Interaction surfaces inaccessible to solvent

**Interaction surface area**

**NON-OBLIGATE COMPLEX**
- weaker interaction
- relatively high $K_d$
- modest, negative $\Delta G^0'$

Properties of Interaction Surface

**SIZE**
- Average $\sim 1600 \, \text{Å}^2$
- ($\sim 800 \, \text{Å}^2$ from each protein)

**SHAPE**
- Relatively flat

**CHEMISTRY**
- Up to $\sim 40$ amino acids
- ($20$ from each protein)
- $\sim 700 \, \text{Å}^2$ non-polar groups
  (modest contribution from hydrophobic effect to $T\Delta S$)
- $\sim 900 \, \text{Å}^2$ polar groups
  (enthalpy contribution hydrogen bonds, electrostatic)

Polar groups important since when proteins X and Y are *not* interacting with each other they have to interact favorably with water

**PERMANENT(OBLIGATE) COMPLEX**
- very strong interaction
- very low $K_d$
- large, negative $\Delta G^0'$

**Average**
- $\sim 3400 \, \text{Å}^2$
- (up to $5000 \, \text{Å}^2$)

**SHAPE**
- More convoluted

**CHEMISTRY**
- $> 40$ amino acids
- (more interactions = more $-\Delta H^0'$)
- greater proportion of non-polar residues
- greater hydrophobic effect as more water molecules displaced.
- So large, negative $\Delta G^0'$ is even more entropy driven
A NON-OBLIGATE COMPLEX
Cytochrome c: Cytochrome c peroxidase

A PERMANENT COMPLEX
Histone H2A:H2B heterodimer
This region forms stable $\alpha$-helix that mediates formation of dimer.

An $\alpha$-helix is just the right size to fit into the major groove of DNA allowing close interactions with bases.

Electrostatic interactions between positively charged amino acids (R, K) and negatively charged phosphate groups help stabilize complex ($\Delta H^0$).

This region is not stably folded when GCN4 is not bound to DNA.

Region becomes folded as $\alpha$-helix upon binding DNA (unfavorable entropy change).
Complementary shape and chemistry (spatial arrangement of chemical groups)
So maximize possible number of favorable interactions to contribute to best $\Delta G$ possible

If the shape and/or chemistry of the interaction surface changes (e.g. by natural or artificial mutation, post-translational modification) the interaction strength will change

Some interactions but shape and chemistry does not entirely fit well so weaker binding

Greater complementarity so more favorable interactions can be made giving greater $\Delta G$ which means stronger binding
A simple bimolecular interaction

\[ P + A \xrightleftharpoons[k_{on}]{k_{off}} PA \]

\[ k_{on} = \text{rate constant for association (on rate)} \quad \text{M}^{-1} \text{s}^{-1} \]
(also called \( k_a \) or \( k_{assoc} \))

\[ k_{off} = \text{rate constant for association (on rate)} \quad \text{s}^{-1} \]
(also called \( k_a \) or \( k_{assoc} \))

Rate of formation of complex PA = \( k_{on} [P]_f [A]_f \)

Rate of breakdown of complex PA = \( k_{off} [PA] \)

Rate of change of PA = \( \frac{d[PA]}{dt} = k_{on} [P]_f [A]_f - k_{off} [PA] \)

At EQUILIBRIUM \( \frac{d[PA]}{dt} = 0 \), so \( k_{on} [P]_f [A]_f - k_{off} [PA] = 0 \)

Thermodynamic Equilibrium Dissociation Constant, \( K_d \)

\[ K_d = \frac{k_{off}}{k_{on}} = \frac{[P]_f [A]_f}{[PA]} \quad \text{Units M} \]

For strongest \( K_d \) (low \( K_d \))

slower off rate (small \( k_{off} \))

faster on rate (large \( k_{on} \))
**SURFACE PLASMON RESONANCE (BIACORE)**

Light (above critical angle of incidence) reflected from interface between media of different refractive index (glass and water)

Light intensity reduced at a specific angle due to interaction with surface plasmons (oscillating charge density in thin gold film), producing a sharp shadow (surface plasmon resonance) due to resonance energy transfer between light wave and surface plasmons

Resonance conditions (angle at which it occurs) are influenced by material adsorbed onto thin metal film (changes refractive index)

Linear relationship between resonance angle and mass concentration of molecules such as proteins, sugars and nucleic acids

SPR signal expressed in Resonance Units (RU)

1 RU = 0.0001° change in resonance angle; equivalent to approx. 1 pg/mm² change in concentration on sensor surface

Requires no labelling of components, can obtain real-time observations of molecular interactions
Initial state - molecule A attached to chip surface (Δ)
- molecule B introduced into buffer flow (○)

Association phase

Dissociation phase

Resonance units

increasing
[molecule B]
in flow

Sensorgrams

association

time

dissociation

http://www.biacore.com/
Attachment of ligand to sensor chip is a critical factor in SPR

- Distance from matrix
- Orientation
- Direct, non-specific binding of target to matrix?

Inhomogeneity of attachment - reduces number of available binding sites

Homogeneity through specific attachment site

Indirect attachment methods - Antibody, GST-fusion protein
Some methods for attaching molecules to sensor chip

**CM5**

- Carboxy-methyl groups on matrix serves as sites of chemical cross-linking
- Can react with: amines, thiols, aldehydes

**Streptavidin-biotin mediated**

- Especially good for nucleic acids/peptides (easy to biotinylate)

**Ni-NTA**

- For example, for His6-tagged proteins

**HPA**

- Customized surface for studies of proteins interacting with membranes/membrane bound ligands
Biacore analysis of Interleukin-2 (IL2) interaction with IL2 receptor

![Graph of IL2 response vs. time at different temperatures]

**Fig. 2.** IL-2–receptor biosensor data collected at different temperatures. IL-2 concentrations were 233, 78, 26, 8.6, 2.9, and 0 nM. Each injection was replicated four times. Running buffer contained 10 mM sodium phosphate (pH 7.4), 150 mM sodium chloride, 0.005% (v/v) P20, and bovine serum albumin (0.1 mg/ml). Binding data were collected with the BIACORE.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Standard deviation (RU)</th>
<th>$k_{on}$</th>
<th>$k_{off}$</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replication</td>
<td>Residual</td>
<td>(M$^{-1}$ sec$^{-1}$)</td>
<td>(sec$^{-1}$)</td>
</tr>
<tr>
<td>5</td>
<td>0.352</td>
<td>0.376</td>
<td>4.66 (4) x 10$^6$</td>
<td>0.0420 (2)</td>
</tr>
<tr>
<td>15</td>
<td>0.355</td>
<td>0.395</td>
<td>6.53 (6) x 10$^6$</td>
<td>0.0682 (4)</td>
</tr>
<tr>
<td>25</td>
<td>0.370</td>
<td>0.410</td>
<td>8.77 (9) x 10$^6$</td>
<td>0.114 (8)</td>
</tr>
<tr>
<td>35</td>
<td>0.365</td>
<td>0.409</td>
<td>1.01 (1) x 10$^7$</td>
<td>0.213 (2)</td>
</tr>
</tbody>
</table>

$^a$ The value in parentheses represents the standard error in the last significant digit.
Factors affecting association, the on-rate ($k_{on}$):
1) Probability of colliding in correct orientation
   - concentration, rate of movement (diffusion), rotational relaxation rate
2) Kinetic energy of collision (depends on temperature)
   - sufficient energy to break interactions with solvent

Factors affecting dissociation, the off-rate ($k_{off}$):
1) Collisions with other molecules
   - outcome depends on energy of collision (kinetic) vs. energy of interaction ($\Delta G$)

Some general observations from measurements of $k_{on}$
Most molecular interactions are diffusion limited:
For most proteins (typical diffusion coefficients 1 - 10 x 10^{-7} cm^2 s^{-1}) this limits fastest possible $k_{on}$ to $\sim 1 \times 10^9$ M^{-1} s^{-1}

For most interactions measured $k_{on}$ is slower than predicted based on diffusion ($1 \times 10^5 - 1 \times 10^7$ M^{-1} s^{-1})

Why?

Orientation of molecules at collision is important

Productive encounter leads to stable complex

Dissociate again

Unproductive encounter - not oriented correctly
SOME VERY FAST INTERACTIONS

For some interactions measured $k_{on}$ is greater than $1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$
(can be up to $1 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1}$)

How do they somehow improve rate of association?
- Increase probability of productive encounter

1) ELECTROSTATIC STEERING

![Diagram of electrostatic steering: Attractive electrostatic force increases speed at which molecules approach each other and can also help to orient molecules correctly.]

Examples: Barnase-Barstar interaction
Acetylcholinesterase - acetylcholine interaction

2) REDUCE DIMENSIONS OF SEARCH

(i) 3 dimensions reduced to 2 dimensions

Protein first binds weakly to membrane and diffuses in plane of bilayer until encounters target

Target protein - an integral membrane protein

Lipid bilayer

(ii) 3 dimensions reduced to 1 dimension

E.g. protein:nucleic acid interactions

Protein scans along linear DNA polymer until it reaches correct target sequence

$k_{on}$ is faster than when target sequence is within longer DNA molecule

Experimental evidence

DNA molecule contains target sequence only

$\text{[Diagram of DNA molecule with } k_{on} \text{] }$
Barnase - a ribonuclease enzyme

Barstar - an inhibitor of Barnase

Active site

Electrostatic potential

Acetylcholinesterase

\[
\text{CH}_3\text{-C-O-CH}_2\text{-CH}_2\text{-N(CH}_3)_3 \rightarrow \text{H-O-CH}_2\text{-CH}_2\text{-N(CH}_3)_3
\]

Acetylcholine

Choline

Active site
Useful literature for lecture 2 (Oct 4, 2004)

**Analytical Ultracentrifugation:**


**Calorimetry:**


Spolar, R. S., and M. T. Record, Jr. (1994). Coupling of local folding to site-specific binding of proteins to DNA. *Science* 263:777-84.

**Surface Plasmon Resonance:** (see also http://www.biacore.com/technology/)


